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Yersinia pseudotuberculosis, iron and disease in birds

by

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Abstract

An epidemiological study was conducted to determine the relative prevalence of yersiniae in different species of wild bird and in the environment. The prevalence of *Y. pseudotuberculosis* in wild birds, determined using bacteriological techniques, was low. *Yersinia pseudotuberculosis* was not isolated from environmental samples. The prevalence of other yersiniae isolated from birds was similar to those isolated from the environment in rural locations but not in urban locations. A concurrent serological survey was carried out on a proportion of the wild birds studied. A high number of serologically positive birds indicated frequent exposure to *Y. pseudotuberculosis*.

Clinical cases of pseudotuberculosis in captive birds occurred in the winter and spring following a period of cold weather. Three outbreaks studied involved passeriforms and were associated with poor management. The sporadic cases studied involved individual columbiforms or psittaciforms with concurrent haemosiderosis.

To allow statistical comparisons of the amount and distribution of stainable iron in histological sections, an image analysis system was developed using an experimental model of haemosiderosis in the chicken. Using this technique for a retrospective study of 180 avian cases, it was found that birds which died from infectious diseases had significantly higher levels of iron in the Kupffer cells than did birds which died from non-infectious diseases. The total amount of hepatic iron was not significantly different between the two groups.

An experimental model was developed in the chicken to examine the effect of parenteral iron on the pathogenesis of pseudotuberculosis. Challenged birds pre-treated with iron-dextran had higher serological titres to *Yersinia* lipopolysaccharide, the organism was more readily isolated from the faeces and there were more intestinal lesions than in challenged chickens pre-treated with dextran or desferrioxamine. However, chickens pre-treated with iron-dextran had fewer bacterial lesions in the liver and spleen.

Intracellular survival of *Y. pseudotuberculosis* and *Y. frederiksenii* *in vitro* was enhanced in iron loaded macrophages. It was also determined that *Y. pseudotuberculosis* was able to acquire iron from normal chicken serum.

Statement

This thesis contains no material that has been used in whole or in part for the award of any other degree or diploma in any educational institution. The nature and extent of any assistance I have received is as stated in the Acknowledgements section of this thesis. Any animal experimentation outlined in this thesis has been approved by the Animal Ethics Committee of Massey University.

Susan Catherine Cork

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‘There cannot be a philosophy, there cannot even be a decent science, without humanity.....the understanding of nature has as its goal the understanding of human nature, and of the human condition within nature’

The Ascent of Man, J Bronowski

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CHAPTER ONE

Review of the literature

Yersinia pseudotuberculosis, iron and disease in birds

General introduction

The provision of suitable ambient conditions of temperature, humidity, pH and nutritional resources for the replication of microorganisms defines their ecological niche for growth. Multicellular organisms, including mammals and birds, also occupy a niche in the natural environment and in turn they provide living space for many microorganisms on their body surfaces (Wake & Morgan, 1986). In general the interaction between the healthy 'host' and the microbial flora occupying the alimentary, respiratory, urogenital canal or epithelial surfaces represent organisms with a complex ecosystem in which both the multicellular host and the microbial flora are also interacting with the environment in which they live. A disturbance in the balance between host and the natural microbial flora may result in disease, either as a result of an increase in the virulence of a facultative pathogen, disruption of the non-specific defences of the host with or without a compromised immune system, or secondary infection with an opportunist pathogen following disruption of the normal microbial flora (Howard, 1991). However, not all infections require a compromised host. There are a small number of microbial organisms which, due to the evolution of specific virulence factors, have become capable of over coming the specific and non-specific immune defences of the multicellular host. The genus *Yersinia* is especially interesting in this regard; *Y. pestis* is essentially an obligate pathogen specifically adapted to arthropod transmission, *Y. pseudotuberculosis* is generally considered a facultative, but potentially very virulent, pathogen whereas *Y. enterocolitica* and the *Y. enterocolitica*-like species represent a range of virulence potential from facultative pathogens to non-pathogenic environmental species (Bercovier & Mollaret, 1984). There is a special interest in the genotypic variation which occurs in this genus and the environmental

factors which determine phenotypic expression of virulence characteristics (Cornelis, 1992). The interaction between host and potential pathogen is complex and the development of predictive models to assist in determining the probable outcome of infection (Smith, 1993) has highlighted the difficulty of determining the relative role of the myriad of variables which interact in the balance between health and disease. (Giffiths, 1993). Along with the effect of temperature on the expression of virulence in yersiniae, the availability of iron is another variable which would appear to have a special importance in the pathogenesis of disease caused by species of this genus (Heesemann *et al.*, 1993).

Based on an interest in wildlife disease surveillance, the evolution of host-pathogen interactions and the immunology of infectious diseases, the following review attempts to draw together information on the various disciplines utilised in veterinary disease investigation and emphasises those factors which effect the interaction between the avian host and *Y. pseudotuberculosis*. Because there is a limited literature on the subject of avian pseudotuberculosis, information has also been reviewed on other infectious diseases in birds and mammals especially where iron availability has a known effect on the course of disease. In this respect the conclusions drawn from the literature are not exclusive to yersiniae.

1-1 The genus *Yersinia*

The genus *Yersinia* (Van Loghem, 1944) is named after Alexander Yersin who first isolated the causal organism of the plague in 1894 (Bercovier & Mollaret, 1984). Although *Y. pestis* and the closely related *Y. pseudotuberculosis* were originally classified in the family *Pasteurellaceae* it is now recognised that these species, along with *Y. enterocolitica* and the environmental '*Yersinia enterocolitica*-like' species are more appropriately placed together in a separate genus within the family *Enterobacteriaceae* along with enteric bacteria such as *Salmonella spp* and *Escherichia coli* (Carter, 1982). The early literature prior to 1960 refers to *Pasteurella pestis* and *P. pseudotuberculosis*. Yersiniae are small, Gram-negative cocco-bacilli which resemble members of the *Pasteurellaceae*. However, yersiniae do not differ from other *Enterobacteriaceae* in their fine structure and overall cell wall composition. The whole-cell lipid composition of all yersiniae investigated exhibits a pattern shared with other *Enterobacteriaceae* (Jantzen & Lassen, 1980). The mol% G + C of *Yersinia* species ranges from 46-50 and is consistent with that for other *Enterobacteriaceae* (Brenner *et al.*, 1977; Brenner, 1978). There are a number of recent additions to the species recognised in the genus *Yersinia* and it is likely that others will be identified as more work is done on this group of organisms (Dolina & Peduzzi, 1993). There was much controversy in the 1980s in reference to the proposed renaming of *Y. pestis* as a subspecies of *Y. pseudotuberculosis*, this was based on the close DNA relatedness of the two species (Bercovier *et al.*, 1980, Mollaret, 1987). The final rejection of this proposal was due to the concern that the former has greater zoonotic potential and that there would be a risk of confusion between cultures in the laboratory. At the present time *Y. pseudotuberculosis* is frequently used in the laboratory as a model for studies on the plague bacillus (Mollaret, 1987). In addition to concerns over the public health risks associated with considering *Y. pestis* and *Y. pseudotuberculosis* as the same species (Williams, 1984) there were also some microbiologists who suggested that the phenotypic characteristics of *Y. pseudotuberculosis* were more similar to those of *Y. enterocolitica* (Brenner *et al.*, 1976) and that this, as well as the similar enteric mode of transmission of these two organisms indicated that there was not enough evidence to consider *Y. pestis* a subspecies of *Y. pseudotuberculosis* (Jones, 1987). However, although *Y. pestis* is well adapted to a life cycle which requires an arthropod vector and is less biochemically diverse than *Y. pseudotuberculosis*, it is important to consider the similarity in genotype between these

pathogenic bacteria. There is recent evidence to support the view that the pathogenesis of systemic infection with *Y. pseudotuberculosis* relies on the expression of similar virulence factors to those identified in *Y. pestis* (de Almeida *et al.*, 1993). It is the recent resurgence of interest in phenotypic expression during *in vivo* growth of pathogenic bacteria which has lead back to the idea that *Y. pestis* and *Y. pseudotuberculosis* are very closely related organisms (Bercovier *et al.*, 1980., Wolf-Watz *et al.*, 1985). In view of the historical importance of *Y. pestis* and the large body of literature available on the subject, this review will consider *Y. pseudotuberculosis* and *Y. pestis* as separate species but will draw comparisons between the two where appropriate.

Yersinia pseudotuberculosis, as a separate species, is fairly homogeneous biochemically but there are at least 9 recognised serotypes (Tsubokura *et al.*, 1984). These have been traditionally represented by roman numerals I-VI with serotypes I, II and IV subclassified into types a and b based on differences in the sugar composition of the polysaccharide chains which contribute to the structure of the lipopolysaccharide cell wall (Wetzler, 1965; Aleksic *et al.*, 1991). Some authors do not distinguish subtypes Ia and b and it is evident that the Japanese work (Tsubokura *et al.*, 1984) proposing a new scheme of serotype description along with the addition of new serogroups has not been widely accepted (Alesksic & Bockermelt, 1984; Alesksic *et al.*, 1991). *Yersinia pestis* has only one recognised serotype and is not generally subclassified although there are variants which have a similar biochemical profile to that of *Y. pseudotuberculosis* (Bercovier & Mollaret, 1984). One of the main distinguishing features of the two species is the lack of urease activity in 99% of *Y. pestis* isolates and lack of motility at 28 °C whereas most *Y. pseudotuberculosis* isolates are urease positive and are motile at 28 °C although not at 37 °C (Carter, 1982). However, recent work has identified several urease negative strains of *Y. pseudotuberculosis* during field studies (Nanni *et al.*, 1991; Iannibelli *et al.*, 1991).

Yersinia enterocolitica is a fairly heterogeneous species which has stimulated a lot of interest in recent years due to public health concerns following several outbreaks of food poisoning (Black *et al.*, 1978; Fukushima *et al.*, 1984) and contamination of blood products (Jones *et al.*, 1993). The species is subdivided into 5 biotypes on the basis of biochemical tests and these are further subdivided into serotypes. Unlike *Y. pseudotuberculosis* the majority of these are not pathogenic although some may be under

certain circumstances (Black *et al.*, 1978; Asakawa *et al.*, 1979; Robins-Browne *et al.*, 1979; Robins-Browne & Prpic, 1985). Several strains of *Y. enterocolitica* have been isolated from faecal samples of birds and mammals although only some serotypes have been implicated in enteric disease (Mair, 1973; Mair & Fox, 1986). Pyrazinamidase activity appears to be a useful biochemical test to distinguish between pathogenic and non-pathogenic strains of *Y. enterocolitica* with the latter showing a positive reaction (Farmer *et al.*, 1992).

Many non-pathogenic strains of *Y. enterocolitica* are abundant in the environment and some of those which were previously classified as '*Yersinia enterocolitica*-like' organisms are now recognised as separate species (Brenner *et al.*, 1980).

Yersinia frederiksenii was officially recognised as a distinct species in 1980 (Ursing *et al.*, 1980) and named after the Danish microbiologist Wilhelm Frederiksen. The biochemical characteristics of this and the other commonly recognised species are outlined in Feeley (1981) and Bercovier & Mollaret (1984). *Yersinia intermedia* was recognised as a separate species in 1980 (Brenner *et al.*, 1980) and *Y. kristensenii* in 1981 (Bercovier *et al.*, 1980). *Yersinia aldovae*, *Y. rhodiae* and other species are also recognised (Bercovier *et al.*, 1978; Wauters *et al.*, 1988., Dolina & Peduzzi, 1993). All are commonly isolated from soil, water, animal and bird faeces and have similar cultural characteristics to *Y. enterocolitica* (Botzler *et al.*, 1968, 1974; Botzler, 1987; Gilmour & Walker, 1988). *Yersinia ruckeri* is the causal agent of red mouth disease in fish (Ewing *et al.*, 1978). This species appears to be less closely related to *Y. enterocolitica* as judged by DNA analysis (Bervovier & Mollaret, 1984). The species referred to as *Y. philomiragia* is possibly not correctly placed in the genus *Yersinia* although it does have a morphological resemblance and some degree of DNA relatedness to *Y. pestis* (Ritter & Gerloff, 1966). It is interesting to note that the DNA relatedness among yersiniaes is 40% or higher except for *Y. ruckeri* which is at most 38% related to other yersiniaes. The DNA of *Y. ruckeri* strains have been shown to be 30% related to *Serratia* species (Ewing *et al.*, 1978). Because of the phenotypic differences between *Y. ruckeri* and other yersiniaes it may eventually constitute a new genus (Bercovier & Mollaret, 1984). It is probably significant that many of the surveys which have been conducted to isolate yersiniaes from a range of environmental and animal sources recognise a certain percentage of untypable '*Yersinia enterocolitica*-like' species (Gilmour & Walker, 1988;

Nanni *et al.*, 1991; Ianabelli *et al.*, 1991). Further phylogenetic studies may help to resolve some of the problems in the classification of presumptive yersiniae but at the present time there does not appear to be a uniform nomenclature reported in the literature.

1-2 Isolation techniques and 'in vitro' characteristics

Isolation of yersiniae from non-contaminated samples, such as blood and lymph nodes, is relatively easy and can be achieved using blood or nutrient agar incubated for 48 hr at 28 °C, or 24 hr at 37 °C followed by 24 hr at room temperature (Bercovier & Mollaret, 1984). A small colony diameter differentiates yersiniae from all other *Enterobacteriaceae*. After incubation for 24-30 hr *Y. pestis* forms minute colonies (0.1 mm) that can barely be discerned with the naked eye. All other yersiniae grown on nutrient agar at 25-37 °C for 24 hr produce visible colonies which reach a diameter of 1.0-1.5 mm at 24 hr and enlarge to 2.0-3.0 mm diameter by 48 hr. When cultured for 48 hr all species dissociate into small (0.5 mm) and large (2.0 mm) colonies. This phenomenon depends on the media used (Brenner, 1981; Feeley, 1981). *Yersinia pestis* and *Y. pseudotuberculosis* give variable growth responses on McConkey agar while all other species grow well on this medium, with colonies reaching a similar size to that observed on nutrient agar. On Salmonella-Shigella agar incubated at 25 °C *Y. pestis* hardly grows at all, whereas all the other species produce pin-point colonies in 24-30 hr. When incubated on this medium at 37 °C, *Y. enterocolitica* is only partially inhibited, whereas all other species are severely inhibited (Bercovier & Mollaret, 1984). Recovery of yersiniae from contaminated samples is more difficult but the chances of successful isolation can be improved by various cold enrichment techniques (Mair & Fox, 1986; Lynch, 1986). A variety of selective media have also been developed for this purpose (Schiemann, 1979) including Cefsulodin-Irgasan-Novobiocin (CIN) agar which has been widely used in human diagnostics especially for the recovery of yersiniae from faecal samples (Schiemann, 1980). Yersiniae produce characteristic pinpoint, dark red colonies when cultured on this media at 28 °C for 24 hours. At 48 hours these colonies develop a bull's-eye appearance. There is some variation between species in appearance and some species are more inhibited than others on this medium (Schiemann, 1980; Fukushima & Gomyoda, 1986). The use of a period of cold enrichment in phosphate buffered saline (PBS) or a broth medium prior to plating on to this selective medium

has become widely accepted and gives a good isolation rate for most yersiniae of interest when compared with other methods (Aleksic & Brokermekt, 1990; Ianabelli *et al.*, 1991). Other faecal and environmental bacterial species may appear similar at 24 hours but not at 48 hours, except some species of *Serratia* and *Citrobacter* (Devenish & Schiemann, 1981; Schiemann, 1982).

There is a fair degree of variability in the biochemical profile of some species and sugar tests are generally left for 5-7 days before they are considered negative (Feeley, 1981; Bisset, 1981). *Yersinia pseudotuberculosis* serotype III has several rhamnose-negative strains and these are often found in clinical isolates. Strains belonging to serotype IV are citrate positive and malonate positive. Up to 5% of *Y. pseudotuberculosis* strains are reported to produce acid from adonitol (Bercovier & Mollaret, 1984). Historically, motility has been used to classify yersiniae, this probably reflects the importance of distinguishing *Y. pestis* from other *Yersinia* species. All yersiniae are non-motile when incubated at 37 °C but are motile at 22-29 °C except *Y. pestis* which is never motile (Carter, 1982). Fresh isolates of *Y. pseudotuberculosis* and *Y. enterocolitica* may require a few subcultures to express their motility. Motile cells have 2-15 peritrichous flagellae characterised by a long wavelength (Nilehn, 1969). Temperature has an important effect on the expression of virulence characteristics in yersiniae and is an important factor in the epidemiology of disease caused by these species in humans and in animals.

Yersinia pestis and *Y. pseudotuberculosis* tolerate a pH range of 5.0-9.6, while other yersiniae can grow in a pH range of 4.0-10.0. The optimum pH range for all species is 7.2-7.4. The possession of a lipopolysaccharide coat allows yersiniae to survive passage through the acidic environment of the stomach following ingestion (Wetzler, 1965)

1-3 Antigenic structure

Bacteria may be differentiated on the basis of antigens which may be either a component of the cell wall or a product of the cell. Secreted protein antigens may only be present under certain conditions of growth and are often associated with virulence (Thal, 1973).

The temperature of culture is especially important when examining virulence

characteristics and antigenicity of yersiniae (Wetzler, 1965). The antigenic structure of some of the yersiniae is complex, but some antigens are shared by *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. The common enterobacterial antigen, which is a 65 Kb protein, has been found in all species so far investigated (LeMinor *et al.*, 1972; Wauters, 1980, 1981).

The somatic antigen of *Y. pestis* is rough (R-antigen) and therefore no serogroups have been described in this species. This R-antigen is also present in *Y. pseudotuberculosis* (Thal & Knapp, 1971). In addition *Y. pestis* and *Y. pseudotuberculosis* share at least 11 out of the 18 antigens described by Lawton *et al.* (1960). The fraction 1 envelope antigen (F1) of *Y. pestis* is best produced when cultures are incubated at 37 °C on protein-rich media (Fox & Higuchi, 1958). This antigen is heat-labile, water-soluble and may be further divided into a carbohydrate protein (F1A) and a carbohydrate-free protein (F1B). Passive haemagglutination with F1 antigen has been used for serological surveys in plague foci. The presence of this antigen has also been demonstrated in *Y. pseudotuberculosis* (Quan *et al.*, 1965). V & W antigens were identified when virulent *Y. pestis* strains were cultured at 37 °C and were found to be associated with the possession of a 45Mda plasmid (Ferber & Brubaker, 1981; Ben Gurion & Scafferman, 1981). In 1980 Gemski *et al.* described the production of V and W plasmid-mediated antigens in *Y. pseudotuberculosis* and *Y. enterocolitica*.

The antigenic scheme for *Y. pseudotuberculosis* was well described by Wetzler (1965) and more recently by Aleksic *et al.* (1991). Thal & Knapp (1971) described an antigenic scheme for *Y. pseudotuberculosis* which comprised 6 serologically different groups based on the thermostable polysaccharide antigens (I-VI) with 16 subgroups (A, B, 2 to 15). Five serological groupings (a to e) can be distinguished on the basis of thermolabile flagellar H antigens. Antigenic relationships have been demonstrated between *Y. pseudotuberculosis* (serogroups II, IV, IVA & VI) and the following organisms: *Salmonella* serogroups B & D, *E. coli* serogroups 017, 055 and 077, and *Enterobacter cloacae* (Mair & Fox, 1986). Wauters *et al.* (1972) described 34 different O antigen and 20 H antigen serogroups in *Y. enterocolitica*. This classification included some serogroups belonging to *Y. intermedia* (017) and *Y. kristensenii* (011, 012, 028). Although not totally specific, these antigens are useful epidemiological markers. Cross reactions also occur between *Y. enterocolitica* serogroup 09 and *Brucella* species (Hurvell &

Lindberg, 1973), and between *Y. enterocolitica* serogroup 012 and *Salmonella* factor 047 (Le Minor *et al.*, 1972; Hurvell & Lindberg, 1973).

1-4 Diagnosis

In humans there has been much interest in developing diagnostic techniques that will allow early identification of infections with yersiniae especially with the risk of blood borne transmission (Ulyatt *et al.*, 1991; Jones *et al.*, 1993; Pietersz *et al.*, 1993). Mair (1968) outlined the basic laboratory procedures for the diagnosis of pseudotuberculosis from clinical cases of the disease. Due to the difficulty in isolating *Y. pseudotuberculosis* from faeces, various serological assays have been developed to assist with diagnosis (Gripenberg *et al.*, 1979; Larson *et al.*, 1985; Kaneko & Manfarne, 1989; Granfors *et al.*, 1989; Stahlberg *et al.*, 1987; Kaneko & Maruyama, 1990). Recent advances have resulted in fairly specific and highly sensitive serological tests for human and animal samples (Herrlinger *et al.*, 1981) although the majority are based on work with *Y. enterocolitica* (Granfors *et al.*, 1981; Hoogkamp-Korstanje & de Koning, 1990; Paerregaard *et al.*, 1991). There has been much debate on the specificity of some immunoassays due to the degree of cross-reaction possible between *Yersinia* spp and other Gram negative bacteria (Corbel, 1979; Bottone & Sheenan, 1983; Appelmelk *et al.*, 1985; Granfors *et al.*, 1988). However, there is a general acceptance that immunoassays such as the enzyme linked immunosorbant assay (ELISA), are valuable research tools to study the pathogenesis of experimental *Yersinia* infection (Heesemann *et al.*, 1988; Hanski *et al.*, 1991) and are useful as a diagnostic tool even though allowance has to be made for possible cross-reactions (Vesikari *et al.*, 1980; Ogata *et al.*, 1987; Fernandez-Lagos *et al.*, 1991; Chart *et al.*, 1991). Combined immunoassays using the serotype-specific lipopolysaccharide (LPS) and the species-specific outer membrane proteins (YOPs) have been used to detect antibodies to virulent yersiniae in infected humans (Fernandez-Lagos *et al.*, 1991) and livestock (Robins-Browne *et al.*, 1993). There have been no serological studies reported on yersiniosis in birds although similar ELISA techniques have been used to screen poultry flocks for infections with salmonellae (Hassan *et al.*, 1990; Hassan *et al.*, 1991; Brigmon *et al.*, 1992; Barrow, 1992).

Sample collection for large scale serological testing of mammals has been made easier

with the use of filter discs (Hamblin & Hedger, 1982; McLean & Hilbank, 1989). These discs have also been used for screening poultry for antibody to salmonellae and other organisms (Bouzoubaa *et al.*, 1992; Minga & Wray, 1992). There has been a lot of work done on the serological diagnosis of some diseases in wild animals including those caused by *Y. pseudotuberculosis* in wild deer (Wetzler, 1965) and *Y. pestis* in various wild animal populations (Gordon *et al.*, 1979; Clover *et al.*, 1989; Williams, 1990). In contrast, although there have been some serological studies done on viral diseases in wild birds (Palmer & Trainer, 1969; Humphery-Smith *et al.*, 1991) these have been limited by the practical problems associated with sample collection. It is hoped that future improvements in diagnostic techniques will allow studies of the health status of free-living animal populations in general.

1-5 Epidemiology

Yersiniosis in mammals and birds is generally caused by *Y. pseudotuberculosis* or *Y. enterocolitica*. It is rarely caused by other species of *Yersinia* except in individual cases where the host is severely debilitated (Robins-Browne *et al.*, 1981). Yersiniosis due to *Y. pseudotuberculosis* tends to be a disease which occurs in the cooler months of the year in populations or individuals under nutritional stress, management stress or with concurrent disease (Obwolo, 1976; Baskin *et al.*, 1977; Obwolo, 1980; Baskin, 1980; Fiennes, 1982; Parsons, 1991). The clinical signs of yersiniosis due to either of these species are very similar in birds and livestock but not in human subjects (Blackmore & Humble, 1983). *Yersinia pseudotuberculosis* (Mackintosh & Henderson, 1984) and *Y. enterocolitica* (Hacking & Sileo, 1974; Kapperud & Olsvik, 1982) have also been isolated from the faeces of apparently healthy birds and mammals. It is generally recognised that yersiniosis is a disease which requires a predisposing factor and control should be based on avoidance of poor management (over crowding, poor ventilation, damp sheds), concurrent disease (parasitism, liver disease, nutritional imbalance), inappropriate feeding regimes and inappropriate changes in intestinal flora following antibiotic therapy (Obwolo, 1976; Fiennes, 1966; Welsh *et al.*, 1992). Potentially pathogenic and environmental species of *Yersinia* are probably transmitted between different species of host through contamination of feed and water supplies (Appelby, 1962; Baskin, 1980). As in other species, evolutionary adaptation to various modes of transmission and the substantial changes between the external environment and that inside the host is an

important factor in the development of phenotypic expression by species of *Yersinia* (Cornelis, 1992; Litwin & Calderwood, 1993).

All species of *Yersinia* grow at temperatures of 4-42 °C with an optimum temperature of 28-29 °C. *Yersinia pseudotuberculosis* is able to withstand freezing and appears to have a selective advantage when stored at refrigeration temperatures over a prolonged period and has been associated with cases of food poisoning following consumption of chilled products (Black *et al.*, 1978; Mair & Fox, 1986; Fukushima *et al.*, 1984). This characteristic may also explain why this species is more commonly isolated in the environment of temperate regions especially during the winter months (Barre *et al.*, 1979; Mackintosh & Henderson, 1984). *Yersinia pestis* also survives well in the environment although it is now more frequently isolated from animal and environmental sources in the tropical and subtropical regions of the world (Hubbert *et al.*, 1966; Epstein, 1991). It has been postulated that the reason for this present distribution was due to the appearance of a variant species, *Y. pseudotuberculosis*, in cooler climates which provided some degree of immunity against *Y. pestis* in rodent populations and consequently prevented the further spread of the plague (Devignat, 1953; Mollaret, 1987; Ell, 1984). Although this argument has some validity considering the close DNA homology between these two species and the number of shared antigens it is considered by some (Bercovier *et al.*, 1980) that if the two are the same species, then *Y. pestis* is a variant of *Y. pseudotuberculosis*.

Disease due to *Y. pestis* has been reported in people and in both wild and domestic mammals in the United States (Hubbert *et al.*, 1966; Olsen, 1970; Eidson *et al.*, 1988) and in people in many Asian and African countries (Ell, 1984; Kilonzo *et al.*, 1993). In wildlife, the disease is called Sylvatic plague reflecting, not only the epidemiology of the disease, but also a political reluctance to acknowledge the presence of the disease and the historical associations of vermin and squalid living conditions with the bubonic plague (Rosser, 1987; Epstein, 1991). The spread of the disease has been constant in some areas and research efforts are still directed towards techniques for rapid diagnosis and the development of new vaccines (Wake *et al.*, 1978; Wake *et al.*, 1983; Alonso *et al.*, 1980).

Unlike other species of *Yersinia*, *Y. pestis* has adapted to a mode of transmission which

relies on the maintenance of infection in reservoir rodent populations and transmission from rodent to rodent via the rodent flea (Hubbert *et al.*, 1966; Wake *et al.*, 1970). Transmission to human subjects occurs by accident when there is crowding in rodent infested areas (Anon, 1907). Despite the fact that *Y. pestis* is highly virulent, with infection often being followed by the death of the host, plague is maintained because transmission between individuals, either by infected fleas or by aerosol, is so efficient. This differs from enteric bacteria which are often facultative pathogens and tend to cause milder disease allowing the host to live and continue to shed organisms into the environment to infect other potential hosts (Robins-Browne *et al.*, 1987; Robins-Browne & Hartland, 1991).

Following reclassification of *Y. pseudotuberculosis* and *Y. pestis* into the genus *Yersinia* and the recognition of *Y. enterocolitica* as a potential pathogen, there has been a lot of interest in determining the epidemiology and pathogenesis of infection caused by these species (Asakawa *et al.*, 1979; Barton, 1992). Many of the *Enterobacteriaceae* colonise the intestine of healthy mammals and birds and some are potentially pathogenic. *Yersinia enterocolitica* is often associated with diarrhoea following enteric colonisation and proliferation (Robins-Browne *et al.*, 1985; Kaneko *et al.*, 1991). *Yersinia pseudotuberculosis* is usually transmitted by the faecal-oral route but is also thought to be transferred through skin abrasions and via inhalation (Wetzler, 1971). Several researchers have investigated whether or not *Y. pseudotuberculosis* can be transmitted by an arthropod vector (Fukushima *et al.*, 1979). Although this has been reproduced on a few occasions it is unlikely that this is a common method of spread (Wetzler, 1965; Wetzler, 1971)).

1-6 Wildlife reservoirs of infection

In the context of this thesis a 'wildlife reservoir' is defined as a population of wild animals which is able to maintain an infectious organism through successive generations without the need for reinfection from an external source (Schurrenberger *et al.*, 1987). This definition is applicable to a range of host-pathogen interactions at the population level. The prevalence of the pathogen in a 'reservoir population' will depend on the population biology of the host, the virulence of the pathogen and the mode of transmission of the disease. For example, rodents are considered to be an important

'reservoir' for *Y. pestis* in plague foci. Infection with *Yersinia pestis* usually has a fatal outcome for individuals in a rodent population. However, even when the disease results in a high mortality rate in one rodent population, the organism can be maintained in the sylvatic cycle via an arthropod vector such as the flea. The flea is able to transmit *Y. pestis* from one rodent to another individual within the same population or to an individual in another population of susceptible hosts (Olsen, 1970; Ell, 1984). In contrast, *Y. enterocolitica* is less virulent and is rarely fatal to the host. It is more frequently associated with enteric disease resulting in malaise and diarrhoea (Slee *et al.*, 1988; Slee & Button, 1990; Robins-Browne & Hartland, 1991). Enteric pathogens such as *Y. enterocolitica* are therefore readily transmitted from host to host by the faecal-oral route via contaminated feed or water (Mair, 1973; Mackintosh, 1993). In most enteric disease, therefore, it would be expected that the prevalence of the organism in the 'reservoir population' would be high (Fukushima *et al.*, 1984; Williams *et al.*, 1978).

Yersinia pseudotuberculosis appears to be intermediate in virulence. During systemic infection, *Y. pseudotuberculosis* shares characteristics common to *Y. pestis* with localisation in regional lymph nodes and proliferation in the spleen followed by rapid dissemination throughout the body. In enteric disease caused by *Y. pseudotuberculosis*, infection is usually restricted to a localised mesenteric adenitis although in some species it is associated with enteric disease similar to that caused by *Y. enterocolitica* (Tobin *et al.*, 1988). Pathogenic organisms adapted to a specific mode of transmission, such as *Y. pestis* which uses an arthropod vector, presumably undergo a high level of selection pressure which finally results in the expression of a very limited range of phenotypic characteristics compared with the broader range of phenotypes seen in other yersiniae many of which are facultative pathogens with the ability to survive for long periods in the environment (Kapperud, 1977; 1981). In this regard *Y. pseudotuberculosis* is a much more diverse organism than *Y. pestis* despite the fact that these two organisms have similar genotypes (Bercovier *et al.*, 1980).

Although many epidemiological surveys have identified yersiniae in soil and water as well as in the faeces of healthy mammals and birds (Hamasaki *et al.*, 1989; Fukushima & Gomyoda, 1991; Fukushima *et al.*, 1991), it would be unwise to assume that the biological cycle of all species in the genus is the same (Kaneuchi *et al.*, 1989). Surveys of the faecal flora of wild mammals and birds (Kawaoka *et al.*, 1984; Kapperud *et al.*,

1981; Kapperud & Olsvic, 1982; Kapperud & Rosef, 1983; Mackintosh & Henderson, 1984; Hacking & Sileo, 1984; Kato *et al.*, 1985; Tanaka *et al.*, 1987; Levre *et al.*, 1989; Hamasaki *et al.*, 1989; Kaneuchi *et al.*, 1989; Fukushima *et al.*, 1990; Fukushima & Gomyoda, 1991; Iannibelli *et al.*, 1991) indicate that many species of environmental and potentially pathogenic yersiniae are commonly isolated from the faeces of healthy animals. Similar studies on the faecal flora of livestock have frequently identified yersiniae (Hodges & Woods, 1974; Hodges & Carman, 1985; Hodges *et al.*, 1984; Bullians, 1987; Henderson & Hemmington, 1983; Henderson, 1984). The significance of isolating potentially pathogenic yersiniae from the faeces of apparently healthy animals is difficult to determine although it has led several authors to conclude that wildlife are reservoirs for *Y. pseudotuberculosis* (Clapham, 1953; Mair, 1973; Fukushima *et al.*, 1987, 1988; Fukushima *et al.*, 1991). It has been suggested that captive animals are infected following the contamination of feed or water with the faeces of wild rodents and birds (Clapham, 1953; Patterson & Cook, 1963; Welsh *et al.*, 1992). *Yersinia pseudotuberculosis* is generally considered to be more prevalent in the environment in the cool wet months of the year (Mair, 1973; Borst *et al.*, 1977; Hamasaki *et al.*, 1989; Hacking & Sileo, 1984), the degree to which this is true for other species is less clear (Levre *et al.*, 1989; Kaneuchi *et al.*, 1989).

In birds, *Y. pseudotuberculosis* is generally not associated with diarrhoea and, as a consequence, faecal shedding is limited except in severe clinical disease (Stovell, 1963). Despite the association of pseudotuberculosis in wild birds with disease in other species (Pullar, 1932; Patterson & Cook, 1963) there has not been any real evidence to suggest that wild birds are a true reservoir for the organism (Levre *et al.*, 1989; Slee, 1993). In many recent surveys the prevalence of this bacterium in the faeces of wild birds has been found to be low (Brittingham *et al.*, 1985; Hamasaki *et al.*, 1989; Iannibelli *et al.*, 1991; Fukushima & Gomyoda, 1991). However, faecal surveys may give an underestimate of the prevalence of infection due to the possibility of intermittent shedding which would not be detected in a single sample, also, the sensitivity of bacteriological methods may be limited due to over growth by more prevalent enteric flora (Barrow, 1992). Serological surveys have been suggested as a more accurate method of determining the prevalence of infection in wild populations and this approach has been utilised in epidemiological surveys of populations in plague foci (Quan *et al.*, 1979).

Yersinia enterocolitica has frequently been isolated from a range of wild and domestic species although pigs are the most common carriers of human pathogenic strains (Fukushima *et al.*, 1983, 1984). It was initially reported that wild birds did not carry *Y. enterocolitica* but more recently a number of isolations have been made (Kapperud & Olsvik, 1982; Ianibelli *et al.*, 1991). Many of the yersiniae isolated from avian sources are biochemically variable and often untypable '*Yersinia enterocolitica*-like' species (Kapperud, 1981; Kapperud *et al.*, 1981; Kapperud & Olsvik, 1982; Iannibelli *et al.*, 1991).

Although *Y. pseudotuberculosis* has often been reviewed alongside *Y. enterocolitica* in the literature it does not appear to conform to what we might expect of a typical 'enteric pathogen' because in people and some other species it is rarely associated with diarrhoea (Mair & Fox, 1986). As a consequence of this it is important to determine the nature and extent of species differences in the susceptibility to and pathogenesis of infection with *Y. pseudotuberculosis* in order to identify which species of mammal or bird, if any, are more likely to meet the criteria required to be a 'reservoir' of infection for this potential pathogen.

1-7 Pseudotuberculosis; the disease

1-7-1 Disease and virulence factors

Disease is generally the result of an imbalance between the host and the pathogen (Smith, 1993). A consideration of the 'triad of disease' is applicable to most bacterial diseases in which the host, the environment and the pathogen interact by way of a complex web of interdependent variables. This could be considered especially relevant in relation to facultative pathogens such as *Y. pseudotuberculosis* which has been implicated as the causative agent in severe systemic infection in a number of host species but is also associated with mild mesenteric adenitis in some hosts, diarrhoea in others and may also be found in faecal samples of clinically normal individuals (Henderson, 1983, 1984). Any factor which favours the survival and multiplication of the pathogen at the expense of the host, or which debilitates the host in some way will tip the balance in favour of the pathogen.

Pseudotuberculosis tends to occur in the cool wet months of the year (Parsons, 1991). Under these conditions the pathogen is more prevalent in the environment (Barre *et al.*, 1979; Mackintosh & Henderson, 1984), surviving well in water and soil when it is wet and cold (Mair, 1973; Barre *et al.*, 1979). The host is often under a degree nutritional stress, fed abrasive feed or has a lowered body temperature if housing or shelter are scarce (Mackintosh, 1988; Mackintosh *et al.*, 1991). A larger dose of organisms coupled with poor immune capacity due to nutritional imbalance and a damaged gut may allow *Y. pseudotuberculosis* to proliferate at the expense of the host. This is thought to explain many of the seasonal outbreaks of the disease in farmed weaner deer in New Zealand (Mackintosh & Henderson, 1984; Henderson, 1983), and in aviary birds (Welsh *et al.*, 1992; Parsons, 1991) and wildlife (Wetzler, 1971). Disease caused by *Y. pseudotuberculosis* is dependent on the number of organisms ingested and their virulence (Wake *et al.*, 1970; Wake, 1984). Virulent yersiniae possess a common virulence plasmid with a molecular weight of 42-48Mda. Most authors consider that the presence of this plasmid is essential for virulence but one report relates a case of pseudotuberculosis in a young boy in which the *Y. pseudotuberculosis* isolate did not possess a virulence plasmid (Fukushima *et al.*, 1991). Although it is possible that the isolate had lost the plasmid on subculture there is some evidence to suggest that chromosomal mediated virulence factors have the major determining effect on the virulence potential of yersiniae (Simonet *et al.*, 1984; Carniel *et al.*, 1992). The 'ail' gene and the 'inv' gene are both associated with the ability of enteropathogenic strains of *Y. enterocolitica* to invade epithelial cells (Auliso *et al.*, 1983; Robins-Browne *et al.*, 1987; Isberg *et al.*, 1987, 1988). In addition there is recent evidence to suggest that the virulence of *Y. enterocolitica* and *Y. pseudotuberculosis* is regulated *in vivo* by a gene associated with iron acquisition (Heeseman *et al.*, 1993; Hagg *et al.*, 1993). This will be considered in more detail later in the review.

1-7-2 The disease

The term 'pseudotuberculosis' has been applied descriptively to a variety of diseases which have 'tuberculosis-like' lesions. Such lesions have been seen commonly in the liver and spleen of wild mammals and birds with chronic granulomatous infections associated with a variety of micro-organisms (Keymer, 1980), including *E. coli* and *Corynebacterium sp.* and, in birds, *Aspergillus spp* fungi. In many cases lesions were not

cultured. The resemblance of the gross lesions to those caused by *Mycobacteria* prompted the use of the term 'pseudotuberculosis' following the identification of the disease by Mallassez & Vignal (1885) who infected guinea pigs with a strain of *Y. pseudotuberculosis* isolated from a child.

Although the non-specific use of the term 'pseudotuberculosis' leads to some confusion when reviewing the very early literature (Wetzler, 1971) the disease caused by *Y. pseudotuberculosis* has been well recorded in recent times (Baskin, 1980; Mackintosh & Henderson, 1984; Slee & Button, 1990; Slee & Skilbeck, 1992, Slee *et al.*, 1988). In rare cases, *Y. enterocolitica* can produce similar lesions (Baskin, 1980) but the variety of diseases and the subsequent autoimmune complications which occasionally follow systemic infection with *Y. enterocolitica* (Rabson *et al.*, 1975; Robins-Browne *et al.*, 1979; Robins-Browne *et al.*, 1987), will not be considered in detail. For further review of *Y. enterocolitica* see Robins-Browne *et al.* (1981) and Swaminathan *et al.* (1982).

Yersinia pseudotuberculosis has been associated with large outbreaks of enteric disease in deer, especially in weaner groups in the autumn (Chapman & Chapman, 1979; Hodges & Woods, 1984; Henderson, 1984) and usually following some identifiable period of 'stress' such as poor feed sources, cold weather or recent transport (Mackintosh, 1988). Outbreaks of pseudotuberculosis also occur in zoo primates (Mair *et al.*, 1970; Hirai *et al.*, 1974; Chang *et al.*, 1980; Plesker & Claros, 1992) ungulates (Baskin, 1980; Wetzler, 1981; Blake *et al.*, 1991) and some rodents also appear susceptible (Kaneko *et al.*, 1978, 1979; Baskin *et al.*, 1977). There are a few reports of the disease in felids (Mair, 1967; O'Sullivan *et al.*, 1976; Spearman *et al.*, 1979) and canids (Fantasia *et al.*, 1993) and numerous reports of the disease in various species of domestic ungulate (Watson & Hunter, 1960; Mair & Harbourne, 1963; Langford, 1969; Mair, 1974; Baskin *et al.*, 1977; De Barcellos & De Castro, 1981; Hanssen, 1982; Karbe & Erickson, 1984; Tsubokura *et al.*, 1984; Griffin, 1987; Callinan *et al.*, 1988; Jerret & Slee, 1989; Brown & Davis, 1989; Jerret *et al.*, 1990; Slee & Button, 1990; Slee & Skilbeck, 1992; Macleod & Patterson, 1992; Hannam, 1993). The disease is an important cause of mortality in aviary birds (Jones, 1980; Keymer, 1980; Fiennes, 1982) and has been reported in many species of wild birds (Keymer, 1980; Fiennes, 1982; Reece *et al.*, 1991; Slee, 1993).

In all species, successful treatment is dependant on early diagnosis, the use of appropriate antibiotics and, in severe enteric cases, electrolytes. For birds, the provision of a warm quiet environment is essential for recovery and the course of action chosen may depend to some extent on the course of the disease (Wetzler, 1971). In some chronic cases antibiotic therapy may not be effective (Flammer, 1991). Generally it is preferable to identify the predisposing factors and implement prophylactic measures to avoid future outbreaks (Welsh *et al.*, 1992). The use of tetracyclines in the water during cool periods is probably not to be advised although this is reported to have been successful in some zoos (Joe Christman, Orana park, personal communication, 1991). The use of vaccines has been attempted in zoo animals (Parsons, 1991), especially primates, and in aviary birds (Zwart, personal communication, 1991) but as yet is not widely accepted. The recent development of a commercial vaccine for deer (Mackintosh *et al.*, 1991) and one for Rainbow trout, (Siwiki & Duvier, 1993) may lead to further developments in this area.

1-7-3 Public health significance

The disease in humans is usually self-limiting with localisation in the mesenteric lymph nodes following the oral route of infection (Knapp, 1958; Mair *et al.*, 1960; Hubbert *et al.*, 1972; Blackmore & Humble, 1987). Patients with liver disease such as cirrhosis or haemochromatosis, or those with thalassaemia or transfusion overload are more likely to develop systemic pseudotuberculosis if infected with the pathogen (Macauley *et al.*, 1967; Yamashiro *et al.*, 1971; Bradley & Skinner, 1974; Marlon *et al.*, 1975; Robins-Browne & Prpic 1985; Griffiths & Bullen, 1987). Diarrhoea may be associated with *Y. pseudotuberculosis* infection (Daniels, 1961; Tobin *et al.*, 1988), although it is not a typical feature of this condition in humans unlike infection with enteropathogenic forms of *Y. enterocolitica* (Toma & Lafleur, 1981; Leino *et al.*, 1987). Pseudotuberculosis in human patients, especially children, should always be considered in the differential diagnosis for appendicitis (Mair & Fox, 1986; Tertti *et al.*, 1989). It is thought that human infection with either *Y. pseudotuberculosis* or *Y. enterocolitica* may be acquired following the consumption of contaminated food or water (Hubbert *et al.*, 1972; Lassen, 1972; Fukushima *et al.*, 1984; Fukushima *et al.*, 1988). Pigs are considered the most common source of human strains of enteropathogenic *Y. enterocolitica* (Tsubokura *et al.*, 1970; 1984). Human pseudotuberculosis is possibly a result of direct contact with carrier

mammals or birds or from contact with environmental sources (Sanekato *et al.*, 1990). Infection with either organism is considered potentially zoonotic in origin (Hurvell, 1981; Grimes, 1987; Fukushima *et al.*, 1989; Fowler, 1989).

1-7-4 The disease in birds; historical overview

The first reports of pseudotuberculosis in birds were those of Zuern (1884) in canaries and those of Nocard (1885) in fowl. Following these initial reports, Muir (1898) published a review of the disease in birds outlining the colony morphology and biochemical characteristics of the aetiological agent and the resultant pathology in naturally occurring cases and in experimental infections. The birds used in the experimental challenge were predominantly passerines but the species used were not specified. Beaudette (1940) updated the early reviews on the subject and also outlined a case report in a blackbird. He described the variable gross and histological lesions found in different birds examined as well as the apparent variation in susceptibility when different species were challenged using a subcutaneous injection of the infectious agent. It was suggested that the domestic hen was relatively resistant to the development of disease when given a subcutaneous injection of *Y. pseudotuberculosis* whereas the pigeon and various passerine species died following the same proportional dose. Clinical disease did not occur in any of the species examined following oral challenge. However, care should be taken when interpreting experimental results reported in the early literature due to the changes in nomenclature and the difficulty in ensuring that the diseases outlined were actually due to *Y. pseudotuberculosis* (Wetzler, 1971). Stovell (1963) gave a comprehensive outline of avian pseudotuberculosis prior to this date in his thesis. He investigated the epidemiology and pathogenesis of pseudotuberculosis in canaries and concluded that canaries infect each other, that they are potential sources of disease for other species, including humans, and that those birds which developed caecal abscesses shed the largest number of organisms (Stovell, 1963). It is generally accepted that for disease to occur there must be predisposing factors which compromise the host's immune responses (Wetzler, 1971; Fiennes, 1982). The majority of recent published reports outline cases of the disease in private aviaries or zoological collections. These are either sporadic cases in individual birds which have concurrent diseases or outbreaks in aviary birds associated with changes in management, cold weather or overcrowding (Wetzler, 1971; Parsons,

1991; Welsh *et al.*, 1992). Most cases of the disease occur in the winter or spring months or following a period of wet cold weather (Mair, 1973, Stovell, 1963; Fiennes, 1982; Parsons, 1991; Slee & Skilbeck, 1992; Welsh *et al.*, 1992). There are several reports of disease caused by *Yersinia pseudotuberculosis* in wild birds. These reports described sporadic cases (Beaudette, 1940; Clapham, 1953; Patterson & Cook, 1963; Slee, 1993, Welsh *et al.*, 1992) and outbreaks of disease resulting in a high mortality (Pullar, 1932; Clarke & Locke, 1962; Mair, 1973).

1-7-5 The pathology and pathogenesis of pseudotuberculosis in birds and other species

The nature and extent of the immune response of the host is determined by 1) the genotype of the host and prior exposure to the pathogen and 2) the antigenic composition and virulence characteristics of the pathogen (Steward, 1989). The genotype of the host determines the major histocompatibility complex and associated differences in tissue type which are known to determine the susceptibility of certain individuals to post-infection complications associated with yersiniosis and other enteric diseases (Bouza *et al.*, 1980; Autenrieth *et al.*, 1991; Arscott *et al.*, 1992). The antigenic nature of the pathogen, which may alter during the course of infection, determines the relative extent of the humoral versus the cell-mediated response (Rook, 1989; Autenrieth *et al.*, 1991).

Yersinia pseudotuberculosis is a facultative intracellular pathogen with the ability to survive extracellularly in some situations. As outlined previously, the expression of YOPs and other plasmid-linked characteristics once within the host, is triggered by environmental signals such as an increase in temperature and a low intracellular calcium concentration (Portnoy *et al.*, 1981, 1984, Portnoy & Martinez, 1985; Rosquist *et al.*, 1988; Straley *et al.*, 1993;). Extracellularly, low iron levels stimulate the expression of other proteins *in vivo* in some strains of yersiniae (Carniel *et al.*, 1989, 1992; Brubaker, 1991; Heeseman *et al.*, 1993). Therefore, in the host, a combination of increased temperature and changes in nutrient availability stimulates a change in the metabolic function of *Y. pseudotuberculosis* and at the same time the release of protein antigens and endotoxin by this organism stimulates a cascade of events in the host species (Kawaoka *et al.*, 1983; Hanski *et al.*, 1991; Autenrieth *et al.*, 1992; Bliska *et al.*, 1993).

Antemortem diagnosis of pseudotuberculosis is difficult due to the non-specific clinical form of the disease (Baskin, 1980; Fiennes, 1982; Kormendy, 1988). The appearance and distribution of the gross lesions are not unique to this disease and even the histological findings may mimic those of other diseases. As a result, culture of affected individuals and/or lesions is essential to make an accurate diagnosis (Wetzler, 1971). It has been reported that the severity and nature of the disease is subject to considerable variation (Wetzler, 1971; Baskin, 1980). For most birds, death is observed within four days from the onset of the clinical signs but in less susceptible birds, such as the domestic hen, the disease may take a more protracted course, of eight to ten days (Muir, 1898; Stafseth, 1962; Wetzler, 1971). In peracute cases, birds may die without any apparent clinical signs and with no specific lesions except those associated with a peracute septicaemia (Wetzler, 1971). Some authors suggest that there are species variations in the form the disease takes (Baskin, 1980).

There has been some attempt to classify the various manifestations of pseudotuberculosis. Wetzler (1971) describes the two forms seen in birds as either acute septicaemia with no characteristic lesions, or the more chronic form in which there are miliary foci of necrosis in the visceral organs especially the spleen and the liver which are often enlarged. Baskin (1980) suggests that the chronic form is more typical in species or individuals which are less susceptible to the disease. Three categories of the disease are described in Baskin's paper 1) the 'peracute' form, 2) the 'subacute' form and 3) the 'chronic' form. In the 'subacute' disease described in birds, enteric lesions are absent or less common than those seen in the 'peracute' form reported in primates and some rodent species. The primary gross findings are disseminated yellow/ grey foci throughout the viscera including the spleen and liver, also the caecal tonsils, kidneys, lungs and skeletal muscle may be involved. Histologically these foci consist of central areas of necrosis with a large number of organisms and an inflammatory reaction with heterophils and occasional giant cells around the periphery. The cellular reaction is variable and, in more acute cases, there is little or no histological evidence of host response around bacterial colonies in the visceral organs (Baskin *et al.*, 1977). It is possible that the wide variation in both the extent and distribution of lesions in birds and mammals infected with *Y. pseudotuberculosis* is a reflection of concurrent disease processes which may influence the course of the infection (Mair, 1973; Keymer, 1980).

It is clear from the wealth of literature on the subject that, although it is not the only factor, iron availability has an important role to play in the outcome of many diseases including pseudotuberculosis (Rabson *et al.*, 1975; Bullen & Joyce, 1982; Robins-Browne & Prpic, 1985; Griffiths & Bullen, 1987; Bullen, 1987). Histological evidence of high levels of iron stored in the tissues and concurrent pseudotuberculosis with florid bacterial lesions in the liver and spleen of human patients, following either iron overload secondary to repeated blood transfusion or disruption of intestinal iron uptake regulatory mechanism, indicates that an alteration in iron metabolism is associated with overwhelming bacterial proliferation in the tissues (Rabson *et al.*, 1975). At the present time, however, there is no clear understanding of the nature or time scale of the mechanisms involved.

1-8 Iron and disease

1-8-1 Iron and the host (normal iron metabolism)

In the normal host, the amount of free iron available is limited, making iron acquisition by invading organisms extremely difficult (Litwin & Calderwood, 1993). The majority of the iron in mammalian or avian hosts is bound to protein carrier molecules in serum and protein complexes in cells. Most intracellular iron is found as haemoglobin, haem, ferritin and haemosiderin. The trace amounts of iron present extracellularly are bound by the high-affinity iron-binding proteins transferrin and lactoferrin (Griffiths & Bullen, 1987). Extracellular haemoglobin and haem have been found to serve as iron sources for many bacteria; however in healthy hosts, they are rapidly bound by two serum proteins, haptoglobin and hemopexin respectively, and are removed from the circulation (Wochner *et al.*, 1974; Eaton *et al.*, 1982). In the normal individual, serum iron-binding proteins are only partially saturated with iron which, in human subjects, is about 33%. The association constant of human transferrin is approximately 10^{36} and the amount of free iron in equilibrium with the proteins is about 10^{-18} M. As a result of this, although there is an abundance of iron present in body fluids, the amount of free iron in circulation is far too low to sustain bacterial growth (Bullen, Rogers & Griffiths, 1978; Bullen, 1981). There are few references describing the specific details of iron metabolism in avian species (Butler *et al.*, 1973) but it is thought that most of the general aspects of iron metabolism described for mammals are applicable to birds

(Lowenstine & Petrak, 1980).

Most of the iron in the body tissues is recycled efficiently through the reticuloendothelial system especially the spleen and the liver. Tissue macrophages have a receptor for transferrin and it is thought that this is important in the transfer of iron from the plasma to the tissues for storage. Iron presented to macrophages *in vitro* without the apoferritin or transferrin carrier may not be phagocytosed (Jones & Summerfield, 1982; Huebers *et al.*, 1988). Haemoglobin from red cells and myoglobin from tissue breakdown is degraded and the iron moiety utilized in tissue repair and red cell production. Absorption of exogenous iron from the diet is also tightly controlled and is regulated to supply the demands of the individual. However, it is well known that calcium in the diet can interfere with iron uptake in the normal individual and that vitamin C enhances iron uptake across the intestinal epithelium (Lynch & Cook, 1980).

In human cases of protein-calorie malnutrition it has been observed that the supplementation of infant diets with minerals including iron, prior to establishing normal protein levels may increase the amount of unbound iron in serum making it more available to potential pathogens (Farmer, 1976; Keusch, 1990). In neonates this has been linked to the development of severe gastroenteritis followed by systemic complications and death (Barry & Reeve, 1977; Becroft *et al.*, 1977).

As a result of these factors the whole diet as well as the protein status of the host must be considered when assessing the dietary iron requirements of a species or an individual (Chandra & Newberne, 1977; Chandra, 1980).

Serum iron binding proteins

The transferrins were discovered following the observation by Schade & Caroline (1944) that raw egg white was inhibitory to bacteria and that this inhibition appeared to be abolished by the addition of iron. Later these workers obtained the first transferrin preparation from a human plasma sample and demonstrated that this substance was also a microbial growth inhibitor (Schade & Caroline, 1946). In subsequent years there has been a large body of work in the area of transferrin biochemistry and physiology as outlined in various texts on the subject (Fletcher, 1989; Broxmeyer, 1989). Transferrins

are found in numerous vertebrate biological fluids and there appears to be little difference in the molecular structure between species (Planas *et al.*, 1961). In blood plasma the presence of transferrin is reflected by total iron (TI) and total iron-binding capacity (TIBC) measurements. These values are commonly used to assess iron status in human patients. In iron overload, TI is greater than or equal to TIBC. This may occur as a primary disorder, as with idiopathic haemochromatosis, or can be secondary resulting from repeated blood transfusions or haemolytic crises. In patients with concurrent liver disease and/or protein deficiency, serum transferrin levels may also be very low resulting in an elevated level of free serum iron. In well developed iron deficiency anaemia in humans, TI is less than one third of TIBC (Broxmeyer, 1989). Similar iron status profiles have been done for birds (Diez *et al.*, 1987) with the discovery that the exact values and relative ratios do differ between species (Lowenstine & Petrak, 1980). The early work of Planas *et al.* (1961) described serum transferrin in the chicken and the duck and noted that there were differences in the values obtained depending on the stage of the breeding cycle in the female bird.

Lactoferrin is another iron binding protein which has a high affinity for iron. Unlike transferrin, lactoferrin is able to bind iron at the low pH conditions associated with necrosis. In mammals polymorphonuclear leucocytes contain high quantities of lactoferrin in their granules (Bullen & Armstrong, 1979; Bullen, 1981). It is thought that degranulation of these cells in inflammatory processes effectively reduces the TI values. During infection, this, along with an elevated plasma TIBC reduces the availability of iron to micro-organisms (Litwin & Calderwood, 1993). Some lactoferrin is found in mammalian monocytes but none in lymphocytes (Bennet & Kokocinski, 1978). The role of lactoferrin in avian infection is less well documented (Powell, 1987). The interaction of lactoferrin with LPS and its role in the development of the hypoferraemia associated with infection has recently been reviewed by Cohen *et al.* (1992).

Intracellular iron binding proteins

Haemosiderin and ferritin are intracellular iron-storage proteins present in most animal tissues. Ferritin is water soluble and of large molecular weight. Haemosiderin is 35 % iron by weight and is concentrated in the lysosomes of cells and is thought to represent

insoluble ferritin aggregates (Mason & Taylor, 1978; Iancu, 1982; Aisen, 1988). Both proteins bind ferric iron which is responsible for the typical diffuse blue colouration observed when histological sections are stained with Prussian blue stain. The storage of excess iron is thought to be the primary function of these proteins (Halliday & Powell, 1988). The ferritin molecule consists of a polypeptide apoferritin portion and an iron hydroxide-phosphate micelle. It is thought that iron may account for 57% of the micellar weight of ferritin (Brock, 1989). In chickens it is thought that the ferritin/haemosiderin ratio in the liver is normally greater than in mammals but the significance of this is not known (Davis *et al.*, 1968). The biosynthesis of the apoferritin/ ferritin subunits is closely regulated by iron as it is observed that when an animal or cell culture is exposed to iron, apoferritin increases. Presumably, in malnutrition states, *de novo* synthesis of apoferritin and other proteins, including the plasma proteins, would be hindered resulting in an alteration in iron metabolism.

Certain iron-chelators are capable of extracting ferric iron directly from ferritin. These include various siderophores derived from bacterial species for example desferrioxamine derived from the actinomycete *Streptomyces pilosus*. This siderophore is available commercially under the trade name Desferal (Ciba Geigy Ltd, Basle, Switzerland) for the treatment of iron overload in humans especially those with idiopathic haemochromatosis (McLaren *et al.*, 1983) but must be used with caution in patients at risk of developing infection with *Y. enterocolitica* and possibly, *Y. pseudotuberculosis* (Robins-Browne *et al.*, 1987).

1-8-2 *In vivo* models

Retrospective studies of human patients with systemic bacterial diseases and concurrent iron overload lead to the hypothesis that an increased availability of iron predisposes the human host to the development of more severe disease (Marlon *et al.*, 1975; Jones *et al.*, 1977; Butzler *et al.*, 1978; Bullen, 1981; Melby *et al.*, 1982; Finkelstein *et al.*, 1983; Chiu *et al.*, 1986; de Sousa, 1989). However, using yersiniosis as an example, it is also apparent that, whereas altered iron metabolism is associated with an increased risk of systemic complications with *Y. pseudotuberculosis* and *Y. enterocolitica*, it is not actually associated with an increased prevalence of the disease (Robins-Browne *et al.*, 1987;

Chandra, 1990). Experimental models comparing the course of parenteral *Y. enterocolitica* and *Y. kristensenii* infection in iron-treated rodents and untreated controls found that iron-treated rodents developed more severe clinical disease than the controls (Robins-Browne & Prpic, 1985). It was also apparent that the relative effect was greater when mice were challenged with *Y. kristensenii* and non-pathogenic *Y. enterocolitica* than when pathogenic yersiniae were used (Robins-Browne *et al.*, 1991). Oral challenge of iron-treated mice with *Y. enterocolitica* resulted in diarrhoea and lethargy but no disease occurred when challenged orally with *Y. kristensenii*. These experiments indicated that the effect of iron on the course of disease was also determined by 1) the route of challenge and 2) other virulence characteristics of yersiniae. The interaction between iron availability in the host and bacterial virulence is complex and therefore the application of apparently simple concepts to the clinical situation is difficult (Sherman, 1990; Griffiths, 1993).

In mammals, the lowering of plasma iron levels associated with fever and the acute-phase immune response was termed nutritional immunity by Kochan (1973). Hill *et al.* (1977) demonstrated that, in chicks with fowl typhoid, iron metabolism was as seriously disturbed as Beisel *et al.* (1974) described in infectious conditions in mammals. Experimental work, using a variety of *in vivo* models, has shown that pretreatment of experimental mammals with parenteral iron dextran increases the severity of disease following bacterial challenge (Robins-Browne & Prpic, 1985; Bullen *et al.*, 1978). However, in the domestic fowl the evidence for this is contradictory leading to the belief that birds differ from mammalian species in regard to the role of iron in the development of bacterial disease (Smith *et al.*, 1977, 1978; Harry, 1979).

In acute systemic disease produced experimentally in chicks (*Gallus domesticus*) by *Salmonella gallinarum*, the injection of iron dextran, but not iron citrate, markedly reduced morbidity and mortality (Smith *et al.*, 1977). In untreated chickens the acute phase of the disease was associated with a moderate anaemia but increased survival was not thought to be associated with correction of this acute phase anaemia (Smith *et al.*, 1977). The higher survival rate of chicks treated with iron dextran was associated with the finding of fewer viable salmonellae in various tissues. A similar result was obtained in chicks challenged with *Mycobacterium avium* (Smith *et al.*, 1978) and *Escherichia coli* (Harry, 1979). It was postulated that infected chicks utilised the additional iron for

antibacterial activity within splenic and hepatic macrophages (Smith *et al.*, 1977). This suggests that the untreated chicks were deficient in iron required for these processes (Smith *et al.*, 1978). Whether or not this would be true for other avian species is unclear.

In another experiment, Hill *et al.* (1977) studied the absorption of labelled dietary iron from the intestine of two-week-old chickens infected with *Salmonella gallinarum*. They found that, during the six day period following infection, the absorption of labelled iron from the gut fell progressively to become eventually half of that in uninfected chicks. During infection there was also a shift in the distribution of the absorbed labelled iron from the liver to the spleen. This lead to the view that during infection there is a redistribution of iron from liver stores for use elsewhere in the body, possibly for erythropoiesis or cell-mediated immune function (Hill *et al.*, 1977).

In mammals hypoferraemia is associated with the acute phase inflammatory response which follows the release of cytokines and other mediators associated with endotoxin production in Gram negative infection (Bone, 1993). Butler *et al.* (1973) examined the role of endotoxin in the production of hypoferraemia in eight to twelve-week-old specific-pathogen-free chicks (SPF). They used an intravenous injection of an *Escherichia coli* (0111:B4) endotoxin and found that there was a rapid but transient hypoferraemia which was maximal after 12-16 hours, was dose dependant and was superimposed on a diurnal variation in the plasma iron level. Recovery was rapid following a single injection and was thought to be associated with a simultaneous increase in the activity of caeruloplasmin which is believed to promote the transference of iron from the reticuloendothelial and hepatic cells to the plasma (Butler *et al.*, 1973).

In addition to iron, Tufft *et al.* (1988) studied the effects of *E. coli* on copper and zinc metabolism in chicks. Acute infection in mammals results in a decrease in serum zinc and an increase in serum copper along with the transient hypoferraemia. It was found that both infection and endotoxin challenge resulted in an increase in serum and bursal copper in chicks along with a redistribution of iron with higher levels occurring in the spleen and a fall in serum iron. Serum zinc levels fell with an associated increase in zinc levels in the liver and spleen. Seven days post-infection, when chicks were recovering,

hepatic and splenic copper and splenic zinc were elevated. Hepatic iron decreased with recovery, whereas splenic iron continued to increase. Tufft *et al.* (1988) concluded that the changes in trace element kinetics observed in the acute phase of infection were, in part, associated with the effect of endotoxin, but that the later responses may be due to other aspects of infection.

In conclusion it should be noted that, although chickens have been described as being resistant to some of the acute and lethal effects of endotoxin (Congleton & Wagner, 1991; Errol *et al.*, 1993) small doses of bacterial endotoxins do result in a marked hypoferemia in chickens (Butler *et al.*, 1973). It is probable that changes in the iron metabolism of chickens, during infection, are mediated by the same factors which alter iron metabolism during infection in mammals (Kluger & Bullen, 1987).

1-8-3 Iron and bacteria (*in vitro*)

Of the many host factors which may trigger expression of different phenotypic characteristics in bacteria, temperature, pH and the availability of iron are some of the most frequently studied *in vitro* (Mellencamp *et al.*, 1981; Bolin *et al.*, 1984; Carniel *et al.*, 1987; Richards, 1989). Many bacteria produce low molecular weight iron chelators (siderophores) when grown under conditions of iron limitation (Neilands, 1981; Finkelstein *et al.*, 1983; Griffiths, 1983; Chart & Griffiths, 1985; Griffiths, 1987; Stuart *et al.*, 1986). Siderophores may enable the bacteria to compete with host proteins for the available iron *in vivo*. An essential part of the 'high affinity' iron uptake systems of some bacteria is the production of outer membrane protein receptors (Bagg & Neilands, 1987; Angerer *et al.*, 1992). These have been well characterised in *E. coli*, *Salmonella spp.* and *Shigella spp.* (Neilands, 1981).

Dho & Lafont (1984) studied the adhesive properties and iron uptake ability in *E. coli* in chicks and were able to demonstrate that the ability to grow in iron-limited conditions was strongly correlated with lethality. The latter property was measured by the ability of *E. coli* strains to grow on transferrin saturated media. In addition they noted that, of the *E. coli* strains tested, 52% of those lethal for chicks, but none of the nonlethal strains, possessed both adhesive and iron uptake abilities. This supports the hypothesis that, along with other chromosomal and plasmid-determined characteristics, the ability

to compete for, and utilise, available iron resources is an important virulence determinant (Chart & Griffiths, 1985).

Iron availability has been shown to have an important role in the phagocytic efficiency of polymorphonuclear leucocytes in mammals (Bullen, 1981; Bullen & Rogers, 1969; Bullen *et al.*, 1974) but, although much has been written on the antibacterial mechanisms of these cells, (Root & Cohen, 1981) there have been relatively few studies on the quantitative relationships between phagocytic cells and bacteria in different species. The outcome of any infection will ultimately depend on 1) the interaction of the bacteria with the specific and non-specific immune mechanisms in the extracellular environment and 2) the intracellular environment within the phagocytic cells. Immune mediated changes in the extracellular environment would include the production of serum opsonins by the host and extracellular nutrient depletion in response to endotoxin. In the intracellular environment bacteria would need to adapt to nutrient depletion, changes in pH and oxidation potential as well as the action of lysosomal enzymes. Bullen (1981) reviews these interactions in detail giving several examples based on *in vivo* and *in vitro* work on *Pseudomonas aeruginosa* infection in rabbits. It was found that the susceptibility of rabbits to this pathogen was increased when the iron-binding capacity of plasma transferrin was saturated. The lethal dose was reduced by 100-fold in rabbits under these conditions (Bullen, Ward & Wallis, 1974). Similar studies in mice showed that the LD₅₀ was reduced by 1,000 fold (Forsberg & Bullen, 1972). Complementary work by Eaton *et al.* (1982) illustrated that the enhanced growth of *E. coli* when haem iron availability was increased could be reversed by the addition of haptoglobin. This protein binds to free haem and makes it unavailable to most bacteria (Litwin & Calderwood, 1993). However, some virulent organisms such as *Yersinia pestis* have developed mechanisms to acquire haem iron from host proteins (Griffiths, 1983; Sikkema & Brubaker, 1987; Sikkema & Brubaker, 1989; Perry *et al.*, 1993).

There is evidence from *in vitro* work that lactoferrin itself is able to act as an antibacterial agent (Bullen, 1981). However, it is difficult to draw definite conclusions about the *in vivo* significance due to the problem of imitating exactly the conditions inside the intact cell where all the bactericidal systems operate simultaneously in a controlled environment (Hanski *et al.*, 1991). Work on *P. aeruginosa* sensitized with

homologous antibody, determined that the addition of iron did not prevent the bactericidal effect of rabbit polymorphonuclear cells (Bullen & Wallis, 1977). It was thought that this was due to the rapid phagocytosis of the bacteria in the presence of antibody, although the results may depend on the form of the iron used (Bullen & Armstrong, 1979). It is interesting to note that the addition of a ferritin-antiferritin complex appeared to reverse the bactericidal effect and stimulate intracellular growth while an apoferritin-antibody complex did not. To fully explore the role of lactoferrin in polymorphonuclear cells it would be necessary to saturate the iron-binding capacity in the cell but, despite some work done on this subject (Gladstone & Walton, 1970, 1971), it is difficult to be sure that the supplementary iron used was assimilated by the cells.

Work on the effect of supplementary iron on macrophage activity and iron metabolism indicates that the form of iron presented to the cells is very important. Macrophages have a transferrin receptor and iron is readily assimilated if it is presented as an apoferritin complex which would normally be the case *in vivo* (Morgan & Baker, 1988). Recent studies have examined the effect of supplementary iron on the *in vitro* bactericidal capacity of cultured macrophages and identified that the addition of a small amount of iron actually enhances the ability of macrophages to kill *Listeria spp.* but that, with an increased supplementation, this is reversed with increased intracellular survival and multiplication (Campbell, 1993). Similar experiments using cultured macrophages have indicated that the same is true *in vitro* for other bacterial species (Zink *et al.*, 1985; Dunlap *et al.*, 1993). What occurs *in vivo* is less easy to interpret.

There has been some work done on the interaction between mammalian macrophages and *Y. pseudotuberculosis* *in vitro* and *in vivo* (Wake & Morgan, 1986; Vessilnova *et al.*, 1992). In squirrels, it was found that *Y. pseudotuberculosis* serotype I was rapidly killed by peritoneal macrophages following experimental intraperitoneal challenge but that serotype III failed to stimulate an immune response and resulted in the death of the host (Vessilnova *et al.*, 1992). *In vitro* it was found that *Y. pseudotuberculosis* was able to survive intracellularly in phagocytic cells and that intracellular conditions of low calcium and an elevation of temperature stimulated the release of YOPs (Straley *et al.*, 1993). Much of the early *in vitro* work examining the effect of iron on the growth of bacteria, used *Y. pseudotuberculosis* or haem deficient mutants of *Y. pestis* and identified

the importance of iron acquisition for growth in these species (Wake *et al.*, 1975). However, there are few recent reports on the effect of iron on the bactericidal activity of phagocytes with regard to *Y. pseudotuberculosis*. Hanski *et al.* (1991) describe experimental work in the mouse examining the intracellular aspects of *Y. enterocolitica* infection in a mouse model but comment that the *in vivo* application of hypotheses developed from *in vitro* models is difficult due to the problem of achieving representative macrophage lines in cell culture.

Bullen *et al.* (1974) suggest that *in vivo*, the rate of destruction of the extracellular bacteria depends on the ratio of bacteria to phagocytic cells, the rate of ingestion of the bacteria and the rate of destruction of the bacteria. In mammals polymorphonuclear cells are active in plasma and inflammatory exudate in which there are unsaturated iron-binding proteins containing transferrin and lactoferrin or both. *In vivo*, the influence of these proteins, in combination with other antibacterial factors can be very important (Bullen, Ward & Wallis, 1974). It is generally thought that avian heterophils are similar to polymorphs in regard to their role in immune defence (Powell, 1987).

In macrophages and other phagocytic cells, the ability to digest endocytosed materials and the killing of phagocytosed bacteria are related but separate processes. Digestion is accomplished by an intracellular reservoir of hydrolytic enzymes. These enzymes are contained in the lysosomes of the cell. Chicken lysosomes contain acid phosphatase, beta-glucuronidase, lysozyme and non-specific esterase activity (Powell, 1987). These enzymes are manufactured in the endoplasmic reticulum and transported to the lysosomes which may then fuse with the primary phagosome containing the endocytosed antigen. This then becomes a secondary phagosome with an acidic environment (Rook, 1989). The effectiveness of this system is dependent on both the virulence characteristics of the pathogen and the degree of activation of the macrophage (Campbell, 1993). Pathogenic bacteria have evolved a number of mechanisms to survive within phagocytic cells, this is achieved by 1) resisting the actions of lysosomal enzymes, 2) prevention of lysosomal fusion or 3) inducing lysis of phagocytic vacuoles (Rook, 1989). Other bacteria, including *Y. pseudotuberculosis* are able to avoid phagocytosis following an initial intracellular localisation (Straley *et al.*, 1993; China *et al.*, 1993). In mammals, intracellular killing appears to be ultimately dependent on oxygen compounds generated from hydrogen peroxide by the lysosomal enzyme

myeloperoxidase. This enzyme contains iron and may be reduced in iron deficiency states which may explain why human patients with clinical anaemia have a high incidence of secondary bacterial infections (Chandra, 1990). Whether or not this is also the case in birds is not fully known although *in vivo* iron deficiency does appear to increase the susceptibility of young chicks to infectious diseases (Powell, 1987).

In conclusion therefore, the results of *in vitro* studies support the hypothesis that it is the balance between the availability of iron to the pathogen and the maintenance of normal cellular functions in the host which is the important factor in determining the role of iron in the outcome of the disease process (Weinberg, 1978; Bullen, 1981; Griffiths, 1993).

1-9 Abnormal and physiological changes in iron metabolism

1-9-1 Human haemosiderosis / Haemochromatosis

Haemochromatosis is defined as an excessive accumulation or storage of iron in tissues other than the reticuloendothelial system such as the heart and the pancreas. In humans this is a genetically linked disease which results in poor regulation of iron absorption across the intestinal wall with resultant accumulation of iron from what would be considered a normal diet (McLaren *et al.*, 1988). There is some confusion in the literature between the terms haemosiderosis and haemochromatosis (MacDonald, 1972; McLaren *et al.*, 1983). It is probably better to confine the use of the latter term to the familial human disease although the term is also used to indicate an advanced form of haemosiderosis in which iron is deposited in tissues other than the reticuloendothelial system and reaches sufficiently high levels in the liver parenchyma to cause clinical disease (Polycose, 1985; Powell *et al.*, 1980). The term haemosiderosis is generally used as a morphological diagnosis indicating that there is excessive stainable iron in the reticuloendothelial system, especially in the liver and spleen. The causes of this are varied and it has been reported in a wide range of mammalian and avian species (Hartley *et al.*, 1959; Powell *et al.*, 1980; Rehag *et al.*, 1980; Gosselin & Kramer, 1983; Gonzales *et al.*, 1984; Borch-Ionson & Nilssen, 1987; Carthew *et al.*, 1993).

1-9-2 Avian haemosiderosis

Iron-containing, brown pigment occurs frequently in the livers of several orders of birds and is well recognised by veterinary pathologists (Lowenstine & Petrak, 1980; Wadsworth *et al.*, 1983; Taylor, 1984; Ward *et al.*, 1988). Despite the numerous reports of this condition over the years there is still no consensus of opinion as to the significance of haemosiderosis in birds and it is thought probable that the causes of this condition are as varied as are the causes in man (Lowenstine & Petrak, 1980).

Although there have been numerous retrospective studies (Kincaid & Stoskopf, 1987; Wadsworth *et al.*, 1983; Taylor, 1984; Lowenstine & Petrak, 1980) and in later years, experimental studies (Ward *et al.*, 1991), there are still many unanswered questions. For example, the 'normal' level of hepatic iron in different species of bird has not been determined nor has the amount of variation between birds from one location to another. It is also not known which species have seasonal fluctuations in the amount of iron stored in the liver and how this affects the distribution of this iron. Of special interest in the context of the present study is the possible link between the amount and distribution of hepatic iron and conditions which predispose birds to microbial disease such as trauma, starvation, cold stress and concurrent parasitism. In mammals, all of these factors are thought to be associated with an alteration in iron metabolism (Lowenstine & Petrak, 1980; de Sousa, 1989).

From reports in the literature it is difficult to determine whether the avian and mammalian forms of haemosiderosis have the same aetiology. This is partially due to the fact that most studies have relied on retrospective material which only allows an assessment of the end point of the condition. However, with the need to improve the understanding of iron overload develops in human haemochromatosis, there have been a few experimental studies in avian and mammalian species (Ward *et al.*, 1991; Gonzales *et al.*, 1984; Gosselin & Kramer, 1983; Iancu *et al.*, 1987; Simpson *et al.*, 1993). Although the disease is now well documented (Powell *et al.*, 1980; Simpson *et al.*, 1993) no animal model has yet been found to produce lesions identical to those associated with the human disease (Ward *et al.*, 1991). However, through this research there is now a lot more known about haemosiderosis and iron overload in these other species.

There have been regular reports of a severe siderotic disease in Myna birds (Lowenstine & Petrak, 1980; Gosselin & Kramer, 1983) and in this species the form of iron overload initially appeared similar to that found in human haemochromatosis. The condition is frequently seen in young and mature birds and can be associated with hepatomegaly and clinical signs of liver disease such as elevated liver enzymes, ascites and jaundice. Iron deposits are found in the liver, spleen, heart, pancreas and lungs (Lowenstine & Petrak, 1980). Although there was some suggestion that the Myna bird chow (Gosselin & Kramer, 1983; Morris *et al.*, 1989) used as the diet for myna birds in many aviaries was excessively high in iron this was not considered to be an important component in the development of the condition. It is still not clear whether a genetic disorder is involved in the Myna bird syndrome although Kincaid & Stoskopf (1987) suggest that the fact that young birds are effected and that there was no correlation between the severity of iron overload and period of captivity supports the view that the condition is congenital in this species.

Studies on other captive avian species support the hypothesis that diet has a major influence in determining the level of hepatic iron stores in birds. Taylor (1984) attempted to compare the relationship between length of time in captivity and the degree of haemosiderosis in fifty avian species necropsied for the Jersey Wildlife Trust. He found that 48 out of 50 birds had histologically stainable iron in the liver, when sections were stained with Prussian blue stain. Taylor (1984) graded the severity of these cases using a semi-quantitative assessment of iron content based on that used by Famm *et al.* (1970) for cases of haemosiderosis in human patients. The grading system used relied on a visual assessment of the size and amount of the iron granules seen in the liver but did not take into account the distribution of iron deposits. From his results Taylor (1984) concluded that hepatic haemosiderosis was common in birds held at the Jersey Wildlife Trust and that the severity of the problem increased with the duration of time the bird had been held in captivity. He considered that this supported the view that the diets of avian species in captivity contained excessive amounts of iron and that measures should be taken to reduce the amount of iron in the diet.

A similar conclusion was reached by Kincaid & Stoskopf (1987) in their study of Tanagers which included four genera and seven species. These birds had been fed a mixed diet including a myna bird chow, which was reported to contain seven to twelve

times the recommended dietary iron requirement for poultry. However, the iron deposits in the tanager livers were equally distributed in hepatocytes and Kupffer cells as well as the reticuloendothelial cells of the spleen and other tissues. Kincaid & Stoskopf (1987) quantified the stainable iron deposits seen in tissues using a modification of the Bothwell technique (Bothwell *et al.*, 1979). Using the technique for assessment of the total iron stores, grades from '0' to '+++' were allocated based on the size and number of stainable iron granules seen histologically using prussian blue stain. Iron stores in a range of different cell or organ types were evaluated. Scores were summated with values from kupffer cells and hepatocytes added separately and averaged to avoid increased weighting of hepatic iron stores. Kincaid & Stoskopf (1987) also observed that periodic acid schiff (PAS) stained sections highlighted positive hepatocellular granules and considered that this represented lysosomal material in direct association with the iron deposits. It was not clear from this work whether or not the stainable iron was causing any damage in the cell types studied. In human haemochromatosis excessive amounts of iron in the cells eventually results in the death of individual hepatocytes and subsequent liver disease although most untreated patients usually die of heart failure before liver disease becomes clinically evident (Nthonli, 1973). Whether or not cell dysfunction occurs in avian haemosiderosis is not clear as although clinical liver disease is associated with the syndrome in myna birds it is not yet clear whether or not the liver disease is primary or secondary to the iron overload (Gosselin, 1981; Gosselin & Kramer, 1983).

Electron microscopic studies have indicated stored iron pigment is both membrane-bound and free in the cytosol as ferritin moieties and larger haemosiderin aggregates (Lowenstine & Petrak, 1980). Ward *et al.* (1991) describe ferritin or haemosiderin granules seen in vacuoles and possibly lysosomes in the hepatocytes of starlings both naturally and following injections of iron dextran. This is similar to what is reported in the human form of iron overload although, in contrast to the syndrome in humans, the same degree of stainable iron storage in birds does not appear to result in a clinical hepatic dysfunction (Ward *et al.*, 1991).

In the majority of the retrospective studies (Lowenstine & Petrak, 1980; Wadsworth *et al.*, 1983; Taylor, 1984; Kincaid & Stoskopf, 1987) there is evidence to suggest that the birds actually died of concurrent diseases such as aspergillosis, candidiasis, mycobacterial

infections and avian malaria. Lowenstine & Petrak, (1980) comment on the possible role of infectious diseases in causing the lesions seen in effected myna birds. They also note that while certain infectious agents are associated with increased storage of iron, birds differ from mammals in that iron is stored primarily in the spleen and not the liver (Lowenstine & Petrak, 1980).

Most of the earlier work done on iron metabolism in birds has dealt with dietary deficiency and homeostasis rather than overload. In addition, much of what has been reported has been based on work done using the domestic chicken (Davis *et al.*, 1968; Butler, 1983). Basic research done on domestic fowl in 1961 by Planas *et al.*, determined that the role of transferrin as a transport protein for serum iron, in the domestic chicken (*Gallus domesticus*) and duck (*Anser anser*), was essentially similar to that in mammalian species, however, it was noted that the laying bird had a much higher level of serum iron than the non-laying female or the male and that the saturation coefficient of transferrin in the laying birds studied was also significantly higher. Other work done at this time suggested that there was also another iron carrier in the blood of birds besides transferrin (Planas *et al.*, 1961). Diez *et al.* (1987) showed that there was a relationship between body iron status and serum ferritin. Normal serum ferritin levels in birds are generally thought to be higher than in mammals although it changes in proportion in a similar way when iron overload occurs. However, other factors, such as endocrine changes associated with the moult and the onset of lay, will also result in changes in serum ferritin levels (Calvo *et al.*, 1982).

Garcia *et al.* (1984) studied the iron content of starlings (*Sturnus vulgaris*) and compared values found for this species with those in poor-flying species such as the domestic chicken (*Gallus domesticus*) and the quail (Garcia *et al.*, 1987). It was noted that the breast muscles and the heart were relatively larger in the starling and that the iron content in the organs was generally two to ten times greater in starlings than in the chicken or quail. This was especially apparent when analysing the iron content liver and the feathers. In particular it was noted that the ration of iron content to body weight was two to four times higher for starlings than the chicken and quail or recorded values for mammalian species. Despite more recent work including that done on starlings (Ward *et al.*, 1991) it is still difficult to be certain that general concepts of what may be 'normal' for birds can be equally applied to all orders of birds or even

individuals within the same species.

In some birds there is a wide variation in dietary requirements for a range of essential nutrients associated with the seasonal changes in physiological demand which occur with the breeding season, the moult and in some species, migration. Diez *et al.* (1987) found a general increase in serum iron, ferritin and tissue ferritin and in iron content in all tissues as ducklings grew up to 8 weeks of age then a fall which was possibly attributable to moulting. Osborn (1979) studied the seasonal changes in the fat, protein and metal content of the liver of the starling and found that there were marked changes in the amounts of zinc, copper, cadmium, mercury and iron in the tissues. These changes were associated with changes in the levels of fat and protein especially in the liver. Ward *et al.* (1991) made detailed measurement of the iron content and distribution of iron stores in the livers of starlings pre- and post-moult and found that the mean levels of hepatic iron were two to three times higher during the post moult (summer/autumn) period than the pre- moult period (spring/winter). They compared the intestinal uptake of labelled dietary iron in starlings treated and untreated with iron dextran and compared the results with those in pre-treated and untreated rats. It was found that untreated starlings in the pre-moult period absorbed more radio-labelled iron than those pre-treated with parenteral iron dextran or those in the post-moult period but that the relative reduction in iron uptake was less than that observed in rats. They concluded that birds had a less tightly regulated iron uptake system than some mammals. However, it should be noted that in this experiment the rats received an oral load of carbonyl iron whereas the birds received an intramuscular dose of iron dextran. Ward *et al.* (1991) also noted that treating starlings with 5mg parenteral iron dextran resulted in a marked decrease in the iron binding capacity of transferrin. This is a similar finding to that reported in cases of haemochromatosis in human patients (Powell *et al.*, 1980). The authors concluded by noting that there were reports of spontaneous haemosiderosis in mammalian species such as the Svalbard reindeer (Borch-Ionhsen & Nilssen, 1987), marmosets (Chalmers *et al.*, 1983) and possibly in lemurs (Gonzales *et al.*, 1984) and that these species may offer an alternative model for the human disease.

1-10 Iron acquisition and other virulence factors of yersiniae

The experimental evidence to support the view that iron acquisition is an important

virulence characteristic of yersiniae is substantial (Carniel *et al.*, 1987; Baumler & Hantke, 1992; Heesemann *et al.*, 1993; de Almeida *et al.*, 1993; Itemann *et al.*, 1993). However, although *in vivo* assays of virulence support this view, the exact mechanism by which the mouse non-lethal yersiniae become lethal to iron loaded mice is still not clear. In this regard, the term 'mouse lethal' is possibly too non-specific to be useful and future *in vivo* assays of virulence to assess the relative importance of iron acquisition would need to determine what actually happens to the exogenous iron when iron treated hosts are challenged. It is apparent that both host and pathogen are potentially able to utilise exogenously supplied iron in the *in vivo* situation, the difficulty in determining the degree of competition between host and pathogen for iron is evident in the conflicting views on the value of results derived from disease models (Payne, 1988; Keusch, 1990; Griffiths, 1993; Perry *et al.*, 1993).

The virulence of *Yersinia enterocolitica* is now known to be closely associated with siderophore production, expression of an iron-repressible outer membrane polypeptide of 65 kDa (Irp 65) and pesticin sensitivity (Pst^s) (Pollack *et al.*, 1986; Heesemann *et al.*, 1993). It was suggested that Irp 65 of *Y. enterocolitica* functions as a receptor for the siderophore Yersiniabactin and possibly also for pesticin. Experimental evidence indicated that the same was true for *Y. pseudotuberculosis* (Heesemann *et al.*, 1993). The relative importance of plasmid mediated virulence factors in disease has recently been reviewed by Straley *et al.* (1993) and will not be described in any detail here. However, it is recognised that expression of YOPs following intracellular growth is an important factor involved in the ability of virulent yersiniae to survive in serum and avoid phagocytosis *in vivo* (Portnoy & Falkow, 1981; Prpic *et al.*, 1985; Robins-Browne *et al.*, 1987).

The possession of specialised iron uptake systems by more pathogenic strains of yersiniae may explain why iron supplementation of mice prior to experimental challenge has less effect on the infective dose than when non-pathogenic yersiniae are used as reported by Robins-Browne & Prpic (1985). Virulent yersiniae, including *Y. pestis* are presumably able to acquire sufficient iron from the host without pretreatment with exogenous iron or siderophores (Robins-Browne *et al.*, 1991). In the gut of the host, however, it is thought that even the non-pathogenic yersiniae are able to obtain sufficient iron from the readily available free iron of the luminal contents to survive but

that additional virulence factors would be required by these organisms to allow invasion of the intestine and systemic spread (Robins-Browne *et al.*, 1987).

Yersinia pseudotuberculosis and certain serotypes of *Y. enterocolitica* (American types, 08, 013, 020 and European serotypes, 03, 09, 05, 27) are enteropathogenic for humans and animals. A prerequisite for expression of pathogenicity is a 70-kilobase virulence plasmid which is commonly present in all enteropathogenic yersiniae and in *Y. pestis* (Cornelis *et al.*, 1987). In addition, there are a group of *Yersinia enterocolitica* serotypes which appear to be non-pathogenic for mice or humans (serotypes 05 and 07, 8). In view of the fact that Heeseman *et al.* (1984) were unable to induce virulence in mouse non-lethal strains by transfer of virulence plasmids from mouse lethal strains it was determined that chromosomal variants were required to produce virulence in mice. These include possession of the 'ail' gene and the 'inv' gene and possibly 'irp 65' or other iron acquisition mechanism. The 'inv' and 'ail' genes of *Y. enterocolitica* are associated with the ability to invade the intestinal wall when given by the oral route (Pepe *et al.*, 1994). This virulence characteristic is not assessed in experiments in which organisms are given parenterally (Robins-Browne & Prpic, 1985).

The 'ail' gene sequences are present only in pathogenic yersiniae. This gene facilitates the adhesion of *Yersinia* bacteria to intestinal epithelial cells while the 'inv' gene is associated with the ability to invade the epithelial cells. Recent research (Pierson & Falkow, 1993) suggests that the 'ail' gene also has a role in the resistance of bacteria to the action of complement in serum. Sequences of the 'inv' gene have also been identified in all yersiniae studied. It should be noted that 'inv' gene expression as well as 'ail' gene expression are temperature dependent (Simonet *et al.*, 1992).

A typical feature of 'mouse virulent' strains is that, following inoculation into mice of a lethal dose, there is rapid dissemination and multiplication of up to 10^9 organisms in the spleen and liver (Autenrieth *et al.*, 1992). Mice pre-treated with iron salts or iron chelators become highly susceptible to the mouse non-lethal group when challenged parenterally (Robins-Browne & Prpic, 1985). However, the effect is less pronounced if mice are given an oral challenge with an increased severity of diarrhoea being the major effect observed in the iron treated mice.

The degree of pathogenicity of yersiniae obviously depends on the efficiency of iron acquisition (Heeseman *et al.*, 1993). In line with this finding is the observation that the virulence of Pgm⁺ (pigment phenotype) *Yersinia pestis* is associated with the ability to store haemin as an endogenous iron source (Perry & Brubaker, 1979; Fetherston *et al.*, 1992). Although the closely related species, *Y. enterocolitica* and *Y. pseudotuberculosis* are unable to store haemin and are therefore phenotypically Pgm⁻, they appear able to utilize haemin as a source of iron (Perry & Brubaker, 1979; Stojiljkovic and Hantke, 1992). In addition, it is recognised that enteropathogenic *Y. enterocolitica* are able to readily utilise exogenous siderophores, such as desferrioxamine (Stuart *et al.*, 1986; Baumler & Hantke, 1992). Some pathogenic strains of *Y. enterocolitica* have been found able to produce siderophores although this remains a controversial area (de Almeida *et al.*, 1993). The early research in this area (Wake *et al.*, 1975; Perry & Brubaker, 1979) was inconclusive which may reflect the fact that many of the siderophores produced by pathogenic yersiniae are unstable and short lived. Heeseman (1987) suggests that there is good evidence that strains of *Y. enterocolitica* and *Y. pseudotuberculosis* produce siderophores *in vivo*. Presumably these strains also express a siderophore uptake system, comprising siderophore production and the corresponding iron repressible outer membrane proteins (Irp) for transporting the chelated iron.

Other characteristics of the pathogen should also be examined. For example, some *Yersinia* Irps have been identified as receptors for exogenous siderophores such as ferrioxamine B (Desferal), (Baumler & Hantke, 1992), haemin (Stojiljkovic & Hantke, 1992) and ferrichrome but although this may allow some yersiniae to utilise the siderophores of other bacteria in the gut this is not necessarily true for all of the pathogenic species (Robins-Browne *et al.*, 1987). Pesticin-sensitivity, another phenotypic characteristic of virulent strains of *Y. pestis*, has also been associated with 'mouse-lethality' in some serotypes of *Y. enterocolitica* and *Y. pseudotuberculosis* (Burrows & Bacon, 1960; Harrison *et al.*, 1980). Pesticin is encoded by a 9.5kb plasmid of *Y. pestis*. The observation that pesticin-sensitivity is blocked when the cells of *Y. pestis* are grown in the presence of iron led to the hypothesis that the receptor for pesticin was analogous to the iron-repressible outer membrane protein that may also act as a siderophore (Hu *et al.*, 1972). If this were true, it is possible that the presence of iron would induce competition at the receptor site at the expense of pesticin. Mutation of *Y. pestis*, *Y. pseudotuberculosis* or *Y. enterocolitica* to pesticin resistant strains (Pst^r), resulted in

reduced virulence for mice after sub-cutaneous challenge but not intravenous inoculation (Une and Brubaker, 1984). Mutation of *Y. pestis* to the Pst^r and Pgm⁻ (pigmentation negative) phenotype results in reduced virulence although this is regained if iron is also added to the culture medium or administered prior to animal challenge (Wake *et al.*, 1975). However, unlike *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* are always Pgm⁻ and although the mutation from Pst^s to Pst^r appears to result in reduced virulence, this is thought to be associated with a reduced ability to invade cells rather than an alteration in the ability to acquire iron (Heeseman *et al.*, 1993). In this respect the route of infection would also have an important effect on the ability of the pathogen to express its virulence potential (Autenrieth *et al.*, 1991).

The use of 'mouse-lethality' assay for determining the virulence of 'enteropathogenic' strains of yersiniae has the potential to cause some confusion when attempts are made to compare the virulence of different species using the results of experiments in which the route of infection is not consistent.

Although it is clear that there is sufficient *in vitro* evidence to support the hypothesis that virulent yersiniae produce iron repressible proteins and possibly a siderophore when exposed to conditions of iron starvation, the time sequence and importance of this *in vivo* is more difficult to determine (Heeseman *et al.*, 1993). It is possible that failure to identify siderophores in all strains of virulent yersiniae is due to the fact that it is difficult to identify these products or that it is difficult to mimic the conditions which stimulate the production of the iron acquisition system. Another factor to be considered is that yersiniae do appear liable to alter their phenotypic expression on sub-culture, depending on the media used and the temperature of incubation (Wake, 1984). The 65 kDa iron repressible protein has never been identified in non-pathogenic yersiniae (Heeseman *et al.*, 1993) but they are known to produce an aerobactin-like siderophore which allows iron acquisition from environmental sources (Robins-Browne *et al.*, 1987).

Despite all the *in vitro* evidence to support the importance of iron uptake as a virulence factor for the pathogenic yersiniae clinical evidence of this is harder to find. Siderophore-producing 'mouse-lethal' strains of *Y. pseudotuberculosis* (I, II, III) have been shown to express Irp65. However, isolates from patients with severe *Y. enterocolitica* 03 or 09 infection with an associated septicaemia and liver abscesses, did

not appear to produce outer membrane proteins which differed from those of isolates causing mild infection (Heesemann *et al.*, 1993). It is possible, however, that different host factors such as iron availability, liver function and host protein status, effect phenotypic expression and therefore *in vitro* studies, in which only one factor is altered prior to harvest of bacteria, may not mimic the *in vivo* situation (Hanski *et al.*, 1991). Therefore, whereas it is clear that iron acquisition is an important feature of virulence in yersiniae other factors, such as the immune status and general physiological state of the host, must also be taken into consideration.

In conclusion, although there has been an increased awareness of the importance of iron acquisition systems and the virulence of yersiniae there has not been a corresponding increase in the understanding of the clinical significance of this despite further progress in the field of molecular biology (Carniel *et al.*, 1992; Heesemann *et al.*, 1993). Numerous reports indicate that iron availability is an important factor in determining the outcome of many infectious diseases (Smith *et al.*, 1981). However, the complexity of the interactions between the host and the potential pathogen which occur alongside alterations in the iron metabolism of both host and pathogen during various stages of the development of infection make it difficult to examine in an *in vivo* model. As a result, although the subject of iron and disease has enjoyed a revival in the last few years there has been limited progress in understanding the clinical implications of this knowledge (Griffiths, 1993).

1-11 Conclusions drawn from the literature

- 1) The belief that wild birds are a 'reservoir' for *Yersinia pseudotuberculosis* despite the reported low prevalence of the organism in the faeces of wild birds in various surveys, deserves further investigation.
- 2) In view of the low prevalence of *Y. pseudotuberculosis* in bacteriological surveys of wild birds and the possibility that bacteriological methods give an underestimate of the true prevalence of the organism in wild birds it appears reasonable to conclude that a concurrent serological survey would be of value.
- 3) There are a wide range of serological tests available for the diagnosis of *Yersinia*

infections in humans but there are no reports of the use of these tests in birds. Due to the apparent lack of consensus in the literature with regard to the value of currently available tests and the known cross-reactivity of yersiniae with other enteric bacteria it would be necessary to evaluate the antigenic structure of *Yersinia pseudotuberculosis* in some detail before a serological screening test could be developed for use in birds.

4) Avian pseudotuberculosis occurs as a sporadic disease in debilitated birds or as an outbreak in susceptible groups of wild or captive birds. The disease occurs following periods of cold weather and is often associated with concurrent disease, crowding and poor nutrition. Treatment of affected birds is difficult and there has been an increased emphasis directed towards the application of prophylactic measures and consequently a need to identify the major predisposing factors.

5) The reasons for differences in the susceptibility of different avian species to infection with *Y. pseudotuberculosis* requires further investigation.

6) Despite the large body of literature on pseudotuberculosis in birds and mammals and the close association between iron availability and the expression of virulence characteristics in pathogenic yersiniae, it is still not clear how iron overload influences the outcome of disease. The observation that haemosiderosis is a common histological finding in birds, especially those with concurrent bacterial infection, suggests that conditions associated with changes in iron metabolism may influence the course of disease in birds.

7) There is some evidence to suggest that the pathogenesis of bacterial diseases such as salmonellosis following exogenous iron overload in chickens is not what would be expected from the results of similar experiments in mammals. Pseudotuberculosis is a disease which could be studied in the chicken to explore the interaction between the avian host, iron and the bacterial pathogen in the attempt to understand the mechanisms of resistance and the role of predisposing factors in the outcome of infection.

8) Despite the large amount of *in vitro* evidence and a number *in vivo* models which support the hypothesis that an increased availability of iron is associated with the

potentiation of bacterial survival, the application of this apparently simple concept has been more difficult. There is a need to develop a disease model to examine the reasons for this lack of progress.

1-12 Aims and scope of the thesis

The present study aims to evaluate the triad of disease as it relates to avian pseudotuberculosis and to consider some of the more general questions arising from the literature review as outlined in section 1-11. The possible interactions between the host, the pathogen and the environment are investigated, and the way in which variables such as season, concurrent disease and differences in species susceptibility have the potential to modify the outcome of infection.

The thesis is divided into two sections, the first examines the role of wild birds in the epidemiology of the yersiniae, describes the development of a serological test and investigates factors which predispose birds to the development of clinical disease. The potential of bacterial virulence factors, such as the carriage of a virulence plasmid and the release of endotoxin, and differences in host susceptibility to disease, to determine the outcome of infection with *Y. pseudotuberculosis* is also examined. In the second section, host factors, which influence the expression of bacterial virulence characteristics, such as temperature and iron availability, are examined experimentally using the methodology developed in section one.

Terms used in the text for which specific definitions have been chosen are listed in appendix (A-1) and the latin names of avian species studied are given in appendix (A-2).

SECTION ONE; The determination of factors predisposing to the development of pseudotuberculosis in avian species and development of a disease model

General introduction

To fully understand how and why certain factors predispose to the development of bacterial diseases in birds it is necessary to consider the interaction between the potential pathogen, the environment and the avian host. It is important to examine how each interacting variable may affect other variables in a given situation.

The following chapters describe studies on certain aspects of the interactions between the host and the environment, the host and the pathogen, and the pathogen and the environment. The latter aspect is especially interesting in relation to the yersiniae which offer a particularly complex example of a virulence regulatory network partially controlled by temperature (Cornelis, 1992). Because it is very difficult to maintain virulent cultures of *Yersinia pseudotuberculosis in vitro* at temperatures greater than 30 °C it was suggested by Wetzler (1965) and later by Levre *et al.* (1989) that this feature alone would make it unlikely that a homeotherm would provide a suitable 'reservoir' host in which to maintain virulent populations of *Y. pseudotuberculosis* without continued reinfection from another source such as the environment (Barre *et al.*, 1979; Fukushima *et al.*, 1991). Temperature and the competition between host and pathogen for nutrients, in particular, iron, will be the basis of this study.

CHAPTER TWO

Wildlife disease Surveillance

Introduction

Despite numerous references (Clapham, 1953; Mair, 1973; Fukushima *et al.*, 1987; Fukushima and Gomyoda, 1991; Fukushima *et al.*, 1991) quoting early reports that wild birds were likely reservoirs of *Yersinia pseudotuberculosis* bacteria (Pullar, 1932; Patterson & Cook, 1963), more recent studies have indicated that the prevalence of *Y. pseudotuberculosis* is not high in samples of faeces collected from wild birds. This is in contrast to the environmental species of yersiniae which are frequently isolated from avian sources (Hubbert, 1972; Kapperud & Rosef, 1983; Kapperud & Olsvik, 1982; Brittingham *et al.*, 1985; Hamasaki *et al.*, 1989; Kaneuchi *et al.*, 1989; Iannibelli *et al.*, 1991). Although the term 'reservoir' population is often used in a rather vague sense to indicate the probable 'source' of an infectious agent, it is more strictly defined as a population in which a micro-organism is maintained indefinitely without the need for reinfection from another source (Schnurrenberger *et al.*, 1987).

The epidemiology of pseudotuberculosis in New Zealand is obscure but appears to follow a seasonal trend with outbreaks of clinical disease in birds occurring in the winter and spring (MAF records, unpublished). The disease in farmed deer is seen more commonly in the autumn and winter period especially in the weaner groups (Mackintosh and Henderson, 1984). Serotype III is thought to be more prevalent in the North Island while serotypes I and II are more common in South Island outbreaks (Henderson, 1983; Henderson & Hemmington, 1983). It has been postulated that serotype I and II outbreaks were associated with wildlife reservoirs whereas outbreaks with serotype III were more frequently associated with livestock reservoirs (Mackintosh, 1988). A survey was conducted by Mackintosh & Henderson (1984) to determine whether wildlife were a reservoir of pathogenic yersiniae for farmed deer at the Invermay research station in the lower South Island. They isolated 8 strains of *Y. pseudotuberculosis* from 158 apparently healthy birds, sampled by necropsy, in the five month period from March to July. Of these isolates four were serotype I, one was serotype II and three were untypable (Mackintosh & Henderson, 1984). There has been no further research

reported on the prevalence of yersiniae in birds in New Zealand although some data is available on rodents indicating that the prevalence of pathogenic yersiniae in rat faeces was low (Armitage, unpublished data).

The hypothesis that pseudotuberculosis in captive mammals and birds is a result of the contamination of food and water by wildlife reservoirs (Mair, 1973; Wetzler, 1971; Fukushima & Gomyoda, 1991) has not been fully tested in either the field or the laboratory. Neither has there been any comparisons made of the relative prevalence of this organism in urban and rural avian populations. To determine whether or not environmental contamination by wild birds could be important in the spread of the disease it was decided to conduct a survey by sampling the faeces of common wild bird species in different locations throughout one year. A concurrent survey was also conducted on environmental samples (soil, water, foliage) collected in the same locations.

Many of the studies on the enteric bacteria of wild birds (Brittingham *et al.*, 1985; Hamasaki *et al.*, 1989; Fukushima & Gomyoda, 1991; Fukushima *et al.*, 1991), are predominantly based on bacterial culture of collected faecal samples or cloacal swabs taken from live birds. In these studies the prevalence of pathogenic yersiniae was found to be low. In contrast the prevalence of pathogenic yersiniae in wild birds is much higher in studies based on necropsy material, such as the study by Mackintosh & Henderson (1984). However, the effect of study site, climate and the species of bird examined, may also account for the difference. The present study attempts to take all of these factors into consideration in the interpretation of the survey results as well as to examine the effect that sampling technique has on the isolation rate.

Materials and methods

2-2-1 Seasonal study

Live birds

A total of 1,370 samples were collected from wild birds and 1,356 samples from non-avian sources (soil, water, foliage, wild mammals) between November 1991 and November 1992. For each season (three month period) at least 300 avian and 300 environmental samples were collected. This sample size was determined from tables (Cannon & Roe, 1982) to give a 99% probability of detecting an estimated prevalence of 1% for isolation of *Yersinia pseudotuberculosis*.

The seasons were determined from the beginning of one month to the end of the third month in the period, as follows, Summer (December 1991-February 1992), Autumn (March 1992-May 1992), Winter (June 1992-August 1992), Spring (September 1992-November 1992).

Necropsy material

In the necropsy study the estimated prevalence of *Y. pseudotuberculosis* was higher (5%) based on other studies (Keymer, 1980) indicating that the collection of 180 samples during the period of a year was sufficient (Cannon & Roe, 1982).

2-2-2 Regional comparisons

Of the 1,370 avian samples 1,190 were collected from live birds and 180 from necropsy samples. All of the necropsy material used in the study was from wild birds inhabiting rural areas. The remainder of the avian material was collected from live birds in rural and urban areas with a similar number of samples collected from each type of area to allow comparisons to be made. Where rural and urban avian populations were to be compared an attempt was made to collect a minimum of 300 samples for each population. In these populations 70-80 samples were collected per three month period. Non-avian material was collected from both rural and urban areas with an equal number of samples collected from each location in each three month period.

Study sites

Samples were collected from both rural and urban bird populations in the lower half of the North Island throughout the year of the survey. Locations were chosen which allowed comparisons to be made between passerine species which had similar dietary habits. Where possible, an equal number of avian and non avian samples were collected from each rural and urban location for each seasonal period.

A Rural (farmland) and Wellington Zoological Gardens

All rural areas chosen were located in the lower North Island of New Zealand and included farm land in the Manawatu and the Wairarapa. An equal number of samples were collected from each type of sample site per seasonal period but not always from the same location. The locations were chosen for the proximity to livestock as follows, 1: The Massey University piggery, 2; The Massey University feed mill, 3: The Massey University dairy unit, 4; The Massey University sheep farm\Deer unit (located on neighbouring properties) 5; The MAF quarantine station, Bulls, Flock House, 6: MAF, Levin kiwi fruit orchard (located near a sheep farm), 7; Feilding agricultural land, 8; Ashurst deer farm, 9, Pahiatua deer farm, 10; Linton rural land, 11; Wellington Zoological Gardens.

For the purposes of the present study the zoo site was considered a rural location due to the presence of a range of other animal species on the land. Non-avian material collected from these sites included soil, foliage, water and wild mammals.

B Urban

The urban study sites included 8 suburban and 2 urban locations of Wellington city. Non-avian material collected from these sites included soil and foliage.

2-2-3 Collection of samples

Methods of collection were dictated by the habits of the birds under surveillance as well as by practical aspects of sample collection and time constraints. This has been taken into consideration in the analysis of the data.

Avian samples

A Faecal samples

In urban Wellington, bird faeces were collected over night on clean plastic sheets placed under starling roost sites. In rural areas samples from a wider range of avian species were collected in this manner. Only fresh moist material was collected for culture. Samples were collected from the sheets using a sterile swab and placed in a labelled plastic bottle containing 5 ml of phosphate buffered saline (PBS). The containers were chilled at 4 °C for 3 weeks for cold enrichment.

B-1 Cloacal swabs

In rural areas of the Lower North Island passerine species were caught in mist nets (0.5x1.0 inch mesh, Avinet, Inc. Dryden, USA) with approval from the Department of Conservation (permit no. 0351). All birds caught were banded with metal bands as part of a national banding scheme, the species of each bird was recorded and where possible the sex and age were determined using plumage and size characteristics. After removal from the net, cloacal swabs were taken using a moistened, sterile, paediatric ear nose and throat (ENT) swab (Medical Wire and Equipment, Bath). The swabs were then placed in labelled plastic containers containing PBS and treated as above.

B-2 Blood samples

Where possible, blood samples were also collected for serology. These were taken by pricking the brachial vein as it crosses the distal humeral condylar surface and collecting a drop of blood on each tag of a 6 tag filter disc (CAHL-virology, Wallaceville, New Zealand). The sample size was restricted to a volume of 400 microlitres collected on filter discs from birds weighing >40g. Of the 540 birds caught in the study, blood

samples were collected from 315 (58%). The details of the serological test will be considered in the next chapter.

C Necropsy material

The third source of material was from gut samples taken from the caecum or large intestine of wild birds presented to the Department of Veterinary Pathology and Public Health for necropsy or from healthy ducks shot during the hunting season. A sterile swab was used to collect material from the large intestine and/or caecum of each bird sampled, this was then placed in a labelled plastic bottle containing 5 ml of PBS and treated as for the faecal and cloacal swabs.

Non-avian material

A Soil

The method used for sampling soil was adapted from the method used by Mair & Fox (1986). From each study site at least 20 x 10 g samples of soil were collected. These were placed in 20 ml of PBS and mixed in a mechanical shaker prior to storage at 4 °C for 10-28 days. Prior to culture the samples were mixed again, the larger particles of soil material were allowed to settle and a 10 microlitre aliquot of the supernatant was plated on to Cefsulodin-Irgasan-Novobiocin agar (CIN, Difco, Detroit).

B Foliage

Foliage was collected by breaking the stalk (of the plant) or branch (of the shrub or tree) at the base and placing representative pieces of leaf or bark material into 10 ml of PBS. Samples which were contaminated with faecal material were included in the study.

C Water

The method used for sampling water was adapted from the method used by Mair & Fox (1986). Water samples were collected as a 100 ml aliquot in 2 x 50 ml syringes and filtered through a 0.45 micron Millipore membrane filter (Field monitor, Millipore corporation, USA). The filter was then placed in 10 ml of PBS and cold-enriched for a period of three weeks. Each 50 ml syringe was used to collect water from the edge and mid flow region of stream sites and from the centre and edge of ponds and water troughs.

D Wildlife

Samples were collected from 65 possums (*Trichosurus vulpecula*), 15 rabbits (*Oryctolagus cuniculus*) and 20 small rodents (16 x *Mus musculus*, 4 x *Rattus rattus*). The possum samples were obtained from necropsy material collected from the Manawatu and Wairarapa during a survey for *Mycobacterium bovis*. In 12 of the animals the ileum, colon and caecum were sampled and from the remaining 53 only caecal samples were available. The rabbit samples were obtained from faecal material found outside the entrance of rabbit burrows located on farm land close to mist netting sites. The rodent samples were collected from the intestinal contents of animals caught in traps close to aviaries on rural property. In the necropsy cases the contents of the intestinal tract or the faecal samples were placed in plastic bottles containing 10 ml of PBS, cold-enriched for 3 weeks and an aliquot plated onto CIN agar.

2-2-4 Bacterial isolation and identification of yersiniae

Each month a batch of samples was cultured to look for the presence of yersiniae. Each sample had been stored in PBS for 10-28 days of cold enrichment at 4 °C prior to plating on CIN agar. After 24 hours of incubation at 28 °C suspect colonies were subcultured in tryptone water and incubated for 6 hours at 28 °C prior to being screened biochemically. *Yersinia* species were identified by standard methods as outlined by Feeley (1981) see appendix (A-3). If no growth was seen after 24 hours the CIN agar plates were incubated for a further 48 hours. *Yersinia pseudotuberculosis* strains isolated from the survey and from clinical isolates were serotyped using the hyperimmune rabbit

serum described in chapter three (3-2-1), in a macroscopic slide agglutination test (MAT). *Yersinia* isolates belonging to the potentially pathogenic species were tested for the presence of virulence plasmids using the technique developed by Portnoy *et al.*, (1981). This will be considered in more detail in chapter four.

2-2-5 Statistical evaluation of results

The chi squared test was used to determine whether or not there was a significant difference between the different parameters, season, location, sampling technique, measured for different populations of birds in rural and urban areas.

Results

2-3-1 Number of samples and location

Due to practical difficulties it was not possible to mist net wild birds in urban areas and the survey design was modified to allow for this with faecal samples accounting for the majority of samples from urban birds. Whereas this may have affected the results, the comparisons between rural and urban avian populations are still considered valid because the number of samples collected from each source was sufficient to determine trends within populations. The following tables outline the number of samples collected per season in each study location.

A-1 Rural (farmland and Wellington Zoological Gardens)

Of the 710 avian samples outlined in Table (2-1), 540 were collected from live birds caught in mist nets. The remaining 170 samples were faecal samples collected from under house sparrow roost sites around farm buildings. The non-avian material included soil, foliage, water and samples from wild animals located in the area.

Table (2-1) Source and number of avian and non-avian samples collected from rural areas between November 1991 and November 1992.

Season	Rural farm land			Zoo		
	Avian	Other	Total	Avian	Other	Total
Summer	190*	176	366	25	15	40
Autumn	125	171	296	30	15	45
Winter	145	161	306	35	15	50
Spring	130	166	296	30	15	45
Total	590	674	1264	120	60	180

* number of samples

B Urban

Samples were collected from under ten starling roost sites in eight similar suburban locations and two urban regions of Wellington city. Of the 480 avian samples collected, 420 (88%) were known to be from starlings (*Sturnus vulgaris*) while the remaining samples were from house sparrows (*Passer domesticus*), blackbirds (*Turdus merula*) and thrushes (*Turdus philomelos*). The non avian samples included 522 soil and 100 foliage samples which were collected from the ground at the same locations.

Table (2-2) Source and number of avian and non-avian samples collected from urban areas between November 1991 and November 1992.

Season	Avian	Non avian	Total
Summer	105*	140	245
Autumn	125	145	270
Winter	115	205	320
Spring	135	132	267
Total	480	622	1102

C Blood samples

Of the 540 birds caught in the study, blood samples were collected from 315 (58%) of the birds.

2-3-2 Summary of yersiniae isolated from different sources

A total of 1,370 avian samples and 1,356 non-avian samples were collected over the period of the survey (November 1991 to November 1992). From these samples 149 strains of yersiniae were isolated, 98 from avian sources and 51 from non-avian sources (Table, 2-3). *Yersinia enterocolitica* accounted for 50 % of the isolates, and 75% of these were classified as biotype 1A, while the biochemical characteristics of the remainder did not fully match any recognised biotype. Of the other 50% of the isolates, 22% were *Y. frederiksenii*, 19% were *Y. kristensenii*, 7.5% were *Y. intermedia* and 1.5% were *Y. pseudotuberculosis*.

Table (2-3) The number and species of yersinia isolated from avian and non-avian sources between November 1991 and November 1992. Figures in brackets indicate total number of samples examined.

<i>Yersinia</i> isolates		Avian sources			Other sources			
<i>Yersinia</i> species	Total	Necrop (180)	Faecal (650)	Cloacal (540)	Soil (1,032)	Foliage (200)	Water (24)	Mamm (100)
<i>enterocolitica</i>	74 **	14	21	6	27	2	1	3
<i>frederiksenii</i>	33	1	23	2	5	1	0	1
<i>kristensenii</i>	29	3	11	6	7	2	0	0
<i>intermedia</i>	11	6	0	3	2	0	0	0
<i>pseudotuberculosis</i>	2	2	0	0	0	0	0	0
Total	149	26	55	17	41	5	1	4

Necrop. = necropsy; Mamm. = Mammals

2-3-3 The effect of sampling techniques on the isolation of yersinia from avian samples

A total of 98 *Yersinia* strains were isolated from 1,370 avian samples. These samples were collected from different populations of wild birds. Availability of material was increased by utilising three different methods of sample collection. During the year of the survey a total of 180 necropsy samples, 650 faecal samples and 540 cloacal swabs were obtained. For each method there were an equal number of samples collected for each three month seasonal period.

Table (2-4) A comparison of the relative isolation rates of different yersiniae in avian material when different sampling techniques are used. Figures in brackets indicate the relative proportion (percentage) of the total isolates for the technique under consideration.

Species of <i>Yersinia</i>	Sampling technique		
	Necropsy	cloacal swab	faecal sample
<i>enterocolitica</i>	14 (54)	6 (35)	21 (38)
<i>frederiksenii</i>	1 (3.8)	2 (12)	23 (42)
<i>kristensenii</i>	3 (11.5)	6 (35)	11 (20)
<i>intermedia</i>	6 (23)	3 (18)	0 (0)
<i>pseudotuberculosis</i>	2 (7.7)	0 (0)	0 (0)
Total number of isolates	26 /180	17/540	55/650

There is a significant difference in the isolation rates of yersiniae when the three different sampling techniques are compared ($df = 2$, $P < 0.001$). The relative proportion of the different species of yersiniae isolated is also significantly different when comparing the prevalence of different species of *Yersinia* in faecal and necropsy samples. The prevalence of *Y. enterocolitica*, *Y. intermedia* and *Y. pseudotuberculosis* was significantly greater in necropsy samples than in faecal samples ($P < 0.05$) whereas the prevalence of *Y. frederiksenii* and *Y. kristensenii* were significantly less ($P < 0.05$). The relative prevalence of yersiniae in cloacal and necropsy samples was similar except for *Y. kristensenii* for which the prevalence was greater in cloacal swabs than in faecal or necropsy samples ($P < 0.05$). However, not all the techniques evaluated samples taken from the same sources of avian material.

From the table it is seen that necropsy results in the isolation of a relatively higher number of yersiniae than the other two techniques. The prevalence of yersiniae isolated from avian necropsy samples is 14% as compared with 3% from cloacal swabs and 8.5% from faecal samples. The latter two sampling techniques used samples obtained from apparently healthy birds. The necropsy cases were birds which had died of causes other than those attributed to *Y. pseudotuberculosis*. There is a significant difference in sensitivity between all three techniques (Faecal > cloacal, $P < 0.001$; Faecal < necropsy, $P < 0.05$; Necropsy > cloacal, $P < 0.05$).

2-3-4 Yersinia isolated from avian sources during the period of the survey (November 1991 to November 1992)

Species difference in faecal yersinia

A total of 25 avian species were sampled during the period of the survey. Of 1,370 avian samples collected 95% were obtained from passerine species of the families Ploceidae, Fringillidae, Sturnidae and Turididae. Approximately 50% of the samples were obtained from rural populations and 50% from urban populations. The majority of the birds in the avian populations studied were introduced species. One hundred native birds were also studied, of these 5% were found to carry yersinia. *Yersinia enterocolitica* was isolated from 3/5 stitchbirds (*Notiomystis cincta*) sampled, 1/14 pukekoes (*Porphyrio porphyrio*) and 1/1 brown kiwi (*Apteryx australis mantelli*). However, although only 1 kiwi was studied in the survey, other work on native birds failed to isolate *Yersinia spp* bacteria from any of 20 Little Spotted Kiwis (*Apteryx owenii*) sampled (see appendix, A-9).

From the results in Table (2-5) it was determined that there was a significantly higher prevalence of yersinia in starlings (*Sturnus vulgaris*) compared with house sparrows (*Passer domesticus*); ($df = 1, P < 0.01$). However, except for *Y. frederiksenii* in which the prevalence was significantly higher in the starlings (*Y. frederiksenii* $P < 0.05$) there does not appear to be a significant difference in the relative proportions of yersinia isolated in the starlings and house sparrows sampled.

Table (2-5) A comparison of the relative prevalence of yersinia isolated from different species of bird.

Species of bird ** (number sampled)	Species of <i>Yersinia</i> isolated					
	<i>pseud</i>	<i>ent</i>	<i>fred</i>	<i>krist</i>	<i>int</i>	total
Introduced Passeriformes						
House sparrow (583)	2 *	15	-	7	9	33
Starling (437)	-	17	20	11	-	48
Black bird (35)	-	1	-	1	-	2
Thrush (25)	-	-	2	1	-	3
Fringillidae *** (67)	-	-	-	-	-	0
Native Passeriformes						
Stitch bird (5)	-	3	-	-	-	3
Anseriformes						
Mallard duck (54)	2	3	-	-	-	5
Gruiformes						
Pukeko (14)	-	1	-	-	-	1
Apterygiformes						
Brown Kiwi (1)	-	1	-	-	-	1
Other species (149)	-	1	1	-	-	2
Total (1,370)	2	41	26	20	9	98

pseud = *Y. pseudotuberculosis*; *ent* = *Y. enterocolitica*; *fred* = *Y. frederiksenii*; *krist* = *Y. kristensenii*; *int* = *Y. intermedia*.

* number of isolates, ** Latin names for the bird species listed in the Table are given in appendix (A-2), *** Greenfinch, Chaffinch and Gold finch

Table (2-6) Comparison of the prevalence of yersinia isolated from starlings and house sparrows using different sampling technique

Sampling technique	Prevalence of yersinia in two species of bird		(df=1)
	House sparrow	Starling	
Faecal sample	1/45 *	45/420	P>0.05
Cloacal swab	11/490	3/16	P>0.05
Necropsy	21/48	0/1	P>0.05
Total	33/583	48/437	P<0.01

* Numerator= number of isolates, denominator = number of samples

From the statistical evaluation in Table (2-6) it is evident that although there appears to be a significant difference in the prevalence of yersinia when comparing the total number of isolates from each of the two species studied ($P<0.01$) this difference is insignificant when comparing results obtained using the same method of isolation.

2-3-5 Seasonal distribution of yersinia isolated from avian sources during the period of the survey

If all of the avian results are considered together there is a significant difference in the prevalence of yersinia in the four seasonal periods ($df = 3$, $P < 0.001$) with a significantly higher prevalence in the winter and summer periods than in the autumn and spring (Table, 2-7). The peak prevalence occurs in the winter and the lowest number of isolates were obtained in the autumn. In addition, during the period of the survey three outbreaks of pseudotuberculosis occurred in captive birds between September and October following a period of cold weather.

Table (2-7) Seasonal distribution of yersiniae isolated from avian sources during the period November 1991 to November 1992

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (41) *	6 **	3	21	11
<i>Y.kristensenii</i> (20)	9	2	7	2
<i>Y.frederiksenii</i> (26)	10	1	13	2
<i>Y.intermedia</i> (9)	4	-	-	5
<i>Y.pseudotb.</i> (2)	-	-	-	2
Total (98)	29/350 ***	6/335	41/365	22/320

* total number of isolates, ** number of yersiniae isolated for each season, *** total number of yersiniae isolated in a season divided by the total number of samples collected.

Yersiniae isolated from faecal samples collected from an urban population of Starlings (*Sturnus vulgaris*)

The overall prevalence of yersiniae isolated from faecal samples collected from the study population was 11.0 % (45/420). Of the 45 *Yersinia* strains isolated from these birds during the year of the survey, 75 % were obtained in the winter. *Yersinia enterocolitica*, *Y. frederiksenii* and *Y. kristensenii* were isolated from faecal samples of starlings and accounted for 34% (15/45), 42% (19/45) and 24% (11/45) of the total number of *Yersinia* isolates respectively. There is a significant increase in the prevalence of yersiniae in the winter period as compared with the autumn ($P<0.001$).

Table (2-8) The seasonal pattern of yersiniae isolated from the faeces of starlings (*Sturnus vulgaris*) roosting in urban Wellington.

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (15) *	0 **	0	15	0
<i>Y.kristensenii</i> (11)	2	1	6	2
<i>Y.frederiksenii</i> (19)	4	1	13	1
<i>Y.intermedia</i> (0)	0	0	0	0
<i>Y.pseudotb.</i> (0)	0	0	0	0
Total (45)	6/115 ***	2/100	34/100	3/105

* total number of isolates, ** number of yersiniae isolated for the three month period season, *** total number of yersiniae isolated in a season divided by the total number of samples collected.

The seasonal pattern of yersiniae isolated from passeriform birds in two locations using the same sampling methods

A Rural farmland in the Manawatu

A total of 12 (2.6%) strains of *Yersinia* were isolated from 460 passeriform birds sampled. The species sampled were predominantly starlings, house sparrows, finches, thrushes and blackbirds. All of the *Y. intermedia*, *Y. kristensenii* and *Y. frederiksenii* isolates were made in the summer period. *Yersinia enterocolitica* was isolated in all seasons except the spring. There were no strains of *Y. pseudotuberculosis* isolated from the passeriform populations studied. The overall prevalence of yersiniae was higher in the summer-autumn period (11/230) than in the winter-spring period (3/230) in the cloacal samples collected from this population ($P < 0.01$).

Table (2-9) The seasonal pattern of yersiniae isolated from cloacal swabs taken from passeriform species caught in rural areas of the Manawatu.

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y. enterocolitica</i> (4) *	2**	1	1	0
<i>Y. kristensenii</i> (3)	3	0	0	0
<i>Y. frederiksenii</i> (2)	2	0	0	0
<i>Y. intermedia</i> (3)	3	0	0	0
<i>Y. pseudotub.</i> (2)	0	0	0	2
Total (14)	10/115 ***	1/115	1/115	2/115

* total number of isolates, ** number of yersiniae isolated for each season, *** total number of yersiniae isolated in a season divided by the total number of samples collected.

B Wellington Zoological Gardens

The overall prevalence of yersiniae isolated from passeriform species caught and sampled at the Wellington Zoological gardens was 6%. *Yersinia enterocolitica* and *Y. kristensenii* were the only two species isolated. In the summer period, 80% of the isolates were obtained which is significantly higher than the number of yersiniae isolated in the winter or spring period ($P < 0.001$).

Table (2-10) The seasonal pattern of yersiniae isolated from cloacal swabs taken from passeriform species caught at the Wellington Zoo

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (2) *	2**	0	0	0
<i>Y.kristensenii</i> (3)	2	1	0	0
<i>Y.frederiksenii</i> (0)	0	0	0	0
<i>Y.intermedia</i> (0)	0	0	0	0
<i>Y.pseudotb.</i> (0)	0	0	0	0
Total (5)	4/15***	1/10	0/35	0/20

* total number of isolates, ** number of yersiniae isolated for each season, *** total number of yersiniae isolated in a season divided by the total number of samples collected.

The seasonal pattern of yersiniae isolated from samples taken from birds submitted for necropsy during the year of the survey.

There was a significantly higher number of yersiniae isolated during the spring period than in the summer, autumn and winter periods ($P < 0.05$) in the necropsy samples examined .

Table (2-11) The seasonal pattern of yersiniae isolated from necropsy samples taken from a range of avian species inhabiting rural coastal regions and farm land.

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (14) *	1**	1	0	12
<i>Y.kristensenii</i> (3)	3	0	0	0
<i>Y.frederiksenii</i> (1)	1	0	0	0
<i>Y.intermedia</i> (6)	1	0	0	5
<i>Y.pseudotb.</i> (2)	0	0	0	2
Total (26)	6/45***	1/45	0/45	19/45

* total number of isolates, ** number of yersiniae isolated for the three month period season, *** total number of yersiniae isolated in a season divided by the total number of samples collected.

The seasonal pattern of yersinia isolated from rural populations of house sparrows using two different sources of material.

A Necropsy

The prevalence of yersinia isolated from necropsy samples taken from house sparrows was 44% as shown in Table (2-12). There were more samples collected in the summer and spring period. Although the number of samples is small the results indicate that there is a significant increase in the prevalence of yersinia isolated from necropsy samples obtained from this population in the spring period as compared with the other seasons ($P<0.05$).

Table (2-12) The seasonal pattern of yersinia isolated from necropsy samples taken from house sparrows (*Passer domesticus*) inhabiting farm land.

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (12) *	1**	0	0	11
<i>Y.kristensenii</i> (1)	1	0	0	0
<i>Y.frederiksenii</i> (0)	0	0	0	0
<i>Y.intermedia</i> (6)	1	0	0	5
<i>Y.pseudotb.</i> (2)	0	0	0	2
Total (21)	3/13***	0/4	0/6	18/25

* total number of isolates, ** number of yersinia isolated for each season, *** total number of yersinia isolated in a season divided by the total number of samples collected.

B Cloacal swabs

The total prevalence of yersinia isolated from cloacal swabs taken from wild house sparrows inhabiting rural areas is less than 2% as shown in Table (2-13). There were no yersinia isolated in the winter and spring. The majority of the *Yersinia* strains (90%) identified from cloacal swabs were isolated in the summer with *Y. intermedia* accounting for over 80%. One strain of *Y. enterocolitica* was isolated in the autumn period. There were no strains of *Y. pseudotuberculosis* isolated.

Table (2-13) The seasonal pattern of yersinia isolated from cloacal samples taken from house sparrows (*Passer domesticus*) inhabiting farm land.

Species of <i>Yersinia</i>	Season			
	Summer	Autumn	Winter	Spring
<i>Y.enterocolitica</i> (1) *	0**	1	0	0
<i>Y.kristensenii</i> (0)	0	0	0	0
<i>Y.frederiksenii</i> (0)	0	0	0	0
<i>Y.intermedia</i> (3)	3	0	0	0
<i>Y.pseudotub.</i> (0)	0	0	0	0
Total (4)	3/100***	1/105	0/100	0/105

* total number of isolates, ** number of yersinia isolated for each season, *** total number of yersinia isolated in a season divided by the total number of samples collected.

2-3-6 Yersinia isolated from non-avian sources during the period of the survey (November 1991 and November 1992).

A total of 51 *Yersinia* strains were isolated from 1,356 non-avian samples. Of these 64% were *Y. enterocolitica*, 14% were *Y. frederiksenii*, 18% were *Y. kristensenii* and 4% were *Y. intermedia*. *Y. pseudotuberculosis* was not isolated from any of the samples collected. There is no significant difference in the prevalence of yersinia from the different samples collected ($df = 3, P > 0.05$).

Table (2-14) The relative prevalence of different yersinia isolated from different non-avian sources during the period of the survey.

<i>Yersinia species</i>	Foliage	Soil	Water	Wild mammal	Total
<i>enterocolitica</i>	2	27	1	3	33
<i>frederiksenii</i>	1	5	0	1	7
<i>kristensenii</i>	2	7	0	0	9
<i>intermedia</i>	0	2	0	0	2
<i>pseudotuberculosis</i>	0	0	0	0	0
Total	5	41	1	4	51

A Environmental samples

There was no significant difference in the prevalence of yersiniae between soil and foliage samples ($df = 1$, $P > 0.5$) nor was there any significant difference in the prevalence of yersiniae in the soil samples studied in the winter-spring period compared with the summer-autumn period ($df = 1$, $P > 0.5$).

B Wild animals

Possums

Only four *Yersinia* strains (three isolates of *Y. enterocolitica* and one of *Y. frederiksenii*) were obtained from the animal samples cultured, all of these were from possums which had been necropsied for another study. All of the 65 brush tailed possums (*Trichosurus vulpecula*) sampled had been caught in rural areas of the Manawatu and Wairarapa. Three strains of *Yersinia* were isolated from the caecum and one from the colon (*Y. enterocolitica*) of the possums studied. Two of these isolates (*Y. enterocolitica*) were made in the summer and the others in the autumn (*Y. enterocolitica*) and spring (*Y. frederiksenii*).

Rabbits

A total of 15 rabbit (*Oryctolagus cuniculus*) faecal samples were studied. No *Yersinia* species were isolated from the samples collected. All of the rabbit samples were collected in the summer period.

Rats and mice

A total of 4 wild rats (*Rattus rattus*) and 16 mice (*Mus musculus*), caught in and around rural aviaries, were necropsied. There were no strains of *Yersinia* isolated from the caecum of these animals.

2-3-7 The seasonal distribution of yersiniae isolated from non-avian samples during the period of the survey

The prevalence of yersiniae isolated from non-avian sources was 3.7% . Of the 51 *Yersinia* strains isolated 56% were collected in the winter period and 32% in the summer period. From the 1,032 soil samples taken 41 *Yersinia* strains were isolated, of these 58% were obtained in the winter months compared with 42% in the summer-autumn period. From the 200 foliage samples collected during the year 4 *Yersinia* strains were isolated, of these 75% were obtained in the spring period. The majority of the foliage samples collected were from urban parks or farm land and had been contaminated with animal faeces. Only one *Yersinia* strain (*Y. enterocolitica*) was isolated from water samples obtained during the survey, this was from a sample taken from an aviary pond collected in the winter following a case of pseudotuberculosis infection in a kaka housed in the aviary. Other samples collected from aviary outbreaks of pseudotuberculosis are not included in the survey data but *Y. pseudotuberculosis* was isolated from a faecal sample which had been lying on the ground 24 hours after the death of the kaka.

Table (2-15) A comparison of the seasonal prevalence of yersiniae isolated from non-avian sources between November 1991 and November 1992

(Total number of samples)	Soil	Foliage	Water	Animal	Total isolates
Summer (331)	13/250	1/50	0/6	2/25	16
Autumn (331)	4/250	0/50	0/6	1/25	5
Winter (381)	24/300	4/50	1/6	0/25	29
Spring (313)	0/232	0/50	0/6	1/25	1
Total (1,356)	41/1,032	5/200	1/24	4/100	51/1,356

2-3-8 The relative prevalence of yersiniae in rural and urban soil

Of the 1,032 soil samples tested there were 41 *Yersinia* strains isolated, of these 27 (66%) were *Y. enterocolitica*. Of the 27 isolates 20 were biotype 1A and the others were untypable.

Table (2-16) A comparison of yersinia isolated from soil collected in rural and urban areas

<i>Yersinia species</i>	Rural areas (510)	Urban areas (522)	Total (1032)
<i>Y.enterocolitica</i>	21	6	27
<i>Y.frederiksenii</i>	5	0	5
<i>Y.kristensenii</i>	3	4	7
<i>Y.intermedia</i>	2	0	2
<i>Y.pseudotb.</i>	0	0	0
Total	31	10	41

Of 41 *Yersinia* strains isolated from soil samples 76% were from samples collected from rural areas compared with 24% from urban areas. *Yersinia enterocolitica* accounted from 60% of the urban soil isolates. In the rural samples 68% of the strains were *Y. enterocolitica*, 16% were *Y. frederiksenii*, 10% were *Y. kristensenii* and 6% were *Y. intermedia*. The total prevalence of yersinia in rural soil samples (6%) is significantly higher than in urban soil samples (2%) ($df = 1$, $P < 0.001$).

2-3-9 Comparative prevalence of yersinia isolated from avian and non-avian sources during the period of the survey (November 1991 to November 1992).

The prevalence of yersinia in birds is significantly higher than was found in environmental samples ($df = 1$, $P < 0.001$). In both the avian and the non-avian sources of material *Y. enterocolitica* accounts for the greatest percentage of the isolates.

Table (2-17) A comparison of the relative prevalence of yersiniae isolated from avian and non-avian sources between November 1991 and November 1992.

<i>Yersinia species</i>	Avian (1,370)	Environmental (1,356)	Total (2,726)
<i>Y.enterocolitica</i>	41 (42*)	33 (65)	74 (50)
<i>Y.frederiksenii</i>	26 (26)	7 (13)	33 (22)
<i>Y.intermedia</i>	9 (10)	2 (4)	11 (7)
<i>Y.kristensenii</i>	20 (20)	9 (18)	29 (19.5)
<i>Y.pseudotuberculosis</i>	2 (2)	0 (0)	2 (1.5)
Total	98	51	149

*Percentage of total isolates from the source indicated.

2-3-10 The relationship between birds and the environment, yersiniae in rural and urban birds and soil

There is no significant difference in the prevalence of yersiniae in samples from the rural birds and the rural soil samples ($df = 1$, $P > 0.05$). However, the prevalence of yersiniae in samples from urban birds (11%) is significantly greater than from urban soil samples (2%) ($df = 1$, $P < 0.001$).

Table (2-18) Comparison of the relative prevalence of yersiniae isolated from rural and urban avian samples and rural and urban soil samples.

<i>Yersinia species</i>	Rural areas		Urban areas	
	Soil	Birds	Soil	Birds
<i>Y. enterocolitica</i>	21	13	6	15
<i>Y. frederiksenii</i>	5	0	0	19
<i>Y. kristensenii</i>	3	1	4	11
<i>Y. intermedia</i>	2	9	0	0
<i>Y. pseudotb</i>	0	2	0	0
Total	31/510	25/458	10/522	45/420

The prevalence of yersiniae isolated from avian sources is significantly higher than from soil in the urban areas sampled ($P < 0.01$). The difference is not significant in the rural

areas sampled ($P > 0.05$).

Of the total 74 *Y. enterocolitica* isolates only 55 strains were typable. These were biotype 1A. In addition to these strains a further 41 '*Yersinia enterocolitica*-like' strains of bacteria were isolated from avian samples. These were not included in the results as it was not possible to classify them into a known *Yersinia* species. All were delayed urease positive and could not be classified as *Citrobacter* or *Serratia species* despite sharing similar colony morphology. Not all of the untyped strains were the same and it is not known if they share similar surface antigens to yersiniae.

Discussion

The use of the term 'reservoir'

Although the term 'reservoir' population has not been specifically defined in many studies examining the role of wildlife in the epidemiology of *Y. pseudotuberculosis*, in the present study the term 'reservoir' population is used to indicate a population in which an organism is maintained indefinitely without the need for reinfection from another source.

The results of the bacteriological survey indicate that the prevalence of *Y. pseudotuberculosis* in the faeces of wild birds is low in the populations studied. The prevalence of other yersiniae isolated from birds is similar to that reported in similar surveys of avian faecal flora in Europe (Brittingham *et al.*, 1985), Japan (Kaneuchi *et al.*, 1989; Hamasaki *et al.*, 1989, Fukushima *et al.*, 1991) and North America (Hacking & Sileo, 1974). It might be expected that the prevalence of *Y. pseudotuberculosis* would be high in the faeces of the bird populations studied if they were a true 'reservoir' of this organism especially in view of the fact that 1) *Y. pseudotuberculosis* is transmitted by the faecal-oral route and 2) the infective dose is high. This is not the situation with some diseases however, for example possums are considered to be an important reservoir for *Mycobacterium bovis* although the prevalence of the organism in these animals is low (Livingston, 1991). In addition to factors such as route of infection and infective dose, the sensitivity of culture techniques must be considered when evaluating the role of 'reservoir' populations in the epidemiology of a disease.

Before evaluating data on the faecal flora of wild bird populations it should be noted that the sensitivity of faecal culture is not known for wild birds and possibly gives an underestimate of the true prevalence of the organism. In addition there is limited data available on the faecal shedding pattern of *Y. pseudotuberculosis* in birds and it is not known whether the organism is shed intermittently as is the case for salmonellae (Cooper *et al.*, 1989). Intermittent shedding of salmonellae by infected chickens makes the interpretation of prevalence surveys for salmonellae difficult where faecal isolation alone is used to determine the level of infection (Barrow, 1993).

In human patients, Mair and Fox (1986) report that faecal isolation is often not possible even in clinical cases. This is not necessarily true for *Y. enterocolitica* which is frequently associated with diarrhoea (Mair and Fox, 1986). Because of the difficulty in culturing *Y. pseudotuberculosis* from faecal samples there has been a lot of research effort directed at developing serological tests for the diagnosis of pseudotuberculosis in human patients (Fernandez-Lagos *et al.*, 1991). Serological tests have also been used for screening of livestock for the presence of antibody to yersiniae (Robins-Browne *et al.*, 1993) but there are no reports of the use of these tests in birds. Where possible, in the present study, blood samples were collected from the live birds caught so that the results of the bacteriological study could be complemented with serological data. The results from the serological survey are discussed in chapter three.

Yersiniae in the faecal flora of wild birds

In the present study most of the avian samples were collected from passeriform species inhabiting rural and urban regions of the lower North Island of New Zealand. No outbreaks of the disease were reported in wild birds or livestock in the rural study sites during the period of the study and no strains of *Y. pseudotuberculosis* were isolated from environmental sources.

In New Zealand, Mackintosh and Henderson (1984) isolated 8 strains of *Yersinia pseudotuberculosis* from 158 apparently healthy birds sampled in the 5 month period from March-July. In contrast, there were no *Y. pseudotuberculosis* strains isolated from 144 birds sampled during the months of August, September and October. The birds sampled were shot on a local refuse tip or in the vicinity of a deer farm in the southern

end of the South Island. Four of the isolates from the intestinal contents and faeces were serotype I, one was serotype II and three others could not be serotyped. The prevalence of *Y. pseudotuberculosis* was found to be 5.3% in ducks, 2.3% in sparrows, 2.3% in sea gulls and 1.7% in starlings. The total prevalence in birds was 2.8% (8/302) compared with <0.1% (2/1,370) in the present study. It is probably significant that of the three methods of sample collection used in the present study, necropsy, cloacal swab and collection of faeces on plastic sheets, the former appeared to be the most sensitive with < 1.0% (1/180) prevalence of *Y. pseudotuberculosis* obtained from birds submitted for necropsy compared with no isolates of this organism by the other two methods. However, from the results of the present study and from the literature it is also likely that the location of the study site has an important effect on the prevalence of yersiniae in the birds which are sampled. The farm used in the Mackintosh and Henderson (1984) study may have constituted a contaminated environment resulting in an increased prevalence of *Y. pseudotuberculosis* in birds. Recent studies evaluating the faecal flora of goats (Lanada, 1990) and a study on yersiniae isolated from the intestinal tracts of wild brown rats shot on a rubbish tip (Armitage, unpublished data), in the same region as the present study, indicated that the faecal flora in these species reflected the yersiniae present in the environment, both in type and in frequency of isolation. The results of the present study indicate that in the wild avian populations studied the prevalence of yersiniae is significantly higher ($P < 0.01$) in avian samples than in the non-avian samples collected. However, this may be a result of a concentration of bacteria in faecal material following passage through the intestinal tract resulting in a more concentrated inoculum (Stovell, 1963; Levre *et al.*, 1989). In this way birds could amplify the number of yersiniae and possibly disseminate potentially pathogenic species, derived from environmental sources or other animal species. In addition it was noted from the results that the relative prevalence of yersiniae in urban starling populations was significantly different from that in urban soil samples ($P < 0.05$) while in the rural areas, the soil and avian samples had a similar pattern of yersiniae ($P > 0.05$). It is possible that the difference between the rural and urban birds is due to the different population biology of birds in these areas. Unfortunately there is insufficient data on each species to allow comprehensive comparisons of differences between avian species but it is likely that the composition of the faecal flora of different passerines would be influenced by differences in diet (Brittingham *et al.*, 1985). It would therefore be unwise to speculate in a general sense about the role of 'wild birds' in the epidemiology

of yersiniae without considering the different species of bird, the population biology of that species and the likely interactions between that species and other species in a given area. All of these factors would probably vary with the season. Fukushima *et al.* (1987) have considered this in their epidemiological studies of *Y. pseudotuberculosis* in Japan where there is a regional variation of plasmid types in human *Y. pseudotuberculosis* isolates. They suggest that there is a wildlife reservoir for certain plasmid types of *Y. pseudotuberculosis* serotype Ib and a porcine source of serotype IV. It is possible that in future the wider application of molecular biology will result in further differentiation between strains of *Y. pseudotuberculosis* which may enhance the understanding of the epidemiology of this micro-organism.

Although *Y. pseudotuberculosis* has been isolated from healthy birds (Fukushima *et al.*, 1991; Mackintosh & Henderson, 1984) it is considered by some authors to be highly virulent for many avian species (Keymer, 1980; Parsons, 1991). In the present study the two isolates of *Y. pseudotuberculosis* recorded in the survey results were obtained from the caecal tonsils of house sparrows found moribund on a pig farm. One isolate was serotype I and the other was serotype II. The birds had been poisoned by a local orchard manager. Both isolates contained a virulence plasmid (see chapter four) but there was no indication that *Y. pseudotuberculosis* caused disease in these two birds as it was not isolated from any other internal organs and no lesions were found in the liver, spleen or intestine. *Yersinia pseudotuberculosis* was not isolated from other birds found dead in the area. The mere isolation of *Y. pseudotuberculosis* from a bird does not necessarily indicate that the bird is suffering from clinical disease, however there is the potential for disease to develop should the host's immune system become compromised (Wetzler, 1971). *Yersinia pseudotuberculosis* was not isolated from cloacal swabs taken from the two house sparrows nor from faecal samples collected from other birds in the area but, in a concurrent study *Y. pseudotuberculosis* was isolated from the tonsils of healthy pigs on the same farm. These isolates were serotype I and II (De Allie, personal communication, 1993). It has often been reported that dead birds from which *Y. pseudotuberculosis* has been isolated, have been found around feeding troughs of livestock suffering from pseudotuberculosis. Pullar (1932) and Clapham (1965) concluded that the birds were the source of the infection. Although contamination of food and water supplies by wild birds and rodents has been implicated as the cause of many zoo and aviary outbreaks of the disease (Appleby, 1962; Welsh *et al.*, 1992) the

actual role of wild birds had not been fully investigated. A possible exception would be the brief report by Patterson and Cook, (1963) which implicated wild pigeons as the 'reservoir' of *Y. pseudotuberculosis* for a guinea pig colony which had been fed contaminated kale from a field in which these pigeons regularly visited. However, Slee (1993) suggests that this may not be the case and that the infected birds are probably reflecting a high prevalence of the organism in the environment.

In the present study *Y. pseudotuberculosis* was not isolated from wild birds caught at the Wellington zoological gardens. The relative prevalence of other yersiniae isolated from cloacal swabs collected from these birds and from birds in rural areas was similar to that found in environmental samples. There had been no problem with pseudotuberculosis at the zoo during the period of the study although *Y. intermedia* had been isolated from the faecal samples of monkeys (Fenwick, personal communication, 1993). At the present time there is insufficient data available to determine whether different yersiniae have a different epidemiology but, from the literature, it appears unlikely that all serotypes of *Y. pseudotuberculosis* have a common animal reservoir. In a comparison between the yersiniae isolated from soil in rural and urban areas it was noted that the relative prevalence of the different species was not significantly different ($P > 0.05$) but that the total prevalence was higher in rural than in urban areas. This may reflect a greater degree of environmental contamination by livestock, and possibly rural birds, which amplify and disseminate yersiniae. However, it should also be noted that in the urban areas sampled the habitat was generally shady and damp whereas the rural areas chosen were much more open. Leaf litter and insect populations as well as soil pH and humidity would presumably be different in each habitat and therefore any future epidemiological study would need to include such measurements in the protocol. For the purposes of the present study the emphasis was on the avian samples and the protocol used was designed to examine the role of wild birds as a reservoir for *Y. pseudotuberculosis*; the non-avian samples were included to broaden the scope of the survey. The results of this work indicate that future studies should concentrate on the interaction between wild birds and the environment and possible species differences in carriage of potentially virulent yersiniae.

Yersiniae in non-avian sources

Japanese workers (Fukushima *et al.*, 1991) have found a higher prevalence of *Y. pseudotuberculosis* in wild mammals than in wild birds and have, in many cases, implicated water as the source of infection for humans and other species (Fukushima *et al.*, 1988, 1989). There have also been numerous reports from Europe (Lassen, 1972, Botlzer, 1987) and North America (Harvey *et al.*, 1976; Botlzer, 1979; Langford, 1972) describing the isolation of yersiniae from water and soil. In New Zealand, Mackintosh and Henderson (1984) isolated *Y. pseudotuberculosis* from the environment and also found that the prevalence of *Y. pseudotuberculosis* was higher in the wild rodents sampled than in wild birds. The significance of this finding is difficult to determine without more knowledge on the faecal shedding patterns in different species, the effect of immunity on faecal shedding and the challenge dose required to set up infection in different wildlife species. In a study of 141 brown rats shot at a refuse tip in the Manawatu, (Armitage unpublished data) no isolates of *Y. pseudotuberculosis* were obtained. In the present study *Y. pseudotuberculosis* was not isolated from any of the wild mammals sampled although 3 strains of *Y. enterocolitica* and 1 of *Y. frederiksenii* were isolated from 65 possums caught in various locations for another study. The role of the possum in the epidemiology of pseudotuberculosis in New Zealand has not been previously examined although it is considered to be an important wildlife reservoir for bovine tuberculosis (Livingstone, 1991). The lagomorph species have been considered important reservoirs for *Y. pseudotuberculosis* in Europe (Mair, 1973) but, in the present study, there were not enough samples to evaluate the role of these as possible reservoir populations in this country. Mackintosh and Henderson (1984) found a high prevalence in lagomorph species but their sample size was also small.

Susceptibility to disease in different avian species

Although the consistently low prevalence of *Y. pseudotuberculosis* in the wild passeriforms examined suggests that it is unlikely that these birds constituted a 'reservoir' of infection this is possibly not the case for other avian species. As with different species of mammals it is possible that some avian species, which are less susceptible to *Y. pseudotuberculosis*, could maintain a low level of infection and provide a constant source of environmental contamination. Pigeons are most often implicated as reservoir hosts for the organism (Patterson & Cooke, 1963; Altman, 1986). However, most avian species, including pigeons, do appear to develop the disease under certain

circumstances such as starvation, concurrent parasitic disease or excessive crowding (Wetzler, 1971; Keymer, 1980). The competition for food and the concurrent cool temperatures which occur in the winter and spring, along with the increased survival of *Y. pseudotuberculosis* in the environment at these times of the year (Barre *et al.*, 1979; Mackintosh & Henderson, 1984) would explain why clinical disease occurred more frequently at these times of the year in birds. Differences in roosting behaviour, diet and level of resistance will also affect the extent to which different avian species are important in the epidemiology of pseudotuberculosis.

It was recognised as early as 1898 that there was a wide variation in species susceptibility to *Y. pseudotuberculosis* and this was later confirmed by Beaudette (1940) and Wetzler (1971). Therefore, the infective dose of *Y. pseudotuberculosis* will probably be different for each species of bird. In addition, the dietary habits and flocking behaviour of some avian species influences both the composition of the normal enteric flora and increases the risk of exposure to potential pathogens (Brittingham *et al.*, 1985). When taking these points into consideration it would seem that although some avian species may fulfil the criteria to be a 'reservoir' for a particular disease, the implication that 'wild birds' are involved is too general to be correct.

In the present study, it was decided to evaluate the relative prevalence of yersiniae obtained from different passeriform species and some native birds. From the available data, it was concluded that there is some species difference in the relative prevalence of yersiniae but that the extent of this difference depends on the location of the study site and the season. Unfortunately there were not enough samples from native birds to allow a comprehensive comparison with the introduced species. However, earlier studies (see appendix. A-9) provided some additional information on these species.

Although no pigeons or parrots were included in the survey these species are commonly presented for necropsy following clinical pseudotuberculosis. Although this may reflect the high number of these species held in captivity it is also possible that wild Columbiforms or Psittaciforms would carry *Y. pseudotuberculosis* and contaminate the environment. In a separate study of the faecal flora of 12 apparently healthy common pigeons (*Columba livia*), 3 birds carried yersiniae (Appendix A-9). One of these isolates was *Y. pseudotuberculosis* serotype II. Of nine healthy native parrots (*Nestor notabilis*,

Nestor meriondelis) surveyed in a concurrent study by the Department of Conservation no yersiniae were isolated from faecal samples collected on Little Barrier Island (appendix, A-9). In addition, it is noted that although *Y. enterocolitica* was isolated from one Brown Kiwi (*Apteryx australis mantelli*) in the survey a separate study of the faecal bacteria of 20 Little Spotted Kiwis (*Apteryx oweni*) found no yersiniae in faecal samples (appendix, A-9).

Passeriforms constitute the order of birds most frequently listed in studies of the faecal flora of wild birds (Brittingham *et al.*, 1985; Levre *et al.*, 1989). As noted earlier the results of these studies are similar to those reported in the present survey where similar sampling techniques are compared. This latter point is important as it is clear from the results of the present study that the sampling method can affect the sensitivity of a bacteriological survey. For example, in the present study, the rate of isolation of yersiniae was found to be greater from faecal samples than from cloacal swabs ($P < 0.01$). Although it is noted that the comparisons between isolation rates obtained using the different techniques were based on examination of material from different birds, the result seems valid considering that the amount of faecal matter collected from the cloaca is often less than that available in faecal material collected from the ground. In the present study samples taken from necropsy cases had the highest isolation rate of yersiniae, but it should also be noted that the faecal and cloacal samples were collected from birds presumed to be healthy while the former were from birds which, although they had died from causes other than pseudotuberculosis, may be considered more susceptible to bacterial disease. Therefore, when comparing the isolation rates of *Yersinia spp* from avian species reported in the literature, it is important to take into consideration the range of sampling techniques used and the source of the sample. For example the study by Mackintosh & Henderson (1984) was based on necropsy material collected from birds and mammals shot for the study whereas many of the other studies on avian enteric bacteria (Brittingham *et al.*, 1985; Hamasaki *et al.*, 1989; Fukushima *et al.*, 1991) including the present one, are predominantly based on culture of collected faecal samples or cloacal swabs taken from live birds. In addition Mackintosh and Henderson (1984) studied birds which inhabited an area which was known to be contaminated with *Y. pseudotuberculosis*.

Bacteriological methods

Iannibelli *et al.* (1991) examined the effect of different culture and isolation techniques on the successful isolation of yersiniae from avian samples and found that the combination of cold enrichment and the use of a selective agar medium compared favourably with other methods. Therefore although many comparative studies have used different culture techniques, for example Mackintosh and Henderson (1984) used direct culture onto MaConkey agar whereas many of the more recent studies used a combination of cold enrichment and selective agar, this does not appear to be a sufficient reason for differences in the prevalence of yersiniae in different studies.

Seasonality

Mackintosh and Henderson (1984) collected environmental samples and were able to isolate *Y. pseudotuberculosis* more readily in the winter than in the summer (unpublished data, Mackintosh & Henderson, 1984). Barre *et al.*, (1979) found that the prevalence of *Y. pseudotuberculosis* was more common in soil samples in the winter, especially when the water table was high. It is widely reported that *Y. pseudotuberculosis* and other potentially pathogenic yersiniae survive well in the environment, especially during the cooler wet months of the year (Mair, 1973). The ability to compete well in cool, wet conditions gives *Y. pseudotuberculosis* a selective advantage over other environmental bacteria in the winter and spring in temperate countries, this adaptation is exploited when using the technique of cold enrichment when attempting to isolate yersiniae from faecal and environmental samples. In the present study it was found that, if all the results were considered together, there was generally a high prevalence of environmental yersiniae isolated from the faeces of birds in the early summer period with a reduction in the late summer and autumn and an increase in the winter. The same trend was seen in yersiniae isolated from the environmental samples collected. It is possible that the fall in autumn follows a lower prevalence of yersinia in the environment after the warmer weather of the summer months. However, when different groups of birds are considered separately a different trend appears. In the starlings, 75% of the *Yersinia* isolates were from samples collected in the winter whereas the majority (83%) of the isolates were obtained in the summer in rural house sparrows. There is insufficient data

in the present study to determine whether or not these variations reflect a difference in the occurrence of the organism in the rural and urban areas although the differences found are statistically significant. Swaminathan *et al.* reviewed reports on the prevalence of yersiniae in Europe and found that there were more yersiniae isolated in the cool months of winter and spring. However, in South Africa it is reported that the prevalence of *Y. enterocolitica* infection is highest during the summer period from December-May which coincides with the wet season (Rabson & Koornhof, 1993). It was suggested that, in the cooler months of the South African regions surveyed, it was too dry for yersiniae to survive in the winter.

2-5 Summary

From the results of the present study the following points can be made.

- 1) The prevalence of *Y. pseudotuberculosis* was low in the wild bird populations studied. This was true even in the winter in areas where there had been outbreaks of the disease in aviary birds.
- 2) Yersiniae are common in the environment and in the faeces of apparently healthy wild mammals and birds. The relative prevalence of yersiniae in samples collected from the wild birds examined in the present study is similar to that reported in surveys overseas.
- 3) Differences in the faecal flora of rural and urban birds probably reflect differences in dietary habits although the effect of different sampling techniques on the sensitivity of bacteriological screening should also be taken into consideration. Differences in the population biology of different wild birds and the effect that sampling technique may have on the results of faecal surveys, make comparisons of results from one study to

another difficult.

4) The location of the study site, the nature of the sample collected and the time of the year have a significant effect on the prevalence of yersiniae. As with many opportunist pathogens it is probably wise to consider the epidemiology of yersiniae in different regions independently especially where the topography and weather patterns differ significantly.

