YERSINIA ENTEROCOLITICA INFECTIONS IN PEOPLE AND OTHER ANIMALS

A NEW ZEALAND STUDY

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A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology

Massey University

July 1997

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ABSTRACT

During the past three decades, *Yersinia enterocolitica* has risen to worldwide prominence from an obscure and taxonomically undefined organism to a common zoonotic pathogen, capable of causing a wide range of clinical syndromes in both animals and people. Prior to this study, however, there was little evidence of the importance of the organism in New Zealand and the overall aim of the thesis was to investigate it's role as a human pathogen in this country and the involvement of animals in the epidemiology of yersiniosis.

Initially, a survey of human infections was initiated with the cooperation of medical laboratories throughout the country, and this eventually continued for eight years. In total, epidemiological data pertaining to 2737 cases was obtained for analysis, including age and sex of patients, details of clinical symptoms, duration of infection, seasonality and the distribution of bioserotypes. Results of the survey showed that yersiniosis was a common human enteric pathogen, with peaks of infection in children under five and young adults. The predominant clinical symptoms were diarrhoea and abdominal pain and the course of infection was usually 1 to 2 weeks. The principal bioserotype combination throughout the study was 4/O:3, however, annual differences were recognised in the incidence of infection with two other bioserotypes, 2/O:5,27 and 2/O:9. No obvious seasonality was detected.

Two other surveys were later initiated, one in which the tonsils of slaughter pigs were examined for the presence of human pathogenic strains of *Y. enterocolitica*, and the other to investigate the faecal carriage of the same strains in a range of domestic animals. As in other countries, pigs were found to be infected with *Y. enterocolitica*, with approximately 24% of pigs harbouring strains of the organisms potentially pathogenic for people, including bioserotypes 4/O:3 and 2/O:5,27. Dogs were the only other animal from which 4/O:3 strains were isolated, however, a wide range of domestic animals were found to carry bioserotypes 2/O:5,27 and 2/O:9.

As human infections with *Y. enterocolitica* have been linked to contact with dogs, a study was designed to examine the carriage and transmission of bioserotype 4/O:3 in a group of 14 young dogs. The animals were separated into 5 groups, 2 containing 4 dogs (Groups I and II) and the others 2 dogs each (Groups III-V). Each of the 4 dogs in Group I, and 2 of the dogs in Group II were challenged orally with the test organism. Regular bacteriological examination of faecal samples from these animals showed that dogs can be readily infected and can excrete the organism for up to 23 days. The 2 in-contact dogs in Group II started to shed the test organism after 5 days. Subsequent transfer of these dogs to Group III and those in Group III to Group IV showed that *Y. enterocolitica* bioserotype 4/O:3 can be transmitted between dogs. At no time did any of the dogs show clinical signs of infection. These findings suggest that dogs can carry *Y. enterocolitica* 4/O:3 asymptomatically and hence might act as a potential source for human infection.

Standard laboratory procedures for the isolation and identification of *Y. enterocolitica* are both time-consuming and insensitive and the development of a rapid molecular method for identification of the organism was attempted. Initially, a non-radioactive DNA probe based on a cloned fragment of the *Yersinia* virulence plasmid was assessed for it's ability to distinguish pathogenic from non-pathogenic strains of the organism. Results using the probe were equivocal, and the polymerase chain reaction (PCR) was adopted as the rapid method of choice. Using the published sequence data from a *Y. enterocolitica* invasion gene (*ail*), present only in pathogenic strains of the organism, primers were designed for use in the PCR. With both extracted DNA and simple broth cultures as the template, the PCR proved to be highly specific and sensitive for pathogenic *Y. enterocolitica*.

The PCR was subsequently adapted for use directly with clinical samples, including tissues and faeces from experimentally infected pigs and dogs. Following initial inhibition of the PCR, two methods were designed that overcame the reduced sensitivity, a nested PCR assay applied to pig tissues and a pre-PCR enrichment step used with faecal samples. The sensitivity of the PCR was comparable to culture, and in some cases was enhanced.

Finally, pulsed field gel electrophoresis was used to examine a total of 602 strains of *Y. enterocolitica* recovered from animals and people during the study, to identify likely reservoirs of infection and to assess the heterogeneity of the organism in New Zealand. Bioserotypes 4/O:3, 2/O:5,27 and 2/O:9 were subdivided into 18, 20 and 40 pulsotypes respectively, with 4/O:3 and 2/O:9 being comparatively homogeneous (approximately 80% of isolates corresponding to one major pulsotype in each) and 2/O:5,27 having a high degree of heterogeneity (approximately 70% of isolates clustered into 6 pulsotypes). The principal pulsotypes in each bioserotype were recovered from a wide range of animal species and from most regions in the country.

ACKNOWLEDGEMENTS

" I get by with a little help from my friends"
John Lennon and Paul McCartney

But all work and no play makes Stan a dull boy, and without the ability to laugh, moan and generally unwind with my "coffee and conference" friends I would indeed have ended up a quivering wreck. A big thankyou, therefore, to Magda, Jacek, young Jane, older Jane, Richard, Suzanne, Laurie, Kylie, Colin (remember the pissed gorilla) and last, but by no means least, to my partner-in-crime, Eamonn. Thanks for the memories!

I would like to pay tribute to my parents-in-law, Danny and Grace, who have been my spiritual mentors and who have always believed in me, thanks for everything.

I have been blessed in my life to have been loved, supported, encouraged and generally pampered by two wonderful parents. I cannot possibly repay them for all they have done for me and only hope that I have made them proud. Perhaps the most effective way that I can show my eternal gratitude to them is by carrying on the tradition and ensuring that my own children have all my support and love whenever they need it.

Finally, my family. Words cannot truly express the debt I owe my wife, Anne, and my children, Jennifer, Aidan and Kirsten. They have lived every day of my PhD with me, sharing my highs and lows, putting up with my moods and always giving me shoulders to lean on. Anne's eternal optimism has constantly encouraged me and her faith in me has helped me believe in myself. Without their love I would not have finished and I hope that I can now relax, put my feet up and rejoin the human race! Asante sana, shukran gazeelan, na penda wewe kabisa x 17!

This thesis is dedicated to the memory of my dear sister-in-law, Grace.

**"Although you are no longer with us,
your light shines on forever."**

The road goes ever on and on
Down from the door where it began.
Now far ahead the road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.

Bilbo Baggins, The Lord of the Rings.

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and thinking what nobody has thought"
Albert Szent-Gyorgyi

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and will never achieve, any results of the slightest value"
Benjamin Jowett

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CHAPTER 1

1. LITERATURE REVIEW

1.1 GENUS *YERSINIA*

The genus *Yersinia* is named after Alexandre Yersin, a Swiss bacteriologist who first isolated the plague bacillus in Hong Kong in 1894, while investigating a catastrophic epidemic of bubonic plague that killed an estimated 60,000 people. He named the bacterium *Pasteurella pestis* due to it's resemblance to other members of the genus *Pasteurella* (Butler, 1994). Although the history of the genus is dominated by the plague bacillus (now termed *Yersinia pestis*), once the taxonomy of the genus *Yersinia* was unravelled, it was found that the first species to have been described was in fact *Y. pseudotuberculosis*. Malassez and Vignal described a disease in guinea pigs in 1883, characterised by nodules in the internal organs resembling tuberculosis (cited by Schiemann, 1989). The organism isolated from the disease was named *Bacillus pseudotuberculosis rodentium* by Pfeiffer in 1889, which later became *Pasteurella pseudotuberculosis* (cited by Bottone, 1981). It was not until 1944, a year after Yersin's death, that the two closely related species were transferred to the new genus *Yersinia*, proposed by Van Loghem (Van Loghem, 1944). The genus *Yersinia* was proposed for inclusion in the family *Enterobacteriaceae* by Frederiksen in 1964 (Bottone, 1981). The relationship between *Y. pestis*, *Y. pseudotuberculosis* and other *Yersinia* species was defined by DNA hybridisation studies in 1980 (Bercovier *et al.*, 1980b).

Members of the genus *Yersinia* are Gram-negative coccobacilli or short rods, ranging in size from 0.5-1μm by 1-3μm. They do not form endospores or true capsules. The production of flagella depends on incubation temperature, being absent at 37°C but occurring at 22°C, except in the case of *Y. pestis* which is non-motile at both temperatures. They grow on simple nutrient media and most will tolerate bile salts, but the growth of pathogenic strains

is improved by blood or tissue extracts. They are facultative anaerobes and are catalase-negative and oxidase-negative. Acid is produced by fermentation of various carbohydrate substrates. Proteolytic activity is variable, as is the production of urease. Nitrates are usually reduced. The DNA base composition is within the range 46-49% GC (Brewer and Corbel, 1986).

The genus *Yersinia* currently contains 11 species including *Y. pestis*, *Y. pseudotuberculosis* (Bercovier *et al.*, 1980b), *Y. enterocolitica* (Bercovier *et al.*, 1980a), *Y. intermedia* (Brenner *et al.*, 1980), *Y. frederiksenii* (Ursing *et al.*, 1980), *Y. kristensenii* (Bercovier *et al.*, 1980c), *Y. aldovae* (Bercovier *et al.*, 1984), *Y. rohdei* (Aleksic *et al.*, 1987), *Y. bercovieri*, *Y. mollaretii* (Wauters *et al.*, 1988b), and *Y. ruckeri* (Ewing *et al.*, 1978).

1.1.1 Taxonomy of *Yersinia enterocolitica*

Y. enterocolitica, the principal agent of human foodborne yersiniosis, has also experienced an identity crisis over the past 60 years, with numerous name changes and taxonomic cleavage. The first isolation of the species was accredited to McIver and Pike, who recovered a small, Gram-negative coccobacillus from a facial abscess of a 53-year-old farmer in 1934 in the USA and named it *Flavobacterium pseudomallei* (cited by Bottone, 1981). Schleifstein and Coleman (1939) compared this isolate with four others from people and later proposed the name *Bacterium enterocoliticum* for this "unidentified microorganism". Over the next three decades the organism was isolated in other countries from animals and people, however, no consensus on it's nomenclature could be met, and it was variously named *Pasteurella pseudotuberculosis* X, '*Pasteurella pseudotuberculosis*' type b, *Pasteurella* X, '*Pasteurella pseudotuberculosis* atypique' and 'Les germes X' (Bottone, 1981). In 1963, Knapp and Thal established the similarity between these strains from America and Europe and in 1964, Frederiksen demonstrated that strains from his collection were distinct from *Y. pseudotuberculosis*, but were similar enough to be designated as a separate species within the genus, which he named *Y. enterocolitica*.

Over the next ten years, particularly in Northern Europe, *Y. enterocolitica* was increasingly isolated from human sources and ultimately became regarded as the medically most important species in the genus. In 1971, Mollaret described 642 cases of yersiniosis diagnosed at the Pasteur Institute in Paris over a four-year period, associated with this organism. In 1974 the World Health Organisation designated the Pasteur Institute as an international reference centre and by 1976, 5800 cases had been recorded (Slome and Black, 1991). It was soon evident, however, that *Y. enterocolitica* represented a very heterogeneous group of bacteria, with those that were biochemically distinct being referred to as *Y. enterocolitica*-like organisms (Bercovier *et al.*, 1980a). In 1980, DNA hybridisation studies were carried out to delineate the species *Yersinia enterocolitica sensu stricto* from these other related species, which were given the names *Y. intermedia*, *Y. frederiksenii* and *Y. kristensenii* (Bercovier *et al.*, 1980a; Bercovier *et al.*, 1980c; Ursing *et al.*, 1980; Brenner *et al.*, 1980). Further species were later separated from *Y. enterocolitica*, *Y. aldovae* in 1984 (Bercovier *et al.*, 1984), and in 1987, *Y. mollaretii and Y. bercovieri* (Wauters *et al.*, 1988b). All the newly created species, other than *Y. enterocolitica*, were predominantly recovered from environmental sources and were believed to be non-pathogenic, however, recent evidence suggests that they may be opportunist pathogens under certain circumstances (Cafferkey *et al.*, 1993; Ursing *et al.*, 1995).

1.1.2 Isolation of *Y. enterocolitica*

Faeces are the most common type of clinical specimen examined for the presence of *Y. enterocolitica* and isolation is relatively straightforward if these or other samples are taken from clinically ill people or animals. The use of enrichment broths such as selenite F has been found to improve the recovery of the organism from faecal specimens (Ratnam *et al.*, 1982) and as this medium is widely used for the enrichment of salmonellae it has been adopted as the enrichment method of choice by many human diagnostic laboratories (McCarthy and Fenwick, 1991). *Y. enterocolitica* grows well on standard plate media such as MacConkey agar, although as growth is slower than other *Enterobacteriaceae*, colonies are easily overlooked when cultivated at 37°C. The performance of media used for the

recovery of *Y. enterocolitica* is therefore improved by incubation at lower temperatures, e.g. 22-28°C, for 48h. A number of selective media designed for the isolation of enteric bacteria have been evaluated for the recovery of yersiniae (Head *et al.*, 1982; Harmon *et al.*, 1983), however, a specialised agar medium, CIN agar, synthesised by Schiemann in 1979, has proved to be the most effective plating medium for these organisms (Schiemann, 1979).

The isolation of *Y. enterocolitica* from asymptomatic people, animal reservoirs, foods or the environment has proved more difficult, due to the presence of small numbers of organisms and their rapid overgrowth by other competitive bacteria. Thus, the recovery of yersiniae from such sources has been widely investigated and many enrichment protocols have been assessed (Schiemann, 1989). Most investigators agree that cold-enrichment techniques, based on the ability of *Y. enterocolitica* to multiply at refrigerator temperatures, are the most successful, and a 3-week enrichment at 4°C in phosphate buffered saline (PBS), pH 7.6, either alone, or supplemented with sorbitol, bile salts or mannitol, has been used for many years for the recovery of yersiniae from a range of samples (Ahvonen, 1972; Greenwood *et al.*, 1975; Pai *et al.*, 1979). Despite it's widespread usage, cold-enrichment in PBS is believed by some workers to preferentially enhance the isolation of environmental strains of *Yersinia* (Van Noyen *et al.*, 1980) and other methods, such as post-enrichment alkali treatment (Aulisio *et al.*, 1980; Doyle and Hugdahl, 1983) and two-step enrichments (Van Noyen *et al.*, 1987b; Toora *et al.*, 1994), have also been devised and evaluated for the preferential recovery of pathogenic strains of *Y. enterocolitica* from mixed cultures. Van Pee and Stragier (1979), evaluated several cold-enrichment media and showed that the enrichment quotient achieved after 3 weeks at 4°C was highly dependent on the initial cell concentration and the medium used. Their findings indicated that media for cold-enrichment should be of a high nutritional value as enrichment in tryptone-soya broth yielded better results than the frequently used PBS. Wauters *et al.* (1988a) used a new enrichment medium, ITC, and found that it was more sensitive for the recovery of *Y. enterocolitica* O:3 strains than either cold- or two-step enrichments.

1.1.3 Identification of *Y. enterocolitica*

Suspect colonies off agar plates are further characterised by inoculating triple sugar iron agar (TSI), two semisolid tubes of motility media and a urea agar slant. All media are incubated at 25°C except one of the motility media, which is incubated at 37°C. The TSI reaction at 24h is an acid slant, acid butt, with no gas or H_2S. *Yersinia enterocolitica* is non-motile at 37°C, motile at 25 C and urea positive (Morris and Feeley, 1976). The identity of *Y. enterocolitica* can be confirmed by the following biochemical tests (incubated at 25°C): sucrose, rhamnose, raffinose, melibiose, alpha-methylglucoside and Simmon's citrate. *Yersinia enterocolitica* is sucrose-positive and negative in the other tests (Schiemann, 1989). Sucrose-negative strains of the organism have been isolated in Japan (Fukushima *et al.*, 1988). The complete biochemical characterisation of *Y. enterocolitica sensu stricto* is described by Bercovier *et al* (1980a). The API 20E microtube biochemical system has been used for the identification of *Y. enterocolitica* with excellent results, providing incubation was performed at 28°C rather than at 37°C as recommended by the manufacturer (Sharma *et al.*, 1990). Unacceptable results were achieved, however, when the system was used with isolates of *Y. intermedia*.

1.1.4 Biotyping of *Y. enterocolitica*

The species *Y. enterocolitica sensu stricto*, as described by Bercovier *et al* (1980a), is biochemically heterogeneous, and a number of biotyping schemes have been proposed. Nilehn (1969), was the first bacteriologist to formally propose a biotyping scheme for the subdivision of *Y. enterocolitica* into five biotypes, based on the following reactions: indole production, attack of aesculin and salicin, lactose oxidation, acid from xylose, trehalose, sucrose, sorbose and sorbitol, ONPG, ornithine decarboxylase, Voges-Proskauer reaction, and nitrate reduction. Although the criteria for inclusion in a biotype have been amended by successive workers (Bercovier *et al.*, 1980a; Wauters *et al.*, 1987), this method for subtyping the species is still widely used today for epidemiological studies (Fukushima *et al.*, 1984c; Adesiyun and Krishnan, 1995). Strains of *Y. enterocolitica* recognised as pathogenic for people are found in biotypes 1B, 2, 3 and 4. Biotype 5 strains have only

been recovered from infections in animals (Wauters *et al.*, 1987). Biotype 1A strains, although originally believed to be environmental in origin and non-pathogenic, have been recovered from patients with gastroenteritis, and their role in such infections is still under debate (Cimolai *et al.*, 1994; Burnens *et al.*, 1996).

1.1.5 Serotyping of *Y. enterocolitica*

In addition to biotyping, the other major phenotypic method used to differentiate strains of *Y. enterocolitica* is serotyping, using antibodies raised in rabbits to lipopolysaccharide O-antigens. The first serotyping scheme was developed by Winblad (1967), and consisted of eight antigenic O-factors. This was expanded in 1971, and later in 1972, to 34 O-factors by Wauters *et al*, and although further O-factors have since been added, their original serotyping scheme is still the most widely used (Wauters *et al.*, 1971; Wauters *et al.*, 1972; Wauters, 1981; Wauters *et al.*, 1991). A revised scheme, based on the work of Wauters *et al*, was proposed by Aleksic and Bockemuhl (1984), who excluded those antigenic factors associated with other closely related species, resulting in the description of 18 serogroups of *Y. enterocolitica* containing 20 O-factors.

It is now well-recognised that the majority of human pathogenic strains of *Y. enterocolitica* correspond to a limited number of serotypes, nevertheless, certain O-factors are found in both pathogenic and environmental strains of the organism. Thus, a combination of biotyping and serotyping is required to differentiate potentially pathogenic from non-pathogenic strains of *Y. enterocolitica*, and both schemes are commonly employed during epidemiological investigations (Fukushima *et al.*, 1985b; 1987). The most commonly recovered bioserotype combinations from human infections are 1B/O:8 (principally in North America), 2/O:5,27, 2/O:9 and 4/O:3 (Mair and Fox, 1986). Although a number of H-antigens have also been identified in *Y. enterocolitica*, they are rarely used to type the species (Aleksic and Bockemuhl, 1987).

1.1.6 Phage typing of *Y. enterocolitica*

While not as widely used as biotyping and serotyping for the phenotypic characterisation of *Y. enterocolitica,* phage typing is still used in reference laboratories to subtype strains of the organism (Bercovier *et al.,* 1978). Three schemes have been developed, one in France (Mollaret and Nicolle, 1965; Nicolle *et al.,* 1976) one in Sweden (Nilehn and Ericson, 1969), and the other in the USA (Baker and Farmer, 1982), however, the French scheme has received the most extensive application. This scheme recognises 10 phagovars, I-XI, with types IX and X sub-divided into subtypes, and type XI being reserved for strains that do not conform to any of these groups. None of these schemes has, however, been developed sufficiently to produce a large number of distinct epidemiological types, with many isolates falling within a single phagovar (Brewer and Corbel, 1986).

One useful application of the French phage typing scheme has been in the subdivision of the most widespread human pathogenic strain of *Y. enterocolitica*, bioserotype 4/O:3, into three geographically defined phagovars. Strains of bioserotype 4/O:3, phagovar VIII have been isolated in Europe and Japan, those of phagovar IXa in South Africa, and those of phagovar IXb in Canada. Isolates from Australia and New Zealand recovered from pigs and people were recently typed at the Pasteur Institute in Paris and were found to belong to phagovar IXb (Pham, personal communication). While most serotype O:3 strains recovered worldwide have been remarkably homogeneous, corresponding to biotype 4, Fukushima *et al* (1984c), described a further three biochemically heterogeneous types within this serotype from Japan, biotypes 4a, 4b and 3b. Kawaoka *et al.* (1987), expanded the number of phages used to subtype Japanese serotype O:3 strains to eight, and although useful epidemiologically, their typing scheme is not used outside Japan.

1.1.7 Virulence factors and virulence testing in *Y. enterocolitica*

Early workers investigating the virulence of *Y. enterocolitica* characterised strains by the production of heat-stable enterotoxin, the ability to penetrate Hela cells, the ability to invoke keratoconjunctivitis in guinea pigs, serum resistance, adult mouse intraperitoneal and

pleural infectivity and lethality in suckling mice (Mors and Pai, 1980; Aulisio *et al.*, 1983). Nevertheless, understanding of the basis of virulence in the organism was limited until Zink *et al.* made a major breakthrough in 1980, with the discovery that the ability to cause conjunctivitis in the guinea pig was associated with the presence of a 41Md plasmid. This was verified by Gemski *et al.* (1980), who isolated a plasmid (pYV) associated with the pathogenicity of strains of *Y. enterocolitica* in mice. Strains harbouring the plasmid were found to be calcium dependent when grown *in vitro* at 37°C, but not at 26°C. The characteristic had previously been correlated with virulence in *Y. pestis* and was considered to be an essential factor in enabling plague strains to resist phagocytic killing and to survive within the reticulo-endothelial system of the host (Burrows and Bacon, 1956; Janssen and Surgalla, 1969). The phenomenon is now believed to be a response to the levels of calcium ions in intracellular and extracellular fluids, ensuring the expression of virulence factors such as outer membrane proteins only in the appropriate mammalian environment. Repression of their synthesis by Ca^{++} suggests that they would only be synthesised in an intracellular situation where the calcium concentrations are low (Cornelis *et al.*, 1987). Tabrizi and Robins-Browne (1992), investigated the effects of plasmid carriage by virulent *Y. enterocolitica* on survival within macrophages, and found that although plasmidless strains could survive phagocytosis, they were subsequently susceptible to killing by polymorphonuclear leucocytes, whereas plasmid-bearing strains were not. Their observations supported the view that suitable conditions for expression of proteins essential for virulence occur within phagosomes of macrophages, i.e. low calcium concentration and a temperature of 37°C.

Portnoy *et al.* (1981), confirmed that strains of *Y. enterocolitica* belonging to the human pathogenic bioserotypes all possessed a plasmid species ranging in mass from 40-48 Md (approximately 70kb) and a period of intensive research into properties of the virulence plasmid followed. Several other properties were subsequently found to be associated with the virulence plasmid, including autoagglutinability, mannose-resistant haemagglutination of guinea pig erythrocytes, capacity to take up Congo red, resistance to the bactericidal activity of serum, hydrophobicity, change of surface charge, appearance of a fibril structure and cytotoxicity for cell cultures (Cornelis *et al.*, 1987). Many of these features are now

known to be associated with the production of a set of proteins termed *Yersinia* outer membrane proteins (Yops), encoded for on genes distributed throughout the plasmid, constituting a thermoactivated regulon controlled by the plasmid gene *vir*F (Cornelis *et al.*, 1989).

As the heterogeneous nature of *Y. enterocolitica* meant that many strains were non-pathogenic, the discovery of the virulence plasmid led to the creation of a number of rapid tests aimed at detecting it's presence. In fact, the first test used to assess virulence based on plasmid-encoded properties was the autoagglutination test devised by Laird and Cavanaugh (1980), prior to the discovery of the plasmid. They showed that virulent strains of *Y. enterocolitica* autoagglutinated in tissue culture medium, but that avirulent strains did not. This test is still in use today, although the finding that it was associated with the presence of a plasmid-encoded polypeptide (P1), now known as the adhesin YadA, has resulted in it's modification into a slide agglutination test, using either rabbit polyclonal antisera raised against the protein or a plant lectin derived from *Mangifera indica* (Skurnik *et al.*, 1984; Sory *et al.*, 1990; Wauters *et al.*, 1995). Another early test that is still in use today is the use of a calcium-deficient medium, magnesium oxalate agar (MOX), to detect virulent strains of *Y. enterocolitica* by their growth restriction at 37°C. This phenotype was first discovered in *Y. pestis* by Higuchi and Smith (1961), and their test was adapted for assessing the virulence of *Y. enterocolitica* by Gemski *et al.* in 1980. Prpic *et al.* (1983), also adapted a *Y. pestis* virulence assay for use in *Y. enterocolitica* virulence studies, the ability of the organism to absorb Congo red dye from agar media (Surgalla and Beesley, 1969). Combined media for the detection of both Congo red uptake and calcium dependent growth have since been devised and are still widely used today (Riley and Toma, 1989; Bhaduri *et al.*, 1991). The binding of virulent *Y. enterocolitica* to another dye, crystal violet, is linked to the presence of the plasmid and has also been used as a virulence assay (Bhaduri *et al.*, 1987).

Due to the propensity of virulent *Y. enterocolitica* to lose the virulence plasmid on repeated subculture, research turned towards a consideration of chromosomally-encoded properties. Investigators soon found that two properties associated by earlier workers with virulence,

namely enterotoxin production and tissue invasiveness, were encoded for in whole or in part by chromosomal genes (Miller and Falkow, 1988; Delor *et al.*, 1990). Production of a heat-stable enterotoxin (YST) similar to that produced by *E. coli* had first been described by Pai and Mors (1978), however, as it was only detectable in supernatants of cultures incubated at 30°C, and was also common in non-pathogenic environmental strains, it was eventually discounted as a potential virulence factor. Delor *et al.* (1990), identified the chromosomal gene *yst* responsible for enterotoxin production and using DNA hybridisation showed that the gene was present in 100% of pathogenic strains of *Y. enterocolitica*, but absent in non-pathogenic strains. They argued that the earlier detection of YST in non-pathogenic strains should be reassessed, as either they produced toxins that were different to YST, or that supposedly non-pathogenic strains could in fact be capable of causing disease. In 1993, Robins-Browne *et al.* examined a small number of *Y. enterocolitica* biotype 1A strains and found that one demonstrated enterotoxic activity, despite failure to hybridise with a *yst* probe. As it had been recovered from a clinical case, they suggested that the novel enterotoxin YST-II may have been responsible for the illness. To date, however, the precise role of enterotoxins in the pathogenesis of yersiniosis remains unclear.

Tissue invasion, either of cultured cell lines or in live animals, has been used for many years to predict the virulence of *Y. enterocolitica*, however, it is only in the last few years that some understanding of the complex nature of this property has been obtained. Miller and Falkow (1988), were the first to demonstrate that two chromosomal loci, *inv* and *ail*, were required for invasion of cell lines *in vitro*. Since then they have established that while *ail* is only carried by pathogenic strains of *Y. enterocolitica*, *inv* can be found in all yersiniae, with the gene only being functional in pathogenic strains (Miller *et al.*, 1989). In addition, it is now known that the plasmid-encoded protein YadA can also promote invasion of epithelial cell lines (Straley *et al.*, 1993). The Ail protein encoded by *ail* also mediates resistance to the bactericidal effect of complement (Carniel, 1995). Although it is accepted that these 3 proteins are responsible for invasion, the precise mechanisms involved have yet to be fully determined. A number of other chromosomally-encoded features of virulent *Y. enterocolitica* have been characterised in recent years, including fimbriae (Iriarte *et al.*, 1993), iron uptake systems (Carniel *et al.*, 1987; Baumler *et al.*, 1993) and urease activity

(De Koningward *et al.*, 1994). A review by Carniel (1995), summarises the recent advances made in these areas.

In order to assess which of the many tests developed to assess the potential virulence of *Y. enterocolitica* were the most reliable, Prpic *et al.* (1985), examined a large collection of isolates, including pathogenic and non-pathogenic strains, for the presence of plasmids and plasmid-associated characteristics. They observed that while Congo red pigmentation was useful for selecting potentially virulent strains, the best *in vitro* predictor of virulence was autoagglutination, followed by calcium dependence. However, they also stated that it was unwise to extrapolate from a single characteristic, as possession of one virulence factor could be misleading. In 1989, Riley and Toma developed CR-MOX agar to ascertain both calcium dependence and Congo red absorption and used it alongside three biochemical tests, pyrazinamidase activity, aesculin hydrolysis and salicin fermentation to examine a collection of strains for potential virulence. Positive results in the three biochemical tests had been shown previously to be associated with environmental strains of *Yersinia* (Kandolo and Wauters, 1985). As a few discrepancies in the results from each of the tests were observed, they recommended the combined use of the four tests to accurately differentiate between pathogenic and non-pathogenic strains. In 1992, Farmer *et al.* further evaluated several simple laboratory tests used to identify pathogenic serotypes of *Y. enterocolitica*, or to indicate their pathogenic potential. They agreed with Riley and Toma that no single test was 100% specific and recommended that a group of tests, including growth on CR-MOX agar, pyrazinamidase production, salicin fermentation-aesculin hydrolysis and xylose fermentation, would allow the best prediction of virulence and would in addition help to identify the bioserotype of the isolate since antisera are not widely available. Chiesa *et al.* (1993), presented data from a study of 1619 strains of *Y. enterocolitica* of human and non-human origin and their results strongly supported the use of a battery of tests, as recommended by Farmer *et al.* (1992), for the assessment of the pathogenic phenotype of the organism.

1.2   *YERSINIA ENTEROCOLITICA* INFECTIONS IN PEOPLE

Clinical isolation of *Y. enterocolitica* (bioserotype 1B/O:8) was first reported by Schleifstein and Coleman (1939), from New York.   In 1966, only 23 human infections were documented in the USA, however, by the end of the 1970s several thousand infections had been reported, mostly from Europe, prompting Bottone (1977), to publish a monograph entitled "a panoramic view of a charismatic microorganism".   Since then a spectacular rise in reported incidence has been noted and the organism has now been isolated in many countries spanning five continents (Cover and Aber, 1989).   *Yersinia enterocolitica* is now well recognised as the aetiological agent of a number of syndromes in people, with one of the earliest reviews of the clinical manifestations of human yersiniosis being written by Nilehn (1967), in Sweden.   Since this excellent review, several others have been published in Europe and the USA documenting many intriguing aspects of the disease (Larsen, 1979; Wormser and Keusch, 1981; Ostroff *et al.*, 1992).   The major syndromes associated with *Y. enterocolitica* infections will be briefly discussed in the following sections.

1.2.1   Gastrointestinal infections

The most common presentation of infection with *Y. enterocolitica* is an acute, self-limited gastroenteritis, occurring in approximately one half to two thirds of all cases.   It is characterised by diarrhoea, fever and abdominal pain, either diffuse, epigastric, or in the right lower quadrant.   The intensity and duration of the diarrhoea are variable, often of short duration but frequently persisting for one to two weeks and occasionally up to one year (Vantrappen *et al.*, 1982; Leino *et al.*, 1987; Ostroff *et al.*, 1992).   Bloody diarrhoea is recognised but usually only in patients under 18-years-old (Ostroff *et al.*, 1992).   The functional impact of the disease results directly from tissue invasion and destruction, with lesions throughout the small and large intestines but most severe in the ileocaecal region (O'Loughlin *et al.*, 1990).   Rarely, sequelae such as intussusception (Burchfield *et al.*, 1983) and perforation of the ileum (Moeller and Burger, 1985) have been recorded.   Excretion of the organism in the stools is reported to occur long after the diarrhoea subsides and is believed to contribute to spread of illness within the family and the community (Marks *et*

al., 1980). Although person-to-person transmission is not well-documented, there are numerous reports of nosocomial outbreaks and familial clusters of infection (Toivanen *et al.*, 1973; Ratnam *et al.*, 1982; Ostroff *et al.*, 1992, Naqvi *et al.*, 1993). Community outbreaks of infection with *Y. enterocolitica* are uncommon and have usually been linked to consumption of contaminated food or water (Eden *et al.*, 1977; Black *et al.*, 1978; Morse *et al.*, 1984), however, in some the source has not been confirmed (Asakawa *et al.*, 1973; Zen-Yoji *et al.*, 1973). In recent years, *Y. enterocolitica* has become increasingly reported as a cause of paediatric diarrhoea, with children under 5 years commonly presenting with a relatively mild gastroenteritis (Kohl, 1979; Lee *et al.*, 1991; Naqvi *et al.*, 1993). In contrast, children over 5 years, in particular school-age children, are more likely to present with fever and abdominal pain which may mimic appendicitis, resulting, in some cases, in unnecessary appendectomy (Zen-Yoji *et al.*, 1973; Black *et al.*, 1978; Morse *et al.*, 1984). Some of the earliest European isolations of *Y. enterocolitica* were from patients suspected of having acute appendicitis, although at surgery the appendix was usually unaffected and mesenteric lymphadenitis or acute terminal ileitis were recognised. (Jansson *et al.*, 1968; Olinde *et al.*, 1984). Following such abdominal infections, symptoms have been shown to be of long duration (Saebo, 1983).

1.2.2   Extra-intestinal infections and post-infection sequelae

Septicaemia is the rarest form of human *Y. enterocolitica* infection and carries the highest mortality rate (Keet, 1974). The earliest review of cases of septicaemia was published by Mollaret *et al.* (1971), who recorded a 50% mortality rate in 17 patients. Septicaemia occurs more frequently in the young and the elderly, and usually involves patients with underlying diseases such as malignancies, diabetes mellitus or other debilitating illnesses (Bouza *et al.*, 1980). A study of *Y. enterocolitica* enteritis in children in the USA identified age as a risk factor for sepsis, with 28% of infants less than 3 months of age affected (Naqvi *et al.*, 1993). Iron overload is known to predispose patients to *Yersinia* septicaemia, with liver disease or blood disorders such as cirrhosis or thalassaemia often involved (Foberg *et al.*, 1986; Arpi *et al.*, 1991). Long-term iron therapy and accidental iron overdosage have also been reported to precipitate septicaemia (Melby *et al.*, 1982; Leighton and MacSween,

1987). Numerous sequelae have been reported following *Y. enterocolitica* bacteraemia, including liver and splenic abscesses (Rabson *et al.*, 1975; Leighton and MacSween, 1987), septic arthritis (Spira and Kabins, 1976), osteitis (Thirumoorthi, 1990), pericarditis (Lecomte *et al.*, 1989), meningitis (Coppens *et al.*, 1995) and mycotic aortic aneurysm (Hagensee, 1994).

Recently, a number of cases of *Y. enterocolitica* septicaemia have been reported following blood transfusion (Aber, 1990; Wagner *et al.*, 1994). In many reports the blood donor was not ill but was assumed to have had an asymptomatic bacteraemia at the time of donation, possibly from a gastrointestinal source. The relatively high frequency of transfusion-mediated infection with *Y. enterocolitica* may be related to two special features not shared with other enteric pathogens: it's ability to grow at refrigerator temperatures and the stimulation of it's growth by exogenous iron. After prolonged storage, blood and blood products will contain sufficient free haemin to provide a good growth medium, allowing profuse multiplication of this iron-dependent organism (Jacobs *et al.*, 1989). Screening of blood donors for gastrointestinal illness has been proposed as a strategy to eliminate carriers of *Y. enterocolitica* (Grossman *et al.*, 1991).

Other extra-intestinal manifestations of *Y. enterocolitica* have been documented, including pharyngitis. In a large multistate outbreak of yersiniosis due to contaminated pasteurised milk, 14 out of 172 patients presented with pharyngitis as the predominant symptom and *Y. enterocolitica* was isolated from throat cultures (Tacket *et al.*, 1983). In another incident, two members of a family were hospitalised with pharyngitis and one subsequently died. Consideration of *Y. enterocolitica* as an aetiologic agent was recommended in the differential diagnosis of patients with pharyngitis whose condition showed deterioration during erythromycin or penicillin therapy (Rose *et al.*, 1987). Since the first documented isolation of *Y. enterocolitica* by McIver and Pike in 1934 from a facial carbuncle (cited by Bottone, 1981), cutaneous infections have been reported uncommonly in the literature. In 1990, Perrot *et al.* described the first case of tenosynovitis caused by *Y. enterocolitica*, resulting from a thorn puncture. Krogstad *et al.* (1992), described cutaneous infections with cellulitis and bullous lesions in three children in the USA who had previously shown

no symptoms of gastrointestinal disease. It was suggested that the lesions might have resulted from either environmental exposure, or from autoinoculation after asymptomatic enteric carriage.

A number of immunological complications are known to occur as sequelae to infection with *Y. enterocolitica*, including reactive arthritis, Reiter's syndrome, anterior uveitis and erythema nodosum (Schiemann, 1989). Reactive arthritis is the most frequent complication of yersiniosis in some countries, and in Scandinavia up to 30% of adults with *Y. enterocolitica* infection later develop polyarthritis (Mair and Fox, 1986). The development of reactive arthritis is largely determined by genetic susceptibility, with the majority of patients belonging to tissue type HLA-B27 (Olson and Finch, 1981). Approximately one-third of arthritis cases later develop Reiter's syndrome, a complex consisting of uveitis, conjunctivitis, balanitis and dermatological lesions (Yu *et al.*, 1985). Erythema nodosum differs from the above syndromes in that it is not correlated with the HLA-B27 antigen and it is predominantly recognised in females over the age of 20 (Schiemann, 1989).

1.2.3 Human infections with *Y. enterocolitica* in New Zealand and Australia

Despite the increased worldwide recognition of the involvement of *Y. enterocolitica* in a range of syndromes, little published information was available on the incidence of human infections in New Zealand. The first record of *Yersinia* infection was in 1963, when Henshall described three cases of mesenteric adenitis in boys, caused by *Pasteurella (Yersinia) pseudotuberculosis*, that had occurred during a two-month period in an Invercargill hospital (Henshall, 1963). Apparently, this was the first isolation of the organism outside Europe. Little is known of the epidemiology except that the boys came from rural areas and had presented with symptoms of appendicitis. No more cases were reported until 1976, when Rose described a case of reactive arthritis and pharyngitis, initially presenting as rheumatic fever, in a 20-year-old laboratory technician who had been working with cultures of *P. (Y) pseudotuberculosis* (Rose, 1976). On questioning, she reported symptoms of malaise and abdominal discomfort over the three-month period prior to her admission to hospital. In 1979, Fallon reported two outbreaks of enteritis that had

apparently occurred in New Zealand in 1975, following the consumption of contaminated rice, however, further details were not recorded and no other reports of these outbreaks have been traced. Also in 1979, Watson *et al.* reported on the results of a survey carried out in Palmerston North to determine the incidence of *Campylobacter jejuni* and *Y. enterocolitica* in cases of acute abdominal disease. *Yersinia enterocolitica* was not isolated during the study and the authors concluded that it was an infrequent cause of enteritis in New Zealand. During the early 1980's only two further cases were recorded, the first in 1981 when a young soldier presented with acute abdominal pain and *Y. pseudotuberculosis* was cultured from abscessed mesenteric lymph nodes at surgery (Malpass, 1981). The infection was considered to have come from drinking stream water while on military exercises. The second case reported in this period was of a 72-year-old man who had died of *Y. enterocolitica* septicaemia, the first well-documented record of the occurrence of this organism in this country (Beeching *et al.*, 1985). At autopsy the patient was found to have haemosiderosis and numerous liver abscesses. Iron metabolism disorders have been well-recognised as a predisposing factor in *Yersinia* septicaemia (Bottone, 1977).

Despite the paucity of reports of *Y. enterocolitica* infection in the literature, figures from the National Health Institute showed that the number of *Y. enterocolitica* isolates received for typing increased gradually from 1981 (Diane Fraser, personal communication, 1988). Between 1981 and 1983, 27 isolates were received, all but one from cases of gastroenteritis. In 1984, 5 *Y. enterocolitica* isolates were received, from Auckland, Dunedin, Christchurch and Wellington. In 1985, 22 *Y. enterocolitica* isolates were received, 16 from Auckland, 2 from Christchurch and one each from Thames, Gisborne, Dunedin and Dannevirke. Three *Y. intermedia* isolates were also submitted from Christchurch, Wellington and Auckland and two isolates of *Y. pseudotuberculosis* from Christchurch and Wellington. A further 27 isolates of *Y. enterocolitica* were received in 1986, the majority from cases of gastroenteritis. These had again been sent in from a number of centres including Auckland, Wellington, Christchurch, Dunedin, Invercargill and Thames. Seven of the isolates had come from hospitalised patients although it is not clear whether the infection was the cause of their hospitalisation. In addition, two *Y. pseudotuberculosis* isolates were received from Christchurch and Invercargill, one from a mesenteric lymph node. In 1987, 38 *Y.*

enterocolitica isolates were received, the majority from Auckland. Unfortunately, no further typing of these isolates was performed, the only evidence of particular serotypes occurring in New Zealand being found in the article by Beeching *et al.* (1985), who stated that O:3, O:5, O:8 and O:9 had been detected, however, no other information was given.

In 1987, two cases of reactive arthritis were reported in the literature, both involving *Y. enterocolitica* O:3, and an apparent increase in the incidence of yersiniosis in the Auckland area was discussed (Ameratunga *et al.*, 1987; Jones and Bruns, 1987). At this point a letter was written to the New Zealand Medical Journal outlining the status of yersiniosis in animals and requesting additional information on the disease in people in order to establish a more accurate picture of the local situation (Fenwick and Morris, 1987). This letter led to the collaboration with Auckland Diagnostic Laboratory and the beginning of the survey under discussion. Notwithstanding the increased interest in *Y. enterocolitica* at this time, it was not until 1991 that further reports of infection were published. Two short communications described the first three cases of transfusion-related septicaemia (one fatal) caused by *Y. enterocolitica* in this country (Ulyatt *et al.*, 1991; Wilkinson *et al.*, 1991). These unusual cases were a harbinger of problems to come in the blood transfusion service and stimulated a renewed interest in yersiniae in the medical profession. Other than the preliminary results of this study (McCarthy and Fenwick, 1991), which will be discussed later, the only other report of *Yersinia* infection in the New Zealand literature described a case of arthritis in a nine-year-old boy that had initially been diagnosed as acute rheumatic fever. Although the boy had suffered a brief episode of abdominal pain, a concurrent pharyngitis had confused the diagnosis, and it was not until rheumatic fever had been ruled out that stool cultures were taken, confirming yersiniosis. The patient's asymptomatic brother was also found to be culture-positive for *Y. enterocolitica* (Lello and Lennon, 1992).

The situation in Australia closely resembles that in New Zealand in that early reports of *Yersinia* infection in the literature described sporadic cases of mesenteric lymphadenitis due to *Y. pseudotuberculosis* (Brown et al., 1966; Hewstone and Campbell, 1970; Zentner, 1971). The first report of *Y. enterocolitica* infection was made in 1972 by Hewstone and

Davidson and described a case of septicaemia and arthritis in a boy with thalassaemia major. The serotype of the isolate was given as Winblad type 1, corresponding to bioserotype 3/O:1,2,3 (Wauters, 1981). However, it was 1979 before the next isolations of *Y. enterocolitica* were recorded, the organism being recovered from only 3 of 3298 specimens during a one-year survey of faecal samples using cold-enrichment followed by culture on *Yersinia*-selective agar (Steele and McDermott, 1979). All of the patients had diarrhoea, but only two of them were assumed to have been infected in Australia, one with bioserotype 3/O:1,2,3 (an unusual pathogenic strain corresponding to the first isolate recovered in Australia) and the other with biotype 1A, commonly regarded as environmental and non-pathogenic. The third patient, who had a bioserotype 4/O:3 infection, had apparently become infected while travelling overseas. No other reports of infection were made until 1983, when Tzipori and Robins-Browne described another case of mesenteric lymphadenitis due to *Y. pseudotuberculosis* that was misdiagnosed as appendicitis. Despite the patient's occupation as a laboratory worker in a veterinary research establishment and the authors' attempts to persuade the clinicians otherwise, the correct diagnosis was reached only after unnecessary laparotomy.

Although still believed to be uncommon in Australia, in 1984 a hospital in Queensland introduced a selective medium for *Yersinia* (CIN) as part of the faecal screening protocol (Sampson and Cope, 1984). Six isolates of *Y. enterocolitica* were subsequently recovered from 231 faecal specimens over a one-year period, all but one of the patients having diarrhoea and abdominal pain, the other having reactive arthritis. Further typing of isolates was not performed. The authors also discussed similar results from concurrent faecal surveys performed at two other Brisbane laboratories and suggested that the use of a selective medium would result in an increased detection rate. Their comments appeared to be justified, because in 1985 three publications were produced that documented cases of reactive arthritis (Hazelton *et al.*, 1985), and abdominal infections (Marriott *et al.*, 1985; Mollee and Tilse, 1985), from Queensland and New South Wales, the majority now involving *Y. enterocolitica* bioserotype 4/O:3, the most common pathogenic strain recovered worldwide. Marriott *et al.* (1985), discussed the epidemiology and clinical symptoms of the disease in 32 children, and emphasised the strong association between

pharyngitis and subsequent enteric symptoms in two-thirds of the patients. In addition, three of the children had presented with "appendicitis" and eight with chronic diarrhoea of more than two weeks duration. In an extension of this study, published in 1987, Marriott further discussed the occurrence of chronic diarrhoea in children, noting that the majority were excreting biotype 1A strains of *Y. enterocolitica* without associated systemic symptoms, in contrast to those infected with biotype 4 strains whose symptoms were considerably more severe.

A further study carried out by Feeney *et al.*, in Queensland in 1987 reported the recovery of *Y. enterocolitica* from 29 patients over a three-year period following the introduction of a selective culture medium. With an isolation rate of 0.9%, *Y. enterocolitica* was the third most common enteric pathogen isolated after *C. jejuni* and *Salmonella* spp. from a predominantly adult population. While most patients showed symptoms of diarrhoea and abdominal pain, 14 were hospitalised, 4 were operated on for possible appendicitis and two had reactive arthritis as their dominant complaint. The authors concluded that *Y. enterocolitica* had emerged as a common cause of diarrhoea in adults in Australia, however, as has been shown in many other countries, the observed increase in incidence was more likely to be a consequence of intensive surveillance and the use of appropriate culture techniques than a true reflection of the situation (Cover and Aber, 1989).

In the summer of 1987-1988, an outbreak of *Y. enterocolitica* enteritis occurred in New South Wales, involving two bioserotypes, 4/O:3 and 1A/O:6,30 (Butt *et al.*, 1991). Of the 11 cases described, 7 involved toddlers with diarrhoea, while the other 4 were young adults, 3 of whom presented clinically with appendicitis. Pasteurised milk samples believed to be the source of infection were examined and strains of bioserotype 1A/O:6,30 were recovered. Outer membrane protein analysis of the O:6,30 isolates from the affected people and the milk showed similar profiles and, although usually considered environmental and non-pathogenic, a strong association with the infection and the clinical signs was suggested. The source of the O:3 isolates was not discovered. Pasteurised milk from New South Wales had been examined previously for *Yersinia* spp. by Hughes (1987), however, only environmental strains of the organism were recovered, the majority of which were untypable.

More recently, the occurrence of a number of cases of transfusion-related septicaemia have further emphasised the similarities between Australia and New Zealand in the emergence of yersiniosis as a serious systemic disease. Seven cases were notified in Australia between 1986 and 1988, five of which were fatal. Details of two of these cases, involving bioserotypes 4/O:3 and 2/O:5,27, were described in detail and guidelines for prevention of similar episodes were discussed (Anonymous, 1988; Elrick, 1988).

1.3 *YERSINIA ENTEROCOLITICA* INFECTIONS IN ANIMALS

The animal kingdom has for many years been regarded as the source of human *Yersinia* infections and intensive surveys were performed on a wide range of animal species in the 1960's and 1970's in an attempt to pinpoint specific reservoirs. Results of many of these surveys, however, showed that while a number of animal species carried strains of *Y. pseudotuberculosis* potentially pathogenic for people, with the exception of pigs and dogs, they usually carried strains of *Y. enterocolitica* that were markedly different to those causing human disease (Swaminathan *et al.*, 1982).

1.3.1 Pigs

The earliest isolation of *Y. enterocolitica* from healthy pigs was by Dickinson and Mocquot in 1961, in a study of the bacterial flora of the alimentary tract (Dickinson and Mocquot, 1961). Although they called the organism they had isolated *Pasteurella pseudotuberculosis* type b, examination of the biochemical reactions of the 16 strains recovered shows that they were indole, xylose, salicin and aesculin negative, and thus almost certainly belong to biotype 4. Since then, slaughterhouse surveys carried out in Europe (Bockemuhl *et al.*, 1979; Hurvell *et al.*, 1979; Christensen, 1980; Nesbakken and Kapperud, 1985; de Boer and Nouws, 1991), North America (Toma and Deidrick, 1975; Schiemann and Fleming, 1981; Kotula and Sharar, 1993; