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FAECAL AND TONSILLAR CARRIAGE OF *YERSINIA* SPP. BY PIGS

A DISSERTATION PRESENTED IN PARTIAL FULFILMENT (25 %) OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY STUDIES IN VETERINARY PUBLIC HEALTH AT MASSEY UNIVERSITY

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MAY, 1997

ABSTRACT

Pigs are considered important reservoirs for pathogenic *Yersinia* spp. which cause disease in humans. Results of many surveys have demonstrated the common occurrence of *Y. enterocolitica* and related species in the tonsils and intestinal tract of healthy slaughter-age pigs. However, the source of infection remains unclear. There is a dearth of information as to when pigs become infected hence, there is a need to conduct further studies not only among slaughter pigs but also among pigs belonging to different age groups on the farm. This study was conducted to determine the faecal and tonsillar carriage of different *Yersinia* spp. by pigs belonging to different age groups, characterise the species isolated by biochemical and serological methods, and determine the *in vitro* virulence characteristics of the isolates by simple assays.

The study was conducted in two phases: first on a known or suspect *Y. enterocolitica*-positive farm within Massey University and second in a slaughterhouse where pigs from the suspect farm are sent for slaughter. A total of 54 faecal samples were collected from pigs belonging to six different age groups including 3-4 days, 2 weeks, 3 weeks, 4 weeks, 8 weeks and 12 weeks together with samples from the sow and one pooled sample from the floor. All of the 54 faecal samples collected were found negative for *Yersinia* spp. after the 21-day cold enrichment, subsequent plating onto CIN agar and use of primary screening tests. In the second phase, a total of 50 samples representing 25 faecal and 25 palatine tonsils were collected from slaughter pigs with ages ranging from 20-24 weeks. Out of the 25 faecal samples, there were two isolates of *Yersinia frederiksenii* and one isolate of the unusual or new biotype of *Yersinia enterocolitica.* Of the 25 samples of palatine tonsils collected, three of the isolates were unusual strains of *Y. enterocolitica* and two were *Y. pseudotuberculosis* Group I or serotype I. All isolates were confirmed by biochemical tests. The two isolates of *Y. pseudotuberculosis* were confirmed as Group I by serological tests. In this study, sample collections were done in the months of August and October when Group I was isolated conforming to the results of De Allie (1994) when serotype I was found only in winter and spring.

The pathogenic potential of the eight isolates of *Yersinia* spp. were tested for *in vitro* virulence associated characteristics using the four simple tests namely salicin fermentation/aesculin hydrolysis, D-xylose fermentation, pyrazinamidase (PYZ) test and Congo Red-magnesium oxalate (CR-MOX). In this study, the unusual strains of *Y. enterocolitica* isolated from the

tonsils and faeces were salicin-aesculin positive, xylose-positive, CR-MOX negative and PYZ negative. Except for PYZ, all three tests suggest the non-pathogenic potential of this unusual strain of *Y. enterocolitica.* The pathogenic potential of *Y. pseudotuberculosis* Group I isolated from the pig tonsils was shown by CR-MOX and PYZ tests. Both isolates were CR-MOX positive and PYZ negative. *Y. pseudotuberculosis* is considered a potential pathogen as shown by *in vitro* virulence tests and should not be overlooked as a causative of human disease.

It was shown in this study that not all pigs are infected with pathogenic and non-pathogenic species of *Yersinia* even if they have been established to harbour the organisms either in their tonsils or faeces. However, pigs sent for slaughter may carry *Yersinia* spp. in their tonsils or faeces suggesting the role of other factors such as management and husbandry practices or contact with other animals during lairage, or from possible cross-contamination during processing at the slaughterhouse. Slaughter pigs are important source of *Y. enterocolitica* infections in humans, hence it is important to prevent or reduce the risk of possible contamination in the slaughterhouse by these organisms. Further investigations are needed to determine the pathogenic potential of the unusual strains of *Y. enterocolitica* and its possible role as a causative agent in human disease. Since *Yersinia* spp. were not recovered from faecal samples of pigs in the farm belonging to different age groups in this study, there is a need to conduct further studies using tonsillar samples or swabs instead of faeces. As shown in this study and other published reports, tonsils yield more isolates of *Yersinia* spp. compared to faecal samples.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the Ministry of Foreign Affairs and Trade (MFAT) for the New Zealand Official Development Assistance-Postgraduate Scholarships (NZODA-PGS) which enabled me to pursue a Masters degree. I also give thanks to the staff of the International Students' Office especially to Ms. Dianne Fountaine-Cody.

I am greatly indebted to Prof. Colin R. Wilks for giving me the opportunity to study and pursue this quest for knowledge. His invaluable guidance, moral support, encouragement and supervision has contributed tremendously to my professional career and development.

I would also like to thank my supervisors, Mr. Per Madie and Dr. Jane Hunter for their outstanding roles in guiding me throughout the study period. I can't thank them enough for their guidance, patience and encouragement in helping me with this study.

I am also very thankful to Mr. Stan Fenwick, a *Yersinia* expert for his valuable contribution, comments and suggestions.

With utmost gratitude and appreciation, I would also like to acknowledge the following:

Dr. Barb Frey for her assistance in helping me to organise the collection of samples at the study farm.

Mr. Peter Wildbore, Ms. Jan Schrama, Ms. Magda Gwodz, Ms. Kylie Walker, Ms. Pam Slack, Ms. Pat Davey and Mr. Faris Sharpe for their friendly assistance during my study.

The two other members of the "Three Musketeer's": Kyung Ho Shin and Durai Subramaniam for their help especially during sample collection.

Mr. Paul Lysagt and the staff of National Pig Breeding Center for their kind permission to collect samples from the farm

I would also like to acknowledge the productive and enjoyable interactions I had with the staff and postgraduate students in the Department of Veterinary Pathology and Public Health and wish to extend special recognition to Assoc. Prof. Maurice R. Alley, Dr. Mark Collett, Dr. Keith Thompson, Dr. John Lumsden, Dr. Joanne Meers, Mr. Stan Fenwick, Dr. Jane Hunter, Assoc. Prof. Tony Charleston, Dr. Bill Pomroy, Mr. Neil Christensen, Prof. Colin R. Wilks and Mr. Per Madie for providing an opportunity to study Diagnostic Pathology, Public Health and Meat Hygiene. It has been a great pleasure for me to work with such people full of dedication towards achieving excellence.

Mrs. Allain Scott, Mrs. Wendy Graham and Ms. Nicola Purdue-Smith for their administrative assistance.

Special thanks is also given to Dr. Heather Simpson for being an adviser who always have a cheerful smile.

I especially wish to acknowledge my husband, Everest for his love, understanding, encouragement, patience, moral support, sense of humour and also for keeping our household in good order while I was busy working with my dissertation. Also to our two daughters, Resalice Anne and Raisa Dana Marie for their love and understanding.

I am also thankful to my foster parents here in New Zealand, Papa Ian and Mama Ruth Mills, and also to our friends, Vince and Heather Norrish for making New Zealand our home away from home.

To my parents, brothers, sisters, in-laws and friends in the Philippines for all the moral support, confidence and believing in my capabilities to pursue this graduate study.

Lastly, I am offering my work to the Lord God Almighty for making all things possible.

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CHAPTER ONE

REVIEW OF LITERATURE

Section A: The Genus *Yersinia*

INTRODUCTION

The genus *Yersinia* comprises a group of Gram-negative, facultatively anaerobic bacilli that share a number of common biochemical, morphological and serological features with other genera of the family *Enterobacteriaceae*. There are at present 11 species recognised within the genus, three of which (*Yersinia pestis, Y. pseudotuberculosis, Y. enterocolitica)* have been shown to be human pathogens (Cornelis *et al.* 1987; Wauters *et al.* 1988; Bissett *et al* 1990) and are classified as the three most important yersiniae (Robins-Browne and Hartland, 1991). *Y. pestis* is the causative agent of bubonic plague (Black Death). The term yersiniosis now refers to infections caused by either *Y. enterocolitica* or *Y. pseudotuberculosis,* the age-old name "plague" being reserved for the disease caused by *Y. pestis* (Mair and Fox, 1986). Both *Y. pseudotuberculosis* and *Y. enterocolitica* in man and animals produce similar clinical signs which are gastrointestinal infections, septicaemia, erythema nodosum, mesenteric lymphadenitis, and reactive polyarthritis. The other strains previously known as "*Y. enterocolitica*-like organisms" are now classified as 7 separate species namely: *Y. frederiksenii, Y. kristensenii, Y. intermedia, Y. aldovae; Y. rhodei, Y. mollaretii* and *Y. bercovieri* (Aleksic *et al.* 1987; Bercovier *et al.* 1984; Brenner *et al.* 1980; Wauters *et al.* 1988). One other species, *Y. ruckeri,* a fish pathogen responsible for red mouth disease in rainbow trout and other fish (Aleksic *et al.* 1987) has been delineated as a separate species. It has only been found in North America and has not been implicated in human disease (Mair and Fox, 1986). Biochemically, *Y. pseudotuberculosis* is a remarkably homogeneous species whereas *Y. enterocolitica* and the former *Y. enterocolitica*-like bacteria constitute a fairly heterogeneous group of bacteria including both well established pathogens and a range of environmental strains ubiquitous in terrestrial and freshwater ecosystems (Bryant, 1983; Kapperud, 1991).

The pathogenic significance of *Y. enterocolitica* is mainly associated with a few serogroups. There is a strong indirect evidence that pigs and food products of porcine origin are the major sources of human infection. Porcine and human strains have been found indistinguishable by

biochemical, serological and phage-typing methods (Hurvell, 1981; Wauters, 1979) hence, pigs are suspected of being the major source of infection for man. *Y. enterocolitica* serogroups 0:3 and 0:9 which have been isolated from pigs are the dominant human pathogens in most parts of the world (Kapperud, 1991; Wauters, 1981). *Y. pseudotuberculosis* although relatively uncommon compared to *Y. enterocolitica,* exhibits similar clinical characteristics in humans. The diseases caused by *Y. pseudotuberculosis* and *Y. enterocolitica* can be regarded as manifestations of a number of primary, acute infections and secondary, immunological complications (Mair and Fox 1986).

HISTORY AND GEOGRAPHICAL DISTRIBUTION

a. *Yersinia enterocolitica*

Yersinia enterocolitica was first associated with human disease in 1939 by Schlefstein and Coleman (as cited in Schiemann 1989) when they described five cultures of bacteria isolated from human infections. Two of the cultures were isolated from facial lesions and three from the intestines of patients with enteritis. They thought the bacteria resembled *Bacillus lignieresi* and *Pasteurella pseudotuberculosis*. Over the next 20 years, there were sporadic reports on the isolation of similar organisms which was then called *Bacterium enterocoliticum* mostly from children with enteritis (Schiemann, 1989). In Europe, the first recognition of human infections with a bacterium that resembled but yet differed in several aspects from *Pasteurella pseudotuberculosis* was made by Hassig *et al.* in 1949 (as cited in Schiemann 1989). Several additional isolations were then reported in which various names were given to the isolated organism (Pasteurella pseudotuberculosis b, Pasteurella X and "Germe X"). The genus *Yersinia* had been proposed to honour the French bacteriologist Yersin, who in 1894 had isolated the plague bacillus during an epidemic in Hong Kong (Schiemann, 1989). *Yersinia enterocolitica* was officially named by Frederiksen in 1964 (Ostroff, 1995). During the last half of the same decade in which the bacterium was named, many reports appeared on the association of this organism with human disease especially enterocolitis. From then on, the organism was isolated with increasing frequency from man and animals and from some foods for human consumption. By 1976 it had become a cause for some concern in both human and veterinary medicine (Morris and Feeley, 1976).

The widespread nature of *Y. enterocolitica* has been well documented. By the mid 1970s, Mollaret *et al.*(1979) had compiled reports of isolates from 35 countries on six continents. The geographical distribution of *Y. enterocolitica* isolation rapidly expanded with reports from Sweden (Winblad *et al.* 1966), Finland (Ahvonen, 1973; Toivanen *et al.* 1973), Belgium (Vandepitte *et al.* 1973; Wauters, 1970), Denmark (Christensen, 1987; Jensen *et al.* 1995), Japan (Zen-Yoji and Maruyama, 1972), Hungary (Szita *et al.* 1973), Czechoslovakia (Rakovsky *et al.* 1973), Africa (Agbolanhor *et al.* 1983), South Africa (Rabson *et al.* 1973), Israel (Shmilovitz and Kretzer, 1978), Canada (Albert and Lafleur, 1971; Lafleur and Martineu, 1973), USA (Bissett, 1976; Weaver and Jordan, 1973; Snyder *et al.* 1982;), UK (Cover and Aber, 1989), Norway (Kapperud, 1986), Australia (Steele and Dermott 1979; Marriott, 1985, 1987), Italy (Chiesa *et al.* 1987; Mingrone *et al.* 1987) and New Zealand (McCarthy and Fenwick, 1990).

According to Mollarett *et al.*(1979), a correlation exists between geographic areas and the serogroups associated with disease in man. There is a distinct geographical distribution of serotypes and biotypes of *Y. enterocolitica* with O:3 and O:9 being the known prevalent pathogenic strains for humans in Europe, Canada and Japan and serotype O:8 in the USA (Bissett *et al.* 1990). Later however, there is an emergence of serotype O:3 over O:8 in human *Y. enterocolitica* infections where the serotypes in fact accounts for the majority of the isolates (Anon., 1990; Ostroff, 1995). Although the reasons are unclear, serogroup shifts are most unique to the United States. In the latter part of the 1980's increases in *Y. enterocolitica* O:9 infections were noted in Great Britain (Prentice *et al.* 1991) and serogroup O:8 has appeared in France, Italy and more recently in Japan (Chiesa *et al.* 1985; Ichinoe *et al.* 1991; Chiesa *et al.* 1991a; Chiesa *et al.* 1991b).

Yersinia enterocolitica infection has become recognised as a major cause of gastro-enteritis in the developed world, particularly in Northern Europe (Cover and Aber, 1989; Black and Slome, 1988). In other parts of Europe and in Japan and Canada, the isolation rate of this bacterium from patients with gastro-enteritis rival those of *Salmonella* (Christensen, 1987; de Groote *et al.* 1982; Hiroshi, 1981; Marks *et al.* 1980; Van Noyen *et al.* 1981). The disease is uncommon in tropical and developing countries (Robins-Browne, 1992), however, there are reports of isolation of *Y. enterocolitica* from pigs in Nigeria (Adesiyun *et al.*1986), and Singapore (Ho and Koh, 1981), and from pigs and humans in Nigeria (Agbonlahor *et al.* 1985;

Ikheloa *et al.* 1992; Lombin *et al.* 1985; Okoroafor *et al.* 1988; Samadi *et al.* 1982). The first report of the isolation of the organism in Trinidad and possibly the West Indies was done by Adesiyun *et al.* 1992. In Latin America, yersiniosis has been reported from Brazil and Argentina involving both *Y. enterocolitica* and *Y. pseudotuberculosis* infection (Falcao 1981, 1987; Mollaret *et al.* 1979).

b. *Yersinia pseudotuberculosis*

Diseases due to *Yersinia pseudotuberculosis* has been recognised since the end of the 19th Century, when in 1883 Malassez and Vignal (cited in Schiemann 1989) isolated the organism from a guinea pig inoculated with pus from a child with tuberculous meningitis. This Gram-negative coccobacillus was then described in more detail and was named *Bacillus tuberculosis rodentium* in 1889 by Pfeiffer. And because of frequent association with guinea pigs used in tuberculosis research, the species designation *"pseudotuberculosis"* came into use (Schiemann, 1989). The first human isolation of *Y. pseudotuberculosis* was done by Saisawa in 1908 (cited in Tsubokura et al. 1970) from a patient with septicaemia. The bacterium was known under various names including *Pasteurella pseudotuberculosis* for nearly 50 years. The organism was considered a pathogen in a variety of animal species. (Tsubokura *et al.* 1970). However, in the early 1950s that status changed abruptly with the reports by Masshof in 1953 and Masshof and Dolle (1953) who isolated this micro-organism from lymph nodes in children undergoing surgery for appendicitis and described the bacteria as the cause of an "abscess-forming reticulocytic lymphadenitis." In 1954, Knapp and Masshoff, confirmed their association with *Y. pseudotuberculosis* infection. Cases were then diagnosed in Germany which were mostly done by serologic means. Subsequent reports of hundreds of cases originated primarily in Europe (Schiemann, 1989). In 1962, Daniels suggested that the organism was responsible for the mesenteric lymphadenitis often observed at laparotomy where the appendix appeared normal. Since then, *Y. pseudotuberculosis* has been associated with a variety of human diseases including mesenteric lymphadenitis, terminal ileitis, arthritis and septicaemia (Mollarett, 1965).

The incidence of human infections with *Y. pseudotuberculosis* has remained far lower in North America than in Europe (Finlayson, 1971; Hubbert *et al.* 1971). Several large outbreaks have also been reported in Japan since 1981 (Inoue *et al.* 1984; Sanbe *et al.* 1987). These reports implicated foods as the vehicles of transmission, however, the foods

responsible were not bacteriologically identified. *Y. pseudotuberculosis* is a well recognised animal pathogen which causes zoonotic infections (Mair, 1975; Paff *et al.* 1976; Saari and Triplett, 1974). The organism has a world-wide distribution and affects a variety of animals including rodents, lagomorphs, carnivores, primates and a wide range of birds (Parsons, 1991). Pseudotuberculosis has also been reported in captive animals in zoological collections (Keymer, 1976; Obwolo, 1976; Baskin *et al.* 1977; Jones 1980), most commonly in birds, rodents and primates. Several studies done in Europe, Canada and Japan have shown that pigs are important reservoirs of *Y. pseudotuberculosis* (Narucka and Westendoorp, 1977; Toma and Deidrick, 1975; Tsubokura *et al.* 1976; Zen-Yoji *et al.* 1974).

GENERAL CHARACTERISTICS OF *YERSINIA* SPP.

The bacterium

The genus *Yersinia* shares many characteristics of the family *Enterobacteriaceae.* They are straight rods or coccobacilli, approximately 0.5-0.8 μm in diameter and 1.3 μm in length, often pleomorphic, Gram-negative, facultatively anaerobic, oxidase-negative and catalase-positive. The organisms can grow on simple media. They are also able to ferment glucose and other carbohydrates with acid production but little or no gas. Phenotypic characteristics are often temperature-dependent, e.g. the organisms are generally non-motile when grown at 37 ° C but motile with peritrichous flagella when grown below 30 ° C (except for *Y. pestis* which is always non-motile). Cultures grown at 25 ° C show peritrichous flagella with one to 18 flagella per cell while few or no flagella are observed in cultures grown at 37 ° C (Nilehn, 1969). An interesting characteristic of most *Yersinia* spp. is the temperature-dependent expression of a number of phenotypic traits such as motility, biochemical properties and virulence-associated markers (Bottone, 1977). In general, more characteristics are expressed by cultures incubated at 25-29 ° C than at 35-37 ° C (Bercovier and Mollaret, 1984).

Biochemically, strains of *Y. enterocolitica* are heterogeneous. Typical strains of *Y. enterocolitica* ferment sucrose but cannot utilise rhamnose, citrate, α-methyl glucose or melibiose (Bottone, 1977). Atypical strains of *Y. enterocolitica* may be divided into four major groups, two of which are sucrose-negative and two of which are rhamnose-positive (Swaminathan *et al.* 1982). On the other hand, *Y. pseudotuberculosis* is a homogeneous species (Mair and Fox, 1986).

Growth requirements

Yersinia enterocolitica is not a nutritionally fastidious organism when grown at 28 °C but may require additional factors for growth at 37 °C (Swaminathan *et al.* 1982). It can grow fairly well on simple media such as nutrient agar becoming confluent within 24 hours. Poor growth on selective media at 35-37 °C is due primarily to increased toxicity of selective agents, and an additional requirement for calcium by plasmid-positive strains (Schiemann, 1989). *Y. enterocolitica* will grow in a simple glucose salts medium but very slowly. The pH range for the survival and growth of *Y. enterocolitica* appears to be 4.6-9.0 (Hanna *et al.* 1977; Stern *et al.* 1980) with the optimum range being pH 7.0-8.0 (Hanna *et al.* 1979). At 4 °C, strains of *Y. enterocolitica* grow slowly at pH values of 5.2 and 5.4 but show heavy growth at pH 5.6-7.6 (Seelye and Yearbury, 1979). Falcao *et al.* (1979) suggested a pH range between 7.6 to 7.9 for optimum growth. *Y. enterocolitica* is a psychrotroph, and this property has provided the most successful basis for isolation of low numbers from mixed cultures present in foods, water and faeces. The success of low-temperature enrichment is not because all other bacteria stop growing or die, but because the growth rates of microbial antagonists are slowed relative to that of *Yersinia enterocolitica* (Schiemann, 1989).

Burrows and Gillett (1966) described the minimal nutritional requirements for growth of *Yersinia pseudotuberculosis*. At 28 °C, *Y. pseudotuberculosis* required no supplement or either thiamine or pantothenate for growth but needed additional nutritional requirements at 37 °C. For *Y. pseudotuberculosis* these were any three or four factors; glutamic acid, thiamine, cystine and pantothenate (Schiemann, 1989).

Both *Y. enterocolitica* and *Y. pseudotuberculosis* can survive better under alkaline conditions than any other Gram-negative bacterium (Aulisio *et al.* 1980; Schiemann, 1983).

Y. enterocolitica is remarkably tolerant to bile salts and other surface active agents (Schiemann, 1980). This tolerance appears to be greater in pathogenic than in non-pathogenic serovars which is an added advantage in isolation media containing bile salts.

Y. enterocolitica is unable to survive heat treatment for 3 minutes at 60 °C (Hanna *et al.* 1977). and is destroyed by standard pasteurisation methods (Francis *et al.* 1980; Hanna *et al.* 1977). The organism appears to withstand freezing well, with numbers decreasing only slightly in chicken meat after 90 days storage at -18 °C (Leistner *et al.* 1975). Hanna *et al.*

(1977) however, found that atypical strains died rapidly during frozen storage of inoculated beef. *Y. enterocolitica* has also been reported to grow and survive for long periods in water and can survive on the outside of milk cartons for more than 21 days (Stanfield *et al.* 1985). There are recent studies which also indicate that *Y. enterocolitica* can proliferate in blood donated for transfusion and stored at +4 °C for more than 3 weeks (Ardiuno *et al.* 1989; Jones *et al.* 1993).

Isolation and Identification

Isolation

Yersinia spp. have been isolated from a wide variety of biological samples obtained from animals, the environment and foods. For many years the majority of *Yersinia* isolates was obtained from animals following necropsy. However, within the last decades, an increasing number of strains have been isolated by workers investigating the carriage of *Yersinia* spp. in apparently healthy domestic and wild animals (Toma and Deidrick, 1975; Kapperud, 1975, 1977, 1981; Brewer and Corbel, 1983). Samples from animals include faeces and caecal contents (Mair and Fox, 1986), throat and rectal swabs from pig carcasses (Greenwood and Hooper, 1985), palatine tonsils from pigs (Hanna *et al.* 1980; Hunter *et al.* 1983; Nesbakken and Kapperud, 1985; De Boer *et al.* 1986). Environmental studies have shown that *Y. enterocolitica* and its related species can be readily isolated from surface and drinking water and from sewage sludge (Saari and Jansen, 1979; Langeland, 1983) while the presence of *Y. pseudotuberculosis* can be demonstrated on the surface and substratum of soil (Barre *et al.* 1977). These organisms have also been isolated from a wide variety of foodstuffs including poultry products and beef (Leistner *et al.* 1975) , pig tongues (Wauters and Janssens, 1976), meat (Hanna *et al.* 1979) and milk (Aldova *et al.* 1975; Schiemann and Toma, 1979). Other samples would include pathological samples such as pus, blood, lymph nodes and faeces (Carniel and Mollaret, 1990).

Cultural methods for the isolation of *Yersinia enterocolitica* from clinical and environmental samples are similar to those commonly used for the isolation of other enteric organisms of public health significance. These include enrichment in liquid media, isolation of pure cultures on selective differential plating media and identification of isolates by biochemical and serological tests (Swaminathan *et al.*1982).

Among the enteric pathogens, *Y. enterocolitica* is unique because of its ability to grow at 4 °C. This property has been utilised by several investigators for the selective enrichment of *Y. enterocolitica* from foods, clinical specimens and environmental samples (Otsuki *et al.* 1973; Tsubokura *et al.* 1973, 1975, 1976; Zen-Yoji *et al.* 1974; Eiss 1975; Greenwood *et al.* 1975; Inoue and Kurose 1975; Leistner *et al.* 1975; Toma and Deidrick 1975; Barre *et al.* 1976; Feeley *et al.* 1976; Hanna *et al.* 1976; Pedersen 1976; Lee 1977).

Faeces is the most common type of clinical specimen examined for the presence of *Y. enterocolitica* (Schiemann, 1989) and can be best recovered from specimens that are incubated at 25 °C or lower. Cold enrichment of highly contaminated specimens such as faeces, and incubating cultures at 4 °C for 1 to 3 weeks in phosphate-buffered saline (PBS) prior to subculture onto enteric media also enhances the recovery of *Y. enterocolitica* (Pai *et al.* 1979). Weissfeld and Sonnenwirth (1982) reported that pre-treatment of faeces with 0.5 % potassium hydroxide at a ratio of 1:2 for 2 minutes, followed by plating onto enteric media resulted in the recovery of the highest number of *Yersinia* spp. isolates. Thal (1978) and Bissett (1981) have presented more detailed reviews on the isolation and identification of *Y. pseudotuberculosis.* As in the case of *Y. enterocolitica,* cold enrichment has been useful for isolating *Y. pseudotuberculosis* (Oberhofer and Podgore 1980 as cited in Schiemann 1989).

Enrichment of food samples, like faecal specimens, has emphasised the use of low temperature enrichment (Schiemann, 1989). Aulisio (1980) observed that *Y. enterocolitica* tolerated short exposures to weak alkali better than other *Enterobacteriaceae* and taking advantage of this property, mixed amounts of cold enrichment cultures from naturally and experimentally contaminated food samples with a solution of 0.5 % potassium hydroxide in 0.5 % potassium chloride before streaking on MacConkey agar. The potassium hydroxide treatment was reported to increase the yield of *Y. enterocolitica* four-fold, and the sensitivity 100-fold.

Most strains of *Y. enterocolitica* will grow on selective enteric media such as Salmonella-Shigella (SS), Xylose-lysine-deoxycholate (XLD), MacConkey, Hektoen, and bismuth sulphite agars. Because of the toxicity of selective agar media at higher temperatures, incubation at 22-28 °C for 48 h is required for growth of *Y. enterocolitica* on these media with the exception of MacConkey agar. Colonies of *Y. enterocolitica* will appear as small, colourless (lactose-negative) colonies on MacConkey and Salmonella Shigella (SS) agars in 48 hours. In some laboratories plates of MacConkey agar inoculated with stool specimens are

routinely incubated at room temperature (Schiemann, 1989). In 1979, Schiemann formulated a new agar medium for the isolation of *Y. enterocolitica* after extensive studies to determine optimum basal requirements of *Y. enterocolitica* and to evaluate the effect of selective chemical agents such as dyes. The new medium, cefsulodin-irgasan-novobiocin (CIN) agar was reported to provide quantitative recoveries of 40 strains of *Y. enterocolitica* representing various serotypes and was reported to be inhibitory to other enteric organisms (Schiemann 1979). Several studies have found CIN agar to be superior for isolating *Y. enterocolitica* from both faeces and foods (Harmon *et al.* 1984; Head *et al.* 1982; Ratnam *et al.* 1983; Walker and Gilmour 1986). The superiority of CIN agar for the recovery of *Y. enterocolitica* from stool suspensions containing 10^2 colony forming units (c.f.u.) or less has been reported by Head *et al.*(1982).

The relative efficacy of three different isolation procedures with regards to the recovery of naturally occurring yersiniae from porcine tonsils were compared by Nesbakken and Kapperud (1985) by : (1) Direct plating on CIN agar ; (2) Pre-enrichment in PBS + 1 % sorbitol (PSB) for 8 days followed by selective enrichment at 4 °C, followed by selective enrichment in modified Rappaport broth (MRB) for four days, at 20 to 25 °C , and (3) Cold enrichment in PSB (three weeks, 4 °C). The results indicated that PSB cold enrichment should be included in any combination regardless of the type of yersiniae sought. This procedure was the most effective single method for the recovery of *Y. enterocolitica* serotype O:3/biotype 4 and *Y. kristensenii.*

Other investigators have recommended the combination of cold enrichment in M/15 phosphate-buffered saline (PBS), pH 7.6, with subsequent plating onto CIN agar for human and animal specimens (Davey *et al.* 1983; Okoroafor *et al.* 1988; Ratnam *et al.* 1983; Van Noyen *et al.* 1987a, 1987b).

Y. pseudotuberculosis will grow on most clinical isolation media, e.g. blood agar and MacConkey, with the exception of bismuth sulphite agar, at both 25 ° and 37 °C and under aerobic and anaerobic conditions (Schiemann 1989). *Y. pseudotuberculosis* is rarely isolated by faecal culture, even in confirmed cases of pseudotuberculous mesenteric lymphadenitis, probably because excretion of the organism has ceased before the onset of pseudoappendicular symptoms. If culture of faeces is attempted, Mair and Fox (1986) recommend the use of lactose-sucrose-urea agar as a selective medium.

Identification

CIN agar provides a unique method of differentiation compared to other media: colonies are 0.5 to 1.0 mm in diameter, slightly raised, smooth and with a distinctive red "bullseye" appearance. Primary screening of suspect colonies from selective media such as CIN may be accomplished by inoculating from each selected colony onto Triple sugar iron (TSI) agar slant and urea agar (Wauters, 1973). A third test is a modification of Edward and Fife's (1961) Lysine-Iron agar (LIA), to Lysine-Arginine-Iron agar (LAIA) (Weagant, 1983). This diagnostic medium provides five biochemical characteristics in a single tube that are suitable for presumptive identification of *Y. enterocolitica.* Typical reactions of *Yersinia* spp. on LAIA are alkaline slant (purple), acid butt (yellow), no hydrogen sulphide (H_2S) production (no darkening of butt) or gas formation after 24 h incubation at 28-29 °C.

Urease-positive strains that show acid butt plus alkaline or acid slant (A/A or K/A), no gas or blackening after 24 h at 28-29 °C on TSI are verified by Gram staining and motility testing at 28 °C and 37 °C (Christensen 1980; Lassen 1975). Urease-negative strains of *Y. enterocolitica* have been reported, but there is no firm evidence that any of these strains are human pathogens (Schiemann, 1989). Identification of *Yersinia* bacteria of the species *Y. enterocolitica* can be completed by doing biochemical tests (incubated at 29 °C): sugars (sucrose, rhamnose, raffinose, melibiose, α-methylglucoside), aesculin, methyl red (MR), Voges-Proskauer (VP) at 29 and 37 °C, Simmon's citrate, ornithine, arginine and lysine. *Y. enterocolitica* is sucrose-positive and negative in all other sugars. The major biochemical tests that differentiate *Y. enterocolitica* from *Y. pseudotuberculosis* are ornithine decarboxylase, sucrose and sorbitol. *Y. enterocolitica* is positive for all three tests, whereas *Y. pseudotuberculosis* is negative. On TSI, *Y. enterocolitica* typically shows acid butt (yellow), acid slant (yellow) , no H_2S and no gas, while *Y. pseudotuberculosis* shows acid butt (yellow), alkaline slant (red), no H_2S and no gas. *Y. pseudotuberculosis* is almost always urease-positive as are most strains of *Y. enterocolitica* (Quinn *et al.* 1994).

Biotypes and serotypes

Yersinia enterocolitica and its related species represent a complex heterogeneous group from the point of view of their chemical and antigenic patterns. The biochemical heterogeneity observed among *Y. enterocolitica* strains led Nilehn (1969) to propose a biotyping scheme for *Y. enterocolitica.* A total of 330 isolates were examined by Nilehn and divided into five

biotypes on the basis of 13 biochemical tests. Wauters (1970) divided *Y. enterocolitica* into four biotypes on the basis of their reactions to indole, xylose, aesculin, and salicin at 37 ° and 22 °C. The biotyping scheme proposed by Wauters *et al.* (1987) involved the addition of new biochemical tests which include pyrazinamidase test, 5-D-glucosidase and proline peptidase. Biotype 1 was divided by these authors to differentiate the non-pathogenic environmental strains (1A) from the North American human pathogenic strains (1B). They also proposed the creation of biotype 6 to accommodate the non-pathogenic *Y. enterocolitica* strains within biotype 3, biotypes 3A and 3B. Further biochemical characterisations have now designated biotype 3A as *Yersinia mollaretii* and biotype 3B as *Yersinia bercovieri* (Wauters *et al.* 1988). Differentiation of species within the genus Yersinia is shown in Table 1 (Bercovier *et al.* 1984; Wauters *et al.* 1988). Biochemical differentiation of the 6 biogroups of *Y. enterocolitica* is shown in Table 2 (Wauters *et al.* 1987)

Serological methods were used in very early investigations to establish antigenic relationships among strains and between related species (Schiemann, 1989). Determination of the antigenic structure will confirm the identity of the organism and indicate the serogroup to which it belongs. Serogrouping is performed by slide-agglutination using single factor O-agglutinating antisera prepared from rabbits hyperimmunised with autoclaved strains of different serogroups
and subgroups (Mair *et al.*1960). *Yersinia enterocolitica* O-antigen representing factor 1 to 57 (Wauters, 1981) are recognised. Slide-agglutination with absorbed, specific O-antisera prepared against serovars O:3, O:5,27, O:8 and O:9 should be sufficient to identify most strains responsible for human yersiniosis (Mair and Fox, 1986).

Yersinia pseudotuberculosis has been classified into 6 serogroups (I-VI) determined by type-specific and heat-stable somatic O antigens. Types I, II, IV and V have subtypes designated as A and B (Bissett 1981; Thal 1979). Serogroup I is very common in animals and has been most frequently associated with human infections (Hallstrom *et al.* 1972; Zaremba and Borowski 1973 as cited by Schiemann, 1989). Serogroups II and III are rare, but the latter group are common in healthy pigs (Mair *et al.* 1979; Tsubokura *et al.* 1973). Serogroups IV and V are rarely encountered (Bradley and Skinner 1974).

Table 1. Biochemical differentiation of species within the genus *Yersinia*

Biochemical Test	*Y. pestis*	*Y. pseudo-tuberculosis*	*Y. enterocolitica*	*Y. frederiksenii*	*Y. intermedia*	*Y. kristensenii*	*Y. aldovae*	*Y. bercovieri*	*Y. mollaretii*	*Y. rhodei*
Indole	-	-	V	+	+	V	-	-	-	-
Ornithine	-	-	+	+	+	+	+	+	+	+
Motility 25 °C to 28 °C	-	+	+	+	+	+	+	+	+	+
Fermentation of sucrose	-	-	+	+	+	-	-	+	+	+
Rhamnose	-	+	-	+	+	-	+	-	-	-
Cellobiose	-	-	+	+	+	+	-	+	+	+
Sorbitol	-	-	+	+	+	+	+	+	+	+
Melibiose	V	+	-	-	+	-	-	-	-	V

+ = 90 % or more strains are positive; - = 90 % or more strains are negative;
V = 11 %-89 % of strains are positive.

Adapted from: Bercovier *et al.* 1984; Wauters *et al.* 1988.
All tests were performed at 25 °C to 28 °C

Table 2. Biochemical differentiation of 6 *Y. enterocolitica* biogroups.

Biogroups	1A	1B	2	3	4	5	6
Lipase	+	+	-	-	-	-	-
Aesculin/Salicin	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Trehalose/Nitrate	+	+	+	+	+	-	+
β-D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	(+)	-
Proline peptidase	V	-	-	-	-	-	+

YERSINIOSIS IN HUMANS

a. *Yersinia enterocolitica*

A wide spectrum of clinical manifestations is caused by *Y. enterocolitica* such as gastrointestinal infections, septicaemia, erythema nodosum, reactive arthritis, myocarditis, organ abscesses, subacute hepatitis and meningitis. Factors such as the age and the physiological state of the host and the pathogenic properties of the particular strain of the organism are important (Gemski *et al* 1980; Gilmour and Walker 1988).

The earliest recognition of *Y. enterocolitica* as a human pathogen was through its association with various gastrointestinal infections such as enteritis, terminal ileitis and mesenteric lymphadenitis (Schiemann 1989). Acute gastro-enteritis or enteritis is the most commonly recognised symptom of clinical yersiniosis (Bottone 1977; Vantrappen *et al.* 1982; Mair and Fox 1986). Although such infection occurs at all ages, the majority of patients are less than five years old (Vandepitte and Wauters 1979). The most common clinical symptoms are fever, abdominal pain and diarrhoea. Nausea and vomiting are rare but may also occur at a low frequency (Bottone 1977; Zen-Yoji 1981; Schiemann 1989; Quinn *et al.* 1994).

Terminal ileitis is a more frequent feature of *Y. enterocolitica* infection; ileal lesions are often severe and may be associated with haemorrhagic necrosis (Mair and Fox 1986). *Yersinia enterocolitica* has been recognised as a cause of terminal ileitis and mesenteric adenitis in human beings since the 1930's, and a number of domestic animals including dogs, cats, cattle, sheep and pigs have been suggested as possible sources of infections (Robins-Browne 1989). It is an invasive enteric pathogen. The bacteria gain access to the small intestine (terminal ileum), penetrate the lamina propria by passing through epithelial cells, and multiply in the Peyer's patches and lymph follicles causing the typical terminal ileitis (Carniel and Mollaret 1990; Robins-Browne 1992). Usually the infection is limited to this area of the intestines, but the organisms may invade the mesenteric lymph nodes and give rise to a systemic infection.

Septicaemia is considered a rare manifestation of infection by *Y. enterocolitica* (Mair and Fox 1986), however, some cases of septicaemia have been reported mainly in elderly subjects (Mair and Fox 1986). Almost all patients who develop systemic yersiniosis have an underlying immune or metabolic disorder, or pre-existing disease (Kapperud and Bergan, 1984). Predisposing conditions include lymphoma, hepatic cirrhosis, uraemia, diabetes and iron overload (Gallant *et al.*1986; Melby *et al.*1982; Rabson *et al.* 1972). Many patients with iron overload receive treatment with desferrioxamine B , a bacterial siderophore which promotes growth of *Yersinia enterocolitica in vitro* and *in vivo*. Thus, desferrioxamine B may add to the risk of systemic yersiniosis developing in patients with siderosis (Robins-Browne *et al.* 1987).
A variety of other illnesses may eventually result from the initial *Y. enterocolitica* gastro-enteritis including painful swellings and blotches (erythema nodosum) mainly on the legs and trunk (Winblad 1981) of females over twenty years old (Mair and Fox 1986). The eruption occurs after a prodromal period lasting 4-14 days.

Of the immunological sequelae of yersiniosis, reactive arthritis is the best described (Robins-Browne 1992). This manifestation is infrequent before the age of 10 years. Most cases have occurred in Scandinavian countries where serogroup O:3 strains and the HLA-B27 histocompatibility antigen are prevalent. Arthritis typically follows the onset of diarrhoea or the pseudoappendicular-like syndrome by two weeks with a range of one to 30 days. Other autoimmune complications of yersiniosis include Reiter's syndrome, iridocyclitis, acute proliferative glomerulonephritis and rheumatic-like carditis.

A number of atypical manifestations may occur in debilitated individuals such as myocarditis, subacute hepatitis, organ abscesses, meningitis and urethritis (Kapperud and Bergan 1984).

b. *Yersinia pseudotuberculosis*

Human infections with *Yersinia pseudotuberculosis* have been reported worldwide, but in most countries the incidence appears to be lower than for *Y. enterocolitica* (Van Noyen *et al.* 1995). Many cases of *Y. pseudotuberculosis* infection have been reported mainly from Europe (Bottone 1977), with a few outbreaks reported in Finland (Tertti *et al.* 1984; 1989) and Japan (Inoue *et al.* 1984; 1988; Sanbe *et al.* 1987; Nakano *et al.* 1989).

Before 1954, only a septicaemic form of *Y. pseudotuberculosis* infection (Schiemann 1989) was recognised. The illness resemblings typhoid fever, occured most commonly in adults with hepatic disease or myoproliferative disorders, and was usually fatal. Gastrointestinal diseases are now recognised more commonly. *Y. pseudotuberculosis* is now associated with a variety of human diseases such as mesenteric lymphadenits, terminal ileitis, arthritis and septicaemia (Mollaret 1965; Mair and Fox 1986).

Manifestations of the disease is usually less severe in man than in animals. The organisms migrate to the ileum and mesenteric lymph nodes, but neither colonises the spleen and liver nor invades the bloodstream (Carniel and Mollaret 1990). Fever occurs frequently but this is usually low grade. The most constant symptom is abdominal pain in the right lower quadrant. According to Mair and Fox (1986), the pain is due to mesenteric lymphadenitis which mimics appendicitis (pseudoappendicular syndrome) and often leads to surgery. After laparotomy, the appendix is found to be normal but is surrounded by enlarged, inflamed and infected mesenteric lymph nodes. Progressively, the infection ascends along the lymphatic route, affecting the distal lymph nodes while the proximal nodes become sterile. The disease is usually self-limiting and the clinical symptoms disappear even without treatment. The infection rarely leads to intestinal necrosis (Ahvonen 1972). Diarrhoea accompanying the abdominal pain is mild and not constantly found.

The most common secondary complications are erythema nodosum and reactive polyarthritis which occur a few weeks after the acute phase. Arthritis is frequently associated with the presence of HLA-B27 antigen (Aho *et al.* 1973). Apart from the polyarthritis associated with epidemic pseudotuberculosis, and the arthralgia present in erythema nodosum, joint involvement is rarely reported as a complication of *Y. pseudotuberculosis* infection (Mair and Fox 1986).

Far-eastern scarlatiniform fever or epidemic pseudotuberculosis, a severe form of pseudotuberculosis, appears to be confined to the far-eastern territories of the former Soviet Union. The disease is characterised by fever, a scarlatiniform rash, arthralgia and acute polyarthritis. Symptoms indicative of fever and gastrointestinal lesions have also been reported (Mair and Fox 1986).

Y. pseudotuberculosis infection in children is not a rare disease in Okayama district, Japan. The clinical manifestations of *Y. pseudotuberculosis* such as mesenteric lymphadenitis and terminal ileitis, occurring mainly in elderly patients are not well known in children (Sato 1987). Based on their study, the clinical features of infection in children were somewhat different from those of European reports wherein the Kawasaki syndrome-like illness or acute renal failure have not been described. The clinical findings in children were similar to the "Far-East scarlet-fever-like disease" that occurred in the USSR. The disease have occurred in epidemics affecting some 5,000 persons in the Far-East (Somov and Martinevsky 1973).

EPIDEMIOLOGY

Reservoirs of *Y. enterocolitica* and *Y. pseudotuberculosis*

1. Animals

For many years, animals have been regarded as the main source of human *Y. enterocolitica* infections. Numerous surveys have been conducted in an attempt to identify specific reservoirs. Results of these surveys have shown that while many animals carry strains of *Y. enterocolitica,* pigs appear to be the principal carriers of the serovars of *Y. enterocolitica* that are pathogenic to humans (Schiemann 1989). Pigs are the principal animal species which have been identified as carriers of serotypes O:3 and O:9 (Carniel and Mollaret 1990) as well as serotype O:5,27 (Hanna *et al.* 1980). Although a definite connection between isolates from pigs and human illness has still to be established, it has been suggested that pigs play an important role in human infection (Pedersen 1979; Wauters 1979). According to Schiemann (1989), the existence of a link between pig carriage and human infections has only been established through epidemiological investigations.

Dogs and cats have been found to harbour *Y. enterocolitica,* particularly serovar O:3 (Fantasia et al. 1985; Yanagawa *et al.* 1978). Japanese studies have also shown that *Y. enterocolitica* may be obtained from 6-27 % of clinically healthy dogs (Yanagawa *et al.* 1978; Fukushima *et al.* 1985). Disease in dogs due to *Y. enterocolitica* appears rare, but enteritis has been reported occasionally (Papageorges *et al.* 1983). There are a few cases of human yersiniosis which have been related directly to contact with infected dogs (Ahvonen *et al.* 1973; Wilson *et al.* 1976).

Y. enterocolitica has also been isolated from cow faeces in the UK (Davey *et al.* 1983) and in Australia (Hughes 1979). However, the strains were identified as *Y. enterocolitica* biotype 1 or one of the *Y. enterocolitica*-like species which are not commonly associated with human gastroenteritis. The bacteria has also been isolated from goats, cows (Inoue and Kurose 1975; Wooley *et al.* 1980), horses and sheep (Nilehn 1969; Wooley *et al.* 1980), monkeys (Nilehn 1969; Van Damme *et al.* 1978; Szita *et al.* 1980), deer (Wetzler and Hubbert 1968) and snails (Botzler *et al.* 1976). The epidemiological significance of the isolation of *Y. enterocolitica* from such animals is still unknown (Swaminathan *et al.* 1982)

Many other animals including wild animals (including rodents), birds and insects have also been shown to harbour *Y. enterocolitica.* The bio-serotypes often isolated are not those commonly associated with human infections, but in Japan, bio-serotype 4/O:3 has been isolated from rats (Kaneko *et al.* 1978) and from both ham and flies in a pig processing plant (Fukushima *et al.* 1979). In Alaska, pathogenic *Y. enterocolitica* was recovered from crabs (Faghri 1984), and *Y. enterocolitica* biotype 2, serotype O:5,27, demonstrating virulence characteristics was recovered from a common garter snake (Kwaga and Iversen 1993). Generally, surveys of free-living animals yield many bacteria identified as *Y. enterocolitica* and related species, but these are rarely recognised as pathogenic forms (Bercovier *et al* 1978; Kapperud 1981; Kapperud and Rosef 1983; Kato *et al.* 1985).

Y. pseudotuberculosis is widely distributed among domestic pets (Fukushima *et al.* 1984; 1985; Mair *et al* 1967; Tsobokura *et al.* 1984; Yanagawa *et al.* 1978; Fukushima *et al.* 1989), farm animals (Tsubokura *et al.*1984), wild animals (Bercovier *et al.* 1978; Fukushima *et al.* 1988; Tsubokura *et al.* 1984) and birds, and occasionally in reptiles (Obwolo 1980). Fukushima *et al.* (1984) reported that *Y. pseudotuberculosis* infection may be related to contact with environmental substances contaminated with *Y. pseudotuberculosis* during the cold months. Pets such as dogs and cats may become sources of infection and excrete up to 10^4

cells per gram of faeces. Contact with infected animals is considered the most common mode of transmission of *Y. pseudotuberculosis* to humans. Animal reservoirs such as rats may also serve as sources of contamination of food and water for human consumption (Kaneko and Hashimoto 1982).

Like *Y. enterocolitica, Y. pseudotuberculosis* has also been isolated from swine (Dickinson and Mocquot 1961; Toma and Deidrick 1975; Tsubokura *et al.* 1976; Zen-Yoji *et al.* 1974; Narucka and Westendoorp 1977) but does not appear to be an important pathogen for these animals (De Barcellos and Castro 1981). The presence of the organism in swine suggests that pork meat may be contaminated. The findings of Shiozawa *et al.* (1988) suggests that retail pork and swine may play an important role in the epidemiology of human infections caused by caused by *Y. pseudotuberculosis.*

2. Water and Environment

Y. enterocolitica and related species are widely distributed in the terrestrial environment including lakes, well and stream waters, which are sources of the organism for warm-blooded animals. There are also a few sporadic reports of yersiniosis where contaminated water has been implicated in disease outbreaks (Lassen 1972; Keet 1974; Anon. 1975; Eden *et al.* 1977; Tacket *et al.* 1985). Although *Yersinia* spp. have been isolated from several water types in many countries, the strains were largely atypical and not belonging to the biotypes associated with disease unless there was obvious evidence of faecal contamination from animals (Fukushima *et al.* 1984; Walker and Grimes 1985). In general, isolates of *Y. enterocolitica* differ from those implicated in human disease (Toma 1973; Schiemann 1987). *Y. pseudotuberculosis* is also widespread in the environment as the result of contamination by faeces of infected animals, mainly rodents and birds (Carniel and Mollaret 1990). The potential for transmission of *Y. pseudotuberculosis* to humans via water contaminated by animals is very real, however, there is no documentation of waterborne cases or outbreaks associated with this species of yersinia (Schiemann 1989).

3. Humans

It is not yet clearly evident as to whether humans serve as reservoirs of *Yersinia* spp. and if the organism is transmitted through the faecal-oral route. Although *Y. enterocolitica* appears to be more animal adapted, it is found more often among human isolates than in other species. In the

USA Shayegani *et al.* (1981), isolated 149 strains of human origin and found that 81, 12, 5.4 and 2 % respectively were *Y. enterocolitica, Y. intermedia, Y. frederiksenii* and *Y. kristensenii.* In Belgium (Wauters 1981) and Japan (Kanazawa and Ikemura 1979) strains of *Y.enterocolitica* have been isolated from apparently healthy individuals. Strains isolated are mainly biotype 1 and are considered clinically unimportant as they do not produce the disease, have similar incidence in both healthy and diseased individuals, are present in small numbers and are transient in the bowel microflora (Wauters 1981). Pathogenic bioserotypes have also been isolated from apparently healthy food handlers in Japan (Asakawa *et al.* 1979). Toivanen *et al.* (1973) identified the potential for person-to-person transmission in a report of six hospital staff members who were infected with *Y. enterocolitica* serovar O:9 after admission of a 9-year old school girl with persistent abdominal pain, diarrhoea and fever. Family members of two infected nurses also developed diarrhoea and abdominal pain. Vandepitte and Wauters (1979) discovered healthy carriers among family members of patients suffering from *Y. enterocolitica* infections or during routine faecal cultures in day-care nurseries. The incidence of infections due to *Y. enterocolitica* in young children who had no known contact with animals indicates that the organism may be transmitted by person-to-person contact (Albert and Lafleur 1971; Lafleur *et al.* 1972; Bergstrand and Winblad 1974; Delorme *et al.* 1974; Dabernat *et al.* 1979).

4. Foods

Y. enterocolitica has been isolated from a wide variety of foods such as milk and milk products, raw meats (beef, pork and lamb) poultry, vegetables and miscellaneous food products (Mollarett 1979). Many of the isolates of *Y. enterocolitica* from foods are biochemically atypical and many do not agglutinate antisera prepared against known serotypes of *Y. enterocolitica.* The pathogenic properties of food isolates of *Y. enterocolitica* have been assessed by a few investigators (Mehlman *et al.* 1978; Lee *et al* 1981; Schiemann 1980). Because of the association between *Y. enterocolitica* O:3 and pigs, the incidence of this organism in pork and pork products has been widely investigated (Gilmour and Walker 1988).

Pigs and pork products are believed to become contaminated during slaughter or by incorporation of tongues in pork products. Strains of *Y. enterocolitica* have been isolated from beef and lamb (Leistner *et al.* 1975) and from various beef products, but these strains were generally considered non-pathogenic. Poultry and poultry products (Norberg 1981; De Boer *et al.* 1982) as well as sea foods (Greenwood and Hooper 1985) have also been found to contain

the bacteria. *Y. intermedia, Y. frederiksenii* and *Y. enterocolitica* biotype 1 were the strains identified from sea foods and were considered of limited public health significance. Raw milk has also frequently yielded bacteria identified as strains of *Y. enterocolitica* which have been occasionally associated with human disease (Schiemann 1978; Greenwood and Hooper 1985; Walker and Gilmour 1986). So far, none has been identified as pathogens (Schiemann 1987; Gilmour and Walker 1988). The occasional isolation of *Y. enterocolitica* in milk is attributed to improper pasteurisation or more likely to post-pasteurisation contamination (Gilmour and Walker 1988; Schiemann 1989). Tofu has been proven to be a vector of yersiniosis. In the Netherlands, 11 % of the samples were contaminated with *Y. enterocolitica* and included strains occasionally associated with disease (Van Kooij and De Boer 1985). So far, no other food reservoir of *Y. enterocolitica* has been identified with the exception of porcine tongues and possibly ground pork (Schiemann 1989).

Y. pseudotuberculosis has been identified as a potential food-borne organism because it is commonly occurring in domestic food animals in which it can cause disease. According to Schiemann (1989), the epidemiological data supporting this conclusion is meager. With the exception of outbreaks occurring in families (Randall and Mair 1962), this kind of epidemiological data for *Y. pseudotuberculosis* is lacking. No epidemics attributable to this micro-organism have been previously reported except the only known epidemic reported by Tertti *et al.* (1988) in Finland where 19 patients were involved in an outbreak of infection caused by *Y. pseudotuberculosis* serotype 3. In spite of active screening of the respective families and environments of the patients, the precise source of infection remains unknown. The potential for milk-borne transmission of *Y. pseudotuberculosis* was brought to attention when Jones *et al.* (1982) isolated *Y. pseudotuberculosis* serovar 1A from mastitic goat's milk. The link between a pig reservoir and transmission to humans is not clear, however, surveys conducted in Japan (Fukushima 1985; Shiozawa *et al.* 1987) have shown that revealed that 2 % of retail pork tongue and 0.8 % of ground pork samples were contaminated with *Y. pseudotuberculosis*. In another study conducted by Shiozawa *et al.* (1988), several virulence factors and plasmid profiles of *Y. pseudotuberculosis* isolated from pork or swine were investigated. The virulence factors of pork and swine isolates were found to be identical to those of human isolates and were considered potentially pathogenic for humans.

Modes of Transmission

The epidemiology of yersiniosis is rather complex and not fully established. Contact with infected animals and people or consumption of contaminated food have been mentioned as the most widely modes of transmission (Sonnenwirth and Weaver 1970; Gutman *et al.* 1973).

Based on clinical and epidemiological data, the most likely portal of entry of *Y. enterocolitica* in both man and animals is the digestive tract. The organism has been isolated from intestinal contents of a large number of animals and stools of affected individuals. Therefore, it has also been presumed that this organism would follow the same epidemiological pattern as the other members of *Enterobacteriaceae,* e.g. various *Salmonella* and *Escherichia* species (Hurvell 1981).

The most frequently documented mode of transmission of *Y. enterocolitica* has been via foods, milk and milk products and water (Cover and Aber 1989). Cooked food can be contaminated by animal faeces, via flies, and occasionally from human sources. Water, milk, cream, pork, tongue, beef, lamb and blood sausage have been incriminated in outbreaks of illness (Quinn *et al.* 1994). Because *Y. enterocolitica* is able to grow at low temperatures, refrigerated foods initially contaminated with a small number of bacteria may serve not only as vehicles of transmission but also as media for growth (Stern and Pierson 1979). A study by Tauxe *et al.* (1987) in Belgium, showed that at least 18 % of children consumed foods containing raw pork, and that this habit was highly associated with yersiniosis. Cross-contamination in the kitchen from raw pork chitterlings (a dish made from pig intestines) to prepared infant foods appeared to be the mode of transmission to infants in an outbreak in the USA (Lee *et al.* 1990).

There seems to be little doubt that the bacteria can also be spread via direct or indirect interhuman transmission, although absolute proof is lacking (Bottone 1977). Evidence supporting such a mode of transmission may be inferred from the marked incidence of yersiniosis in young children who have not had animal contact (Zen-Yoji and Maruyama 1972; Albert and Lafleur 1971; Lafleur *et al.* 1972; Delorme *et al.*1974; Bergstrand and Windblad 1974), reports involving familial and interfamilial outbreaks (Gutman *et al.*1973; Szita *et al.* 1973; Ahvonen and Ross 1973) and epidemics in confined settings (Ahlqvist *et al.* 1971; Olsovsky *et al.* 1975; Toivanen *et al.* 1973). According to Bottone (1977), under the latter two conditions, as individuals become symptomatic in a sequential rather than sporadic order, person-to-person transmission is indicated rather than transmission through animal or food

sources. The existence of asymptomatic carriers of *Y. enterocolitica* also supports an interhuman mode of transmission (Mollaret 1972).

Faecal-oral spread by handling infected pigs, dogs and cats has been reported (Carniel and Mollaret 1990). Serotypes O:3 and O:9 may also spread from person-to-person by the faecal-oral route, and this transmission from asymptomatic carriers could be a possible explanation of some sporadic cases.

Transmission by blood transfusion has also been reported (Cover and Aber 1989). Use of contaminated blood has led to 10 cases of bacteraemia due to *Y. enterocolitica* with seven deaths in the USA since 1987, four of the deaths were due to *Y. enterocolitica* O:3 (Li *et al.* 1993). *Y. enterocolitica* present during transient bacteraemia in the donor may multiply in blood products when stored at 4 °C, without manifestly altering their appearance. Accordingly, contaminated blood may be infused directly into patients during blood transfusion. In this manner of administration, even environmental strains of *Y. enterocolitica* could be as lethal as the enteropathogenic strains (Robins-Browne 1992). According to Jacobs *et al.* (1989), a septic shock syndrome produced during transfusion is fatal in 70 % of cases.

The principal natural route of infection by *Y, pseudotuberculosis* seems to be the faecal-oral one, as supported by studies in animals and man (Obwolo 1980). It has been frequently identified as a food-borne pathogen and it is commonly occurring in food animals but relatively few epidemiological data exists. According to Carniel and Mollaret (1990), the cycle of transmission is very simple: infected faeces from contaminated animals are spread in the environment, other animals become infected by ingestion of contaminated water and grass and in turn further spread the organisms throughout the environment. Most often, human cases occur by consuming raw contaminated vegetables (Aldova *et al.* 1979). The organism is also transmitted directly from animals (pets) to humans (Fukushima *et al.* 1984) or from humans to humans via dirty hands (Carniel and Mollaret 1990)

Seasonal prevalence

Compared with other enteric pathogens, infections due to *Y. enterocolitica* appear to peak in the autumn and winter months (Swaminathan *et al.* 1982). These findings are documented in several studies. Rakovsky *et al.* (1973) reported that 67.5 % of human cases of *Y.*

enterocolitica infections in Czechoslovakia occurred between December and March. In Belgium and Sweden, infections due to *Y. enterocolitica* occur most frequently during October (Vandepitte *et al.*1970; Wauters 1970; Arvastson *et al.* 1971;Vandepitte *et al.* 1973; Vandepitte and Wauters 1979). In Romania and Hungary (Rusu *et al.* 1973; Szita and Svidro 1976) infections peak between November and March while in West Germany, the peak is between September and December (Bockemuhl *et al.* 1979). The seasonal incidence of yersiniosis during the autumn and winter months in European countries (Mollaret 1972; Delorme *et al.* 1974; Arvastson *et al.* 1971) has been shown to coincide with the domestic slaughter of pigs in households during festive occasions (Wetzler and Hubbert 1968). There is also an increased incidence of clinical infections due to *Y. pseudotuberculosis* during the cold season. There are two reasons for this: first, the bacteria survive and multiply better in the environment at low temperatures; second, animals which are healthy carriers of the organism excrete large numbers in their faeces after stress caused by cold weather and starvation (Carniel and Mollaret 1990).

Food-borne outbreaks

Even in the absence of any strong epidemiological evidence, *Y. enterocolitica* has been suggested as an important micro-organism in foodborne disease outbreaks. Four disease outbreaks reported in the literature have implicated *Y. enterocolitica* as the causative agent. The source of infection was not identified in the three outbreaks that occurred in Japan. The other outbreak which occurred in Canada in 1975 was reported to be due to the consumption of raw milk (Anon. 1976). However, the serotype of *Y. enterocolitica* isolated from the raw milk was different from the affected children with gastro-enteritis. The evidence is insufficient to implicate *Y. enterocolitica* as the causative agent. Food has not been identified as the vehicle in any outbreak in those countries where yersiniosis is endemic and where the common serovars are O:3 and O:9 (Schiemann 1989). Actual foodborne outbreaks caused by *Y. enterocolitica* are comparatively rare (WHO, 1987) except in the USA where, the epidemiology of yersiniosis has been characterised by foodborne outbreaks (Aulisio *et al.* 1983; Black *et al.* 1978; Shayegani *et al.* 1982; Morse *et al.* 1984; Tacket *et al.* 1984; 1985). The food vehicles were identified as chocolate milk, powdered milk, chow mein probably contaminated by a food handler, tofu or soybean curd manufactured with contaminated water, pasteurised milk indirectly involving pigs (probably faecal contamination), bean sprouts immersed in well water and chitterlings. Fukushima *et al.* (1984) suggested that the main source of contamination of raw milk is not the mammary gland but probably faeces. Four of these documented outbreaks

have involved serovar O:8 which appears to be restricted to North America. The fifth outbreak which implicated the chitterlings was caused by serotype O:3, a serotype which has been rarely isolated in the USA (Lee *et al.* 1990).

YERSINIOSIS IN NEW ZEALAND

Human infections

The incidence of human yersiniosis has dramatically increased in this country in the last five years and *Y. enterocolitica* is now considered the second most common enteric pathogen recovered after *Campylobacter jejuni.* The strains most commonly recovered are bioserovars 4/O:3, 3/O:5,27 and 2/O:9 (Mc Carthy and Fenwick 1990). Clinical features of infection include diarrhoea and reactive arthritis following enteritis (Ameratunga *et al* 1987; Jones and Bruns 1987). Lello and Lennon (1992) described a syndrome in a young boy which was initially diagnosed as acute rheumatic fever but later known to be caused by *Y. enterocolitica.* The patient had complained of a sore throat prior to admission. Six cases of transfusion-related sepsis have been recorded in New Zealand. Three of the cases were fatal (Wilkinson *et al.* 1991; Ulyatt *et al.* 1991). *Y. enterocolitica* is one of the common organism implicated in blood transfusion related sepsis (Wilkinson *et al.*1991). It has been shown that low numbers of *Y. enterocolitica* can grow in blood at 4 °C. In New Zealand, blood is kept at 4 °C for up to 35 days. In two of the cases reported by Wilkinson *et al.* (1991), the blood was 15 to 24 days old at the time of transfusion. In an experiment conducted by Ardiuno *et al.* (1989), *Y. enterocolitica* organisms were inoculated into two units of blood to obtain a concentration of 0.1-1.0 colony forming units per ml. Endotoxin was first detected three weeks after inoculation and the concentration paralleled the lag phase of growth. *Y. enterocolitica* relies on free iron for growth (Perry and Brubaker 1979). Prolonged storage of blood is associated with progressive haemolysis releasing iron and this may explain the prolonged lag phase observed in the experiments (Wilkinson *et al.* 1991).

The current rate of yersiniosis in New Zealand is difficult to estimate. From June 1996, the disease has been specially listed in the schedule of notifiable diseases but not all laboratories culture the organism (McNicholas *et al.* 1995 as cited by Wright *et al.* 1995). In a large Auckland community laboratory which has been routinely culturing stool specimens for *Yersinia* since 1987, *Yersinia* was the most common pathogenic enteric bacterium detected

after *Campylobacter*. In 1994, the incidence of yersiniosis surpassed salmonellosis in both Auckland and Eastern Bay of Plenty (EBOP) regions. According to Wright *et al.* (1995), if the EBOP rate of 84 cases per 100 000 were applied nationally, there could be almost 3000 cases of yersiniosis a year in New Zealand. Also in this study, symptoms of yersiniosis were indistinguishable from those associated with other enteric pathogens, and in 64 % of cases reported, patients were ill for more than a week.

In another study on yersiniosis conducted in Auckland (Fenwick and McCarthy 1995), a bimodal age distribution of cases was observed between 1988 and 1993 with peaks of infection in the 0-4 year olds and young adults between 20 and 23 years of age. Males outnumbered females in all except for these two age groups. The 1994 data from the same source show a similar age and sex distribution.

Y. enterocolitica serotypes O:3, O:5,27, O:8 and O:9 have been detected in New Zealand (Beeching *et al.* 1985). *Y. enterocolitica* serotype O:3 biotype 4 (biotype 4 strains invariably serotype as O:3 and vice versa) accounts for over 90 % of cases of human yersiniosis in New Zealand (Fenwick and McCarthy 1995). This type is present in New Zealand pigs at a relatively high prevalence rate, as it is in many other pig-producing countries (De Allie 1994). In a pilot study conducted at a local pig slaughterhouse to ascertain the prevalence of infection in these animals, it was found out that 24 % of 200 bacon-weight pigs were carrying strains of *Y. enterocolitica* potentially pathogenic for people (Fenwick 1996).

In a survey of 203 samples of ready-to-eat meat products from New Zealand retail outlets, *Y. enterocolitica* was isolated from 3.4 % of the foods tested (Hudson *et al.* 1992). Only cooked foods were contaminated suggesting that contamination occurred after cooking as a result of poor hygiene practices. All but one of the isolates were considered pathogenic.

There are few reports of human infections with *Y. pseudotuberculosis* in New Zealand. The first human case of mesenteric lymphadenitis due to *Y. pseudotuberculosis* in New Zealand was reported by Henshall (1963), which incidentally was the first reported isolation of *Y. pseudotuberculosis* as a human pathogen outside Europe. Later, *Y. pseudotuberculosis* was associated with a case of reactive arthritis in New Zealand, although this was only demonstrated serologically (Rose 1976). Another case of mesenteric lymphadenitis was reported in a 19 year old soldier (Malpass 1981).

Animal infections

In New Zealand, *Y. enterocolitica* has been isolated from from farmed deer (Henderson 1984), sheep (Bullians 1987), goats (Buddle *et al.* 1988; Lanada 1990) and pigs (De Allie 1994) and is a frequent cause of enteritis and death in domestic animals, particularly goats and cattle, however, the strain most commonly involved, which is bioserotype 5/O:2,3 is considered non-pathogenic for man (Slee and Button 1990; Buddle *et al.* 1988).

Yersinia enterocolitica has been involved in a variety of clinical syndromes in domestic animals in New Zealand. The organism has been shown to cause diarrhoea among hoggets (McSporran *et al.* 1984) and suppurative enteritis in cattle and sheep (Belton and McSporran 1988). An outbreak of diarrhoea with three deaths among 22 three-month-old kids placed in a shed containing pigs, was due to *Y. enterocolitica* (Orr *et al.* 1987) . A strain of *Y. enterocolitica* serotype O:5,27 has been isolated from slaughtered deer in New Zealand (Bcsi 1992), but no history or clinical signs were reported.

Animal reservoirs for *Y. enterocolitica* infection in this country are being extensively studied. While several surveys have shown that many animals carried strains of *Y. enterocolitica,* the pig was the only domestic animal which consistently carried those bioserotypes potentially pathogenic for man. Work currently in progress at Massey University is aimed at whether such strains are indistinguishable from those isolated from human sources when typed by more specific molecular methodology (McCarthy *et al.* 1995). Yersiniosis in pigs will be dealt with separately in section B. Dogs and cats were found to harbour similar strains from an occasion but not to the same extent as pigs. Carriage and transmission of *Y. enterocolitica* serotype O:3 biotype 4 in dogs were conducted by Fenwick *et al.* (1994). Their findings suggest that dogs can carry this biotype asymptomatically and hence might act as a potential source of infection for people and other animals.

The first recorded outbreak of *Y. pseudotuberculosis* infection in farmed red deer occurred in this country in 1978 (Beatson and Hutton 1981). Since then yersiniosis due to *Y. pseudotuberculosis* serotypes I, II, and III has become one of the common causes of death in farmed deer in New Zealand (Beatson 1984; Mackintosh and Henderson 1984a, b; Wilson 1984). Outbreaks of the disease can affect up to 40 % of animals in a group, although usually less than 20 % are affected (Mackintosh 1992). While this organism has caused tremendous problems for the deer industry, *Y. pseudotuberculosis* has also been shown to cause disease in

other domestic species such as cattle (Hodges *et al.* 1984), and in sheep where the organism has been associated with abortion (Hartley and Kater 1964).

VIRULENCE ACTIVITY

Not all strains of *Y. enterocolitica* can cause human disease. There are strains which are known to be pathogenic but have not always been positive in virulence assays. According to Farmer *et al.* (1992) virulent strains of *Y. enterocolitica* contain a 70-kilobase virulence plasmid (45 MDas) and the loss of this plasmid is accompanied by a loss in the ability to invade tissues. The pathogenic significance of *Y. enterocolitica* is associated with only a few serogroup-biovar combinations and therefore differentiation between pathogens and non-pathogens has relied upon serogrouping and biotyping of isolates. A number of studies have shown an excellent correlation between the serotype and biotype of *Y. enterocolitica* and its ability to cause infections of the intestinal tract and to invade tissue (Bottone 1981; Butler 1983; Cornelis *et al.* 1987; Miller *et al.* 1988, 1989; Zink *et al.* 1980). Accordingly, a number of different *in vitro* and *in vivo* tests have been proposed for differentiation of pathogenic and non-pathogenic variants (Prpic *et al.* 1985; Robins-Browne *et al.* 1989; Schiemann 1989; Wachsmuth *et al.* 1984). These tests include: salicin fermentation-aesculin hydrolysis, autoagglutination at 37 °C, calcium-dependent growth restriction at 37 °C, binding of Congo red dye, pyrazinamidase test, use of DNA probes, resistance to the bactericidal effect of normal human serum, and various cell and animal culture models. The majority of these tests are based on properties associated with 40-50 MDa virulence plasmid which is a prerequisite but not sufficient in itself, for virulence within the genus *Yersinia* (Cornelis *et al.* 1987; Portnoy and Martinez 1985). The properties concerned are subject to problems of gene expression *in vitro* and the sensitivity and specificity of several tests have been questioned (Prpic *et al.* 1985; Wacsmuth *et al.* 1984). There is also the possibility of the plasmid being lost during prolonged storage or subculturing (Kapperud 1991).

a. *IN VITRO* TESTS

Salicin fermentation-aesculin hydrolysis: Salicin and aesculin are both β-glucosides that vary in the chemical structure of the compound attached to D-glucose by a beta-linkage. Both compounds are hydrolysed by the enzyme β-glucosidase. Both tests are routinely carried out during biochemical characterisation of yersiniae. Pathogenic strains of *Y. enterocolitica* are

salicin-aesculin negative and non-pathogenic strains are positive in both tests. Aesculin and salicin have been advocated for the differentiation of the environmental from pathogenic strains (Shayegani *et al.* 1981). A rapid reaction for these substrates within 24 h is only seen in strains of biogroup 1. Nevertheless, some strains have delayed reactions. Wauters *et al.* (1987) did not consider aesculin hydrolysis to be a particularly useful test and cautioned that a positive reaction is dependent on both temperature and time of incubation. Farmer *et al.* (1992) found that incubation at 25 °C with a final reading at two days accurately differentiated pathogenic from non-pathogenic serotypes. The results of salicin-aesculin tests at 36 °C were not as helpful in identifying strains of non-pathogenic serotypes because positive reactions often occurred after 48-hours. It may not be necessary to do aesculin hydrolysis routinely since it is more difficult to read and correlates 100% with salicin fermentation.

Calcium-dependent growth at 37 °C: The requirement of calcium for the proper growth of some *Yersinia* strains has been shown to be associated with the virulence plasmid (Gemski *et al.* 1980). This characteristic is however, dependant on temperature and can only be demonstrated if incubated at 37 °C. A culture of *Y. enterocolitica* is grown on a medium such as magnesium oxalate (MOX) agar, tryptic soy agar with added magnesium chloride and sodium oxalate. Mair and Fox (1986) used magnesium oxalate to demonstrate calcium dependency at 37 °C for some strains of *Y. enterocolitica*. Strains that contain the virulence plasmid are calcium-dependent at 37 °C and thus fail to grow or tend to produce small colonies (Gemski *et al.* 1980; Noble *et al.* 1987).

Congo Red uptake: This test demonstrates the ability of plasmid-bearing yersiniae to bind Congo red. In cultures of *Y. enterocolitica* grown at 37 °C on tryptic soy agar with added Congo red dye, strains which contain the virulence plasmid take up the red dye and appear as red colonies (Prpic *et al.* 1983). Riley and Toma (1989) modified MOX to include Congo red (CR) which allows visualisation of calcium-dependent growth and uptake of Congo red dye on the same plate. This combination is referred to as Congo red-magnesium oxalate (CR-MOX) agar. Isolates were first grown on 5 % sheep blood agar for 24 h. Individual colonies were then picked off and subcultured onto CR-MOX agar plates. These plates are then incubated at 37 °C, and observed for the presence of small red colonies at 24-48 h. Freshly isolated strains of pathogenic yersiniae contain the virulence plasmid and are CR-MOX-positive. When the plates are kept for several additional days at 37 °C, it is often possible to observe the development of

a large colourless colony from a small red colony due to loss of plasmid in some cells. Non-pathogenic strains are CR-MOX-negative producing only large colourless colonies.

In one of the studies conducted, only 15 of the 63 strains of pathogenic serotypes produced small red colonies on CR-MOX agar (Farmer *et al.* 1992). These results were in agreement with those done by other workers (Kandolo and Wauters 1985; Kay *et al.* 1983; Riley and Toma 1989; Wachsmuth *et al.* 1984), who have noted that strains that have been stored or subcultured several times have often lost the *Yersinia* virulence plasmid. After 24-30 h of incubation, it is usually possible to identify a pathogenic serotype and determine the percentage of population (colonies) that still contain the plasmid.

Pyrazinamidase test: Pyrazinamidase activity has also been proposed to separate non-pathogenic from pathogenic strains whether or not they actually harbour the virulence plasmid. Kandolo and Wauters (1985), used this test to characterise 381 strains of yersiniae, and found an excellent correlation between virulence and a negative test for pyrazinamidase, which is indicated by non-colouration of the medium. *Y. pestis and Y. pseudotuberculosis* were pyrazinamidase-negative, as were strains of *Y. enterocolitica* which belonged to bioserogroups usually associated with human disease. This test measures the enzyme pyrazine-carboxylamidase (pyrazinamidase) which was used to characterise the mycobacteria and corynebacteria (Farmer *et al.* 1987). The test is simple, reproducible and shows a striking correlation between potential pathogenicity and lack of pyrazinamidase activity independent of the virulence plasmid. The absence of pyrazinamidase activity appears to be a characteristic of pathogenic strains of *Y. enterocolitica* (Kandolo and Wauters, 1985), although it does not differentiate plasmid-positive and plasmid-negative strains. In general, pyrazinamidase-negative virulent strains are also usually aesculin-negative, whereas pyrazinamidase-positive strains are usually aesculin-positive (Cornelis *et al.* 1987). Farmer *et al.* (1992) found that there is 91 % agreement between the pyrazinamidase and salicin-aesculin tests. Both tests can be used routinely, and strains that provide conflicting results can be studied by other methods.

Autoagglutination: Laird and Cavanaugh (1980) developed a method to detect the production of yersinia outer membrane protein (YOPS) and expression of surface fimbriae. A culture of *Y. enterocolitica* is inoculated into two tubes of tissue culture media, e.g. Earle's minimum essential medium plus Earle's salts and glutamine, where one tube is incubated at 37 °C and the other at 22 °C. Autoagglutination at 37 °C but not at 22 °C is usually associated with the

secretion of YOPS and surface fimbriae found in pathogenic yersiniae. According to Farmer *et al.* (1992), other broth media can also be used such as MR-VP broth. Autoagglutination is easy to do and is easily adaptable to the daily routine since MR-VP broth is readily available in most laboratories. As with CR-MOX agar, old strains of pathogenic serotypes are usually negative. In their study, only 8 of 63 strains were positive. Autoagglutination was usually clear and easy to read, however, the tubes should be handled gently because the agglutinated cells are easily re-suspended, giving uniform turbidity that would be read as negative. Although autoagglutination can be a very useful test for fresh isolates, the same authors do not use this method routinely, because CR-MOX agar provides the same information and has several advantages.

Detection of specific virulent O antigen serotypes: The phenotypic heterogeneity in *Y. enterocolitica* has prompted the development of several schemes for routine subdivision of the bacterium on the basis of serogroups, biovars and phagevars (Kapperud and Bergan 1984; Mollaret *et al.* 1979; Wauters *et al.* 1987). *Y. enterocolitica* has been classified into approximately 60 serogroups on the basis of O antigens (Wauters 1987). However, the strains associated with disease in man or animals belong to only a few serogroups. Thus, O:3, O:5,27; O:8 and O:9 are the most important causative agents in man. The serogroups commonly associated in pathological processes in man or animals belong to distinct serogroup-biovar-phagevar combinations (Mollaret *et al.* 1979). Chiesa *et al.* (1993) agree that simple tests such as pyrazinamidase and salicin-aesculin are primary tools to identify *Y. enterocolitica* isolates as a pathogenic or non-pathogenic biotype, while O typing is important in understanding the ecology and epidemiology of the disease.

DNA colony hybridisation: This a new technique for detecting pathogenic bacteria in foods that has the advantage of identifying specific genotypes (Schiemann 1989). A DNA probe for *Y. enterocolitica* has been developed from the virulence plasmid (Hill *et al.* 1983). DNA hybridisation depends upon isolated clones of the organism sought, and this, as in culture techniques, requires at least some degree of selectivity in recovery media. Therefore, the success of this methodology will depend in part on the traditional methods of selective enrichment and isolation.

Serum resistance: One indication of bacterial virulence is resistance to killing by normal serum (Taylor 1983). Nilehn (1973) first reported that *Y. enterocolitica* was more resistant to

serum destruction when grown at 25 °C than at 37 °C, and that serum resistance correlated with bacterial clearance *in vivo*. Serum resistance appears to be related to outer membrane proteins (Balligand *et al.* 1985). Pai and De Stephano (1982) found a different influence with temperature, and that loss in serum resistance was accompanied by loss of virulence in rabbits and mice and also calcium dependence at 37 °C. Other investigators (Chiesa and Bottone 1983) could not relate serum resistance to any virulence marker.

Other tests: These tests include invasion of Hela cells (Devenish and Schiemann 1981), plasmid encoded outer membrane proteins (Skurnik 1985), and disturbance of the normal chemiluminescent response of human neutrophils (Lian and Pai, 1985). Studies by Schiemann and Devenish (1981) and Pai and De Stephano (1982) indicate that at least two factors are required for virulence in *Y. enterocolitica*: the ability to penetrate the mucosal surface of the intestine (measured by the HeLa cell infectivity test) and the presence of virulence factors (measured by the autoagglutination test). In general, most of the above *in vitro* tests have been linked to the carriage of a plasmid 40-42 MDa plasmid first described by Zink *et al.* (1980). No single *in vitro* assay is a reliable indicator of pathogenic strains of *Y. enterocolitica* but the combination of biochemical identification to the species level and total plasmid profile, when associated with restriction endonuclease digests is predictive of *Y. enterocolitica* virulence as measured by lethality in mice.

b. *IN VIVO* TESTS

Y. enterocolitica infection is contracted primarily by the oral route, therefore, animal studies on pathogenicity using this route of infection may accurately describe pathogenesis in humans (Schiemann 1989). Carter (1981) has reviewed the various laboratory models used for describing the pathogenicity of *Y. enterocolitica*. The pathogenic potential of *Y. enterocolitica* has been assessed by using animal models including the Sereny test or guinea pig conjunctivitis model (Sereny 1955), the suckling mouse assay for heat stable enterotoxin (Pai and Mors 1978), mouse intraperitoneal challenge (Aulisio *et al.* 1983), and mouse diarrhoea and splenic infection following oral challenge (Bakour *et al.* 1985). Animal models have limitations because of cost and lack of appropriateness, except in specialised facilities (Noble *et al.* 1987).

Zink *et al.* (1980) first demonstrated that isolates of *Y. enterocolitica* serovar O:8 associated with human infections produced positive results in the Sereny test i.e., keratoconjunctivitis.

Later, Gemski *et al.* (1980) reported that *Y. enterocolitica* serovar O:8 carried a 52.2 ± .1 Mda plasmid that was required for development of keratoconjunctivitis in the guinea pig eye. This plasmid also coded for calcium-dependent growth at 37 °C which could be demonstrated in cultures on calcium-deficient magnesium oxalate (MOX) agar. The association between calcium dependence and positive Sereny test for serovar O:8 was confirmed by Schiemann and Devenish (1980), however, these workers observed that the infection was confined to the conjunctiva and did not involve the cornea as in classic keratoconjunctivitis.

Pai and Mors (1978) and Pai *et al.* (1978) first demonstrated that *Y. enterocolitica* produces a heat-stable enterotoxin *in vitro* that is detectable by the suckling mouse assay and in rabbit ileal loop.

The laboratory mouse has been used frequently to study the pathogenicity of *Y. enterocolitica.* North American serovars O:8 and O:21 carrying the virulence plasmid are highly pathogenic for adult mice by intraperitoneal injection or by oral inoculation at slightly higher doses, by subcutaneous injection at considerably higher doses (Quan *et al.* 1974) or by infection through drinking water (Carter 1975; Laird and Cavanaugh 1980; Schiemann and Devenish 1982; Schiemann 1981). Virulence in the mouse is related to calcium dependence, which is plasmid mediated (Gemski *et al.* 1980). Lethal infections with serovars O:3, O:9, and O:5,27 have been reported when the dose is above 10^7 bacteria administered intravenously (Maruyama *et al.* 1979). These serovars will also produce lethal infections in suckling mice infected intraperitoneally (Aulisio *et al.* 1983).

Bakour *et al.* (1985) developed a mouse model using spleen counts for differentiating plasmid-positive from plasmid-negative strains of serovars O:3, O:9, O:5,27 and non-pathogenic serovars after oral infection through drinking water over 24 h. The results were more reproducible when the mice were stressed by holding them for 6 days at 4 °C before the spleen was removed and bacterial counts were completed. Plasmid-negative and non-pathogenic serovars were never found in the spleen under these conditions.

RAPID DIAGNOSTIC METHODS

The method most commonly applied for the detection of *Y. enterocolitica* in foods and other samples includes cold pre-enrichment, selective enrichment and biochemical confirmation. The

entire procedure may take more than three weeks to complete. In situations when there is a need to arrive at a quick diagnosis, one cannot therefore rely on this method, Hence, the development of rapid and specific detection methods is necessary. There is also a need for rapid diagnostic methods because the development of isolation procedures which clearly differentiate pathogenic from non-pathogenic strains has proved difficult (Kapperud 1991).

A few rapid methods have recently been developed for the detection of pathogenic species of *Yersinia* from clinical samples and food. Using a synthetically produced oligonucleotide probe, it was found that the prevalence of pathogenic yersiniae in Norwegian pork products was substantially higher than previously demonstrated by conventional methods (Kapperud *et al.* 1990). A simple colony immunoblotting method using monoclonal antibodies (MABS) was developed to detect *Y. enterocolitica* serotype O:3 in the faeces of pigs (Li *et al.* 1992). The use of indirect immunofluorescence has been used successfully to detect *Y. enterocolitica* O:3 cells in the tonsils of pigs (Shiozawa *et al.*1991) and *Y. enterocolitica* O:3, O:5, O:8 and O:9 from histological preparations of human clinical specimens (HoogKamp-Korstanje *et al.* 1986). Several reports have also described the use of the polymerase chain reaction (PCR) for detection of *Y. enterocolitica* species from various sources including humans, animals, food and water (Fang *et al.* 1992; Fenwick and Murray 1991; Ibrahim *et al.* 1992; Kapperud *et al.* 1993; Kwaga *et al.* 1992; Nakajima *et al.* 1992; Wren and Tabaqchali 1990). According to Saiki *et al.* (1988), the PCR has the potential of providing a rapid, specific as well as a sensitive method of detection of pathogenic bacteria. However, PCR necessitates concentration of bacteria from the sample, as only a small volume of material can be analysed. Rasmussen *et al.* (1995) recommends the use of immunomagnetic separation (IMS) prior to PCR to concentrate the bacteria. Moreover, IMS eliminates inhibiting material both in the removal of the supernatant fluid and due to the washing of immunomagnetic particles. The authors concluded that IMS-PCR can be used to detect *Y. enterocolitica* after pre-enrichment.

TREATMENT, PREVENTION AND CONTROL

Treatment

Specific treatment of infection is seldom required since yersiniosis is usually self-limiting. Gastrointestinal symptoms usually subside in 2-3 weeks without recourse to antibiotics. When the disease is mild such as with enterocolitis or uncomplicated pseudoappendicular syndrome, antimicrobial chemotherapy is not useful. However, systemic infections or extra-intestinal

infections should be treated and antibiotic therapy is indicated in patients with septicaemia, intra-abdominal sepsis or severe terminal ileitis (Mair and Fox 1986). The antibiotic of choice should be based on the results of sensitivity tests. Before the results of antimicrobial-susceptibility tests, a combination of doxycycline and an aminoglycoside is recommended for patients with septicaemia (Horstein *et al.* 1985). Most strains produce β-lactamases which renders them resistant to "older" penicillins and first- generation cepahalosphorins, including ampicillin and cephalothin (Robins-Browne 1992). Two beta-lactamases, A and B have been identified in *Y. enterocolitica*: beta-lactamase A hydrolyses a variety of penicillins and cephalosporins, and beta-lactamase B is a strong cephalosphorinase but does not hydrolyse many of the penicillins (Cornelis *et al.* 1973). In contrast, *Y. pseudotuberculosis* is susceptible to penicillins because it lacks beta-lactamase (Kanazawa and Kuramata 1974). Ampicillin, cephalothin, and carbenicillin are also found to be active against *Y. pseudotuberculosis* (Mair and Fox 1986).

Y. enterocolitica is usually susceptible to the aminoglycosides (gentamicin, kanamycin, streptomycin), polymixin, chloramphenicol, trimethoprim-sulfamethoxazole and newer beta-lactam antibiotics (Scribner *et al.* 1982) especially ceftriaxone, cefotaxime, ceftizoxime, and cefmenoxime (Hornstein *et al.* 1985). Anecdotal evidence suggests that patients with prolonged diarrhoea or generalised infection will respond to treatment with trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol or an appropriate β-lactam antibiotic. A synergistic action between sulfamethoxazole and trimethoprim against *Y. enterocolitica* has been observed (Hammerberg *et al.* 1977). Isolates of *Y. enterocolitica* are highly susceptible to aminoglycosides *in vitro,* but patients treated with these agents may respond poorly, which may be due to a large proportion of bacteria being located intracellularly (Robins-Browne 1992).

In pigs, there is at present no general indication for the treatment of *Yersinia* infections since clinical signs are so rare. In the study conducted by De Allie (1994), there was no evidence to suggest that the pathogenic strains of *Yersinia* isolated from the tonsils of pigs are pathogenic for pigs. But the disease has been identified as a rare cause of sporadic enteritis in pigs in New Zealand (Gill 1996). Experimental infection of colostrum-deprived newborn pigs with a human isolate of *Y. enterocolitica* biotype 4 serotype O:3 has produced clinical disease which include anorexia, vomiting and diarrhoea (Shu *et al.* 1995). Studies *in vitro* suggest that isolates are

often sensitive to oxytetracycline, furazolidone, neomycin, sulphonamides, and spectinomycin. Tetracyclines have also been used in feed to eliminate infection (Taylor 1992).

Prevention and Control

The most important reservoirs of *Yersinia* spp. are animals particularly pigs and occasionally pet dogs in the case of *Y. enterocolitica,* and all kinds of domestic and free-living species for *Y. pseudotuberculosis.* Avoiding contact with animals that are obviously ill is a reasonable precaution for preventing human infections (Schiemann 1989). Morita *et al.* (1968) found that *Y. pseudotuberculosis* infections could be prevented by excluding birds and rodents from contact with humans. In New Zealand, since 1993, the number and size of outbreaks of yersinia enteritis in young deer have declined because of improved management techniques such as indoor wintering and the widespread use of a formalin-killed vaccine which contains *Y. pseudotuberculosis* serotypes I, II and III (Gill 1996). The results indicated a good protection of calves against clinical yersiniosis if two doses of vaccine are given or administered 3 to 4 weeks apart in autumn (Mackintosh 1993). However, according to Orr (1995), vaccination may not be successful in the face of a severe challenge but may reduce the severity of the outbreak.

Present epidemiological evidence suggests a link between the pig reservoir of *Y. enterocolitica* and human infection. In endemic regions, where pigs are the major reservoir of infection preventive measures could be taken to reduce the bacterial load in slaughtered animals. Since spread of *Y. enterocolitica* from pig to pig appears to occur from contact with faeces, hygiene coupled with housing groups of pigs in separate drainage areas is likely to reduce infection. Control of flies and rodents and disinfection of pens before restocking also appear to reduce transmission (Taylor 1992). At the farm level, newborn piglets are easily colonised and become long-term pharyngeal and intestinal carriers, without signs of illness (Schiemann 1989).

Prevention of human yersiniosis relies chiefly on good hygienic practices, especially during food processing and handling. During slaughtering and processing, bacteria present in the oral cavity or intestinal contents may easily contaminate the carcasses and the slaughterhouse environment. The application of Hazard Analysis Critical Control Points and Good Manufacturing Practices (HACCP/GMP) during pig slaughter and processing must be focused on limiting such contamination. In this context, *Y. enterocolitica* presents a very special problem because it is a psychrophile and able to survive and propagate at temperatures

approaching 0 °C. Hence, chilling of food products can not be considered an effective control measure (Kapperud 1991). The introduction of *Y. enterocolitica* into refrigerated foods, especially through cross-contamination or by handlers may occur leading to multiplication during extended refrigerated storage or if not done previously (Schiemann 1989). Since water can be a vehicle for transmitting *Y. enterocolitica* (Toma and Diedrick 1975; Botzler *et al.* 1976; Harvey *et al.* 1976; Highsmith *et al.* 1977; Jansen and Saari 1977; Kapperud 1977; Caprioli *et al.*1978; Schiemann 1978), the use of untreated or contaminated water in food manufacturing will always be accompanied by a certain risk.

In general, standard measures to prevent and control zoonotic salmonellosis can also be applied to prevent and control yersiniosis (WHO 1987). These measures include three main lines of defence:

- the pathogen-free breeding and rearing of slaughter animals
- the improvement of hygiene during transportation, slaughtering and processing including different means of decontamination; and
- education of all categories of people involved in production, processing and final preparation of food products.

Until more is known about the epidemiology of yersiniosis in New Zealand, the following general precautions are recommended (McCarthy *et al.* 1995):

- wash hands prior to food handling and eating, after handling raw meat, and after animal contact
- prepare foods in a clean environment
- cook meat , especially pork thoroughly
- protect water supplies from human and animal excreta
- exclude people with diarrhoea from food handling

Since *Y. enterocolitica* is widespread in nature in both living and non-living systems, the general methods of environmental hygiene and sanitation of food and water should be applied in the control of animal diseases caused by this organism (Morris and Feeley 1976).

PUBLIC HEALTH IMPLICATIONS

A review of several studies concerning different aspects of the epidemiology of human pathogenic *Yersinia enterocolitica* pinpoints that it is an important foodborne pathogen (Cover and Aber 1989; Schiemann 1989; Doyle 1990; Kapperud 1991) which has caused several outbreaks of disease (Black *et al.* 1978; Greenwood and Hooper 1990; Tacket *et al.* 1985; Aber *et al.* 1982; Maruyama 1987; Lee *et al.* 1990). The organism has been isolated from many foodstuffs including pork and pork products as well as milk and dairy products. Pigs are implicated as reservoirs for human pathogenic strains (Pedersen 1979; Andersen *et al.* 1991; Kapperud 1991) and some isolates from pigs are found indistinguishable from similar human isolates by currently available methods (Kapperud 1990; Blumberg 1991). New sensitive methods such as DNA-DNA hybridisation have revealed a high contamination of pork products by pathogenic *Y. enterocolitica* (Nesbakken *et al.* 1991). These products may be important vehicles of infection. *Y. enterocolitica* O:3 is the serotype most commonly associated with illness in Northern Europe (Mollaret *et al.* 1979; Verhaegen *et al.* 1991; Prentice *et al.* 1991) and is now also recorded frequently in the United States (Bottone *et al.* 1987; Bisset *et al.* 1990). The highest isolation rates in pigs have been from the tonsils and oral cavity as well as intestinal contents hence, the organism may be readily spread from the throat and faeces to the rest of the carcass during slaughter. Changes in slaughterhouse practices as well as modification of the preparation of food for human consumption may substantially reduce the risk of exposure to this microorganism.

The consequences of yersiniosis are severe and can include prolonged acute infections, pseudoappendicitis and long-term sequelae such as reactive arthritis and erythema nodosum (Mair and Fox 1986; Cover and Aber 1989). *Y. enterocolitica* infection is a financial and public health problem of greater magnitude than the actual number of cases would suggest (Ostroff *et al.* 1992).

OTHER *YERSINIA* SPP.

Yersinia enterocolitica has become well established as an enteric pathogen since it was first described in 1939 (Bottone 1981, 1983; Butler 1983; Cornelis. *et al.* 1987). However, there is considerable confusion in the literature because not all strains of this species cause intestinal infections. Unlike *Salmonella* and *Shigella* species which are intrinsic pathogens (essentially all

strains can cause enteric infections), there is strain-to-strain variation in the pathogenicity of *Y. enterocolitica* (Bottone 1981; Butler 1983; Cornelis *et al.* 1987; Kay *et al.* 1983; Wachsmuth *et. al.* 1984).

Previously classified *Y. enterocolitica*-like organisms such as *Y. frederiksenii, Y. kristensenii, Y. intermedia, Y. aldovae, Y. rhodei, Y.mollaretti* and *Y. bercovieri* are now classified as seven separate species. They are gram-negative bacteria of world-wide distribution frequently isolated from environmental, animal and human sources. The environmental strains constitute a spectrum of phenotypic variants which display a variety of antigenic factors. There is growing evidence that such environmental yersiniae may not be pathogenic for man (Van Noyen *et al.* 1981), although some environmental strains may occasionally act as opportunistic microorganisms leading to atypical clinical manifestations (Wauters 1981; Kapperud 1991).

Noble *et al.* (1987), however, found that strains of other *Yersinia* species had one or more properties perhaps suggestive of pathogenicity, but these results have not been confirmed. At present there is no convincing evidence that strains of other *Yersinia* species are enteric or invasive pathogens. Further studies are needed to determine whether they have a role in human disease as opportunistic pathogens.

Section B: Yersiniosis in Pigs

INTRODUCTION

Healthy pigs have been found to carry strains of *Y. enterocolitica* pathogenic to humans in frequencies ranging from 25 to 80 % (Wauters 1970; Pedersen 1979; Christensen 1980; Doyle *et al.* 1981; Nesbakken and Kapperud 1985). Pathogenic strains of *Y. enterocolitica* serotype O:3; O:5,27; O:8 and O:9 and also *Y. pseudotuberculosis* have frequently been isolated from the intestine and oral cavity of pigs (Christensen 1980; De Boer *et al.* 1986; Doyle *et al.* 1981; Harmon *et al.* 1984; Hurvell 1981; Schiemann 1980; Schiemann and Fleming 1981; Asplund *et al.* 1990; Tsubokura *et al.* 1976). There has also been reports that the major source of yersiniosis in humans may be pigs, since strains isolated from raw retail pork are indistinguishable from those isolated from humans with yersiniosis (Fukushima and Tsubokura 1985; Fukushima *et al.* 1987; Shiozawa *et al.* 1987).

The significance of pigs as a source of *Y. enterocolitica* infections in man was established by epidemiological studies (Tauxe *et al.* 1987; Ostroff *et al.* 1994). In some investigations a significant correlation has been shown between the consumption of pork and the occurrence of yersiniosis in humans (Christensen 1987; Tauxe *et al.* 1987). An outbreak of yersiniosis among infants in the United States in which pig chitterlings were implicated was reported by Lee *et al.* (1990). Many of the affected infants were being cared for by a surrogate mother (usually the grandmother) and this person was frequently noted to bottle feed the infant while cleaning and preparing pork intestines, known as chitterlings at the kitchen sink. A prospective case-control study in Norway conducted by Ostroff *et. al.* (1994) suggests a link between yersiniosis and consumption of undercooked pork and sausage products, and untreated water. According to Samadi *et al.* (1992), *Y. enterocolitica* infections in humans is apparently rare in non-pork eating Moslem countries which also supports the role of pork as the vehicle of infections.

Porcine and human strains were found to be indistinguishable by biochemical, serological and phage-typing methods (Wauters 1970, 1979; Hurvell 1981). Furthermore, typing methods based on detection of heterogeneity in plasmid and chromosomal DNA has so far been unable to distinguish between human and porcine strains (Nesbakken *et al.* 1987; Kapperud *et al.* 1990). Nesbakken *et al.* (1987) demonstrated that the virulence plasmids of both porcine and human isolates have identical restriction patterns. Later, Kapperud *et al.* (1990) found that both human and porcine isolates exhibit the same chromosomal restriction fragment length

polymorphism. Blumberg *et al.* (1991) showed that strains isolated from humans and chitterlings made from pig intestines epidemiologically associated with the source of the outbreak mentioned above had identical ribotype patterns. According to Nesbakken *et al.* (1991), new sensitive methods such as DNA-DNA hybridisation have revealed a high contamination of pork products by pathogenic *Y. enterocolitica,* making these products important vehicles of infection.

Studies in Europe, Canada and Japan have shown that pigs are also important reservoir for *Y. pseudotuberculosis* (Narucka and Westendoorp 1977; Toma and Diedrick 1975; Tsobokura *et al.* 1976; Zen-Yoji *et al.* 1975). The presence of the organism in pigs suggests that pork may be contaminated. Studies conducted in Japan have shown that retail meat was contaminated with *Y. pseudotuberculosis* (Shiozawa *et al.* 1988). *Y. pseudotuberculosis* serotype 4b has been isolated from a retail shop in Japan (Fukushima 1985). These authors have pointed out that there is a close relationship between the presence of *Y. pseudotuberculosis* in pigs and contamination of pork by this organism, suggesting that pork may be an important source of *Y. pseudotuberculosis* infection. However, because of very limited epidemiological studies, the link between a pig reservoir and transmission to humans with *Y. pseudotuberculosis* still remains unclear.

YERSINIA INFECTIONS

a. Y. enterocolitica

Y. enterocolitica is found world-wide and has been reported in pigs in many countries (Bockemuhl *et al.* 1979; Cantoni *et al.* 1979; De Barcellos and Castro 1981; Doyle *et al.* 1981; Schiemann and Fleming 1981; Hunter *et al.* 1983). According to Christensen (1980), not all pig herds are infected. The incidence of *Y. enterocolitica* carriage by pigs varies among herds (Fukushima *et al.* 1983) and geographic location. Feed has been found to be infected, and studies of the dissemination of infection in pig facilities indicate that infection is transmitted from contaminated pens in which the yersiniae can persist for more than 3 weeks. It appears that transmission from pig to pig is via faecal contamination of pens, water and feed (Taylor 1992). Maintenance of *Y. enterocolitica* in pigs appears to depend more upon animal contact than any environmental source, and colonisation has been related to the level of hygiene associated with pig husbandry (Aldova *et al.* 1980). Young pigs become carriers within 1-3 weeks after entering contaminated pens, remaining colonised for long periods without re-infection (Fukushima *et al.* 1984a). Older pigs without any apparent signs of infections will excrete *Y. enterocolitica* for 3-7 weeks after experimental challenge, then show cross-

protection between serovars O:3 and O:5,27 upon re-challenge. New-born pigs are easily colonised and become intestinal and pharyngeal carriers but are very resistant to disseminated infections and disease (Schiemann 1989). Pigs appear to maintain the organism without signs of illness (Fukushima *et al.* 1983).

Y. enterocolitica has been shown to infect pigs orally, to multiply and be present in the faeces within 2-3 weeks of infection and to disappear from the faeces within 30 weeks (Fukushima *et al.* 1984a). Studies by Erwerth and Natterman (1987) suggest that oral infection is followed by establishment of infection in the tonsils and the development of enteritis in the ileum and large intestine. Similar colonisation was reported by Schiemann (1988).

Y. enterocolitica is shed in the faeces of infected pigs for up to 30 weeks and has been shown to be transmitted to human food and elsewhere on farms by flies (Fukushima *et al.* 1979). Other studies suggest that faeces can remain infected for up to 12 weeks and that in suitable substrates, the organism may multiply at 22-24 °C.

The organism has been isolated abundantly in culture from outbreaks of diarrhoea in weaned pigs from which no other infectious agents could be recovered (Taylor 1992). Mild fever (39.4 °C, 103 °F) was present and the diarrhoea contained no blood or mucus and was dark in colour. Blood-stained mucus may also be found in some diarrhoeic patients and on solid faeces passed by pen mates. *Y. enterocolitica* has also been isolated from the rectal mucosa in cases of rectal stricture (Taylor 1992).

Most human pathogenic *Y. enterocolitica* are able to colonise the lymphoid tissue and gastro-intestinal tract of pigs without causing any clinical signs (Fukushima *et al.* 1984; Schiemann 1988), but a study from China (Zheng 1987) reporting the isolation of *Y. enterocolitica* from pigs with diarrhoea suggests that the bacteria may also be capable of producing overt illness in pigs. A prevalence of 49.4 % (60 out of 124 samples) from the faeces of diarrhoeic pigs was reported. Fifty eight of the isolates (96.6 %) were shown to belong to serotypes O:3 or O:9. The clinical signs are not distinctive, but the occurrence of mild fever with blood and mucus on solid faeces can indicate yersiniosis in the absence of swine dysentery (Taylor 1992).

Erwerth and Natterman (1987) have described in detail the lesions caused by *Y. enterocolitica* infection which consist of catarrhal enteritis in the small and large intestines. Microcolonies of

the organism can be seen in the disrupted intestinal epithelium and in pigs with rectal lesions, bacterial penetration and inflammation reach the muscularis mucosae.

b. Y. pseudotuberculosis

Y. pseudotuberculosis is less frequently identified in pigs than *Y. enterocolitica* and is less often demonstrated in America than in Europe or Japan (Taylor 1992). It is commonly found in rodents, which probably represent the main source of infection for pigs. Although isolations of *Y. pseudotuberculosis* from healthy pigs have been reported (Mair *et al.* 1979; Tsubokura *et al.* 1984), its association with pigs appears to be rare (Harper *et al.* 1990). The organism has been recovered from a clinical case in Canada (Langford 1972), from diarrhoeic pigs in Brazil (De Barcellos and De Castro 1981), from sick and dead pigs in New Zealand (Hodges *et al.* 1984), from 8-12 weeks old pigs in Australia (Slee and Button 1990), from pigs with chronic diarrhoea in Argentina (Fain Binda *et al.* 1992), and in Japan from pigs with diarrhoea and oedema of the eyelids, lower face, and lower parts of the abdomen (Morita *et al.* 1968). *Y. pseudotuberculosis* serotype III was the most frequently isolated strain from the reports described above.

Animals infected with *Y. pseudotuberculosis* had watery, non-bloody diarrhoea. Gross lesions in pigs include intestinal intussusception, and thickening of the mucosa of the spiral colon with associated fibrin tags (Slee and Button 1990). Lesions due to generalised infection with *Y. pseudotuberculosis* have been described in pigs (Fain Binda *et al.* 1992; Morita *et al.* 1968). The lesions were miliary grey-white spots in the liver and spleen and swollen, grey-white mesenteric lymph nodes, resembling those caused by *Y. pseudotuberculosis* in other species. A catarrhal and diptheritic change was described in the colon and rectum, and oedema and ascites also occurred. Microscopic lesions in the large intestines showed central caseous necrotic foci, surrounded by lymphocytes, plasmacytes and macrophages in the submucosa, with oedema and chronic inflammatory infiltrates in the mucosa. *Y. pseudotuberculosis* was isolated from the liver, spleen, lungs, duodenum, rectum and mesenteric lymph nodes. It can also be isolated from inflammatory lesions of the rectal mucosa (Taylor 1992).

Anatomical Distribution

Y. enterocolitica has been isolated from the intestinal tract and oral cavity of pigs in several countries (Christensen 1980; Hurvell 1981; Mollaret 1979). Numerous studies have shown that

pigs may be asymptomatic carriers of serotype O:3 and O:9, which are the most important causal agents of human *Y. enterocolitica* enteritis in Europe (Kapperud 1985; Pedersen 1979; Schiemann and Fleming 1981; Wauters 1979).

Y. enterocolitica O:3 is often found in the intestinal contents and faeces of pigs. A study conducted by Shiozawa *et al.* (1991) in Japan found that 24.3 % of 140 pigs were carriers of the organism in the caecum. The bacterium has also been isolated from pig carcasses (Nesbakken 1988; Nesbakken *et al.* 1994) in Norway. Nesbakken (1988) isolated *Y. enterocolitica* O:3 from the surface of 19 of 30 pig carcasses with the highest frequency from the anterior part of the carcass. In contrast, Christensen (1987) isolated *Y. enterocolitica* O:3 from 31 of 100 pigs examined with an even distribution on the carcass in Denmark. Andersen (1988) isolated pathogenic *Y. enterocolitica* from carcasses with varying frequencies depending on the evisceration technique used. Manual evisceration was found to correspond with high frequencies of contamination: 26.3 % on the medial aspect of the hindlimb and 12.9 % on the split sternum. The use of a mechanised bung cutter was found to reduce the rate of contamination, especially when the use of the bung cutter was supplemented by enclosing the anus and rectum in a plastic bag to minimise faecal contamination (Nesbakken *et al.* 1994). The organism was recovered from only 0.8 % of carcasses when the plastic bag technique was employed. *Y. enterocolitica* O:3 was recovered from 10 % of pig carcasses when eviscerating procedures did not include the use of plastic bag technique (Nesbakken *et al.* 1994).

In contrast, De Boer and Nouws (1991) did not isolate pathogenic yersiniae from samples of 210 pig carcasses or from 20 samples of pig head meat, suggesting that contamination of carcasses during the slaughtering process with yersiniae from either faecal or tonsillary region did not seem to occur frequently in the Netherlands. Differences in sampling techniques, enrichment and/or slaughtering practices may explain these results.

There are therefore variations in the frequency of isolation of microorganism from different sites of pigs in different countries (Asplund *et al.* 1990; DeBoer and Nouws 1991). Other authors have demonstrated that *Y. enterocolitica* inhabits the tonsils and the tongue in addition to the gastrointestinal tract making both sites important for the recovery of the organism (Nesbakken and Kapperud 1985; Christensen 1987; Kapperud 1991).

Y. pseudotuberculosis has also been consistently recovered from the intestinal contents and tonsils of healthy slaughtered pigs in various parts of the world (Toma and Diedrick 1975; Tsobokura *et al.* 1976; Weber and Knapp 1981; Zen-Yoji *et al.* 1974).

Seasonal and Geographical Occurrence

Y. enterocolitica has been isolated from humans in many countries of the world, but it seems to be found most frequently in cooler climates (WHO 1981; 1987). Relatively little is known about the incidence in tropical countries (Kapperud 1991).

There are numerous reports on the seasonal incidence of yersiniae infections in slaughtered pigs (Bockemuhl *et al.* 1979; Tsubokura *et al.* 1973, 1976; Weber and Knapp 1981; Zen-Yoji *et al.* 1974). As for *Y. enterocolitica,* no seasonal variation has been reported in earlier publications (Nilehn 1969). Later, many workers, however, reported that *Y. enterocoltica* infections were more prevalent in autumn and winter (Ahvonen 1973; Arvastson *et al.* 1971; Winblad 1973) or winter or early spring (Rakovsky *et al.* 1973; Vandepitte *et al.* 1973).

The literature on the rate of isolation of harmful *Yersinia* strains from pigs indicates that there is a definite, climatically determined north/south gradient (Bulte *et al.* 1992). The psychrotrophic *Yersinia* strains are found more often in slaughter pigs in Scandinavia than in Central European countries. In Central Europe, the rate at which harmful *Yersinia* strains were detected was 10.5 % for tongues and 14. 2 % for tonsils, which is well below the ratesfound in Scandinavian countries which were 20 % and 57. 7 % respectively. The rate at which the organisms are excreted in the faeces is low in all countries at 2.4 to 2.7 %.

According to Tsubokura *et al.* (1976), the isolation of *Y. enterocolitica* from the caecal contents of pigs was dependent on season. The organisms were more frequently isolated in winter to early spring than in summer. However, no relationship could be established between rate of isolation and serogroup distribution. In a study conducted by Bockemuhl *et al.* (1979), the lowest incidence in faecal samples was observed during the summer months (August, 1976, 0 %) but increased steadily to a maximum in spring (April 1977, 71.2 %). With one exception, serotype O:3 and O:9 were only isolated during October to December. Monthly variation of infection in pig faeces were also studied by Weber and Knapp (1981). Their study showed a peak in January (winter) with 14 % (13 of 95) infected, with a mean of 2.7 % (33 of 1206) infected throughout the year.

In a study conducted in New Zealand, yersiniae were isolated from pigs throughout the year with peak prevalence in the colder months. Seasonal differences in the occurrence of serotypes O:3 and O:5,27 were not significant, with both being found throughout the year with lowest isolation rates in summer (De Allie 1994).

Y. pseudotuberculosis infections and isolations from animals and pork were found in the colder months in Japan (Fukushima *et al.* 1987). Based on the observations of Slee and Button (1990), the seasonal occurrence of *Y. pseudotuberculosis* infection was the same for sheep, goats and pigs, being restricted to the winter and spring months. De Allie (1994) observed seasonal variation in the isolation of serotypes of *Y. pseudotuberculosis*. Serotypes I and II were only detected in winter and spring. Serotype III was detected throughout the year with a peak in autumn and low in summer.

With regards to geographical occurrence, there are few studies that compare the prevalence of *Y. enterocolitica* in tropical and temperate countries. In a study conducted by Adesiyun *et al.* (1992), a prevalence of 2 % for *Y. enterocolitica* in rectal swabs of piglets in Trinidad was reported which was significantly lower than the 16. 1 % found for rectal swab samples in another study conducted in the same country by Adesiyun and Krishnan (1995). Studies in similarly warm tropical environments have recorded considerably lower prevalence of *Y. enterocolitica* infection such as 1.6 % found in rectal and tongue swabs in the Zaria area of Nigeria (Lombin *et al.* 1985) and the 0.53 % reported for slaughter pigs in Plateau area of Nigeria (Okoroafor *et al.* 1988). Compared to reports from temperate regions, the prevalence of 21. 3 % reported by Adesiyun and Krishnan (1995) is considerably lower than the 43.4 % found in Norwegian pigs (Nesbakken and Kapperud 1985) and the 51.6 % found in pigs in the USA (Doyle *et al.* 1981).

CARRIER STATUS OF *YERSINIA* SPP. IN PIGS

Tonsillar Carriage

The highest isolation rates in pigs have been from the tonsils and oral cavity. Several investigators in Europe (de Boer *et al.* 1986, 1991), USA (Doyle and Hugdahl 1983; Doyle *et al.* 1989; Schiemann and Fleming 1981) and Japan (Fukushima *et al.* 1989; Shiozawa *et al.* 1987, 1988) have reported that pathogenic strains of *Yersinia* are carried on the tongue and in the throat of healthy pigs, suggesting potential dissemination from the tonsils to other

pharyngeal regions, and to the carcass during slaughter and processing. It is well documented that *Y. enterocolitica* O:3 can be isolated from approximately 25-50 % of tonsils and tongues of healthy pigs (Andersen *et al.* 1991; Christensen 1980; Doyle *et al.* 1981; Nesbakken and Kapperud 1985; Pedersen 1979; Schiemann and Fleming 1981; Wauters 1979). Nesbakken (1985) was able to isolate *Y. enterocolitica* O:3 from 68 % of 47 pigs by enrichment of tongues and tonsillar tissues.

In the lower North Island of New Zealand, *Y. enterocolitica* is the most common species of *Yersinia* isolated from the tonsils of pigs sent for slaughter. *Y. enterocolitica* biotype 4 serotype O:3 and *Y. enterocolitica* biotype 3 serotype O:5,27 are the most common pathogenic variants of *Y. enterocolitica* harboured in the tonsils of these animals in New Zealand (De Allie 1994).

Y. pseudotuberculosis is also a frequent inhabitant of the tonsils. Serotypes I, II and III were detected with serotype III being the most consistent isolate (De Allie 1994).

Faecal carriage

Isolation from intestinal contents has been reported to be of much lower frequency, approximately one-tenth of that found in tongues and tonsils (Aldova *et al.* 1980; Esseveld and Goudzwaard 1973; Pedersen and Winblad 1979; Szita *et al.* 1980; Toma and Deidrick 1975; Tsubokura *et al.* 1973; Weber and Lembke 1981; Zen-Yoji *et al.* 1974). However, higher frequencies of isolation from faeces have also been reported. Fukushima *et al.* (1983) found that 7.1 % of faecal samples from five farms were infected, with as much as 14.6 % infected in one of the farms. In Japan, Maruyama (1987) examined caecal contents at 41 abattoirs and found a constant high percentage of pigs infected. Out of 9423 pigs examined, 1114 (11.8 %) were found to be infected with the human pathogenic serotypes O:3, O:9 or O:5,27. In a study conducted by Andersen (1988) in Denmark, out of total 1458 faecal samples, 24.7 % were positive for *Y. enterocolitica* 4/O:3. Zheng (1987) in China and Bockemuhl *et al.* (1979) in Germany reported findings of 48.4 % and 27.4 % respectively. In another study conducted in Japan, Shiozawa *et al.* (1991) found that 24.3 % of 140 pigs were carriers of the organism in the caecum, with counts varying from less than 300 up to 100 000 bacteria per gram of intestinal content. In a study conducted by Adesiyun and Krishnan (1995), it was clearly evident that rectal swabs yielded significantly more *Y. enterocolitica* isolates than either tongue swabs or tonsils. This finding is at a slight variance with the reports of De Boer and Nouws (1991) in theNetherlands where 17 % and 20 % of rectal and tongue swabs, respectively were

positive for *Y. enterocolitica.* The rather lower isolation rate found in the tonsillar tissue compared to the rectal swabs may be explained, in part by the fact that tonsils were subjected to the high scalding temperature prior to sampling while the rectal samples were not (Adesiyun and Krishnan 1995). Also in their study, the fact that over a six-week period of sampling, *Y. enterocolitica* serotype O:3 was repeatedly recovered from rectal swabs of pigs suggests that this serotype can be readily recovered from faeces.

CONTAMINATION DURING PIG SLAUGHTERING

Slaughter animals are recognised reservoirs of a number of human pathogens and following slaughter, carcass contamination often occurs through faecal materials, hides and pelts (Nesbakken and Kapperud 1985; Grau 1986; Asplund *et al.* 1990). Pig slaughter is an open process with many opportunities for the contamination of the pork carcass with potentially pathogenic bacteria (Borsch *et al.* 1996).

During lairage, pathogenic bacteria may spread from infected to non-infected pigs as shown for *Yersinia* spp. by Fukushima *et al.* (1990). During subsequent slaughtering procedures, pathogenic bacteria may spread to the carcass from the intestines, stomach contents, and oral cavity/oesophagus. The critical operations are circumcision of the rectum, removal of the intestinal tract and removal of the pluck set. In Denmark, Norway and Sweden, it is common practice to use plastic bags to seal off the rectum after loosening the rectum. This procedure reduces dissemination of organisms present in the faeces to the carcass and subsequently to the cut meat and can be considered of great significance for the production of pork. Pathogenic bacteria such as *Yersinia* spp. are present in high numbers on tonsils. The incidence of *Yersinia* spp. on tonsils, carcass fore-end and liver/diaphragm in a Danish study was found to be 56 %, 40 % and 25 % respectively (Christensen and Luthje 1994). After changing pluck removal, so that the tongue was left untouched in the non-split head until the head was removed from the carcass, contamination was reduced. Meat inspection procedures concerning the head represent a cross-contamination risk. Pathogenic bacteria such as *Yersinia* spp. may be transferred from the tonsillary region to the other parts of the carcass by the knives and hands of butchers and meat inspection personnel (Nesbakken 1988). Furthermore, bacterial growth will occur during storage of the pork. *Y. enterocolitica* being a psychrotroph is able to grow on meat stored at chill temperatures, but in general, the growth rate is dependent also on other environmental factors such as pH value and relative humidity (Palumbo 1986; Wallentin Linberg and Borsch 1993). The major contamination source of *Y. enterocolitica* is the pig, and the contamination of

carcass with this bacteria may be limited, provided that strict slaughtering procedures are used (Borsch *et al.* 1996).

CHAPTER TWO

FAECAL AND TONSILLAR CARRIAGE OF *YERSINIA* SPP. BY PIGS

INTRODUCTION

Yersiniosis is an important zoonotic disease caused by pathogenic strains of *Yersinia enterocolitica* and *Y. pseudotuberculosis*. Since the late 1960's, *Y. enterocolitica* has increasingly become recognised as a cause of food borne disease in humans (Taylor, 1992). The organism is associated with the following clinical manifestations: acute and chronic gastro-enteritis, mesenteric lymphadenitis which may result in pseudoappendicular syndrome; sepsis of the very young, the aged and immunocompromised; and post-infection syndromes such as reactive arthritis and erythema nodosum (Cover and Aber 1989). *Y. pseudotuberculosis* is similar to *Y. enterocolitica* in clinical characteristics, but is relatively uncommon. Both species of *Yersinia* have been isolated from pigs and reports of association between infection and clinical disease in these animals are increasing (Hurvell 1981).

The source of human infection varies, but the pig is considered to be an important reservoir for human *Y. enterocolitica* infection (Hurvell, 1981; Schieman, 1989; Kapperud, 1991). Although pigs are considered as natural reservoirs and play an important role in the transmission of human yersiniosis, the source of infection in pigs themselves remains unclear. There are many reports in the literature of surveys conducted on pig carcasses, offal and faeces for the presence of *Y. enterocolitica* (Doyle *et al.* 1991; Schieman and Fleming, 1981; Hunter *et al.* 1983). Results of these surveys have stimulated further studies suggesting that infection is distributed world-wide in pigs, and serotypes considered pathogenic to man are commonly present. Many studies have also shown that pigs may be asymptomatic carriers of strains which belong to the same serotypes, biotypes as those associated with human disease (Wauters, 1979; Hurvell, 1981). The majority of human *Y. enterocolitica* infections are caused by O:3/biotype 4. The strains associated with disease in man and animals belong to only a few serogroups. Serogroups O:3, O:5,27, O:8 and O:9 are considered the most important causative agents of yersiniosis in man (Kapperud 1991). These strains are more commonly found in pigs than in any other species (Carniel and Mollaret 1990; Hanna *et al.* 1980).

Y. enterocolitica is a faecal commensal but there are certain serotypes that inhabit the oral cavity (Kapperud, 1991). *Y. enterocolitica* 0:3 is often found in the intestinal contents and

faeces of pigs. A study conducted by Shiozawa *et al.* (1991) found that 24.3 % of 140 pigs were carriers of the organism in the caecum. Several researchers (Asplund *et al.* 1990; de Boer and Nouws 1991) have found some variations in the frequency of isolation of microorganisms from different sites in pigs.

This study was undertaken to determine the faecal and tonsillar carriage of different species of *Yersinia* by pigs belonging to different age groups and characterise the species isolated by biochemical and serological methods.

MATERIALS AND METHODS

Sample size and collection

The study was conducted in two phases: first in a known or suspect *Yersinia enterocolitica*-positive farm within Massey University and second, in an abattoir where pigs from the suspected farm are sent for slaughter. The period of study was from August to November 1996. Faecal samples were collected from pigs of six different age groups, including 3-4 days, 2 weeks, 3 weeks, 4 weeks, 8 weeks and 12 weeks. A total of 54 samples was collected representing seven samples from the litter of each age group, one sample from the sow and one pooled sample from the floor of the pen (environmental sample). Based on previous studies, it was assumed that a 30 % prevalence of *Yersinia* infection was present in this farm. Based on a table for determination of sample sizes at the 95 % level of confidence, for an infinite population size at a 30 % level of infection, a total of nine animals or samples per herd were required to detect at least one infected animal from a herd. Faecal samples were collected in sterile bottles with scoops and rectal swabs were placed in transport medium. In the abattoir, a total of 50 samples (representing 25 faecal and 25 palatine tonsils) were collected from slaughter pigs with ages ranging from 20-24 weeks. The pigs were subjected to the standard slaughtering procedures (electrical stunning, exsanguination, scalding, dehairing, evisceration), as applied in licensed New Zealand pig abattoirs. Faecal samples were taken from the lower portion of the large intestines. The posterior parts of the tongue, tonsils and pharynx were removed as one block from the pluck following evisceration and placed into a sterile plastic bag, labelled individually and placed in a chilly bin for transport to the laboratory. Sample collections were done in such a way that faeces and tonsils correspond to the same animal undergoing evisceration. It was noted, however, that the tonsils had already been cut during the evisceration process, so it was not possible to get the whole pair of palatine tonsils. After

collection, faecal samples or rectal swabs and tonsils were transported immediately to the microbiology laboratory at the Department of Veterinary Pathology and Public Health for processing. Tonsils were stored at 4 °C for 1-3 hours before processing.

Cold enrichment

Samples were processed individually and aseptically for cold enrichment. Special precautions were taken to prevent cross-contamination of samples. Faeces and tonsils were processed in separate rooms. For faecal samples, a 1:10 dilution was followed where approximately 1 g of faeces was mixed in 9 ml of M/15 (PBS), pH 7.6 (Appendix) to make a 1:10 dilution. Faecal suspensions were placed in sterile screw capped universal bottles, labelled and stored for 21 days at 4 °C. A positive control of the faeces was also done however, the number of *Y. enterocolitica* cells per g of sample was not determined. The tonsils were processed under a safety hood. Tonsils were trimmed to remove excess non-tonsillar tissues. Sterile disposable blades were used and on each occasion instruments such as scissors and forceps were disinfected with 95 % alcohol and flamed in a Bunsen burner between samples. Tissues were sliced and placed in a stomacher bag, to which was added 25-30 ml of M/15 (PBS), pH 7.6. Again, on each occasion a disposable pipette was used. This was then homogenised in a stomacher "400" apparatus (Colworth 400, London), for 10-15 minutes. After homogenisation, the suspensions were transferred to sterile screw-capped universal bottles, labelled and stored in a similar manner to the faecal samples.

Cultivation after cold-enrichment

After 21 days of incubation at 4 °C , a loopful of suspension from each sample was plated onto Cefsulodin Irgasan Novobiocin (CIN) agar (DIFCO, U.S.A.) (Schiemann 1979). (Appendix). Plates were incubated at 29 °C and examined after 24-48 hours for growth and cultural characteristics of the bacteria.

Typical *Yersinia* colonies (pink to dark red "bull's-eye" colonies surrounded by a transparent border, varying in size from pin-point at 24 hours to approximately 2.5 mm in diameter at 48 hours) were picked off and sub-cultured onto blood agar (BA) and MacConkey agar plates (Appendix). Colonies of *Yersinia* spp. are small, colourless, non-lactose fermenters on MacConkey agar. Only non-haemolytic cultures on BA were used. Oxidase test was also done

on BA plates. From BA plates, cultures were then transferred onto Bijoux bottles containing peptone water, incubated for 24 hours and used as inoculum for further characterisation.

Isolation and screening of *Yersinia* spp.

For screening *Yersinia* spp., triple sugar iron agar (TSI), lysine iron agar (LIA) (Weagant 1981) and urea slants were inoculated with the inoculum and incubated at 29 °C for 24-48 hours. At this point the mortality test was performed. Isolates that gave either an acid slant/acid butt (A/A) or an alkaline slant/acid butt (K/A) reaction on TSI, and an alkaline slant/acid butt on LAIA, and were urea positive (pink colour) were considered presumptively *Yersinia.* Cultures which have at least one of the following reactions were rejected: hydrogen sulphide or H_2S production (blackening on TSI), excessive gas production (bubbles or cracks on the medium) or non-fermentation (TSI), non-fermentation (LIA) and urea negative. From this, one drop of the inoculum was transferred to Bijoux bottles each containing 4 ml of tryptone water (TW) and further incubated for 24-48 h. This was used as the inoculum for biochemical characterisation and subsequent identification of the isolates (See Table 3 for the flow diagram used to isolate *Yersinia* spp).

Identification and serotyping of *Yersinia* isolates

Yersinia spp. were identified on the basis of morphological, biochemical and cultural parameters as described elsewhere (Bercovier *et al.* 1980). Each isolate was subjected to the following biochemical tests at 29 °C: ornithine decarboxylase, methyl red, Voges-Proskauer (MRVP) (also at 37 °C), indole production, acid production from the following sugars: sucrose, L-rhamnose, D-melibiose, D-trehalose, D-xylose, salicin and aesculin hydrolysis. All tests were read daily for two days .

Serotyping was carried out by slide agglutination. All isolates identified as *Yersinia spp.* were stored in 15 % glycerol broth at -70 °C.

Table 3. Flow diagram used in the isolation of *Yersinia* spp. from tonsils and faeces of pigs.

Palatine tonsils → 25-30 ml M/15 (PBS, pH 7.6) in "stomacher"

↓

Faeces (1:10 dilution in M/15) → Enrichment PBS, 4 °C for 21 days

↓

After 21 days, 0.1 cm^3 suspension cultivated on CIN agar plates at 29 °C for 18-48 hours

↓

Sub-culture, BA 29 °C for 24 hours (non-haemolytic)

↓

Tryptone water 29 °C for 24 hours

↓

Screening → TSI slants 29 °C for 24-48 hours
LIA slants 29 °C for 24-48 hours
Urea slopes 29 °C for 24-48 hours

↓ Motility 29 °C; 37 °C 24-48 hours

Biotyping → Sucrose
Rhamnose
Melibiose
Trehalose
Xylose
Aesculin
Ornithine
Indole

↓ MRVP 29 °C
MRVP 37 °C
Salicin
Other biochemical media

Serotyping → Specific antisera

↓

Yersinia spp.

↓

Storage (15 % GB) -70 °C

CIN= Cefsulodin Irgasan Novobiocin
BA= Blood Agar
GB= Glycerol Broth
PBS= Phosphate Buffered Saline

TSI= Triple Sugar Iron
LIA= Lysine Iron Agar
MRVP= Methyl Red-Voges Proskauer

Pathogenicity/Virulence tests

To differentiate pathogenic from non-pathogenic strains, salicin fermentation and aesculin hydrolysis were used. All *Yersinia* spp. isolates were tested for potential virulence by the pyrazinamidase (PYZ) activity test and Congo Red Magnesium Oxalate (CR-MOX).

Salicin fermentation and aesculin hydrolysis are tests routinely carried out during biochemical characterisation of isolates of *Yersinia* spp. Salicin fermentation and aesculin hydrolysis were determined by inoculating 2-3 drops of test strain into Bijoux bottles containing 3 ml of the preparation or onto aesculin agar slopes (Appendix) which were then incubated at 29 °C and read after 24 and 48 hours. For salicin fermentation, a positive reaction is indicated by a pink to red colouration of the preparation while a negative reaction is indicated by no colour change. A positive reaction for aesculin hydrolysis was determined by blackening of the medium while no colour change in the medium indicates a negative reaction. Pathogenic strains of *Yersinia* spp. are salicin-aesculin negative while non-pathogenic strains are positive in these two tests.

The pyrazinamidase (PYZ) test was performed as described by Kandolo and Wauters (1985). (Appendix). All *Yersinia* spp. isolated in this study were tested for potential virulence by PYZ activity test. Overnight broth cultures of *Yersinia* spp. were inoculated over the entire slant of PYZ agar, incubated at 25 °C for 48 hours, and tested with freshly prepared solution of 1% ferrous ammonium sulphate. A pink to brownish discolouration on the slant indicated a positive result and no colour change was associated with a negative result.

All *Yersinia* isolates were grown on 5 % sheep blood agar for 24 hours. From these plates, individual colonies were picked off and subcultured onto CR-MOX agar plates, and were incubated at 37 °C at 24 and 48 hours and observed for the presence of red colonies. CR-MOX positive reaction is indicated by small red colonies. Freshly isolated strains of pathogenic yersiniae are CR-MOX positive and contain the virulence plasmid. Further incubation of plates for several days would show the development of large, colourless colonies from the small red ones due to loss of plasmid. Negative results for CR-MOX were shown by the presence only of large colourless colonies.

RESULTS

Results of this study are presented in Table 4. All of the 54 faecal samples from pigs belonging to six different age groups were found negative for *Yersinia* spp. Some plates showed colonies resembling "bulls-eye", which however, were already found to be more than 2.0 mm in diameter after only 24 hours of incubation. On TSI slants, typical reactions were acid slant/acid butt (A/A) with excessive gas formation and all were urea negative. No further biochemical tests were done since the tests conducted were primary screening tests and negative for *Yersinia.*

Of the 25 samples of tonsils, three isolates were identified as unusual *Yersinia enterocolitica* or *Y. enterocolitica* new biotype and two were *Y. pseudotuberculosis* Group I. Of the 25 faecal samples, there were two isolates of *Y. frederiksenii* and one isolate of an unusual *Y. enterocolitica.* It was interesting to note that this single faecal isolate identified as being an unusual strain came from the same animal from which this strain was also isolated from the tonsils. Table 5 shows the results of all biochemical tests used in the identification of *Yersinia* spp. from pigs. Table 6 shows the biochemical profile of three *Yersinia* spp. isolated from the tonsils and faeces of pigs in this study. Table 7 shows the biochemical tests used to distinguish the unusual strains of *Y. enterocolitica* from other *Yersinia* spp.

The reactions of eight isolates of *Yersinia* spp. using the four simple tests (salicin fermentation/aesculin hydrolysis, D-xylose fermentation, pyrazinamidase test and CR-MOX) described by Farmer *et al.* 1992 are presented in Table 8. The unusual strains of *Y. enterocolitica* isolated were salicin-aesculin positive, D-xylose positive, CR-MOX negative and PYZ negative. The first three tests would indicate non-pathogenicity, however, PYZ test was negative which indicates virulence. *Y. pseudotuberculosis* Group I isolates were salicin negative, aesculin positive, CR-MOX positive and PYZ negative. Except for aesculin, the three other tests confirms the isolate to be pathogenic/virulent. *Y. frederiksenii* isolates were positive for salicin, aesculin and PYZ tests but negative for CR-MOX, all indicating that this strain is non-pathogenic or avirulent.

Table 4. Summary of results of *Yersinia* study conducted at the pig farm and abattoir

Age of pigs	No. and type of samples	Results
FARM		
3-4 Days	9 *	-
2 Weeks	9	-
3 Weeks	9	-
4 Weeks	9	-
8 Weeks	9	-
12 Weeks	9	-
Total	54	
ABATTOIR	25 tonsils	3 *Y. enterocolitica* "new biotype" 2 *Y. pseudotuberculosis* group I
20-24 Weeks	25 faeces	1 *Y. enterocolitica* "new biotype" 2 *Y. frederiksenii*
Total	50	

*= faecal samples from 7 piglets, 1 sow and 1 pooled sample from floor (environmental sample)

- = negative

Results of biochemical tests used in the identification of *Yersinia* spp. from pigs

Reference (Animal #)	22 T	34 T	36T	34 F	16 T	38 T	24 F	44 F
Source	tonsils	tonsils	tonsils	faeces	tonsils	tonsils	faeces	faeces
TSI	A/A	A/A	A/A	A/A	K/A	K/A	A/A	A/A
LIA	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Urea	+	+	+	+	+	+	+	+
Motility 29 °C 37 °C								
MR 29 °C 37 °C	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
VP 29 °C 37 °C	+ -	+ -	+ -	+ -	- -	- -	+ -	+ -
Ornithine	-	-	-	-	-	-	+	+
Nitrate	+	+	+	+	+	+	+	+
Simmon's citrate	-	-	-	-	-	-	-	-
Indole	+	+	+	+	-	-	+	+
MacConkey Agar	lactose -	lactose -	lactose -	lactose -	lactose -	lactose -	lactose -	lactose -
Blood Agar	N/H	N/H	N/H	N/H	N/H	N/H	N/H	N/H
Sucrose	+	+	+	+	-	-	+	+
Trehalose	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	+	+	+	+
Melibiose	+	+	+	+	+	+	-	-
Raffinose	+	+	+	+	-	-	-	-
α-Methyl-Glucoside	-	-	-	-	-	-	-	-
Sorbose	+	+	+	+	-	-	+	+
Sorbitol	+	+	+	+	-	-	+	+
Cellobiose	+	+	+	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	-	-	+	+
Lactose	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	-	-	-	-
Lysine	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-
Aesculin	+	+	+	+	+	+	+	+
Identification	YE	YE	YE	YE	YP	YP	YF	YF
Biotype/Serotype	new	new	new	new	group I	group I		

A/A = acid/acid
K/A = alkaline/acid
+ = positive
- = negative
N/H = non-haemolytic

YE= *Yersinia enterocolitica*
YP = *Yersinia pseudotuberculosis*
YF = *Yersinia frederiksenii*
MR= Methyl Red
VP= Voges-Proskauer

TSI= Triple Sugar Iron
LIA= Lysine Iron Agar

Table 6. Biochemical Profile of three *Yersinia* spp. isolated from tonsils and faeces of pigs

Test	*Y.enterocolitica* (new biotype)	*Yersinia pseudotuberculosis*	*Yersinia frederiksenii*
Ornithine	-	-	+
Voges-Proskauer	+	-	+
Indole	+	-	+
Aesculin (24 h)	+	+	+
Sucrose	+	-	+
Rhamnose	-	+	+
Raffinose	+	-	-
Melibiose	+	+	-
α-methyl glucoside	-	-	-

+ = positive - = negative

Table 7. Biochemical differentiation of the unusual strains of *Y. enterocolitica* from other *Yersinia* spp.

Test	Unusual YE Strain	YE	YK	YM	YB	YF	YI	YA	YR	YP
Ornithine	-	+	+	+	+	+	+	+	+	-
Voges-Proskauer	+	+/-	-	-	-	+	+	+	-	-
Indole	+	V	V	-	-	+	+	+	-	-
Aesculin	+	V	-	-	-	V	+	-	-	-
Sucrose	+	+	-	+	+	+	+	-	+	-
Rhamnose	-	-	-	-	-	+	+	+	-	V
Raffinose	+	-	-	-	-	-	-	+	V	-
Melibiose	+	-	-	-	-	-	+	-	V	V
α-methyl glucoside	-	-	-	-	-	-	+	-	-	-

YE= *Y. enterocolitica*; YK= *Y. kristensenii*; YM= *Y. mollaretii*; YB=*Y. bercovieri*; YF= *Y. frederiksenii*;
YI= *Y. intermedia*; YA= *Y. aldovae*; YR= *Y. rohdei*; YP= *Y. pseudotuberculosis*

+ = positive reaction; -= negative reaction; V= variable reaction; +/-= most strains positive

Table 8. Reactions of 8 isolates of *Yersinia spp* from pigs using 4 simple virulence tests as described by Farmer *et al.* (1992)

Species (Animal No.)	Source	Salicin Fermentation	Aesculin Hydrolysis	PYZ Test	CR-MOX
Y. enterocolitica new biotype (22T)	Tonsils	+	+	-	-
Y. enterocolitica new biotype (34T)	Tonsils	+	+	-	-
Y. enterocolitica new biotype (36T)	Tonsils	+	+	-	-
Y. enterocolitica new biotype (34F)	Faeces	+	+	-	-
Y. pseudotuberculosis Group I (16T)	Tonsils	-	+	-	-
Y. pseudotuberculosis Group I (38T)	Tonsils	-	+	-	-
Y. frederiksenii (24F)	Faeces	+	+	+	-
Y. frederiksenii (44F)	Faeces	+	+	+	-

PYZ = pyrazinamidase test
CR-MOX = Congo Red-magnesium oxalate agar
+ = positive
- = negative

DISCUSSION

Results of this investigation have shown that the rate of infection of pigs with *Y. enterocolitica* at the study farm with a prevalence of 50 % has decreased since the study of Fenwick which was carried out at the same farm in 1988-89. All of the 54 faecal samples in the present study yielded negative results. The non-isolation of *Yersinia* spp. from faecal samples may be due the small number of samples collected. Another reason for the negative results is the possibility that the micro-organisms may have been significantly reduced or eliminated from this farm by good husbandry practices. The study farm is now considered a nucleus breeding unit of high health status and with a high standard of hygiene and animal husbandry (Frey, personal communications). According to Ostroff (1995), different farm sizes and animal husbandry techniques have an effect on the prevalence of yersiniosis. Isolation and sampling techniques may also have a significant effect on isolation rates (Doyle *et al.* 1981). Faecal and/or rectal swabs samples may yield less *Y. enterocolitica* isolates than either tongue or tonsil samples. This finding seems to agree with the findings of other investigators (Aldova *et al.* 1980; Essevald and Goudzwaard 1973; Pedersen and Winblad 1979; Szita *et al.* 1980; Toma and Diedrick 1975; Tsubokura *et al.* 1973; Weber and Lembke 1981; Zen-Yoji *et al.* 1974), that the frequency of isolation was approximately ten times greater from the tongues and tonsils than from faeces. In a study conducted by Nielsen *et al.* (1996), all of the 25 pigs experimentally inoculated with *Y. enterocolitica* O:3 were faeces culture positive from day 5 to 21 post infection (p.i.), whereas shedding of bacteria declined to < 10 % of the pigs at day 49 p.i. and to 0 % at day 68 p.i. During necropsy at day 70 p.i., *Y. enterocolitica* O:3 was isolated from the tonsils of 20 inoculated pigs, whereas the rest of the gastrointestinal tract and associated lymph nodes were culture negative. It is well known that pigs may carry *Y. enterocolitica* O:3 in the tonsils for months after faecal excretion has stopped (Wauters and Pohl 1972; Kapperud 1991).

When pigs from the study farm were sent to slaughter, both faeces and tonsils yielded *Yersinia* spp. It was interesting to note in this study that *Yersinia* spp. could be more readily recovered from slaughter pigs than from piglets on the farm. It is therefore also possible that the age of pigs may be an important factor in the prevalence of the infection in these animals. There is also the possibility that the pigs were only infected with a small number of viable cells of *Yersinia* spp., too few to be detected in the faeces by cold enrichment. Based on the study done by Fukushima *et al.* (1989), *Yersinia*-positive farms can be divided into infected or uninfected

farms, depending on the level of *Yersinia* organisms in the caecal contents of the pigs, the former group containing animals with 10^2 or more organisms per g and the latter group containing animals with less than 10^2 organisms per g. In an experimental study conducted by Nielsen *et al.* (1996), the in-feed oral inoculation of pigs with 10^8 colony forming units (c.f.u.) of *Y. enterocolitica* O:3 successfully led to infection and excretion by all inoculated pigs. There is also the possibility that pigs may have been infected only during the time spent at the slaughterhouse. Most of the studies conducted previously by other investigators (Doyle *et al.* 1981; Fukushima *et al.* 1990; Kwaga *et al.* 1990; Kapperud 1991; de Boer and Nouws 1991; Kotula and Sharar 1993) were on slaughter pigs and not on pigs at the farm level. According to Fukushima *et al.*(1989), pigs are often contaminated with *Yersinia* from the faeces of infected pigs and from contaminated floors, during the time spent in slaughterhouse lairage. Results of another study conducted by Fukushima *et al.* (1990), suggest that most serotypes were horizontally transferred from infected pigs to other pigs during the 20 h period when pigs were all fasting and eating the faeces of other pigs from the lairage floor and drinking water that had been washed off from the pig's body.

Still another possibility is the evolving nature of yersiniosis as ascribed by examining published reports of serogroup distributions and shifts in the USA (Lee *et al.* 1991; Bisset 1979; Quan 1979; Shayegani and Parsons 1987; Bottone 1983, 1987; Bisset *et al.* 1990; Metchock *et al.* 1987), Great Britain (Prentice *et al.* 1991) and in France, Italy and Japan (Chiesa *et al.* 1987; Ichinohe *et al.* 1991; Chiesa *et al.* 1991a; 1991b). This could perhaps explain the finding of the unusual strain of *Y. enterocolitica* isolated from pigs from this farm. Bioserovars 4/O:3, 3/O:5,27 and 2/O:9 are the most common strains recovered in humans in New Zealand (MacCarthy and Fenwick 1990). Of these bioserovars O:3 and O:5,27 are also commonly found in slaughter pigs (De Allie 1994). Within the last two years at the Department of Veterinary Pathology and Public Health, a number of atypical *Yersinia enterocolitica* strains have been isolated from cattle, sheep and goats and subsequently from humans. According to Fenwick *et al.* (1996), all isolates were extensively defined biochemically, and although their profiles were consistent with *Yersinia* (Farmer *et al.* 1985), they did not appear to be related to any known species and did not possess the virulence markers. This present study is the first report of unusual strains of *Yersinia enterocolitica* isolated from pigs in New Zealand. Several biochemical tests were used to distinguish the unusual strains from other *Yersinia* spp. as described by Fenwick *et al.* (1996). One key test was ornithine, as all *Yersinia,* with the exception of *Y. pseudotuberculosis* and a few atypical strains of *Y. enterocolitica,* are ornithine-positive. Other key tests that differentiated the unusual strains from other *Yersinia*

spp. were acid production from the carbohydrates melibiose, raffinose and sucrose, and negative reactions for rhamnose and α-methylglucoside. Fenwick *et al.* (1996) also observed two different biochemical profiles in their study. Of the twenty five strains, twenty one strains belonged to phenotype I, which was characterised by negative ornithine test and a positive indole reaction. The four remaining strains belonged to phenotype II, and they were ornithine positive and indole negative. In this study, the three isolates recovered from the tonsils and one isolate from the faeces of pigs belonged to phenotype I.

To determine their pathogenic potential, the four isolates of unusual strains of *Y. enterocolitica* were subjected to the four simple tests described by Farmer *et al.* (1992). These include: salicin fermentation/aesculin hydrolysis, D-xylose fermentation, pyrazinamidase (PYZ) test and Congo Red-Magnesium Oxalate (CR-MOX). According to Riley and Toma (1989), the combined use of CR-MOX, salicin-aesculin and PYZ tests provides a method for accurately differentiating between pathogenic and non-pathogenic *Y. enterocolitica* strains. Generally, *Y. enterocolitica* has the following pattern: (1) salicin-aesculin positive strains are always non-pathogenic and the vast majority are also pyrazinamidase positive, (2) salicin-aesculin negative, pyrazinamidase negative strains are always pathogenic and (3) salicin-aesculin negative, pyrazinamidase positive strains although uncommon are non-pathogenic. The CR-MOX test is a simple method to screen for plasmid-containing pathogenic *Y. enterocolitica* and yields results in 24 h. A strain which has lost the plasmid will be CR-MOX negative, but if it is a potential pathogen it will be salicin-aesculin negative and PYZ negative. In this study, the unusual strains of *Y. enterocolitica* isolated from the pig's tonsils and faeces are salicin-aesculin positive, xylose positive, and pyrazinamidase negative. Kandolo and Wauters (1985) found an excellent correlation between virulence and a negative test for pyrazinamidase. *Y. pestis, Y. pseudotuberculosis* and strains of *Y. enterocolitica* which belonged to bio-serogroups usually associated with human disease were pyrazinamidase negative. The pyrazinamidase test is usually positive in saprophytic strains of *Y. enterocolitica* but was found to be negative in the unusual strain of *Y. enterocolitica* isolated in this study. This finding agrees with the observations of Fenwick *et al.* (1996), where unusual strains of *Y. enterocolitica* isolated from the faeces of ruminants with clinically apparent enteric infections were also pyrazinamidase negative. These strains, with their lack of plasmid-associated virulence markers are probably environmental in origin, however, since they have not yet been isolated from environmental samples this remains speculation (Fenwick *et al.* 1996).

Two isolates of *Yersinia pseudotuberculosis* serotype I were recovered from the pig's tonsils in this study. In a study conducted by De Allie (1994), *Y. pseudotuberculosis* serotypes I, II and III were isolated from the tonsils of slaughter-age pigs. *Y. pseudotuberculosis* serotype I was isolated only in the months of July, August and October (winter and early spring), serotype II between the months of June and November, and serotype III throughout the year except in the month of February, with the highest peak in autumn and lowest in the spring and summer. In this study, sample collections were done in the months of August and October when serotype I was isolated conforming to the results of De Allie's investigations. All of the 25 faecal samples from slaughter age pigs in this study were negative for *Y. pseudotuberculosis*. As mentioned in the review of literature, according to Mair and Fox (1986), *Y. pseudotuberculosis* is rarely isolated by faecal culture, even in confirmed cases of pseudotuberculous mesenteric lymphadenitis. The reason for this was probably that excretion of the organism had ceased before the onset of pseudoappendicular symptoms.

The pathogenic potential of *Y. pseudotuberculosis* group I isolated from the tonsils of pigs in this study was shown by CR-MOX and PYZ tests. Both isolates were CR-MOX positive and PYZ negative indicating that the isolates are potential pathogens. As discussed in the literature review, *Y. pseudotuberculosis* is associated with a variety of human diseases such as mesenteric lymphadenitis, terminal ileitis, erythema nodosum, reactive arthritis and septicaemia (Mollaret 1965; Mair and Fox 1986). Studies carried out in Europe, Canada and Japan have shown that pigs are an important reservoir of *Y. pseudotuberculosis* (Narucka and Westendoorp 1977; Toma and Diedrick 1975; Tsubokura *et al.* 1984; Zen-Yoji *et al.* 1974). Therefore, the presence of this organism in the tonsils of slaughter pigs suggests that pig carcasses and pork products could become contaminated and act as a source of infection to man.

Among the other *Yersinia* spp, there were two isolates of *Y. frederiksenii* recovered from faeces of pigs. These are generally regarded as environmental strains and not usually associated with disease in people (Van Noyen *et al.* 1981). In a study conducted in Brazil between mid 1980 and 1985 (as cited in Falcao 1987), all strains of *Yersinia* were isolated from food and the environment with the exception of *Y. frederiksenii* as were only isolated from pig's faeces. According to Ursing *et al.* (1980), isolation of *Y. frederiksenii* from human stools usually reflects only transient carriage. It should be considered an opportunistic pathogen for humans and the clinical significance should be weighed in view of available epidemiological data. In the UK, during the 10-year period 1984-1993, when all faecal and food samples were routinely

submitted to the Public Health Laboratory, results have shown an expectedly high faecal carriage rate of *Y. frederiksenii* (29 %) and low faecal carriage of *Y. intermedia* (2 %) compared with the prevalence of these strains in foods (17 and 22 % respectively). According to Greenwood (1995), although the significance of these species in human infection is debatable, these figures suggest that strains of *Y. frederiksenii* are capable of greater persistence in the human gut than strains of *Y. intermedia.*

CONCLUSION

From this study the following conclusions can be drawn. Even though pigs have been established to harbour *Yersinia* spp. in their tonsils or faeces, not all pigs are infected with pathogenic and non-pathogenic species of yersiniae. Pigs sent for slaughter carry *Yersinia* spp. in their tonsils and faeces as in other countries. Although faecal samples are considered common clinical specimens and more convenient to collect for isolation of *Yersinia* spp., the tonsils should be the preferred sample for isolation. Results of this study in conjunction with other published reports show that *Yersinia* spp. are more commonly isolated from the tonsils than from faecal samples. As more researchers study *Yersinia,* there is a possibility that more isolates or strains will be discovered as shown by the emergence of unusual strains or new biotypes of *Y. enterocolitica.* Although these unusual strains isolated from pigs are closely related to *Y. enterocolitica* and show a biochemical profile similar to those isolated by Fenwick *et al.* (1996), the definitive status of these new isolates have yet to be determined. *Y. pseudotuberculosis* is considered a potential pathogen as shown by *in vitro* virulence assays such as the pyrazinamidase test and Congo Red Magnesium Oxalate (CR-MOX) and should not be overlooked as a causative agent of human disease. This strain should also be considered a potential risk for people in direct contact with infected pigs, such as veterinarians, pig farmers and abattoir workers as it is apparently similar to strains previously isolated from human cases of enteric infections.

RECOMMENDATION

Because slaughter pigs have been established as an important source of *Y. enterocolitica* infections, it is important to prevent or at least attempt to decrease the degree of contamination in the slaughterhouse. In the study slaughterhouse, the tonsils were not always removed intact together with the pluck, having already been sliced, with portions remaining in the pig's head. This is a potential source of contamination especially if the knives are not adequately sterilised.

Pathogenic strains of *Yersinia* spp. from the tonsils and intestines are likely to contaminate the carcasses and the slaughterhouse environment. The application of Hazard Analysis Critical Control Point (HACCP) and Good Manufacturing Practice (GMP) in pig slaughter must be focused on limiting this spread (Borsch *et al.* 1996). It is therefore recommended that emphasis be placed on the following critical points during slaughtering and dressing (Kapperud 1991; Troeger 1994; Borsch *et al.* 1996) :

(i) scalding
(ii) singeing
(iii) circumanal incision and removal of the intestines
(iv) excision of the tongue, pharynx and particularly the tonsils
(v) post-mortem meat inspection procedures which involve incision of mandibular lymph nodes
(vi) deboning of the head.

Tonsils should be removed intact together with the pluck and examined separately to prevent contamination of the carcass. As practised in Scandinavian countries, the incorporation of the plastic bag technique to seal off the rectum after it has been freed would also help in preventing the dissemination of *Y. enterocolitica* which are spread via the faeces. The use of a bung cutter and sealing of the rectum with a plastic bag is practised in New Zealand during beef slaughter but not in pig slaughter.

Yersinia infections in pigs have traditionally been diagnosed by culture of samples obtained from tonsils and faeces. In the Nordic countries, the recommended culture method for *Yersinia enterocolitica* takes at least four days for a positive diagnosis and three weeks for a negative diagnosis (Anon. 1987). Most laboratories follow the 21-day cold enrichment method to isolate the organisms from clinical specimens and food samples. Hence, there is a need to develop cheap, rapid, sensitive and less time-consuming non-culture methods for detection of *Yersinia* infections especially in a large scale surveillance of pig populations.

Age is considered an important factor for *Y. enterocolitica* colonisation of pigs but no conclusion could be drawn from this study as to the age at which pigs become infected. Although different age groups were used, part one of this study was limited to isolation of *Yersinia* spp. from faecal samples and not tonsillar samples. In addition to this, the number of samples collected was very small and was limited to only one farm. Future studies should still

be aimed at determining the age when pigs become colonised with *Y. enterocolitica* in their tonsils. It is also not clear at the moment whether the presence of pathogenic *Yersinia* organisms is related to certain conditions during growth and fattening. The nil isolation rate in this particular farm which was previously found to be Yersinia-positive provides some encouragement that good husbandry procedures and control procedures might be effectively implemented to reduce or eliminate pathogenic serotypes of yersiniae.

Research efforts should be directed towards intervention studies and development of methods to reduce colonisation of pigs on the farm, contamination at the slaughterhouse, growth during refrigeration of foodstuffs and storage and spread within the kitchen.

APPENDIX

Preparation of media used for the isolation, identification, virulence tests and storage of *Yersinia* isolates

Aesculin Hydrolysis

1. To 1 litre of distilled water, add the following materials:

   1 gm Aesculin (DIFCO)

   0.5gm Ferric Iron Citrate

   25 gm Heart Infusion Broth (DIFCO)

   15 gm Bacto Agar (DIFCO)

2 Dissolve by gentle heating.

3. Mix thoroughly and dispense 3 ml amounts in Bijoux bottles.

4. Sterilise by autoclaving for 15 minutes at 121 °C.

5. Cool in a slanting position and store at +4 °C.

Blood Agar

This medium comprises a salt base and a blood base.

Salt Base

1. To 1 litre of distilled water add 15.0 gm Bacto Agar (DIFCO) and 5.0 g m Sodium Chloride (NaCL), and dissolve by heating

2. Autoclave for 15 minutes at 121 °C.

3. Cool to 45-50 °C.

4. Dispense 10 ml amounts into each petri dish and allow to cool and dry.

Blood base

1. To 1 litre of distilled water, add 44.0 gm of Columbia Blood Agar Base (DIFCO) and dissolve by heating.

2. Autoclave for 15 minutes at 121 °C.

3. Cool to 45-50 °C.

4. Add 5 % (W/V) of sheep blood.

5. Dispense 10-15 ml amounts over the already prepared salt base.

6. Allow to cool and dry, and store at +4 °C.

Carbohydrates (Sugars)

1. To 1 litre of distilled water, add the following materials:

   10 gm peptone (DIFCO)
   3 gm Meat Extract (GIBCO, BRL, Scotland)
   5 gm Sodium Chloride
   10 ml Andrades Solution

2. Dissolve by stirring and adjust pH to 7.2.

3. Dispense in 200 ml bottles.

4. Autoclave for 15 minutes at 121 °C.

5. Add 20 ml (10 %) Seitz filtered carbohydrates*

6. Dispense aseptically in 3 ml amounts into Bijoux bottles. Store at 4 °C.

* The following were used: Comprises of sucrose, trehalose, rhamnose, melibiose, raffinose, α-methyl-glucoside, sorbose, sorbitol, cellobiose, maltose, xylose, arabinose, salicin and lactose.

Cefsulodin-Irgasan-Novobiocin Agar (CIN)

(Schiemann 1979)

The preparation of this medium is achieved by the combination of the *Yersinia* selective agar base and the *Yersinia* antimicrobic supplement Cefsulodin Novobiocin (CN).

1. To 1 litre of distilled water, add 59.5 gm of *Yersinia* Selective Agar Base (DIFCO) and dissolve completely by boiling.

2. Sterilise by autoclaving for 15 minutes at 121 °C.

3. Cool to 45-50 °C.

4. Aseptically add 10 ml rehydrated *Yersinia* Antimicrobic Supplement CN (DIFCO, USA).

5. Mix thoroughly avoiding the formation of air bubbles, and dispense 15-20 ml amounts into sterile petri dishes.

6. Allow to cool and dry, store at +4 °C.

Congo Red - Magnesium Oxalate Agar (CR-MOX)

(Riley and Toma 1989)

1. To 825 ml of distilled water, add 40 gm of Tryptic Soy Agar (DIFCO) and dissolve by boiling.

2. Autoclave for 15 minutes at 121 °C.

3. Cool the molten medium to 55 °C, and add the following solutions:

   80 ml of 0.25 M Magnesium Chloride (2.38 gm/100 ml)
   80 ml of 0.25 M Sodium Oxalate (3.55 gm/100ml)
   10 ml of 20 % D-galactose
   5 ml of 1 % Congo red

4. All solutions are sterilised by autoclaving for 15 minutes at 121 °C, except for D-galactose solution which is filter-sterilised.

5. After thorough mixing, dispense 15-20 ml amounts into sterile petri dishes.

6. Allow to cool and dry. Store at +4 °C.

Decarboxylase Test Broth

1. To 1 litre of distilled water add 10.5 gm Moeller's decarboxylase base medium and dissolve by gentle heating.

2. Dispense into 200 ml bottles.

3. To each 200 ml amount, add 2 gm L-Arginine (SIGMA), or 2 gm L-lysine (SIGMA), or 2 gm L-ornithine (SIGMA), whichever is required.

4. Adjust pH to 6.0.

5. Dispense 3 ml amounts into Bijoux bottles.

6. Autoclave for 15 minutes at 121 °C. Store at +4 °C.

Glycerol Broth (15 %)

1. To 300 ml of distilled water add the following materials:

   2.4 gm Nutrient Broth
   45 ml Glycerol

2. Mix together (don't need to heat)

3. Dispense 3 ml amounts into Bijoux bottles.

4. Autoclave for 15 minutes at 121 °C. Store at +4 °C.

Lysine Iron Agar (LIA)

(Weagant 1983)

1. To 1 litre of distilled water, add the following materials:

   34.5 gm Lysine iron Agar (DIFCO)

2. Dissolve by heating.

3. Dispense in 6 ml amounts into screw-capped polycarbonate tubes.

4. Autoclave for 15 minutes at 121 °C.

5. Cool in slanting position. Store at +4 °C.

MR-VP Medium

1. To 1 litre of distilled water, dissolve 17 gm Bacto MR-VP Medium (DIFCO).

2. Dispense into test tubes in 10 ml amounts.

3. Sterlize by autoclaving for 15 minutes at 121 °C.

Methyl Red Test

To 5 ml of culture add 5 drops of methyl red solution. The indicator solution is prepared by dissolving 0.1 gm Bacto Methyl red in 300 ml of 95 % alcohol and diluting to 500 ml in distilled water. A positive reaction is indicated by a distinct red colour, showing the presence of acid. A negative reaction is indicated by a yellow colour.

MacConkey Agar

1. To 1 litre of distilled water, suspend the appropriate amount of medium and heat to boiling with gentle swirling to dissolve completely.

2. Add 10 gm of lactose to Bacto MacConkey Agar Base and swirl the flask to dissolve completely.

3. Sterilize by autoclaving for 15 minutes at 121 °C.

4. Cool to 45-50 °C.

5. Dispense in approximately 20 ml amounts into sterile petri dishes.

6. Allow to cool and dry, store at +4 °C.

M/15 Phosphate-Buffrered Saline pH 7.6 (PBS)

1. Prepare solution A by dissolving 9.07 gm of Potassium Di-hydrogen Phosphate (KH_2PO_4) in 1 litre of distilled water.

2. Prepare solution B by dissolving 11.87 gm of Disodium Hydrogen Phosphate ($Na_2HPO_4\ 2H_2O$) in 1 litre of distilled water.

3. Mix 128 ml of solution A and 872 ml of solution B, and add 9.0 gm of sodium chloride (NaCL).

4. Autoclave for 15 minutes at 121 °C. Store at +4 °C.

Pyrazinamidase Slopes

(Kandolo and Wauters 1985)

1. Prepare buffer solution by dissolving 2.37 gm of Tris-Maleate in 500 ml of distilled water.

2. Add the following materials:

   15 gm Tryptic Soy Agar (DIFCO)
   1.5 gm Yeast Extract (DIFCO)
   0.5 gm Pyrazinecarboxamide (MERCK)

3. Dissolve by boiling in buffer.

4. Dispense 5 ml amounts into screw-capped polycarbonate tubes.

5. Sterilise by autoclaving for 15 minutes at 121 °C

6. Cool in a slanting position, and store at +4 °C.

Simmons Citrate Agar

1. To 1 litre distilled water, add 24.2 gm Bacto Simmons Citrate Agar.

2. Heat to boiling to dissolve completely.

3. Mix thoroughly and dispense 3 ml amounts in Bijoux bottles.

4. Sterilize by autoclaving for 15 minutes at 121 °C.

5. Cool in slanting position and store at +4 °C.

Triple Sugar Iron Agar (TSIA)

1. To 1 litre of distilled water, add 65.0 gm TSIA (DIFCO) and dissolve by heating.

2. Dispense 6 ml amounts into screw-capped polycarbonate tubes.

3. Autoclave for 15 minutes at 121 °C.

4. Cool in a slanting position, and store at +4 °C.

Tryptone Water

1. To 1 litre of distilled water, add 20 g m Tryptone (DIFCO) and dissolve by gentle heating

2. Dispense in 3 ml amounts into Bijoux bottles.

3. Autoclave for 15 minutes at 121 °C, and store at +4 °C.

Urea Agar

This medium comprises an agar base and a urea base.

Agar Base

1. To 1 litre of distilled water, add 15.0 gm Bacto Agar (DIFCO), and dissolve by

heating.

2. Dispense into 200 ml bottles.

3. Autoclave for 15 minutes at 121 °C.

4. Cool at 45-50 °C

Urea base

1. To 100 ml distilled water, add 29.0 gm Bacto Urea Agar (DIFCO) and mix to dissolve.

2. Filter sterilise and store at 4 °C.

To prepare the medium, add aseptically 20 ml of Bacto Urea Agar Base to 200 ml Agar Base. Mix thoroughly and dispense 3 ml amount into each sterile Bijoux bottle. Cool in a slanting position. Store at +4 °C.

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