In conclusion, any wildlife survey should consider the population biology of the species to be examined. To be an effective 'wild life reservoir' for *Y. pseudotuberculosis* it would be expected that either 1) the host population would not be severely affected by the potential pathogen and would shed bacteria over a long period of time either continuously or intermittently, or 2) individuals in the host population may be susceptible to the disease but would shed large numbers of organisms infecting other individuals in the population before they died. The first option is possibly more common but the latter could be effective if the bacteria were shed for a sufficient period of time or the infective dose was low. In the second option the spread of the disease is dependent on the population dynamics of a species being conducive to the spread of infection such as in birds which share a communal roost site. At the present time not enough is known about the faecal shedding pattern of *Y. pseudotuberculosis* or the pathogenesis of pseudotuberculosis in different avian species to determine which species of wild birds, if any, could constitute a 'reservoir' of infection although it is known that many species of bird can act as amplifier hosts or mechanical carriers of the organism (Stovell, 1963). This will be examined further in chapter four. Due to 1) the limited amount of information available on the use of serological screening for disease surveillance in wild bird populations and 2) the need to develop improved techniques for the study of the pathogenesis of disease in birds, it was decided to develop a simple serological test. Because this study aims to design protocols for sampling rare and endangered birds or wild species which must be sampled and released, it was necessary to develop a test which could be used with small amounts of blood. For this reason blood samples were collected on filter discs. The application of this test and of similar serological tests could greatly improve the sensitivity of disease surveillance of wild birds for infectious agents such as *Y. pseudotuberculosis* which can be difficult to isolate from faecal material. This forms the subject of the next chapter.

CHAPTER THREE

Antigen studies and serology

Introduction

This chapter describes 1) the development of a serological test to detect the presence of antibodies to *Yersinia pseudotuberculosis* serotypes I, II and III in small samples of blood collected from different species of bird and 2) the application of this test to evaluate the level of exposure to *Y. pseudotuberculosis* in the wild birds sampled in the survey outlined in chapter two. The lack of commercial anti-IgG conjugates for use with serum from different avian species directed an attempt to develop a 'blocking' ELISA for the surveillance study in order to avoid the problems in interpretation of results from a 'direct' ELISA test. The specificity of the available anti-chicken-IgG conjugate is not fully known for each species of interest. This is a particular concern because there is a wide variation in the nature of antigenic epitopes on the IgG of different avian species (Enberg *et al.*, 1992; Higgins & Warr, 1993).

Much of the early literature on the subject of avian serology has been re-evaluated in the attempt to develop a sensitive and specific screening test for *Salmonella* infection in the chicken (Barrow, 1992). Thain & Cullen (1978) and Williams & Whittemore (1975) considered some of the problems associated with an age related degree of serological response in chickens. The correlation between serological titres measured using different tests available for the serological diagnosis of *Salmonella* infection in chickens was also examined (Williams & Whittemore, 1976). Garren and Hill (1959) noted that there was some breed difference in the antibody response to an oral challenge with *Salmonella spp.* in chickens. The White Leghorn was found to produce lower levels of agglutinating antibodies than heavier breeds such as the Rhode Island Red. Although it was generally believed that the White Leghorn was more resistant to *Salmonella* infection the relationship between resistance and low agglutination titres was not clear (Garren & Hill, 1959). Chart & Rowe, (1992) and Barrow (1992) concluded that the ELISA test using the 'O' antigen of the *Salmonella* lipopolysaccharide (S-LPS) cell wall, produced the most sensitive diagnostic test for chickens in experimental situations and in the field.

There have been many modifications to the recommended protocol for serological diagnosis of yersiniosis in human patients and this is possibly due to the inherent problems associated with the use of any single serological test (Bottone & Sheenan, 1983; Maki-Ikola *et al.*, 1991). Problems have arisen in both the sensitivity and the specificity of traditional tests based on agglutination reactions. This is a well recognised problem in bacterial serology due to the convergent evolution of antigenic types in organisms which share a similar ecological niche (Schniatman & Klena, 1993).

In common with many of the *Enterobacteriaceae* the somatic or 'O' antigen forms part of the lipopolysaccharide coat of *Y. pseudotuberculosis*. It is part of this fraction which determines the serotype specificity (Mair & Fox, 1986; Fernandez-Lagos *et al.*, 1991). The rest of the lipopolysaccharide coat is composed of common polysaccharide chains and lipid A. Lipid A is common to all Gram negative bacteria and is the major component of the 'endotoxin' present in the cell walls of all the *Enterobacteriaceae*. Lipid A is highly immunogenic and, in some susceptible host species, low doses can also result in endotoxic shock (Bone, 1993). It is thought that tumour necrosis factor (TNF) and interleukin-1 (IL-1) are major mediators of these reaction and may be important in the outcome of overwhelming Gram negative infections (Bucklin *et al.*, 1993). Lipid A is responsible for the cytokine-mediated acute phase inflammatory response identified in Gram negative infections (Bone, 1993).

It has long been recognised that *Y. pseudotuberculosis* serotype II has sugar components in the 'O' antigen which are common to *Salmonella* group B and that *Y. pseudotuberculosis* serotype IV has sugar components in the 'O' antigen common to *Salmonella* group D (Wetzler, 1965, 1971; Mair & Fox, 1986). However, the degree of observed cross-reactivity with other *Enterobacteriaceae* depends on the purity of the antigen used in the test. This is because of the cross-reactive enterobacterial protein antigen present in all common enteric species (Hofstra & Dankert, 1979; Taguchi *et al.*, 1991). Most animal species will have been exposed to these common cross-reactive antigens and thus have some degree of non-specific antibody reaction (Mair & Fox, 1986). In consideration of the wide variety of bacteria to which wild birds are likely to have been exposed, it was important to explore the possible problems in specificity which may be inherent in a serological test used for surveillance of antibodies to *Y.*

pseudotuberculosis in wild birds. Recent improvements in immunoassay technology such as the enzyme linked immunosorbant assay (ELISA) and the acceptance of the technique of collecting small amounts of blood on filter discs (Minga & Wray, 1992; Barrow *et al.*, 1992) have greatly simplified serological surveillance in wildlife.

In view of the wide range of ELISA tests currently available and the confusion in the literature associated with the value of different serological tests available for the diagnosis of yersiniosis (Bottone & Sheeman, 1983; Heeseman, 1990) it was decided to examine the possible reasons for the variable success of present serological tests. Through preliminary work on the antigenic properties of different bacterial preparations it was possible to develop an ELISA for detecting specific antibodies to *Y. pseudotuberculosis* serotypes I, II and III. Following evaluation of this test in experimental rabbits and chickens it was hoped that the test could then be modified to examine serum samples from other species of bird potentially exposed to the different serotypes of *Y. pseudotuberculosis* in the wild.

At the present time there is no suitable serological test for screening wild birds for bacterial disease nor is there data available on the serological response of chickens, or other birds, following infection with *Y. pseudotuberculosis*. Therefore, in addition to the provision of an epidemiological tool to supplement the bacteriological screening of wild birds, it was necessary to develop a serological test to monitor seroconversion following oral infection in the chicken.

Materials and methods

Recipes for solutions and buffers not defined in the text can be found in the appendix (A-4).

3-2-1 Development of an enzyme-linked immunosorbant assay (ELISA) to determine the presence of antibody to *Y. pseudotuberculosis* serotypes I, II and III.

Preparation of bacterial antigen

The sources of bacteria used in the preparation of antigen are shown in appendix (3-3-1). Bacterial strains were stored in 10% glycerol broth at -70 °C and thawed prior to plating onto blood agar. All cultures were screened to check for purity and for their correct species identification. Inocula on blood agar plates were incubated at 28 °C for 48 hours, harvested into 10 ml of physiological saline broth, centrifuged at 10,000 rpm and the organisms washed prior to antigen preparation.

Formalin fixed antigen (F)

The pellet of bacteria was re-suspended in 10.0 ml of 0.01 % formalin and divided into 10 x 1 ml aliquots prior to storage at 4 °C. This antigen was used for agglutination tests and as an antigen for comparative ELISA work and immunoblot studies.

Heat killed antigen (HK)

The pellet of bacteria was re-suspended in 10.0 ml physiological saline and autoclaved for 2 hours at 121 °C. This antigen was used to immunise rabbits and chickens for the production of hyperimmune sera. In addition aliquots of this antigen were used for comparative ELISA work and immunoblot studies.

Lipopolysaccharide antigen (LPS)

Bacteria were prepared as for the heat killed antigen, divided into 1.0 ml aliquots, centrifuged for 10 minutes at 10,000 rpm and re-suspended in 1.0 ml of SDS-PAGE sample buffer (see appendix A-4). To each 100 microlitre aliquot an equal volume of 10 mg/ml proteinase-K was added. The mixture was incubated at 60 °C in a water bath for 2 hours, boiled for 15 minutes and dialysed against distilled water for 5 days. This method is a modification of that outlined by Chart & Rowe, (1992). The LPS antigen was used for comparative ELISA work and immunoblot studies in addition to the examination of the response to endotoxin in the chicken.

Production of serotype specific antisera

Rabbits

The production of typing antisera in adult New Zealand White rabbits was based on a protocol developed at the Pasteur institute, Paris (E. Carniel, personal communication). Two New Zealand White rabbits were used for each of 6 serotypes of *Yersinia pseudotuberculosis* and *Y. enterocolitica* serotype 0.3. The procedure is outlined in Table (3-1) and involved the injection of washed heat killed bacteria (HK antigen) into the ear vein of the rabbit with a sequential increase in the size of the dose over a period of three weeks. Twenty millilitres of blood was collected by cardiac puncture of anaesthetised rabbits 10 days after the final injection. The rabbits were returned to the breeding colony after blood collection. The blood was stored at 4 °C overnight prior to centrifugation to separate the serum from the blood clot. Serum was collected and stored in 1.0ml aliquots at -70 °C. The agglutination titre of each hyperimmune serum was determined by tube agglutination as described by Mair (1973). This serum was used for serotyping clinical isolates, as a primary antibody in immunocytochemical tests and as a positive control serum in ELISA studies and immunoblot work.

Table (3-1) Protocol for the production of hyperimmune serum in adult New Zealand rabbits and two-week-old male white leghorn chickens.

Day	Rabbit	Chicken
0	Collect pre-inoculation serum	Collect pre-inoculation serum
1	Inject 0.5 ml heat killed antigen into the marginal ear vein	Inject 0.5 ml heat killed antigen into the wing vein
5	Inject 1.0 ml	Inject 1.0 ml
9	Inject 2.0 ml	Inject 1.0 ml
13	Inject 4.0 ml	Inject 1.0 ml
23	Collect blood by cardiac puncture of anaesthetised rabbits	Collect blood by exsanguination of stunned birds

Chickens

The procedure used for the production of hyperimmune serum in the chickens was similar to that in the rabbits and is outlined in Table (3-1). Two-week-old male White Leghorn chickens were used for the production of each bacterial strain. Heat killed *Y. pseudotuberculosis* serotypes I,II and III, *Y. enterocolitica* 0.3, and the environmental yersiniae, *Y. kristensenii* and *Y. frederiksenii* were prepared as outlined previously. Blood was collected by exsanguination of stunned birds 10 days after the final injection, this was stored at 4 °C overnight and centrifuged to separate the serum from the blood clot. Aliquots of 1.0 ml were stored at -70 °C after agglutination titres to the homologous antigen had been determined using tube agglutination as for the rabbit serum. This serum was used as a positive control serum in ELISA work and for immunoblot work. In addition, it was attempted to prepare hyperimmune serum to proteinase-K digested lipopolysaccharide in chickens, using the same protocol as for heat killed antigen, by inoculating chickens with the purified (LPS) antigen.

Agglutination reaction

Serum samples to be tested were incubated at 56 °C for 30 minutes to destroy complement. Serial two fold dilutions of hyperimmune chicken or rabbit serum were made and 0.5 ml aliquots were added to 0.25 ml of 0.1 % formalin fixed antigen. Samples were incubated at room temperature in test tubes and read after 10 minutes.

Positive samples were those in which agglutination had occurred with the result that there was a clump of material seen at the base of the tube. Each sample was tested in triplicate. The titre was the highest dilution of serum which resulted in agglutination.

ELISA procedure

Indirect ELISA

The indirect ELISA was based on the procedure of Fernandez-Lagos *et al.* (1991) with minor modifications. The test was carried out in 96-well, flat-bottom, polystyrene microtitre plates (Nunc, Inter Med, Denmark) and all the components of the test were added in 100 micro-litre volumes. The plates were always incubated at room temperature (20-25 °C). ELISA plates were coated with 1/100 dilution of prepared antigen (F,HK or LPS) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 and left overnight at 4 °C. The optimum concentration of antigen was determined using chequer board titrations of antigen and positive control antiserum. The next day plates were washed three times in PBS containing 0.05 % Tween 20 (PBS-Tween). Plates were stored at 4 °C until required. Prior to the addition of test serum, plates were washed in PBS-Tween and incubated at room temperature with blocking buffer (PBS 0.05% Tween 20 + 3% milk substitute, Coffee-mate, Carnation). Test and control sera were diluted in blocking buffer and titrated across the plate. Antisera were incubated with antigen for 2 hours and plates were then washed three times with PBS-Tween. Commercial anti-IgG peroxidase conjugate was added at a predetermined concentration (1/10,000 for goat anti-rabbit-IgG, Sigma Immunochemicals, A-0545; 1/1,000 for sheep anti-chicken-IgG, The Binding Site, Birmingham) diluted in blocking buffer. The peroxidase conjugate was incubated with the attached antibody for 1 hour and plates were washed three times in PBS-Tween prior to the addition of substrate (o-Phenylenediamine Dihydrochloride, OPD; Sigma, P-8287 4mg/10ml PBS with the addition of 3 microlitres of hydrogen peroxide/10 ml). After 10 minutes incubation in the dark the colour change in each well was stopped by the addition of 1.0M sulphuric acid and optical densities were read at a wavelength of 492 nm by an automated ELISA reader (340 ATC-labinstruments Geo.m.b.H, Untersbergstrabe 1, Salzburg). The titre of each hyperimmune serum with the homologous antigen was determined. Cross reactions between different bacterial preparations and heterologous hyperimmune rabbit

and chicken serum were evaluated using bacterial antigen, prepared using different methods (F, HK or LPS) for the yersiniae of interest and *Salmonella* groups B and D.

3-2-2 Evaluation of the 'indirect' ELISA test using sera from an experimentally infected group of chickens

Thirty, one-day-old male White Leghorn chickens were purchased from a commercial company and tested by faecal culture to make sure they were free of *Yersinia spp.* and *Salmonella spp.* These were raised in five groups of six birds. The birds in five of these groups were given a single oral dose of *Y. pseudotuberculosis* serotype II which had been isolated from a the spleen of a lorikeet (LORIKEET-MUVPPH-92). Birds were dosed at two weeks of age with serial dilutions (10^4 - 10^8) of the challenge isolate. Blood samples were collected prior to oral challenge and every two days following challenge for twenty days. Samples were collected from each bird on a filter disc which contained six segments. For ELISA tests it had been previously determined, using discs coated with hyperimmune rabbit and chicken blood, that two segments incubated overnight with 100 microlitres of blocking buffer eluted the equivalent of 1 microlitre of serum under the conditions stated. This was determined by assessing the dilution of serum which gave an equivalent optical density reading to the serum eluted from the discs. Samples were labelled and stored at 4 °C until the end of the experiment when all samples could be tested together. ELISA plates (Nunc, Denmark) were coated with *Y. pseudotuberculosis* serotype II LPS antigen as outlined in the previous section. On each test plate serial dilutions of hyperimmune chicken sera were used as a positive control serum. The optical density readings for a 1/100 dilution of each serum tested is given in Table (3-3). The optical density readings of 1/100 dilution of serum samples from the experimental birds prior to oral *Yersinia* infection were determined and were used as negative control sera on all ELISA tests.

3-2-3 The development of a 'blocking ELISA' to measure the antibody titre to different serotypes of *Y. pseudotuberculosis* in different species of bird.

ELISA plates (Nunc, Denmark) were coated with a 1/100 dilution of HK antigen as outlined for the 'indirect' ELISA. Plates were washed three times in washing buffer (PBS-0.05% Tween 20) and incubated for 30 minutes with blocking buffer (PBS-0.05% Tween 20, 3% milk substitute). The optimum concentration for the primary (chicken or rabbit) and secondary (rabbit or chicken) antisera was initially determined in an 'indirect' ELISA with most of the chicken antisera used at a concentration of 1/10 and the rabbit antisera at a concentration of 1/10,000. Sera were diluted in blocking buffer and added as 100 microlitre aliquots to each well. Serial two fold titrations were made across the plate of the primary antisera and the secondary antisera were used at a single concentration in order to determine the ability of the primary antiserum to block binding of the second antiserum. On each plate, serial dilutions of primary and secondary hyperimmune sera against the homologous antigen were made to provide a standard curve for each antiserum on each test plate. This also allowed a determination of the variation of antigen coating on each plate. In each 'blocking test' the primary antibody was incubated with the antigen for 1 hour, the plate was washed three times in blocking buffer and the secondary antibody was added. The secondary antibody was incubated with antigen not already bound by the primary antibody, for 1 hour. Plates were washed three times prior to the addition of species- specific IgG peroxidase conjugate, incubated for 1 hour and washed again three times. It had been previously determined that anti-chicken IgG did not recognise rabbit IgG and that anti-rabbit IgG did not recognise chicken IgG. One hundred microlitres of substrate (OPD, Sigma, P-8287) was added to each well on the plates and incubated in the dark for 15 minutes. The reaction was stopped with 1M sulphuric acid. Plates were read using the automated SLT 340 ATC system (Labinstruments, Salzberg) at a wavelength of 492 nm. The 'blocking test' was optimised using positive and negative control sera to achieve optical density readings in the range 1.000-2.500.

3-2-4 Use of the 'indirect' ELISA test for measuring serum samples on discs from chickens and other species of bird.

Blood was collected on filter discs from the chickens used for the production of hyperimmune serum, from experimental chickens prior to and following oral challenge with *Y. pseudotuberculosis* serotype II and from wild birds caught in mist nets during the survey described in chapter 2. The filter discs were purchased from Wallaceville Animal Health Laboratory (Upper Hutt) and the disc ELISA technique used, was based on that described by Chart & Rowe, (1992). For each bird one or two 6 tag discs were used to collect blood following a small needle prick in the brachial vein at the radio-ulna joint. Each small tag was saturated in whole blood. ELISA plates were coated with a 1/100 dilution of *Y. pseudotuberculosis* LPS antigen (serotypes I, II and III). For each test, plates were initially incubated with blocking buffer and washed three times with washing buffer (PBS-0.05% Tween-20) between each step. Serial titrations of known positive and negative control chicken serum were added to each plate to give a standard curve for the test antigen. For the disc test in the survey two disc tags per bird were added to a well in each of the three serotype tests (*Y. pseudotuberculosis* serotypes I, II and III). Templates were prepared for each plate to identify the samples. One hundred microlitres of phosphate buffered saline (PBS) was added to each well and incubated overnight at room temperature. The next day the plates were washed prior to the addition of anti-chicken-IgG peroxidase conjugate (The binding site, Birmingham). Following incubation at room temperature for 1 hour, plates were washed, and substrate was added and optical density values read as outlined earlier.

3-2-5 The use of the direct ELISA to determine the affinity of commercial anti-chicken IgG for IgG of other avian species

Serum samples, taken from wild birds, were eluted from blood coated discs overnight, as described earlier. Aliquots of 100 microlitres of a 1/100 dilution of each serum sample were transferred to each well on labelled Nunc ELISA plates. Templates were made to allow identification of the source of each serum sample and the plates were left overnight to incubate at room temperature. Chicken serum and serum eluted from blood coated discs was titrated along the first two rows of each plate to act as a positive control and rabbit serum was used at a 1/100 dilution as the negative control. The

following day the plates were washed three times in PBS-Tween. Conjugate (The binding site, Birmingham) was added at a 1/500 dilution and incubated at room temperature with the serum coated plates for 1 hour. The plates were washed three times in PBS-Tween before the addition of substrate (OPD). Plates were incubated for ten minutes in the dark before the colour reaction was stopped using sulphuric acid and optical density readings were read on an automatic ELISA reader as described earlier.

3-2-6 SDS-PAGE gel electrophoresis and Western blot procedures.

The following procedures were used for determining the differences in antigenic structure of different preparations of bacterial antigen, the specificity of hyperimmune serum raised against heat killed bacterial antigen and the specificity of commercial anti-chicken IgG peroxidase conjugate.

Method 1 was used for the majority of SDS-PAGE gel electrophoresis and Western blot work in the study but where greater clarity of bands was required the second method, a system with greater resolution, was used.

Method 1

SDS-PAGE gels were run with each of the samples tested using the method of Laemmli (1970). Antigens (F, HK and LPS) were initially run on a polyacrylamide gradient gel (4-20 %) to determine the optimum gel concentration for later work. It was decided to use a 4 % stacking gel with a 15% running gel in the mini cell system (Bio-rad Mini Protean II, Bio-rad laboratories). Standard molecular weight markers (Sigma SDS-200 KIT, MW 29, 000 to 205,000) were run on each gel. Ten microlitre volumes of each sample were added per well. Gels were run for 45 minutes at 200 V and stained with either Coomassie brilliant blue (Sigma, R-250) or a silver stain based on the method of Tsai & Frasch (1982). Western blots were made of each gel using the method of Towbin *et al*, (1979) in the mini cell system and a transfer time of 2 hours at 100 V. Blots were transferred onto 0.2 micrometre nitrocellulose paper (cellulose nitrate, Micro filtration systems, A020A330R) and soaked in blocking buffer (PBS 0.05% Tween 20 + 3% milk substitute, Coffee mate, Carnation). Each blot was washed three times in

PBS 0.05% Tween 20 prior to incubation in hyperimmune serum. The agglutination titre was determined for each hyperimmune serum against the homologous antigen. Antisera were diluted in blocking buffer and incubated with the blot for 1-2 hours. Blots were then washed three times in washing buffer (PBS-0.05% Tween 20) prior to incubation with a commercial anti-IgG peroxidase conjugate. Sheep anti-chicken IgG (The binding site, Birmingham) was used at a dilution of 1/200 and goat anti-rabbit IgG (Sigma Immunochemicals, A-0545) was used at a concentration of 1/500. After incubating overnight with conjugate, blots were washed three times in washing buffer. Substrate solution, DAB (Diaminobenzidine 5mg/10 ml PBS + 20 microlitres of hydrogen peroxide + 20 microlitres of 1% Nickel-Cobalt solution) was added and the blot was allowed to develop for 2-3 minutes prior to washing with water.

Method 2

A series of 15 % polyacrylamide running gels with a 5% separating gel were prepared and allowed to set with 10 or 15 well combs prior to loading the antigen preparations. The loaded gels were then run at 50 V for the first hour and 100 V for 15 hours overnight at 10 °C in a cooling system. Fifty to one hundred microlitres of antigen sample or 20 microlitres of serum samples diluted in SDS-PAGE buffer were added to each well. Standard molecular weight markers were used. For the serum sample SDS-PAGE a commercial preparation of chicken IgG (Sigma Immunochemicals, I-4881) was prepared and run along with the chicken and other avian serum samples. The reservoir buffer used was a 1M solution of Tris-glycerine-10% SDS (pH 8.3). Two identical gels were run at the same time and one was stained with either Coomassie blue or by the silver stain method (Tsai & Frasch, 1982) and the other was transferred onto nitrocellulose for immunoblotting (Trans-blot system). The transfer time was 4 hours or overnight at 3 °C using a constant voltage of 50 V for the shorter period of time and 35 V for overnight transfer. The transfer buffer used was TGM (Tris-glycine-methanol, pH 8.3). Blots were washed in TBS-Tween washing buffer three times and then incubated with blocking buffer (3% casein hydrolysate in distilled water). Blots were stained with Ponceau red to determine transfer and samples were labelled. Hyperimmune serum was diluted in 1% w/v fish gelatin in TBS. Generally rabbit hyperimmune serum was used at 1/100 and chicken serum at a 1/10 dilution. Blots were incubated with the primary antiserum for 2 hours and where applicable, the secondary

antibody for 2 hours. Blots were washed three times for ten minutes in washing buffer between each step. For immunoblots of serum samples the primary antibody was the sheep antichickens-IgG conjugate (The Binding Site, Birmingham) and this was used at a concentration of 1/200. The substrate used was 4-chloro-1-Naphthol (60mg/ml) dissolved in 19 ml cold methanol added to 100 ml Tris and 60 microlitres of hydrogen peroxide. The blot was developed in the substrate for 5 to 15 minutes and washed in distilled water when the bands were obvious.

3-2-7 The use of Lectins to determine the location of polysaccharide antigen on western blots of proteinase-K digests of *Y. pseudotuberculosis* serotype II antigen (LPS).

SDS-PAGE gel electrophoresis was carried out as described above with the addition of 100 microlitres of S-LPS antigen prepared using two strains of *Y. pseudotuberculosis* serotype II (IP 2935, YPS-LORIKEET) added to each of two wells. Western blotting was carried out as in method two. Commercial biotinylated Lectins (TP, Sigma L-3134; BSI, Sigma L-3759) were prepared at a concentration of 0.05 mg/ml and diluted at a 1/4 concentration in casein hydrolysate. Blots were cut into strips according to the antigen of interest and incubated in the lectin mixture for 2 hours. The lectins used were chosen because of their affinity for sugar fractions known to be present in the 'O' antigen of *Y. pseudotuberculosis* (Davies, 1958; Samuelson *et al.*, 1974). The lectin TP (*Tetragonolobus purpureas*) has an affinity for alpha-L-fucose (*Y. pseudotuberculosis* serotype IIa) and BSI (*Bandeiraea simplicifolia*) has an affinity for alpha-D-galactose (*Y. pseudotuberculosis* IIa & b). To increase the sensitivity of the reaction it was decided to mix the available lectin solutions together. Following incubation the blots were washed in washing buffer three times. Blots were then incubated in a 1/400 dilution of Streptavidin-biotinylated horseradish peroxidase complex (Sigma, 50 microlitre/20 ml) for 2 hours. Following washing the blot was developed in amino-ethyl carbazole (AEC). This was prepared by adding 2 ml of 10 mg/ml of AEC in acetone (di-methylformamide) to 50 ml of acetate buffer (50 mM acetate solution, pH 5.0) followed by the addition of 7 microlitres of hydrogen peroxide. The blot was allowed to develop for 8 minutes and then washed in washing buffer.

Results

During the development of a disc ELISA test for the serological screening of avian species it was necessary to examine the antigenic components in different preparations of yersiniae and how these related to the antigenic characteristics of other enteric bacteria. The data generated during the initial test development described in sections 3-3-1 to 3-3-3 is relevant to the application of *Yersinia* serology in all host species. The result sections 3-3-4 and 3-3-5 deal specifically with the use of the test in avian species.

3-3-1 Preparation of hyperimmune serum in rabbits and chickens

The titres for each hyperimmune serum tested against homologous antigen in either the agglutination test or the ELISA test were higher in the rabbits than in the chickens ($P < 0.001$) in which hyperimmune serum had been raised using a similar immunisation protocol. The sera outlined in Table (3-2) were used for serotyping *Y. pseudotuberculosis* isolates and as positive control sera in the ELISA test. Cross reactivity in the ELISA test was minimised when sera were used at the dilutions indicated in Table (3-3). Because the rabbit sera had higher titres than the chickens for all the antigens tested the rabbit hyperimmune sera were used in the antigen studies. The source of the bacterial isolates used in this chapter are listed in the appendix (A-6).

Table (3-2) A comparison of agglutination titres for hyperimmune chicken and rabbit sera produced for the present study and determined by serial titration against the homologous antigen.

Antigen (HK) *	Agglutination titre			
	Chicken 1	Chicken 2	Rabbit 1	Rabbit 2
<i>Y. pseudotuberculosis</i> I	1/128	1/64	1/512	1/256
<i>Y. pseudotuberculosis</i> II	1/32	1/64	1/256	1/128
<i>Y. pseudotuberculosis</i> III	1/32	1/32	1/1024	1/512
<i>Y. enterocolitica</i>	1/32	1/128	1/256	1/128
<i>Y. pseudotuberculosis</i> IV	ND	ND	1/512	1/512
<i>Y. pseudotuberculosis</i> V	ND	ND	1/64	1/64
<i>Y. pseudotuberculosis</i> VI	ND	ND	1/16	1/16
<i>Y. frederiksenii</i>	<1/2	1/8	ND	ND
<i>Y. kristensenii</i>	1/16	1/8	ND	ND

* Heat killed antigen. ND = Not done

Table (3-3) Titres of hyperimmune chicken and rabbit sera produced for use in immunoblotting and ELISA, determined by serial titration in an 'indirect' ELISA test against the homologous antigen.

Antigen (HK)	Titre *			
	Chicken 1	Chicken 2	Rabbit 1	Rabbit 2
<i>Y. pseudotuberculosis</i> I	1/100	1/10	1/5000	1/100
<i>Y. pseudotuberculosis</i> II	1/20	1/10	1/2000	1/200
<i>Y. pseudotuberculosis</i> III	1/40	1/10	1/10,000	1/1000
<i>Y. enterocolitica</i> 0.3	1/10	1/10	1/200	1/200
Antigen (LPS)				
<i>Y. pseudotuberculosis</i> II	1/1	1/1	ND	ND

* Titre = highest dilution of serum that gave an optical density reading > 1.000 at a wavelength of 492 nm in the 'indirect ELISA' and had the least reactivity with heterologous antigen preparations. ND = not done.

3-3-2 Determination of the affinity of commercial anti-chicken IgG for IgG of other avian species using ELISA and immunoblot studies

Use of the 'direct' ELISA

The optical density values derived from direct ELISA tests using plates coated with sera from different species of bird are shown in Table (3-4). Standard curves were derived for determining the optimal concentration of commercial anti-chicken IgG to use in the test and the optical density values obtained for antigen derived from chicken sera collected on discs was found to be significantly higher than those for other species tested. Samples from all the species were collected on discs and treated in the same way for the test.

Table (3-4) A comparison of the optical density values obtained using the 'direct' ELISA test to evaluate the binding of commercial anti-chicken-IgG to sera from different avian species and rabbit sera which were coated on an ELISA plate at 1/100 dilution.

Number of samples	Optical density readings		Species
	Range	Mean	
25	1.035-1.289	1.111	<i>Gallus domesticus</i> *
25	2.012-2.863	2.484	<i>Gallus domesticus</i> **
25	1.025-1.312	1.211	<i>Passer domesticus</i>
20	0.657-0.923	0.810	<i>Turdus merula</i>
25	0.634-0.912	0.846	<i>Carduelis chloris</i>
5	0.068-0.172	0.125	<i>Hemiphaga novezeelandiae</i>
5	0.245-0.546	0.424	<i>Nestor notabilis</i>
5	0.002-0.176	0.078	<i>Trichoglossus haematodus moluccanus</i>
5	0.728-0.937	0.869	<i>Vanellus novaehollandiae</i>
25	0.082-0.088	0.088	<i>New Zealand White rabbit</i>

* Control chickens, ** Immunised chickens.

The sheep antichickens IgG (The Binding Site) used in the present study at a dilution of 1/500 recognises and binds to antigenic components in the chicken samples tested. The higher optical density readings for the immune sera would indicate that there was more IgG in these samples. This was confirmed by immunoblot studies following SDS-PAGE of control and immune chicken sera (see later). Immunoblot studies also

indicated that the conjugate was binding to the IgG component in the serum and not serum albumin. The high optical densities recorded in Table (3-4) for passerine species indicate that the commercial anti-chicken IgG recognises antigenic components in the passerine sera examined. In comparison the optical density values for the pigeon and psittacine samples examined indicate a very limited or negligible amount of cross reactivity with chicken IgG in these species. The low optical density readings for the rabbit sera indicates that the sheep anti-chicken IgG conjugate is not recognising rabbit IgG.

Immunoblot studies to determine the specificity of commercial anti-chicken IgG

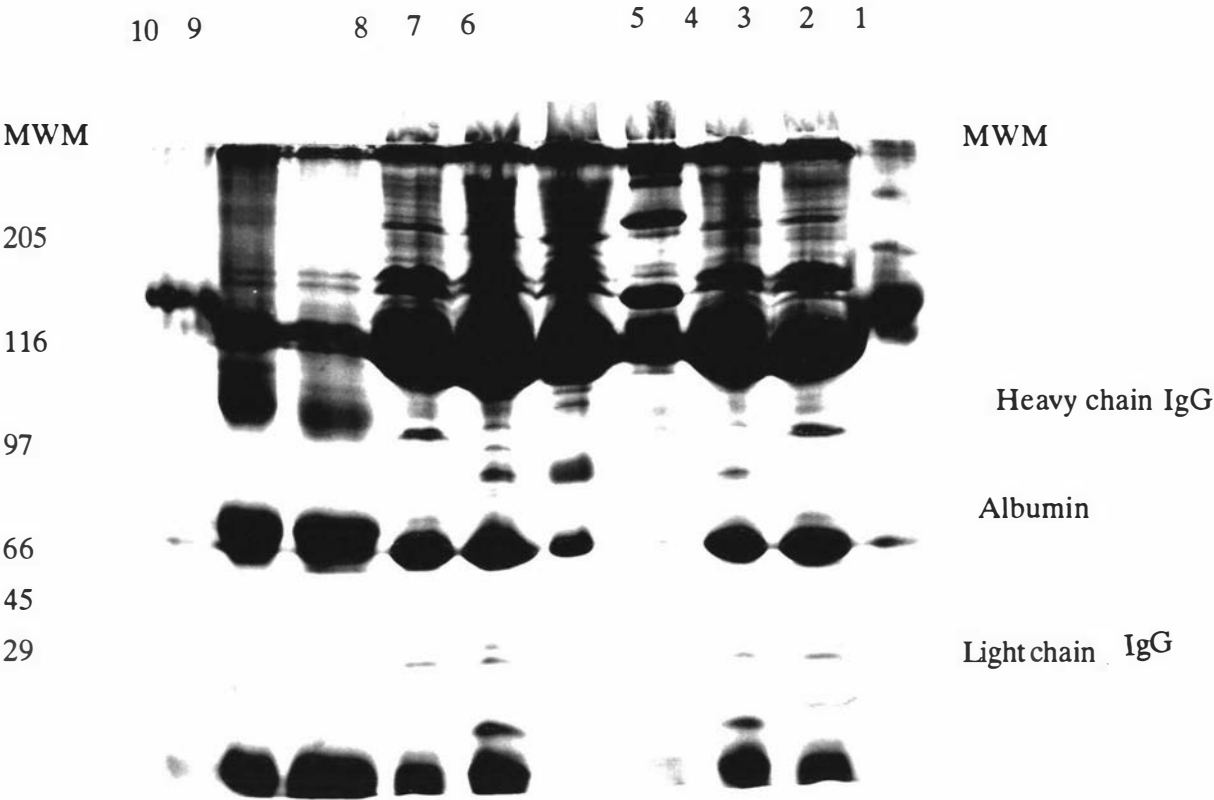
SDS-Page gel electrophoresis of avian serum samples

It can be seen from figure (3-1) that the heavy and light chains of IgG molecules and the serum proteins have similar molecular weights in all the bird sera examined. All have a common albumin band at 67 Kd. Densitometry of this and other Coomassie blue stained gels indicated that the amount of protein present in 2 discs was similar to that present in 1 micro-litre of serum from the same bird.

Immunoblot of SDS-Page gel probed with commercial anti-chicken IgG

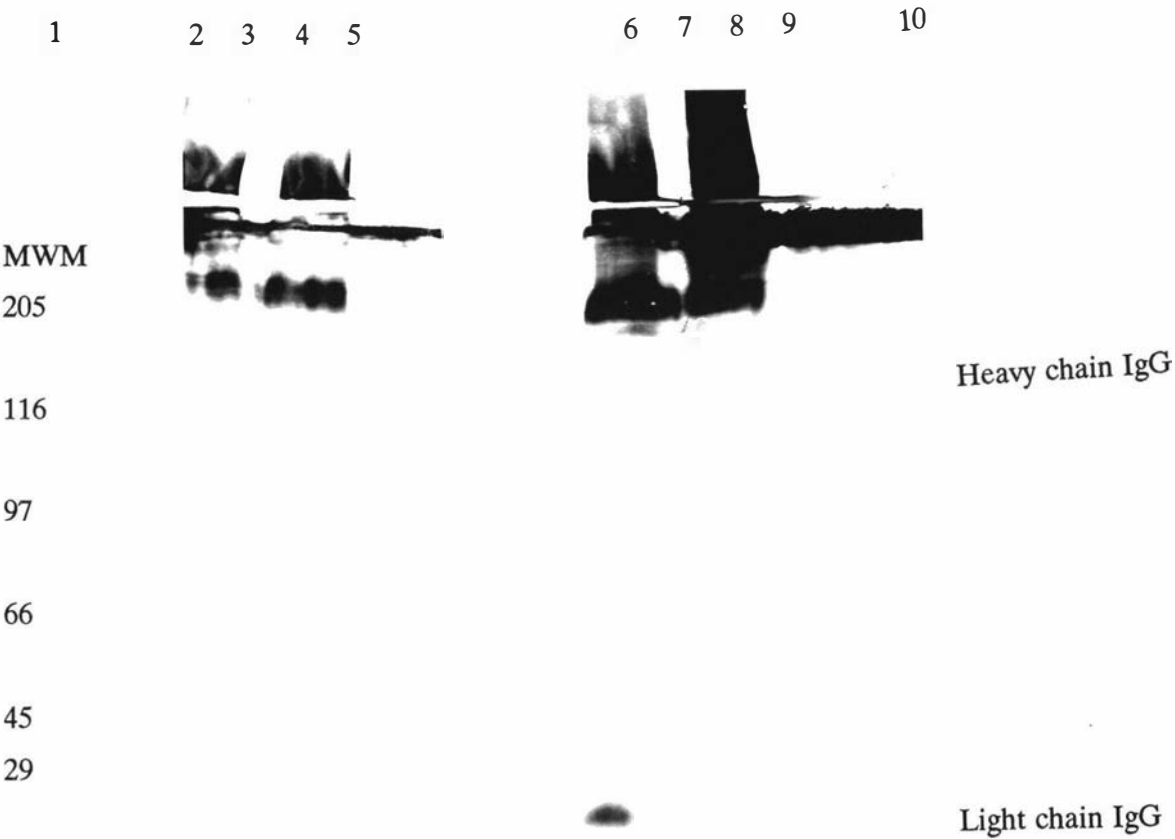
Immunoblot studies (figure, 3-2) were unable to demonstrate that commercial anti-chicken IgG conjugate recognised IgG from other species of bird. The conjugate was found to be specific for chicken IgG within the limitations of sensitivity of this test. The conjugate used at a concentration of 1/200 did not react with albumin in any of the species but there was some degree of reaction with material in the wells which had failed to migrate in the gel.

Figure (3-1) A comparison of SDS-PAGE gel electrophoresis band patterns of sera from different avian species run on a 15% polyacrylamide gel and stained with Coomassie blue stain.



Column 1; Molecular weight markers (Sigma), 2; Chicken serum (control), 3; Chicken serum (immune), 4; Pigeon serum, 5; Pigeon serum (discs), 6; Chicken serum (immune-discs), 7; Chicken serum (control-discs), 8; House sparrow (discs), 9; Kea (discs), 10; Molecular weight markers (Sigma, SDS-200 Kit).

Figure (3-2) Immunoblot of the gel in figure (3-1) probed with a commercial anti-chicken IgG to determine the degree of cross reactivity between IgG in different species.



Column 1; Molecular weight markers (Sigma), 2; Chicken serum (control), 3; Chicken serum (immune), 4; Pigeon serum, 5; Pigeon serum (discs), 6; Chicken serum (immune-discs), 7; Chicken serum (control-discs), 8; House sparrow (discs), 9; Kea (discs), 10; Molecular weight markers (Sigma, SDS-200 Kit). H = Heavy chain IgG, L = Light chain IgG

Evaluation of the validity of using serum eluted from discs

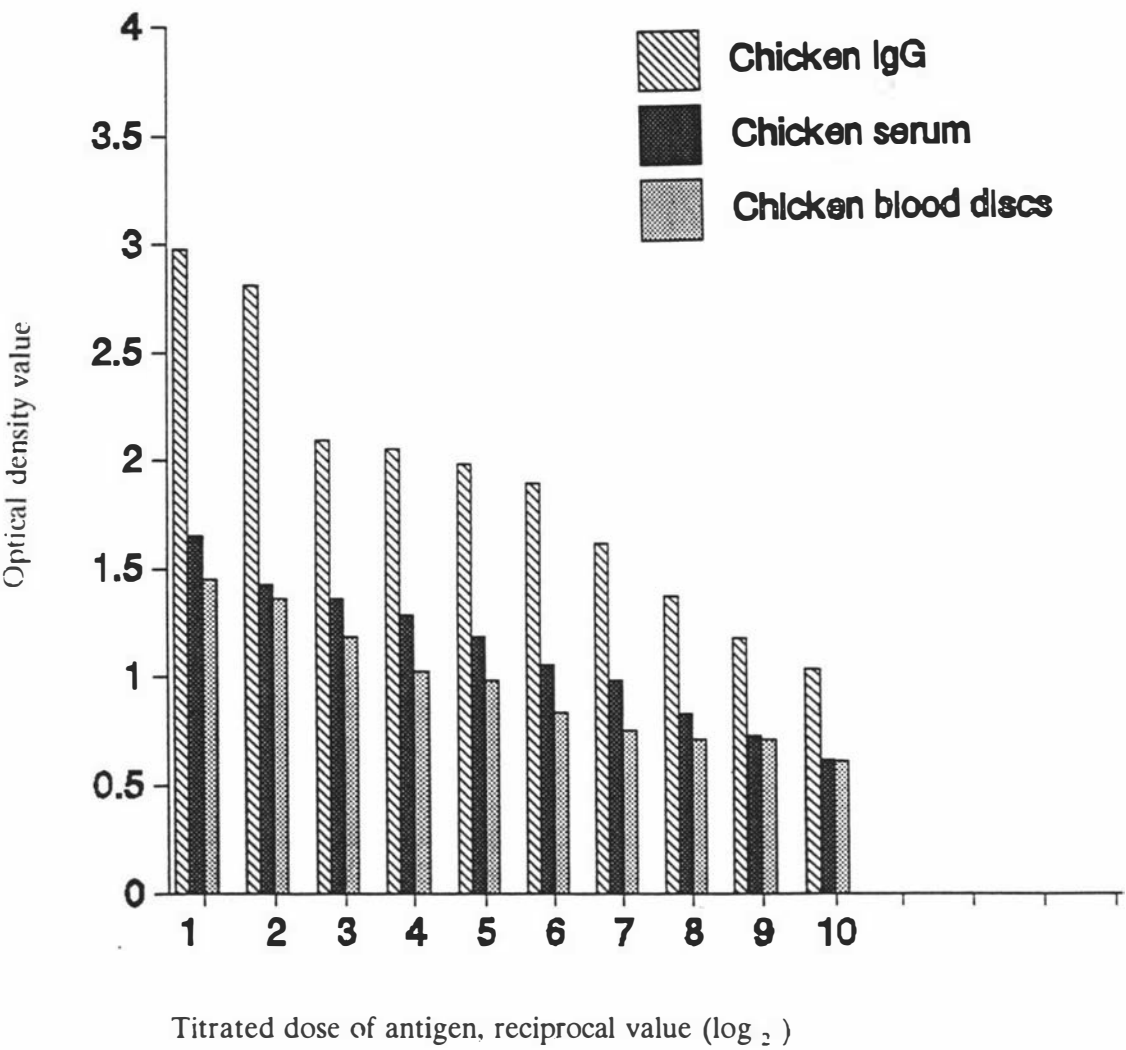
The specificity and sensitivity of commercial anti-chicken IgG for IgG in serum discs compared with serum from the same bird obtained by centrifugation to separate serum from whole blood. Both serum and eluted discs gave detectable reactions at equivalent dilutions of 1/100. The commercial anti-chicken IgG recognised IgG in all three sample preparations.

3-3-3 Determination of the specificity of the 'indirect' ELISA test using hyperimmune sera raised in chickens and rabbits.

Immunoblot work

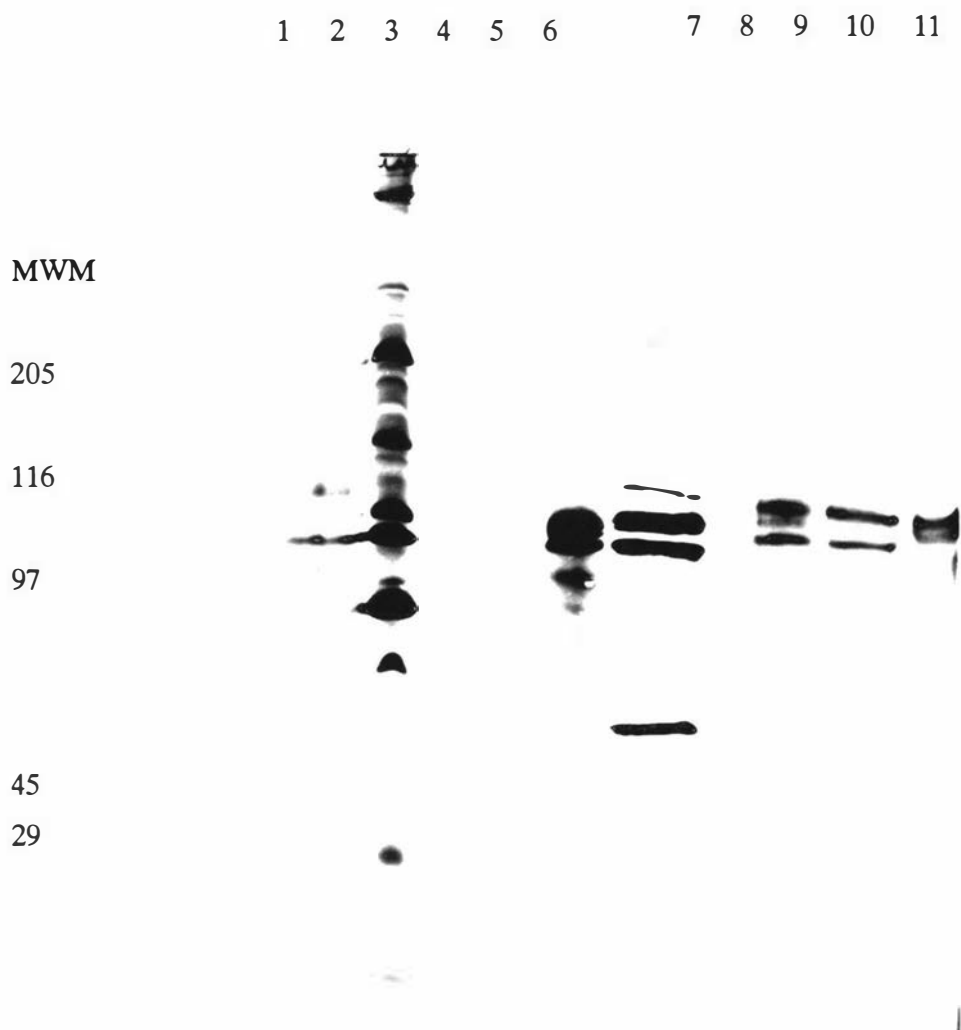
In figure (3-4) it can be seen that rabbit hyperimmune serum raised against a crude HK preparation of *Y. pseudotuberculosis* serotype II antigen cross reacts with antigens in HK preparations of other bacteria. The common Enterobacterial protein antigen is present in all the heat treated preparations and has been recognised by the serum. The antigenic profile of formalin preparations is more complex with several different epitopes recognised in serotype II *Y. pseudotuberculosis* but fewer in serotype III. The LPS antigen for both serotype II and III *Y. pseudotuberculosis* was not recognised in this blot. It was noted that the rabbit antisera reacted with the protein antigen of both *Salmonella* group B and D. The polysaccharide antigen of *Salmonella* group B and of *Y. pseudotuberculosis* was not recognised in the immunoblot. Another blot was prepared of the same antigen preparations and probed with lectin to determine whether the polysaccharide had been transferred. This blot is seen in figure (3-5) and illustrates the binding of lectin bound conjugate to the lipopolysaccharide fraction of a proteinase-K digest of two strains of *Y. pseudotuberculosis* serotype II.

Figure (3-3) A comparison of optical density values obtained in the 'direct' ELISA test when commercial anti-chicken-IgG (Sigma) was incubated with a plate coated with serial titrations of a commercial chicken IgG (Sigma) *, chicken serum ** and chicken serum eluted from blood coated discs. ***



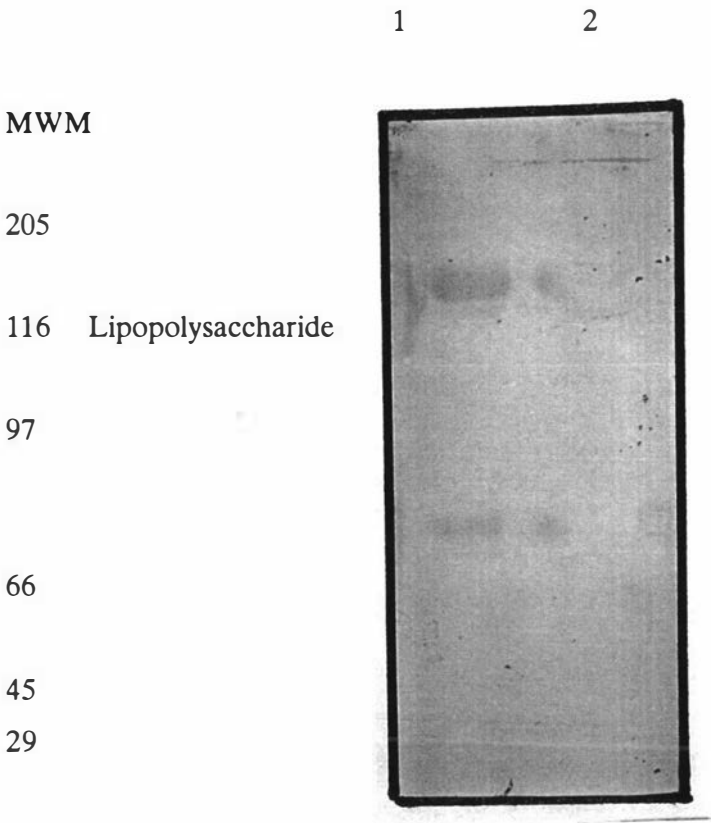
*Chicken IgG (Sigma) prepared as an initial 1/500 dilution. **Chicken serum prepared as an initial 1/100 dilution. ***Chicken blood on discs prepared as an equivalent 1/100 dilution of serum. ****The antigen coating the plate was serially titrated in steps of 2. Commercial anti-chicken IgG peroxidase conjugate (The binding site) was used as the primary antibody in the test. All values are the mean of five separate tests.

Figure (3-4) Immunoblot of different bacterial antigen preparations run in a 15% SDS PAGE gel and probed with rabbit hyperimmune serum raised against heat killed (HK) *Y. pseudotuberculosis* serotype II (IP-2935) antigen.



Column 1; Molecular weight markers (Sigma), 2; *Y. pseudotuberculosis* II (HK) (IP-2935), 3; *Y. pseudotuberculosis* II (F) (IP-2935), 4: *Y. pseudotuberculosis* II (LPS) (IP-2935), 5; *Y. pseudotuberculosis* II (LPS) (LORIKEET-MUVPPH); 6; *Y. pseudotuberculosis* I (HK) (IP-2941), 7; *Y. pseudotuberculosis* III (F) (IP-2889), 8; *Y. pseudotuberculosis* III (LPS) (IP-2889), 9; *Salmonella typhimurium* Group B (HK) (ATCC 14028), 10; *Salmonella panama* Group D (HK) (CDC 186), 11. *Y. frederiksenii* (HK) (LANADA-MUVF).

Figure (3-5) Lectin probed western blot of the lipopolysaccharide antigen (LPS) of *Y. pseudotuberculosis* serotype II (IP-2935) and *Y. pseudotuberculosis* serotype II (LORIKEET-MUVPPH) using the biotinylated lectins, TP and BSI (Sigma).

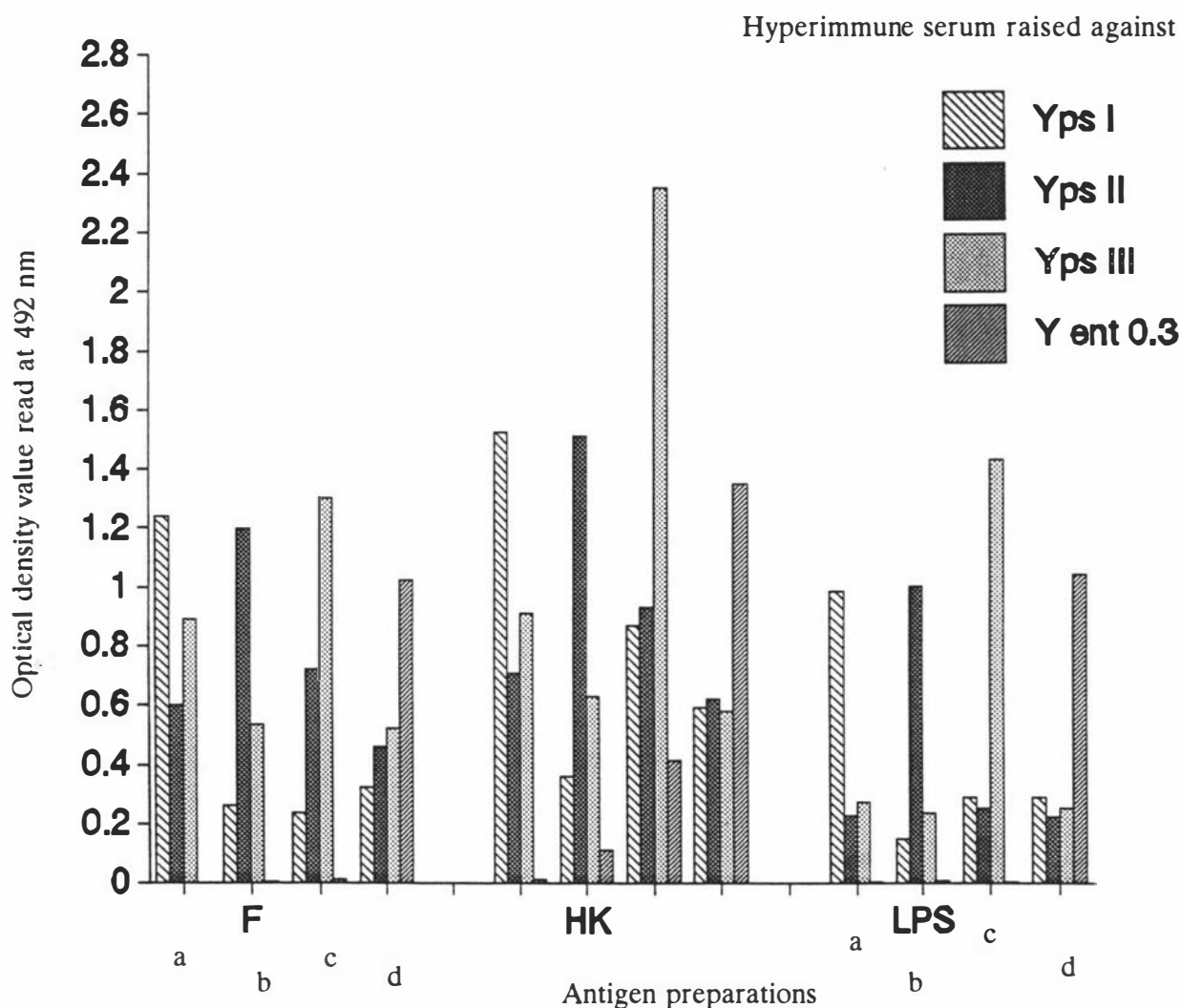


1 represents an LPS preparation of *Y. pseudotuberculosis* serotype II (IP-2935), 2 represents an LPS preparation of *Y. pseudotuberculosis* serotype II (Lorikeet-MUVPPH)

Cross reactivity of hyperimmune sera raised in rabbits and chickens determined by the 'indirect' ELISA

The optical density readings for rabbit and chicken hyperimmune serum incubated in an ELISA test with different antigen preparations are shown in figure (3-6) and figure (3-7) respectively. There is a degree of cross reactivity in the 'indirect ELISA' test whichever antigen preparation is chosen but the LPS preparation results in a more specific test. The heat killed antigen gives a more sensitive test but there is an associated reduction in specificity. The reasons for this were examined in the immunoblot studies which indicated that there was a common immuno-dominant antigen in the heat killed preparations as illustrated in figure (3-4).

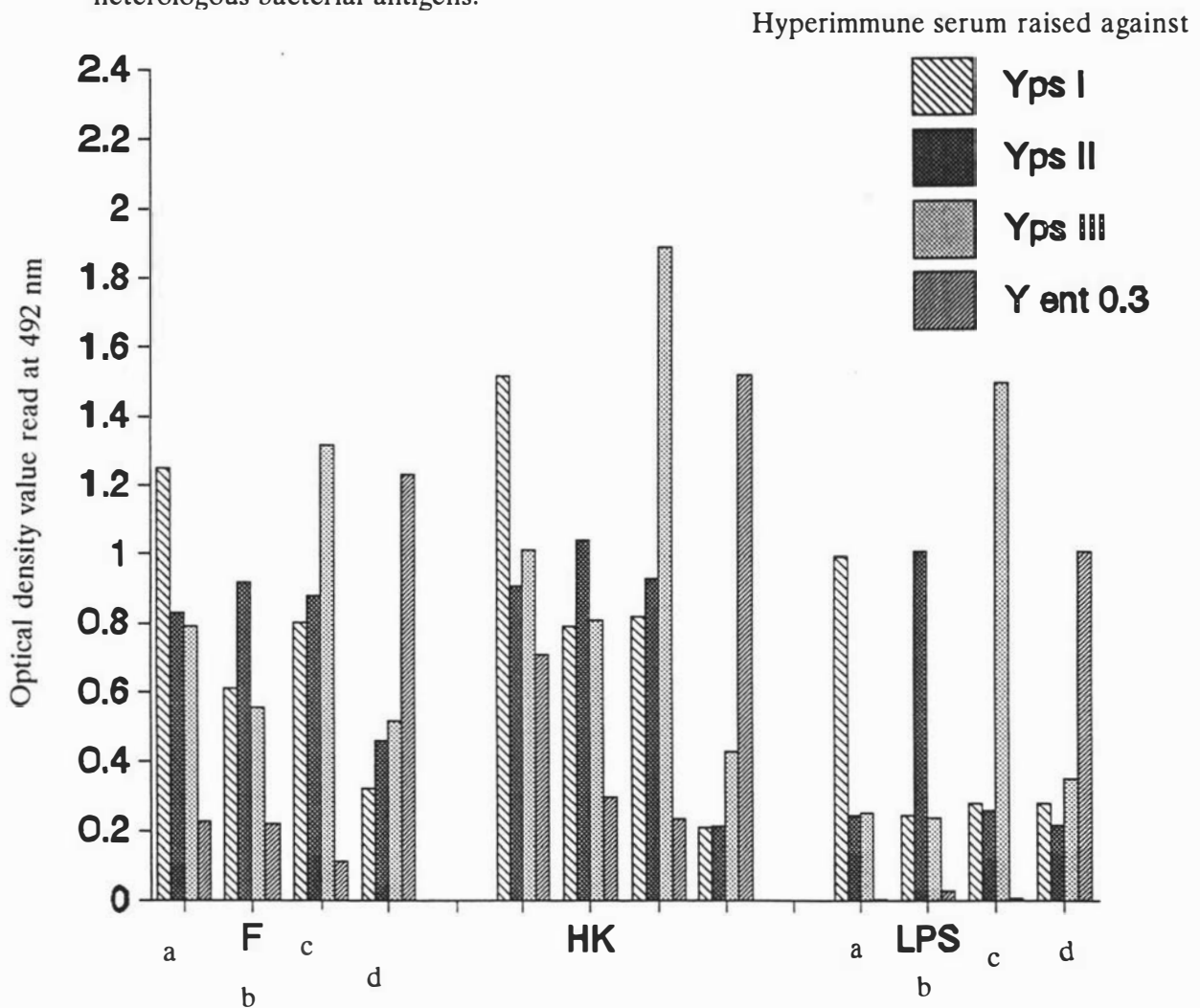
Figure (3-6) A comparison of the optical density values* for different rabbit hyperimmune sera** raised against heat killed (HK) bacterial antigen when used in an 'indirect' ELISA test and incubated with different preparations of homologous and heterologous bacterial antigens.



a = *Y. pseudotuberculosis* serotype I; b = *Y. pseudotuberculosis* serotype II; c = *Y. pseudotuberculosis* serotype III; d = *Y. enterocolitica* 0.3.

*Mean of 5 optical density readings for the same test. All values were repeatable with a range ± 0.050 . **Hyperimmune sera were used at a concentration determined by indirect ELISA of homologous heat killed antigen as shown in Table (3-2). ***Antigen preparations are described in the materials and methods, HK=heat killed antigen, F=formalin fixed antigen, LPS=lipopolysaccharide antigen.

Figure (3-7) A comparison of the optical density values* for different chicken hyperimmune sera** raised against heat killed (HK) bacterial antigen when used in an 'indirect' ELISA test and incubated with different preparations of homologous and heterologous bacterial antigens.



a = *Y. pseudotuberculosis* serotype I; b = *Y. pseudotuberculosis* serotype II; c = *Y. pseudotuberculosis* serotype III; d = *Y. enterocolitica* 0.3.

*Mean of 5 optical density readings for the same test. All values were repeatable with a range ± 0.050 . **Hyperimmune sera were used at a concentration determined by indirect ELISA of homologous heat killed antigen as shown in Table (3-2). ***Antigen preparations are described in the materials and methods, HK=heat killed antigen, F=formalin fixed antigen, LPS=lipopolysaccharide antigen.

3-3-4 Determination of positive/negative cut-off value in the LPS disc ELISA.

Experimental evaluation of the LPS ELISA

A total of 30 serum samples from *Salmonella* and *Yersinia* free birds were examined by LPS ELISA. Results are shown in Table (3-5). Equivalent tests for specific pathogen free (SPF) sera indicated a high titre of antibodies reactive with *Y. pseudotuberculosis* serotype II and the optical density values in the ELISA were higher than those for 1/100 dilutions of pre-inoculation sera as shown in Table (3-5). It was not possible to determine whether or not the SPF flock was free of infection with bacteria which had an antigenic similarity to serotype II *Y. pseudotuberculosis* and therefore it was decided not to use these sera as negative control sera in the ELISA test.

Table (3-5) Determination of positive/negative cut-off points in the LPS ELISA for *Y. pseudotuberculosis* serotype I, II and III; examination of pre-inoculation sera from chickens used in experimental challenge.

ELISA LPS	Number of sera tested	Mean	SD	Range	Mean + 2SD	Mean + 4SD
Yps I	30	0.098	0.05	0.023-0.197	0.198	0.298
Yps II	30	0.154	0.06	0.088-0.297	0.274	0.394
Yps III	30	0.084	0.04	0.012-0.142	0.164	0.244

For experimental sera the cut-off point for each antigen was selected as the mean plus 2 x standard deviations (SD) in order to decrease the number of false positives while maintaining sensitivity. Results for the survey work are presented with cut off points of mean plus 2 x SD and mean plus 4 SD to allow for the possibility of potential cross reactivity in the sera of free ranging birds ie 0.274 and 0.394 respectively in the serotype II assay ie a loss of sensitivity was being accepted in favour of enhanced specificity.

3-3-5 Evaluation of the serological response to LPS antigen in chickens experimentally infected with *Yersinia pseudotuberculosis* using the 'indirect' ELISA.

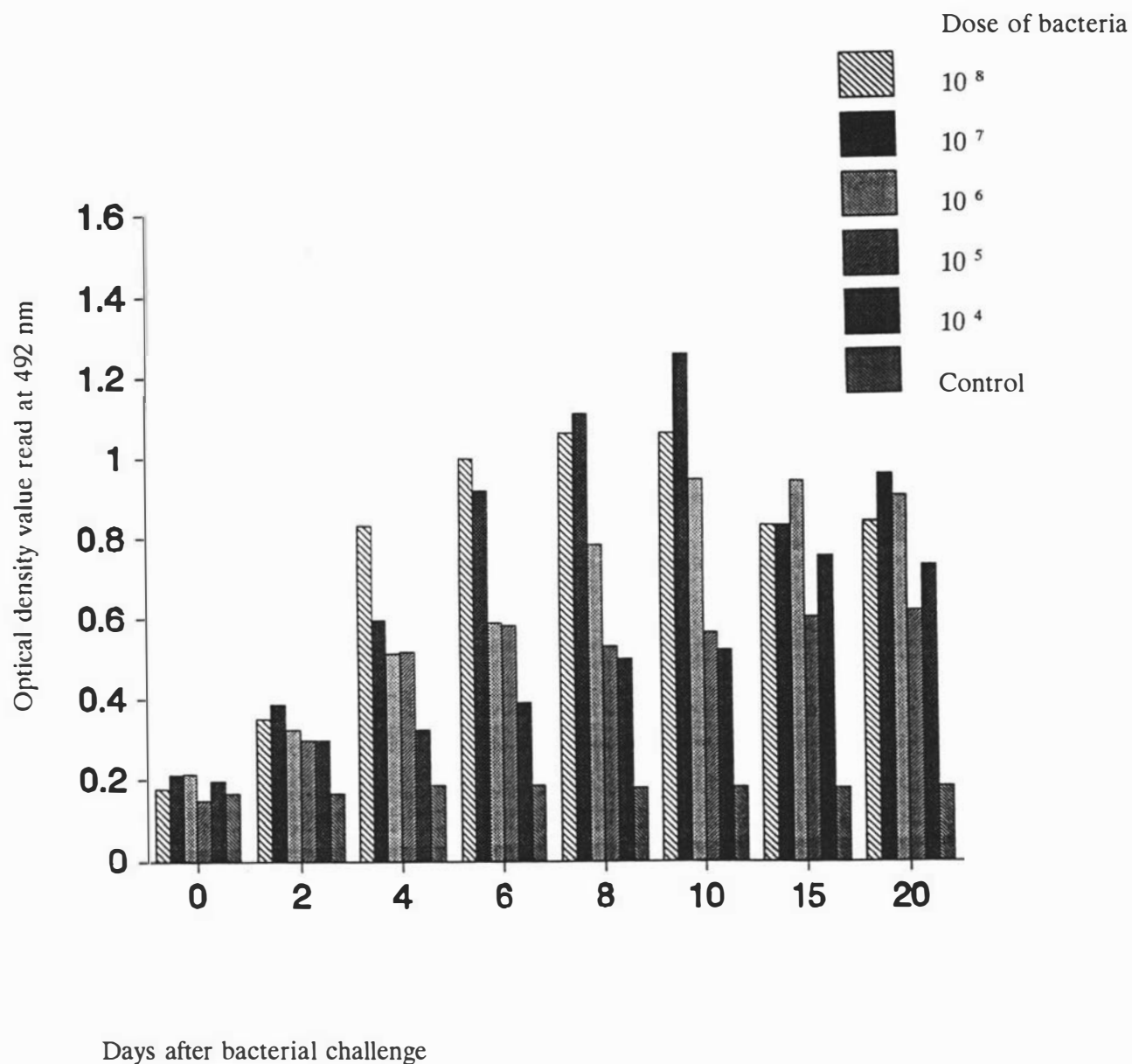
There appears to be a dose related serological response to a single oral dose of *Y. pseudotuberculosis* serotype II when optical density values are determined for a 1/100 dilution of sera in an 'indirect' ELISA using *Y. pseudotuberculosis* serotype II (LPS) as the antigen. This is illustrated in figure (3-8). The optical density readings rise steadily from day 2 to day 6 following oral challenge reaching a peak level at day 10 and then falling between day 10 and day 20. Serial dilutions of the same serum samples indicated that the rise in optical density at 10 days was associated with an increase in titre from 1/100 to 1/200. At 20 days serum samples, as well as blood coated discs, were collected from birds. Serial titration of serum samples in the ELISA test indicated that the titres were correlated with the agglutination titres and ELISA titres determined using serum eluted from blood coated discs (Table, 3-6).

Table (3-6) A comparison of the titres* measured by ELISA, disc ELISA and agglutination, for sera collected from chickens 20 days following oral challenge with *Y. pseudotuberculosis*.

Dose of bacteria given	Serological test		
	Agglutination	ELISA (serum)	ELISA (discs)
10^{-8}	1/64	1/512	1/400
10^{-7}	1/32	1/128	1/200
10^{-6}	1/32	1/64	1/100
10^{-5}	1/16	1/64	1/100
10^{-4}	1/4	1/32	ND
Control	<1/2	<1/2	ND

* Mean of five values

Figure (3-8) A comparison of the optical density values*, measured in an indirect ELISA, for chicken serum eluted from blood coated discs, collected at intervals from chickens subjected to oral challenge with a single titrated dose of *Y. pseudotuberculosis*.



* Average of 2 values for each sample, the individual optical density values for each bird can be found in appendix (A_16).

3-3-6 Serological survey of wild birds

The results of the serological survey are outlined in Tables (3-9) to (3-11) for *Y. pseudotuberculosis* serotypes I, II and III respectively. The optical density values for each individual bird tested are given in appendix (A-10). All of the samples were collected from birds in rural areas. The species sampled are outlined in Table (3-7). *Yersinia pseudotuberculosis* was not isolated from any of the birds in the serological survey. The prevalence of antibody to *Y. pseudotuberculosis* antigen in these birds was 90% overall with a similar percentage of seropositive birds in all species sampled except in the final group which was composed of various non- passerine species in which the prevalence of seropositive birds was 36 % (Table, 3-8).

Table (3-7) Avian species from which cloacal swabs and blood samples were taken for concurrent examination.

Species sampled	Samples collected from Mist netted birds	
	Cloacal swabs taken	Blood samples taken
House sparrow	383	198
Finch*	67	36
Thrush	25	24
Starling	16	12
Blackbird	35	31
Other	147	17
Total	540	315

* Fringillidae, Emberizidae & Prunellidae.

Table (3-8) Prevalence of positive serological reaction to *Y. pseudotuberculosis* serotypes I, II and III in birds that were also sampled for faecal culture.

Species	Serotype of <i>Y. pseudotuberculosis</i>			
	Total*	I	II	III
House sparrow	187/198 (94%)	167**	181	184
Finch ***	33/36 (92%)	29	31	32
Thrush	22/24 (92%)	20	21	19
Starling	8/12 (75%)	7	8	6
Black bird	29/31 (94%)	23	28	27
Other	5/14 (36%)	4	5	4
Total	284/315 (90%)	250 (79%)	274 (87%)	272 (86%)

* Number of birds positive for antibody to any serotype out of total number tested.

** Number of birds positive for antibody to this serotype. *** Fringillidae, Emberizidae & Prunellidae.

There was a high prevalence of seropositive birds in all the passerine species examined (75% - 94%). The birds in the 'other' category included mallard ducks (*Anas platyrhynchos*), pukekoes (*Porphyrio porphyrio*) and a kingfisher (*Halcyon sancta vagans*) and the number of seropositive samples was relatively smaller (36 %)

There were no *Y. pseudotuberculosis* strains isolated from the birds sampled in the serological study although environmental yersiniae were isolated from 3% of the birds. It is noted however that no yersiniae were isolated from five of the species of finch examined (*Fringilla coelabs*, *Carduelis carduelis*, *Carduelis chloris*, *Emberiza citrinella* & *Prunella modularis*) but that, as with the other passeriforms studied, the number of seropositive birds is high in these species.

Table (3-9) A comparison of the prevalence of positive serological reactions to *Y. pseudotuberculosis* serotype I in different locations and seasons during the year.

Location	Winter/spring	Summer/autumn	Total
MU piggery	9/9 (100%)	0/0	9/9 (100%)
MU feedunit	12/12 (100%)	26/29 (90%)	38/41 (93%)
MU sheep farm	23/23 (100%)	27/31 (87%)	50/54 (93%)
Levin orchard	22/25 (88%)	0/0	22/25 (88%)
Flockhouse	0/0	11/11 (100%)	11/11 (100%)
Linton rural	3/3 (100%)	1/18 (6%)	4/21 (19%)
Ashurst deer	2/2 (100%)	18/21 (86%)	20/23 (87%)
Field. rural	5/5 (100%)	12/12 (100%)	17/17 (100%)
Foxton rural	0/0	7/7 (100%)	7/7 (100%)
Wgt Zoo	21/25 (84%)	54/55 (98%)	75/80 (94%)
Other	18/19 (95%)	8/8 (100%)	26/27 (96%)
Total	115/123 (93%)	164/192 (85%)	279/315 (89%)

Table (3-10) A comparison of the prevalence of positive serological reaction to *Y. pseudotuberculosis* serotype II in different locations and seasons during the year.

Location	Winter/spring	Summer/autumn	Total
MU piggery	8/9 (89%)	0/0	8/9 (89%)
MU feedunit	10/12 (83%)	23/29 (79%)	33/41 (80%)
MU sheep farm	19/23 (83%)	21/31 (68%)	40/54 (74%)
Levin orchard	21/25 (84%)	0/0	21/25 (84%)
Flockhouse	0/0	11/11 (100%)	11/11 (100%)
Linton rural	3/3 (100%)	1/18 (6%)	4/21 (19%)
Ashurst deer	2/2 (100%)	13/21 (62%)	15/23 (7%)
Field. rural	4/5 (80%)	12/12 (100%)	16/17 (94%)
Foxton rural	0/0 (100%)	7/7 (100%)	7/7 (100%)
Wgt Zoo	19/25 (76%)	53/55 (96%)	72/80 (90%)
Other	18/19 (95%)	7/8 (88%)	25/27 (93%)
Total	104/123 (85%)	148/192 (77%)	252/315 (80%)

Table (3-11) A comparison of the prevalence of positive serological reaction to *Y. pseudotuberculosis* serotype III in different locations and seasons during the year.

Location	Winter/spring	Summer/autumn	Total
MU piggery	9/9 (100%)	0/0	9/9 (100%)
MU feedunit	12/12 (100%)	28/29 (97%)	40/41 (98%)
MU sheep farm	23/23 (100%)	31/31 (100%)	54/54 (100%)
Levin orchard	23/25 (92%)	0/0	23/25 (92%)
Flockhouse	0/0	11/11 (100%)	11/11 (100%)
Linton rural	3/3 (100%)	1/18 (6%)	4/21 (19%)
Ashurst deer	2/2 (100%)	19/21 (90%)	21/23 (91%)
Field. rural	5/5 (100%)	12/12 (100%)	17/17 (100%)
Foxton rural	0/0	7/7 (100%)	7/7 (100%)
Wgt Zoo	22/25 (88%)	54/55 (98%)	76/80 (95%)
Other	18/19 (95%)	8/8 (100%)	26/27 (96%)
Total	117/123 (95%)	160/192 (83%)	277/315 (88%)

The prevalence of antibody to *Y. pseudotuberculosis* serotype I, II and III antigen (LPS) is high in all areas studied with the exception of the Linton location in the summer/autumn period. For all three serotypes there is no significant difference ($P>0.05$) between the two seasonal periods indicated. Although the prevalence of serologically positive birds appears to be higher in the winter/spring period than in the summer/autumn period this is not the case if the Linton results are not included in the analysis. If this is done for all three serotypes it is found that the prevalence of seropositive birds is similar in the two seasonal periods examined. It is noted that the Wellington Zoo results are not significantly different from the results from the rural areas.

Discussion

In the present study, the development of a screening test for sera collected from wild birds followed the development of a new disc ELISA used for assessing the serological response of chickens following oral infection with *Y. pseudotuberculosis*. The development of this ELISA test involved extensive studies on the cross reactivity of different antigen preparations. This preliminary work helped to explain why there are conflicting opinions reported in the literature on the value of serological diagnosis and the on the difficulties which arise when such tests are used to screen populations for bacterial diseases. Although much of what was learned in the application of ELISA methodology to chickens was similar to that reported in the literature for salmonellae (Barrow, 1992), there were some difficulties which were unique in application to wild life studies. In addition, with recent interest in importance of endotoxin on the course of Gram negative diseases (Bone, 1993), it was decided to examine the role of LPS on the clinical course of avian pseudotuberculosis. It is thought that species differences in susceptibility to LPS may be important in determining the susceptibility to pseudotuberculosis.

The discussion is divided into four sections each of which outlines a major development made in this chapter

3-4-1 Antigens of *Y. pseudotuberculosis* and factors determining the sensitivity and specificity of a serological test

Much of the conflict of opinion with regard to the value of serological tests currently used in the diagnosis of human yersiniosis, is based on the wide range of possible cross reactions and the apparent lack of specificity. This is especially relevant when such tests are used as screening tools because there is often no information on the previous exposure of individuals to enteropathogenic yersiniae or other organisms. In common with all the Enterobacteriaceae, *Yersinia pseudotuberculosis* and *Y. enterocolitica* have a lipopolysaccharide coat made up of the serotype specific 'O' antigen and lipid A. Yersiniae have historically been considered facultative intracellular pathogens and share

many virulence factors with members of the Enterobacteriaceae but the extracellular phase of replication is also considered important (Fukushima *et al.*, 1991). Following infection, the humoral response to both the lipopolysaccharide (LPS) antigen and *Yersinia* outer membrane proteins (YOPs) of pathogenic yersiniae has been found useful for monitoring the course of the disease in human patients (Gripenberg *et al.*, 1979; Martinez *et al.*, 1991; Maki-Ikola *et al.*, 1991). Possession of the LPS coat by Gram negative bacteria aids passage through the acidic environment of the stomach (Schniatman & Klena, 1993) and this possibly explains why many common enteric pathogens have developed similar surface antigens through the course of evolution (Wetzler, 1965). The similarity in LPS coating as an adaptation of enteric passage characterises the phenotypic similarity between *Y. pseudotuberculosis* and *Y. enterocolitica* and other members of the enterobacteriaceae. The development of specific screening tests for viral diseases and some bacterial diseases such as that caused by *Y. pestis* has been less difficult due to the antigenic uniqueness of the more highly specialised pathogens which are adapted to a specific host species or mode of transmission (Quan *et al.*, 1985; Thomas *et al.*, 1988, 1992).

3-4-2 Development of a serological test for the measurement of IgG antibody to *Y. pseudotuberculosis* serotypes I,II and III in birds.

The development of an ELISA for the serological diagnosis of human yersiniosis has been reviewed recently by several authors (Granfors, 1989; Fernandez-Lagos *et al.*, 1991; Robins-Browne *et al.*, 1993). Due to the lack of agreement on the value of the available serological tests reported in the literature (Fernandez-Lagos *et al.*, 1991) and because of problems associated with the reliability of serological diagnosis of *Yersinia* infections it was decided to examine the possible reasons for these problems.

As a result of work on the antigenic structure of yersiniae it was possible to develop an ELISA test which could be used specifically to measure IgG antibody to *Y. pseudotuberculosis* in birds. The current tests had been found to have a limitation in specificity due to the antigenic similarity between *Yersiniae* and other common Gram

negative bacteria. In the present study it was necessary to have a highly specific and sensitive tool in order to detect low antibody titres resulting from previous exposure to *Y. pseudotuberculosis* rather than the high titres which correspond with a recent infection. When compared with traditional agglutination tests the ELISA test was found to be highly sensitive and specific. However, in the field situation, the specificity is probably reduced due to the wide range of bacteria with antigenic similarity to which wild birds are constantly exposed.

The present study found that the use of the HK (heat killed) antigen preparation provided the most sensitive assay when used in the 'indirect' ELISA test, this finding was in agreement with Chart & Rowe (1992). This sensitivity was partially due to the protein component which remained in the crude HK antigen preparation as seen in the immunoblot (figure, 3-5). SDS-PAGE gels stained with Coomassie blue support this hypothesis as it was found that some bands of the crude HK antigen stained with this dye which has an affinity for proteins whereas the lipopolysaccharide (LPS) preparations did not stain with Coomassie blue when run on the same gel. Silver staining which is more sensitive for protein as well as having an affinity for lipopolysaccharide (Moller *et al.*, 1993) did stain bands in the LPS preparation. Immunoblot studies using hyperimmune rabbit serum that had been raised against the crude HK preparations, indicated that formalin fixed antigen resulted in a more specific reaction when probed with heterologous antisera than did the heat killed preparation. It also gave a stronger reaction than proteinase K digests of LPS antigen. For this reason F (formalin fixed) antigen was used for agglutination tests as described by Mair and Fox (1986). Lectin-probed blots of LPS antigen indicated that the bands stained in the silver stained SDS-PAGE gels and the bands recognised by hyperimmune serum raised against the crude HK antigen preparation were sugar residues associated with the 'O' antigen. In theory the LPS antigen should be serotype specific as outlined by Davies (1958) and Westphal *et al.* (1952) but it is known that the 'O' antigens of *Y. pseudotuberculosis* serotype II and IV cross react with the 'O' antigens of *Salmonella* group B and D respectively, and in addition share epitopes with strains of *E. coli* (Wetzler, 1965; Mair and Fox, 1986).

The specificity of the test was found to depend on the purity of the antigen preparation used. Under experimental conditions when comparing different antigen preparations in the 'indirect' ELISA, the proteinase K digest LPS preparation of *Y. pseudotuberculosis* was found to result in less cross reactivity than the F and Hk preparations. This was confirmed by immunoblot studies and agrees with what is reported for ELISA tests developed for the diagnosis of *Salmonella spp.* infection in chickens (Barrow, 1992, Cooper *et al.*, 1989). Fernandez-Lagos *et al.* (1991) outlined the Westphal method of LPS antigen preparation based on phenol extraction (Westphal *et al.*, 1952). This method has been used by many groups to produce a serotype specific antigen for ELISA work but Granfors *et al.* (1989) indicated that they found the heat killed preparation produced a test with equivalent specificity to the LPS preparation and argued that the labour involved in the preparation of the LPS antigen was not necessary. Much of the recent interest in developing a simple and cost effective ELISA for screening chicken flocks for *Salmonella* has also arisen because of the need to find a sensitive and specific antigen (Barrow, 1992). Chart & Rowe, (1992) developed a simplified method of purifying crude heat killed preparations of bacteria using a proteinase K digest followed by dialysis aimed at removing remaining proteins. In the present study this method has proved to be simple and effective and has resulted in a serotype specific antigen for use in an 'indirect' ELISA for *Y. pseudotuberculosis*.

The use of blood coated paper discs for the screening of chicken flocks for antibody to *Salmonella spp.* was outlined by Minga & Wray (1992), who found that the discs provided a reliable and efficient method of collecting samples which did not suffer significant loss of titre following storage and handling. These discs were used in ELISA tests and were found to yield similar results when compared with serum samples from the same bird (Cooper *et al.*, 1989; Humphrey *et al.*, 1991). In the present study, serum collected from hyperimmune chickens and rabbits and serum eluted from blood coated discs collected from the same animals were used in the same ELISA test and optical density readings of serial dilutions of serum were compared. This was done to determine the amount of serum eluted from discs under the conditions used in the ELISA procedure. In all cases it was found that the amount of serum eluted from 2 discs incubated overnight in a plate with 100 microlitres of blocking buffer was equivalent to a 1/100 dilution of serum from the same animal. This indicated that 2

discs eluted the equivalent of 1 microlitre of serum. Although this result is not in agreement with that reported in the literature for the work on Aujeszky's disease (Banks, 1985) this is possibly because the conditions of the present test differ from those used in the automated systems as used in the Aujeszky's disease eradication programme. It is probable that the amount of serum eluted from the blood soaked discs will depend on the incubation times used and the blood samples collected. Cooper *et al.* (1989) found that serological titres for *Salmonella enteritidis* derived from filter discs were half those determined from serum samples from the same bird. In the present study the titres derived from discs were similar to those from serum when adjusted to the equivalent starting concentration.

3-4-3 The use of the 'indirect' ELISA to determine the serological response of chickens following oral challenge with *Yersinia pseudotuberculosis*

The 'indirect' LPS ELISA was used to monitor the IgG response of experimentally infected chickens over a period of time following a single oral challenge dose of bacteria. It was found that using blood coated discs collected from individual birds the IgG response to the LPS antigen of *Y. pseudotuberculosis* serotype II was dose related and that it peaked between day 6 and day 10 before levelling out at day 15 and falling slightly by day 20.

In agreement with Cooper *et al.* (1989) the ELISA test was found to be more sensitive than the agglutination test. Williams & Whittemore (1976) compared the sensitivity of different agglutination tests for the diagnosis of *Salmonella typhimurium* in chickens and found that the microantiglobulin test (MAG) was the most sensitive. In the present study the tube microagglutination test (MAT) was used for comparison. There is some disagreement over the reliability of the different agglutination tests with some variation in results depending on the antigen used and the experience of the technician in the interpretation of the results (Barrow, 1992). There is a general acceptance that ELISA tests have the capacity to provide a more sensitive, specific and repeatable means of detecting specific antibody to different bacterial pathogens but that the interpretation of results from these tests must be based on an understanding of the limitations of serology in the field situation (Chart & Rowe, 1992).

The magnitude of the serological response in experimentally challenged chickens has been shown to depend on the invasiveness of the challenge strain and on its ability to multiply in the tissues of the host in order to stimulate the cytokine release and inflammatory processes associated with the acute-phase inflammatory response (Cooper *et al.*, 1993; Bone, 1993). However, the genotype of the host is also an important moderating factor (Congleton & Wagner, 1991). Garren and Hill (1959) outline the difference in agglutinating antibody titres of two strains of chicken in response to inoculation with live and inactivated *Salmonella gallinarum* cultures. They reported that the White Leghorn was inherently resistant to infection with *Salmonella gallinarum* and that the antibody response to the bacterium was lower than that found in the more susceptible Rhode Island Red chickens. However, the relationship between low antibody titre and resistance is not fully apparent. It was initially suggested that the inherent resistance may have been due to enteric mechanisms which prevent bacterial replication in the gut and therefore limit the spread of bacteria into the circulation. However, Garren and Barber (1955) had noted that the White Leghorn chicken did develop granulomas in the viscera following oral challenge with *Salmonella gallinarum*. This does not however rule out the possibility that the spread of infection is limited by enteric mechanisms but possibly the limited antigenic stimulation results from other moderating factors which may differ between breeds of chicken (Davis *et al.*, 1968). In the present study there was a measurable antibody response to *Y. pseudotuberculosis* serotype II LPS antigen following oral challenge with the homologous strain but this was not marked and may indicate limited antigenic stimulation associated with resistance to infection at the level of the gut. However, it was also noted that hyperimmune serum raised in White Leghorn chickens by the parenteral route, using a series of injections of a heat killed bacterial preparation, resulted in significantly lower titres (MAT and ELISA) than those measured in rabbits when using a similar protocol. The significance of this in natural infections is difficult to determine. The relationship between endotoxin and the acute phase inflammatory response, including the hypoferraemia of infection, will be further discussed in chapter six.

3-4-4 Application of a screening ELISA for serological surveys of wild bird populations

In birds the epidemiology of different serotypes of *Y.pseudotuberculosis* and other yersiniae may be sufficiently different to warrant a determination of the species and serotype involved. This is not always possible using bacteriological methods due to the short shedding time following infection in some cases (Mair & Fox, 1986; Granfors *et al.*, 1989; Fernandez-lagos *et al.*, 1991). There is evidence to suggest that the serological response to YOPs is a useful screening test for the diagnosis of yersiniosis in people (Maki-Ikoli *et al.*, 1991). This is based on the fact that all virulent yersiniae contain a virulence plasmid and express YOPs during the course of infection (Straley *et al.*, 1993). There are merits in this approach as it reduces the requirement for numerous serotype specific tests, but it is not suitable for epidemiological surveys or in cases where it is important to distinguish between infection with *Y. pseudotuberculosis* and *Y. enterocolitica*. For the purposes of the present study the LPS ELISA was considered the most suitable test for serological screening of wild birds but could be improved by the additional use of a YOP based ELISA to screen samples prior to using the LPS ELISA and therefore increase the specificity of the test further.

Attempts to develop a 'blocking' ELISA for use with blood coated discs were not successful. This was largely due to the restriction of volume of blood (1 disc/bird = 6 tags) which can be taken from small birds (< 100g) and the need to use only 2 tags per well in the ELISA to allow testing for *Y.pseudotuberculosis* serotypes I, II and III. In the disc ELISA test the blood coated discs are soaked overnight in 100 microlitres of PBS-Tween buffer, and it was previously determined that this resulted in the elution of 1 microlitre of serum. Therefore in the 'indirect' and 'blocking ELISA' protocols, the samples are tested at a 1/100 dilution. Attempts to use a smaller volume of buffer to elute the serum resulted in inconsistent results compared with the good correlation determined when comparing results of disc-eluted serum at a 1/100 dilution and a 1/100 dilution of chicken serum derived from the same bird using traditional blood collection techniques. In the experimental situation it was determined that hyperimmune chicken serum used at a 1/100 dilution was unable to block hyperimmune rabbit serum raised

against the homologous antiserum, this was true even when the rabbit serum was used at a 1/10,000 dilution. Lowering the concentration of the rabbit serum further resulted in lower optical density readings but did not allow a clear distinction between positive and negative controls. When rabbit antiserum was used as the primary antibody and chicken antiserum as the second antibody it was found that a 1/10 dilution of the rabbit hyperimmune serum resulted in 33% blocking of a 1/100 dilution of the chicken serum compared with the positive control. This was not increased if the chicken serum was used at a higher dilution. It had previously been determined that the anti-rabbit IgG did not recognise the chicken IgG and that the anti-chicken IgG did not recognise the rabbit IgG. From the results it was apparent that to achieve > 65% blocking of the secondary antibody it was necessary to use the primary antibody undiluted. This was not possible for the disc ELISA. In addition to this, the fact that even the high titre rabbit serum was unable to effectively block low titre chicken hyperimmune serum at 1/100 dilution may suggest that some form of antibody displacement was occurring in the system or that the different IgG of the different species were recognising different epitopes of the antigen.

The restriction on sample volume was determined by the body weight of the bird sampled. One disc was determined to hold 60 microlitres of blood, considering a further 60 microlitres was often lost from the bird at the time of collection each bird gave at least 120 microlitres of blood per sample. It is generally recommended that no more than 10% of a bird's blood volume should be taken at any one sampling and for a 40g house sparrow this would limit the maximum sample volume to 400 microlitres. The survey work was performed on wild birds which were caught in mist nets, banded and released after sampling.

Using the 'indirect' ELISA, it was found that the prevalence of seropositive birds was very high with similar results for all three serotypes. This could indicate that the wild birds studied had been exposed to all three serotypes of *Y. pseudotuberculosis*, that there was a lot of cross reactivity between the three serotypes or that the birds were exposed to other bacteria with similar antigenic components. More work needs to be done to examine both the serological response of wild birds to bacterial infection and on the antigenic structure of the common faecal bacteria of different species bird.

The prevalence of faecal isolation of *Y.pseudotuberculosis* in the same populations was 0% although other *Yersinia spp.* had been isolated from the birds sampled. It is probable that wild birds are continually exposed to *Y. pseudotuberculosis* through the food and water supply but that they do not shed the bacteria for a prolonged period following infection. This view is supported by the experimental infections in canaries reported by Stovell (1963) and in chickens (see chapter four). In birds which develop clinical disease, however, large numbers of bacteria are shed leading to environmental contamination and possibly rapid reinfection and dissemination of infection to other birds in a group (Wetzler, 1971). In the wild birds sampled it is also possible that the development of an antibody response was associated with a reduction in the numbers of pathogenic bacteria in the gut to a level that was not detectable by faecal culture or that the bacteria resided intracellularly.

Another explanation for the disparity between the large number of seropositive birds and the low faecal prevalence is that either the faecal isolation methods were not sensitive enough for *Y. pseudotuberculosis* giving an under-representation of the true level or that the serological test was not specific enough for the purpose of serological screening. The faecal isolation procedures have been discussed in chapter two, although it is recognised that *Yersinia* selective agar, CIN, is inhibitory to some serotypes of *Y. pseudotuberculosis* (Fukushima *et al.*, 1986) the procedure used involved plating on CIN following 3 weeks of cold enrichment. Iannibelli *et al.* (1991) found this procedure to be as sensitive as others available, in this regard the results of this study are similar to those of similar studies overseas. The possibility that the serological test used in the present study is not specific enough for use in the field situation is difficult to evaluate as it is not possible to identify all the bacteria with which wild birds may come in to contact and as a result it is difficult to identify all possible antigenic cross reactions. The test had a high degree of specificity when the sera of chickens immunised with a specific heat killed bacterial antigen were compared with the sera of chickens immunised with the HK antigen of a heterologous organism in the 'indirect' ELISA. Immunoblot

work confirmed this finding and indicated that the test was able to distinguish between antisera raised against different serotypes of *Y. pseudotuberculosis* and *Salmonella* groups B & D which are known to share common antigens with *Y. pseudotuberculosis* serotypes II and IV respectively.

Comparing the prevalence of seropositive birds in different locations it was found that the results were similar in each of the 10 rural locations except for the Linton area. There does not appear to be an explanation for this anomaly as all the tests were performed on the same day and under the same conditions and there was no difference in the species of bird sampled in this area. A comparison of the prevalence of seropositive birds in the Summer/Autumn period as compared with the Winter/Spring period indicated that there was generally a trend for a higher prevalence of seropositive birds in the Winter/Spring period, however, this was not statistically significant and the difference was not apparent if the Linton results were not included. Although more outbreaks of the clinical disease are associated with the Winter/Spring period in birds the sero-prevalence of antibody to *Y. pseudotuberculosis* does not indicate a pattern in exposure. However, it is not clear what the duration and magnitude of the serological response to *Y. pseudotuberculosis* is in wild species. It is clear from these results that much more work is required to evaluate the extent of species specificity in the development of the humoral response of birds to Gram negative infections as it is already evident that there are measurable differences between breeds of chicken in this regard (Garren & Hill, 1959) and possibly also in the duck (Higgins & Warr, 1993).

The specificity of commercially available anti-chicken IgG was evaluated in order to determine the validity of the 'indirect' ELISA for other species of bird. Direct ELISA tests using plates coated with antiserum from different passeriform species, psittaciforms, columbiforms and galliforms indicated that the commercial conjugate reacted with the serum from passeriform species and to a lesser extent with that from the psittaciforms and the columbiforms examined. Serum from control and hyperimmune chickens was used in the test for comparison. The optical density values for serial dilutions of these samples were greater than those from the other species. Immunoblot studies confirmed this finding but failed to demonstrate that the conjugate

recognised the heavy and light chains of IgG of passeriform serum despite the relatively high optical density values achieved in the 'direct' ELISA. Immunoblot studies indicated that the commercial sheep anti-chicken-IgG conjugate recognised the light and heavy chains of chicken IgG and did not cross react with other serum proteins in the chicken. It was also evident that the conjugate did not cross react with the albumin band of other avian species. There was some cross reactivity in some immunoblots with high molecular weight products which had failed to migrate down the gel. Although the immunoblots did not provide evidence that the commercial anti-chicken-IgG recognised IgG of other avian species there was evidence of binding with sera of other avian species in the ELISA. It was decided therefore to use the 'indirect' ELISA for antibody to *Y. pseudotuberculosis* with all of the sera collected but to remain cautious in the interpretation of the results. For larger columbiforms and psittaciforms the 'blocking' ELISA may be useful if larger volumes of serum could be collected.

Wildlife disease surveillance is a challenging prospect requiring considerable preparation and planning (Brittingham *et al.*, 1985; Kaneuchi *et al.*, 1989). Sample collection is not always straight forward and an understanding of the population biology of the species of interest is essential in the interpretation of the results. When considering avian disease surveillance, particularly in relation to seasonal trends in microbial flora it is important to take into consideration changes in diet associated with regional food availability and physiological alterations associated with moult and breeding season. The failure to isolate *Y. pseudotuberculosis* from faecal samples is probably an indication that the organism is not shed for long periods of time following infection (Stovell, 1963). In contrast, the high prevalence of serologically positive birds probably indicates a high degree of exposure to *Y. pseudotuberculosis* or to organisms with similar antigens. In consideration of the failure to demonstrate unequivocally that IgG of non-galliform species was being recognised in the assay it is possible that all or part of the reaction with these species is non specific. Wild birds would be exposed to a wider range of bacteria than birds in experimentally controlled conditions and this factor must be taken into account when comparing the specificity claimed for serological tests in the field with birds under controlled conditions (Cooper *et al.*, 1991). A large body of experimental work on the use of the LPS ELISA to screen chickens for antibodies to

Salmonella species in the field as compared with the experimental situation was comprehensively reviewed by Barrow (1992). He concluded that serological testing was valuable to determine the antibody status of the flock but was not necessarily reliable for determining whether an individual bird was seropositive or seronegative. If this is true for the *Yersinia* ELISA, the survey results may be an accurate reflection of the extent of exposure to *Y.pseudotuberculosis* in wild birds. The similar prevalence of seropositive birds to all serotypes may indicate exposure to all three serotypes or to cross reacting antigens. This is an area of research which requires further experimental evaluation with both *in vivo* and *in vitro* investigation of the nature of the serological response to bacterial organisms in a range of avian species. At the present time however, the lack of species specific conjugates limits the wide application of present serological tests designed to screen small amounts of blood. In addition, the complexity of the lipopolysaccharide antigens of bacterial species and the difficulty in defining the antigenic components using conventional SDS-PAGE staining protocols and immunoblotting may partially explain why the development of sero-epidemiology for bacterial diseases is so far behind that already available for viral diseases (Dorst, 1991).

During the period of the survey several small outbreaks of pseudotuberculosis occurred in aviaries in the locations of the study as well as four sporadic cases. The epidemiology of pseudotuberculosis in birds is still unclear but from the results of chapter two and three it is apparent that healthy birds do not generally excrete the organism in large numbers and that the majority of birds have been exposed to the organism. Barre *et al.*, (1979) and more recently Fukushima *et al.* (1991) suggested that the environment may provide a reservoir for *Y.pseudotuberculosis* infection. The high prevalence of environmental yersiniae in the faeces of wild birds examined along with the high rate of seroconversion to potentially pathogenic yersiniae suggests that where a high rate of carriage of *Y. pseudotuberculosis* is reported in populations of wild birds this may actually reflect a high rate of environmental contamination rather than indicating that carrier birds are the cause of the high prevalence of the organism in the environment. In the present study the survey was carried during one twelve month period from November 1991 to November 1992, it had been a fairly warm year and there were no isolations of *Y. pseudotuberculosis* from environmental sources in the locations studied.

This is in contrast to the study of Mackintosh and Henderson (1984) who isolated the organism from both the environment and avian species caught at the study site. It appears that the organism survives well in cool wet environments common in the winter and spring (Barre *et al*, 1979, Wetzler, 1971) but concurrent seasonal changes which may be occurring in the avian populations at this time have not been examined and could be relevant to the epidemiology of the disease. In the next chapter the hypothesis that there are species differences in pathogenesis of pseudotuberculosis in birds and the factors which may be linked to differences in species susceptibility to oral challenge with *Y. pseudotuberculosis*, will be examined.

Summary

- 1) An 'indirect' ELISA test has been developed which has a high degree of sensitivity and specificity under laboratory conditions.
- 2) Experimentally infected chickens developed a dose related serological response to the LPS antigen of *Y. pseudotuberculosis* following a single oral dose of live organisms.
- 3) Hyperimmune sera raised in chickens against heat killed bacterial antigen had a lower titre than that raised against the same antigen in rabbits. Chickens given parenteral injections of purified LPS failed to develop a measurable antibody titre. This is indicative of a low immunogenicity of LPS to chickens.
- 4) The specificity of serological tests developed under laboratory conditions is difficult to determine under field conditions. This is because the antigenic structure of enteric yersiniae is very similar to that of other members of the Enterobacteriaceae.
- 5) There is not enough known about the serological response of avian species to bacterial infection. To investigate this further it would be necessary to develop species-specific conjugates in-order to evaluate the serological response of a wide range of avian species following infection with *Y. pseudotuberculosis* using ELISA techniques.
- 6) There are a high number of birds which are seropositive to *Y. pseudotuberculosis* serotypes I, II and III in the wild populations studied. Despite the limitations of applying 'indirect' ELISA test for serological screening it is thought that this result is probably representative of the true sero-prevalence in these populations reflecting a high level of exposure to the bacteria, but not necessarily evidence that the birds are persistently infected.

CHAPTER FOUR

Pseudotuberculosis, the disease

Introduction

There have been many reviews which describe naturally occurring pseudotuberculosis in wild and domestic birds (Wetzler, 1971; Obwolo, 1980; Keymer, 1980; Parsons, 1991) but there has been little experimental work reported on the disease in birds except for the studies carried out in canaries by Stovell (1963) and later quoted by Wetzler (1971). The early literature on the subject of pseudotuberculosis in birds is confusing, with little or no differentiation made between natural cases in wild birds and experimental situations in various species. In many of the early experimental reports (Muir, 1898, Pfaff, 1905) the source of the challenge bacterium, the size of the inoculum, and route of infection were incompletely described and the species of bird often not stated (Wetzler, 1971). Despite this, much data can be derived from the work of Muir (1898), Rieck (1889) and Pfaff (1905) which was reviewed by Beaudette (1940) in an article written in response to his identification of the disease in a blackbird. There are several general articles on the subject of pseudotuberculosis in mammals (Baskin *et al.*, 1977; Baskin, 1980; Wetzler, 1971) in which there is a recognised species difference in the susceptibility to systemic infection and consequently in the pathogenesis of the disease. In birds, there also appears to be a species difference in susceptibility with passerine species generally considered highly susceptible (Muir, 1898; Pfaff, 1905; Beaudette, 1940) and chickens relatively resistant (Wetzler, 1971).

There have been reports of fatal infection in finches and 'small song birds' following subcutaneous injection of 0.5 ml of a bacterial broth containing an isolate from a recent clinical case of *Y.pseudotuberculosis*. Disease was not produced if the same dose was given orally (Muir, 1898). In similar experiments a 1.0 ml parenteral dose of the same organism that caused disease in passerine species produced no disease in chickens (Pfaff, 1905). Stovell (1963) conducted several trials in canaries to study the pathogenesis of the disease and faecal shedding following oral doses of *Y.pseudotuberculosis*. He concluded that canaries infect each other, that they were potential sources of disease for other species, and that those birds shedding the greatest

number of bacteria in their faeces were those which had developed caecal abscesses. In addition Stovell (1963) studied three outbreaks of the disease in aviary canaries. Cold weather, recent change in diet and mixing of new male birds prior to the breeding season were considered important in precipitating the outbreaks. Obwolo (1976) and Fiennes (1982) identified similar 'stress' factors associated with outbreaks of pseudotuberculosis in cage birds and other animals.

The factors predisposing individual birds to the development of systemic pseudotuberculosis are less well established although liver disease and haemochromatosis are often regarded as factors predisposing to the development of systemic complications in *Y. pseudotuberculosis* infections in people (Robins-Browne *et al.*, 1987; Rabson *et al.*, 1975; Robins-Browne & Hartland, 1990). Intestinal trauma associated with a change in diet, parasitism or cold stress are reported to be important predisposing factors in deer and zoo animals (Mackintosh & Henderson, 1984; Parsons, 1991). In birds there is some evidence to suggest that this is also the case (Fiennes, 1982; Wetzler, 1971). In yersiniae, as with other potentially pathogenic organisms, it would seem reasonable to conclude that the virulence of the infective agent, the dose of the inoculum and the route of the challenge are all important factors in determining whether or not an infection will become established in the host.

This chapter deals with three issues with the aim of 1) comparing the *in vitro* virulence characteristics of clinical isolates of *Y. pseudotuberculosis* with those isolated in the field survey; 2) determining what management factors are associated with outbreaks of pseudotuberculosis in cage birds; and 3) determining what factors predispose individual wild and captive birds to the development of systemic pseudotuberculosis following enteric infection. The study includes clinical material from retrospective cases of the disease in birds and experimental bacterial challenge in the chicken.

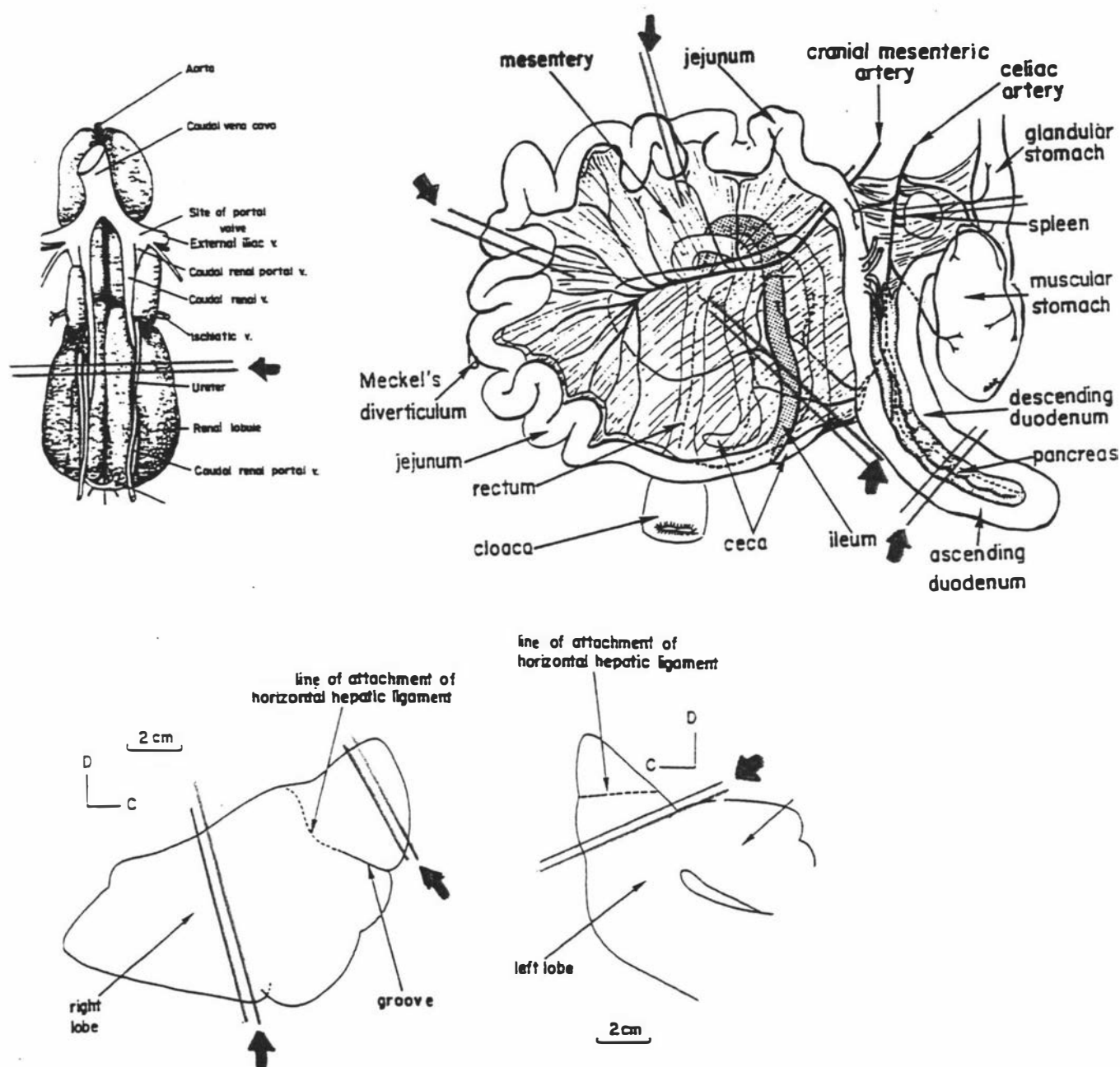
Materials and methods

4-2-1 Experimental pseudotuberculosis in the chicken

The birds used for this experiment are described in chapter three (see section 3-2-2). In addition to the collection of blood outlined for serology, faecal samples were collected prior to oral challenge and every two days following challenge until the end of the experiment. Twenty days after oral challenge all of the birds were euthanased with an overdose of halothane anaesthesia (Fluothane, ICI Pharmaceuticals, Great Britain) and necropsy was performed. Samples of the visceral organs were taken (see figure, 4-1) and fixed in 10% buffered formalin. Histological sections were prepared and stained as described below. Immunocytochemical tests were performed on histological sections prepared from tissues taken from each bird.

Serum samples and sections of fresh liver were also collected from each bird at day 20 and were frozen at -20 °C for biochemical assessment, the procedures used for these tests and the results are described more fully in chapters five and six. Briefly, serum iron, serum transferrin and total iron binding capacity were measured (see section 6-2-2) and an assessment of total liver iron was measured using a calibrated spectrophotometer (see section 5-2-1). Concurrent serological studies for this group of birds were outlined in chapter three.

Figure (4-1) Sections taken from the viscera of experimental birds and clinical cases at necropsy.



Samples were taken from the liver, spleen, proventriculus, duodenum, pancreas, jejunum, colon, caecum, caecal tonsils, and kidney (figure, 4-1) as well as from the heart and lungs, and fixed in 10% buffered formalin. Intestinal sections were taken as cross sections with some dissection to identify lesions not included in the sample site. the arrows indicate the area from which sections were taken.

4-2-2 Retrospective studies

Necropsy material and paraffin blocks of sections were obtained from cases recorded and filed in the Department of Veterinary Pathology and Public health and from Ministry of Agriculture and Fisheries (MAF) diagnostic laboratories as well as from private aviary owners and veterinary clinics. This material was treated as described below and sections were stained for examination. In the majority of these cases, however, no bacterial isolates were available.

4-2-3 Necropsy procedures and histological staining

Experimental birds, and clinical cases of pseudotuberculosis submitted for necropsy, were processed as described below. A clinical history was taken for each bird. All gross findings were recorded.

Tissue preparation

Formalin-fixed tissues were processed overnight in a Shandon Citadel 2000 (Watson Victor Ltd) tissue processor. Paraplast tissue-embedding medium (Oxford labware) was used to embed the processed tissues in the Shandon Histocentre. Sections were cut at 3 microns using a Leitz Wetzler microtome. Sections were floated out onto a water bath, mounted on a glass slide and dried at 60 °C. Serial sections were taken from each block of tissue and stained with Haematoxylin and Eosin (Gill et al, 1974); Gram-Twort (Twort, 1924) and Perls' Prussian Blue stain for ferric iron (Perls, 1967). Sections of Liver and spleen were also stained with a combined Perls' iron-Gram stain, Periodic Acid-Schiff (PAS) (McManus, 1946) and a modified Prussian blue stain. The modified staining procedures are described below. In addition 3-5 micron sections were cut for immunocytochemical studies.

Modified staining techniques

Combined Perls' iron-Gram stain

This method combines the Perls' iron stain (Perls, 1867) with the Gram-Twort stain (Twort, 1924) with a washing step between the two. The technique allows bacterial lesions and stainable ferric iron to be visualised on the same slide. Ferric iron appears blue and Gram negative bacteria are red using this stain.

Modified Prussian blue stain

This method, based on that described by Schmeltzer (1933) is designed to identify both ferrous and ferric iron in tissue sections and cytology smears. The tissues were dewaxed and rehydrated prior to treatment with 10% ammonium sulphide for two hours. Sections were washed in distilled water and treated with a filtered solution of equal parts of 2% hydrochloric acid and 10% potassium ferricyanate for 25 minutes. A 10% solution of ferricyanate was found to give the same results as the 20% solution recommended in the original method and results from sections incubated for 25 minutes were preferable to those incubated for 15 minutes. Sections were washed and counterstained in 1% neutral red for 5 minutes and washed and dehydrated prior to mounting in DPX (BDH Ltd, Poole).

Various combinations of the above staining methods were compared in similar sections using image analysis.

4-2-4 Assessment of the amount of stainable iron in histological sections using visual evaluation

The visual assessment of stainable iron was based on the modified Bothwell technique reported by Kincaid & Stoskopf (1987). The amount of stainable iron in each section was determined at 40x magnification with the intensity of the colour subjectively graded as highly intense (+++), intense (+++), less intense (++), trace (+) and negative (-) depending on the amount and distribution of colour in the tissues. The distribution of the stainable iron in the liver was divided into Kupffer cell iron and hepatocyte iron.

4-2-5 Assessment of stainable iron in histological sections using image analysis

An image analysis system was developed using VIDAS (Kontron Elektronik, GmbH, Germany). The hardware consisted of a microscope (Nikon, Japan), frame grabber and computer screen linked to a hard drive and disc drive system. The software package was commissioned for the purpose of the study and was based on a standard VIDAS colour-linked module modified to assess the intensity and area of blue stained regions on representative stained sections. Each set of slides was given a code number and each file was labelled according to the programme option chosen before loading on to duplicate discs.

Number of sections and field of view

A total of five sections from the liver, and in some cases two sections from the other organs, of each clinical case and experimental birds were examined using image analysis to quantify the area and intensity of Prussian blue staining. Slides were viewed at 40 x magnification using a microscope (Nikon, Japan) set at a standard light intensity reading of 165 lux. Five fields of view were analyzed per section and the mean value recorded along with the standard deviation for each slide.

Calibration

The area and intensity values set by the programme were based on arbitrary units on a scale of 0-100,000,000 providing a quantitative indication of relative values. This was checked by comparing values from slides which had been visually determined as having high stain intensity (++++) and those with low stain intensity (+) in Prussian blue stained slides from clinical cases.

Example of measurements and calculations.

In stained liver sections taken from a native pigeon the visual stain intensity '++++' equated to an image analysis reading of '9.86' expressed in logarithmic form ($\log_{10}$).

In stained liver sections taken from a kaka the visual stain intensity '+' equated to an image analysis reading of '5.62' expressed in logarithmic form.

The calculations were as follows;

Native pigeon

Stain Intensity (163,476 $\pm$ 4,000) $\times$ Area (43,902 $\pm$ 2,000) = Total iron (7,176,900,000 $\pm$ 8,000,000); in the result table this is expressed in $\log_{10}$, ie. 9.86 $\pm$ 0.001.

Kaka

Stain Intensity (1,311 $\pm$ 200) $\times$ Area (320 $\pm$ 50) = Total iron (419,520 $\pm$ 10,000); expressed as a $\log_{10}$ the value is 5.62 $\pm$ 0.01.

The error is minimal in relation to the total value and it was decided not to include it in the general result analysis. For each case the final value is the mean of five readings.

Choice of programme option

Three programme options were available in the iron assessment programme.

1: Integrated intensity and area

This option was used to measure the area and intensity of blue staining in the field of view and recorded each value as a total value for the field of view captured by the frame grabber. Slides stained with the Perls' iron stain or the modified Prussian blue stain were analyzed using this option.

2: Area and intensity of iron and of bacteria

This option was used to measure the intensity and area of blue staining in the field of view and the area and intensity of pink staining in either the total field of view or in an area outlined by a cursor. Slides stained with the combined Perls' iron-Gram Twort stain were analyzed using this option. In all of the slides examined using this option the area of bacteria to be considered for analysis was outlined by a cursor. The intensity value for the pink stained area was discounted as this could not be calibrated sufficiently to be quantitative.

3: Macpath

This option was used to measure the area and intensity of blue staining within selected areas of the field of view as outlined by a cursor. Perls' iron-stained sections were analyzed using this option and the cursor was used to delineate the Kupffer cell and hepatocyte stainable iron and evaluate each separately. Area and intensity values were compared for each field of view on each slide.

4-2-6 Immunocytochemistry

Sections were dewaxed in two changes of xylene and rehydrated through two changes of absolute alcohol followed by immersion in 70% alcohol. The sections were then washed in distilled water for 10 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide (9 ml in 300 ml PBS) for 30 minutes. Sections were then washed in running tap water for three minutes and washed in PBS (phosphate buffered saline) for 10 minutes. A trypsin digest (0.1%) was used on the formalin-fixed sections to release cross-linked proteins. A volume of trypsin solution sufficient to cover each section was left to incubate for 7-10 minutes before the sections were washed in PBS for ten minutes. Non-specific binding was blocked by incubating the sections with a 10% solution of normal goat serum (0.1 ml goat serum mixed with 0.9 ml of PBS 0.05% Tween-20-3% milk substitute) for 5 minutes. These sections were then incubated with the primary antibody for 1 hour. Hyperimmune rabbit sera was used for immunocytochemistry, the working dilution for the primary antibody depended on the serotype of *Y. pseudotuberculosis* causing the lesion. Preparation of hyperimmune serum was described in section (3-2-1). Serum was diluted in blocking buffer containing PBS 0.05% Tween-20-3% milk substitute. Following incubation, sections were washed three times for five minutes in PBS. Sections were incubated in the second antibody for 30 minutes. Commercial goat anti-rabbit IgG-peroxidase conjugate was used at a concentration of 1/1,000 diluted in blocking buffer. Sections were washed in three changes of PBS for five minutes before the addition of peroxidase substrate. The substrate was prepared by the addition of 5 mg DAB (diaminobenzidine, Sigma chemical Co. USA) to 10 ml PBS followed by 12 microlitres of hydrogen peroxide and 50 microlitres of 1% Cobalt chloride. The sections were incubated with the substrate for 5 minutes prior to washing and counterstaining with Meyer's haemalum for 30 seconds, washing in Scott's tap water for 1 minute and rinsing. Sections were then dehydrated and mounted in DPX. Positive and negative controls were run in each batch of slides used for immunocytochemical staining with either the primary or the secondary antibody left out of the protocol.

Determination of the specificity of the immunocytochemistry technique

Where the serotype of *Y. pseudotuberculosis* associated with liver and spleen lesions in retrospective clinical material was known, the primary antibody chosen was the rabbit antiserum raised against the homologous heat-killed antigen. The staining intensity was compared visually, following the use of heterologous antisera as the primary antibody. In cases where the serotype of *Y. pseudotuberculosis* was unknown each of the three serotypes were evaluated using immunocytochemical staining. The staining intensity resulting from the use of the three different hyperimmune sera as the primary antibody, was compared.

In addition, slides of liver and spleen sections from avian cases of bacterial diseases other than pseudotuberculosis, but which gave similar histological lesions, were also processed and stained as described above to determine the specificity of the test.

Expression of the results

The amount of bacterial antigen present in liver and spleen tissue was expressed as the intensity of the brown to dark brown colour which developed at the sites of antigen-antibody reactions. The intensity of the colour was subjectively graded as highly intense (++++), intense (+++), less intense (++) , trace (+) and negative (-) depending on the amount and distribution of colour in the tissues.

4-2-7 Isolation and identification of bacterial strains

Experimental birds

Prior to and following oral challenge with serial dilutions of the challenge isolate (LORIKEET-MUVPPH-92, see sections (3-2-2) and (4-2-8), faecal samples were screened for yersiniae and salmonellae as outlined for the survey samples. One gram of faecal material from each bird was collected from the floor of each cage. The cages had plastic floors and were washed with Virkon (Antec International, no. 2164851) daily. The faecal samples were added to 10 ml PBS and processed as described in chapter two. Isolates were stored at -70 °C for *in vitro* virulence testing and plasmid isolation and

compared with isolates derived from natural cases of the disease and from the two birds outlined in the survey work.

Survey samples

In the survey, two isolates of *Y.pseudotuberculosis* were recovered from the intestinal tract of two house sparrows which had been found dead at a piggery. The intestinal contents were scraped into a sterile 10 ml vial of PBS (phosphate buffered saline) using a sterile swab.

Clinical cases

Necropsy was performed on birds which died of the disease and sections of liver, spleen, lung and intestinal contents were taken for bacteriological examination. Visceral organs were handled aseptically and were flamed, cut with a scalpel blade and swabbed. Swabs were moistened with sterile PBS prior to plating on CIN and blood agar. Samples were screened for *Escherichia coli*, *Salmonella spp*, *Pasteurella spp* and *Yersinia spp*. *Yersinia* were identified as outlined in chapter two and *Y.pseudotuberculosis* isolates were stored for serotyping and virulence testing.

4-2-8 Storage and handling of the challenge isolate (LORIKEET-MUVPPH-92)

This isolate was obtained from the spleen of a lorikeet (*Trichoglossus haematodus moluccanus*) which died of clinical pseudotuberculosis. The isolate was screened and stored at -70 °C as outlined above. This isolate was thawed after a month in storage and plated onto blood agar to check purity. The plate was incubated at 28 °C for 48 hours and harvested into 10 ml of heart infusion broth. This broth was incubated at 28 °C for 24 hours and 10 microlitre aliquots were plated onto each of 20 blood agar plates. These were incubated overnight at 28 °C and each was harvested into 2 ml of tryptone water containing 15% glycerol. After 6 hours at 28 °C, 1 ml aliquots were pipetted into 1.5 ml plastic micro-centrifuge tubes (Treff lab. Switzerland), labelled and frozen at -70 °C. These aliquots were stored for later use as challenge bacteria in the experiments outlined in chapter six and for the *in vitro* work outlined in chapter seven. Each 1 ml aliquot contained approximately 2×10^7 viable bacteria as determined by

colony counts of 10 microlitre loops of serial dilutions plated out on CIN. Six aliquots randomly selected, were checked for contaminants and estimates of viable colony counts/ml were made for each aliquot tested. All aliquots yielded pure cultures of *Y.pseudotuberculosis* serotype II. In the six aliquots tested the bacteria contained a virulence plasmid, grew well on CIN, were urease positive and were positive for the *in vitro* virulence characteristics outlined in the next section.

4-2-9 *In vitro* virulence tests

Autoagglutination test

This test was based on the protocol designed by (Laird & Cavanaugh, 1980). The medium was prepared by mixing 1000 ml of distilled water with 9.72 gm of Modified Minimum Essential Medium Eagle (MEM, Flow laboratories, UK) and stirring for 30 minutes. The final pH of the medium was adjusted to between 7.0 and 7.5 after the addition of 1 gm of sodium bicarbonate (NaHCO_3). The solution was filter sterilised following the addition of 100 ml of foetal bovine serum to make a 10 % solution. The medium was stored in aliquots of 200 ml in sterile bottles at 4 °C until required. The medium was dispensed as a 2 ml aliquot into a small test tube. The tube was then inoculated with a single bacterial colony from the test culture which had been grown on trypticase soy agar. After 24 hr incubation at 37 °C the tube was checked. A positive result is indicated by a layer of irregularly-edged flocculant growth at the bottom of the tube. The medium often remains clear. Agglutination is correlated with virulence. Negative strains remain suspended in the medium making a homogeneous cloudy suspension.

Calcium dependency test

This test was based on the use of Magnesium oxalate agar (MOX) as described by (Mair & Fox, 1986). This was prepared by adding 2.68 gm of sodium oxalate, 4.06 gm of magnesium chloride and 39 gm of Columbia base agar to 1000 ml of distilled water and mixing well. The mixture was then boiled and sterilised by autoclaving at 121 °C for 15 minutes. When cooled the medium was poured into sterile petri dishes. A colony of

the test bacteria incubated at 28 °C for 24 hours on blood agar was isolated and plated onto the test medium. After 24 hours incubation at 37 °C the plates are checked. MOX- positive strains produce both small (< 0.5 mm) and large colonies (> 0.5 mm) on this medium whereas MOX- negative strains produced only large colonies (0.5-2.0mm diameter).

4-2-10 Plasmid isolation

The following protocol was modified from that reported by Portnoy *et al.* (1981). *Yersinia* isolates stored in a solution of tryptone water containing 15% glycerol were thawed and plated onto blood agar, incubated at 28 °C for 24 hours and harvested into 2 ml of brain heart infusion broth (BHI). The cells in the broth were grown to a density of 10^8 - 10^9 . These were incubated at 28 °C for 24 hours and the cells were harvested by centrifugation, washed twice with 50 mM Tris buffer (pH 8.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA) and re-suspended in 40 microlitres of the same solution (TE). Cells were transferred with a Pasteur pipette in to 1.5 ml plastic microcentrifuge tubes containing 60 microlitres of lysis buffer (TE + 4% sodium dodecyl sulphate, SDS) pH 12.4. The tube contents were mixed by inversion of the tubes and incubated in a water bath for 20 minutes at 37 °C. The pH was adjusted to 8.0 by the addition of 130 microlitres of 2 M Tris-hydrochloride (pH 7.0). Single stranded deoxyribonucleic acid (DNA) was precipitated by the addition of 160 microlitres of 5M NaCl. The tubes were chilled in a water bath for 2 hours and centrifuged for 5 min at 15,000g in a microcentrifuge (Eppendorf model, Kempthorne Medical supplies). The supernatant fluid was decanted into another tube and the plasmid DNA precipitated by the addition of 0.55 ml of cold isopropanol. Samples were frozen at -70 °C for 2 hours and the precipitate was collected after centrifugation for 3 minutes in the microcentrifuge. The supernatant was discarded and the precipitate in the tubes was dried overnight in the 37 °C incubator. The following day the sediment was re-suspended in 30 microlitres of TE. One half of each prepared sample, along with samples prepared from *Escherichia coli* which contained plasmids of known molecular weight, were subjected to electrophoresis in a Tris-borate-buffered agarose gel. The gel was stained with ethidium bromide and photographed.

Results

4-3-1 Experimental pseudotuberculosis in chickens

Clinical signs

The birds in the groups which received the largest doses of bacteria (10^7 to 10^8), showed a transient loss of appetite and mucoid diarrhoea in the first three days following infection. There were no further clinical signs noted in these birds except for a mild intermittent diarrhoea. Birds in groups receiving 10^4 to 10^5 bacteria, showed no clinical signs of infection except for occasional loose faeces. Birds in the uninfected control group also occasionally had loose faeces.

Faecal shedding times

Table (4-1) indicates the faecal shedding times of the five birds in each group. The duration of faecal shedding was correlated with the size of the inoculum with birds receiving the highest dose shedding for at least ten days while birds receiving the lowest dose shed for no more than two days.

Table (4-1) A comparison of the number of birds shedding bacteria in each treatment group and the duration of faecal shedding in two-week-old chickens following oral challenge with a single dose of *Y. pseudotuberculosis* serotype II.

Day following challenge	Dose of inoculum (colony forming units)					
	10^8	10^7	10^6	10^5	10^4	Control
0	0	0	0	0	0	0
1	5	5	5	5	5	0
2	5	5	5	5	3	0
3	5	5	5	4	0	0
4	5	5	5	1	0	0
5	5	5	5	0	0	0
6	5	3	4	0	0	0
7	5	4	1	0	0	0
8	5	5	0	0	0	0
9	5	0	0	0	0	0
10	3	0	0	0	0	0
15	0	0	0	0	0	0
20	0	0	0	0	0	0

Gross and histological findings

As shown in Table (4-1), the birds in the groups receiving the highest dose of bacteria (10^7 - 10^8) were lighter in body weight at the end of the experiment than the birds in groups receiving lower doses of bacteria (10^4 - 10^6). Birds in the control group were the heaviest on average although there was variation within groups. There were no gross lesions found in experimental chickens examined 20 days following oral inoculation although in some infected birds the caecal tonsils appeared larger than in the control birds. Histological examination and immunocytochemical studies on the visceral organs including serial sections of the caecal tonsils did not demonstrate the presence of lesions in any of the experimental birds.

Table (4-2) Body weights of experimental birds 20 days after an oral infection with *Y.pseudotuberculosis* serotype II.

Dose of inoculum	Mean weight	Range
10^8	300 g	250-375
10^7	346 g	260-395
10^6	335 g	260-420
10^5	348 g	290-380
10^4	352 g	340-365
Control	372 g	290-490

4-3-2 Retrospective cases of pseudotuberculosis in avian species

Bacteria isolated from clinical cases in captive birds

All of the 11 isolates examined were from captive birds (Table, 4-3). All isolates were serotype II. The faecal isolate was cultured from a sample which had been collected from the aviary floor one day after the death of the bird. All deaths occurred following a period of cool, wet weather in the Spring of 1992 and 1993. The outbreaks of the disease in passerine species occurred in association with changes in management as outlined in Table (4-4) and involved the death of 55% - 65% of the birds in the aviary. The individual birds involved in the sporadic cases noted above had concurrent hepatic haemosiderosis. Other retrospective clinical material available indicated similar findings of concurrent hepatic haemosiderosis as well as the presence of intestinal parasites in two cases and liver disease in three as outlined in Table (4-5).

Table (4-3) Source and serotype of *Y. pseudotuberculosis* strains isolated from clinical cases of pseudotuberculosis in captive birds submitted for necropsy in the period between November 1991 and November 1993.

Species of bird *	Mortality	Number submitted	Source of isolate	Serotype	Month
Kaka	Sporadic	1	Faeces	II	September
Lorikeet	Sporadic	1	Liver	II	September
Native pigeon	Sporadic	1	Liver	II	September
Canary	25/50	3	Spleen	II	August
Canary	12/22	3	Spleen	II	August
Zebra finch	16/25	2	Spleen	II	September

* Latin names can be found in appendix (A-2)

Yersinia pseudotuberculosis was also isolated from the intestinal contents of two house sparrows found moribund at a pig farm (chapter two). Serotype I and II were identified and both contained virulence plasmids as outlined in Table (4-10). These isolates could not be distinguished biochemically from the isolates obtained from the clinical material and were positive for the *in vitro* virulence tests.

Necropsy and histopathology

A: Gross findings

Outbreaks in passeriforms

In the six canaries (*Serinus canaria*) and two zebra finches (*Poephila guttata*) examined, enteritis with enlargement of the caecal tonsils was the predominant finding. There was enlargement of the spleen and liver in five of the canaries and in one finch. There were numerous miliary white foci in the liver of two canaries and in the spleen of one. In each of the three outbreaks in passeriforms only a sample of the population was submitted for necropsy. The history in each case indicated that the birds had died over a period of one to four days with no clinical signs observed prior to death. In the finch aviary there was no mortality in budgerigars (*Melopsittacus undulatus*) housed in the same cage.

Sporadic cases in columbiforms and psittaciforms

In five psittaciforms and four native pigeons (*Hemiphaga novaeseelandiae*) examined in this study or described on file, the predominant lesions were hepatomegaly and splenomegaly with numerous small foci of necrosis in these tissues (figures, 4-7, 4-8 & 4-11). In two of the parrots there were lesions in the lung and kidney. The history in each of the sporadic cases indicated that the birds had become lethargic and lost interest in food over a period of a week and appeared 'fluffed-up' (figure, 4-6). Diarrhoea was not a common clinical finding. In one case a single Kaka (*Nestor meridionalis*) in a group of six had died. This bird had been recently transported and had been chased by the other birds. In this referred case only formalin fixed material was available for study and *Y. pseudotuberculosis* had not been isolated from the lesions of the bird prior to fixation. However, the organism was identified from a faecal sample collected from the aviary and immunocytochemical studies of the fixed material indicated that the faecal strain and the antigenic material in the lesions were the same serotype. During a visit to the aviary the remaining five birds were sampled and found to be negative for the bacterium on faecal culture. A heavy burden of intestinal parasites was identified in a rainbow lorikeet (*Trichoglossus haematodus moluccanus*) which had concurrent haemosiderosis and was in very poor condition when presented for necropsy. In five out of the nine sporadic cases of pseudotuberculosis examined, the clinical history indicated that the course of the disease had lasted for ten days or longer.

B: Histopathology

Passeriforms

In the canaries and the Zebra finches examined there were well developed granulomas identified in the caecal tonsils. In one canary there was a severe enteritis with bacterial colonies in the lamina propria of the intestinal wall, adhesions between intestinal loops and pancreatic involvement. The extent of the bacterial lesions is illustrated in figures (4-3) to (4-5) using immunocytochemical techniques to highlight the bacterial colonisation of the serosal surface. This colonisation extended over the surface of the proventriculus, gizzard and between intestinal loops. Lesions were also identified in the liver, spleen (figure, 4-2) and lung of this bird. The intestinal lesions in the other birds

examined were less severe but in all cases the spleen contained numerous foci of necrosis with little cellular response. In one finch there was a large granuloma in the liver but this did not stain with immunocytochemical techniques. Intestinal parasites (ascarids) were identified in three of the birds examined.

Columbiforms and psittaciforms

In each of the cases examined the lesions were characterised by miliary foci of necrosis in the liver and spleen. The lesions varied in size with a cellular response evident in 30% of the lesions. Giant cells were not a feature in all cases but well formed granulomas were prominent in the livers of three of the four pigeons and three of the five parrots examined (figure, 4-10). Lesions in the lung and kidneys involved bacterial colonies with little cellular response. In the reticuloendothelial organs there was a gradation of lesions with three different types recognised; 1) small lesions containing large numbers of bacteria and little cellular response, 2) intermediate lesions surrounded by heterophils and, in haematoxylin and eosin stained slides, with an amorphous eosinophilic centre and 3) large granulomas with a central zone of necrosis surrounded by giant cells. Gram stained sections demonstrated that, in the intermediate lesions, the bacteria were spread around the periphery and in the granulomas there were few bacteria present. Immunocytochemistry confirmed these observations and also highlighted the presence of bacteria inside macrophages in the spleen and liver. In mature lesions there appeared to be a transitional zone with more bacteria dying or being removed and replaced with necrotic cellular debris. Macrophages in the liver and spleen contained stainable iron in all of these birds with iron also present in the hepatocytes of the liver. In some sections the bacteria could be seen in the macrophages along-side stainable iron in the lysosomes of the cell (figure, 4-9 and 4-14).

4-3-3 Predisposing factors associated with outbreaks of pseudotuberculosis in captive birds

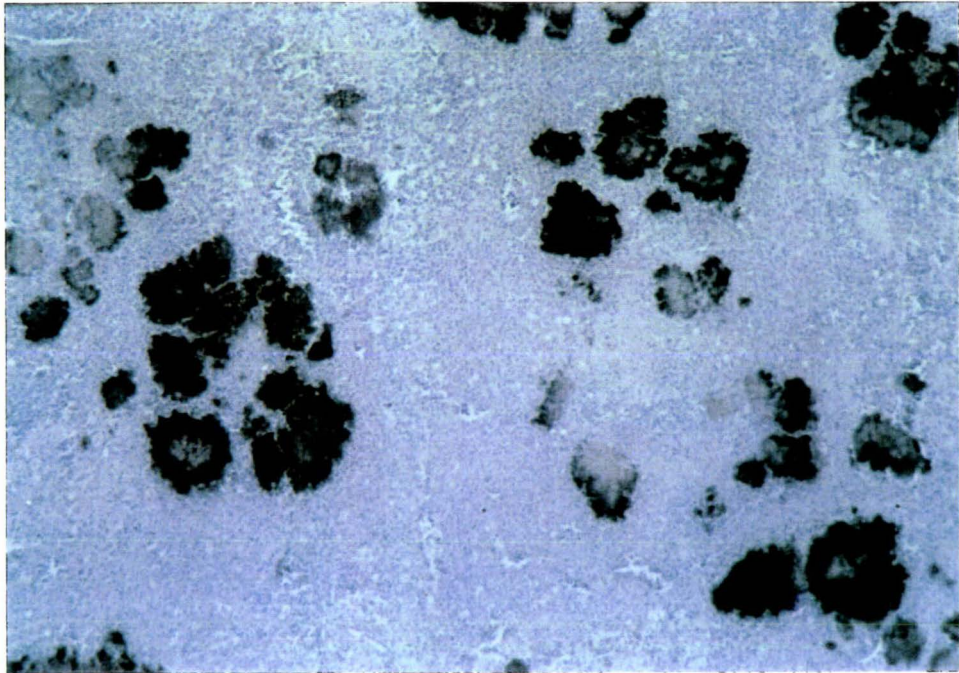
Changes in management such as modification of the diet, introduction of new birds and new personnel were identified as major predisposing factors in the three outbreaks studied.

Table (4-4) Management changes associated with outbreaks of pseudotuberculosis in passerine species maintained in aviaries.

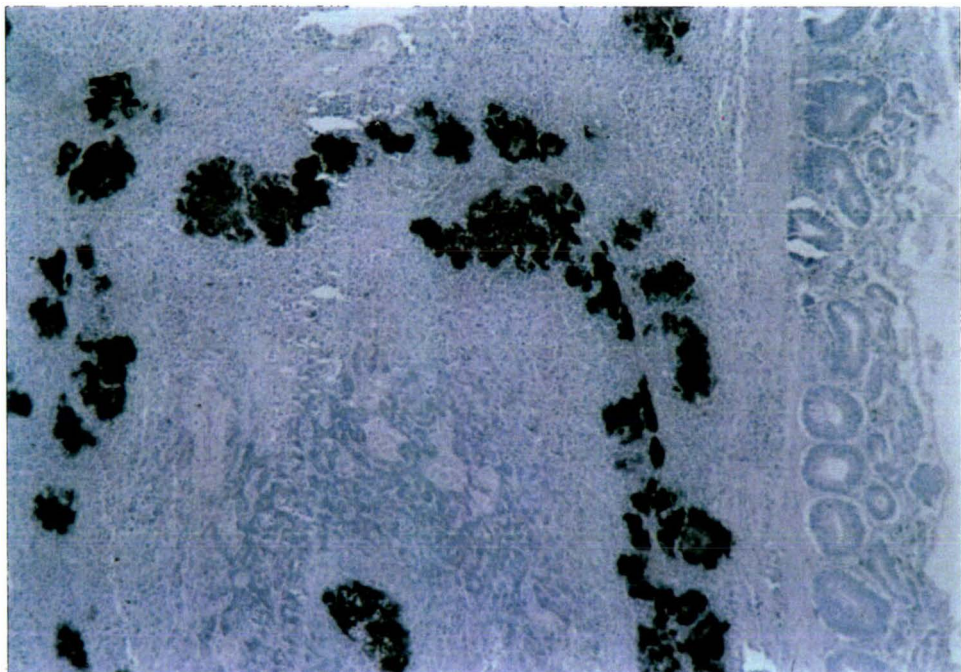
Species	Mortality	Management
Canary	55%	Poor insulation of aviary, recent change in diet
Canary	60%	Recent change in diet, new male birds mixed with flock
Finch	65%	Recent change in diet, change in feeding routine

4-3-4 Concurrent diseases associated with the development of systemic pseudotuberculosis in captive psittacine and columbiform species.

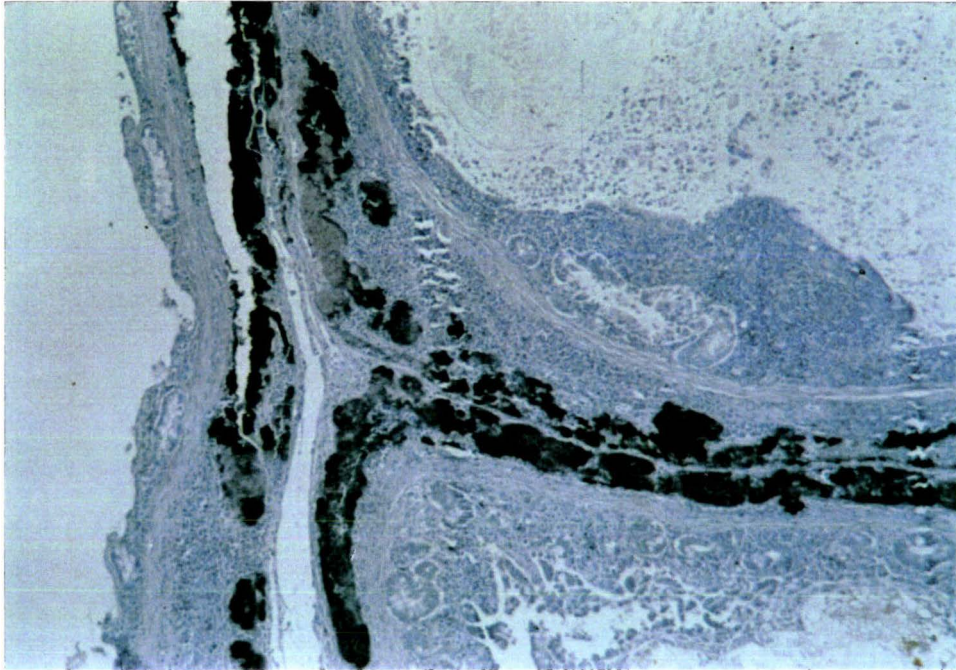
In Table (4-5) it is seen that intestinal parasites, starvation and hepatic haemosiderosis are frequently reported in birds which have died of systemic pseudotuberculosis. The distribution of iron in the liver of these birds was predominantly in the Kupffer cells but in the native pigeons and the lorikeet there was also a significant proportion in the hepatocytes. The relative distribution of iron in representative slides is indicated in Table (4-7).



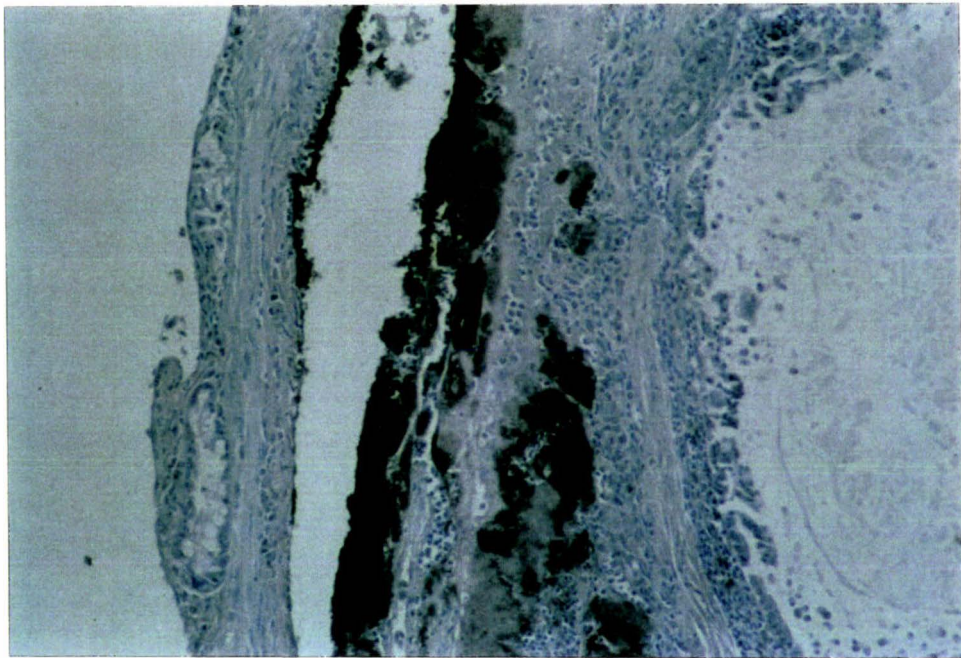
4-2 Immunocytochemical staining of a section of spleen taken from a canary which died following infection with *Y. pseudotuberculosis* serotype II. x 10



4-3 Immunocytochemical staining of a section of jejunum taken from a canary which died following infection with *Y. pseudotuberculosis* serotype II. x 10



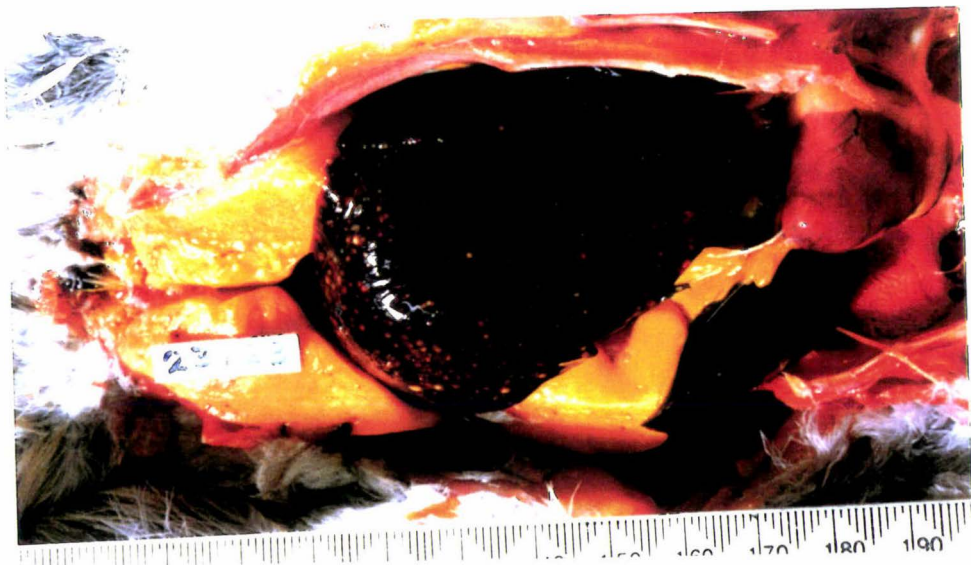
4-4 Immunocytochemical staining of a section of proventriculus taken from a canary which died following infection with *Y. pseudotuberculosis* serotype II. x 10



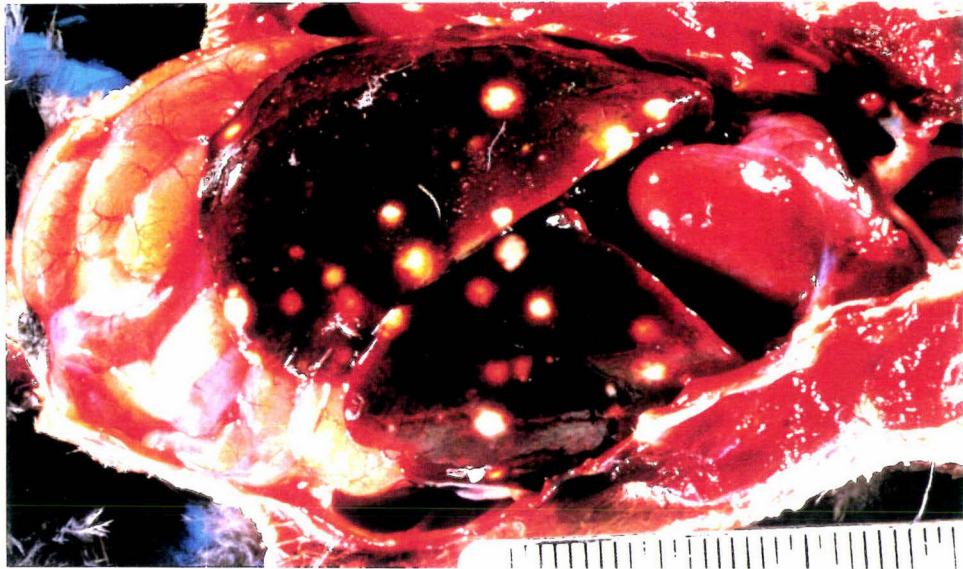
4-5 Immunocytochemical staining of a section of proventriculus taken from a canary which died following infection with *Y. pseudotuberculosis* serotype II. x 10



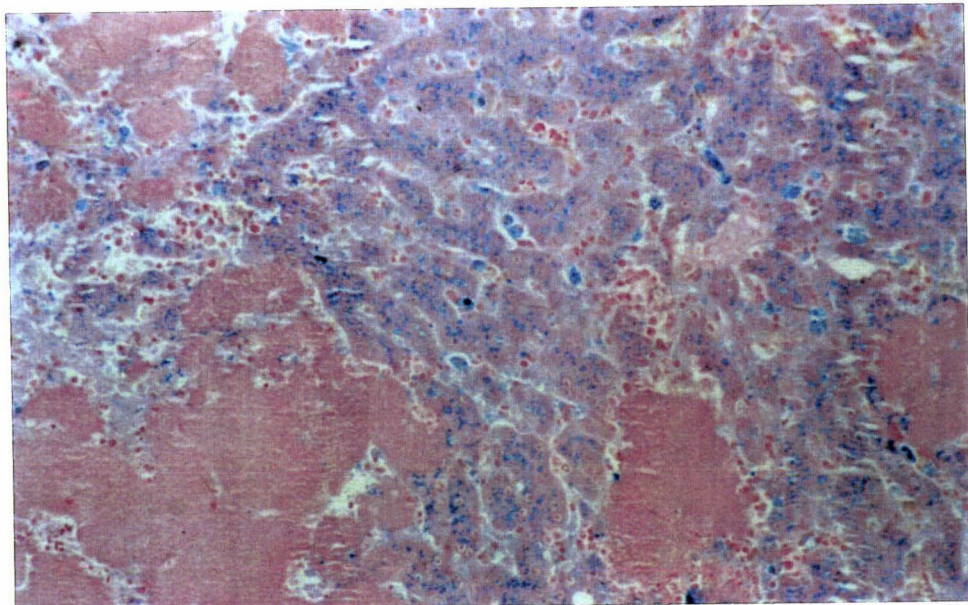
4-6 A native pigeon (*Hemiphaga novaeseelandiae*) showing the non-specific 'fluffed-up' appearance often associated with lethargy, and weight loss in clinical pseudotuberculosis.



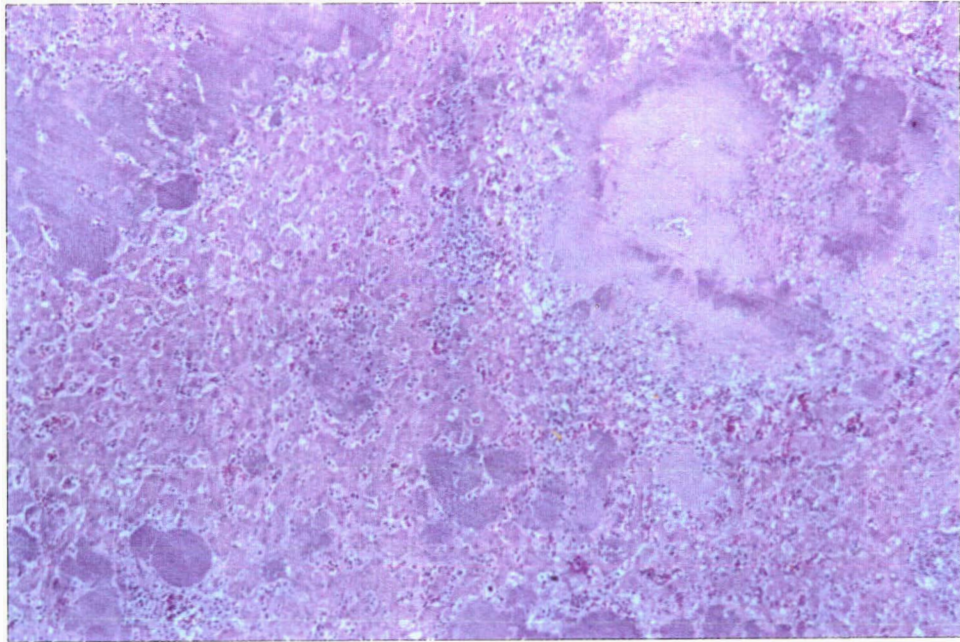
4-7 Gross picture of the enlarged dark liver of a native pigeon which died following infection with *Y. pseudotuberculosis* serotype II. There are numerous miliary foci of necrosis scattered throughout the hepatic parenchymal tissue. The dark colour of the liver indicates that this bird has concurrent haemosiderosis.



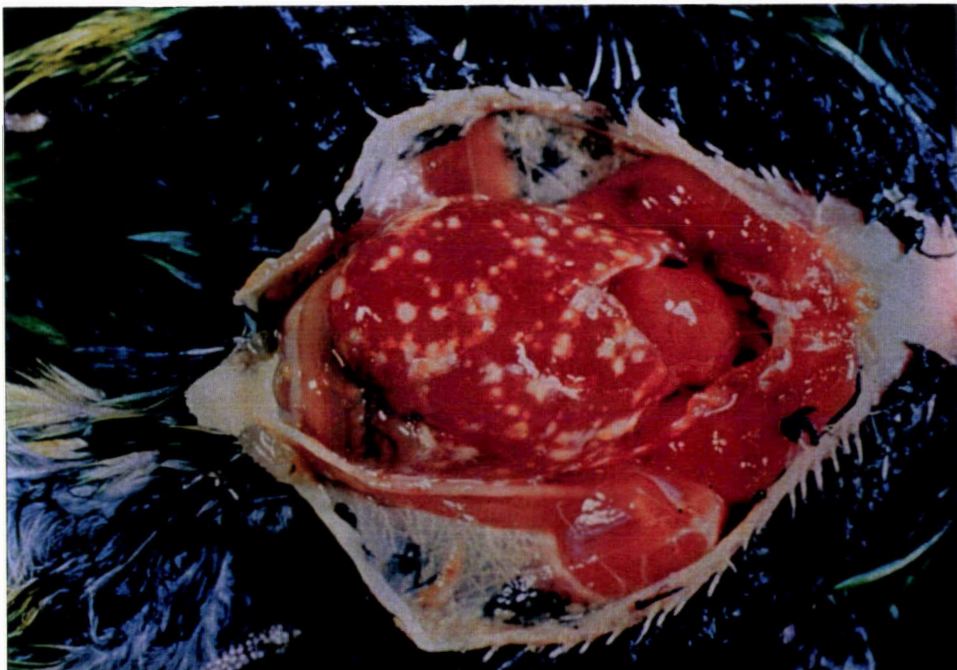
4-8 Gross picture of the enlarged bronzed liver of a rainbow lorikeet (*Trichoglossus haematodus mollucanus*) which died following infection with *Y. pseudotuberculosis* serotype II. There are numerous foci of necrosis scattered throughout the hepatic parenchymal tissue.



4-9 Histological section of the liver illustrated in figure (4-8) showing large areas of necrotic tissue and numerous bacterial colonies. Iron pigment is stained blue and is present in both hepatocytes and Kupffer cells. Perls' iron stained section x 20



4-10 Histological section of the liver seen in figure (4-11) showing a well defined granulomas and numerous foci of necrosis containing bacteria. H & E stained section x 10



4-11 Gross picture of the enlarged liver of a Kakariki (*Cyanoramphus novaezelandiae*) which died following infection with *Y. pseudotuberculosis* serotype II. There are numerous foci of necrosis scattered throughout the hepatic parenchymal tissue.

Table (4-5) Source of histological material from clinical and retrospective cases of avian pseudotuberculosis examined and stained for the purpose of the present study

Species	Concurrent findings	Source
Kaka	Hepatic haemosiderosis	AHL-B*
Lorikeet	Intestinal parasites, hepatic haemosiderosis	MU-VPPH
Kea	Hepatic haemosiderosis, trauma	AHL-B
Budgerigar	Intestinal parasites	MU-VPPH
Kakariki	Intestinal parasites, hepatic haemosiderosis	AHL-B
Native pigeon	Hepatic haemosiderosis, starvation	MU-VPPH
Native pigeon	Hepatic haemosiderosis	OTO*
Native pigeon	Hepatic haemosiderosis, trauma	OTO
Native pigeon	Hepatic haemosiderosis, starvation	MU-VPPH

* AHL-B = Alistair Johnstone. Batchelar Animal Health Laboratory. Palmerston North: OTO= Megan Clemance. Otorohanga kiwi house: MU-VPPH. Department of Veterinary Pathology and Public Health. Massey University.

Table (4-6) Retrospective material reviewed from post mortem reports of avian and mammalian pseudotuberculosis.

Species	Concurrent findings	Source
Native pigeon	Hepatic haemosiderosis, hepatomegaly intestinal parasites	AHL-B
Native pigeon	Hepatic haemosiderosis, fatty liver, trauma	AHL-B
Saddleback	Hepatic haemosiderosis, hepatomegaly	AHL-R***
Stitch bird	Hepatic haemosiderosis	AHL-B
Kiwi	No comments available	AHL-B
Romney lamb (<i>Ovis aries</i>)	Iron pigment in the liver, premature lamb	MU-VPPH
Red deer (<i>Cervus elaphus</i>)	Enteritis, hepatic iron pigment	MU-VPPH
Red deer	Enteritis, hepatic iron pigment	MU-VPPH
Red deer	Enteritis, hepatic iron pigment	MU-VPPH

*** AHL-R = John Howell, Animal Health Laboratory, Ruakura.

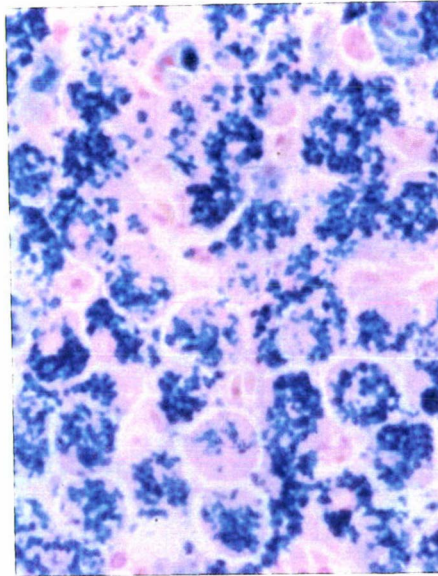
From the results in Table (4-6) it is noted that a significant amount of stainable iron appears to be common in the hepatic tissue of red deer (*Cervus elaphus*). The iron in the liver of the lamb (*Ovis aries*), which was premature and died soon after birth, was present in both the hepatocytes and Kupffer cells. *Yersinia pseudotuberculosis* serotype III was isolated from the lamb and two of the deer. Serotype I was isolated from the other deer examined. In red deer, unlike the avian cases, diarrhoea was a common clinical sign prior to death. In two of the three deer examined there was iron pigment identified in the lamina propria of the intestine and there were numerous bacteria in the intestinal lumen.

4-3-5 Immunocytochemistry and staining results

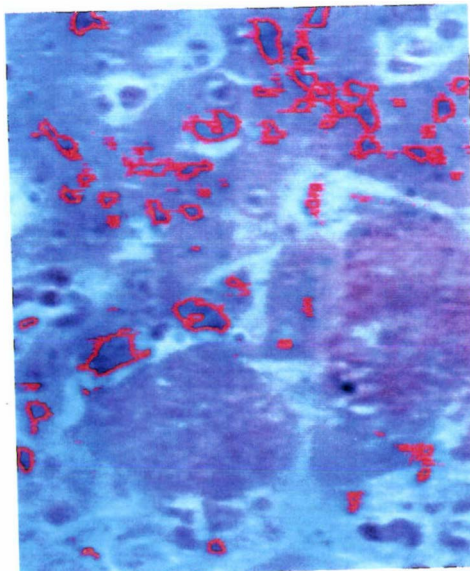
Sensitivity and specificity of the immunocytochemistry technique

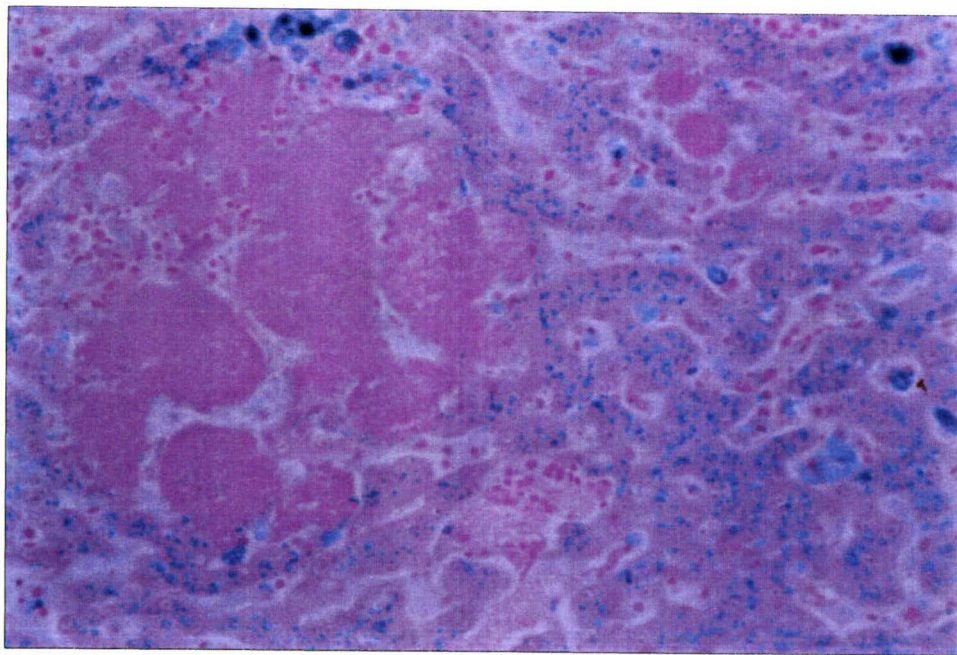
It was found that the immunocytochemistry technique was sensitive and specific for the staining of *Yersinia* species bacteria in histological sections. Rabbit hyperimmune serum raised against heat killed bacterial antigen, when used as the primary antibody in immunocytochemical staining, resulted in a more specific reaction than the Gram stain which does not allow the identification of bacterial species in histological sections. The sensitivity of the immunocytochemistry method and the Gram stain were similar. Both methods were suitable for outlining the extent and distribution of bacteria in different types of lesion as outlined in Table (4-7). The immunocytochemistry technique used was not totally serotype specific for *Y. pseudotuberculosis* but there was a difference in staining intensity with the greatest intensity resulting from the use of the homologous antiserum and a lesser response when heterologous antisera were used as outlined in Table (4-8).

4-12 Photograph of the 'image grabber' screen showing the amount of stainable iron seen in a Perls' iron stained hepatic section taken from a stitchbird (*Notiomystis cincta*) with haemosiderosis. x 40.



4-13 Photograph of the 'image grabber' screen showing the image analysis evaluation of stainable iron in a sector of the stained section seen in figure 4-14





4-14 Perls' iron stained section of the liver of a rainbow lorikeet showing a well defined bacterial lesion and iron in hepatocytes and Kupffer cells. Perls' iron stain x 40. The image analysis outline of this section is illustrated in figure (4-13).

Table (4-7) A comparison of the amount and distribution of stainable iron and bacterial antigen in stained liver sections of birds with histological lesions in the liver.

Species of bird +	Diagnosis	Perls' iron Ferric iron (Fe ³⁺)*	Bacterial antigen	
			Gram stain**	Immunocytochemistry***
Canary	Pseudotb.	+K	+	+++
Canary	Pseudotb.	+K	+	++
Canary	Pseudotb.	+K	++	+
Canary	Pseudotb.	+K	+	+
Canary	Pseudotb.	+K	+	++
Zebra finch	Pseudotb.	+K	+	+
Native pigeon	Pseudotb.	+++K\++H	+++	++++
Native pigeon	Pseudotb.	+++K\+++H	+++	++++
Native pigeon	Pseudotb.	+++K\++H	+++	+++
Lorikeet	Pseudotb.	+++K\++H	+++	++++
Kaka	Pseudotb.	++K\++H	+++	+++
Budgerigar	Pseudotb.	+K	+	+
Rosella	Tuberculosis	+K\+H	-	-
Spur winged plover	Bact. hepat.	+K\+H	++	-
Storm petrel	Bact. hepat.	+K\+H	++	-

+ Latin names for the species of bird listed can be found in appendix (A-2). Pseudotb. = Pseudotuberculosis; Bact. hepat. = Bacterial hepatitis; K = Kupffer cell; H = Hepatocyte. '++++' = strong staining reaction; '-' = negative staining reaction. * Perls' iron stained sections, intensity of reaction determined subjectively with either Kupffer cell (K) distribution or hepatocyte (H) distribution. ** Gram stained sections, amount of bacteria stained in lesions. *** Immunocytochemistry, intensity of staining in lesions.

Serotype specificity of immunocytochemical staining

The intensity of the staining reaction in the liver and spleen with the homologous antisera used as the primary antibody in the immunocytochemical test is shown in figures (4-2) to (4-5). It was found that there was enough cross reactivity between heterologous serotypes to allow the technique to work without prior knowledge of the serotype of the causative agent but that the results were better if the homologous antiserum was chosen.

Table (4-8) Comparison of the staining intensity in different sections of tissue, infected with *Y. pseudotuberculosis* serotype II, incubated with homologous and heterologous primary antisera.

Antisera	Location of lesions			
	Liver	Spleen	Intestine	Caecal tonsil
Yps I	+	+	-	+
Yps II	+++	+++	+	++
Yps III	+	+	-	+
Yent 0.3	-	-	-	-

Visual assessment of Perls' iron stained sections

A visual assessment of the amount and distribution of stainable iron in liver sections from different avian cases is given in Table (4-7). This will be examined in more detail in chapter five. Briefly, in the experimental chickens there was no visually determined stainable iron in the liver, spleen or other organs examined. In the retrospective cases there was a significant amount of stainable iron in the spleen and liver of pigeons and parrots studied which had pseudotuberculosis. In the passerine species there was stainable iron present in the reticuloendothelial cells of the spleen and liver but not in the hepatocytes.

Image analysis and determination of number and distribution of bacterial lesions in liver sections stained with a combined Perls' iron-Gram Twort stain.

The results of image analysis of sections of liver stained with Perls' iron stain and combined Perls' iron-Gram Twort stain are given in Table (4-9). Representative sections of slides examined are illustrated in figures (4-12) and (4-13). It was found that in the chronic cases of pseudotuberculosis in the native pigeons and psittaciform species there was a significant amount of stainable iron in the liver. Although the number of lesions did not appear to be related to the amount of iron present the size of the lesions was greater in birds with high levels of stainable iron in the liver. In the passeriform species examined there was very little stainable iron present in the liver and this

appeared to be restricted to the Kupffer cells. The hepatic lesions in these birds were numerous but small. In the passerines the largest lesions occurred in the spleen.

Table (4-9) The use of Image analysis to quantify the relative amount of iron and the size of bacterial lesions in Perls' iron-Gram Twort stained liver sections from clinical cases of pseudotuberculosis in birds

Species of bird *	Stainable hepatic iron			Lesion		
	Intensity	Area	Total	Total area	Average area	No
Rainbow lorikeet	3.6	3.1	6.7	3.9	3.2	5
Kaka	2.6	3.0	5.6	4.3	3.9	6
Budgerigar	3.0	3.4	6.4	2.0	1.0	10
Native pigeon	4.8	4.6	9.4	3.7	3.1	5
Native pigeon	4.3	4.6	8.7	5.4	4.9	5
Native pigeon	3.4	4.9	8.3	4.7	3.8	12
Canary	1.9	2.1	4.0	3.9	1.0	15
Canary	1.9	2.1	4.0	4.0	1.1	13
Canary	1.6	2.2	3.8	3.4	1.2	9
Canary	1.6	2.1	3.7	3.9	1.2	10
Canary	1.8	2.4	4.2	3.3	1.1	11
Zebra finch	2.0	2.2	4.2	3.2	1.0	12

* Latin names for the birds listed can be found in appendix (A-2).

From the results in Table (4-9) and the visual assessment of lesions it is apparent that the relationship between the amount of stainable hepatic iron (area and intensity) and the area of bacterial lesions is not a simple one. The bacterial lesions in the pigeons are larger and contained more bacterial antigen than in the canaries and the finch when sections were examined using immunocytochemical techniques. There is some species difference in both the distribution and degree of iron in the liver. In the canaries, the zebra finch and the budgerigar examined, the iron was in Kupffer cells only (figure, 4-5). In the Kaka the iron was predominantly in Kupffer cells but also in hepatocytes to a lesser extent. In the Rainbow lorikeet and the native pigeons examined the distribution of iron was in both Kupffer cells and hepatocytes (figure, 4-14). The relative distribution of iron in the livers of different species of bird will be considered in more detail in chapter five.

4-3-6 Virulence characteristics of *Yersinia* species isolated from avian samples

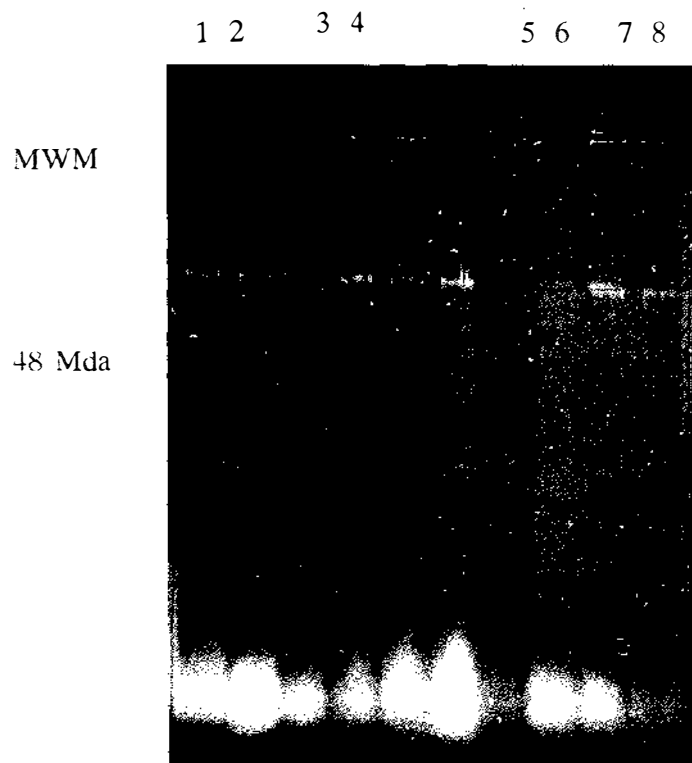
Virulence plasmids (48 MDa) were extracted from all of the *Yersinia pseudotuberculosis* isolates including the samples isolated during the survey. These are illustrated in figure (4-15). In comparison the non-pathogenic yersiniae, *Y. frederiksenii* and *Y. enterocolitica* biotype 1A isolates did not contain virulence plasmids. The presence of a plasmid was correlated with calcium dependency at 37 °C (MOX), autoagglutination and pigmentation on congo red agar (CRMox) agar at 37 °C.

Table (4-10) *In vitro* virulence characteristics of *Y. pseudotuberculosis* in comparison with non pathogenic yersiniae isolated from avian material and other sources

Isolate	Serotype	Invitro characteristics		P	Identification
		Autoaggl.	Ca dependency		
<i>Y.pseudotb</i>	I	+	+	+	YPS-HSP-5M PIGGERY
<i>Y.pseudotb</i>	II	+	+	+	YPS-HSP-4M PIGGERY
<i>Y.pseudotb</i>	II	+	+	+	YPS-KAKA-2-SEPT.92
<i>Y.pseudotb</i>	II	+	+	+	YPS-22920 FINCH
<i>Y.pseudotb</i>	II	+	+	+	YPS-22739 CANARY
<i>Y.pseudotb</i>	II	+	+	+	YPS-LORIKEET
<i>Y.pseudotb</i>	II	+	+	+	YPS-22877 CANARY
<i>Y.pseudotb</i>	II	+	+	+	YPS-23723 PIGEON
<i>Y.enter.</i>	1A	-	-	-	KAKA-AV-W
<i>Y.fred.</i>	NA	-	-	-	YFRED- 23111

P = virulence plasmid (see figure 4-15); NA = information not available

Figure (4-15) Plasmid content of *Yersinia pseudotuberculosis* strains isolated from avian sources.



- 1) YPS-HSP-5M PIGGERY; 2) YPS-HSP-4M PIGGERY; 3) YPS-KAKA-2-SEPT.92;
4) YPS-22920 FINCH; 5) YPS-22739 CANARY; 6) YPS-LORIKEET; 7) YPS-22877
CANARY; 8) YPS-23723 PIGEON

Discussion

Stovell (1963) conducted a series of experiments using canaries to study the faecal excretion of *Y. pseudotuberculosis* following a single oral inoculation. His data indicated that 16 out of 20 experimental canaries shed from 10^4 - 10^7 viable bacteria per day for 3-19 days. The 12 experimental birds which survived, ceased shedding the bacteria 20 days following the last oral inoculation. In the present study it was found that two-week-old chickens given a single oral inoculation of *Y. pseudotuberculosis* serotype II excreted viable bacteria for two to ten days. The chickens which shed bacteria for the longest period were those which received the highest initial dose. Unlike the canary study there were no mortalities and clinical signs of the disease only occurred for a brief period of time and only in the high-dose groups. Necropsy of birds in all groups at day 20 indicated that there were no gross lesions except for some enlargement of the caecal tonsils in a third of the birds. This may indicate antigenic stimulation in these birds but it was not possible to demonstrate the presence of bacterial antigen using immunocytochemistry. Stovell (1963) found that canaries which died during the experimental infection had enlarged caecal tonsils and that *Y. pseudotuberculosis* could be readily isolated from these lesions. It was concluded from this finding that canaries which had caecal abscesses shed the largest number of bacteria and were a source of infection for other canaries as well as being responsible for environmental contamination. In chickens this may also be true but, as reported in the literature (Muir, 1898; Pfaff, 1905; Wetzler, 1971), chickens are less susceptible to the disease. A larger dose of bacteria ($>10^8$) given over an extended period of time may be required to establish infection in the White Leghorn breed chosen for this study especially in a controlled experimental situation in which 'stress' factors are limited.

The pattern of faecal shedding and the results of the concurrent serological study, discussed in chapter three, indicate that the response of White Leghorn chickens to enteric infection with *Y. pseudotuberculosis* is similar to that measured in experimental *Salmonella enteritidis* infection. The latter has been reviewed recently by Chart *et al.* (1993) and discussed by Barrow (1992). In addition, in *Salmonella gallinarum* infection, there was found to be a dose related duration of faecal shedding in chickens following a single oral dose of bacteria and no obvious clinical signs or necropsy findings (Cooper *et al.*, 1991). The results of the present study indicate that the same occurs in *Y.*

pseudotuberculosis infection in the chicken. The serological response to *Y. pseudotuberculosis* antigen, in common with what is found for *Salmonella gallinarum*, did not follow the pattern of faecal excretion but appeared to peak at six to ten days in all groups with a less well defined dose related pattern. Agglutinating antibodies to *Salmonella* were present for five months after the infection had been identified on faecal culture in some chickens in flocks where field infection was occurring (Chart *et al.*, 1991).

In the present study, the challenge inoculum was given as a single dose to apparently healthy chickens. The *Y. pseudotuberculosis* strain used was isolated from the spleen of a lorikeet. This strain was serotype II and contained a virulence plasmid. The possession of the 48 Mda plasmid in this isolate and other clinical isolates was found to correlate well with the expression of *in vitro* virulence characteristics such as calcium dependency at 37 °C and autoagglutination. *Yersinia pseudotuberculosis* serotype II was the serotype most frequently isolated from clinical cases of the disease in birds in the present study. In North America and Europe, serotype I is reported to be more commonly associated with disease outbreaks and sporadic cases of the disease in birds (Wetzler, 1971; Keymer, 1980; Parsons, 1991). Stovell (1963) used a clinical isolate from an outbreak of disease in a canary colony for his experimental challenge strain, this was serotype I. The *in vitro* virulence characteristics of the two serotypes are indistinguishable (Wetzler, 1965) although a recent review by de Almeida *et al.*, (1993) indicates that the 'iron repressible protein' gene (irp 23) which is associated with iron acquisition may be different in *Y. pseudotuberculosis* serotype II. There has not been any work to fully evaluate the significance of their results in the clinical situation and it may be that the strains used in the study were not representative of the serotype II group (de Almeida *et al.*, 1993). *Yersinia pseudotuberculosis* serotype II is subclassified into group a and b by the Japanese (Tsubukura *et al.*, 1984) on the basis of variation in the 'O' antigen. There has been no evidence to suggest that the two sub-groups differ in pathogenicity although this possibility can not be ignored in relation to possible species difference in susceptibility. The lorikeet strain (LORIKEET-VPPH-92) used for the present study was compared with the standard type strain (IP 2935) from the Pasteur Institute. The strains were morphologically similar, shared the same biochemical characteristics and contained virulence plasmids of the same molecular

weight. Lectin probes indicated that the lorikeet strain was serotype IIa but unfortunately it was not possible to determine the subtype of the Pasteur institute strain. In the experimental challenge faecal isolates were compared with the challenge isolate and it was found that following passage through the intestinal tract the bacteria still contained the virulence plasmid. In addition the Kaka isolate, which had been subcultured from the faeces of a bird which had died the previous day was also found to have retained the virulence plasmid. This suggests that although *Y. pseudotuberculosis* bacteria are frequently reported to lose the virulence plasmid on subculture when stored *in vitro*, this may not be an important factor in field situations.

In the present study, an *in vitro* experiment which examined the survival of *Y. pseudotuberculosis* serotype I, II and III in distilled water indicated that the bacteria survived for 18 months when stored at 4 °C and it was still possible to extract plasmids from the cultures (see appendix A-11). However, when stored at 28 °C the bacteria were overgrown by contaminants and could not be cultured. This adds support to the suggestion that environmental conditions are important in the maintenance of the disease in mammals and birds (Barre *et al.*, 1979; Fukushima *et al.*, 1991) with more outbreaks of disease occurring following cool, wet weather (Mackintosh and Henderson, 1984; Fiennes, 1982; Parsons, 1991).

It has been reported several times that *Y. pseudotuberculosis* is more readily isolated from the environment in the winter and spring (Swaminathan *et al.*, 1982; Mackintosh and Henderson, 1989). However, this does not explain why some birds in an aviary develop clinical disease while others remain healthy.

Stovell (1963) examined three outbreaks of pseudotuberculosis in North American canary colonies and noted that cold weather, reproductive stress, over heating and poor ventilation were important in precipitating the disease. In one case paprika had been added to the diet of show canaries to enhance their colour. It was suggested that because paprika was a bowel irritant this may have predisposed the birds to the development of the disease (Stovell, 1963). In the present study three outbreaks of the disease were studied which involved captive finches and canaries. In all three incidents there were management factors which appeared to have precipitated the disease resulting in high mortality. In none of the cases was it possible to isolate the causative

agent from the environment or from faecal samples from recovered birds. The outbreaks were controlled by changes in management and antibiotic therapy.

In the sporadic cases studied, there appeared to be individual birds which were more susceptible to the disease because there was no evidence to suggest that the other birds were not exposed to the same infection. It is possible that there is a species difference with regard to susceptibility but where individuals share the same environment and food sources, as was the case with the kaka, it would appear that certain predisposing factors may potentiate the development of clinical disease. In the native pigeons and in most of the psittaciforms studied, there was a significant amount of haemosiderin in the liver distributed in both the hepatocytes and the Kupffer cells. The lesions in the liver of these birds appeared more florid and larger than those in the canaries which had only limited stainable iron in their livers which was restricted to the reticuloendothelial cells. In people the association between stainable iron in the liver, iron availability and the development of systemic pseudotuberculosis has been well documented (Rabson *et al.*, 1975, Robins-Browne *et al.*, 1987).

From the results of this chapter it is evident that the possession of a virulence plasmid by a strain of *Y. pseudotuberculosis* does not necessarily indicate that infection will result in disease. It is clear that predisposing factors are important in the development of clinical disease and that there is a wide variation in species and individual susceptibility to *Y. pseudotuberculosis*. Concurrent parasitism, poor nutrition and cold weather are implicated in the majority of clinical cases of the disease in mammals and birds.

Summary

1) An immunocytochemical test has been developed to detect the presence of LPS antigen in bacterial lesions. This test allows identification of both the species and the serotype of *Y. pseudotuberculosis* in lesions and is therefore more specific than the chemical staining.

2) Chickens challenged with oral doses of *Y. pseudotuberculosis* serotype II did not develop clinical disease and, even at the highest dose of 10^9 organisms, ceased shedding *Yersinia* in the faeces within 15 days. At necropsy *Y. pseudotuberculosis* was not cultured

from the intestinal tissue nor were there any lesions found in other organs 20 days following challenge.

3) Clinical cases of pseudotuberculosis were examined in passeriforms, columbiforms and psittaciforms. Necropsy and histological examination supported the hypothesis that there are species differences in the pathogenesis of pseudotuberculosis but that this also depends on concurrent disease

4) Outbreaks of the disease examined in captive passeriforms were associated with poor management and occurred after periods of cold wet weather. In the passeriforms there was a large amount of antigen present in the caecal tonsils and in the spleens of the finches and canaries examined. The clinical history and the necropsy findings were consistent with a diagnosis of acute overwhelming septicaemia.

5) Sporadic cases of the disease examined were associated with concurrent disease such as parasitism, starvation and chronic debilitation. In the individual columbiforms and most of the psittaciforms examined the lesions were predominantly in the liver and spleen although some birds also had lesions in the kidney, lung and intestine. The lesions were well walled off with giant cell formation and well developed granulomas in the spleen and liver. These lesions are consistent with a more chronic disease often associated with species less susceptible to the disease. Birds which were in-contact with clinically effected birds did not develop the disease although the causative agent was isolated from faecal samples.

5) In the chronic cases of the disease the presence of excess hepatic iron was associated with large florid lesions in the liver. Immunocytochemical examination indicated that these lesions contained a large amount of bacterial antigen.

6) In both the acute and chronic forms of pseudotuberculosis there was an association between the amount of iron present in the Kupffer cells and the development of disease. To examine this further it is necessary to determine the factors affecting the amount of iron in the livers of birds and whether or not species differences in iron metabolism are important in 1) determining species susceptibility to pseudotuberculosis or 2) could be associated with the seasonal factors which affect this susceptibility.

In the next chapter the link between iron availability and stainable iron in the livers of birds is examined. From this it is hoped that a disease model can be developed to examine the possible link between iron availability and the development of systemic *Y. pseudotuberculosis* infections in birds. Quantitative studies of the amount and distribution of stainable iron in the livers of birds and the possible significance of haemosiderosis in regard to infectious disease will form the basis of the next chapter.

CHAPTER FIVE

Haemosiderosis

Introduction

Iron-containing brown pigment occurs frequently in the livers of birds of several orders and families (Lowenstine & Petrak, 1980) and has been observed in both wild and domestic birds in regions all over the world (Ward *et al.*, 1991). The distribution of this histologically stainable iron is variable (Taylor, 1984) indicating that a range of aetiological factors are probably involved in the development of haemosiderosis (Lowenstine & Petrak, 1980). Unfortunately there is little information available on the normal iron metabolism of different avian species (Wadsworth *et al.*, 1983). In some species of bird, especially migratory species, seasonal changes in tissue iron levels occur as part of the normal physiological cycle and are associated with the breeding season and the moult (Osborn, 1979; Osborn and Young, 1985). In a recent study by Ward *et al.*, (1991) it was shown that the regulation of iron uptake at the intestinal level in some avian species was not as tightly regulated as it is in some mammals. However, although some authors favour the hypothesis that dietary overload is responsible for the development of haemosiderosis in many avian species (Kincaid & Stoskopf, 1987) the distribution of iron in the liver tissue is not the same as that seen in human dietary overload (Iancu, 1982; Iancu *et al.*, 1987) or in the early stages of primary haemochromatosis (Powell *et al.*, 1980). There has not been any conclusive evidence that the presence of stainable iron in the liver tissue of birds has any clinical significance although it has been linked to the presence of concurrent infectious (Lowenstine & Petrak, 1980) and neoplastic diseases (Hill *et al.*, 1986). It is also noted that a similar amount of stainable iron in the liver of a human patient would be considered abnormal (Kincaid & Stoskopf, 1987).

Although the presence of histologically stainable iron in the livers of birds is not generally associated with hepatic disease the possible exception to this is the 'haemochromatosis syndrome' seen in the Indian Hill Myna bird (*Gracula religiosa*) (Gosselin, 1981; Gosselin & Kramer, 1983). The term haemochromatosis is used in this

context to describe an excessive accumulation of iron in tissues other than the reticuloendothelial system. Although often associated with hepatic fibrosis, the condition may follow hepatic disease rather than be a cause of it (Gosselin, 1981; Morris *et al.*, 1989). In the literature the terms haemosiderosis or secondary haemochromatosis are used to describe the presence of histologically stainable iron limited to the reticuloendothelial system and hepatic parenchyma (Powell *et al.*, 1980). Although both terms have been used in reference to iron overload in birds (Gosselin & Kramer, 1983; Lowenstine & Petrak, 1980; Ward *et al.*, 1991) the term haemosiderosis will be used in this study. Haemosiderosis used as a descriptive term does not infer a particular pathogenesis to the condition unlike the term haemochromatosis which may be confused with the primary idiopathic disease in human patients which has a genetic basis and results in iron overload secondary to poor control of iron uptake at the level of the intestinal epithelium (Nhonoli, 1973).

Experimental work done in the starling (Ward *et al.*, 1991) and retrospective studies of a number of avian species (Taylor, 1984; Lowenstine and Petrak, 1980; Wadsworth *et al.*, 1983) and reindeer (Reimers *et al.*, 1982; Borch-Ionsen & Nilssen, 1987) indicate that changes in liver iron are related to fluctuations in dietary iron and physiological changes which occur in the breeding season and moult. The moult is dependent on an alteration in the amount of circulating thyroid hormone, and this in turn has an important influence on the regulation of serum transferrin (Osborn, 1979). Experimental work on dietary overload and the injection of parenteral iron in rats (Iancu *et al.*, 1987) has linked saturation of serum transferrin with hepatic siderosis.

There are numerous factors influencing iron metabolism in birds and it was decided to do a retrospective study on avian cases of haemosiderosis in an attempt to identify some of the aetiological factors involved along with the presence of any concurrent diseases. In addition an experiment was designed to induce haemosiderosis and study the distribution of parenterally injected iron dextran in liver tissue, in the domestic chicken (*Gallus domesticus*), a species which has low levels of tissue iron under normal circumstances. From this work it was hoped that a model could be developed for examining the clinical significance of iron overload in infectious diseases in addition to determining the significance of haemosiderosis in the avian cases of pseudotuberculosis outlined in chapter four.

Materials and Methods

5-2-1 Experimental haemosiderosis in the chicken

Experimental birds and experimental procedure

Twelve one-day-old male White Leghorn chickens were purchased from a commercial source (Golden lay poultry producers). Faecal samples from these birds were bacteriologically screened for *Yersinia* and *Salmonella* species bacteria. The chicks were divided into two groups and housed in plastic bottomed cages. Water was supplied *ad lib.* and the birds were fed a commercial chick rearing mash (Massey University feed unit). At two weeks of age the birds were divided into four groups of three birds. One bird in each group received an intravenous injection of dextrose saline in a 0.2 ml volume and served as a control. The remaining two birds in each group received an intravenous injection of 10 mg (0.2ml) of iron dextran (Imferon; Fisons, Loughborough) on day one. This dose was based on that used by Ward *et al.* (1991) for experimental studies of haemosiderosis in starlings (*Sturnus vulgaris*). In one group, birds were sacrificed 24 hours after treatment, in the other groups birds were sacrificed 48 hours, 6 days and 10 days following treatment. Treated and control birds were euthanased by an overdose of halothane anaesthesia and necropsy was performed as previously described. Serum was collected from each bird for serology and serum iron biochemistry.

Histology and staining

Histological sections were prepared from visceral tissues including the bone marrow from all twelve birds. Liver aspirates were also taken from the iron-treated birds and fixed on slides. All sections were stained with haematoxylin and eosin and Perls' iron stain. Sections were taken from the same tissues in each of the birds. Liver sections were taken from three areas of the liver, the caudal edge, longitudinally through the midline and a tangential section through the cranial edge. Each liver section was stained with Perls' iron stain and modified Perls' iron stain. The amount and distribution of stainable iron in each section of tissue was evaluated visually and by image analysis using the methods outlined in chapter four (section 4-2-5).

Biochemical assessment of iron in liver tissue

Sections of liver were taken from each experimental bird and frozen at -20 °C prior to biochemical assessment of total iron content. The analysis was carried out by the Wallaceville Animal Health Laboratory, Upper Hutt, using acid digestion followed by atom absorption spectrophotometry.

Calibration of image analysis values

A Total stainable iron

A quantitative assessment of the amount and distribution of stainable ferric (Perls' iron stain) and ferric + ferrous iron (modified Perls' iron stain) was obtained using image analysis of liver sections from each experimental bird. The values for total stainable iron in each section were determined by adding the products of area (A) multiplied by average intensity (I) for the Kupffer cells and the hepatocytes.

Total stainable iron = Hepatocyte iron (A x I) + Kupffer cell iron (A x I).

The final value for each bird was determined as the mean value obtained from analysis of five fields of view on each of five sections from different areas of the liver. Only sections with uniform staining were used for analysis and for each section the five fields of view were randomly selected within five sectors drawn on the slide.

These values were then expressed as logarithms ($\log_{10}$) and compared with biochemical iron measurements, determined as micro-moles per gram, for each bird. Determination of the quantitative difference in hepatic iron levels between treated and control chickens was achieved by comparing the total amount of hepatic iron determined by biochemical analysis.

B Distribution

The relative distribution of iron following injection was determined using image analysis to assess the amount of stainable iron in the hepatocytes compared with that in the Kupffer cells. This was achieved using a manual system 'Macpath' (see chapter four) to outline the Kupffer cells in the section so that calculations for the iron content of Kupffer cells and hepatocytes could be determined separately.

Kupffer cell iron = Area (Kupffer cell iron) x average Intensity (Kupffer cell iron)

Hepatocyte iron = Area (hepatocyte iron) x average Intensity (hepatocyte cell iron)

All results were expressed in $\log_{10}$ form and compared to values obtained in the retrospective cases.

5-2-2 Retrospective cases of haemosiderosis in birds

Source of material

Necropsies were performed on 180 birds submitted to the Department of Veterinary Pathology and Public Health over a period of two years (September 1991 to 1993). The cases were submitted from captive breeding establishments and wild life monitoring schemes run by the New Zealand Department of Conservation in addition to cases from private aviculturalists and veterinarians. The majority of cases were native birds and the remainder were introduced species and cage birds. There were a total of 40 different avian species, representing six of the major avian orders, included in the study. These are detailed in appendix (A-13) which lists both the common and latin names used in the text.

Assessment of hepatic iron stores

Gross and histological findings and the relevant clinical history were recorded for each case. Sections of tissue were cut and stained according to diagnostic requirements to establish the cause of death. In many cases the cause of death was not established but a diagnostic comment is given where appropriate. In each case one or more extra liver

sections were cut and stained with Perls' iron stain. Representative sections of liver tissue were examined visually and subjected to image analysis to allow a quantitative assessment of the extent and distribution of hepatic iron stores.

5-2-3 Expression of the results

Because the image analysis values are empirical it was considered appropriate to scale values in logarithmic form for statistical analysis. Using the logarithmic scale, values range from 0.00 - 20.00.

A summary of representative clinical data is presented in the results of this chapter with each value representing the mean of five readings. The equivalent data for the other cases examined can be found in appendix (A-13).

Because the values are expressed in log 10 form they are added when multiplication of the original value was required, for example when calculating the total amount of iron in hepatocytes or Kupffer cells. When addition of the original value was required, for example, when deriving the total stainable hepatic iron from the sum of the Kupffer cell iron and hepatocyte iron, the value was converted from the log 10 form for the calculation and the product converted back to logarithmic form after the calculation had been made.

Chicken

$$\text{Kupffer cell iron} = A (2.26) \times I (1.60) = \text{Total} (3.86)$$

$$\text{Hepatocyte iron} = A (4.27) \times I (3.46) = \text{Total} (7.73)$$

log₁₀ values calculated to two-decimal places

$$3.86 = \log_{10} 7.244 \div \cdot 100$$

$$7.73 = \log_{10} 53.703.179 \div \cdot 1.000.000$$

$$\text{Kupffer cell iron (7.244)} + \text{Hepatocyte iron (53,703,179)} = \text{Calculated total iron (53,710,423)}$$

$$\text{Total hepatic iron (7.73)} = \text{Kupffer cell iron (3.86)} + \text{Hepatocyte iron (7.73)}$$

Statistical analysis

The data were evaluated using a two-way analysis of variance for the total iron, hepatocyte iron and the Kupffer cell iron in each order of birds. The difference in the amount and distribution of stainable hepatic iron in birds within each order which died of infectious diseases was compared with non-infectious diseases using the students t-test. In this context the term 'infectious' was used to include any disease caused by a microbial or parasitic agent, the term 'non-infectious' was used to include all other diseases. The raw data is available in appendix (A-13) along with the species sampled and the diagnostic comments for each case examined. A summary of the variance and standard deviation for each data set is given in appendix (A-12).

Results

5-3-1 Experimental evaluation of the use of image analysis for assessment of the amount of iron stored in the livers of birds

Experimental haemosiderosis in the chicken

In the birds which received an intravenous injection of 10 mg iron dextran there was a transitory period of panting and lethargy followed by resumption of normal behaviour within 5 minutes. No birds died during the experiment. All birds were negative for *Y.pseudotuberculosis* on serology and by faecal culture. *Salmonella* species were not isolated from the faecal samples of day old birds.

Necropsy and histology

There were no gross abnormalities detected at necropsy although the livers of treated birds were dark in colour compared to control birds. Histological sections stained with Perls' iron stain (for ferric iron) or modified Perls' iron (for ferric and ferrous iron) appeared visually similar. Image analysis results (Table 5-1) indicate that the modified Perls' iron test is less sensitive for ferric iron resulting in lower intensity values ($P < 0.01$). There is no significant difference ($P > 0.05$) in the relative hepatocyte/Kupffer

cell distribution of iron using the two techniques or in the area measurements. Therefore it was decided to use the Perls' iron stain for all of the retrospective material.

Table (5-1) A comparison of the amount and distribution of stainable iron in histological sections, stained for ferric iron and for a combination of ferric and ferrous iron, taken from the liver tissue of experimental chickens examined at 1,2, 6 and 10 days following a single injection of 10 mg iron dextran.

Days following treatment	Fe3+ Kupffer cell		Fe3+/Fe2+ Kupffer cell		Fe3+ Hepatocyte		Fe3+/Fe2+ Hepatocyte	
	A	I	A	I	A	I	A	I
1	3.16	2.65	2.38	0.00	2.52	2.88	2.36	0.00
1	3.95	4.20	2.90	0.00	3.40	2.60	3.40	0.00
2	4.59	3.71	4.56	3.14	4.85	4.21	4.84	3.95
2	4.50	3.84	4.55	3.16	4.85	4.14	4.85	3.79
6	2.26	1.60	2.28	1.90	4.27	3.46	4.27	3.22
6	2.20	2.45	2.18	1.95	4.36	3.61	4.38	3.29
10	1.30	1.00	1.00	0.00	3.69	3.14	3.69	0.00
10	0.00	0.00	0.00	0.00	3.11	3.59	3.11	0.00

*Image analysis values for area and intensity are empirical values on a scale of 0 - 100,000,000 and are expressed in this table in a logarithmic form. A=area, I=intensity.

The distribution of iron in the liver of chickens following parenteral administration of iron dextran

The following comments apply to Perls' iron stained sections. There was no stainable iron detected visually in the tissues of the four control birds nor was there any stainable iron detected by image analysis. In all eight of the treated birds which received a parenteral dose of 10 mg iron dextran there was visually detectable iron in the liver but not in the spleen, bone marrow, intestinal tract or muscle. In heart sections taken from two birds there were macrophages laden with haemosiderin located near to the heart valve. In the liver, stainable iron was present in the Kupffer cells at 24 hours, and in the hepatocytes at 48 hours, following treatment. Birds examined six and ten days

following treatment had stainable iron in both the hepatocytes and the Kupffer cells but predominantly in the hepatocytes. The maximal extent of hepatic iron loading, determined both visually and by image analysis, was evident at 48 hours. There was quantitatively less stainable iron present in the liver tissue 10 days following injection of iron dextran. This is outlined and compared with image analysis results in Table (5-2)

Table (5-2) Comparison of the area, intensity of staining and relative distribution of stainable iron in histological sections of liver taken from chickens 1,2,6 and 10 days following a 10 mg injection of iron dextran, and stained with Perls' iron stain.

Day following treatment	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
0	0.00	0.00	0.00	0.00	0.00	0.00
0	0.00	0.00	0.00	0.00	0.00	0.00
1	3.16	2.65	5.81	2.52	2.88	5.40
1	3.95	4.20	8.15	3.40	2.60	6.00
2	4.59	3.71	8.31	4.85	4.21	9.05
2	4.50	3.84	8.34	4.85	4.14	8.99
6	2.26	1.60	3.86	4.27	3.46	7.73
6	2.20	2.45	4.65	4.36	3.61	7.97
10	1.30	1.00	2.30	3.69	3.14	6.83
10	0.00	0.00	0.00	3.11	3.59	6.71

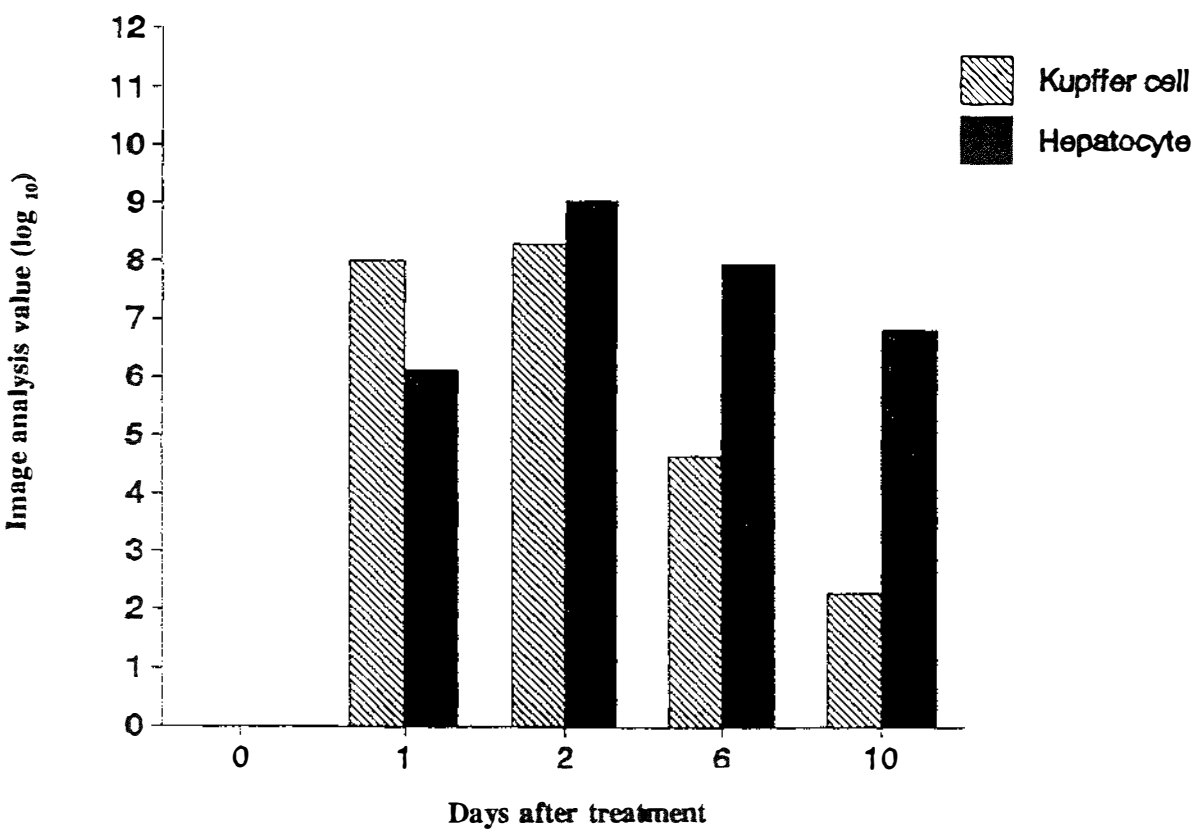
The total amount of stainable iron in the Kupffer cells is calculated by multiplying the average intensity value by the area of stainable iron in Kupffer cells. The same calculation is made for the hepatocytes. It is apparent that there is a significant amount of variability between individual birds within each treatment group but that this is less than the difference between treatment groups. The total quantitative value for stainable ferric iron in histological sections of the experimental birds is compared with the biochemical values in Table (5-3). The total value is calculated as the sum of the mean values for the hepatocytes and Kupffer cells in each section examined.

Table (5-3) Comparison of the total amount of iron detectable in hepatocytes and Kupffer cells, measured biochemically, and the mean value for stainable iron in liver sections, determined by image analysis.

Days after iron treatment	Total stainable Fe ³⁺ iron	Biochemical estimate (μ mole Fe/ gram)
0	0.00 +/- 0.00	190
0	0.00 +/- 0.00	160
1	5.90 +/- 0.30	700
1	8.20 +/- 0.20	760
2	9.10 +/- 0.28	1030
2	9.10 +/- 0.25	Not tested
6	7.70 +/- 0.30	530
6	8.00 +/- 0.05	750
10	6.80 +/- 0.35	420
10	6.70 +/- 0.15	390

The results are illustrated graphically in figure (5-1) showing the distribution of stainable iron in the hepatocytes and Kupffer cells at 1, 2, 6 and 10 days following a parenteral injection of 10 mg iron dextran. A representative Perls' iron stained liver section from a chicken six days following treatment is illustrated in figure (5-2)

Figure (5-1) The distribution of stainable hepatic iron following parenteral treatment with 10 mg of iron dextran (Imferon). The raw data can be found in appendix (A-13).



Each value represents the mean of five values obtained by image analysis of sections from two chickens.

5-3-2 The use of image analysis to quantify the amount of stainable iron present in stained histological sections of avian liver tissue

The results in Table (5-3) show that there was a good correlation ($P < 0.05$) between the amount of stainable iron measured in hepatic sections and the biochemical assessment of iron two to ten days following injection of exogenous iron dextran into domestic chickens. However, one day following injection the value for stainable iron was lower than expected if the two values were directly correlated. The amount of biochemically detectable iron and the amount of stainable iron is highest on day two and falls to lower values by day ten.

The 'Macpath' programming option allowed Kupffer cell and hepatocyte iron to be measured independently. In the first 24 hrs after injection there was more stainable iron detected in the Kupffer cells than in the hepatocytes ($P < 0.01$) of iron dextran treated chickens. After 48 hrs there was significantly more stainable iron detected in the hepatocytes than in the Kupffer cells ($P < 0.01$).

From image analysis of histological sections derived from control chickens it was determined that biochemically measured hepatic iron in the range of 120-190 micromol/l in chickens is not associated with histologically detectable iron in Perls' iron stained liver sections.

Comparison of image analysis values for experimental chickens and those derived from retrospective cases of other species.

The values for total stainable iron and the relative distribution of iron stores in the livers of experimental chickens which had been given an exogenous supply of iron, fell within the middle range of values recorded for the retrospective avian cases (Table, 5-4). The experimental birds did not develop the severe degree of siderosis seen in some species. However, the amount present was considered significant in comparison with the control birds which had no stainable iron in liver tissues, refer to Table (5-2). The degree of siderosis produced in an experimental chicken six days after receiving an injection of 10 mg iron dextran is shown in figure (5-2). Representative Perls' iron

stained hepatic sections of other birds listed in Tables (5-4) and (5-6) are illustrated in figures (5-3) to (5-5)

Table (5-4) Comparison of the area, intensity of staining and relative distribution of stainable iron in histological sections of liver in retrospective cases taken from chickens 2 and 6 days following a 10 mg injection of iron dextran, and stained with Perls' iron stain with that in retrospective cases of haemosiderosis from other species.

Species of bird	Kupffer cell			Hepatocyte			Treatment/ concurrent disease
	A	I	T	A	I	T	
* Chicken	2.26	1.60	3.86	4.27	3.46	7.73	10 mg ID (6d)
Chicken	2.20	2.45	4.65	4.36	3.61	7.97	10 mg ID (6d)
Chicken	4.59	3.71	8.31	4.85	4.21	9.06	10 mg ID (2d)
Chicken	4.50	3.84	8.34	4.85	4.14	8.99	10 mg ID (2d)
Lorikeet	5.00	5.65	10.65	4.40	4.00	8.05	Pseudotuberculosis
Kaka	4.83	3.97	8.70	2.02	1.02	3.04	Pneumonia
NZ pigeon	6.05	6.40	12.45	2.45	2.00	4.45	Pseudotuberculosis
NZ pigeon	6.00	6.90	12.90	3.55	2.12	5.67	Starvation
NZ shoveller	5.39	3.00	8.39	5.90	4.00	9.90	Starvation
Scaup	4.35	4.45	8.70	2.00	2.84	4.84	Starvation
Tui	5.70	5.00	10.70	5.30	3.59	8.89	Aspergillosis
Stitch bird	4.40	5.10	9.50	5.20	3.20	8.40	Aspergillosis
Canary	2.85	1.56	4.36	1.95	0.75	2.70	Pseudotuberculosis

A = Area, I = Intensity, T = Total; ID = iron dextran

* Latin names for the birds listed are found in appendix (A-2).

5-3-3 The distribution of iron in liver tissue and factors associated with haemosiderosis in birds

Haemosiderosis was reported as an histological finding in fifty (28%) of the one hundred and eighty necropsy cases examined. The degree of stainable iron detected visually in the liver ranged from mild to severe (see figures, 5-3 to 5-5). In ten of these fifty cases the extent of iron storage in the hepatocytes was considered severe enough for hepatic haemosiderosis to have been recorded as a diagnostic finding (figure, 5-5). In these cases the livers appeared bronze in colour on gross examination (figure, 4-11).

There was some species variation in the level and distribution of stainable iron with the more severe cases occurring in psittaciform and columbiform species. Anseriformes and some native passerine species such as the saddleback (*Philesturnus carunculatus*) and the stitch bird (*Notiomystis cincta*) were also noted to have severe hepatic siderosis at necropsy. Introduced granivorous passeriforms such as the house sparrow (*Passer domesticus*) and the greenfinch (*Carduelis chloris*) had little or no stainable iron in the hepatic parenchymal tissue and a limited amount of stainable iron in the Kupffer cells of the liver. In these birds there was frequently a greater amount of iron present in the reticuloendothelial cells of the spleen than in the liver. In the other species examined the stainable iron was generally restricted to the liver except in the mallard (*Anas platyrhynchos*) and several psittaciformes in which there was evidence of erythrophagocytosis in the hepatic Kupffer cells and in splenic macrophages. However, in the passeriforms, the amount of stainable iron visible in the spleen appeared to bear no relationship to the amount of stainable iron present in the liver. In all the species examined, except for the native pigeon (*Hemiphaga novaeseelandiae*), there were individuals examined which had little or no stainable iron in the liver or other tissues.

The range and mean values for the total stainable iron present in histological sections of representative cases of avian haemosiderosis is outlined in Tables (5-4) and (5-6) and illustrated by figures (5-2 to 5-4). The histological appearance of iron pigment in H & E, Perls' iron and PAS stained hepatic sections is illustrated in figures (5-5) to (5-7). The section shown was taken from a saddleback *Philesturnus carunculatus* which had a significant amount of parenchymal iron pigment. In H & E stained sections this iron pigment appears brown in colour and in Perls' iron stained sections this pigment stains blue. Bile pigment also appears brown in H & E stained sections but does not stain with Perls' iron stain. In the PAS stained section the iron granules appear dark brown or purple and are seen to be bound within lysosomal structures.

Seasonal pattern

In the anseriformes examined there appeared to be an association between the occurrence of hepatic haemosiderosis and season. All of the male and female mallard ducks examined in the post-breeding moulting period had a significant amount of stainable iron in both the hepatocytes and Kupffer cells. In contrast only two out of

fourteen ducks examined outside the post-breeding moulting period had significant stainable iron stores in the liver.

Concurrent disease

In 79 (44%) out of the 180 birds examined, infectious diseases were diagnosed. In most of these cases there was detectable iron in the reticuloendothelial cells of the liver and of the spleen. In 50% of the birds there was also a significant degree of hepatocyte iron especially in those birds which had been ill for some time prior to death. The most severe haemosiderosis occurred in birds of several species which had been rehabilitated following a history of trauma or starvation. In 75% of these cases there were concurrent parasitic and microbial infections. The range and mean values for the total stainable iron in the liver sections of birds representing the five major avian orders are illustrated in Table (5-5) along with a comparison between birds with concurrent infectious and non infectious diseases.

Table (5-5) A comparison of the amount of total stainable iron, measured using image analysis, in histological sections of liver tissue taken from birds representative of different avian orders which died from 'infectious' and 'non-infectious' causes.

Order	Non-infectious disease		Infectious disease	
	Range	Mean (n)*	Range	Mean (n)
Psittaciform	0.00-10.65	5.50 (10)	6.50-10.65	8.40 (15)
Columbiform	5.65-12.9	8.50 (5)	9.76-12.45	10.70 (3)
Passeriform (native) **	4.56-10.96	8.70 (15)	9.50-10.70	9.80 (10)
Passeriform (introduced)	0.00-5.20	4.31 (30)	4.20-6.60	5.30 (35)
Anseriform	0.00-9.90	6.70 (32)	8.40-9.70	9.40 (4)
Galliform	0.00-5.32	2.00 (6)	1.56-5.98	3.92 (6)
Others	1.00-10.90	10.50 (3)	1.56-10.32	5.60 (6)
Total (180)	0.00-12.90	5.30 (101)	4.34-12.45	6.30 (79)

* (n) = number of birds examined; ** The native passeriforms which have a fruit or nectar based diet are considered separately from the introduced passeriforms which are predominantly granivorous or insectivorous.

The values for the variance and standard deviation of these results can be found in appendix (A-12). The results are presented graphically in figures (5-8) to (5-10)

Species difference in the distribution of stainable iron in relation to different concurrent diseases.

The results of the image analysis procedure which was used to determine the total amount of stainable iron (area and intensity) for the 180 representative avian samples is given in appendix (A-13) along with a comment on any concurrent disease noted at necropsy. The distribution of iron stores is illustrated for representative cases in figures (5-3 to 5-5) and corresponding image analysis for iron distribution is given in Table (5-6).

Table (5-6) A comparison of the relative distribution of stainable iron in representative liver sections, determined by image analysis, of birds from different species with concurrent diseases

Species*	Stainable iron (Fe^{3+})			Concurrent disease
	Kupffer cell	Hepatocyte	Total	
Rainbow lorikeet	10.65	8.05	10.65	Pseudotuberculosis
Kaka	8.70	3.04	8.20	Pneumonia
Kea	8.56	3.00	8.46	Anaemia
Native pigeon	12.45	4.45	12.45	Pseudotuberculosis
Native pigeon	12.90	5.67	11.35	Starvation
NZ pigeon	9.89	5.36	7.78	Ingluveitis
NZ shoveller	8.39	9.90	8.80	Starvation
Scaup	8.70	4.84	8.85	Starvation
Weka	1.00	10.90	10.90	Visceral gout
Storm petrel	10.32	8.60	9.45	Focal hepatitis
Tui	10.70	8.89	11.0	Aspergillosis
Saddleback	9.82	10.96	10.96	Hepatopathy
Stitch bird	9.50	8.40	8.71	Aspergillosis
House sparrow	5.50	1.30	5.50	Atoxoplasmosis
Canary	4.36	2.70	4.56	Pseudotuberculosis
Canary	5.30	2.12	5.23	Pseudotuberculosis
Pheasant	5.32	3.98	5.32	Carcinoma

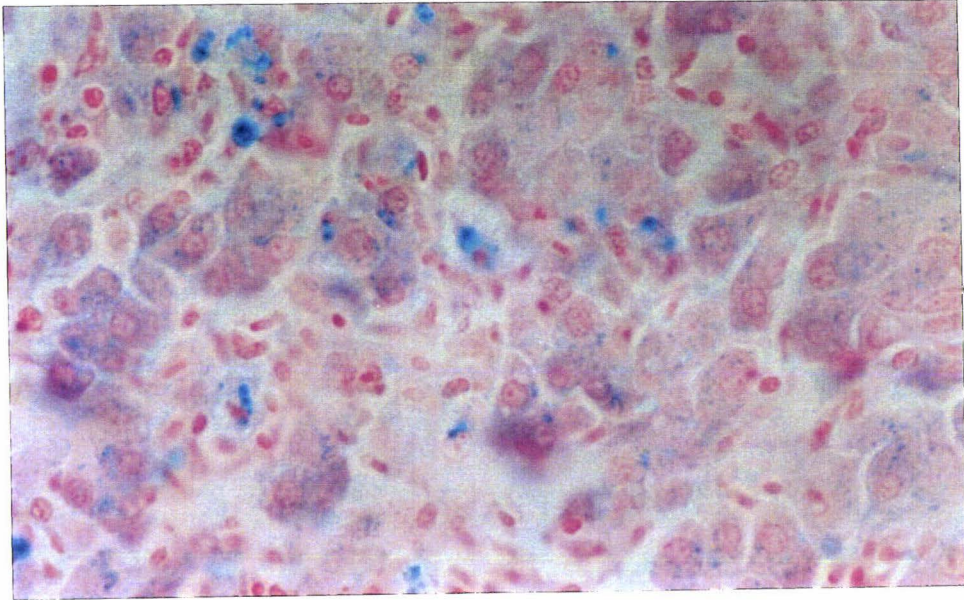
*Latin names for the birds listed are given in appendix (A-2).

Diet

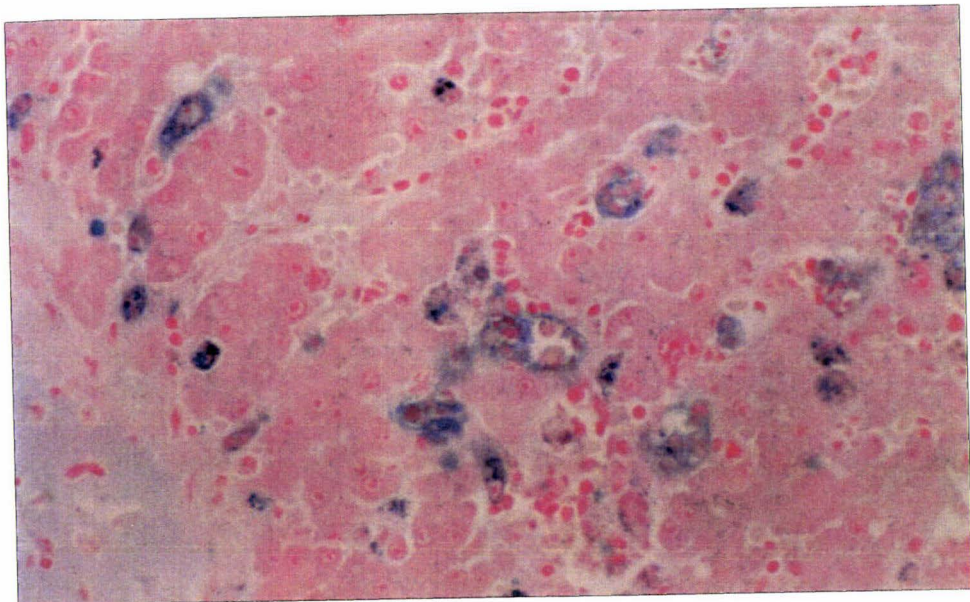
Unfortunately insufficient data was submitted with the clinical histories to determine whether or not there was a relationship between the iron content of the diet and the degree of hepatic haemosiderosis in the wild birds examined. However, it was noted that native birds such as the stitch bird (*Notiomystis cincta*) (5/12), tui (*Prosthemadera novaeseelandiae*) (4/6), native pigeon (*Hemiphaga novaeseelandiae*) (8/8) and saddleback (*Philesturnus carunculatus*) (3/4) which feed on iron-rich berries and leaves had severe haemosiderosis whereas seed-eating passeriforms such as the house sparrow had limited stainable iron in the liver. Wekas (*Gallirallus australis*) (3/3), which eat a variety of foods had clearly defined periportal siderosis with iron in hepatocytes and not Kupffer cells. In the present study this distribution was unique to this species.

Iron in other tissues

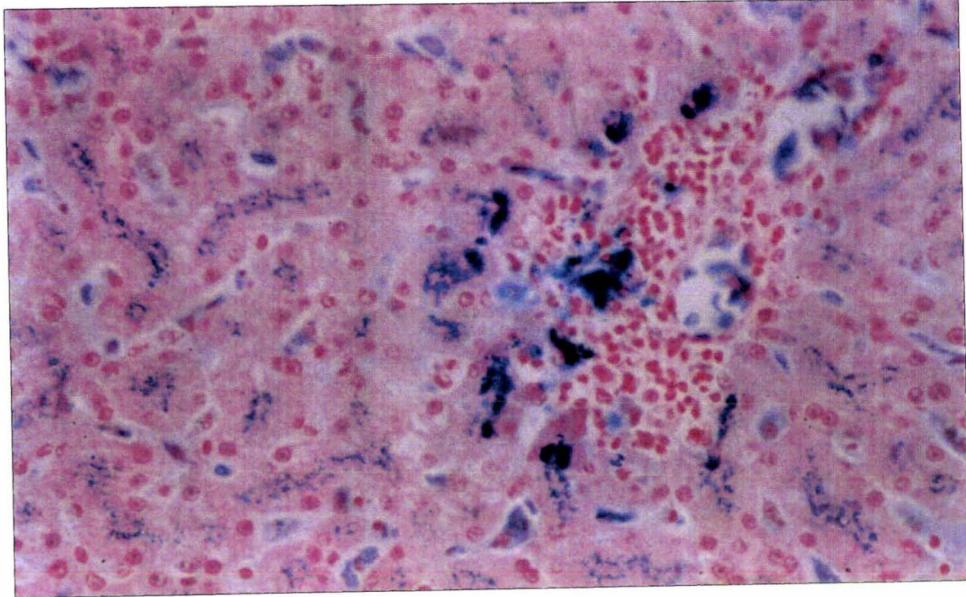
In two birds, a saddleback and a stitchbird, the degree of haemosiderosis was severe. In these cases there was evidence of necrosis of individual hepatocytes which may, in time, have resulted in clinical liver disease. The amount of iron visible in some hepatocytes was considered excessive enough to have resulted in the death of the cell. Sections taken of all other tissues and stained for iron failed to demonstrate siderosis in any other organ except for a small amount in the reticuloendothelial cells of the spleen. In two out of ten Regent parrot chicks (*Polytelis anthopeplus*) examined there was stainable iron in the intestinal epithelium, both of these birds died of *Escherichia coli* septicaemia following an enteritis.



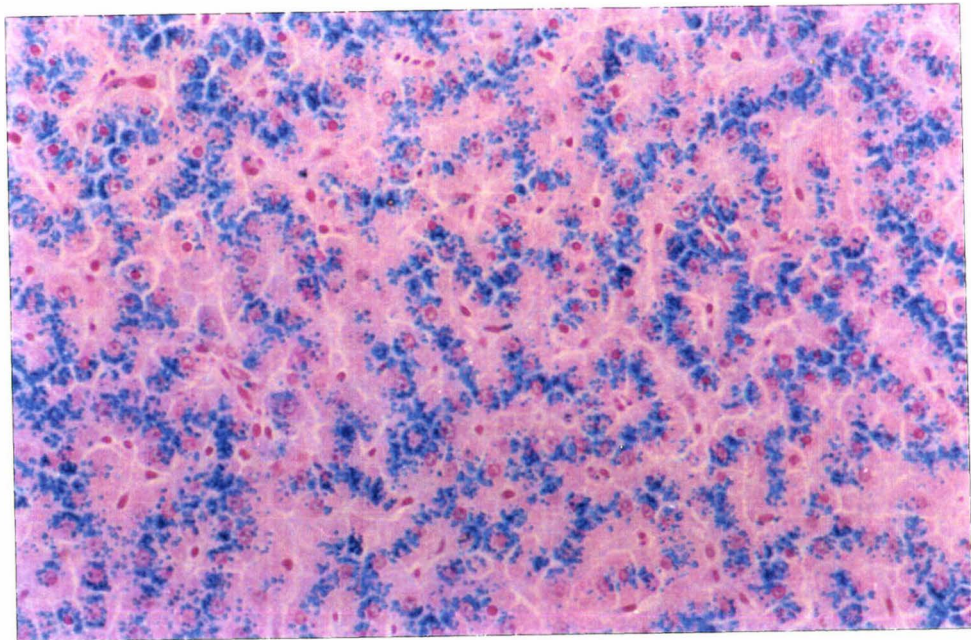
5-2 Histological section of the liver of a chicken taken 6 days following an injection of 10 mg iron dextran. Iron pigment can be seen in the hepatocytes and the Kupffer cells as blue staining granules. Perls' iron stained section. x 40.



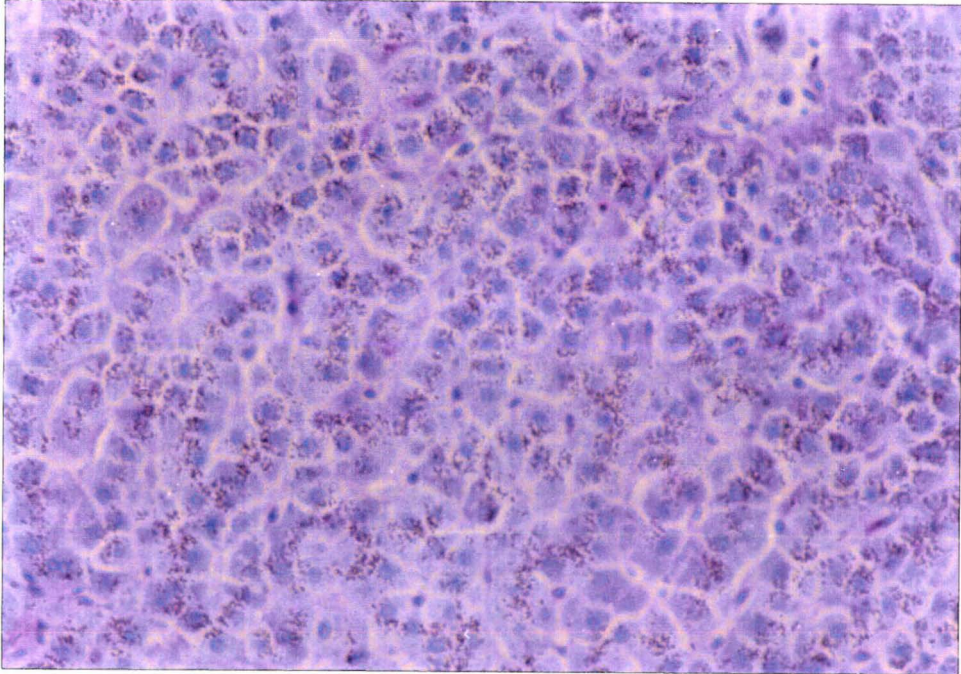
5-3 Histological section of the liver of a Regent parrot (*Polytelis anthopeplus*) which had concurrent fungal disease. Iron can be seen in the Kupffer cells, there is also evidence of erythrophagocytosis. Perls' iron stain. x 40.



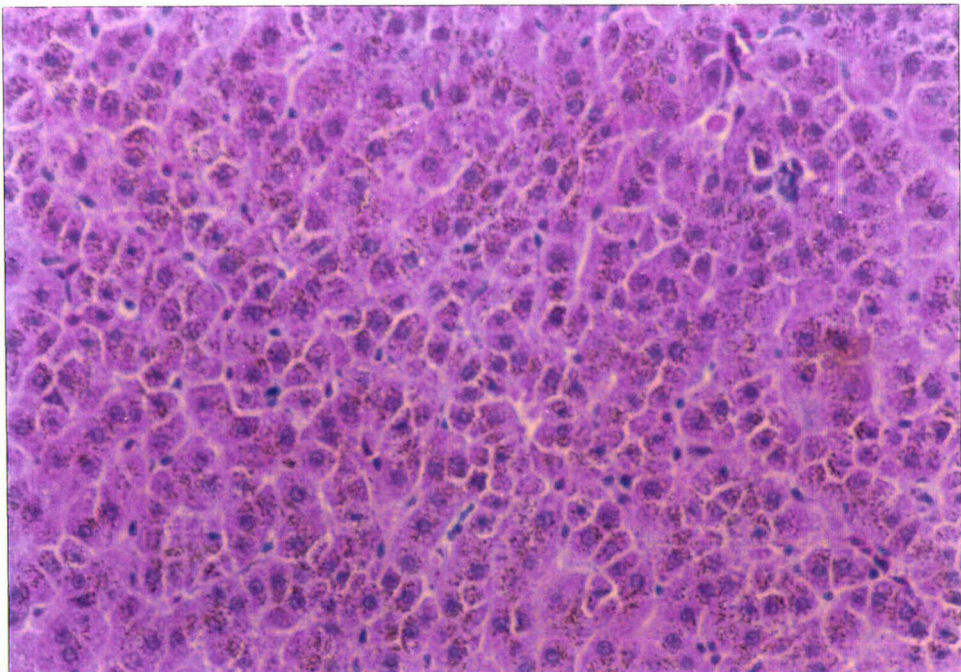
5-4 Histological section of the liver of a black swan (*Cyngus australis*) which died following trauma. Iron pigment can be seen in Kupffer cells surrounding the blood vessel and is also seen scattered diffusely throughout the hepatic parenchymal tissue. Perls' iron stain. x 40.



5-5 Histological section of the liver of a saddleback (*Philesturnus carunculatus*) which died from unknown causes. Iron pigment can be seen scattered throughout the hepatic parnechymal tissue but there is little iron in the Kupffer cells. Perls' iron stained section. x 20.



5-6 PAS stained section of the liver of the saddleback illustrated in figure (5-5). Iron pigment appears dark brown in PAS stained sections and can be seen within the purple staining lysosomal structures of hepatocytes.

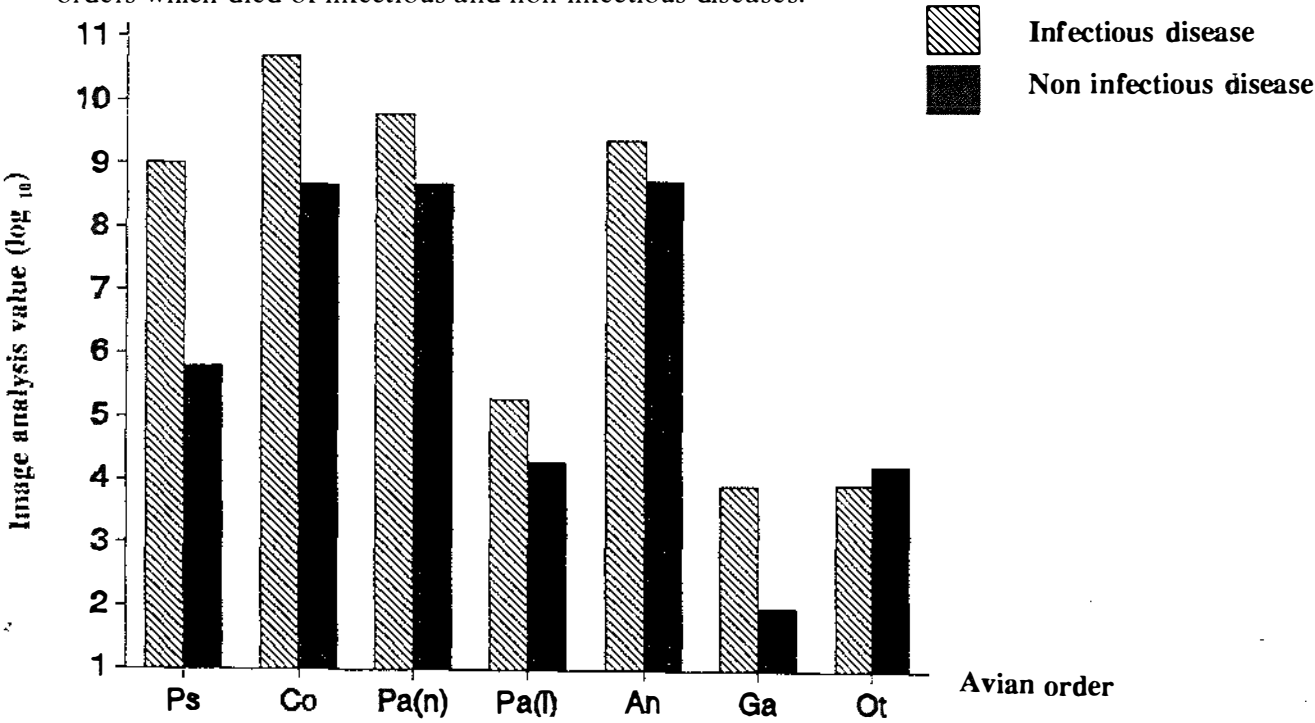


5-7 H&E stained section of the liver of the saddleback illustrated in figure (5-5). Iron pigment and bile appears brown in H&E stained sections. Perls' iron stain stains loosely bound ferric iron blue in histological sections and is semi-quantitative.

5-3-4 Statistical evaluation of the relationship between the amount and the distribution of iron levels in histological sections of liver tissue and infectious disease.

The results for the stainable iron measured in all of the birds examined are presented graphically in figures (5-8) to (5-10). The image analysis data for other species is given in appendix (A-13). The variability in the image analysis values for different sectors on the same section was not significant ($P>0.05$). The analysis of variance of image analysis data from each order of birds is presented in appendix (A-12). The following bar graphs and the summary of results relate to a two-way analysis of variance of each data set. The student's t-test allowed both between and within order comparisons using this data.

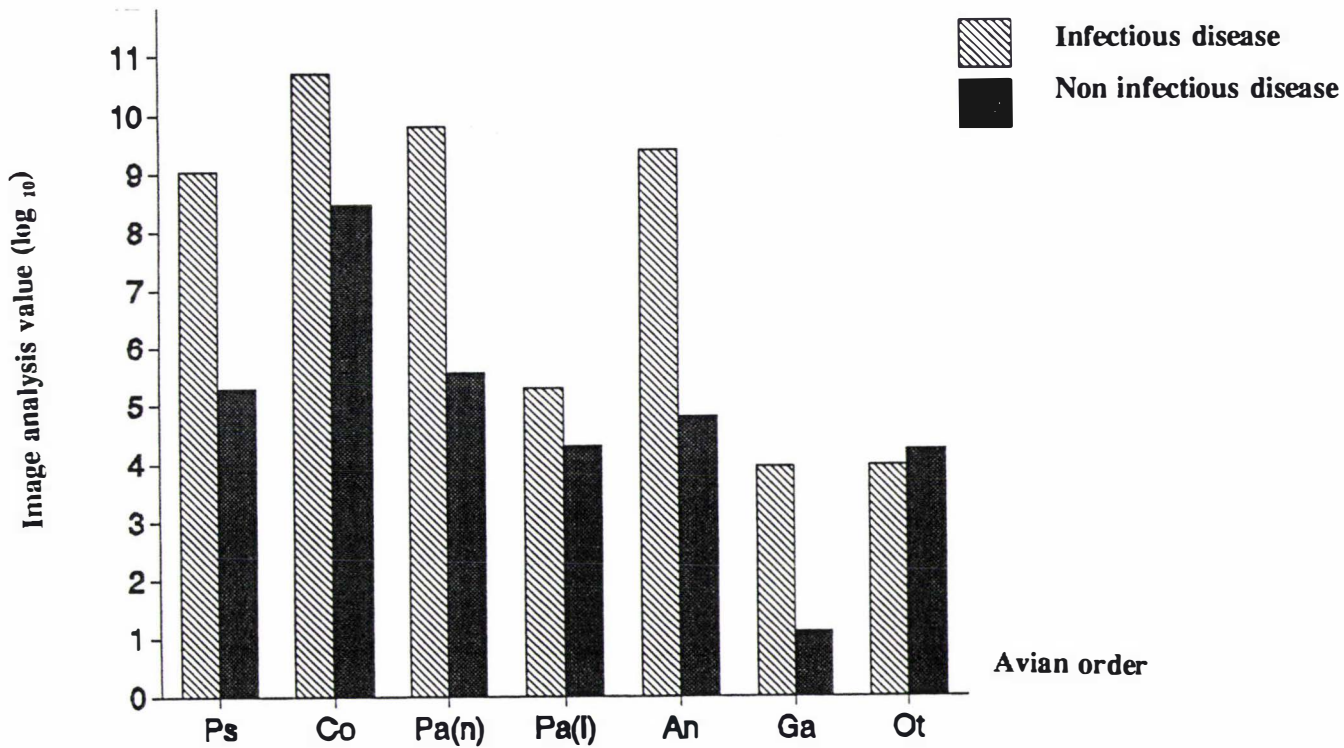
Figure (5-8) A comparison of the total stainable hepatic iron in birds from different orders which died of infectious and non-infectious diseases.



Ps=psittaciformes n=25 (15/10); Co=columbiformes n=8 (3/5); Pa(n)=native passeriformes n=25 (10/15); Pa(i)=introduced passeriformes n=65 (35/30); An=anseriformes n=36 (4/32); Ga=galliformes n=12 (6/6); Ot=others n=9 (6/3). Numbers within parentheses represent the number of birds in which death was attributed to infectious/non-infectious diseases.

Only in the psittaciformes was there significantly more total stainable iron ($P<0.005$) in those with infectious disease than in those with non-infectious disease.

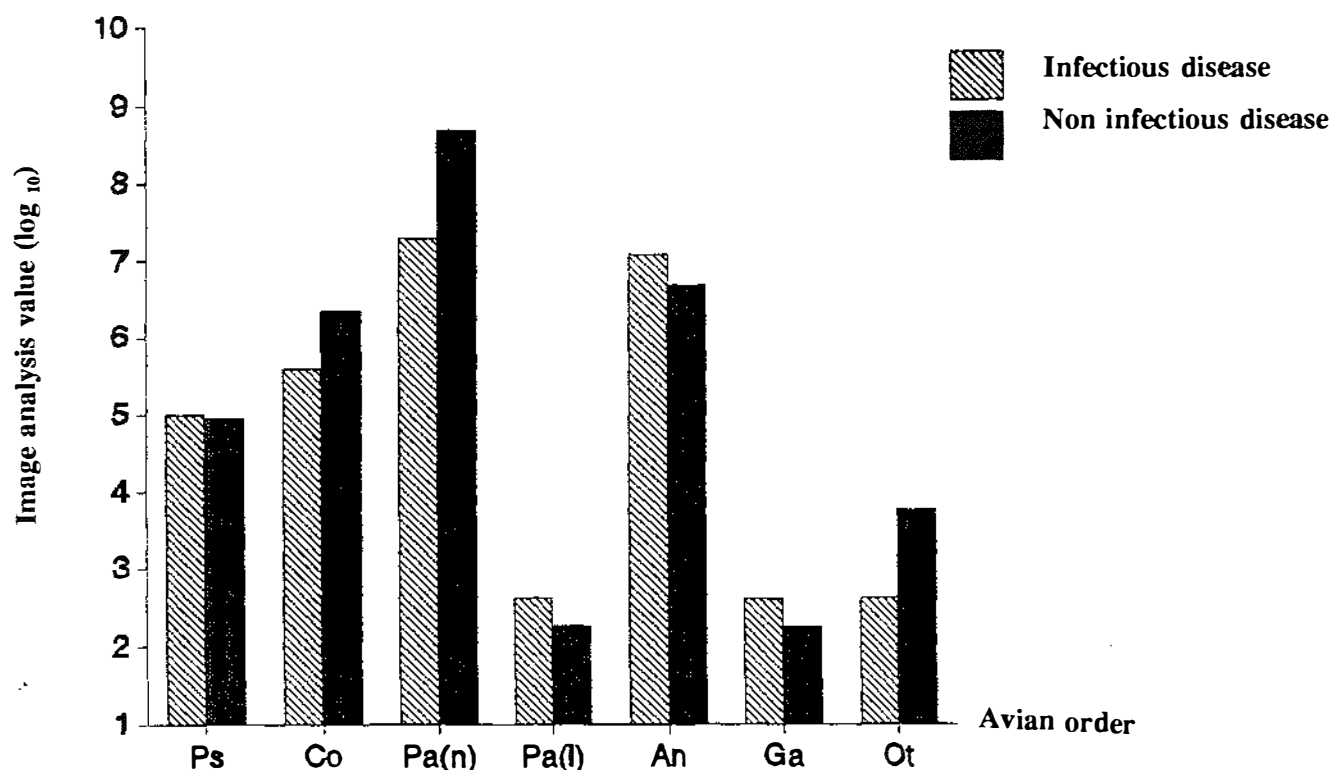
Figure (5-9) A comparison of the total stainable Kupffer cell iron in birds from different orders which died of infectious and non-infectious diseases.



Ps=psittaciformes n=25 (15/10); Co=columbiformes n=8 (3/5); Pa(n)=native passeriformes n=25 (10/15); Pa(i)=introduced passeriformes n=65 (35/30); An=anseriformes n=36 (4/32); Ga=galliformes n=12 (6/6); Ot=others n=9 (6/3). Numbers within parentheses represent the number of birds in which death was attributed to infectious/non-infectious diseases.

In all orders of bird, except in the 'other' category, there is significantly more stainable iron in the Kupffer cells of birds which died of an infectious cause than in those which died from non-infectious causes ($P < 0.05$). See also appendices (A-12) and (A-13).

Figure (5-10) A comparison of the total stainable hepatocyte iron in birds from different orders which died of infectious and non-infectious diseases.



Ps= psittaciformes n=25 (15/10); Co=columbiformes n=8 (3/5); Pa(n)=native passeriformes n=25 (10/15); Pa(i)=introduced passeriformes n=65 (35/30); An=anseriformes n=36 (4/32); Ga=galliformes n=12 (6/6); Ot=others n=9 (6/3). Numbers within parentheses represent the number of birds in which death was attributed to infectious/non-infectious diseases.

There is no significant difference in the amount of stainable iron in the hepatocytes of birds within each order which died of infectious compared with those which died of non-infectious causes. From the values given in appendix (A-12) it is evident that there is a significant difference in the amount of hepatocyte iron when comparing the mean values between different orders of bird ($P < 0.05$) and that the galliforms and the granivorous passeriforms have the lowest values. The birds with the highest values for stainable hepatocyte iron are the native passeriforms and the native columbiforms.

Summary of results

There is a difference in the amount and distribution of iron in the liver tissue of different species of bird presented for necropsy. Available data for the 180 cases examined is listed in the appendix (A-13), the results are presented graphically in figures (5-7) to (5-9) and the statistical evaluation of this data is presented in appendix (A-12). The native pigeon had the most severe siderosis and this was associated with starvation, trauma and infectious disease. Anseriform species demonstrated a wide degree of variation in the amount and distribution of stainable iron. The most severe siderosis was seen in a shoveller which died of starvation. In chapter four it was noted that in columbiforms and some psittaciforms, microbial lesions were more widespread in the liver tissue of birds which had pseudotuberculosis with concurrent haemosiderosis. In the granivorous passeriforms examined haemosiderosis was not present. In all of the cases of avian pseudotuberculosis examined iron was present in the Kupffer cells.

The results presented in Appendix (A-13) and illustrated in figures (5-7) to (7-9) indicate that there is a statistically significant difference in the amount of total iron in the livers of birds in different orders and that the galliform group generally has a low value of stainable iron in stained hepatic section. In contrast the native pigeon and the native passeriforms appear to have a high level of stainable iron in stained hepatic sections.

When comparing birds within orders, the difference in the amount of stainable hepatocyte iron in the livers birds which died of infectious as compared with non-infectious causes, is not significant. In contrast, the amount of stainable iron in the Kupffer cells is significantly greater ($P < 0.05$) in the livers of birds which died of infectious vs non-infectious causes except in the 'others' group which was composed of several species of bird.

Discussion

The results of the present study indicate that histologically stainable iron is a common finding in avian liver sections and is often associated with concurrent infectious and non-infectious disease processes. This finding is in agreement with other studies (Lowenstine & Petrak, 1980; Wadsworth *et al.*, 1983). Of the 180 birds examined in the present study the majority were from the wild or were wild birds held in captivity for captive breeding programmes. In all of the species examined there were some individuals which had little or no stainable iron in the liver sections examined.

Although there are several retrospective studies on the subject of avian haemosiderosis in the literature (Taylor, 1984; Lowenstine and Petrak, 1980), the present study is possibly the first to attempt to quantitatively assess hepatic iron stores in histological material. The limitations of using the Perls' iron staining technique to quantify tissue iron are acknowledged (Stevens, 1982). However, although the Prussian blue reaction will only outline loosely bound ferric iron, the modified test which can also identify ferrous iron has proved to be less sensitive. The injection of iron dextran into two-week-old chickens produced a satisfactory experimental model for haemosiderosis in this species and allowed an assessment of the correlation between the total stainable iron determined by image analysis, and the total iron determined biochemically. This is not always possible in retrospective studies as liver samples may not be available or there may not be sufficient hepatic tissue for biochemical assessment.

In the experimental model it was possible to assess the relative amounts of exogenously supplied iron present in the liver at different time intervals following an intravenous injection of iron dextran. From this it was determined that, in chickens, an intravenous dose of 10 mg iron dextran is initially assimilated from the blood stream into the Kupffer cells and then into the hepatocytes within 48 hours. In the two-week-old chickens used in the experimental model, this iron appears to have been mobilised from the liver stores in the period following maximal hepatic storage, with the resultant reduction of the stored iron within the ten days following treatment. This was despite the fact the birds were healthy and had been fed on a complete poultry ration containing more than the minimum iron requirement. This finding may indicate that either the experimental chickens had utilised the exogenous iron over the period of the

experiment or that the iron had been redistributed to other tissues. There was no stainable iron detected in the bone marrow or other tissues examined from these birds, therefore, if it had been redistributed to these tissues the iron was present in an unstainable form. It is also possible that the exogenous iron was utilised for erythropoiesis, this possibility could be evaluated using radiolabelled iron. Similar experiments performed in starlings indicated that a supply of parenteral iron dextran resulted in a reduced uptake of radiolabelled dietary iron from the gut (Ward *et al.*, 1991). In post-moult starlings, with naturally high liver iron, the absorbed dose of radiolabelled dietary iron was also less, suggesting that the high tissue iron has a negative feed back on iron absorption from the diet (Ward *et al.*, 1991). This would be expected from what is known about iron absorption in poultry (Hill *et al.*, 1977). However, the results of Ward *et al.* (1991) indicated that in starlings the uptake of iron was less regulated than in experimental mammals subjected to the same experiment.

To avoid seasonal variability in hepatic iron content, the White Leghorn chicken, a breed in which there is no stainable iron seen in livers of healthy birds, was chosen for the experimental model. Young male birds were chosen in order to avoid the possible effect of hormonal regulation of ovarian activity and any subsequent metabolic changes, including iron mobilisation, which may occur in female chickens (Planas *et al.*, 1961). The amount and distribution of stainable iron in the liver of the experimental chickens following injection of iron dextran was compared with that seen in the retrospective cases of haemosiderosis in other species. It has been reported in other studies that galliformes have low levels of tissue iron compared to other species (Osborn, 1979). This has been confirmed by the results of the present study. Biochemical assessments of hepatic iron indicate that apparently normal, two-week-old male White Leghorn chickens have hepatic iron levels of 120 - 190 micromol/gram. This value is similar to that reported by Osborn (1979) and significantly less ($P < 0.01$) than the value recorded for pre or post-moult starlings (Osborn, 1979). It has also been noted that the levels of iron and other metals measured in the tissues of starlings varied significantly over the course of a year (Ward *et al.*, 1991; Osborn, 1979). The complex interaction between trace metals in the body where the level of one may directly or indirectly alter the level of another should also be taken into consideration when considering iron metabolism. In the present study hepatic and serum copper and zinc levels were not assessed but

future biochemical evaluation of the haemosiderosis model developed in the chicken should examine these elements as it is known that they are also important in the development of metabolic and infectious disease.

In the present study avian material was collected from 40 different species of birds representing six common orders. Histological material from these birds was examined over a period of two years with a similar number of samples collected in each seasonal period. In some species of bird examined there appeared to be a significant difference in the amount of stainable hepatic iron in birds which died in the post-moult period. Osborn (1979) noted that, in seasonal breeding birds such as starlings, hepatic iron stores rise in the autumn following the summer moult. It was suggested that this increase may reflect the increased haemopoietic activity of some birds at the time of the moult and is associated with changes in the levels of thyroid hormone (Diez *et al.*, 1986). It was also noted that there was an increase in the amount of serum protein iron carriers underlining the possibility that there are physiological changes in tissue iron levels which are associated with this annual event. Osborn (1979) also measured changes in liver weight, protein levels, copper and zinc. It was found that changes in copper and zinc loads corresponded with changes in liver weight, serum protein copper did not appear to alter much during the year but serum protein zinc levels were slightly elevated just prior to the moult in the spring (Osborn, 1979). In the retrospective material available for the present study there was insufficient data to enable seasonal comparisons to be made within most of the species studied. In the anseriformes, however, samples from mallard ducks had higher levels of stainable iron in the liver in the autumn period than in the spring ($P < 0.01$). It is probable that similar physiological variations in hepatic iron stores occur in the other species examined although it may be less marked in species which are less seasonal or those which do not migrate such as some of the passeriforms and the galliforms.

In addition to hormonal changes, the seasonal variation in hepatic iron levels observed in starlings (Osborn, 1979) and also in reindeer (Borch-Johnsen & Nilssen, 1987; Borch-Johnson *et al.*, 1989) has been shown to be influenced by diet. In a study of 77 Svalbard reindeer it was found that during the winter season the body weight decreased about 43% in females and 39% in males. Liver weight was reduced about 65% in both sexes with an iron content of 29 ± 13 mg iron/100 g wet weight in the autumn and $291 \pm$

52 mg iron/100g wet weight in the winter. Borch-Johnsen & Nilssen (1987) observed that the increase in liver iron content in the winter was associated with ingestion of forage plants containing high levels of iron. They also noted that animals with a very high iron concentration of the rumen content usually had massive siderosis in both the parenchymal and non-parenchymal cells of the liver. In contrast, siderosis was restricted to non-parenchymal cells in starving animals with normal rumen iron concentration. Concurrent studies on the degree of saturation of serum transferrin indicated that this was significantly greater in deer feeding on iron-rich forage. This finding supports the view that parenchymal iron loading follows dietary excess whereas the translocation of iron from catabolized blood and lean tissue is associated with iron loading in the non-parenchymal cells. Experiments performed in rats indicated that animals loaded with an oral dose of carbonyl-iron stored the extra iron in the parenchymal cells of the liver with a distinctive periportal pattern (Iancu & Shiloh, 1988). Ward *et al.* (1991) observed that a similar histological pattern was seen in the starlings examined in the post moult period.

In human patients the storage of excess iron in the liver is thought to be associated with hepatic disease when it becomes extreme, however, even in these cases the fibrosis and cirrhosis may have been present prior to the build up of iron in the tissues (Iancu *et al.*, 1987). The clinical significance of haemosiderosis in birds is still not clear (Ward *et al.*, 1991). In human haemochromatosis there is a concurrent reduction in the iron-binding capacity of serum transferrin (Powell *et al.*, 1980). This condition results in a greater amount of unbound iron in the blood stream and is thought to be associated with an increased risk of bacterial septicaemia (Robins-Browne *et al.*, 1987). Systemic yersiniosis due to *Y. pseudotuberculosis* or *Y. enterocolitica* is reported to be more common in these patients due to the increased availability of iron (Weinberg, 1978). Desferal, a commercial siderophore is often used to chelate excess iron in haemochromatosis or cases of transfusion haemosiderosis in human patients (Pollycose, 1985). This treatment has been suggested for use in myna birds suffering from hepatic iron overload and, although the aetiology is not fully understood, it has been suggested that it has a genetic basis like primary haemochromatosis in humans (Gosselin, 1981; Morris *et al.*, 1989). In the present study the New Zealand native pigeon and native passeriforms which feed on plant material and fruits, had a significantly higher prevalence of severe haemosiderosis ($P < 0.01$) than did birds such as chickens and granivorous passerines

such as the house sparrow. Although there is insufficient data in the present study to determine the effect of diet on hepatic iron stores it appears from the literature available on birds (Hill *et al.*, 1977; Butler, 1983; Kincaid and Stoskopf, 1987) and mammals (Hartley *et al.*, 1959; Borch-Johnson *et al.*, 1987; Iancu *et al.*, 1987) that both the amount and form of dietary iron is an important factor in determining hepatic iron stores. In humans, the form of haemosiderosis reported in Bantu tribes in Africa was associated with alcoholism and drinking from iron cooking pots, because the acidic pH of the alcohol increases intestinal absorption of the metal (Nhonoli, 1973). Vitamin C also enhances iron absorption across the intestinal tract (Lynch & Cook, 1980) and the tannins in plant leaves decrease it (Gonzales *et al.*, 1984). From this it is clear that dietary analysis should always take the whole formulation into account rather than metal content in isolation (Carmichael *et al.*, 1975; Taylor, 1984; Spelman *et al.*, 1989).

Lowenstine and Petrak (1980) examined liver sections from myna birds using electron microscopy and found that the iron pigment was located within membrane bound lysosomal structures and free in the cytosol as ferritin moieties and larger haemosiderin aggregates. In the present study periodic acid schiff (PAS) stained sections indicated that the majority of the iron in the liver sections examined was in lysosomal structures.

In human haemochromatosis considerable research efforts have been directed towards the identification of a mechanism of liver damage and Witzleben & Buck (1971) hypothesised that it was secondary to peroxidation as a result of the iron overload. Stainable iron is the ferric form which is loosely associated with tissue proteins as ferritin or haemosiderin. It is thought that haemosiderin is a metabolite of ferritin and that a build up of the former is more frequently associated with liver disease (MacDonald, 1972). It is probable that there are numerous mechanisms which will result in an alteration in iron metabolism in humans and in birds (Lowenstine and Petrak, 1980). What is clear, even at the present time is that the normal hepatic mechanisms for metabolising iron are incompletely understood (Weinberg, 1984).

There is a lot of evidence from retrospective clinical studies and from *in vitro* work indicating that iron availability is an important determining factor in the outcome of host-pathogen interactions (Kochan, 1973; Weinberg, 1984; Bullen, 1987). The limitations of retrospective studies are apparent in the present study and are due 1) to

lack of information about the exact progress of the concurrent disease processes and 2) to how microbial agents interact with the iron metabolism of the host *in vivo*. Histological observations are useful but they only represent a single point in time at the end of the disease process and give no indication of how the host and the microbial agent interacted as the availability of iron altered. The availability of iron to micro-organism will increase in conditions where serum transferrin is saturated (Weinberg, 1978). In these conditions liver iron levels will also be increased with predominantly reticuloendothelial cells loading.

From the results of the present study and from other reports in the literature, it appears that although hepatic haemosiderosis is frequently only a histological finding and not associated with overt liver disease, it is often associated with concurrent malignant and infectious diseases (Hill *et al.*, 1986). The histological description of excess stainable iron in the liver is probably a reflection of an altered iron metabolism associated with increased turnover of tissue iron. This alteration may occur following starvation or trauma as well as changes in metabolism associated with seasonal physiological changes (Osborn, 1979).

There is evidence in the literature supporting the hypothesis that hypochromic anaemia associated with iron deficiency is one of the factors which eventually disrupts the efficiency of cell mediated immune defences in chronic infections (Chandra & Newberne, 1977; Chandra, 1990; Weinberg, 1984). This may be important in chronic infections in iron deficient hosts such as the chicken. In contrast there is a large body of experimental evidence from both *in vitro* and *in vivo* studies to suggest that in many host species an increased availability of iron in the host during infection favours the survival and replication of the invading pathogen at the expense of the host (Griffiths, 1993). It is thought that the increased saturation of serum transferrin which is associated with the haemosiderosis of iron overload, may interfere with the hypoferraemia of infection. The lowering of serum iron in the acute phase of infection occurs along with fever and is well recognised as a non-specific host defense mechanism. In view of the observed link between iron and infectious diseases, including pseudotuberculosis, in many avian species a disease model was developed to examine this experimentally. The relationship between iron and disease in birds forms the subject of Chapters six and seven.

Summary

1) A quantitative image analysis system was developed. This was achieved using an experimental model of haemosiderosis in the chicken. Development of this model allowed an assessment of the correlation between stainable hepatic iron in histological sections and total hepatic iron assessed biochemically and an assessment of the distribution of stainable iron in the liver following parenteral administration of iron dextran.

2) In the healthy chicken there was no stainable iron in the liver but, following parenteral administration of iron dextran, stainable iron was present in the Kupffer cells within 24 hours and was redistributed to the hepatocytes within 48 hours. The total amount of stainable iron was maximal 48 hours following injection and had fallen significantly ten days following administration.

3) Haemosiderosis is a common histological finding in the livers of birds. Retrospective analysis of total stainable iron in avian liver sections indicated that species in the order galliformes had very low levels of iron in hepatocytes in comparison with the native columbiforms and native passeriforms. Granivorous passeriforms and many members of the order psittaciformes had moderate levels of iron in hepatocytes. It was found that, within each order, birds which died from infectious diseases had significantly higher levels of iron in the Kupffer cells than birds which died from non-infectious diseases but that the total amount of hepatic iron was not significantly different between these two groups. The total amount of iron in liver tissue was independent of disease possibly reflecting differences in iron metabolism and diet.

SECTION TWO; Iron and disease, application of a disease model

General introduction

Despite a heightened interest in the role of iron in disease and the large amount of *in vitro* evidence to support the hypothesis that the survival of many microbial pathogens is enhanced by an increase in the availability of iron (Griffiths & Bullen, 1987; Litwin & Calderwood, 1993), few questions have been answered with regard to the clinical significance of this finding (Griffiths, 1993). It is likely that *in vitro* studies are unable to mimic the wide range of possible interactions between host and pathogen *in vivo* (Hanski *et al.*, 1991). In addition, from the results of mammalian *in vivo* models it is clear that the progression of the experimental disease was accompanied by an alteration in the host's iron metabolism. This, along with the expression by the micro-organism of a range of virulence characteristics stimulated by changes which occur within the living host, produces a situation far more complex than can be examined in a single experiment (Bullen, 1981; Wake & Morgan, 1986). In consideration of the complex interaction between the host, iron availability and the virulence potential of the pathogen it is not surprising that some models have produced conflicting results.

The following chapter outlines the application of an *in vivo* model using the domestic chicken (*Gallus domesticus*) to study the effect of iron availability on the development of pseudotuberculosis. These chickens were also the source of tissues and serum for the *in vitro* studies outlined in chapter seven. There are several reasons why the White Leghorn chicken was selected for the model, 1) it had been determined in chapter five that this breed has low tissue iron levels with little or no Prussian blue stainable iron in histological sections, therefore the effect of treatment with parenteral iron dextran on hepatic iron levels would be readily visible, 2) there is base-line physiological data already available for this breed, 3) commercial White Leghorn chickens are genetically very homogeneous, and as a result, the within group variation in response to given treatments not great thus allowing the use of small study groups. In addition to the above considerations, and in view of the apparent contradictions which have come out of the early work studying the effect of parenteral iron on the course of disease in chickens, it was necessary to develop an experimental model which would allow a comparison with the results of other work.

CHAPTER SIX

The effect of parenteral iron dextran on the outcome of pseudotuberculosis in the chicken

Introduction

There are numerous retrospective and experimental reports describing the deleterious effect of an increased availability of iron on the outcome of host-pathogen interactions in mammals (Bullen, 1981). It is probable that iron availability also has a direct role in determining the outcome of infectious disease in birds. However, there are conflicting views as to the role iron availability plays in affecting the outcome of host-pathogen interactions in chickens (Smith *et al.*, 1977). Pretreatment of chickens with iron dextran has been reported to enhance the long term survival of chickens infected with *Escherichia coli* (Harry, 1979) and to enhance resistance to oral infection with *Salmonella gallinarum* in the acute phase of infection (Smith *et al.*, 1977). On the other hand, observations made from retrospective studies of other species of bird indicate that iron loading of hepatic Kupffer cells, associated with haemosiderosis, starvation, erythrophagocytosis or tissue breakdown, is often severe in birds with concurrent bacterial, fungal or parasitic disease (Lowenstine and Petrak, 1980). Although it is possible that there are other factors associated with microbial infection in mammals and birds which are also indirectly associated with altered iron metabolism there is evidence from *in vitro* work to support the hypothesis that competition between host and pathogen for available iron is an integral factor in determining the outcome of infectious diseases (Griffiths & Bullen, 1987).

In view of the apparent contradictions in the literature with regard to the effect of iron on the outcome of infectious disease in chickens (Smith *et al.*, 1977, 1978; Harry, 1979), it became necessary to design an experiment to answer some of the questions posed by this previous work. In addition, it was important to understand why previous disease models, which examined the potential role of iron in host-pathogen interactions had failed to predict the outcome of disease in the clinical situation (Griffiths, 1993).

To allow a comprehensive evaluation of 1) the possible difference between chickens and mammals in their utilisation of iron during infection and 2) the possibility that iron is

a limiting factor for virulent *Yersinia pseudotuberculosis* during the early stages of infection, the experiment was designed to allow comparisons to be made with the rodent models which examined the effect of parenteral iron dextran, with and without exogenous siderophores on the outcome of infection with yersiniae (Robins-Browne *et al.*, 1987). In most of the disease models designed to examine the effect of parenteral iron dextran on the course of disease in chickens challenged with bacteria, the outcome was assessed during and at the end of the experiment using quantitative microbiological assessment of bacterial numbers in different organs along with mortality rates and the development of clinical signs. Similar methods were used in the rodent work but none of the studies evaluated the host response to infection in any detail.

The following experiment attempts to identify whether or not an increase in iron availability in the early stages of infection, has any effect on the pathogenesis of pseudotuberculosis in healthy chickens. The experiments outlined in the following chapter use the experimental methodology developed in section one of this thesis to evaluate the host response to infection. Using a combination of serology, histology and serum biochemistry it was hoped that a more complete understanding of the role of iron in host-pathogen interactions could be gained. The *in vitro* studies which were conducted concurrently are described in chapter seven.

Materials and methods

6-2-1 Iron and exogenous siderophores

Iron dextran

Iron dextran (Imferon, Fisons) was given intravenously as a 10 mg (0.2 ml) dose to 14-day-old chickens weighing approximately 120 g. It had been determined (chapter five) that 10 mg of this product, given intravenously to 14-day-old male chickens results in the storage of stainable iron in the liver and an increase in biochemically measurable iron with maximal hepatic iron loading two days following injection. For this reason the bacterial challenge was given two days after injection.

Desferrioxamine

Desferrioxamine (Desferal; Ciba-Geigy Ltd, Basle, Switzerland) is a siderophore derived from *Streptomyces pilosus* (Keberle, 1964). A 10% solution was prepared by dissolving 500mg of desferrioxamine in 5 ml sterile distilled water. A 10 mg (0.1 ml) dose was given intramuscularly to birds 24 hours after treatment with 10 mg iron dextran or 10 mg dextran T10 (Pharmacia, Uppsala, Sweden).

6-2-2 Experimental birds and procedure

Thirty, one-day-old male chicks were purchased from a commercial breeder of White Leghorn layers. These chicks were raised in plastic bottomed cages, given water *ad lib* and fed a commercial poultry grower ration produced by Massey University feed mills. At 2 weeks of age the birds were divided into 5 groups of 5 birds. Faecal samples were taken from each bird and tested for *Salmonella* spp. and *Yersinia* spp. prior to the start of the experiment. Prior to oral challenge, blood samples were collected from each bird and stored on filter discs.

Treatment groups

Four groups of five birds were given the treatment regimens outlined in Table (6-1) prior to oral infection with *Y. pseudotuberculosis* bacteria. The remaining group of ten birds was divided into four groups and individual birds were treated as outlined in Table (6-1), this control group was not exposed to *Y. pseudotuberculosis* bacteria.

Table (6-1) Treatment regimens given to experimental and control groups of chickens.

Treatment group	Number of birds	Day of treatment			
		0	1	2	2-10
Iron dextran (ID)	5	0.1 ml ID	None	Y.ps*	Y.ps ~
Iron dextran (ID)	4	0.1 ml ID	None	None	None
Iron dextran + Desferal	5	0.1 ml ID	0.2 ml De	Y.ps	Y.ps
Iron dextran + Desferal	2	0.1 ml ID	0.2 ml De	None	None
Desferal (De)	5	0.1 ml D	0.2 ml De	Y.ps	Y.ps
Desferal (De)	2	0.1 ml D	0.2 ml De	None	None
Dextran (D)	5	0.1 ml D	None	Y.ps	Y.ps
Dextran (D)	2	0.1 ml D	None	None	None

* *Y. pseudotuberculosis* was given as a 1.0 ml aliquot containing 10⁸ viable colony forming units. ~ *Y. pseudotuberculosis* added to the drinking water daily as aliquots of 10⁷ bacteria in 1.0 ml of saline.

Bacterial challenge and sample collection during the course of the experiment

On day two of the experiment, as outlined in Table (6-1), each of the 20 birds in the four experimental groups were given an oral inoculation of *Yersinia pseudotuberculosis* serotype II (LORIKEET MPPH-92) as a 1.0 ml aliquot containing 10⁸ viable colony forming units. The challenge bacteria used for this experiment were from the same batch prepared and frozen in 1 ml aliquots at -20 °C for the previous oral challenge experiment (chapter four). Samples from this aliquot store were also plated on blood agar and CIN agar, harvested into tryptone water prior to incubation at 28 °C and prepared for plasmid isolation (Portnoy *et al.*, 1992) and biochemical screening to test viability and species characteristics of the challenge organism. Aliquots of 1 ml of a saline solution containing 10⁷ *Y. pseudotuberculosis* serotype II was added daily to the water in each cage of birds in the experimental group in order to maintain bacterial challenge throughout the experiment. It had previously been determined that although there was a slight increase in water intake two days following oral challenge there was no significant difference in water intake between groups of healthy birds or between infected birds in the same treatment group (P>0.05) with each bird taking an average of 20 ml of water per day. The control birds were housed in a separate room to prevent the risk of aerosol spread of *Y. pseudotuberculosis* from the infected birds. The control group received no oral bacterial challenge.

Faecal shedding

Faecal samples were collected on days 0, 6, 8 and 10 from each bird exposed to *Y. pseudotuberculosis*. One gram of faeces was added to 10 ml of PBS and cold enriched for 3 weeks at 4 °C before isolation on CIN agar was attempted. Faecal bacteria isolated during the course of the experiment were screened biochemically and stored in glycerol broth at -20 °C as described previously. All *Yersinia pseudotuberculosis* isolates were subjected to serotyping and examined for plasmid carriage.

Serology

Six and ten days following the first oral inoculation with bacteria, blood samples were collected from all 25 birds. Blood was collected on filter discs and stored at 4 °C for serological testing using the disc ELISA test which had been developed in chapter three. The antigen used was a proteinase-K digest of the lipopolysaccharide antigen from the challenge isolate (YPSII-LORIKEET-VPPH-92) prepared as described previously (section 3-2-1). A 'cut-off' OD value of 0.274 (mean of control plus 2 standard deviations) was used.

Necropsy and sample collection

All 30 birds were euthanased 10 days after the initial oral challenge with bacteria. Each bird was necropsied and any gross lesions noted. Sections of liver were labelled and frozen at -20°C for biochemical assessment of iron content. Histological sections were prepared from a selected range of tissues as outlined in chapter four. Sections were stained for both iron and bacteria using the combined Perls' iron-Gram Twort stain described previously (chapter 4). Liver and spleen iron stores were assessed visually and using image analysis. The location, size and morphology of lesions seen histologically were described. Image analysis was also used to assess lesion size and distribution. Unstained sections were prepared for immunocytochemical analysis, to assess the amount of antigen present in lesions, using the method described in chapter four.

Liver iron assay

Samples of liver were sent to the Wallaceville Animal Health laboratory for biochemical analysis of total iron content (see chapter five)

Serum biochemistry

Blood, collected from the brachial vein of each bird immediately prior to euthanasia, was chilled for 24 hours prior to centrifugation to separate the serum from the red cells. The packed cell volume (PCV) was recorded as an average value for each group of birds by collecting a sample of blood from each bird in microhaematocrit tubes (Type II microhematocrit capillary tubes, American Scientific Products) and spinning in a haematocrit centrifuge. A larger amount of blood was collected from each bird using a 1.00 ml syringe followed by transfer to a 2.5 ml Eppendorf tube, the blood was allowed to clot overnight and the serum removed prior to storage at -20 °C. Where there was only a small amount of serum collected, the samples from each group were pooled. Biochemical testing was done at the Palmerston North Hospital laboratory using assay techniques based on human serum iron profiles. Serum transferrin was measured using an immunological turbidity test. Serum ferritin was measured using an immunoassay (ES 300, Boehringer Mannheim). Serum iron was separated from serum transferrin in the ferric form using guanidine chloride and reduced to the ferrous form by ascorbic acid. A dual wavelength colorimetric assay was used to determine the total amount of iron in the assay following the addition of ferricyanate.

For each group, 100 microlitres of serum was tested for levels of serum iron, serum transferrin and serum transferrin saturation. Some samples were also tested for serum ferritin levels.

Results

6-3-1 Clinical signs of disease in different treatment groups

Infected birds that had been pre-treated with iron dextran, irrespective of whether or not they had also received desferrioxamine, developed a blood-tinged mucoid diarrhoea and a transient pyrexia with an associated panting and polydipsia within 24 hours of the initial bacterial challenge. Infected birds, pre-treated with dextran or desferrioxamine alone, showed no clinical signs of disease. All birds were clinically normal two days following the initial dose of bacteria. One bird died six days after the initial bacterial challenge as a result of complications associated with inhalation pneumonia, this bird was in the iron dextran plus desferrioxamine treatment group. Necropsy findings indicated that the bird had inhaled *Y. pseudotuberculosis* and had bacterial lesions in the primary bronchi and throughout the lung parenchyma, there were no lesions found in other visceral organs.

6-3-2 Faecal shedding of *Yersinia pseudotuberculosis* in chickens pre-treated with iron dextran and/ or desferrioxamine.

Yersinia pseudotuberculosis was isolated from birds in all of the experimentally infected groups within six days following the initial bacterial challenge but not from any of the experimental chickens prior to bacterial challenge nor from any of the uninfected control birds treated with iron dextran and/ or desferrioxamine. Throughout the experiment, *Y. pseudotuberculosis* was readily isolated from the faeces of birds given iron dextran. Isolation of the challenge strain from birds in the group given dextran alone was more difficult. On day ten *Y. pseudotuberculosis* could not be isolated from any of the birds in the group given dextran alone but was isolated from all five birds in the iron dextran treated group. This difference is statistically significant ($P < 0.025$). None of the other differences noted were statistically significant using the chi-squared test.

Table (6-2) A comparison of the prevalence of *Yersinia pseudotuberculosis* in the faeces of groups of chickens treated with iron dextran or dextran, with or without desferrioxamine, and exposed to *Y. pseudotuberculosis* in the drinking water

Treatment	Day following initial bacterial challenge			
	0	6	8	10
Iron dextran	0/5	3/5	5/5	5/5
Desferrioxamine	0/5	3/5	3/5	1/5
Iron dextran + desferrioxamine	0/5	3/4*	4/4	2/4
Dextran	0/5	2/5	4/5	0/5

* One bird in this group died of aspiration pneumonia

6-3-3 Serological response

All of the experimental chickens which were challenged with repeated oral doses of *Y. pseudotuberculosis* serotype II developed a serological response to the lipopolysaccharide antigen of the homologous bacterium 10 days following initial bacterial challenge see figure (6-1). At day six the number of seropositive birds in groups pre-treated with iron dextran was greater than in the dextran treated group although this difference was not significant using the chi-squared test ($P>0.05$). However, ten days following oral challenge, the iron treated birds also had higher serological titres than those which did not receive iron. At the end of the experiment all of the control birds pre-treated with iron dextran and/or desferrioxamine were seronegative in the ELISA test.

Table (6-3) A comparison of the number of seropositive birds in each group of chickens treated with iron dextran or dextran, with or without desferrioxamine, and exposed to *Y. pseudotuberculosis* in the drinking water.

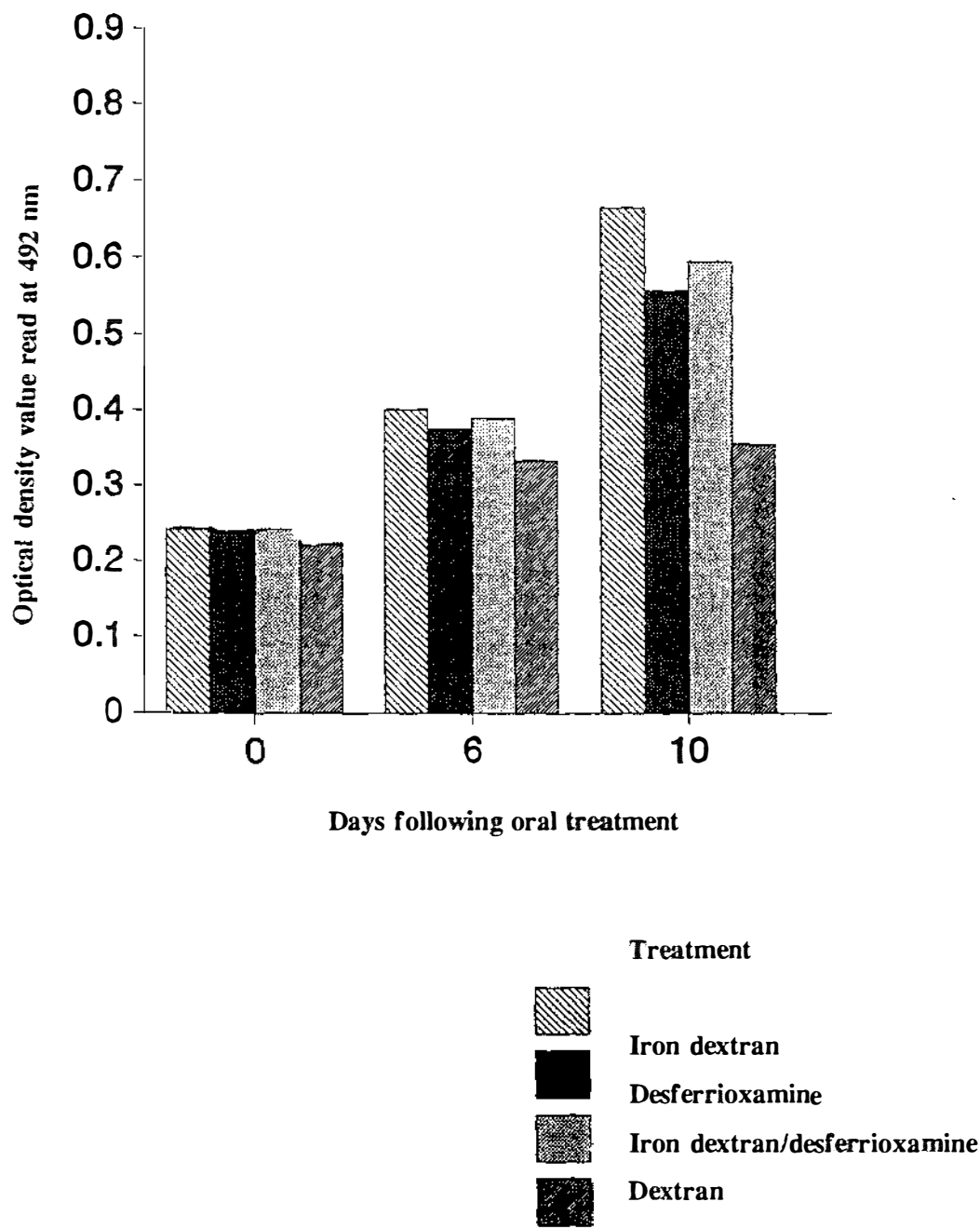
Treatment	Day following oral dosing		
	0	6	10
Iron dextran	0/5	3/5	5/5
Desferrioxamine	0/5	2/5	5/5
Iron dextran + desferrioxamine	0/5	2/4	4/4
Dextran	0/5	0/5	5/5

A seropositive bird is a bird with an optical density > 0.274 (see section 3-3-4)

Table (6-4) A comparison of the serological titres against the lipopolysaccharide antigen of *Y. pseudotuberculosis* serotype II measured by ELISA in chickens treated with iron dextran or dextran, with or without desferrioxamine, and exposed to *Y. pseudotuberculosis* serotype II in the drinking water for 10 days

Treatment	Serological Titre				
	<1/100	1/100	1/200	1/400	1/800
Iron dextran	-	-	3/5	1/5	1/5
Iron dextran + desferal	-	1/4	1/4	2/4	-
Desferal	-	-	3/5	2/5	-
Dextran	-	3/5	2/5	-	-

Figure (6-1) A comparison of the serological response of chickens challenged orally with *Y. pseudotuberculosis* following treatment with iron dextran with or without desferrioxamine or dextran as measured by the LPS-ELISA. See also appendix (A-16).



6-3-4 Serum and tissue biochemistry

A Serum iron

It was noted that the serum samples from birds in the group treated with desferrioxamine and dextran were very haemolysed, unfortunately this haemolysis artificially elevated the iron profile test results in this and some of the other groups as indicated in Table (6-5).

Table (6-5) A comparison of serum iron profiles measured in control and challenged chickens treated with iron dextran or dextran, with or without desferrioxamine,

Treatment		Serum iron profile			PCV
	Y.ps	S.Tr ⁺	S.ir ⁺⁺	TIBC ⁺⁺⁺	
Iron dextran	+	79.73*	50.2*	18.1*	45%
Iron dextran	-	9.25	15.1	2.1	45%
Iron dextran + desferal	+	10.57	23.5	2.4	39%
Iron dextran + desferal	-	106.16*	64.4*	24.1*	39%
Desferal	+	13.67	18.1	3.1	38%
Desferal	-	20.26	28.5	4.6	35%
Dextran	+	100.0*	48.5*	22.7*	45%
Dextran	-	6.17	21.3	1.4	46%

* Haemolysis may have elevated the value; + = Serum transferrin g/l; ++ S.ir = Serum iron μ mol/l; +++ TIBC = Total iron binding capacity μ mol/l.

All samples were taken from birds ten days after the initial treatment regimen, all were collected and handled in the same manner and tested at the same time. Each serum transferrin, serum iron and TIBC value represents that of a pooled serum sample from each group of birds and as a result analysis of these results was limited to general comparisons between treatment groups.

Control groups

Serum transferrin and total iron binding capacity are higher in the control group treated with iron dextran than in that treated with dextran. The value for serum iron is lower in the iron dextran treated birds. In the control group treated with the serum

transferrin levels are very high with a corresponding elevation of TIBC. The value for serum iron in this group is also high. Unfortunately, due to haemolysis in a number of the samples taken for analysis, the iron profile values are artificially high, as a result of this the values for serum iron, TIBC and serum transferrin in the desferal/iron dextran treated control group can not be compared.

Bacterial challenge groups

Despite the artificially elevated readings associated with the haemolysis of some samples, a few comparisons can be made in this group. If the infected and control desferal treated birds are compared it is noted that serum transferrin, TIBC are higher and serum iron levels are lower in the infected birds. In earlier experiments (chapter three) the injection of heat-killed bacterial antigen, or oral infection with *Yersinia pseudotuberculosis*, resulted in an elevation of serum transferrin and TIBC and a fall in serum iron values, the results are shown in Table (6-6).

Table (6-6) A comparison of serum iron profiles measured in chickens injected with purified LPS, heat killed bacteria, untreated controls and chickens orally challenged with *Y. pseudotuberculosis*

Treatment (two birds per group)	Serum iron profile			PCV
	S.Tr ⁺	S.ir ⁺⁺	TIBC ⁺⁺⁺	
Purified LPS	7.65	20.0	1.8	44%
Heat treated bacteria	9.25	15.1	2.1	41%
Experimental infection	7.04	18.2	3.2	43%
Control	6.31	23.5	1.4	45%

Packed cell volume

Due to the effect that haemolysis has on the PCV, only the values for the control groups can be compared. The PCV for the desferal treated group was lower than for the dextran and iron dextran treated birds (P<0.05).

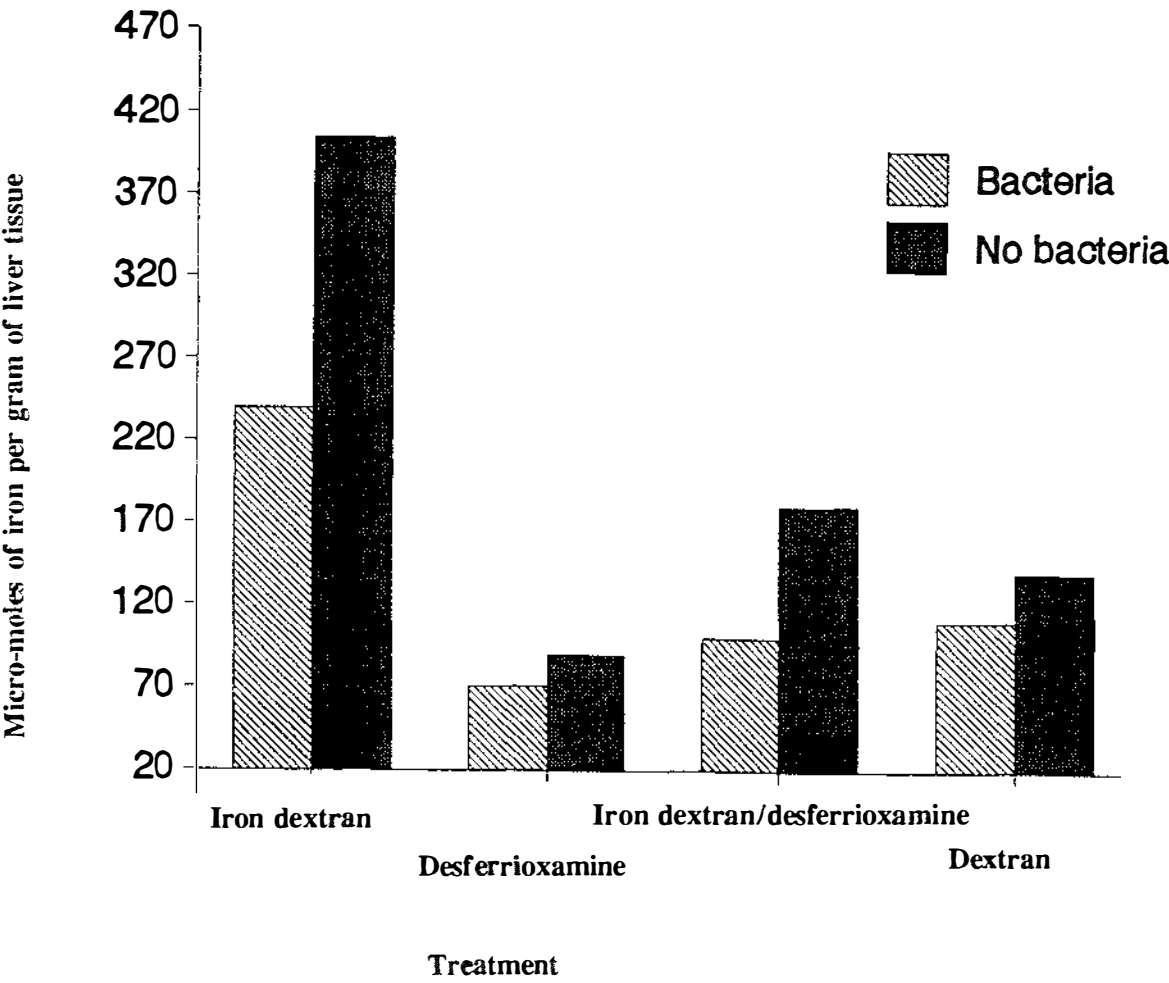
B- Liver iron

In all the treatment groups the amount of iron in the liver tissue of infected birds was less than that in the control birds ($P<0.05$). The results are represented graphically in figure (6-2).

Table (6-7) A comparison of the biochemically measured iron content in the livers of two-week-old male chickens treated with iron dextran or dextran, with or without desferrioxamine, 10 days after oral challenge with *Yersinia pseudotuberculosis* serotype II

Treatment	μ mol iron /gram		Number of birds
	Range	Mean	
Iron dextran + bacteria	160-320	232	5
Iron dextran	390-420	415	3
Desferal + bacteria	52-95	77	5
Desferal	80-100	90	2
Iron dextran + Desferal + bacteria	78-290	170	5
Iron dextran + Desferal	130-230	180	2
Dextran + bacteria	88-130	107	5
Dextran	120-160	140	5

Figure (6-2) A comparison of the hepatic iron content in control and challenged chickens measured ten days following parenteral treatment with iron dextran or dextran, with or without desferrioxamine.



6-3-5 Necropsy and histology

Gross findings

Birds in the infected groups were lower in body weight than the birds in the uninfected control groups 10 days following initial dosing ($P < 0.05$). There were no significant gross findings noted in birds from the dextran treated group except for a slight enlargement of the caecal tonsils in one bird and a moderately enlarged spleen in another. In both the infected and control birds treated with desferrioxamine the livers were pale and enlarged. Birds which received iron dextran generally had dark livers. In birds which received desferrioxamine in addition to iron dextran there was a variation in hepatic size and colour resulting in a mottled appearance.

Table (6-8) A comparison of the body weight and the necropsy findings of control and challenged chickens treated with iron dextran or dextran, with or without desferrioxamine.

Treatment	Weight range (g)	Gross findings			No.
		spleen	liver	Caecum	
Iron Dextran + Y.ps*	176-217	NVL**	Dark	Dark	5
Iron Dextran	281-320	NVL	Dark	NVL	3
Iron dextran+De+ Y.ps	175-232	NVL	Mottled	Dark	5
Iron dextran + De	251-264	NVL	Mottled	NVL	2
Desferal (De) + Y.ps	173-215	NVL	Pale	NVL	5
Desferal (De)	238-254	NVL	Pale	NVL	2
Dextran + Y.ps	166-210	NVL	NVL	NVL	5
Dextran	320-335	NVL	NVL	NVL	3

*Y. ps = *Yersinia pseudotuberculosis*; NVL = No visible lesions

Histological lesions

Infected birds pretreated with iron dextran with or without desferal had more severe intestinal lesions than those pretreated with dextran with or without desferal but the latter groups had more severe lesions in the liver and spleen. Birds given desferal alone had some lesions in the spleen. Those birds given iron and desferal in combination had

lesions in the liver, spleen and intestine. Representative lesions are illustrated in figures (6-3 to 6-6). In the iron dextran treated birds there were small granulomatous lesions in the lamina propria of the large intestine. In some sections there was also extensive sloughing and loss of the epithelial surface of villae as seen in figure (6-6). This was associated with haemorrhage into the lumen of the intestine in some birds. In birds which had hepatic lesions these were small and disseminated throughout the parenchyma. In one of the dextran treated birds there were several small hepatic lesions but in the other birds there was only a single lesion in the sections examined. Examples of these lesions are given in figures (6-3) to (6-5). In the centre of the abscess there was an area of cellular debris with pyknotic nuclei bordered by a few infiltrating lymphocytes and macrophages. The lesions were well defined with some fibrous reaction evident. The splenic lesions were larger than the hepatic lesions in birds which had abscesses in both organs. These lesions were most pronounced in the dextran treated birds as illustrated in figure (6-5). In most sections the granulomas were well formed with a large number of macrophages surrounding a zone of necrosis. Occasional multinucleated giant cells were present in some sections. In Gram stained sections there appeared to be few bacteria present in these lesions.

Number of birds with lesions

There are significantly more birds with splenic lesions in groups not receiving iron-dextran than in those groups which did receive iron-dextran ($P < 0.05$). There is no statistically significant difference between treatment groups in the number of birds with hepatic lesions ($P > 0.05$). However, there are a greater number of birds with intestinal lesions in the iron-treated groups than in the groups not receiving iron ($P < 0.01$).

Table (6-9) A comparison of the number of birds in each group of chickens with lesions in the liver, spleen and intestine following treatment with iron dextran or dextran, with or without desferrioxamine, and challenged with *Yersinia pseudotuberculosis*,

Treatment	Number of birds with lesions in visceral organs		
	Liver	Spleen	Intestine
Iron dextran	0/5	0/5	2/5
Iron dextran + desferrioxamine	1/4	2/4	2/4
Desferrioxamine	1/5	2/5	0/5
Dextran	2/5	5/5	0/5

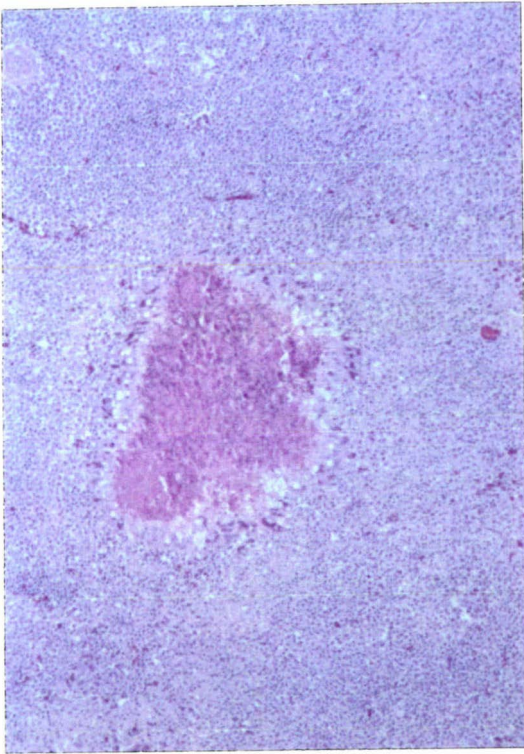
Number of lesions

There was a significant difference in the number of splenic lesions when comparing the different treatment groups ($df = 3$, $P < 0.001$). Birds in the dextran treated group had significantly more lesions in the spleen than birds in the other groups ($P < 0.001$). There were significantly more intestinal lesions in birds treated with iron dextran than in those not given iron ($df = 1$, $P < 0.01$). There was no significant difference between treatment groups in the number of hepatic lesions ($P > 0.05$).

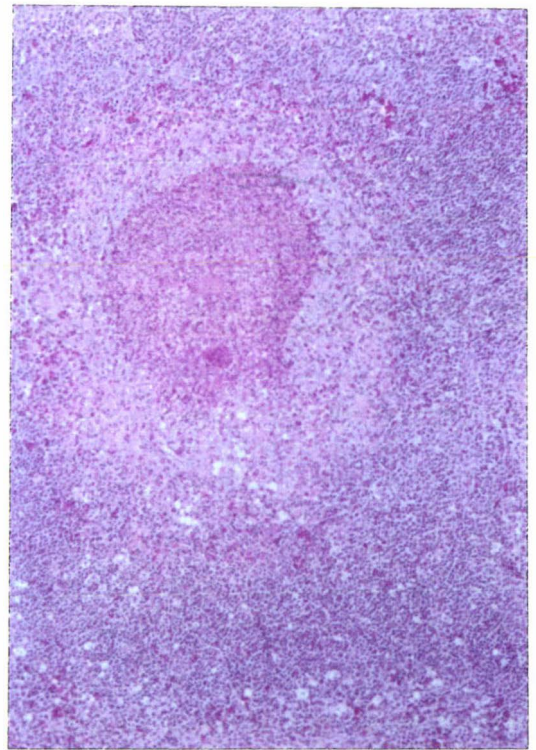
Table (6-10) A comparison of hepatic iron levels and the number and distribution of histological lesions in the liver, spleen and intestine of birds treated with iron dextran or dextran, with or without desferrioxamine, and challenged with *Y. pseudotuberculosis*

Treatment	Organ		
	Liver	Spleen	Intestine
Iron dextran	0*	0	4
Iron dextran + desferrioxamine	1**	5	3
Desferrioxamine	1*	2	0
Dextran	3*	17	0

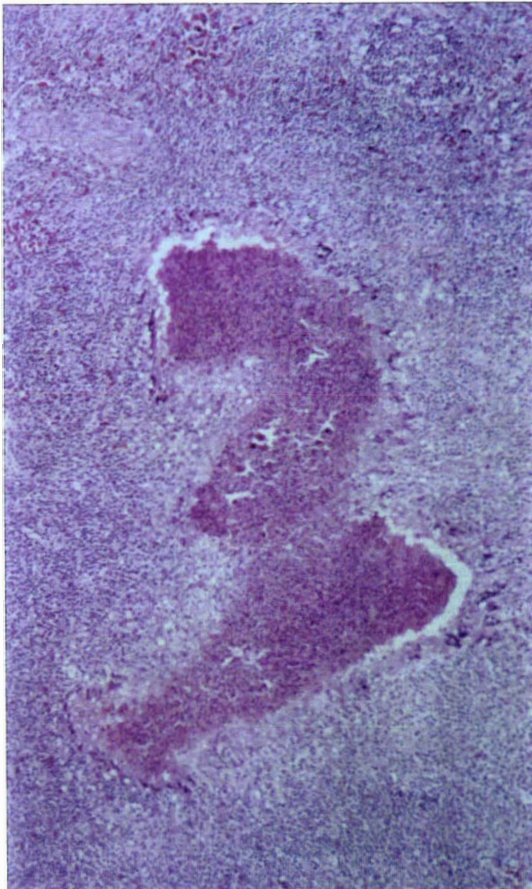
* Number of lesions in 25 slides (5 slides/bird); ** Number of lesions in 20 slides (5 slides/bird).



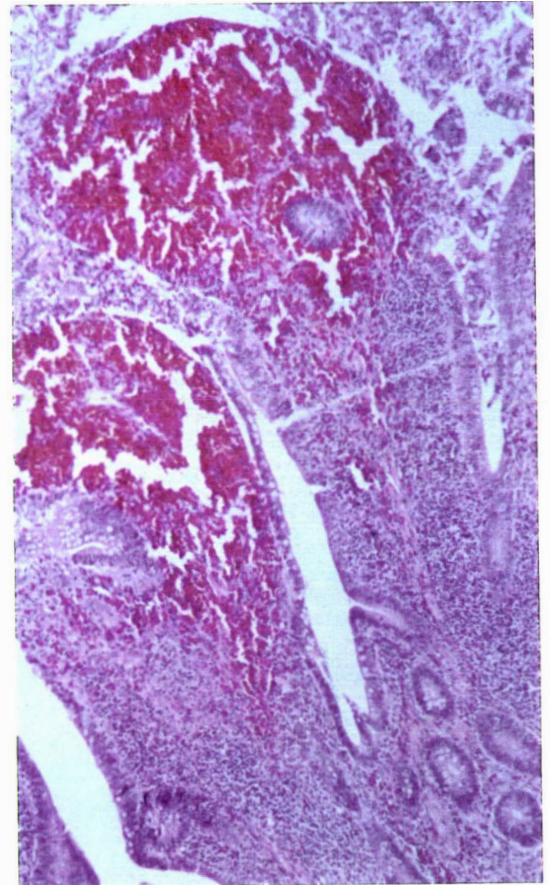
6-3 Histological section taken from the spleen of a chicken challenged with *Y. pseudotuberculosis* following treatment with iron dextran. H & E. x 10 (see text for description).



6-4 Histological section taken from the spleen of a chicken challenged with *Y. pseudotuberculosis* following treatment with desferrioxamine. H & E. x 10 (see text for description).



6-5 Histological section taken from the spleen of a chicken challenged with *Y. pseudotuberculosis* following treatment with dextran. H & E. x 10 (see text for description).



6-6 Histological section taken from the intestine of a chicken challenged with *Y. pseudotuberculosis* following treatment with iron dextran. H & E. x 10 (see text for description).

Assessment of the amount of stainable and biochemically measured iron in the liver tissue of infected as compared with control chickens

None of the birds in the experimental groups pretreated with iron dextran and infected with *Y. pseudotuberculosis* had any evidence of stainable iron in the liver at necropsy. This is in contrast to the control birds given an injection of iron dextran and not exposed to *Y. pseudotuberculosis* which still had evidence of stainable iron in the liver tissue 10 days following treatment. The results of biochemical analysis of liver tissue from infected and uninfected birds are seen in Table (6-11). Using analysis of variance on these results it is shown that, for birds in all treatment groups, the amount of iron measured in hepatic tissue is lower in the infected birds than in the non-infected birds ($P < 0.05$).

Table (6-11) A comparison of the amount and distribution of iron in the livers of two-week-old male chickens treated with iron dextran or dextran, with or without desferrioxamine, 10 days after challenge with *Yersinia pseudotuberculosis* serotype II

Treatment	μ mol/g	Distribution of iron		No.
		Kupffer cell	Hepatocyte	
Iron dextran + bacteria	160-320	*0.00-2.10	*0.00-0.70	5
Iron dextran	390-420	0.00-2.30	5.71-6.83	4
Desferal + bacteria	52-95	0.00-0.00	0.00-0.84	5
Desferal	80-100	0.00-0.00	0.00-0.00	2
Iron dextran+De+bacteria	78-290	0.00-0.00	0.60-1.77	5
Iron dextran+Desferal	130-230	0.00-0.00	0.60-0.69	2
Dextran + bacteria	88-130	0.00-0.00	0.00-1.12	5
No treatment	120-160	0.00-0.00	0.00-0.00	2

* The range of image analysis reading are given.

Assessment of the amount of antigen in visceral lesions using immunocytochemistry.

Chickens which developed lesions in the liver and spleen following oral challenge with very large doses of *Y. pseudotuberculosis* had little antigen in the lesions when stained using immunocytochemistry.

Discussion

In the present study, it was decided that repeated oral challenge was more appropriate, than a single inoculum, in a disease model which aims to mimic the situation which is likely to occur in natural cases of the disease. A similar protocol was adopted by Robins-Browne & Prpic (1985) in a rodent model using oral challenge with yersiniae. In the present study it was possible to infect all of the experimental chickens with *Yersinia pseudotuberculosis* by the oral route, allowing between group comparisons, and an evaluation of the effect of different treatment regimens on the outcome of the infection. In contrast to birds which received a single oral challenge of the same bacteria (chapter four) all of the continuously challenged birds developed lesions. This is similar to the findings of Stovell (1963) who examined the effect of repeated oral challenge with *Y. pseudotuberculosis* in canaries.

Before comparing the results of the present study with those of studies reported in the literature, it should be noted that any observed difference between iron-treated animals and controls depends on 1) the iron and protein status of the host, 2) the ability of the pathogen to compete with the host for iron, 3) the route of challenge. The dose of bacteria used along with the other virulence characteristics of the pathogen are also important. The latter point is especially important when comparing the relative effect of iron in experiments using different species of *Yersinia* (Bullen, 1987).

In the present study there is experimental evidence to support the hypothesis that iron-treated birds initially develop more severe disease than untreated birds when challenged with *Y. pseudotuberculosis*. Iron-treated birds challenged with *Y. pseudotuberculosis* developed clinical signs of disease such as diarrhoea, lethargy and anorexia. These birds also developed higher serological titres to the LPS antigen of the challenge bacterium and *Y. pseudotuberculosis* was more readily isolated from their faeces than from the faeces of untreated birds. After the initial clinical disease noted in the iron-dextran treated groups, the birds appeared clinically normal, except for one bird in the iron dextran /desferrioxamine treated group which died six days after the initial bacterial challenge. At necropsy this bird was found to have bacterial lesions throughout the lungs but no lesions were found in the other visceral organs. *Yersinia pseudotuberculosis* was cultured from the lung lesions and it was concluded that the bird probably died

following inhalation of the challenge organism, therefore this chicken was not included in the statistical analysis of the experimental results. In the early stages of infection, following oral challenge with bacteria, it is possible that parenteral iron dextran, supplied to chickens prior to infection, counteracts the hypoferraemia which develops as part of the non-specific acute phase inflammatory response. As a result treatment with iron dextran may initially exacerbate infection with *Y. pseudotuberculosis*.

Similar results are reported in mammalian models, for example in an experiment which challenged rodents orally or parenterally with *Y. enterocolitica* it was found that pre-treatment with iron dextran resulted in more severe clinical disease (Robins-Browne & Prpic, 1985). It was noted, however, that the relative difference between iron treated and untreated groups was greater when mice were challenged with non-virulent yersiniae than when virulent strains were used. This finding would be consistent with virulent yersiniae being more efficient at competing with the host for available iron while non-virulent yersiniae can only grow rapidly and produce disease when there is readily available iron. *Yersinia pseudotuberculosis* is considered to be more virulent than many strains of *Y. enterocolitica* (Wake & Morgan, 1986; Autenrieth *et al.*, 1991) and therefore it is likely that this *Y. pseudotuberculosis* is able to acquire iron readily from the normal mammalian or avian host but that this will be limited in the low iron host or during the hypoferraemic state in acute infection. This hypothesis would explain why human patients suffering from haemochromatosis and iron overload are more susceptible to the development of systemic yersinia infection even though the prevalence of the disease is not greater in these patients. The results of *in vitro* work using human immune serum indicated that the growth of *Y. pseudotuberculosis* was inhibited but that the inhibitory effect was reversed if iron-dextran was added indicating that iron availability was a limiting factor in immune serum. In contrast, the growth of *Y. pseudotuberculosis* and other bacteria in immune serum from patients with haemochromatosis was not inhibited (Wake & Morgan, 1986; Griffiths & Bullen, 1987).

In the present study, histological examination of tissues from all of the challenged chickens indicated that the number of intestinal lesions was greatest in the iron dextran-treated groups. This may have resulted from an increased number of bacteria proliferating in the intestine and could be associated with an increased availability of iron in the intestinal contents. It is well recognised that intestinal absorption of dietary iron is reduced in chickens given parenteral sources of iron dextran (Hill *et al.*, 1977).

Robins-Browne *et al.* (1991) suggested that a readily available pool of iron may explain why non-virulent yersiniae were able to survive in the intestinal lumen of healthy individuals. It is possible that an increase in this iron pool, along with alterations in normal intestinal flora associated with it, may favour the replication of virulent yersiniae *in vivo*. The serological response of the iron-treated chickens suggests that the number of bacteria taken up by the intestinal lymphoid tissue was greater than in chickens not treated with iron-dextran. Unlike the models which determined that desferrioxamine potentiated the development of yersiniosis in mice, treating chickens with desferrioxamine did not appear to have a significant effect on the course of pseudotuberculosis. This may be due either to the ability of *Y. pseudotuberculosis* to acquire iron without an exogenous siderophore (Heeseman *et al.*, 1993) or due to differences between mice and chickens in their response to desferrioxamine, although other factors may also be involved.

In contrast to the increased severity of clinical disease in the iron-treated chicken, these groups had fewer lesions in the liver and spleen than those in the groups not treated with iron. This result is similar to that which Smith *et al.* (1977) found in chickens challenged with *Salmonella gallinarum*. Although they did not examine tissues histologically they compared bacterial counts from homogenised tissues and blood samples and determined that there were fewer bacteria present in the livers and spleens of birds treated with iron than in those untreated. Smith *et al.* (1977) suggested that the apparent protective effect of exogenous iron dextran indicated that the domestic fowl had a high iron requirement during infection and utilised the extra supply in host defence or for erythropoiesis.

A possible explanation for the lower number of bacterial lesions in the spleens and livers of birds treated with parenteral iron dextran is that it is a consequence of enhanced phagocytic and bactericidal capacity with early restriction of infection by the reticuloendothelial system in iron-treated chickens (Smith *et al.*, 1977). Although there have been *in vivo* assays to assess the efficiency of phagocytosis in squirrels following experimental *Y. pseudotuberculosis* infection (Vesselinova *et al.*, 1992) few studies have been able to evaluate the way in which iron availability effects this process (Keusch, 1990). This is because it is difficult to determine the mechanisms involved in the interaction between iron, host and pathogen *in vivo* due to the inherent complexity of

iron metabolism in the host and the alterations which occur during infection. The competition between host and pathogen for iron is a dynamic process, the outcome of which is influenced by a number of other variables. In the present study, by pre-treating chickens with iron dextran and or dextran and desferrioxamine it was hoped that, by comparing the outcome of challenge in each treatment group, the possible potentiation of disease as a result of a relative increase or decrease in tissue and serum iron could be evaluated. Although it was found that increasing the amount of iron in the serum of the chicken initially resulted in a more severe clinical infection the histological findings indicated that the interaction was more complex. Ten days following the initial challenge the iron-treated birds had fewer visceral lesions than the untreated birds suggesting that the iron may have enhanced the ability of the chicken to limit infection. Consideration must be given to both the effect of iron on the intracellular and extracellular specific and non-specific immune mechanisms of the chicken and to the ability of the challenge organism to acquire iron from the intracellular and the extracellular iron-binding proteins of the chicken host. The lack of data reported in the literature on 1) the effect of iron loading chicken phagocytic cells on the survival and replication of yersiniae, and 2) the ability of yersiniae to acquire iron from chicken serum, prompted the development of an *in vitro* system. This is described in chapter seven.

The possibility that there are host differences with regard to the effect of changes in body iron and susceptibility to disease, can not be ignored. From the results of chapter five, it would appear that the order galliformes may not be representative of other avian orders with regard to susceptibility to iron overload. The White Leghorn chicken is a species with limited iron reserves and is considered relatively resistant to pseudotuberculosis and it is for these reasons that it is particularly suitable for the development of a model designed to assess the effect of administering exogenous iron dextran on the development of pseudotuberculosis. As with any other *in vivo* model, it is important to determine the relative importance of each different variable in the system.

Disease model

To fully appreciate the outcome of the experiments described it is necessary to 1) identify which of the variables may be host dependant, for example the initial iron status of the host is determined by genetic factors and diet and 2) to determine the ability of the pathogen to acquire iron from the host. The competency of *Yersinia* organisms to acquire iron has been correlated with their ability to cause disseminated rather than localized infection (Weinberg, 1978). In addition highly virulent strains of yersiniae may have an LD50 so low that there would be no significant reduction following treatment with exogenous iron. This possibility was used to explain why the effect of pre-treating rats with non-pathogenic *Y. frederiksenii* strains resulted in a greater reduction in LD 50 than when a pathogenic strain of *Y. enterocolitica* was used (Robins-Browne et al, 1979). Although it was previously thought that it may mask the extent of the effect of iron on virulent yersiniae it is also possible that in rats, during infection, iron is not a limiting factor to the growth of these organisms *in vivo*.

Host factors

In all of the birds orally challenged with *Yersinia pseudotuberculosis*, including those given iron dextran, there was no stainable iron in the liver at the time of euthanasia. The observed fall in the amount of hepatic iron following bacterial infection was supported by biochemical assessment of hepatic iron which indicated a reduction of 15-40 micromol per gram of tissue in challenged birds as compared with unchallenged controls. This finding supports the hypothesis that iron metabolism is altered during bacterial infection in the chicken. It was suggested by Hill *et al.*, (1977) that hepatic iron is redistributed to the spleen during infection. In the present study it was not possible to verify this finding because the spleens from infected birds did not contain stainable iron and after the histological material had been prepared there was insufficient tissue to analyse biochemically. It is possible that the splenic iron content had increased but that the iron was either not in the ferric form or was tightly bound to serum proteins unlike the loosely bound ferric iron associated with ferritin and haemosiderin stored in the liver.

Chickens given a series of intravenous injections of a crude heat-killed preparation of

Yersinia pseudotuberculosis or purified lipopolysaccharide developed hypoferraemia. A similar hypoferraemia was observed in birds following an oral challenge of *Y. pseudotuberculosis*, this is probably the result of the release of antigenic material from bacterial cells and it seems to occur very early in the course of the infection. It may correspond with the rapid uptake of bacteria by the enteric lymphoid system as is thought to occur in infections with salmonellae in chickens (Barrow, 1992). In addition, intravenous challenge with heat-killed salmonellae or purified lipopolysaccharide has given a similar hypoferraemic response (Barrow, 1992; Chart *et al.*, 1991). In these birds, as with experimentally infected mammals, hypoferraemia is associated with a rise in serum transferrin levels and total iron binding capacity (TIBC) which is typical of the non-specific phase of the acute phase inflammatory response (Weinberg, 1978).

In mammals it is known that following a single intravenous injection of lipopolysaccharide or endotoxin the resulting elevation of temperature and hypoferraemia are transitory and follow the release of cytokines and interleukin mediators from activated monocyte-macrophages (Myszewski & Stern, 1991; Kelso, 1993). In enteric infections, lymphoid tissue in the gastrointestinal tract may be especially important in antigen presentation (El-Marghi & Mair, 1979; Van der Waail, 1993). In the present study, it was found that continued exposure of the healthy chicken to pathogenic *Yersinia pseudotuberculosis*, under some circumstances, resulted in clinical disease. Therefore repeated challenge with high doses of bacteria along with increasing the availability of iron resulted in an imbalance between the chicken host and the potential pathogen. The outcome being manifested by clinical disease. To what extent the exogenous supply of iron initially benefits the pathogen at the expense of the host by enhancing the growth of the pathogen or by interfering with the cell-mediated or humoral responses of the host is still unclear (Chandra, 1990; Keuch, 1990; Griffiths, 1993).

It is also not clear from the results of the *in vivo* experiment just what effect desferrioxamine has on the outcome of *Y. pseudotuberculosis* in chickens. It would initially appear that treating birds with desferrioxamine had no significant effect and that the increased severity of disease in the desferrioxamine/iron dextran treated group was due to iron dextran. However, although exogenous siderophores may not be important

in the acquisition of iron by *Y. pseudotuberculosis* it is also possible that the effect of desferrioxamine was masked by other factors. This possibility is explored further in chapter seven because it is important to determine the relative effect of desferrioxamine on the growth of the bacteria compared with the possibility that desferrioxamine alters the iron metabolism of the host. This point is important because desferrioxamine (Desferal) is generally used as a clinical treatment of human haemochromatosis or haemosiderosis which is secondary to transfusion iron overload, to chelate excess iron (Jones *et al.*, 1977; Robins-Browne & Prpic, 1987). It is in these patients that the complication of systemic yersiniosis is important. Previous reports suggest that both *Y. enterocolitica* or *Y. pseudotuberculosis* have the potential to be involved (Griffiths & Bullen, 1987) but although there are a few speculative reports from retrospective studies to sustain this view (Rabson *et al.*, 1975; Sherman, 1990), the only experimental evidence supporting the suggestion that infection with virulent yersiniae has been exacerbated by the use of desferrioxamine, refers to infection with *Y. enterocolitica* (Robins-Brown & Hartland, 1991).

Pathogen

Recently Fukushima *et al.* (1991) challenged the idea that *Y. pseudotuberculosis* is an intracellular pathogen and commented that, although the bacteria are able to survive phagocytosis and the microbicidal actions of phagocytic cells, the deciding factor on the outcome of infection is the ability of the bacteria to replicate extracellularly. Virulence is therefore dependent on the ability of yersiniae to survive serum killing and the effect of antibody as well as the ability to compete with the host for available serum iron (Simonet *et al.*, 1984; Straley *et al.*, 1993). In contrast, other studies support the view that intracellular survival and replication are important in the pathogenesis of the disease associated with virulent yersiniae (Macauley *et al.*, 1967; Marlon *et al.*, 1975; Cornelis, 1992; Iteman *et al.*, 1993). In addition, it is also apparent from retrospective work in people that cellular iron overload may promote replication of *Y. pseudotuberculosis* in the reticuloendothelial system (Rabson *et al.*, 1975). This could explain why lesions of pseudotuberculosis are often larger and more florid in the livers of birds with hepatic siderosis.

In addition to iron availability other factors effect the pathogen once it has gained entry

into the host, among these is increase in temperature. *Yersinia pseudotuberculosis* appears to have a high iron requirement at temperatures greater than 37 °C (Heeseman, 1987; Heeseman *et al.*, 1993). Most strains are haem pigment negative (pgm-) and, unlike *Y. pestis*, have a limited capacity to utilise haem iron although most virulent strains appear to contain the *irp* (iron repressible protein) gene which may code for an iron receptor for siderophores produced by virulent strains of *Y. enterocolitica* and *Y. pseudotuberculosis* (Yersibactin) (de Almeida *et al.*, 1993; Iteman *et al.*, 1993). Robins-Browne & Prpic (1985) found that the relative effect of desferal or iron dextran in lowering the LD₅₀ of yersiniae introduced orally or intraperitoneally to rodents was more pronounced when normally avirulent species, such as *Y. frederiksenii*, were used as the challenge bacteria. This supports the view that in virulent yersiniae the development of alternative iron acquisition mechanisms is an important aspect of pathogenicity enabling *Y. pseudotuberculosis* to acquire iron from the serum of the host.

Another point to consider in species susceptibility to bacterial infection is the possibility that some bacteria are host-specific due to their inability to compete with serum transferrin for iron except in the host species to which the bacterium has adapted. This is true for *Neisseria spp.* which have adapted to human hosts with the ability to compete for iron bound to human transferrin but not to transferrin of other species (Griffiths, 1993; Gray-Owen & Schryvers, 1993). It is not fully understood whether or not some *Yersinia* species are host specific. It is possible that there are strain differences in the virulence expression of pathogenic yersiniae which become apparent when different host species become infected. If it does occur, host specificity in strains of *Y. pseudotuberculosis* could be due to differences in iron acquisition ability (Roeser, 1980; de Almeida *et al.*, 1993).

Despite their limitations, *in vitro* studies are a valuable supplement to *in vivo* work and allow individual parameters in host-pathogen interactions to be measured. This will be considered in the next chapter.

Summary

In view of the observed link between the availability of iron in the host and the development of infectious disease, including pseudotuberculosis in many avian species, an experimental model to examine this was developed in the chicken. The chicken was chosen as a model for several reasons, 1) there was already a lot of base-line data available for this species, 2) it was necessary to compare this model with others already developed in the chicken and 3) because healthy chickens have no stainable iron in the liver it is easy to monitor the effect of infection on the hepatic iron levels in different treatment groups.

1) Following treatment with iron dextran, chickens challenged orally with *Y. pseudotuberculosis* developed more severe disease than those not given iron dextran. These birds showed clinical signs of disease, had the highest serological titres, there were intestinal lesions and *Y. pseudotuberculosis* was more readily isolated from the faeces.

2) The number of splenic lesions in chickens which were treated with iron dextran was less than in those not treated. This can be explained by the fact that the normal chicken has low reserves of iron and requires additional iron to maintain the efficiency of cell-mediated immunity. If the experiment had continued for a longer duration it is possible that the iron treated birds would overcome infection whereas the mortality in untreated birds would be high.

3) Treatment of chickens with the exogenous siderophore, desferrioxamine, did not affect the outcome of infection. Therefore it was concluded that *Y. pseudotuberculosis* does not require exogenous siderophores to acquire iron *in vivo*.

4) Ten days following oral challenge, chickens treated with iron dextran had no stainable iron in the liver. This was in contrast to birds treated with iron dextran and not challenged. Chickens in all treatment groups which were challenged with an oral dose of *Y. pseudotuberculosis* had lower levels of biochemically assessed iron in the liver than did birds not challenged.

5) Chickens given an oral dose of *Y. pseudotuberculosis* or given a parenteral dose of bacterial endotoxin develop hypoferramia associated with an increase in serum transferrin, an increase in total iron binding capacity and a decrease in serum iron. Chickens are therefore similar to mammals in their response to endotoxin.

In conclusion, it is apparent that 1) parenteral administration of iron dextran initially results in a more severe infection in the chicken, 2) during infection in the chicken there is a reduction in the amount of hepatic iron and 3) the exogenous supply of iron is utilised by the chicken as infection develops. It is possible that chickens are not representative of other avian species in regard to this disease.

It is apparent that *Y. pseudotuberculosis* is able to acquire iron from the normal chicken during infection. This is possibly not true in the early stages of the disease when the serum iron content is low, the administration of iron dextran at this time may therefore potentiate the development of this disease in the acute stages of infection. The growth of *Y. pseudotuberculosis* in serum is examined in the next chapter along with the intracellular growth of yersiniae in iron loaded macrophages.

CHAPTER SEVEN

Iron, yersiniae and the host immune response; *in vitro* studies**Introduction**

There is a large body of evidence, derived from *in vitro* studies, to support the hypothesis that iron loading of phagocytic cells results in the enhanced survival of intracellular bacteria (Bullen *et al.*, 1974; Bullen & Joyce, 1982; Keown *et al.*, 1983; Griffiths & Bullen, 1987). However, it is also known that small amounts of iron can actually enhance the bactericidal capacity of the phagocytic cell (Sherman, 1990; Dunlap *et al.*, 1993). Intracellular killing appears to be ultimately dependent on oxygen compounds generated from hydrogen peroxide by the lysosomal enzyme myeloperoxidase. This enzyme contains iron and may be reduced in iron deficiency states (Chandra, 1990). Much of the work has been done with cell cultures derived from mammalian tissues but recently there has been more interest in examining the interaction between chicken macrophages, iron and salmonellae (Barrow, 1993).

Smith *et al.* (1977) suggested that the apparent protective effect resulting from parenteral administration of iron dextran to chickens prior to bacterial challenge, may have been due to enhanced phagocytic killing capacity of reticuloendothelial cells. This hypothesis would seem reasonable if the level of iron available to chicken reticuloendothelial cells is insufficient to maintain optimum phagocytic activity during infection. Iron dextran given to a host deficient in iron can be readily utilised by the host and therefore not be available to the invading micro-organism. If this were true then it is likely that bacterial challenge of iron-replete birds, following treatment with iron dextran, would result in an increased amount of iron available to the micro-organism. Enhanced bacterial growth *in vivo*, following treatment with iron dextran, has been found to increase the severity of disease in iron-treated mammals exposed to salmonellae (Kluger & Rothenberg, 1979) or yersiniae (Robins-Browne *et al.*, 1987). However, it is difficult to predict to what extent the outcome of loading phagocytic cells with iron is due to the enhancement of the growth of intracellular bacteria as compared to the impairment of the bactericidal capacity of the iron-loaded host cell (Kaplan & Basford, 1979; Finkelstein *et al.*, 1983; Noble *et al.*, 1987; Green *et al.*, 1988). The net effect that loading the host with iron will have on the outcome of a disease, will also

depend on whether or not iron is a limiting factor to the *in vivo* growth of the pathogen.

It was apparent from the retrospective studies on avian species (chapter five) that an increase in the amount of iron in the Kupffer cells was associated with the presence of microbial disease, including pseudotuberculosis. In view of this association and the apparent similarity between the bactericidal functions of avian and mammalian phagocytic cells (Powell, 1987; Myszewski & Stern, 1993), it appears likely that chicken phagocytic cells which are overloaded with iron *in vitro* will favour the survival of intracellular bacteria. In the chicken, it was difficult to produce iron overload *in vivo* due to the low iron content of normal chicken tissues. However, it was decided that an *in vitro* system using cultured chicken macrophages could be used to achieve saturation of phagocytic cells with iron similar to that observed in other species of bird described in the retrospective study (chapter five).

In addition, the extracellular survival of bacteria is also considered to be important in the development of systemic disease and is thought to be enhanced in conditions which are associated with an increase in serum iron (Rabson *et al.*, 1975; Smith *et al.*, 1981; Mellencamp *et al.*, 1981; Melby *et al.*, 1982; McLaren *et al.*, 1983). It is apparent that pathogenic bacteria have evolved a number of mechanisms to allow acquisition of iron from the host *in vivo* and as a result are able to survive extracellularly. Bacteria which are less pathogenic often require an increased availability of iron before overcoming host defences. This possibly explains why the virulence of relatively non-pathogenic yersiniae is enhanced in mice which have been pre-treated with iron dextran whereas that of pathogenic yersiniae is not (Robins-Browne & Prpic, 1985). No equivalent experiments have been reported in mice using *Y. pseudotuberculosis* but with this organism it is likely that exogenous iron is not necessary for extracellular survival of this organism. However, the relative effect may depend on the host species and the strain of *Y. pseudotuberculosis* used. In the present study, it was decided to examine the relative effect of exogenous iron dextran on the challenge strain of *Y. pseudotuberculosis* used in the *in vivo* work outlined in chapter six.

In this chapter an *in vitro* system is described which examines the hypotheses that 1) the intracellular growth of yersiniae in iron loaded chicken phagocytes is enhanced and 2) that the ability to acquire iron from serum is an important virulence characteristic of *Y. pseudotuberculosis*.

Materials and methods

7-2-1 Phagocytosis assay

Collection of chicken spleen tissue

Spleen tissue was harvested from a total of six male White Leghorn chickens. All birds were tested for the presence of faecal *Salmonella spp.* and *Yersinia spp.* Two birds were euthanased at 3 days of age and the remaining birds were maintained on a commercial pelleted chicken ration and housed in plastic cages until required. After initial testing of the *in vitro* system the remaining four birds were euthanased at 10 days of age. Following euthanasia the spleen was removed from the bird using aseptic technique.

Harvesting spleen cells

The capsule of the spleen was incised and forceps were used to release the cells into a sterile petri dish containing 2.0 ml of Eagle's minimum essential medium (MEM, Sigma M-0643) and 10% specific-pathogen-free (SPF) chicken serum. Cells were dispersed in the medium by drawing them in to a 10 ml syringe containing the same medium, and expelling with moderate pressure. The same syringe was later used to collect the cells from the petri dish. The medium and cell mixture were then added to 10 ml of sterile MEM + 10% SPF chicken serum, this was gently mixed and a 100 microlitre aliquot was removed for counting the yield of viable cells.

Estimation of the number of viable cells

A 100 microlitre aliquot of cells suspended in MEM + 10% serum was added to 100 microlitres of vital blue dye (0.2% Trypan blue, BDH, Poole). The number of cells in 10 microlitres was estimated by counting the number of unstained cells in the microcytometer grid. Red cells were not included. From this calculation it was determined that there were approximately 12×10^7 cells in 10 ml of the preparation.

Cell culture

Spleen cells were cultured in 96-well Nunc (Nuncclon, Denmark) cell culture plates. Approximately 12×10^5 cells were added with 100 microlitres of the culture medium (MEM + 10% chicken serum) per well. Cells were incubated at 37 °C in a CO₂ incubator. The plates were checked for any change in pH and signs of contamination every three days initially. After 10 days the culture medium was changed and the cells were washed using culture medium and an antibiotic solution containing equal parts of 10⁶ IU/ml Penicillin G (Biochemie); 1% Streptomycin, Boehringer Mannheim GmbH and 5% Kanamycin (Sigma). Plates were then viewed under an inverted microscope. Only wells containing a carpet of adherent cells with the morphological features of macrophages, were used for the experiment.

Slide preparations

In addition to the above procedure, 100 microlitres of the cell preparation was added to each of ten culture slides. Any procedure carried out with the culture plates was repeated with the slide preparations. At the end of the experiment the cells on the slides were fixed and stained with vital blue dye to allow a visual assessment of cell viability and phagocytic activity.

Phagocytosis of exogenous iron in cell culture

Iron dextran (Imferon) was added as a 1% solution to half of the wells on each cell culture plate and five of the slide preparations. The plates were left to incubate overnight at 37 °C and the following day the cells were washed with fresh culture medium and antibiotics.

Preparation of yersiniae

Pure cultures of *Y. pseudotuberculosis* serotype II and *Y. frederiksenii* were prepared from stock culture. The *Y. pseudotuberculosis* isolate chosen was the challenge isolate which was described in chapter four. The *Y. frederiksenii* (Y. FRED. 23111) organism chosen, had been isolated from a healthy bird and did not express plasmid related virulence

characteristics. A description of this isolate was also given in chapter four.

These bacteria were grown overnight on blood agar plates incubated at 28 °C and harvested into 2 ml tryptone water. After a further overnight incubation at 28 °C serial dilutions of the suspension were plated onto selective media (CIN) using a 10 microlitre loop. The colonies which were visible after 48 hours incubation at 28 °C on CIN agar were counted. It was determined that a viable colony count of 2×10^7 organisms/ ml could be produced using this method.

Phagocytosis of bacteria in cell culture

A total of 5×10^6 colony forming units (250 microlitres of tryptone water suspension) were added to each well of the cell culture plate. Plates were divided in two with *Y. pseudotuberculosis* added to one side of the plate and *Y. frederiksenii* added to the other side. An equal number of bacteria were added to the wells containing iron-treated cells and to those containing untreated cells at a ratio of approximately five bacteria per cell (5 : 1.2). Bacterial cells were also added to the slide preparations. Bacteria and cells were incubated at 37 °C for periods of 0 minutes, 1 hour, 2 hours, 3 hours and 4 hours. Following incubation, cells were washed with culture medium and antibiotic solution containing equal parts of 10^6 IU/ml Penicillin G (Biochemie) 1% Streptomycin (Boehringer Mannheim GmbH) and 5% Kanamycin (Sigma) to remove extracellular bacteria. The plates were then washed three times in sterile phosphate buffered saline (pH 7.4). Cells were lysed by the addition of 20 microlitres of saponin (1%) per well to release intracellular bacteria. Plates were incubated for 2 hrs at 37 °C to ensure complete lysis of cells. Saponin solution was also added to a series of 100 microlitre suspensions of bacteria to determine the effect of saponin on the bacterial cells.

Radiolabelled Uracil (H^3) uptake

The scintillation system was calibrated for yersiniae by recording the scintillation counts obtained for serial titrations of a known number of Uracil- H^3 (Amersham Life Sciences, USA) labelled yersiniae. Each titration had five replicates and the values presented in the results are the mean values for these five replicates.

The scintillation counts measured the uptake of a labelled metabolite (Uracil- H^3). The label was prepared as a 1/20 dilution of the stock solution (50 micro-Ci/ml) and 10 microlitres was added to each well. Plates were incubated for 2 hours to allow viable yersiniae to take up the label.

Labelled bacterial cells were harvested from each well on the plate, row by row, using a cell harvester (LKB Wallac cell harvester, 1295-00). The amount of radioactivity present in yersiniae harvested from each well onto a filter paper-square was measured using an automated scintillation counter system (1205 Betaplate Liquid Scintillation Counter, Pharmacia).

Expression of the results

The reading for each well containing iron-treated phagocytic cells was compared with the corresponding well from untreated phagocytic cells. In the results section the mean and standard deviation of the scintillation counts are given for each series of five replicates. The individual assay readings and their statistical analysis are given in appendix (A-14). A two-way analysis of variance was used to evaluate the results of each assay and the results of the iron-treated cells are compared with those of the untreated cells using the student's t test.

7-2-2 Serum resistance assay

Collection and preparation of serum from chickens

Serum was aseptically collected from healthy two-week-old chickens before and after the injection of heat killed *Yersinia* antigen as outlined in chapter three. Hyperimmune sera were raised against the heat stable antigen of *Y. pseudotuberculosis* serotype II and *Y. frederiksenii* (chapter three) and serum with agglutinating titres greater than 100 for the homologous antigen was achieved. Pre-inoculation sera or that collected from SPF chickens were used as the control, this sera did not agglutinate bacteria at a dilution of 1/10. Hyperimmune and control sera were stored in 1 ml aliquots at -20 °C until required. Thawed aliquots of both hyperimmune sera and pre-inoculation sera were heat treated at 56 °C for 30 minutes to remove complement activity. Serum was also

obtained from the specific pathogen free (SPF) flock at Wallaceville Animal Health Laboratory.

Preparation of yersiniae

See previous section (7-2-1)

Preparation of media and culture plates for the assay

Ten, 48 well plastic cell culture plates (Nunc, Denmark) were divided into two lots of five replicates and labelled appropriately. A template was drawn with exactly the same plan for each of the ten plates (figure, 7-1). Five plates were labelled for incubation at 28 °C and five for incubation at 37 °C.

A total of eight different media were prepared. Minimum Essential Medium Eagle (MEM, Sigma, M-0643) was used as a base for all the media preparations. The composition of each medium is summarised in Table (7-1). The control serum (S) used was chicken serum which contained transferrin (Tr) at a level of 6.31 g/l see chapter six; Table (6-6).

Table (7-1) Summary of the content of each medium prepared for the serum experiment

Medium (10 ml)	Content				
	Iron availability (IA)			Opsonin(O)	
	ID(mg)	STr	Si(mg)	Ab	C
MEM + Control S	0.00	+	0.00	-	+
MEM + Control(heated) S	0.00	+	0.00	-	-
MEM + Hyperimmune S	0.00	+	0.00	+	+
MEM + Hyperimmune(heated) S	0.00	+	0.00	+	-
MEM + Iron Dextran	1.00	-	0.00	-	-
MED + Iron Dextran + S	1.00	+	0.00	-	+
MEM + Desferal + S	1.00	+	1.00	-	+
MEM + Iron Dextran De + S	1.00	+	1.00	-	+

Ab = antibody; C = complement; ID = Iron dextran; STr = serum transferrin; Si = siderophore (Desferrioxamine).

Iron availability

A volume of 0.2 ml (10 mg) iron dextran (Imferon) was added to each 10 ml aliquot of culture medium. The basis of this calculation was for the iron dextran to be equivalent to the 10 mg dose given per 10 ml of blood given to the experimental chickens described in chapter six. A volume of 0.1 ml (10 mg) desferrioxamine (Desferal) was added to each 10 ml aliquot of culture medium based on similar criteria. Changes in pH were counteracted by the addition of buffered MEM + 10% SPF chicken serum to give a pH of 7.4 in each well.

Chicken serum

Serum was filter sterilised using a 0.2 micron filter (MFS, Microfiltration systems, USA) immediately prior to its use. Heat-treated serum and untreated immune and non-immune serum was added to sterile MEM to give a 10% preparation.

Procedure of the bacterial assay

Media preparations were added to wells as 0.9 ml aliquots using a sterile pipette. The media added to the plate were duplicated on each side as illustrated in figure (7-2). The plates were carefully prepared in a biological safety cabinet (Gelman Sciences, Australia) and covered with a sterile lid. When all the plates were prepared, 100 microlitres of bacterial suspension containing approximately 6.5×10^6 ($\pm 0.5 \times 10^6$) viable bacteria were added to each 1 ml well.

Figure (7-1) Template of assay wells containing modifications of minimum essential medium (MEM) such as the addition of serum (S), iron dextran (ID) or an exogenous siderophore (De). The serum added was either a control serum from an SPF chicken or hyperimmune serum from an immunised chicken (I), this was either heated (H), to destroy complement, or stored at 4 °C prior to use.

MEM +S	MEM+S (H)	MEM+S (I)	MEM+S (H:I)	MEM + ID	MEM + S + ID	MEM + S + De	MEM + S+IDDe
							Y.psII
							Y.psII
							Y.psII
							Y.fred
							Y.fred
							Y.fred

After the overnight incubation of five plates at 28 °C and the other five at 37 °C, serial 100 fold dilutions of the suspension in each well were plated on selective media (CIN) using a 10 microlitre loop. It had previously been determined that a viable colony count of approximately 2×10^8 yersiniae per ml was obtained following incubation of inoculated bacteria in tryptone water overnight at 28 °C and it was therefore calculated that an initial dilution of $1/10^4$ of the bacterial suspension from each of the wells would be appropriate to allow a countable number of colonies to be quantified visually. However, aliquots of $1/10^2$ and $1/10^3$ dilutions were also plated and, where counting was possible, a viable colony count was made.

Presentation of the results

The raw data for the colony counts of bacteria is listed in the appendices. Approximately 6.5×10^6 bacteria were added per well. Colony counts are given as the mean count for 5 plates, along with the standard deviation, and a two-way analysis of variance performed on each data set. The student's t test was used to evaluate differences between data sets.

Results

7-3-1 Calibration and standardisation of the phagocytosis assay

From Table (7-2) it can be seen that the scintillation counts obtained from the wells to which less than 10^4 bacteria had been added are not significantly different from those to which no bacteria were added ($P>0.05$) indicating there is a limitation to the sensitivity of the test. Concurrent plate counts indicated that a value of greater than 50 in the scintillation system was consistent with a viable colony count of greater than 10^3 bacteria, when yersiniae were exposed to the conditions of the test and with an incubation time of two hours. There was no significant difference in the scintillation counts of *Y. pseudotuberculosis* and *Y. frederiksenii* when the values for each titration were compared ($P>0.05$).

Table (7-2) A comparison of the scintillation counts recorded for titrations of Uracil-H³ labelled *Yersinia frederiksenii* and *Y. pseudotuberculosis* incubated for 2 hours in MEM at 37 °C.

	Number of bacteria added ($\log_{10}$)							Blank
	8	7	6	5	4	3	2	0
Y. ps	655*	872	356	145	84	35	38	33
Y. fred	754	875	352	153	82	41	36	41

* mean value for five replicates

There is a good correlation ($P < 0.01$) between the values obtained in the scintillation counter and the number of bacteria when added in the range of 10^4 to 10^7 per well.

The effect of saponin, iron and the uptake of Uracil-H³ on bacterial viability

The data presented in Table (7-2) is presented graphically in figure (7-2) along with data obtained from a similar experiment in which iron-dextran was added to the medium. Each point on the graph represents the mean scintillation count of five replicate wells, statistical analysis of the results can be found in appendix (A-14). The addition of iron-dextran to the medium resulted in a significant increase in the scintillation counts recorded for both *Y. frederiksenii* and *Y. pseudotuberculosis* ($P < 0.05$).

Figure (7-2) Standard curves showing the scintillation counts obtained following the addition of different numbers of yersiniae to wells of media with and without the addition of iron dextran and incubated for two hours with Uracil-H³.

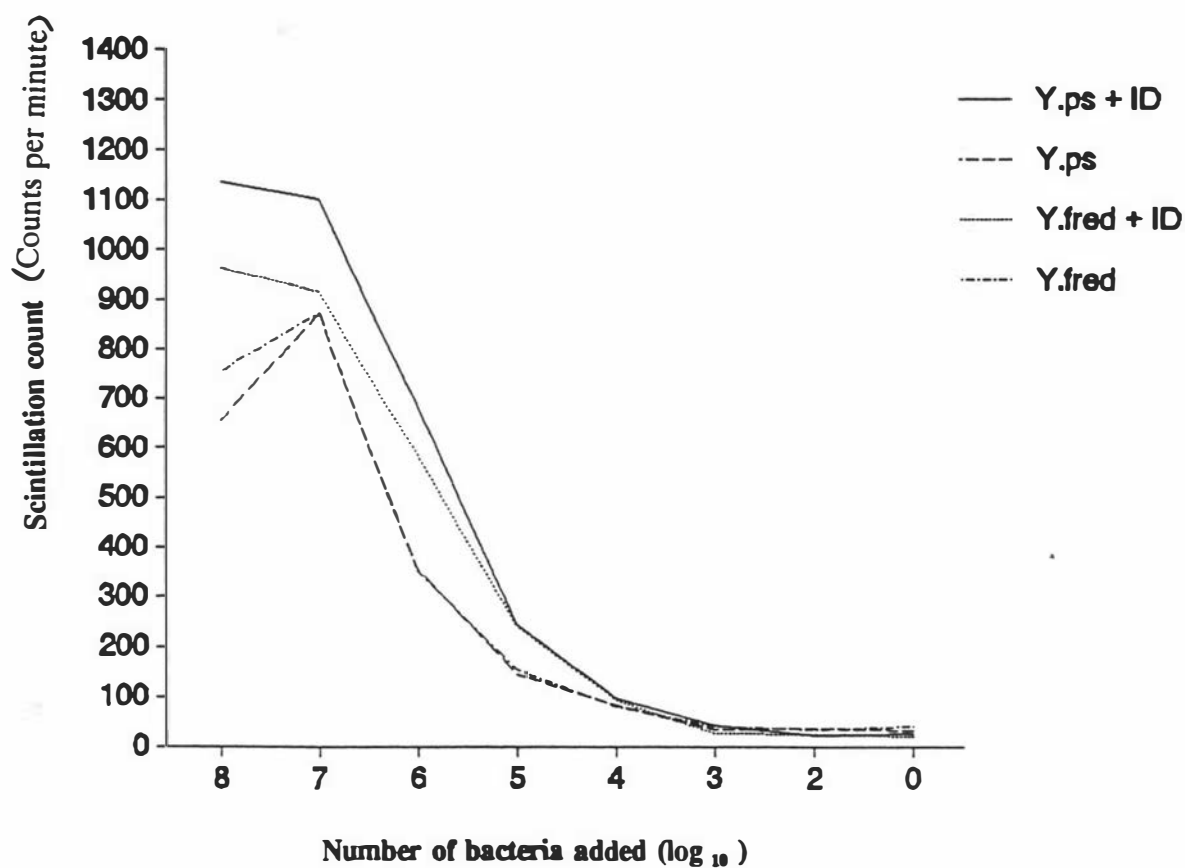


Table (7-3) A comparison of the scintillation counts obtained for titrated yersiniae incubated for 2 hours with radiolabelled Uracil-H³ in untreated medium, medium with saponin added and medium with iron dextran added

	Number of bacteria added (log ₁₀)			
	7	6	5	4
<i>Y. ps</i>	872*	356	145	84
<i>Y. fred</i>	875	352	153	82
	Saponin treated yersiniae			
<i>Y. ps</i>	870	356	142	80
<i>Y. fred</i>	856	350	156	89
	Iron dextran treated yersiniae			
<i>Y. ps</i>	1102	686	244	98
<i>Y. fred</i>	916	587	241	94

* Mean value of five replicates (counts per minute)

From the results in Table (7-3) it can be seen that there is no significant reduction in the scintillation counts obtained from saponin treated yersiniae compared with untreated yersiniae ($P>0.05$). This was verified by incubating yersiniae in media, with and without, saponin. Aliquots of the medium containing yersinia were plated on CIN agar and the number of colonies growing on the plate after 24 hours incubation, were counted. There was no significant difference in the number of colony forming units detected after 24 hours ($P>0.05$) between the saponin treated and untreated yersiniae.

Summary of results

The scintillation system using saponin to lyse phagocytic cells and an incubation time of two hours to allow bacterial uptake of labelled Uracil-H³ at 37 °C was found to be suitable for enumerating released intracellular yersiniae in the subsequent experiments. The addition of iron dextran to the medium resulted in a significant increase in the scintillation counts indicating that iron was a limiting factor in the replication of yersiniae under the conditions of the test at 37 °C. To avoid the complication of extracellular proliferation of yersiniae, the protocol was revised in subsequent assays of

intracellular yersiniae to include an extra washing step and the use of antibiotics to kill extracellular yersiniae prior to the lysis of phagocytic cells.

7-3-2 The survival and replication of yersiniae following phagocytosis by chick spleen macrophages *in vitro*.

Bacteria were added to macrophage cultures at a ratio of 5:1 giving about 2×10^6 yersiniae added per well. It was found that the number of intracellular bacteria released at the end of the experiment was related to the amount of time the phagocytic cells were incubated with the bacteria as shown in Table (7-4).

The effect of pretreating phagocytic cells with iron on the survival and replication of intracellular yersiniae.

When a two-way analysis of variance and the student's t test was used it was found that the scintillation counts were significantly higher ($P < 0.001$) following lysis of iron loaded phagocytic cells than following lysis of untreated phagocytic cells at all times of incubation with *Y. pseudotuberculosis*. This relative increase in the scintillation counts obtained for *Y. frederiksenii* released for iron loaded as compared with untreated phagocytic cells was less pronounced ($P < 0.05$). In addition, the scintillation count for released intracellular *Y. frederiksenii* following 60 minutes incubation was significantly less for iron loaded chick spleen phagocytes than for untreated phagocytes ($P < 0.05$).

The results are given in Table (7-4) and the statistical analysis of the results can be found in the appendix (A-14). There is some variability between replicates as indicated by the standard deviation but this was found to be less than the variability between treatment groups. In Table (7-4) the scintillation count recorded at time '0' represents the negative control value corresponding to the 'back ground' value obtained in the scintillation system when no yersiniae are present. The scintillation count obtained from the total number of yersiniae added to the system (1×10^7) is given at the top of the table for both *Y. pseudotuberculosis* and *Y. frederiksenii*. The scintillation counts obtained from 60 to 240 minutes incubation with iron loaded and untreated phagocytic cells, reflect the number of viable intracellular yersiniae released following lysis of phagocytic cells at those times. This is dependent on 1) the phagocytic capacity of the

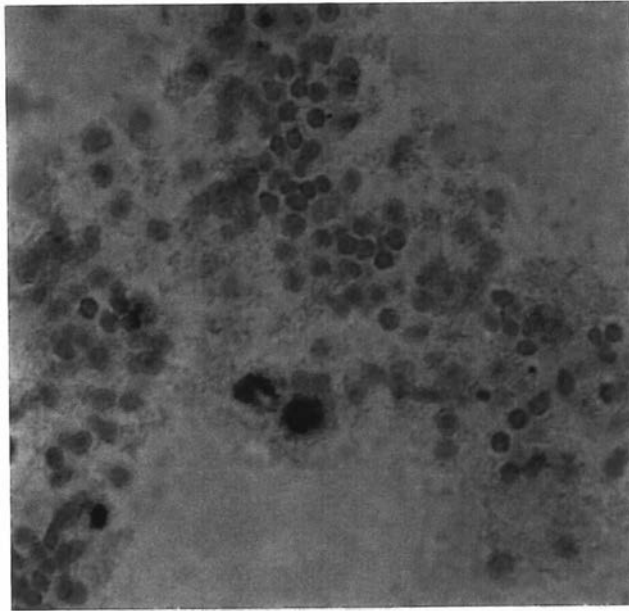
chick spleen cells 2) the bactericidal capacity of the chick spleen cells and 3) the net outcome of survival and replication of intracellular yersiniae. The appearance of iron loaded chick spleen phagocytes is illustrated in figure (7-4) following fixation and staining with Perls' iron stain on a slide.

Table (7-4) A comparison of the scintillation counts recorded for intracellular yersiniae released from iron treated and untreated chick spleen macrophages

Time *	<i>Y.pseudotuberculosis</i>		<i>Y.frederiksenii</i>	
	No iron	Iron	No iron	Iron
	659 +/- 89 **		640 +/- 43	
0 minutes	31 +/- 8	33 +/- 6	42 +/- 3	25 +/- 2
60 minutes	240 +/- 21	617 +/- 71	599 +/- 11	401 +/- 23
120 minutes	323 +/- 26	675 +/- 31	250 +/- 38	384 +/- 17
180 minutes	264 +/- 3	542 +/- 84	282 +/- 9	345 +/- 21
240 minutes	150 +/- 27	470 +/- 47	209 +/- 8	300 +/- 10

* Incubation time (macrophage cultures + bacteria; 37 °C) ; ** The initial value is the reading recorded for the total number of yersiniae added (10^7) prior to incubation. Each value represents the mean count for five replicates +/- standard deviation.

The scintillation counts for the number of intracellular *Y. pseudotuberculosis* bacteria released from iron loaded chick spleen macrophages as compared with untreated chick spleen macrophages following different periods of incubation is illustrated in figure (7-3)



7-4 Stained slides showing fixed chick spleen cells including macrophages following phagocytosis of iron dextran which was added to a suspension of chick spleen cells. Perls' iron stained section. x 1000.

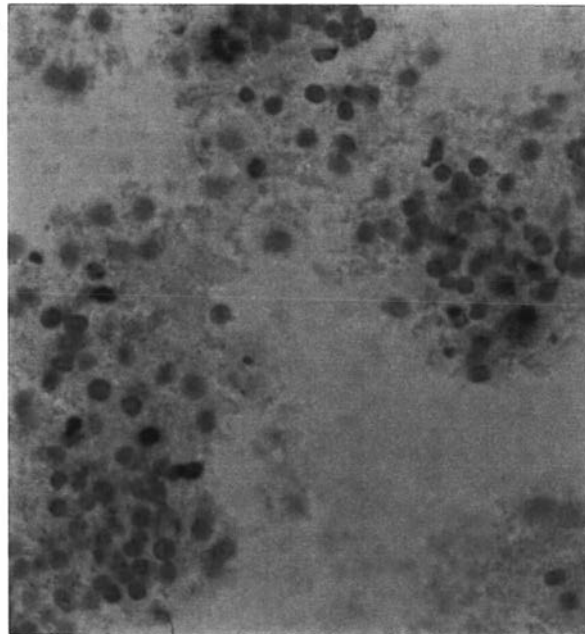
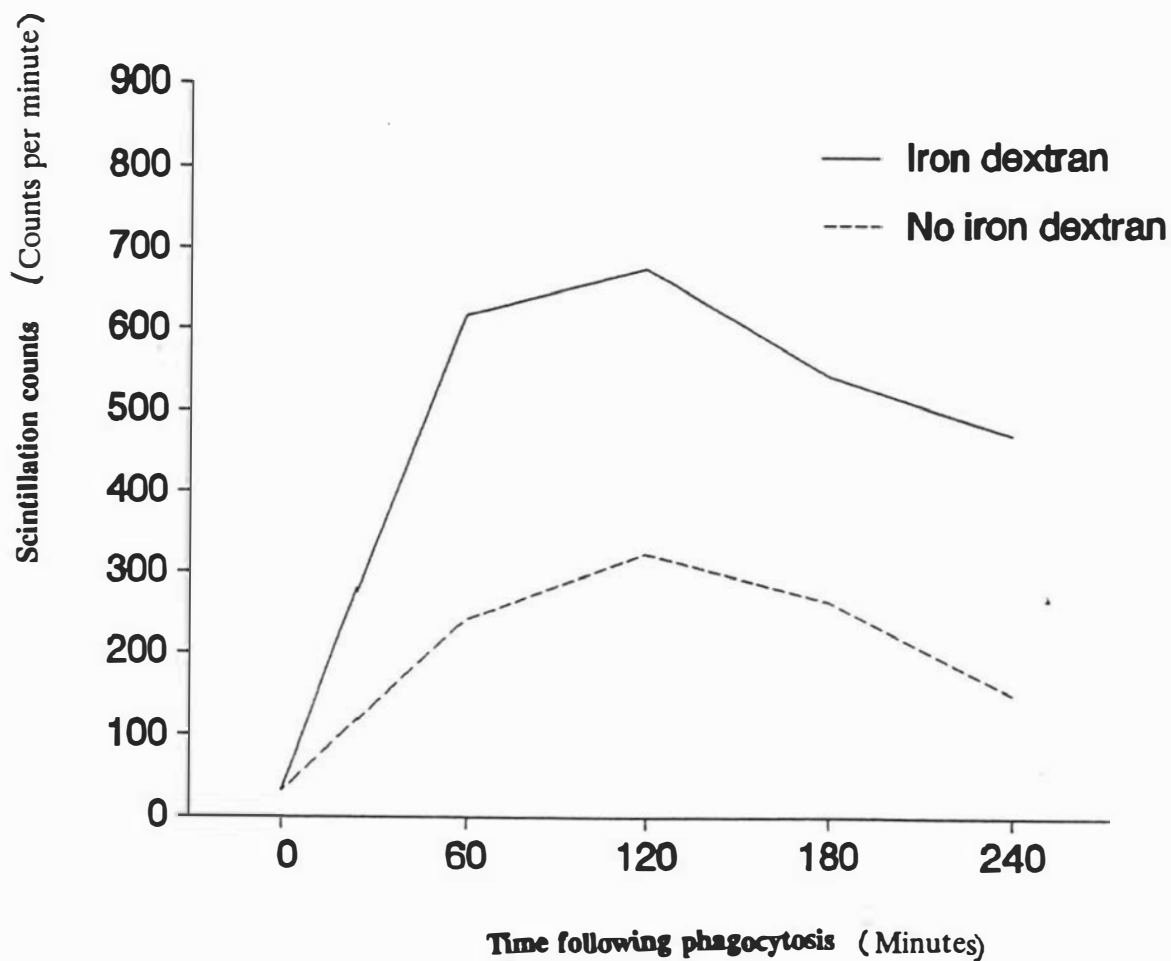


Figure (7-3) A comparison of the number of viable intracellular *Y. pseudotuberculosis* bacteria released following lysis of iron-loaded and untreated chick spleen macrophages as determined by the scintillation system.



Summary of results

The number of intracellular yersiniae released from chick spleen macrophages increases with the time of incubation and is greater when macrophages are loaded with iron dextran.

7-3-3 The growth of *Yersinia pseudotuberculosis* in serum under different conditions

The statistical evaluation of the serum assay data is available in appendix (A-15)

The effect of temperature on the assay results

From the results in Table (7-5) it can be seen that at 28 °C there was a mean value of 540 colony forming units derived from a 10 µl of a 10⁻⁴ dilution of MEM medium containing *Y. pseudotuberculosis* following 24 hr incubation. The colony count obtained following plating of an aliquot of medium containing *Y. pseudotuberculosis* was significantly less when the medium was incubated at 37 °C than when the same medium was incubated at 28 °C ($P < 0.001$). Observations of the cultures made under the phase contrast microscope demonstrated that the bacteria were less motile at 37 °C. The effect of altering the iron availability of the medium on the growth of *Y. pseudotuberculosis* was more marked when the incubation temperature was 28 °C than at 37 °C

The effect of serum, complement and antibody

The addition of serum to the medium reduces the colony counts at both the temperatures tested although this was only significantly different at 28 °C ($P < 0.05$). The addition of heated serum resulted in a smaller reduction in the colony count at both temperatures when compared with the addition of unheated control serum but again the difference was only significant at 28 °C ($P < 0.01$). Hyperimmune serum added to the medium resulted in a significant reduction in the colony count when the medium was incubated at both 37 °C ($P < 0.005$) and 28 °C ($P < 0.05$). The addition of heated hyperimmune serum to the medium also reduced the colony counts obtained following incubation at 28 °C and 37 °C but this was not significantly greater than the effect of control serum ($P > 0.05$)

The effect of iron availability

The addition of iron dextran to medium containing serum resulted in higher colony counts than those obtained from medium that had untreated serum added but the difference was not significant at 28 °C and only marginally significant at 37 °C ($P>0.05$). The addition of desferrioxamine to medium containing serum resulted in a significant reduction in the colony count at 37 °C ($P<0.001$) but not at 28 °C ($P>0.05$).

The addition of iron dextran to the medium containing control serum resulted in a greater colony count when compared with the addition of desferrioxamine at 37 °C ($P<0.005$) but not at 28 °C ($P>0.05$). At neither temperature did the addition of desferrioxamine and iron dextran in combination result in a significantly greater colony count than the addition of iron dextran alone.

Table (7-5) A comparison of the colony counts obtained from a 10 microlitre aliquot of *Yersinia pseudotuberculosis* following 24 hr incubation in different media at 28 °C and 37 °C plated as a 10^{-4} dilution

Medium	Colony forming units *	
	37 °C	28 °C
MEM + Serum	128* +/- 39	456 +/- 47
MEM + Serum (Heated)	140 +/- 42	469 +/- 35
MEM + Hyperimmune S	49 +/- 9	397 +/- 29
MEM +Hyperimmune(Heated) S	100 +/- 29	417 +/- 42
MEM + S + Iron Dextran	160 +/- 32	462 +/- 41
MEM +S+Desferal (Si)	54 +/- 12	424 +/- 42
MEM +S+IronDextran+De(Si)	162 +/- 35	466 +/- 40
MEM	168 +/- 53	540 +/- 47

* Mean count of five replicates representing the number of colonies on CIN media grown from a 10 µl loop of bacterial broth +/- standard deviation.

addition of a combination of desferrioxamine and iron dextran did not result in a significantly higher colony count than was obtained following the addition of iron dextran alone ($P>0.05$).

Table (7-6) A comparison of the colony counts obtained from a 10 microlitre aliquot of *Yersinia frederiksenii* following 24 hr incubation in different media at 28 °C and 37 °C plated as a 10^{-4} dilution

Medium	Colony forming units *	
	37 °C	28 °C
MEM + Serum	105 +/- 32	456 +/- 44
MEM + Serum (Heated)	162 +/- 31	454 +/- 34
MEM + Hyperimmune S	50 +/- 11	335 +/- 31
MEM +Hyperimmune(Heated)S	164 +/- 40	375 +/- 37
MEM +S+Iron Dextran	176 +/- 42	756 +/- 45
MEM +S+Desferal (Si)	98 +/- 31	486 +/- 45
MEM +S+Iron Dextran+De(Si)	152 +/- 41	743 +/- 48
MEM	231 +/- 47	850 +/- 52

* Mean count of five replicates representing the number of colonies on CIN media grown from a 10 µl loop of bacterial broth +/- standard deviation.

7-3-4 The growth of *Yersinia frederiksenii* in serum under different conditions

The statistical evaluation of the serum assay results is available in appendix (A-15)

The effect of temperature

From the results shown in Table (7-6) it can be seen that at 28 °C there was a mean value of 850 colony forming units derived from a 10 µl of a 10⁻⁴ dilution of the MEM media containing *Y. frederiksenii* following 24 hr incubation. There is a significant reduction in the colony count obtained when the medium was incubated at 37 °C ($P < 0.001$) compared with that obtained when the medium was incubated at 28 °C. Observations under the phase contrast microscope indicated that the bacteria were less motile at 37 °C. Increasing the iron availability of the medium enhanced the growth of *Y. frederiksenii* to a greater extent at 28 °C than at 37 °C. In contrast the inhibitory effect of immune serum on the growth of *Y. frederiksenii* is greater at 28 °C.

The effect of serum, complement and antibody

The addition of serum to the medium significantly reduced the colony counts at both temperatures but the difference was greater at 28 °C ($P < 0.001$) compared with 37 °C ($P < 0.01$). The addition of heated serum resulted in a smaller reduction in the colony count compared with the addition of control serum and the difference was only significant at 28 °C ($P < 0.01$). Hyperimmune serum added to the medium resulted in a reduction in the colony count at both temperatures with a greater reduction than with control serum at the equivalent temperature, 37 °C ($P < 0.001$) and 28 °C ($P < 0.01$). The colony counts obtained after the addition of heated hyperimmune serum to the medium were not significantly different from those obtained following the addition of heated control serum at 37 °C but they were significantly lower when incubated at 28 °C.

The effect of iron availability

The addition of iron dextran to medium containing serum resulted in higher colony counts when media were incubated at either 28 °C or 37 °C ($P > 0.05$). The addition of iron dextran resulted in a greater increase in colony count than did the addition of desferrioxamine to the medium at 37 °C ($P < 0.01$) and at 28 °C ($P < 0.001$). The

Summary of the results

A-Phagocytosis assay

- i) Splenic phagocytes derived from chickens are able to phagocytose iron dextran (figure, 7-4)
- ii) *Yersinia pseudotuberculosis* and *Y. frederiksenii* are able to survive intracellularly following phagocytosis by chicken macrophages.
- iii) The survival and/or replication of intracellular yersiniae is enhanced when phagocytic cells are loaded with iron.

B-Serum assay

- i) The growth of yersiniae is inhibited by the presence of chicken serum in the medium, this effect is more marked for *Y. frederiksenii* than *Y. pseudotuberculosis* and is reduced by the addition of iron dextran to the medium.
- ii) The survival and replication of extracellular yersiniae is decreased by an elevation in temperature and the presence of immune serum. When hyperimmune serum was heated to remove complement the inhibitory effect is greatly decreased.
- iii) The addition of desferrioxamine has no effect on the growth of *Y. pseudotuberculosis* in the serum assay but the addition of iron dextran resulted in an increase in growth at 37 °C.

Discussion

7-4-1 Phagocytosis assay

In the present study it was found that pretreating chicken phagocytic cells with iron dextran resulted in an increase in the number of intracellular yersiniae released following cell lysis. In this regard the *in vitro* results for yersiniae and chicken phagocyte systems are similar to those derived from systems designed to examine the effect of iron on the interaction between mammalian phagocytic cells and salmonellae (Dunlap *et al.*, 1993), *Listeria* (Campbell, 1993), *Corynebacterium spp* (Zink *et al.*, 1985) and other bacteria (Finkelstein *et al.*, 1983; Griffiths, 1993).

In the scintillation system the final number of bacteria released from the cells is determined by 1) the number of bacteria phagocytosed, 2) the number of bacteria surviving the bactericidal mechanisms of the phagocytic cell and 3) the ability of the bacteria to multiply intracellularly. A limitation of this system is the failure to indicate of the extent to which the increased number of released bacteria is due to intracellular replication of the bacteria or the impaired bactericidal capacity of the phagocytic cell.

The relative effect of iron in suppressing the bactericidal capacity of the phagocyte compared with an enhancement of bacterial survival and replication is difficult to determine and requires an accurate measurement of the stored iron in the bacteria prior to the assay, the level of iron in the bactericidal enzymes and the optimum conditions for phagocytic efficiency. At the present time, even the most elaborate experiments have failed to explain the exact mechanism by which intracellular iron enhances survival of potentially pathogenic bacteria (Zink *et al.*, 1985).

7-4-2 Serum resistance

Complement

Hanski *et al.* (1991) found that pretreatment of mice with thiouracil to destroy complement had no effect on the course of *Y. enterocolitica* 0.8 infection. In addition, it was noted that *in vitro*, mouse serum had no effect on the growth of either plasmid-positive or plasmid-negative strains of *Yersinia* whereas human serum was inhibitory to

both and especially the plasmid negative strain (Hanski *et al.*, 1991). In the present experiment chicken serum inhibited the growth of both *Y. frederiksenii* and *Y. pseudotuberculosis* when grown *in vitro* at 37 °C but the relative reduction in colony count is greater for *Y. frederiksenii*.

From this study it is apparent that the presence of chicken serum significantly inhibits the growth of *Y. frederiksenii in vitro* even at 28 °C, this is similar to that which is found for other non-pathogenic bacteria (Kluger & Rothenberg, 1979; Perry & Brubaker, 1979; Robins-Browne *et al.*, 1991). In contrast the presence of serum did not have a significant effect on the growth of *Y. pseudotuberculosis* at 28 °C unless hyperimmune serum was used. The growth of *Y. pseudotuberculosis* was inhibited when serum was added to the medium at 37 °C. The removal of complement, by heating the serum at 56 °C for 30 minutes, reduced this inhibitory effect but not to a significant extent. Heating hyperimmune serum significantly reduced the inhibitory effect of the addition of immune serum on the growth of *Y. pseudotuberculosis* at 37 °C but not at 28 °C. This finding was similar but less significant for *Y. frederiksenii* and may indicate that, at body temperature, complement has a synergistic effect enhancing the ability of antibody to inhibit the proliferation of extracellular yersiniae.

Temperature

Temperature also has an important effect on the ability of yersiniae to multiply. In both pathogenic and non-pathogenic yersiniae, motility and growth are inhibited at 37 °C. This has been noted previously (Wake *et al.*, 1975) and would appear to be an important determinant in the long term survival of the organism *in vitro* and presumably in the host (Jones *et al.*, 1977; Keown *et al.*, 1983; Keusch, 1990).

Iron availability

In vivo, the availability of iron in the serum of the host is normally very limited but will be elevated where serum transferrin is saturated as in iron overload or haemolytic disease (Neilands, 1981; Bullen, 1981; Mellencamp *et al.*, 1981). Serum iron is reduced in hyperimmune chicken serum (Butler *et al.*, 1973). It is known that non-pathogenic yersiniae are able to utilise environmental sources of iron through a variety of iron

uptake systems and many of the non-pathogenic environmental yersiniae are now known to produce siderophores such as aerobactin (Stuart *et al.*, 1986). Until recently, it had been thought that the pathogenic yersiniae were unable to produce siderophores although an iron uptake gene had been identified which was thought to be associated with virulence in *Y. enterocolitica* and possibly *Y. pseudotuberculosis*. Although the exact mechanism by which this acts is still not fully understood the association between the ability to acquire iron and virulence in yersiniae is becoming well established (Sikkema & Brubaker, 1989; Heeseman *et al.*, 1993; Robins-Browne *et al.*, 1991). In the present study it was found that the growth of *Y. frederiksenii* was inhibited in normal chicken serum and that this effect was reversed following the addition of iron to the medium, this effect was most marked at 28 °C. The growth of *Y. pseudotuberculosis* was not significantly inhibited in chicken serum at 28 °C but the inhibitory effect at 37 °C was reversed by the addition of iron dextran. Research into the effect of hyperimmune serum on the growth of *Pasteurella*, and other bacteria, found that the inhibitory effect of serum and hyperimmune serum could be partially reversed by the addition of iron dextran to the medium indicating that iron may be an important limiting factor to the growth of bacteria in serum (Bullen & Rogers, 1967; Bullen *et al.*, 1974; Smith *et al.*, 1981; Brock & Ng, 1983). The results of the present study support this view but also indicate that the relative effect depends on the temperature of incubation and the genotype of the bacterium.

It is not known if the *Y. pseudotuberculosis* strain used in the present study is representative of the species but it was chosen because it was the same isolate used for the *in vivo* studies described in chapter six. It was considered important to compare the effect of iron *in vitro* on this pathogenic strain with the effect of iron *in vitro* on a strain that was non-pathogenic under field conditions. Although it would have been interesting to compare a plasmid-positive and plasmid-negative strain of *Y. pseudotuberculosis*, it was apparent that factors other than the carriage of a plasmid were important in determining differences in iron metabolism and pathogenesis *in vivo* (Heeseman *et al.*, 1993; de Almeida *et al.*, 1992). *Yersinia frederiksenii* was chosen as the non-pathogenic strain because it was found to be common in the environment and in the faeces of apparently healthy wild birds.

It appears that it is the organisms ability to acquire iron from a given medium rather

than the absolute amount present which determines the growth of yersiniae in any particular environment. The bacteriostatic effect of chicken serum on *Yersinia pseudotuberculosis* appears to be less dependent on the limitation of iron than for *Y. frederiksenii*. From the *in vitro* experiments it appears that *Y. pseudotuberculosis* is probably able to acquire sufficient iron from normal serum by competing with serum transferrin. This would possibly explain the reason why chickens not treated with exogenous iron dextran still developed systemic lesions when challenged with *Y. pseudotuberculosis*. It is probable that systemic spread would not have occurred if the birds had been challenged with *Y. frederiksenii*. In this regard, the outcome of the *in vitro* experiment is in agreement with the predictions of Robins-Browne & Prpic (1985). The addition of desferrioxamine to the medium did not appear to enhance the growth of the strains of yersiniae used in the present study although it has been reported that some strains of *Y. pseudotuberculosis* and *Y. enterocolitica* can utilise exogenous siderophores including desferrioxamine (Stuart *et al.*, 1986). The results of the present study indicated that the addition of iron dextran and desferrioxamine to medium containing chicken serum increased the growth of two strains of yersiniae but that the effect was not significantly greater than when iron dextran alone was added. Desferrioxamine added alone to the medium appeared to inhibit the growth of *Y. pseudotuberculosis* especially at 37 °C. The reason for this inhibitory was not clear although it may indicate that the available iron is chelated into a form which the particular strain of *Y. pseudotuberculosis* used for the experiment was unable to use. There may be differences between strains of *Y. pseudotuberculosis* in this regard although other workers have also failed to demonstrate the ability of *Y. pseudotuberculosis* to utilise some siderophores (Wake & Morgan, 1986; Chambers & Sokol, 1994).

At the present time it is not known whether there are species specific strains of *Yersinia pseudotuberculosis* (de Almeida *et al.*, 1993). If such strains do exist they may vary in their ability to acquire iron from serum proteins of different host species as is the case with *Neisseria* (Griffiths, 1993). In addition it is probable that the ability of *Y. pseudotuberculosis* to acquire iron from chicken serum would depend on the total iron binding capacity of the serum which, as discussed in chapter six, alters during the course of infection. It is possible that during the acute-phase inflammatory response, the hypoferraemia associated with a rise in serum transferrin, would reduce the ability of

Y. pseudotuberculosis to acquire iron extracellularly. This would explain the initial potentiation of disease *in vivo* in the iron-dextran-treated chickens. The relative effect of an increase in serum opsonins and a reduction in serum iron, as factors inhibiting the growth of yersiniae in immune serum would be difficult to determine.

7-4-3 *In vivo* application

The difference in the ability of the pathogenic and the non-pathogenic strains of yersiniae to survive intracellularly *in vitro* was less than would be expected from their behaviour *in vivo*. This is possibly due to the fact that *in vivo*, pathogenic yersiniae express virulence factors such as YOPs which are only released following the intracellular stage and are important in systemic spread (Fukushima *et al.*, 1991; Autenreith *et al.*, 1991). *In vivo*, virulent yersiniae are thought to express virulence characteristics following calcium deprivation and elevated temperature as occurs after cell passage from the intestinal M-cells prior to entry into the host's systemic circulation (Robins-Browne *et al.*, 1987). Hanski *et al.* (1991) found that plasmid-containing *Yersinia enterocolitica* 0.8 were more virulent than plasmidless strains when inoculated into the Peyer's patches of mice, than when introduced intraperitoneally. This finding that intracellular signals may be important in the expression of full virulence potential in yersiniae. *In vivo*, *Y. pseudotuberculosis* would express YOPs following entry into the host whereas *Y. frederiksenii* would not, consequently the limited difference between the two species observed in the *in vitro* phagocytosis assay may be an underestimate of the expected differences *in vivo*. For example, the *in vitro* phagocytosis assay in the present study does not allow expression of variables such as resistance to phagocytosis which, in virulent yersiniae, is associated with the expression of some YOPs (Simonet *et al.*, 1990; Straley *et al.*, 1993). Fukushima *et al.* (1991) suggest that the extracellular phase of infections may be of special importance in infection with *Y. pseudotuberculosis* which may spread extracellularly following initial invasion and intracellular multiplication in the intestinal lymphoid tissue. In contrast, *Y. frederiksenii* would be readily phagocytosed and bacterial multiplication limited early in the course of infection unless intracellular iron favoured intracellular multiplication and an increase in the number of bacteria sufficient to overcome phagocytic capacity. This suggestion is consistent with the results of the present study which showed that the bacteriostatic effect of serum was more marked for *Y. frederiksenii* than for *Y. pseudotuberculosis*.

The chick spleen cells used for the assay were obtained from birds which were 4 to 20 days old. Kodoma *et al.* (1976) found that although splenic macrophages were weakly bactericidal to *S. pullorum* at hatching, this property increased with age. The results of the present study indicate that the phagocytic cell populations derived from the chicks were able to recognise and phagocytose bacteria and were also able to readily assimilate and store iron as illustrated in combined Gram-Prussian blue stained slides of cultured cells. In each of the assays the cells which were loaded with iron were incubated with the iron dextran for 2 hours and culture plates were thoroughly washed several times prior to the addition of the bacteria 24 hours later. Earlier experiments in which the yersiniae were incubated with iron dextran prior to phagocytosis indicated that there was no significant difference in scintillation counts obtained from intracellular yersiniae following release from chick spleen phagocytes ($P > 0.05$; data not shown). From this result it was determined that the intracellular iron in the phagocytic cells was responsible for the observed differences in intracellular replication and survival, rather than any effect of stored iron in the yersiniae studied. In addition the experimental protocol used was designed to examine the effect of iron loading chicken macrophages on the survival of yersiniae, the treatment and source of the yersiniae used was the same in all the *in vitro* studies therefore it is unlikely that the iron content of the bacteria would have been a significant variable in the outcome of the assay.

Experiments using *Corynebacterium equi* (*Rhodococcus equi*) (Zink *et al.*, 1985) indicated that intra-bacterial stored iron was utilised by the non-pathogenic bacteria when an exogenous siderophore such as desferrioxamine was added but that virulent bacteria could utilise intra-bacterial iron without the supply of a siderophore. This may be an important factor in the reduction of the LD 50 of non-pathogenic yersiniae *in vivo* when desferal, with or without iron dextran, is given parenterally to mice. This has been shown for strains of *Y. enterocolitica* and *Y. frederiksenii* but not for *Y. pseudotuberculosis* (Robins-Browne *et al.*, 1987). The results of this present *in vitro* study suggest that it is probable that *Y. pseudotuberculosis* is able to compete with the host for iron using iron acquisition mechanisms which are associated with virulence in the species.

Within the limitations of the *in vitro* studies outlined in this chapter, it has been possible to answer the questions posed at the end of chapter six. It is clear that an increased pool of iron in phagocytic cells does enhance the ability of both pathogenic and non-

pathogenic intracellular yersiniae to survive and replicate *in vitro*. It is also apparent that an increased availability of iron in serum increases the number of yersiniae which are able to survive and replicate. However, the most significant effect in the serum study appears to be inhibition of bacterial growth at 37 °C. In a pathogen which causes disease in homeothermic species which have a high body temperature, this may at first appear surprising. However, as discussed by Cornelis (1992), an increase in temperature is one of the factors thought to trigger the expression of virulence in pathogenic yersiniae. In agreement with other studies on the subject (Cornelis *et al.*, 1987; Robins-Browne *et al.*, 1987; Hanski *et al.*, 1991) the results of the present study would support the hypothesis that the availability of iron is an important factor in deciding the outcome of host-pathogen interactions but that the relationship is complex. The relative effect of iron availability as compared with other important variables which may upset the balance between host and pathogen is often difficult to establish but should always be considered.

Summary

Phagocytosis assay

- 1) Loading chicken spleen cell cultures with iron dextran results in the presence of stainable iron within phagocytic cells, therefore chicken phagocytic cells are able to phagocytose iron dextran *in vitro* when grown in MEM and chicken serum.
- 2) The beta plate scintillation system was calibrated for enumerating intracellular yersiniae following release from lysed phagocytic cell. The system was suitable for this purpose when used within the criteria set down in the protocol described.
- 3) Iron-loading of chicken macrophages prior to the addition of bacteria to the medium resulted in an increased number of intracellular yersiniae present at the end of the assay, therefore the presence of iron favoured the intracellular survival of yersiniae. In this regard iron loaded chicken macrophages are similar to mammalian macrophages.

Serum assay

- 4) The *in vitro* growth of yersiniae was reduced by factors such as incubation in medium at 37 °C, the presence of complement and the addition of hyperimmune serum
- 5) Chicken serum inhibited the growth of *Y. frederiksenii* in medium but had less affect on *Y. pseudotuberculosis*. Addition of iron dextran to the serum enhanced the growth of *Y. frederiksenii* but not *Y. pseudotuberculosis* possibly indicating that *Y. pseudotuberculosis* was able to obtain sufficient iron from chicken serum.
- 6) Iron acquisition is an important virulence characteristic of *Yersinia pseudotuberculosis*.

Conclusion

Despite the limitations of the *in vitro* system, it was determined that 1) iron-loading of phagocytic cells resulted in the increased survival of intracellular yersiniae and 2) that in normal chicken serum *Y. pseudotuberculosis* was able to acquire sufficient iron for growth but that *Y. frederiksenii* required added iron for growth.

The *in vitro* studies support the hypothesis that iron availability is an important factor in determining the outcome of host-pathogen interactions. The *in vivo* studies indicate that, while the host requires sufficient iron for immune function, too much iron may impair both the specific and non-specific host defences. This was well illustrated in the chicken model. The pathogen requires sufficient iron for growth and different bacteria have developed mechanisms to acquire iron *in vivo*.

Although the low iron reserves of the chicken suggest that it is possibly not representative of other species it is still probable that the concepts developed in this work are more generally applicable.

CHAPTER EIGHT

General Discussion

The aim of this study was to evaluate a range of possible interactions between the host, the pathogen and the environment with particular reference to *Y. pseudotuberculosis*. It was hoped that consideration of these interactions could be used to predict the outcome of infection. Due to the complexity of the interactions involved in host-pathogen interactions the experimental aspects of the study were confined to an examination of specific aspects identified as being important in the retrospective and epidemiological studies. For example, the observed increase in the prevalence of sporadic cases and outbreaks of pseudotuberculosis following cool, wet weather may have been the result of an increased survival rate of psychrophilic *Yersinia pseudotuberculosis* in the environment, with a consequent increased challenge to the host. Alternatively, it may have been due to the increased susceptibility of the host to disease associated with starvation or chilling in cooler months. As it was likely that both factors influenced the outcome of the disease in birds, the epidemiological study was carried out in conjunction with retrospective and experimental studies which identified some of the factors thought to predispose birds to the development of pseudotuberculosis.

During the specific study of avian pseudotuberculosis, general protocols were developed which could be applicable to the study of other avian diseases. Much of the data generated in the present research project resulted from the application of specifically developed methods derived from experimental work with the domestic fowl. These were used to assist in the interpretation of a large amount of retrospective and field data gathered from wild avian species. Difficulty in collecting sample material and the fact that base line data are often unavailable for avian species has previously imposed limitations on studies of wild or rare birds. New techniques described in this thesis allow a more meaningful interpretation of results derived from retrospective material and a more quantitative evaluation of field data collected from wild birds. Internationally, there is an increasing awareness amongst biologists of the need to gather information on the diseases of wild and rare avian species. It is anticipated that many

of the protocols derived from this study will find application in future investigations of diseases in wild birds.

Although each chapter in this thesis includes an independent discussion of the results, it was considered appropriate to reconsider the relative importance of each component of the thesis. In order to make the discussion easier to follow it is broken up into six sections and concludes with suggestions for future research.

8-1 The role of wild birds and the environment in the epidemiology of *Yersinia pseudotuberculosis*

Bacteriological study

From the results of this study it is apparent that, although wild birds are continually exposed to yersiniae in the environment, the prevalence of pathogenic yersiniae in faecal samples taken from these birds is low. The prevalence of other yersiniae in the samples collected was similar to that found by other investigators (Brittingham *et al.*, 1985; Hamasaki *et al.*, 1989; Levre *et al.*, 1989; Fukushima *et al.*, 1991; Fukushima & Gomyoda, 1991). Intermittent shedding or latent carriage of *Y. pseudotuberculosis* could explain the low rate of isolation of the organism in these studies. However, in the present study, this seems unlikely in consideration of the large number of birds sampled and the consistently low number of isolates recovered throughout the year.

In order to be most useful, bacteriological surveys of wildlife diseases should include concurrent sampling of the species of interest, other species and the environment in the locations chosen for the survey. The sampling techniques should be consistent throughout the survey and although from the present study it would appear that necropsy is the most sensitive technique, faecal sampling does provide representative results for determining the faecal flora of wild bird populations. Cloacal samples are less reliable due to the small amount of faecal material collected and the reduced success in culturing the organism. The advantage of taking cloacal samples is that the identity of the bird is known and the results of culture can be correlated with blood samples taken from the same individual.

Yersinia pseudotuberculosis has been isolated from apparently healthy wild birds (Mackintosh & Henderson, 1984; Altman, 1986; Kaneuchi *et al*, 1989) and clinically affected birds can shed large numbers of the organism resulting in environmental contamination (Stovell, 1963; Altman, 1986) however, this may not be true for all species of bird. The higher prevalence of *Y. pseudotuberculosis* in some columbiforms (appendix A-9) supports the view that some species of bird may be important in the epidemiology of the disease as proposed by Altman (1986) and indicates that general statements referring to 'wild birds' in general are inappropriate in epidemiological studies. The low prevalence of *Y. pseudotuberculosis* in the wild birds surveyed in the present study suggests that these populations would need continual reinfection from environmental sources to maintain *Y. pseudotuberculosis* in the enteric flora. However, it is not known how sensitive bacteriological methods are for detecting the carriage of organisms. The low prevalence of *Y. pseudotuberculosis* in this and in other studies reviewed could be an underestimate of the true prevalence. This has been shown for salmonellae (Barrow, 1992). For this reason it was decided to develop a serological screening test to assess exposure to serotypes of *Y. pseudotuberculosis* in wild birds.

The use of a lipopolysaccharide-based ELISA for serological screening

The development of a serological test for screening wild birds for the presence of antibody to three common serotypes of *Y. pseudotuberculosis* (I,II & III) followed the development of a disc ELISA test for chickens. The application of this test to wild birds was difficult in practice due to the need to increase its specificity without a significant loss of sensitivity and to determine whether or not it was capable of detecting antibody in the small amounts of serum which can be collected from some avian species. The lack of commercial species-specific conjugates, the wide degree of cross-reactivity between yersiniae and other enteric bacteria in these birds and the difficulty of obtaining known positive and negative serum samples from species other than the domestic fowl makes the evaluation of a field trial difficult. The 'indirect' LPS ELISA test was found to be sensitive and specific when used for monitoring the serological response of chickens following experimental oral challenge with *Y. pseudotuberculosis*.

The use of serological screening in the present study indicated that there was a very high prevalence of seropositive birds. This, coupled with a low prevalence of faecal isolates

from the wild birds sampled, supports the hypothesis that wild birds are continually exposed to *Y. pseudotuberculosis* but do not readily shed the bacteria for prolonged periods of time. Although the limitations of screening tests in the field should be taken into consideration, the value of the data should not be dismissed. Similar limitations exist for other serological tests developed for enteric bacteria and these tests are widely used (Barrow *et al.*, 1992; Barrow, 1993). In both the agglutination tests and the more recently developed ELISA tests used for screening chickens for salmonellae, it is well recognised that although these tests are not accurate for assessing the *Salmonella* status of an individual bird they can give an indication of the *Salmonella* status of chickens at the population level (Cooper *et al.*, 1989; Barrow, 1992). This is probably also true for the serological test developed in this study with regard to *Y. pseudotuberculosis*. It is unlikely that these tests could be improved using the technology currently available (Barrow, 1992). This is because some aspects of bacterial serology have intrinsic problems associated with similarities in antigen structure between bacterial species (Taguchi *et al.*, 1991). Concurrent serological tests which use both LPS and genus-specific virulence associated characteristics, for example *Yersinia* outer membrane proteins (YOPs), may partially overcome the problem of cross-reactivity, (Fernandez-Lagos *et al.*, 1991; Robins-Browne *et al.*, 1993; Maki-Ikola *et al.*, 1991). However, the application of these multiple tests to samples collected from wild birds has some limitations due to 1) the small amount of blood which can be collected from small wild birds and 2) the lack of information available on the extent and duration of the serological response of birds to YOPs during infection. Further research directed at the application of serological screening for bacterial diseases in wild birds should concentrate on the development of species-specific conjugates for use in the ELISA test. In addition experimental evaluation of the serological responses of species other than chickens to different antigens is necessary.

8-2 Disease, immune mediators and serological response

In the present study, during the production of hyperimmune serum for reference positive control sera, it was found that, 1) injections of crude, heat-killed antigen resulted in low serological titres for LPS and 2) purified proteinase-K digests of LPS resulted in very low titres in the chicken. Results from immunoblot studies indicated that the difference in antigenicity was associated with the reduction in the protein

content of the purified LPS. There are no published reports on the serological response of chickens to oral infection with *Y. pseudotuberculosis* although it is well recognised that chickens produce significantly lower serological titres to parenteral heat killed preparations of yersiniae (Wetzler, 1965) and salmonellae (Cooper *et al.*, 1993; Brito *et al.*, 1993) than do rabbits when used for the production of hyperimmune sera.

It is accepted that LPS, as a component of endotoxin, is an important mediator in the development of endotoxic shock and can result in the death of some susceptible species (Congleton & Wagner, 1993). During bacterial infections in mammals it is known that the acute phase inflammatory response coincides with the release of cytokines, interleukin-1 and tumour necrosis factor in response to the release of Lipid A from Gram negative bacteria (Evans *et al.*, 1993). Although not all the mechanisms involved are fully understood, there is evidence to suggest that an exaggerated response to endotoxin by the host can be fatal. This is illustrated by the fact that humans experiencing endotoxic shock may be successfully treated by neutralising the endotoxin or by giving exogenous antibody which is directed against inflammatory proteins (Bone, 1993). The exact mechanisms involved in this response in birds are not known but it is thought that cytokines are also involved in avian species (Higgins *et al.*, 1993).

In view of the fact that in-bred lines of chicken do not appear to be as susceptible to some bacterial diseases as are some other avian species (Garren & Hill, 1959; Alder, 1993), it would be interesting to examine whether or not the degree of the immune response of passeriforms, columbiforms and psittaciforms to LPS is related to susceptibility to the endotoxin-mediated effects of *Y. pseudotuberculosis*. The possible effect of genotype on the nature and extent of the immune response to endotoxin was not evaluated in this study but has been evaluated in recent work on breed differences in chickens with regard to the carriage of salmonellae and the difficulty in identifying carriers (Barrow, 1992; Cooper *et al.*, 1993).

Serological studies of chickens experimentally infected with *Y. pseudotuberculosis* indicated that there was a dose-dependent serological response to lipopolysaccharide. This was demonstrated in birds orally infected with a single dose of *Y. pseudotuberculosis*. The birds sero-converted by day four, reached a maximum titre by the sixth day and levelled off at day ten. This is similar to the serological response

reported by Barrow *et al.* (1992) for experimental *Salmonella* infections in chickens.

The influence of the host genotype in determining the outcome of a disease and how host factors determine the course of a disease is an area of research which has raised more interest in the last few years and will probably attain a greater importance as more is known about immunity in different species (Vidal *et al.*, 1993; Collins, 1993). Another area of research which has attained a higher profile in recent scientific literature is the role of iron metabolism in determining the course of infectious disease (Griffiths, 1993). The importance of the acute-phase inflammatory response in the early stages of infection has already been discussed. Fever and hypoferraemia associated with this host response are thought to limit the ability of the invading pathogen to survive extracellularly. In many infectious diseases, including pseudotuberculosis, the causative agent has developed virulence characteristics to overcome the iron restriction imposed by the host (Litwin & Calderwood, 1993). The remainder of this thesis concentrated on developing an avian model for this disease based on information derived from retrospective studies and from the experimental literature already available.

8-3 Pseudotuberculosis, the disease and predictive models

Pseudotuberculosis, in humans, is a disease which becomes systemic only in patients with a concurrent debilitating disease. Many of the diseases implicated are associated with an alteration in the levels of serum iron and iron carrying proteins and include thalassaemia and haemochromatosis (Rabson *et al.*, 1975; Robins-Browne *et al.*, 1979, 1987; Melby *et al.*, 1982).

With the aim of determining the significance of the effect of iron on the outcome of pseudotuberculosis in birds, it was necessary to take into consideration 1) the conflicting views on the role of iron availability in the course of infectious disease in the chicken, 2) the apparent association between haemosiderosis and infectious disease in birds and 3) the importance of iron acquisition in the virulence of yersiniae. Before the experimental results and retrospective studies in this thesis are discussed it is important to note that much of the data have been generated alongside recent developments in molecular biology.

Disease and predictive models

There has been a lack of agreement among scientists with regard to the relative importance of potential virulence characteristics of pathogenic bacteria. Many animal disease models have been disappointing when used to predict the outcome of clinical disease in people (Chandra, 1990; Griffiths, 1993). Much of the problem associated with drawing conclusions from some of the *in vivo* studies has arisen through the use of a mode of transmission which does not reflect the natural situation. This posed particular problems when it came to assessing the virulence potential of *Y. pseudotuberculosis* because most of the research on the pathogenesis of yersiniae has been carried out in rodents using *Y. enterocolitica* to study enteric disease or *Y. pestis* to study systemic disease. Groups working on *Y. enterocolitica* have drawn their conclusions from work based on oral or parenteral transmission, and those working on *Y. pestis* have drawn their conclusions from parenteral transmission experiments. There have been comparisons made, based on retrospective studies, between *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* but very little experimental work has been directed specifically at *Y. pseudotuberculosis* (Vessilnova *et al.*, 1992) except when injected parenterally as a model for *Y. pestis* research (Wake & Morgan, 1976). It is apparent that *Y. pseudotuberculosis* shares characteristics with both *Y. pestis* and *Y. enterocolitica* and, although it is phenotypically similar to the latter when associated with enteric disease, its closer genetic similarity to *Y. pestis* is manifest during systemic infection. This is especially apparent in debilitated hosts or following parenteral transmission (Robins-Browne *et al.*, 1989).

The limitations of many disease models may partially explain why the *Yersinia* model, of host-parasite relationships, developed by Wake & Morgan (1986) has never gained the wide acceptance it deserves. Although the complexity of their model may limit its widespread use, the information obtained from this work with regard to the interaction between iron and *Y. pestis* has provided a valuable resource for the present study. When the expression of virulence in *Y. pestis* mutants, under conditions of iron limitation and iron supplementation was examined, it became apparent that virulence expression in *Y. pseudotuberculosis* may be more dissimilar to *Y. enterocolitica in vivo* than was previously thought. This is because *in vivo*, other factors often mask the difference. Consequently, experiments interpreting 'end-point' results, such as the

mouse lethality assay, may draw the wrong conclusions with regard to the effect of different treatments on the course of a disease. In addition, it is important to point out that factors other than iron availability may have an overriding effect on the outcome of infection with yersiniae. It is this factor that makes it difficult to study host-pathogen interactions *in vivo* (Griffiths & Bullen, 1987).

8-4 Avian haemosiderosis, a retrospective study

In the present study it has been noted that in some species of bird examined at necropsy infection with *Y. pseudotuberculosis* occurred concurrently with haemosiderosis. However, it was not known whether an increased availability of iron was directly responsible for the increased susceptibility to the development of systemic pseudotuberculosis or if both haemosiderosis and the presence of the infectious agent were linked to some other unknown factor.

In a comparison between different orders of birds it was noted that the galliforms generally, had the lowest levels of stainable hepatic iron. In healthy chickens there is no stainable iron in the liver tissue. This supports the suggestion that the chicken is a species with low hepatic iron reserves (Garcia *et al.*, 1984; Garcia *et al.*, 1987) and low levels of tissue iron compared to species such as the starling which are strong flyers (Osborn, 1979; Wadsworth *et al.*, 1983). In some species there is an alteration in iron metabolism associated with the seasonal changes in hormone levels which regulate the breeding cycle, moult and migration (Diez *et al.*, 1986). In highly seasonal species such as some passeriforms and anseriforms, it would appear that increased iron stores, associated with changes in diet, metabolic state and seasonal changes in hormone levels, are possibly quite normal at certain times of the year (Ward *et al.*, 1991).

Statistical evaluation of image analysis data indicated that the amount of Prussian blue stainable iron in Kupffer cells was significantly greater in birds which died from an infectious disease when compared with those which died from non-infectious causes. The relationship between the relative amount of hepatic iron and infectious disease is not a simple one, in that the total stainable iron in liver sections was not significantly different between birds which died from infectious and non infectious causes and the amount of stainable iron in the hepatocytes was frequently lower in birds which had an

infectious disease.

It is generally recognised that retrospective studies of histological material are limited by the fact that only the end point of a disease can be observed. Nevertheless, the information gained from this material gives an insight into species differences in hepatic iron levels as well as the value of assessing the distribution of iron in hepatic tissues for determining the significance of the stainable iron seen at necropsy. The results of this study support the hypothesis that there is a link between changes in iron metabolism and the development of infectious disease in birds. With confirmation of the significance of iron in the pathogenesis of disease in birds, the nature of this interaction can be examined *in vivo*.

8-5 Experimental evaluation of the role of iron in the development of infectious disease in birds

The experimental model developed using the chicken in the present study was based on that developed by Robins-Browne & Prpic (1985) for examining the effect of parenteral iron on the development of *Y. enterocolitica* infection in mice but modified to allow comparison with previous models developed in the chicken (Smith *et al.*, 1978). The reasons for the choice of an oral route of challenge and the development of the methods used to evaluate the host-pathogen interaction have already been discussed.

It was found that chickens pre-treated with iron dextran initially developed more severe clinical disease following an oral infective dose of *Y. pseudotuberculosis* than did birds pre-treated with dextran or the chelating agent, desferrioxamine, alone. The serological response to lipopolysaccharide was higher in chickens from the iron-treated groups than in chickens from the untreated groups. In addition, iron-treated chickens had an increased rate of faecal excretion of viable challenge bacteria, they had mucoid blood tinged diarrhoea and at necropsy there was evidence of gut lesions and haemorrhage in the intestinal lumen. These results indicate that a larger number of bacteria were replicating in the gut of the iron-treated chickens than in the untreated birds which did not show clinical disease and had few intestinal lesions at necropsy. In contrast, the number and extent of granulomatous lesions in the liver and spleen was greater in the birds not treated with iron.

Chickens given exogenous iron absorb less iron from the diet when given a normal ration (Hill *et al.*, 1977). This is also seen in mammals, including humans, where iron absorption across the intestinal epithelium is closely regulated. In cases of haemochromatosis this mechanism fails (Powell *et al.*, 1980) and this failure may partially explain why systemic complications from enteric diseases are more common in human patients suffering from this disease. It should also be noted that birds challenged with bacteria had lower levels of iron stored in the liver than did the control birds in each treatment group. This indicates that the chicken may draw on iron reserves during infection as postulated by Smith *et al.* (1978).

In chickens it appears that the availability of iron does have an effect on the pathogenesis of pseudotuberculosis but the high number of splenic lesions in the dextran treated chickens suggests that *Y. pseudotuberculosis* is able to acquire sufficient iron from the un-treated host to replicate extracellularly following systemic spread. In iron-treated chickens there is also some evidence that the proliferation of *Y. pseudotuberculosis* in the intestine was greater than in dextran treated chickens given the same oral challenge of bacteria. This is possibly due to increased iron levels of the luminal contents associated with a reduction in the absorption of iron across the intestinal wall in iron-treated chickens (Hill *et al.*, 1977). These observations illustrate the fact that when an exogenous supply of iron is given prior to bacterial challenge, consideration must be given to 1) the effect an increased availability of iron will have on the host, and 2) the relative effect this will have on the pathogen. Obviously any predictive model needs to carefully assess the iron status of both host and pathogen. Failure to take this into consideration is probably the reason why it has been difficult to apply what is known about experimental models examining iron and disease to the clinical situation.

Because both the pathogenesis of the host-pathogen interaction and the host-iron metabolic interaction do not develop in a mutually independent way it is not possible to determine a predictive model applicable to all situations (Griffiths, 1993). The following hypotheses were derived from the retrospective and experimental studies, 1) that the intracellular survival of *Y. pseudotuberculosis* is enhanced in iron-loaded macrophages, 2) that the extracellular survival of *Y. pseudotuberculosis* is enhanced in an iron-loaded serum, and 3) that the availability of iron in serum would have a greater

effect on the growth of non-pathogenic yersiniae than on pathogenic yersiniae.

8-6 Iron and Yersiniae, *in vitro* work

Pre-treating phagocytic cells with iron dextran prior to the addition of yersiniae to the media resulted in an increased number of intracellular bacteria. From this work it was apparent that the phagocytic cells derived from chick spleen tissue produced a result similar to that reported in the literature when mammalian phagocytic cells were used (Zink *et al*, 1985). Therefore, with regard to the relative effect of iron dextran on the bactericidal capacity of phagocytic cells, mammalian and chicken phagocytes appear to act in a similar way. Because there is little information available on the relative effect of desferrioxamine and iron dextran on the outcome of experimental pseudotuberculosis *in vivo* or *in vitro*, it was decided to develop an *in vitro* system to examine this.

The results indicated that temperature had an influence on the relative effect of altering the availability of iron and that *Y. pseudotuberculosis* and *Y. frederiksenii* differed in some respects. It was found that chicken serum inhibited the growth of *Y. frederiksenii* when incubated at both 28 °C and 37 °C and that this was partially prevented by the addition of iron dextran to the medium. In contrast, the addition of iron dextran to the medium had no significant effect on the growth of *Y. pseudotuberculosis* at 28 °C and only a marginally significant enhancing effect at 37 °C. The addition of desferrioxamine had no significant effect on the growth of *Y. pseudotuberculosis in vitro* therefore, it is apparent that *Y. pseudotuberculosis* was able to acquire sufficient iron from chicken serum without exogenous siderophore and is also able to grow in serum without a supply of exogenous iron. It was found that other factors, such as the presence of serum opsonins, inhibited the growth of this organism in serum and that the relative effect of these factors was more pronounced at higher temperatures.

To summarise, evidence from *in vitro* studies support the hypothesis that in birds, as in mammals, iron-loaded phagocytic cells favour the survival and replication of intracellular yersiniae. In addition, *Y. pseudotuberculosis* is able to survive and replicate in normal chicken serum although this survival was limited in immune sera. This may be explained by the fact that serum transferrin is elevated in immune sera and as a result the available serum iron is reduced. This, in addition to the presence of serum opsonins,

may be responsible for the inhibitory effect of immune serum on the growth of *Y. pseudotuberculosis*. *Yersinia frederiksenii* is able to grow under the same conditions as *Y. pseudotuberculosis in vitro* but chicken serum appears to inhibit the growth of this non-virulent strain. This inhibitory effect is reversed by the addition of iron dextran suggesting that iron may have been a limiting factor.

Whilst noting the importance of iron availability to the pathogen, it should also be remembered that a deficiency of iron may affect the immune efficiency of the host. In the experimental disease model pseudotuberculosis in the chicken is initially potentiated by the administration of exogenous iron but during the course of the disease the chicken utilises the additional iron source. The chicken is therefore able to compete with the pathogen for this resource. In clinical cases of pseudotuberculosis, other factors such as concurrent disease, low host-protein status and lack of prior exposure to the pathogen, are all involved in determining the outcome of infection.

8-7 Further research

Further *in vitro* studies need be done to examine the combined effect of iron and opsonins on the phagocytic and bactericidal capacity of splenic and blood macrophages as well as heterophils. However, none of the present *in vitro* systems will allow the main questions to be answered, for example, determination of the relative effect of the iron availability on the cell-mediated immune system of the host and of the relative amount of serum iron available to pathogenic bacteria during the acute phase of the disease. It is clear that each of the major variables in the equation are mutually dependent and it is not possible to examine one without a full consideration of the other. The complexity of the immune response and possible host differences in both the time scale and the extent of specific and non-specific responses to the invading bacterium requires a complex model to be formulated which can take into account possible species differences. In this regard, further *in vivo* experiments using an avian model which has a high level of tissue iron such as the Greater Indian Hill myna (*Gracula religiosa*) or starling (*Sturnus vulgaris*) may provide some informative comparisons. With preparatory work having already been completed using chickens, the complication of natural alteration in iron levels which occurs in other avian species could be quantified and accounted for, thus allowing similar experiments which artificially manipulate iron

levels to be carried out.

In addition there are many more questions which need to be answered with regard to the epidemiology of yersiniae in mammalian and avian species. Comprehensive environmental and faecal sampling along with concurrent serological tests would be required to evaluate the true nature of the epidemiology of different serotypes of *Y. pseudotuberculosis*. The complexity of studying the epidemiology of a facultative bacterial pathogen has been highlighted in the present study and partially explains why few of the questions with regard to pseudotuberculosis have as yet been answered despite the wealth of literature already available on the subject. Retrospective and experimental investigations are an important supplement to disease investigation. It is clear from the data generated from this study that more information on specific potential host species, such as susceptibility to the disease, challenge dose required, faecal shedding pattern following infection and the population biology of each species, is needed before a full understanding of the role of wild birds in the epidemiology of *Y. pseudotuberculosis* is possible.

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Appendix (A-1) Glossary of terms used in the text

Anseriforms = Water fowl, members of the order Anseriformes

Avian = Of, pertaining to, or caused by birds

Columbiforms = Pigeons and doves, members of the order Columbiformes

Facultative pathogen = A potentially pathogenic organism capable of existing under differing conditions and of causing disease under certain circumstances.

Galliforms = Includes the domestic fowl or chicken, members of the order Galliformes

Haemochromatosis = A chronic disease characterised pathologically by excessive deposits of iron in the body and clinically by hepatomegaly with cirrhosis, skin pigmentation, diabetes mellitus, and frequently cardiac failure. The idiopathic form of the disease is heritable. The term has also been applied to severe iron overload associated with erythropoietic and dietary factors and iron overload associated with repeated blood transfusions. In this thesis the term haemochromatosis has been used for the idiopathic form of the disease only.

Haemosiderin = An iron-containing glycoprotein pigment found in liver and in most tissues, representing colloidal iron in the form of granules much larger than ferritin molecules. It is insoluble in water and differs from ferritin in electrophoretic mobility. Pathologic accumulations are known to occur in a number of disease states.

Haemosiderosis = Deposition of haemosiderin in body tissues without tissue damage, reflecting an increase in body iron stores. In severe cases, haemosiderosis is difficult to distinguish histologically from haemochromatosis as it appears that the distinction between the two conditions is a matter of degree and is not a reflection of the aetiology.

Iron dextran = A colloidal solution of ferric hydroxide in complex with partially hydrolyzed dextran of low molecular weight; used parenterally for treatment of iron deficiency anaemias.

Infection = The invasion of a host by organisms such as bacteria, fungi, viruses, protozoa, helminths or insects, with or without manifest disease.

Infectious disease = A disease due to invasion of the body by pathogenic organisms, which subsequently grow and multiply.

Non-infectious disease = A disease due to factors other than infectious organisms.

Opportunist pathogen = A potentially pathogenic organism incapable of inducing disease in a healthy host, but able to produce infections in a less resistant or injured host.

Pseudotuberculosis = An infection caused by *Yersinia pseudotuberculosis*, occurring in many animals, including rodents and birds, and which may produce severe disease with septicaemia in debilitated individuals.

Passerine = Perching birds, members of the order Passeriformes, also referred to in the text as passeriforms

Pathogen = An infectious agent which is capable of causing disease.

Psychrophilic bacteria = Cold-tolerant organisms which develop best at temperatures between 15 °C and 20 °C but are able to compete well at lower temperatures.

Reservoir population = A population of animals which is able to maintain an infectious agent from generation to generation without reinfection from an outside source.

Siderophores = Low molecular weight, high-affinity iron chelators, produced by many bacteria, which are able to successfully compete with host-binding proteins for iron. The majority of bacterial siderophores are derivatives of catechol or hydroxamic acid.

Virulence = The disease producing capacity of a pathogenic agent.

Appendix (A-2) Common and latin names of avian species examined in the present study, the scientific names of other species cited in the text are given, where possible, when the species is first mentioned.

Order	Family	Species	Common name
Psittaciformes	Psittacidae	<i>Nestor meridionalis</i>	Kaka
		<i>Nestor notabilis</i>	Kea
		<i>Strigops habroptilus</i>	Kakapo
		<i>Cyanoramphus unicolor</i>	Antipodes Island parakeet
		<i>Cyanoramphus auriceps</i>	Yellow-fronted parakeet
		<i>Cyanoramphus x</i>	Hybrid parakeet
		<i>Cyanoramphus novaezelandiae</i>	Red-fronted parakeet
		<i>Platycerus eximulus</i>	Eastern Rosella
		<i>Polytelis anthopeplus</i>	Regent parakeet
		<i>Melopsittacus undulatus</i>	Budgerigar
	Loridae	<i>Trichoglossus haematodus mollucanus</i>	Rainbow lorikeet
Columbiformes	Columbidae	<i>Columba livia</i>	Feral rock pigeon
		<i>Hemiphaga novaeseelandiae</i>	New Zealand native pigeon
Galliformes	Phasianidae	<i>Gallus domesticus</i>	Chicken
		<i>Phasianus colchias</i>	Ring-necked pheasant
Coraciiformes	Alcedinidae	<i>Halcyon sancta vagans</i>	Kingfisher

Order	Family	Species	Common name
Passeriformes	Ploidae	<i>Passer domesticus</i>	House sparrow
	Prunellidae	<i>Prunella modularis</i>	Hedge sparrow
	Emberizidae	<i>Emberiza citrinella</i>	Yellow hammer
	Fringillidae	<i>Fringilla coelebs</i>	Chaffinch
		<i>Carduelis chloris</i>	Greenfinch
		<i>Carduelis carduelis</i>	Goldfinch
		<i>Serinus canaria</i>	Canary
	Sturnidae	<i>Sturnus vulgaris</i>	Starling
		<i>Gracula religiosa</i>	Greater Indian hill myna
	Turdidae	<i>Turdus philomelos</i>	Thrush
		<i>Turdus merula</i>	Black bird
	Eopsaltriidae	<i>Petroica australis</i>	North Island Robin
	Zosteropidae	<i>Zosterops lateralis</i>	Silver eye
	Meliphagidae	<i>Notiomystis cincta</i>	Stitchbird
		<i>Prothemadera novaeseelandiae</i>	Tui
	Callacida	<i>Philesturnus carunculatus</i>	Saddleback
	Estrilidae	<i>Poephila guttata</i>	Zebra finch
Anseriformes	Anatidae	<i>Anas platyrhynchos</i>	Mallard
		<i>Anas rhynchotis</i>	New Zealand Shoveller
		<i>Aythya novaeseelandiae</i>	New Zealand Scaup
		<i>Hymenolaimus malacorhynchos</i>	Blue duck
		<i>Branta canadensis</i>	Canada goose
		<i>Cygnus atratus</i>	Black swan

Appendix (A-3) Biochemical tests used to identify yersiniae from avian and non-avian sources in the present study

Table (1) Differential characteristics of the genus *Yersinia* and biochemically similar genera (tests performed at 37 °C unless otherwise noted), adapted from Mair & Fox (1986).

Characteristics	Genus of bacteria						
	<i>Yer</i>	<i>Cit</i>	<i>Ser</i>	<i>Ent</i>	<i>Esc</i>	<i>Kle</i>	<i>Pro</i>
Christensen's Urea	D	D	D	D	-	D	+
Simmons' citrate	-	+	+	+	-	D	D
Motility 28 °C	+*	+	+	+	+	-	+
Motility 37 °C	-	+	+	+	D	-	+
Voges-Proskauer 28 °C	D	-	+	+	-	D	D
Arginine dihydrolase	-	+	-	D	-	-	-
Lysine decarboxylase	-**	u-	D	D	+	D	-
Ornithine decarboxylase	D	+	D	+	D	-	D
Phenylalanine deaminase	-	-	-	-	-	-	+
H ₂ S (Klinger's)	-	D	+	-	-	-	D
Gas from glucose fermentation	-/W	+	D	+	+	D	+

Yer. = *Yersinia*; *Cit.* = *Citrobacter*; *Ser.* = *Serratia*; *Ent.* = *Enterobacter*; *Esc* = *Escherichia*; *Kle* = *Klebsiella*; *Pro* = *Proteus*. ' + ' = /> 90% of strains +ve; ' - ' = /> 90% of strains -ve; D = different reactions in different species in the genus; W = weak.

* except *Yersinia pestis*; ** except *Yersinia ruckeri*.

Table (2) Characteristics differentiating the species of the genus *Yersinia* (tests performed at 37 °C unless otherwise noted), adapted from Mair & Fox (1986).

Characteristics	Species of <i>Yersinia</i>						
	<i>pest</i>	<i>pseu</i>	<i>ent</i>	<i>fred</i>	<i>int</i>	<i>kris</i>	<i>ruck</i>
Motility	-(-)*	-(+)	-(+)	-(+)	-(+)	-(+)	d
Ornithine decarboxylase	-	-	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-	+
Voges-Proskauer	(-)	-(-)	-(+)	-(+)	-(+)	-(-)	-(d)
Indole	-	-	d	+	+	d	-
Urease	-	+	+	+	+	+	-
Simmon's citrate	-(-)	-(-)**	-(-)	-(d)	d(+)	-(-)	-(+)
Acid production							
D-Cellobiose	-	-	+	+	d(+)	+	-
Sucrose	-	-	+	+	+	-	-
L-Rhamnose	-	+	-	+	d(+)	-	-
D-Melibiose	d	+**	-	-	+	-	-
D-Raffinose	-	d	-	-	d(+)	-	-
A-Methyl-D-glucoside	-	-	-	-	+	-	-
L-Sorbose	-	-	+	+	+	+	-
D-Sorbitol	-	-	+	+	+	+	+
Maltose	-	+***	+	+	+	+	+

pest = *Y. pestis*; *pseu* = *Y. pseudotuberculosis*; *ent* = *Y. enterocolitica*; *fred* = *Y. frederiksenii*; *kris* = *Y. kristensenii*; *int* = *Y. intermedia*; *ruck.* = *Y. ruckeri*. ' + ' = /> 90% of strains are +ve; ' - ' = /> 90% of strains are negative. * symbols in parentheses indicate reactions at 28 °C. ** strains belonging to serotype IV are citrate +ve; *** many animal strains in serogroup III are melibiose and maltose -ve.

Differentiation of the biotypes of *Y. enterocolitica* was performed by the system outlined by Mair & Fox (1986).

Appendix (A-4) Recipes for buffers and media not listed in the text**PBS (Phosphate buffered saline)**

NaCl	8.00g
KCl	0.20g
Na ₂ HPO ₄ ·2H ₂ O	1.15g
KH ₂ PO ₄	0.20g

Dissolved in 1 litre of distilled water

ELISA and Immunoblot washing solution (pH 7.2)

NaCl	20.20g
Na ₂ HPO ₄ ·2H ₂ O	1.15g
KH ₂ PO ₄	0.20g
Tween 20	0.5ml

Dissolved in 1 litre of distilled water

ELISA coating buffer 0.05M Carbonate-Bicarbonate Buffer (pH 9.6)

Na ₂ CO ₃	1.59g
NaHCO ₃	2.93g

Dissolved in 1 litre of distilled water

ELISA substrate buffer, 0.1M Citric Acid-Phosphate Buffer (pH 5.0)

Citric acid, H ₂ O	7.30g
Na ₂ HPO ₄ · 2H ₂ O	11.86g

Dissolved in 1 litre of distilled water

ELISA Enzyme substrate (pH 5.0)

OPD (ortho-phenylene diamine, 2HCl)	8.0 mg
Citric Acid-Phosphate Buffer	15.0ml
30% H ₂ O ₂	5 micro-litres

SDS-PAGE gel electrophoresis**Electrode Buffer (pH 8.3)**

Tris base	9.00g
Glycine	43.2g
SDS	3.00g

Dissolve in 600 ml distilled water to make the stock solution and dilute 70 ml of the solution in 280 ml of distilled water prior to use.

SDS-PAGE sample buffer

Tris 1.51g
Glycerol 20.0ml

Dissolve with 35ml distilled water, adjust to pH 6.75 with HCl, add;

SDS 4.00g
2-Mercaptoethanol 10.0ml
Bromophenol Blue 0.002g

Dilute with distilled water to 100 ml. Boil samples for 15 minutes prior to use.

Acrylamide solutions

1.5 M Tris HCl, pH 8.8 (27.23g Tris base, adjust to pH 8.8 with 1 N HCl and make to 150 ml with distilled water)

0.5 M Tris HCl, pH 6.8 (6.00g Tris base, adjust to pH 6.8 with 1 N HCl and make to 60 ml with distilled water)

10% SDS (dissolve 10.00g SDS in distilled water and bring to 100ml)

Acrylamide solution (Acrylamide/Bis, 40%; Bio-Rad, Richmond), adjusted to a 30% stock solution.

Separating gel			
Concentration	10%	12%	15%
Distilled H ₂ O	4.05 ml	3.35 ml	2.00 ml
1.5M Tris/HCl pH 8.8	2.50 ml	2.50 ml	2.50 ml
Add 100 micro-litres of 10% SDS			
Acryl/Bis 30%	3.30 ml	4.00 ml	4.95 ml
De-gas for 15 minutes			
Add 10 % Ammonium persulphate (50 micro-litres) and TEMED (5 microlitres)			

Stacking gel	
Concentration	5%
Distilled H ₂ O	6.1 ml
1.5M Tris/HCl pH 8.8	2.50 ml
Add 100 micro-litres of 10% SDS	
Acryl/Bis 30%	1.30 ml
De-gas for 15 minutes	
Add 10 % Ammonium persulphate (50 micro-litres) and TEMED (5 microlitres)	

Staining solution

Coomasie Blue R 250	0.1 g
Methanol	20.0 ml
Glacial Acetic acid	14.0 ml

Make up to 200 ml in distilled water

Destaining solution

10 % Glacial acetic acid
30 % Ethanol
60 % H₂O

Western Blotting

Transfer Buffer

Tris	4.55g
Glycine	21.6g
Methanol	200 ml

Make upto 1.5 l with distilled water

Appendix (A-5) Antigenic scheme for *Yersinia pseudotuberculosis*, adapted from Wetzler (1971) with new serotypes proposed by Tsubokura *et al* (1984) given in parentheses.

Serotype	Subtype	O-antigen (thermostable)	H-flagellar (thermolabile)
I (1)	A (a)	1,2,3,13*	a,c
	B (b)	1,2,4,13	a,c
II (2)	A (a)	1,5,6,13 (16)	a,d
	B (b)	1,5,7,13 (16,17)	a,d
	(c)	(1,5,7,11,18) 13	ND
III (3)	toxic	1,8,13	a
	non-toxic	1,8,13	a
IV (4)	A (a)	1,9,11,13	a,b
	B (b)	1,9,12,13	a,b,d
V (5)	A (a)	1,10,13	a,a,e (b)
	B (b)	(1,10,15)	a
VI (6)		(1,13)	a
(7)		(1,13,19)	ND
(8)		(1,13,20)	ND

* sero-factor 13 occurs irregularly in all serotypes and may cross react with *Y. enterocolitica*, *Shigella flexneri* and *Bordetella bronchiseptica*; sero-factor 1 is a rough somatic antigen of *Y. pseudotuberculosis* which is shared with *Y. pestis*, *Shigella flexneri* and *Escherichia coli* (Wetzler, 1965, 1971).

Appendix (A-6) Source and identification of bacterial strains used in the present study

Origin of isolate	Bacterial species (serotype)	Identification
Institut Pasteur*	<i>Yersinia pseudotuberculosis</i> (I)	IP 2941
Institut Pasteur	<i>Y.pseudotuberculosis</i> (II)	IP 2935
Institut Pasteur	<i>Y.pseudotuberculosis</i> (III)	IP 2889
Institut Pasteur	<i>Y.pseudotuberculosis</i> (IV)	IP 2687
Institut Pasteur	<i>Y.pseudotuberculosis</i> (V)	IP 2821
Institut Pasteur	<i>Y.pseudotuberculosis</i> (VI)	IP 1554
Institut Pasteur	<i>Y.enterocolitica</i> (0.3) biotype 4	IP 134
MUVPPH	<i>Y.enterocolitica</i> biotype 1A	KAKA-AV-W
MUVF	<i>Y.enterocolitica</i> biotype 1A	Y.ENT 1A LANADA
MUVF	<i>Y.frederiksenii</i>	Y.FRED. LANADA
MUVPPH	<i>Y.frederiksenii</i>	Y.FRED. 23111
MUVPPH	<i>Y.pseudotuberculosis</i> (I)	YPS-HSP-5M PIGGERY
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-HSP-4M PIGGERY
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-KAKA-2-SEPT.92
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-22920-FINCH
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-22739-CANARY
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-LORIKEET
MUVPPH	<i>Y.pseudotuberculosis</i> (II)	YPS-22877-CANARY
MUVF	<i>Y.kristensenii</i>	YK-LANADA
MUVF	<i>Y.intermedia</i>	YI-LANADA
NZCDC	<i>Salmonella panama</i> (Group D)	CDC 186
NZCDC	<i>S.typhimurium</i> (Group B)	ATCC 14028

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NZCDC = New Zealand Communicable Disease Centre. **** MUVPPH = Department of Veterinary Pathology and Public Health; Massey University. MUVF = Massey University Veterinary faculty.

Appendix (A-7) Yersinia isolated from avian sources in the survey described in chapter two.

Table (1) Yersinia isolated from avian sources during the survey in the summer period (November 1991 to February 1992) listed in chronological order of sample collection.

Location	Species of bird	Sampling method	Yersinia species
Manawatu	<i>Passer domesticus</i>	Necropsy	<i>Y.kristensenii</i>
Manawatu	<i>Passer domesticus</i>	Cloacal	<i>Y.kristensenii</i>
Palmerston North	<i>Turdus philomenos</i>	Necropsy	<i>Y.kristensenii</i>
Manawatu	<i>Passer domesticus</i>	Cloacal	<i>Y.kristensenii</i>
Palmerston North	<i>Turdus merula</i>	Necropsy	<i>Y.kristensenii</i>
Pahiatua	<i>Anas platyrhynchos</i>	Faecal	<i>Y.frederiksenii</i>
Manawatu	<i>Turdus philomenos</i>	Cloacal	<i>Y.frederiksenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faecal x3	<i>Y.frederiksenii</i>
Manawatu	<i>Zosterops lateralis</i>	Cloacal	<i>Y.enterocolitica</i>
Palmerston North	<i>Passer domesticus</i>	Necropsy	<i>Y.enterocolitica</i>
Ashurst	<i>Anas platyrhynchos</i>	Faeces	<i>Y.enterocolitica</i>
Massey University	<i>Porphyrio melanolas</i>	Cloacal	<i>Y.enterocolitica</i>
Wellington Zoo	<i>Passer domesticus</i>	Cloacalx2	<i>Y.enterocolitica</i>
Manawatu	<i>Passer domesticus</i>	Cloacal	<i>Y.kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faecal x2	<i>Y.kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faecal	<i>Y.frederiksenii</i>
Manawatu	<i>Anas platyrhynchos</i>	Faecal x2	<i>Y.frederiksenii</i>
Linton	<i>Zosterops lateralis</i>	Cloacal	<i>Y.frederiksenii</i>
Palmerston North	<i>Turdus philomelos</i>	Necropsy	<i>Y.frederiksenii</i>
Manawatu	<i>Passer domesticus</i>	Cloacalx3	<i>Y.intermedia</i>
Manawatu	<i>Passer domesticus</i>	Necropsy	<i>Y.intermedia</i>
Wellington Zoo	<i>Passer domesticus</i>	Cloacal	<i>Y.kristensenii</i>

Table (2) *Yersinia* isolated from avian sources during the survey in the autumn period (March 1992 to May 1992) listed in chronological order of sample collection.

Location	Species of bird	Sampling method	<i>Yersinia</i> isolate
Palmerston North	<i>Passer domesticus</i>	Cloacal	<i>Y. enterocolitica</i>
Palmerston North	<i>Sturnus vulgaris</i>	Faecal	<i>Y. enterocolitica</i>
Wellington Zoo	<i>Passer domesticus</i>	Cloacal	<i>Y. kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces	<i>Y. frederiksenii</i>
Kapiti	<i>Apteryx australis mantelli</i>	Necropsy	<i>Y. enterocolitica</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces	<i>Y. kristensenii</i>

Table (3) *Yersinia* isolated from avian sources during the survey in the winter period (June 1992 to August 1992) listed in chronological order of sample collection.

Location	Species of bird	Sampling method	<i>Yersinia</i> species
Wellington	<i>Sturnus vulgaris</i>	Faecal	<i>Y. enterocolitica</i>
Waikanae	<i>Turdus merula</i>	Cloacal	<i>Y. enterocolitica</i>
Wellington	<i>Sturnus vulgaris</i>	Faecal x3	<i>Y. kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces x10	<i>Y. frederiksenii</i>
Manawatu	<i>Anas platyrhynchos</i>	Necropsy	<i>Y. enterocolitica</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces	<i>Y. frederiksenii</i>
Palmerston North	<i>Sturnus vulgaris</i>	Faeces	<i>Y. frederiksenii</i>
Kapiti	<i>Notiomystis cincta</i>	Faeces x3	<i>Y. enterocolitica</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces x3	<i>Y. kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faeces	<i>Y. frederiksenii</i>

Table (4) *Yersinia* isolated from avian sources during the survey in the spring period (September 1992 to November 1992) listed in chronological order of sample collection.

Location	Species of bird	Sampling method	<i>Yersinia</i> isolate
Wellington	<i>Sturnus vulgaris</i>	Faecal x2	<i>Y. kristensenii</i>
Wellington	<i>Sturnus vulgaris</i>	Faecal	<i>Y. frederiksenii</i>
Massey piggery	<i>Passer domesticus</i>	Necropsy x11	<i>Y. enterocolitica</i>
Wellington	<i>Passer domesticus</i>	Faeces	<i>Y. frederiksenii</i>
Massey piggery	<i>Passer domesticus</i>	Necropsy x5	<i>Y. intermedia</i>
Massey piggery	<i>Passer domesticus</i>	Necropsy x2	<i>Y. pseudob</i>

Appendix (A-8) Yersinia isolated from non-avian sources in the survey described in chapter two.

Table (1) Yersinia isolated from non-avian sources during the summer period of the survey (November 1991 to February 1992)

Location	Sample source	Sample	<i>Yersinia species isolated</i>
Rongatea	Possum	Caecum	<i>Y.enterocolitica</i>
Manawatu	Farm land	Soil x 8	<i>Y.enterocolitica</i>
Manawatu	Farm land	Foliage	<i>Y.enterocolitica</i>
Manawatu	Possum	Colon	<i>Y.enterocolitica</i>
Manawatu	Farm land	Soil x 3	<i>Y.frederiksenii</i>
Feilding	Farm land	Soil x 2	<i>Y.intermedia</i>

Table (2) Yersinia isolated from non-avian sources during the autumn period of the survey (March 1992 to May 1992)

Location	Sample source	Sample	<i>Yersinia species isolated</i>
Manawatu	Farm land	Soil	<i>Y.kristensenii</i>
Wellington	Zoo	Soil	<i>Y.enterocolitica</i>
Rongotea	Possum	Caecum	<i>Y.enterocolitica</i>
Manawatu	Farm land	Soil x 2	<i>Y.kristensenii</i>

Table (3) *Yersinia* isolated from non-avian sources during the winter period of the survey (June 1992 to August 1992)

Location	Sample source	Sample	<i>Yersinia species isolated</i>
Feilding	Aviary	Water	<i>Y.enterocolitica</i>
Manawatu	Farm land	Soil x 8	<i>Y.enterocolitica</i>
Manawatu	Farm land	Foliage	<i>Y.enterocolitica</i>
Waikanae	Aviary	Soil x 2	<i>Y.enterocolitica</i>
Wellington	Urban area	Soil x 6	<i>Y.enterocolitica</i>
Manawatu	Farm land	Soil x 2	<i>Y.frederiksenii</i>
Manawatu	Farm land	Foliage	<i>Y.frederiksenii</i>
Feilding	Garden	Foliage	<i>Y.kristensenii</i>
Wellington	Urban area	Soil x 4	<i>Y.kristensenii</i>
Wellington	Urban area	Foliage	<i>Y.kristensenii</i>

Table (4) *Yersinia* isolated from non-avian sources during the spring period of the survey (September 1991 to November 1992)

Location	Sample source	Sample	<i>Yersinia species isolated</i>
Rongotea	Possum	Caecum	<i>Y.frederiksenii</i>

Appendix (A-9) *Yersinia* isolated from birds sampled by the Department of Conservation during disease surveillance for *Chlamydia psittaci* and other disease screening procedures during 1992-1993.

<i>Avian species</i>	Location	<i>Yersinia species</i>	No. positive/ No. collected
<i>Columbia livia</i>	Christchurch	<i>kristensenii</i>	2/12
<i>Columbia livia</i>	Auckland	<i>pseudotuberculosis</i>	1/12
<i>Nestor meridionalis</i>	Orana park	<i>none</i>	0/9
<i>Apteryx spp</i>	Kapiti	<i>none</i>	0/20
<i>Niomystis cincta</i>	Kapiti	<i>frederiksenii</i>	3/5
<i>Hemiphaga novaezealandiae</i>	Orana park	<i>pseudotuberculosis</i>	3/9

Appendix (A-10) Optical density values measured at 492nm for sera of wild birds in the serological survey as measured by the LPS ELISA (*between plate variation corrected in reference to a standard curve).

Location	Date	Bird	Yps I	Yps II	Yps III
MUpig*	22-10-92	1. B/b1	0.654	0.932	0.762
		2. HSP3	0.596	0.931	0.562
		3. C1	0.834	0.932	0.723
		4. HSP 1	0.692	0.292	0.517
		5. HSP 5	0.524	0.726	0.654
		6. HSP 4	0.602	0.801	0.536
		7. Thr C1	0.354	0.832	0.712
		8. B/b	0.628	0.664	0.612
		9. B/b	0.354	0.434	0.392
Levin **	10-10-92	1. Chaff.	0.768	0.883	0.656
		2. GF2	0.576	0.465	0.512
		3. St2	0.597	0.527	0.983
		4. GF3	0.692	0.576	0.592
		5. St1	0.492	0.385	0.214
		6. St2	0.723	0.677	0.226
		7. GF5	0.841	0.679	0.723
		8. B/b1	0.475	0.564	0.623
		9. B/b3	0.982	1.080	0.412
		10. Bb	0.913	1.802	0.712
		11. Bb2	0.613	0.535	0.461
		12. GF8	0.815	1.125	1.342
		13. GF4	0.745	1.080	0.321
		14. GF1	0.725	1.126	0.820
		15. GF7	0.681	1.141	0.926
		16. GF6	0.715	0.809	0.734
		17. Bb3	0.698	0.726	0.691
Levin **	29-09-92	18. A13	0.572	0.428	0.891

		19. A9	0.391	0.468	0.592
		20. A8	0.516	0.681	0.546
		21. A7	0.265	0.382	0.315
		22. A6	0.276	0.382	0.324
		23. A5	0.347	0.369	0.321
		24. A4	0.287	0.681	0.268
		25. A3	0.342	0.428	0.351
MU sheep	08-10-92	1. B81	0.426	0.567	0.456
		2. C26	0.792	0.543	0.824
		3. C2	0.523	0.452	0.423
		4. C51308	0.785	1.078	0.729
		5. B63509	0.824	0.823	0.715
		6. C51307	0.735	1.036	0.813
		7. C51309	0.546	1.054	0.264
		8. C51308	0.874	1.029	0.271
		9. Bb1	0.561	0.921	0.654
		10. Bb2	1.560	1.321	0.724
		11. HSP1	0.983	1.216	0.892
		12.C51310	0.824	0.342	0.973
		13.D16002	0.571	0.378	0.397
		14.B34	0.465	0.546	0.496
		15.St6	0.613	0.823	0.716
		16.B67	0.453	0.916	0.783
		17.chaff.	0.635	0.523	0.657
		18.B89	0.672	0.243	0.456
		19.Th7	0.735	0.341	0.376
		20.HSp1	0.463	0.652	0.612
		21.HSP2	0.517	0.539	0.546
		22.Fin.	0.527	0.412	0.502
		23.HSP3	0.375	0.615	0.476
MUfeed	07-10-93	1. B63513	0.839	0.926	0.893

		2. B63508	0.916	1.538	0.945
		3. HSP 1	0.816	0.279	0.347
		4. HSP8	0.482	0.531	0.487
		5. HSP7	0.819	0.623	0.782
		6.HSP79	0.519	0.752	0.698
		7.HSP34	0.693	0.712	0.709
		8.HSP24	0.591	0.693	0.691
		9.Th9	0.517	0.532	0.562
		10.O18	0.387	0.491	0.532
		11.HSP91	0.308	0.312	0.314
		12.HSP67	0.617	0.756	0.576
Linton		1.Bb5	0.538	0.523	0.498
		2.O7	0.816	0.913	0.934
		3.Th8	0.536	0.413	0.451
Ashdeer		1.O3	0.581	0.429	0.529
		2.B657	0.826	0.639	0.629
Field.		1.B/b	0.308	0.312	0.378
		2.Ch	0.438	0.421	0.486
		3.Th	0.506	0.521	0.576
		4.St	0.609	0.612	0.618
		5.HSP	0.587	0.531	0.576
WGTZOO	31-08-92	1. 5GZ	1.936	1.631	0.925
		2. 4GZ	0.983	1.753	0.982
		3. 3GZ	1.935	1.926	1.098
		4. 1GZ	0.917	0.921	1.009
		5. D17	1.937	1.513	0.354
		6. 1GZ	0.287	0.360	0.489
		7. 2GZ	0.452	0.323	0.324
		8.B/b	0.467	0.563	0.418
WGTZOO	28-06-92	9. B176	0.934	1.623	0.517
		10. B181	1.093	1.059	0.498

		11. G9Z	0.956	0.921	0.998
		12. B180	1.056	1.021	0.427
		13. G5Z	1.453	1.059	0.829
		14. G3Z	0.987	1.062	0.265
		15. B176	0.859	1.239	0.729
		16. G6Z	0.067	0.112	0.165
		17. B179	0.253	0.129	0.173
		18. G2Z	0.098	0.056	0.176
		19.HSP	0.513	0.459	0.561
		20.HSP	0.309	0.312	0.311
		21.HSP	0.465	0.436	0.465
		22.HSP	0.623	0.523	0.728
		23.HSP	0.791	0.719	0.560
		24.HSP	0.713	0.812	0.783
		25.HSP	0.465	0.529	0.598
Other 9*	24-08-92	1. RL	0.187	0.127	0.243
		2.HSP	0.376	0.432	0.423
		3.Ch.	0.475	0.512	0.487
		4.GZ	0.365	0.396	0.396
		5.HSP	0.397	0.398	0.399
		6.HSP	0.476	0.412	0.407
		7.HSP	0.527	0.436	0.529
		8.HSP	0.456	0.459	0.598
		9.St	0.409	0.418	0.467
		10.HSP	0.390	0.398	0.398
		11.HSP	0.576	0.523	0.564
		12.HSP	0.423	0.491	0.500
		13.HSP	0.476	0.493	0.512
		14.HSP	0.519	0.518	0.576
		15.O1	0.507	0.512	0.523
		16.B/b	0.598	0.563	0.545

		17.HSP	0.409	0.418	0.465
		18.HSP	0.597	0.629	0.512
		19.HSP	0.496	0.531	0.523
MUsheep	23-12-92	1. D13	0.987	0.213	1.008
		2. C7	0.652	0.216	0.983
		3.Bb	0.524	0.563	0.453
		4.HSP	0.519	0.519	0.564
		5.HSP	0.482	0.496	0.598
		6.HSP	0.478	0.498	0.324
		7.HSP	0.578	0.516	0.465
		8.HSP	0.516	0.529	0.365
	30-01-92	9. B85	1.098	0.982	1.009
	31-01-92	10. B81	0.979	0.993	0.938
		11. C26	1.692	0.965	1.893
		12. B83	0.927	0.001	0.839
		13. B80	0.835	0.342	0.729
		14. B88	0.528	0.342	0.786
		15. B96	0.728	0.438	0.829
	29-01-92	16. B66	0.865	0.568	0.915
		17. B65	0.573	0.531	0.673
		18. B64	0.358	0.413	0.418
		19. B66	0.598	0.521	0.618
		20. B62	0.600	0.610	0.659
		21. B63	0.652	0.732	0.735
		22. C24	0.796	0.893	0.687
		23. B7	0.894	0.983	0.988
		24. B93	0.652	0.652	0.798
		25. B89	0.693	0.783	0.799
		26. B79	0.485	0.563	0.564
		27. B78	0.198	0.132	0.154
		28. B103	0.131	0.133	0.276

		29. B93	0.254	0.250	0.198
		30. B90	0.243	0.258	0.498
		31. C26	0.387	0.362	0.398
MUfeed	20-02-92	1. B146	0.837	0.932	0.897
		2. B148	0.783	0.825	0.698
		3. B140	0.945	0.916	0.917
		4. B138	0.671	0.853	0.976
		5. B159	0.316	0.321	0.583
		6. B149	0.245	0.258	0.398
	22-01-92	7. Bb	1.982	0.921	0.498
		8. B142	0.564	0.312	0.917
	19-02-92	9. B128	1.045	0.932	0.927
		10.HSP	0.623	0.767	0.387
		11.HSP	0.452	0.563	0.728
		12.HSP	0.647	0.782	0.915
	26-02-92	13. B151	1.342	0.835	1.036
		14. B133	0.734	0.453	0.985
		15. B134	0.576	0.553	0.683
		16. B157	0.635	0.653	0.482
		17. B156	0.683	0.733	0.826
		18. B128	0.251	0.216	0.281
		19. B158	0.165	0.153	0.176
		20. B152	0.735	0.753	0.693
		21. B155	0.724	0.853	0.729
		22. B154	0.462	0.562	0.629
		23. B150	0.934	0.992	0.891
		24. B153	1.097	1.236	0.988
		25. B149	0.987	1.025	0.938
		26. B153	0.376	0.722	0.265
		27. B131	0.564	0.323	0.643
		28. B72	0.376	0.873	0.376

		29. B32	0.354	0.342	0.376
Linton	08-02-92	1. St1	1.879	1.653	0.982
		2. D28	0.143	0.129	0.176
		3. B113	0.065	0.053	0.013
		4. B27	0.187	0.151	0.176
		5. B117	0.193	0.179	0.198
		6. B106	0.076	0.056	0.092
		7. B104	0.092	0.068	0.037
		8. D25	0.088	0.044	0.054
		9. D25	0.091	0.024	0.012
		10. B108	0.056	0.048	0.054
		11. B114	0.058	0.022	0.086
		12. B105	0.043	0.037	0.067
		13. B120	0.045	0.037	0.048
		14. B121	0.098	0.103	0.104
		15. B123	0.047	0.053	0.035
		16. B124	0.056	0.053	0.057
		17. B122	0.067	0.066	0.068
		18. C30	0.017	0.135	0.154
Ashdeer	02-02-92	1. B/b	0.176	0.146	0.156
		2. HSP	0.354	0.235	0.312
		3. B/b	0.387	0.354	0.312
		4. HSP	0.673	0.342	0.546
		5. HSP	0.476	0.423	0.424
		6. HSP	0.675	0.621	0.576
		7. HSP	0.786	0.721	0.756
		8. HSP	0.637	0.534	0.605
		9. St	0.645	0.523	0.657
		10. HSP	0.698	0.624	0.756
		11. B/b	0.763	0.523	0.645
		12. HSP	0.803	0.753	0.723

		13. HSP	0.818	0.853	0.812
		14. HSP	0.698	0.535	0.576
		15. HSP	0.576	0.635	0.645
		16. HSP	0.276	0.212	0.213
		17. HSP	0.308	0.312	0.312
		18. HSP	0.387	0.313	0.311
		19. HSP	0.406	0.413	0.409
		20. HSP	0.487	0.513	0.518
		21. B/b	0.265	0.329	0.356
WGTZOO	03-01-92	1. Bb	0.487	0.963	0.998
		2.GF	0.503	0.492	0.523
		3.Bb	0.625	0.631	0.678
		4.GZ2	0.623	0.529	0.536
		5.HSP	0.723	0.678	0.768
		6.HSP	0.576	0.523	0.564
		7.HSP	0.798	0.845	0.846
		8.HSP	0.768	0.792	0.725
		9.HSP	0.724	0.683	0.658
		10.HSP	0.913	0.831	0.834
		11.HSP	0.988	0.863	0.834
	08-02-92	12. B106	1.024	0.951	0.265
		13. B104	1.376	0.923	0.267
		14. B104a	1.698	0.989	0.298
		15. B114	0.928	0.962	0.675
		16.HSP	0.634	0.651	0.657
		17.HSP	0.602	0.532	0.647
		18.B/b	0.522	0.498	0.456
		19.HSP	0.398	0.353	0.376
		20.HSP	0.623	0.652	0.645
		21.HSP	0.645	0.891	0.823
		22.HSP	0.598	0.883	0.881

		23.B/b	0.287	0.492	0.453
		24.HSP	0.487	0.531	0.511
		25.St	0.619	0.589	0.534
		26.HSP	0.498	0.623	0.613
		27.GF	0.798	0.815	0.813
		28.B/b	0.729	0.813	0.809
		29.HSP	0.387	0.392	0.345
		30.HSP	0.798	0.816	0.812
		31.HSP	0.573	0.635	0.546
		32.HSP	0.529	0.435	0.354
		33.B/b	0.612	0.525	0.365
		34.HSP	0.598	0.429	0.243
		35.HSP	0.518	0.413	0.416
		36.B/b	0.597	0.526	0.512
		37.HSP	0.537	0.513	0.498
		38.HSP	0.509	0.492	0.453
		39.HSP	0.598	0.486	0.476
		40.HSP	0.498	0.682	0.587
		41.HSP	0.619	0.536	0.528
		42.Th	0.598	0.581	0.598
		43.HSP	0.539	0.593	0.498
		44.HSP	0.598	0.618	0.612
		45.HSP	0.645	0.629	0.587
		46.Th	0.564	0.593	0.487
		47.HSP	0.507	0.493	0.498
		48.HSP	0.657	0.632	0.598
		49.HSP	0.798	0.734	0.698
		50.HSP	0.587	0.645	0.453
		51.HSP	0.795	0.873	0.748
		52.HSP	0.637	0.841	0.685
		53.HSP	0.717	0.832	0.824

		54.HSP	0.439	0.648	0.598
		55.HSP	0.573	0.783	0.726
Flock		1.GF	0.576	0.581	0.564
		2.TH	0.657	0.554	0.553
		3.GF	0.498	0.563	0.538
		4.HSP	0.675	0.612	0.589
		5.Bb	0.598	0.595	0.546
		6.HSP	0.587	0.583	0.586
		7.GF	0.587	0.561	0.534
		8.GF	0.678	0.609	0.592
		9.Th	0.645	0.599	0.598
		10.GF	0.976	0.883	0.912
		11.HSP	0.954	0.845	0.934
Field.		1.HSP	0.698	0.632	0.687
		2.O5	0.675	0.745	0.687
		3.GF	0.856	0.838	0.845
		4.HSP	0.895	0.821	0.812
		5.HSP	0.745	0.763	0.734
		6.HSP	0.698	0.789	0.734
		7.HSP	0.698	0.693	0.654
		8.HSP	0.735	0.713	0.687
		9.GZ	0.897	0.821	0.798
		10.GZ	0.798	0.754	0.723
		11.HSP	0.657	0.832	0.835
		12.HSP	0.798	0.785	0.723
Foxton		1.HSP	0.786	0.693	0.586
		2.HSP	0.897	0.873	0.834
		3.HSP	0.576	0.510	0.510
		4.Th	0.798	0.716	0.623
		5.HSP	0.896	0.811	0.723
		6.HSP	0.678	0.652	0.654

		7.HSP	0.698	0.813	0.745
Other		1.HSP	0.679	0.745	0.624
		2.HSP	0.325	0.321	0.342
		3.HSP	0.478	0.416	0.415
		4.HSP	0.412	0.423	0.412
		5.Th	0.450	0.452	0.436
		6.HSP	0.390	0.351	0.398
		7.HSP	0.333	0.425	0.423
		8.HSP	0.546	0.726	0.697

Appendix (A-11) The survival of *Yersinia pseudotuberculosis* serotypes I, II and III in phosphate buffered saline and distilled water at 4 °C and 28 °C.

Materials and methods

Type cultures of *Yersinia pseudotuberculosis* were obtained from the Pasteur Institute in Paris. Three serotypes, *Y. pseudotuberculosis* I (IP2941), II (IP2935) and III (IP2889), were plated onto blood agar and incubated at 28 °C for 48 hours prior to harvest. Twelve aliquots of 3.0 ml tryptone water were prepared and samples of *Y. pseudotuberculosis* serotype I, II or III, were added to each of four bottles labelled for the three serotypes. Bacteria were incubated overnight at 28 °C and the following day 1.0 ml of tryptone water, containing approx. 10^8 yersiniae, was taken from each bottle and added to 10.0 ml sterile preparations of either phosphate buffered saline (PBS) or distilled water as outlined in Table (1). For each serotype two 10.0 ml preparations of PBS and two 10.0 ml preparations of distilled water were inoculated with approx. 10^8 bacteria. One of each preparation was then incubated at 4 °C and the other at 28 °C. The 10.0 ml preparations were sampled monthly for twelve months to determine how long *Yersinia pseudotuberculosis* was able to survive in PBS or distilled water at two different temperatures. The monthly samples were taken using a 10 microlitre loop, the bacteria were then plated onto selective agar (CIN) and incubated at 28 °C for 48 hours. Plasmid preparations were made from some of the resulting cultures using the method of Portnoy *et al.* (1981) as outlined in chapter four.

Table (1) Summary of the treatment of samples and codes used in the experiment.

Preparation (incubation temperature)	<i>Yersinia pseudotuberculosis</i>		
	I	II	III
PBS (4°C)	PB1a	PB2a	PB3a
Distilled water	DW1a	DW2a	DW3a
PBS (28°C)	PB1b	PB2b	PB3b
Distilled water	DW1b	DW2b	DW3b

Results

The results of the experiment are represented in Table (2). It was found that;

- 1) all three serotypes of *Y. pseudotuberculosis* tested survived in PBS and distilled water for at least six months at both 4 °C and 28 °C
- 2) the number of yersiniae present per millilitre after six months storage *in vitro* was less than the number initially added to the preparations
- 3) *Y. pseudotuberculosis* survived for a longer period of time in either preparation when stored at 4 °C compared with 28 °C
- 4) plasmids can be isolated from strains of *Y. pseudotuberculosis* stored for 6 months or longer *in vitro*.

Table (2) The survival, growth and plasmid carriage of *Y. pseudotuberculosis* serotypes I, II and III, stored in two different preparations at 4°C and 28°C over a twelve month period.

Code	Samples taken at 6 months		No. months sample +ve
	Approx. no. of bacteria	Plasmid present	
DW1a	10 ⁵ /ml	+	> 12 months
DW2a	10 ⁵ /ml	+	> 12 months
DW3a	10 ⁵ /ml	+	> 12 months
DW1b	10 ³ /ml	ND*	8 months
DW2b	10 ³ /ml	+	10 months
DW3b	10 ⁴ /ml	ND	10 months
PB1a	10 ⁵ /ml	ND	> 12 months
PB2a	10 ³ /ml	+	12 months
PB3a	10 ⁴ /ml	ND	10 months
PB1b	10 ² /ml**	ND	7 months
PB2b	10 ³ /ml**	ND	7 months
PB3b	10 ⁴ /ml**	ND	7 months

* Plasmid preparation not performed due to small yield of bacteria. ** heavy growth of contaminants.

Conclusion; *Y. pseudotuberculosis* can survive for longer than 12 months at 4 °C in distilled water or PBS and still retains the virulence plasmid.

Appendix (A-12) Statistical evaluation of the relationship between the amount and distribution of iron levels in histological sections of liver tissue and infectious disease.

Table (1) Analysis of variance of image analysis values for the total stainable iron in Perls' iron stained hepatic sections of birds in different orders (see section, 5-3-4)

Order	n	Statistical values			
		Mean	SD	Var	CSS
Psittaciform (inf)	15	9.03	1.14	1.30	18.2
Psittaciform (non inf)	10	5.81	2.75	7.59	68.3
Columbiform (inf)	3	10.7	1.52	2.30	4.60
Columbiform (non inf)	5	8.69	2.62	6.86	27.4
Passeriform native (inf)	10	9.8	0.55	0.30	2.74
Passeriform native (non)	15	8.7	1.92	3.71	52.0
Passeriform int. (inf)	35	5.3	0.83	0.69	4.11
Passeriform int. (non)	30	4.3	2.20	4.68	23.4
Anseriform (inf)	4	9.4	0.67	0.45	1.34
Anseriform (non inf)	32	8.77	2.44	5.96	178.8
Galliform (inf)	6	3.94	1.49	2.22	11.1
Galliform (non inf)	6	2.00	1.87	3.48	17.4
Others (inf)	6	3.97	1.54	2.37	11.87
Others (non inf)	3	4.26	2.86	8.17	16.34

inf. = birds which died of an infectious disease; non-inf. = birds which died of a non-infectious cause, n = number of birds examined, SD = standard deviation, Var = variance, CSS = sum of the squares

Table (2) Analysis of variance of image analysis values for the stainable Kupffer cell iron in Perls' iron stained hepatic sections of birds in different orders (see section, 5-3-4)

Order	n	Statistical values			
		Mean	SD	Var	CSS
Psittaciform (inf)	15	9.03	1.14	1.3	18.2
Psittaciform (non inf)	10	5.29	2.93	8.6	77.49
Columbiform (inf)	3	10.7	1.52	2.30	4.60
Columbiform (non inf)	5	8.47	2.87	8.25	33.0
Passeriform native (inf)	10	9.8	0.55	0.30	2.74
Passeriform native (non)	15	5.56	1.76	3.09	43.21
Passeriform int. (inf)	35	5.3	0.83	0.69	4.12
Passeriform int. (non)	30	4.3	2.16	4.68	23.4
Anseriform (inf)	4	9.4	0.67	0.45	1.34
Anseriform (non inf)	32	4.8	2.55	6.33	71.79
Galliform (inf)	6	3.94	1.49	2.22	11.09
Galliform (non inf)	6	1.1	3.44	11.86	59.29
Others (inf)	6	3.97	1.54	2.37	11.87
Others (non inf)	3	4.22	2.84	8.08	16.15

inf. = birds which died of an infectious disease; non-inf. = birds which died of a non-infectious cause, n = number of birds examined, SD = standard deviation, Var = variance, CSS = sum of the squares

Table (3) Analysis of variance of image analysis values for the stainable hepatocyte iron in Perls' iron stained hepatic sections of birds in different orders (see section, 5-3-4)

Order	n	Statistical values			
		Mean	SD	Var	CSS
Psittaciform (inf)	15	4.99	1.68	2.82	39.5
Psittaciform (non inf)	10	4.94	2.34	5.48	49.3
Columbiform (inf)	3	5.58	1.25	1.57	3.13
Columbiform (non inf)	5	6.34	1.23	1.51	6.02
Passeriform native (inf)	10	7.28	1.64	2.69	24.2
Passeriform native (non)	15	8.68	1.97	3.87	54.2
Passeriform int. (inf)	35	2.62	1.01	1.01	6.07
Passeriform int. (non)	30	2.25	1.47	2.17	10.86
Anseriform (inf)	4	7.07	1.49	2.21	6.63
Anseriform (non inf)	32	6.67	3.46	11.96	131.56
Galliform (inf)	6	2.60	1.07	1.15	5.77
Galliform (non inf)	6	2.24	1.87	3.48	17.4
Others (inf)	6	2.62	1.59	2.54	12.68
Others (non inf)	3	3.75	2.59	7.22	14.44

inf. = birds which died of an infectious disease; non-inf. = birds which died of a non-infectious cause, n = number of birds examined, SD = standard deviation, Var = variance, CSS = sum of the squares

Appendix (A-13) Image analysis data: Chapter five

Table (1) Stainable iron in hepatic sections of chickens treated parenterally with 10 mg iron dextran. see section (5-3-1)

Slide	Kupffer cell iron *		Hepatocyte iron *	
	Mean (A)	Mean (I)	Mean (A)	Mean (I)
1-1	3.16+/-0.30	2.65+/-0.20	2.52+/-0.08	2.80+/-0.10
1-2	3.95+/-0.15	4.20+/-0.20	3.40+/-0.10	2.60+/-0.20
2-1	4.59+/-0.20	3.71+/-0.25	4.85+/-0.20	4.21+/-0.28
2-2	4.50+/-0.35	3.84+/-0.13	4.85+/-0.15	4.14+/-0.31
3-1	2.26+/-0.30	1.60+/-0.21	4.27+/-0.30	3.46+/-0.24
3-2	2.20+/-0.21	2.45+/-0.12	4.36+/-0.25	3.61+/-0.25
4-1	1.30+/-0.32	1.00+/-0.08	3.69+/-0.12	3.14+/-0.16
4-2	0.00+/-0.00	0.00+/-0.00	3.11+/-0.13	3.59+/-0.15

* Values are expressed as log₁₀ values.

Table (2) The amount and distribution of stainable iron measured in hepatic sections of birds from different orders. see section (5-3-3) which died from infectious and non-infectious causes.

A-Psittaciform (infectious diseases)

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
A-1	3.50	3.00	6.50	2.90	2.50	5.40
A-2	3.56	4.00	7.56	2.79	3.00	5.79
A-3	3.85	4.05	7.95	2.10	2.02	4.12
A-4	3.78	4.02	7.98	2.80	1.09	3.89
A-5	4.82	4.02	8.84	2.70	1.00	3.70
A-6	4.83	3.97	8.70	2.02	1.02	3.04
A-7	4.56	4.30	8.86	3.00	1.86	4.86
A-8	5.26	4.30	9.56	2.05	1.80	3.85
A-9	5.35	4.10	9.45	2.98	2.00	4.98
A-10	5.32	4.28	9.60	2.30	1.59	3.89
A-11	5.45	4.45	9.90	2.07	2.60	4.67
A-12	5.30	4.30	9.60	2.56	2.00	4.56
A-13	5.20	4.80	10.00	2.00	2.70	4.70
A-14	5.45	4.90	10.35	5.30	4.05	9.35
A-15	5.00	5.65	10.65	5.70	3.00	8.05

A-Psittaciformes; Non-infectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
A-16	0.00	0.00	0.00	0.00	0.00	0.00
A-17	1.90	1.08	2.98	4.34	1.02	5.36
A-18	2.08	1.90	3.98	3.98	1.00	4.98
A-19	3.16	1.40	4.56	2.78	2.00	4.78
A-20	2.90	2.10	5.00	3.67	2.00	5.67
A-21	2.78	2.00	4.98	3.00	2.80	5.80
A-22	4.60	2.40	7.00	4.34	1.00	5.34
A-23	5.56	3.00	8.56	1.80	1.20	3.00
A-24	2.80	2.40	5.20	4.62	0.60	5.12
A-25	5.65	5.00	10.65	7.35	2.00	9.35

B-Columbiform-infectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
B-1	7.56	2.20	9.76	4.92	2.00	6.92
B-2	7.67	2.22	9.89	3.12	2.24	5.36
B-3	6.05	6.40	12.45	2.45	2.00	4.45

B-Columbiform-noninfectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
B-4	2.65	2.00	4.85	3.63	2.02	5.65
B-5	6.30	2.02	8.32	3.60	1.71	5.31
B-6	5.78	2.20	7.98	6.30	2.00	8.30
B-7	6.28	2.00	8.28	4.78	2.00	6.78
B-8	6.00	6.90	12.9	3.55	2.12	5.67

C-Passeriformes-Native birds-infectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
C-1	4.40	5.10	9.50	5.20	3.20	8.40
C-2	7.54	2.02	9.56	5.69	1.10	6.79
C-3	7.32	2.13	9.45	3.02	1.10	4.12
C-4	7.28	1.90	9.18	3.89	2.00	5.89
C-5	7.04	2.30	9.34	3.45	2.25	5.70
C-6	7.56	2.04	9.60	5.70	1.60	7.30
C-7	7.46	2.20	9.66	5.50	2.36	7.86
C-8	8.56	2.00	10.56	5.85	3.00	8.85
C-9	8.05	2.40	10.45	6.90	2.08	8.98
C-10	5.70	5.00	10.70	5.30	3.59	8.89

C-Passeriformes-Native birds-noninfectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
C-11	3.25	2.25	4.50	2.50	2.06	4.56
C-12	3.86	2.70	5.56	4.50	2.20	6.70
C-13	3.95	2.00	5.95	4.10	2.02	6.12
C-14	3.78	1.20	4.98	3.00	2.70	5.7
C-15	2.80	1.04	3.84	5.67	3.03	8.70
C-16	1.90	1.80	3.70	9.00	1.30	10.30
C-17	3.56	1.30	4.86	7.90	1.06	8.96
C-18	2.56	2.00	4.56	7.65	1.20	8.85
C-19	1.85	1.60	3.45	7.00	1.98	8.98
C-20	4.50	2.10	6.60	7.25	2.64	9.89
C-21	3.60	2.05	5.65	7.81	2.10	9.91
C-22	5.35	2.10	7.45	8.30	2.09	10.39
C-23	5.79	2.10	7.89	8.29	1.60	9.89
C-24	2.56	2.00	4.56	8.00	2.30	10.30
C-25	5.42	4.40	9.82	8.90	2.06	10.96

C-Passeriformes-introduced birds-infectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
C-26-30	3.80	0.40	4.20	1.00	0.23	1.23
C-31-35	2.85	1.56	4.36	1.95	0.75	2.70
C-36-40	3.00	2.30	5.30	1.82	0.40	2.12
C-41-45	2.80	2.50	5.30	1.00	0.70	1.7
C-46-50	2.54	3.30	5.84	1.45	2.35	3.70
C-51-55	2.30	2.20	5.50	1.00	0.30	1.30
C-56-60	4.80	1.80	6.60	1.90	1.06	2.96

C-Passeriformes-introduced birds-noninfectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
C-61-65	0.00	0.00	0.00	0.00	0.00	0.00
C-66-70	3.20	1.06	4.26	1.00	0.70	1.70
C-71-75	3.45	2.01	5.46	2.02	1.10	3.12
C-76-80	3.12	2.48	5.60	1.45	1.45	2.7
C-81-85	3.80	1.52	5.32	1.40	0.30	1.70
C-86-90	3.65	1.55	5.20	2.80	1.50	4.30

D-Anseriformes-infectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
D-1	5.80	2.60	8.40	5.40	2.00	7.40
D-2	5.70	4.02	9.72	5.09	3.70	8.79
D-3	5.78	4.00	9.78	4.00	1.19	5.19
D-4	5.10	4.60	9.70	5.09	1.80	6.89

D-Anseriformes-noninfectious diseases

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
D-5	0.00	0.00	0.00	0.00	0.00	0.00
D-6	2.00	0.56	2.56	1.24	1.00	2.24
D-7	2.05	1.90	3.95	3.82	2.00	5.82
D-8	2.90	1.08	3.98	6.20	2.50	8.70
D-9	4.35	4.45	8.70	2.00	2.84	4.84
D-10	5.39	3.00	8.39	5.90	4.00	9.90
D-11	2.56	2.30	4.86	1.67	1.29	3.96
D-12-16	1.85	1.60	3.45	7.90	1.75	9.65
D-17-21	4.50	2.10	6.60	7.82	2.00	9.82
D-22-26	4.35	3.50	7.85	7.81	2.10	9.91
D-27-31	4.70	4.39	3.00	5.90	4.00	9.90
D-32-36	4.89	3.00	7.89	7.00	2.89	9.89

E-Galliform-infectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
E-1	0.90	0.66	1.56	0.30	0.35	0.65
E-2	1.30	1.02	3.32	1.80	0.51	2.31
E-3	3.70	1.08	4.78	1.80	1.50	3.30
E-4	2.90	1.38	4.28	2.00	1.78	3.78
E-5	1.50	1.20	3.70	1.80	0.87	2.67
E-6	3.68	2.30	5.98	1.00	1.89	2.89

E-Galliform-noninfectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
E-7	0.00	0.00	0.00	0.00	0.00	0.00
E-8	0.30	0.02	0.32	1.00	0.31	1.31
E-9	0.68	0.30	0.98	0.90	0.40	1.30
E-10	0.10	0.18	0.28	0.98	0.20	1.18
E-11	0.90	0.29	1.19	1.07	1.00	2.07
E-12	2.00	1.98	3.98	4.30	1.02	5.32

F-Other infectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
F-1	1.00	0.56	1.56	0.30	0.35	0.65
F-2	1.20	1.90	3.10	1.11	1.20	2.31
F-3	2.03	2.00	4.03	1.80	1.50	3.30
F-4	2.97	1.10	4.07	2.78	1.00	3.78
F-5	5.00	5.07	10.07	4.07	4.60	8.67
F-6	5.82	4.50	10.32	6.48	2.12	8.60

F-Other Noninfectious disease

Slide	Kupffer cell			Hepatocyte		
	Area	Intensity	Total	Area	Intensity	Total
F-7	0.60	0.40	1.00	6.40	3.25	9.65
F-8	0.95	0.05	1.00	7.10	3.80	10.90
F-9	1.30	0.02	1.32	6.40	2.20	8.60

Appendix (A-13) Species of bird and diagnostic comments for the avian cases identified in the image analysis Tables (1) and (2).

Table (3) Experimental haemosiderosis , see section (5-3-1)

Slide	Chicken	Day following treatment
1-1	Group 1	1 day
1-2	Group 1	1 day
2-1	Group 2	2 days
2-2	Group 2	2 days
3-1	Group 3	6 days
3-2	Group 3	6 days
4-1	Group 4	10 days
4-2	Group 4	10 days

Table (4) Retrospective cases, see Table (2) and section (5-3-3)

A-Psittaciforms, infectious diseases

Slide	Species	Diagnostic comments
A-1	<i>Melopsittacus undulatus</i>	Pseudotuberculosis
A-2	<i>Polytelis anthopeplus</i>	Enteritis, colisepticaemia
A-3	<i>Polytelis anthopeplus</i>	Enteritis, colisepticaemia
A-4	<i>Polytelis anthopeplus</i>	Enteritis, colisepticaemia
A-5	<i>Polytelis anthopeplus</i>	Pneumonia, enteritis
A-6	<i>Nestor meridionalis</i>	Pneumonia
A-7	<i>Nestor meridionalis</i>	Aspergillosis
A-8	<i>Cyanoramphus x</i>	Pneumonia
A-9	<i>Cyanoramphus auriceps</i>	Aspergillosis
A-10	<i>Cyanoramphus unicolor</i>	Aspergillosis
A-11	<i>Cyanoramphus unicolor</i>	Aspergillosis
A-12	<i>Cyanoramphus unicolor</i>	Aspergillosis
A-13	<i>Cyanoramphus unicolor</i>	Aspergillosis
A-14	<i>Cyanoramphus unicolor</i>	Aspergillosis
A-15	<i>Tricoglossus haematodus moluccanus</i>	Pseudotuberculosis, intestinal parasites

A-Psittaciforms, non-infectious diseases

Slide	Species	Diagnostic comments
A-16	<i>Polytelis anthopeplus</i>	Trauma
A-17	<i>Strigops habitulus</i>	Trauma
A-18	<i>Strigops habitulus</i>	Aspiration pneumonia
A-19	<i>Strigops habitulus</i>	Aspiration pneumonia
A-20	<i>Cyanoramphus auriceps</i>	Trauma
A-21	<i>Cyanoramphus auriceps</i>	Trauma
A-22	<i>Cyanoramphus auriceps</i>	Starvation
A-23	<i>Nestor notabilis</i>	Aneamia, no diagnosis
A-24	<i>Nestor notabilis</i>	Trauma
A-25	<i>Nestor notabilis</i>	Anaemia, no diagnosis

B-Columbiforms, infectious and non-infectious diseases

Slide	Species	Diagnostic comments
B-1	<i>Hemiphaga novaeseelandiae</i>	Ingluveitis, gangrene
B-2	<i>Hemiphaga novaeseelandiae</i>	Ingluveitis
B-3	<i>Hemiphaga novaeseelandiae</i>	Pseudotuberculosis
B-4	<i>Hemiphaga novaeseelandiae</i>	Trauma
B-5	<i>Hemiphaga novaeseelandiae</i>	Starvation
B-6	<i>Hemiphaga novaeseelandiae</i>	Trauma
B-7	<i>Hemiphaga novaeseelandiae</i>	Starvation
B-8	<i>Hemiphaga novaeseelandiae</i>	Starvation

C-Native passeriforms, infectious disease

Slide	Species	Diagnostic comments
C-1	<i>Notiomystis cincta</i>	Aspergillosis
C-2	<i>Notiomystis cincta</i>	Aspergillosis
C-3	<i>Notiomystis cincta</i>	Aspergillosis
C-4	<i>Notiomystis cincta</i>	Aspergillosis, mites
C-5	<i>Notiomystis cincta</i>	Aspergillosis, mites
C-6	<i>Notiomystis cincta</i>	Aspergillosis, hepatitis
C-7	<i>Petroica australis</i>	Bacterial hepatitis
C-8	<i>Prosthemadera novaeseelandiae</i>	Bacterial hepatitis, pneumonia
C-9	<i>Prosthemadera novaeseelandiae</i>	Aspergillosis
C-10	<i>Prosthemadera novaeseelandiae</i>	Aspergillosis

C-Native passeriforms, non-infectious diseases

Slide	Species	Diagnostic comments
C-11	<i>Prothemadera novaeseelandiae</i>	Trauma
C-12	<i>Petroica australis</i>	Trauma
C-13	<i>Petroica australis</i>	Starvation
C-14	<i>Notiomystis cincta</i>	Trauma
C-15	<i>Notiomystis cincta</i>	Starvation
C-16	<i>Notiomystis cincta</i>	Poisoning
C-17	<i>Notiomystis cincta</i>	Aspiration pneumonia
C-18	<i>Notiomystis cincta</i>	Aspiration pneumonia
C-19	<i>Notiomystis cincta</i>	Exposure, chick
C-20	<i>Prothemadera novaeseelandiae</i>	Starvation, haemosiderosis
C-21	<i>Prothemadera novaeseelandiae</i>	Starvation, haemosiderosis
C-22	<i>Philesturnus carunculatus</i>	Starvation, haemosiderosis
C-23	<i>Philesturnus carunculatus</i>	Trauma, haemosiderosis
C-24	<i>Philesturnus carunculatus</i>	Hepatopathy, haemosiderosis
C-25	<i>Philesturnus carunculatus</i>	Hepatopathy, haemosiderosis

D-Introduced passeriforms, infectious and non-infectious diseases

Slide	Species	Diagnostic comments
C-26-30	<i>Passer domesticus</i>	Atoxoplasma
C-31-35	<i>Serinus canaria</i>	Pseudotuberculosis
C-36-41	<i>Fringillidae</i>	Pseudotuberculosis
C-42-46	<i>Passer domesticus</i>	Poisoning, enteritis
C-46-50	<i>Carduelis chloris</i>	Coccidiosis
C-51-55	<i>Passer domesticus</i>	Atotoxoplasma, poisoning
C-56-60	<i>Passer domesticus</i>	Pneumonia, atoxoplasma
C-61-65	<i>Sturnus vulgaris</i>	Trauma, starvation
C-66-70	<i>Turdus merula</i>	Trauma, starvation
C-71-75	<i>Turdus philomelos</i>	Trauma, starvation
C-76-80	<i>Passer domesticus</i>	No diagnosis, poisoning
C-81-85	<i>Fringillidae</i>	Poisoning
C-86-90	<i>Fringillidae</i>	Poisoning

D-Anseriforms, infectious and non-infectious diseases

Slide	Species	Diagnostic comments
D-1	<i>Anas platyrhynchos</i>	Bacterial hepatitis
D-2	<i>Branta canadensis</i>	Enteritis, hepatitis
D-3	<i>Cygnus australis</i>	Hepatitis, trauma
D-4	<i>Cygnus australis</i>	Hepatitis, pneumonia, trauma
D-5	<i>Anas platyrhynchos</i>	Trauma
D-6	<i>Hymenolaimus malacoryhchos</i>	Liver rupture
D-7	<i>Anas platyrhynchos</i>	Trauma
D-8	<i>Anas platyrhynchos</i>	Trauma
D-9	<i>Aythya novaeseelandiae</i>	Starvation
D-10	<i>Anas rhynchos</i>	Starvation
D-11	<i>Anas platyrhynchos</i>	Control bird
D-12-16	<i>Anas platyrhynchos</i>	Control bird
D-17-21	<i>Anas platyrhynchos</i>	Control bird
D-22-26	<i>Anas platyrhynchos</i>	Control bird
D-27-31	<i>Anas platyrhynchos</i>	Control bird
D-32-36	<i>Anas platyrhynchos</i>	Control bird

E-Galliforms, infectious and non-infectious diseases

Slide	Species	Diagnostic comments
E-1	<i>Gallus domesticus</i>	Egg peritonitis
E-2	<i>Gallus domesticus</i>	Coccidiosis
E-3	<i>Gallus domesticus</i>	Yolk sac infection
E-4	<i>Gallus domesticus</i>	Yolk sac infection
E-5	<i>Gallus domesticus</i>	Yolk sac infection
E-6	<i>Gallus domesticus</i>	Egg peritonitis
E-7	<i>Gallus domesticus</i>	Control bird
E-8	<i>Gallus domesticus</i>	Control bird
E-9	<i>Gallus domesticus</i>	Control bird
E-10	<i>Gallus domesticus</i>	Control bird
E-11	<i>Chrysolophus pictus</i>	Ovarian carcinoma
E-12	<i>Chrysolophus pictus</i>	Carcinoma

F-Other species, infectious and non-infectious disease

Slide	Species	Diagnostic comments
F-1	<i>Apteryx oweni</i>	Aspergillosis
F-2	<i>Megadyptes antipodes</i>	Bacterial hepatitis, oil ingestion
F-3	<i>Eudyptula minor</i>	Bacterial hepatitis, ophthalmitis
F-4	<i>Puffinus griseus</i>	Pneumonia, poisoning
F-5	<i>Porphyrio mantelli</i>	Focal hepatitis
F-6	<i>Pelagodroma marine maesnaine</i>	Focal hepatitis
F-7	<i>Gallirallus australis</i>	Siderosis
F-8	<i>Gallirallus australis</i>	Siderosis, visceral gout
F-9	<i>Gallirallus australis</i>	Siderosis, visceral gout

Appendix (A-14) Statistical analysis of the scintillation count data from the phagocytosis assay and calibration of the scintillation system described in chapter seven

Table (1) Phagocytosis assay, section (7-2-1); *Yersinia frederiksenii*

Time	Number	Statistical variables			
		Mean	SD	Variance	CSS
0	5	41.8	2.59	6.7	26.8
60	5	599	11.36	129	516
120	5	249.4	37.79	1427.8	5711.2
180	5	282	9.14	83.5	334
240	5	209	7.58	57.3	230
X	5	640	43.45	1887.5	7550

Table (2) Phagocytosis assay, section (7-2-1); *Y. frederiksenii* with iron loaded phagocytic cells

Time	Number	Statistical variables			
		Mean	SD	Variance	CSS
0	5	25	1.58	2.5	10
60	5	401	22.57	509.5	2038
120	5	384	16.88	285	1140
180	5	345	20.53	421.5	1686
240	5	300	10.49	110	440
X	5	640	43.45	1887.5	7550

Table (3) Phagocytosis assay, section (7-2-1); *Yersinia pseudotuberculosis*

Time	Number	Statistical variables			
		Mean	SD	Variance	CSS
0	5	31	8.03	64.5	258
60	5	240	21.24	451	1804
120	5	323	26.1	679.5	2718
180	5	264	2.74	7.5	30
240	5	150	26.68	712	2848
X	5	659	89.40	7991.5	31966

Table (4) Phagocytosis assay, section (7-2-1) *Y. pseudotuberculosis* with iron loaded macrophages

Time	Number	Statistical variables			
		Mean	SD	Variance	CSS
0	5	33	5.7	32.5	130
60	5	617	70.57	4980.5	19922
120	5	675	31.33	981.5	3926
180	5	542	84.00	7048	28192
240	5	470	47.02	2210.5	8842
X	5	659	89.40	7991.5	31966

Table (5) Calibration and standardisation of the scintillation counting system for *Yersinia pseudotuberculosis*.

Number of bacteria added (log 10)							
8	7	6	5	4	3	2	Blank
650	840	356	148	90	31	38	33
663	872	360	140	78	34	35	32
655	970	357	143	81	40	38	29
640	700	300	152	67	23	23	36
648	978	407	145	104	47	56	35

Table (6) Calibration and standardisation of the scintillation counting system for *Yersinia pseudotuberculosis* with iron dextran added to the medium.

Number of bacteria added (log 10)							
8	7	6	5	4	3	2	Blank
1110	1261	690	240	95	43	20	28
905	1050	580	265	101	44	25	30
1260	969	578	258	90	45	26	32
1265	1110	683	231	81	40	24	28
1135	1120	899	226	123	46	20	24

Table (7) Calibration and standardisation of the scintillation counting system for *Yersinia frederiksenii*

Number of bacteria added (log 10)							
8	7	6	5	4	3	2	Blank
750	875	350	151	80	51	36	41
769	798	348	154	91	38	37	39
718	810	352	162	78	39	29	38
691	898	351	172	81	43	30	45
842	994	359	126	80	41	48	42

Table (8) Calibration and standardisation of the scintillation counting system for *Yersinia frederiksenii* with iron dextran added to the medium.

Number of bacteria added (log 10)							
8	7	6	5	4	3	2	Blank
960	912	580	240	90	25	20	21
900	913	578	242	89	35	21	23
898	952	567	198	78	26	21	30
873	894	542	230	91	21	36	31
1189	909	668	295	122	33	32	15

Appendix (A-15) Statistical analysis of the colony counts for *Y. pseudotuberculosis* and *Y. frederiksenii* obtained in the serum assay described in chapter seven.

Serum experiment (see section 7-2-2): *Yersinia pseudotuberculosis* 37 °C

Variable	N	Statistical values			
		Mean	SD	Var	CSS
MEM	5	168	53.3	2846	11384
MEM + serum (S)	5	128	38.8	1506.5	6026
MEM + serum (heated)	5	140	41.7	1739.5	6958
MEM + hyperimmune S	5	49	8.7	76.5	306
MEM + hyperimmune (heated) S	5	100	29.0	841.5	3366
MEM + S + iron dextran	5	160	32.1	1030	4120
MEM + S + Desferal (Si)	5	54	11.8	138.5	554
MEM + S + iron dextran + De (Si)	5	162	35.1	1232.5	4930

Serum experiment (see section 7-2-2): *Yersinia pseudotuberculosis* 28 °C

Variable	N	Statistical values			
		Mean	SD	Var	CSS
MEM	5	540	46.7	2184	8736
MEM + Serum	5	456	47.2	2232	8928
MEM + Serum (heated)	5	469	35.1	1238	4952
MEM + Hyperimmune S	5	397	28.8	831.5	3326
MEM + Hyperimmune (heated) S	5	417	42.0	1767.5	7070
MEM + S + Iron dextran	5	462	40.9	1669	6676
MEM + S + Desferal (Si)	5	424	41.8	1748.5	6994
MEM + S + Iron dextran + De (Si)	5	466	39.6	1571	6284

Serum experiment, see section (7-2-2); *Yersinia frederiksenii* 37 °C

Variable	N	Statistical values			
		Mean	SD	Var	CSS
MEM	5	231	47.4	2246.5	8986
MEM + Serum	5	104.8	32.1	1033.2	4132.8
MEM + Serum (heated)	5	162.4	30.5	931.3	3725.2
MEM + Hyperimmune S	5	50.2	11.0	121.2	484.8
MEM + Hyperimmune (heated) S	5	164	39.8	1587.5	6350
MEM + S + Iron dextran	5	176	42.1	1772	7088
MEM + S + Desferal (Si)	5	98	31.1	967.5	3870
MEM + S + Iron dextran + De (Si)	5	151.6	40.8	1662.3	6649.2

Serum experiment, see section (7-2-2); *Yersinia frederiksenii* 28 °C

Variable	N	Statistical values			
		Mean	SD	Var	CSS
MEM	5	850	51.8	2688	20752
MEM + Serum	5	456	44.0	1938.5	7754
MEM + Serum (heated)	5	454	33.6	1130	4520
MEM + Hyperimmune S	5	335	30.7	943	3772
MEM + Hyperimmune (heated) S	5	375	37.4	1400	3600
MEM + S + Iron dextran	5	756	44.7	2000.5	8002
MEM + S + Desferal (Si)	5	486	45.2	2046	8184
MEM + S + Iron dextran + De (Si)	5	743	48.2	2323	9292

Appendix (A-16) Optical density values obtained in the 'indirect' LPS-ELISA for sera collected from chickens challenged with *Y. pseudotuberculosis* serotype II

Table (1) Optical density values obtained in the 'indirect' LPS-ELISA for sera collected from the chickens described in section 3-3-5 figure (3-8)

Day after challenge	Dose of bacteria (log ₁₀)					Control
	8	7	6	5	4	
0	0.231	0.134	0.143	0.146	0.216	0.297
	0.245	0.234	0.258	0.165	0.256	0.177
	0.132	0.176	0.198	0.164	0.156	0.159
	0.143	0.348	0.276	0.153	0.193	0.096
	0.145	0.132	0.196	0.128	0.165	0.109
2	0.381	0.392	0.224	0.287	0.289	ND
	0.256	0.347	0.232	0.311	0.345	
	0.363	0.392	0.225	0.247	0.349	
	0.479	0.435	0.384	0.395	0.282	
	0.277	0.374	0.600	0.245	0.231	
4	0.695	0.625	0.389	0.349	0.333	ND
	0.659	0.519	0.468	0.428	0.366	
	1.132	0.625	0.449	0.480	0.385	
	0.832	0.577	0.535	0.733	0.294	
	0.845	0.632	0.726	0.602	0.245	
6	0.762	0.923	0.460	0.378	0.348	ND
	0.702	1.643	0.563	0.489	0.387	
	1.245	0.584	0.612	0.534	0.371	
	1.378	0.668	0.644	0.789	0.492	
	0.917	0.787	0.671	0.724	0.367	
8	0.932	1.365	0.561	0.282	0.594	ND
	0.786	1.749	0.836	0.375	0.435	
	1.321	0.783	0.781	0.504	0.487	
	1.382	0.835	1.049	0.798	0.529	
	0.910	0.831	0.703	0.702	0.451	
10	0.981	1.351	0.761	0.324	0.587	0.333
	0.761	1.820	0.896	0.346	0.526	0.146
	1.347	0.912	0.952	0.833	0.512	0.165
	1.397	0.965	1.225	0.744	0.531	0.103
	0.890	0.934	0.897	0.582	0.454	0.156
15	0.943	0.891	0.863	0.584	0.592	ND
	0.756	0.937	0.968	0.349	0.534	
	0.981	0.927	0.983	0.593	0.523	
	0.821	0.874	0.965	0.349	0.567	
	0.672	0.548	0.945	0.581	0.455	
20	0.922	0.756	0.853	0.566	0.620	0.351
	0.735	0.787	0.872	0.350	0.561	0.145
	0.981	0.785	0.982	0.621	0.543	0.136
	0.921	0.758	0.911	0.436	0.541	0.123
	0.659	0.642	0.923	0.585	0.413	0.172

ND = Not done

Table (2) Optical density values obtained in the 'indirect' LPS-ELISA for sera collected from the chickens described in section 6-3-3, figure (6-1)

Day after challenge	Treatment groups				
	Iron dextran	Desferal	Iron dextran/Desferal	Dextran	Control
0	0.251	0.245	0.224	0.199	0.203
	0.216	0.232	0.222	0.211	0.179
	0.266	0.245	0.256	0.229	0.197
	0.222	0.265	0.254	0.231	0.183
	0.293	0.212	0.293	0.173	0.131
6	0.357	0.338	Died	0.259	ND
	0.392	0.374	0.346	0.263	
	0.420	0.416	0.398	0.331	
	0.373	0.376	0.448	0.331	
	0.394	0.392	0.332	0.263	
10	0.582	0.585	Died	0.456	ND
	0.665	0.529	0.531	0.447	
	0.754	0.645	0.594	0.434	
	0.594	0.477	0.527	0.436	
	0.584	0.443	0.456	0.453	

ND = Not done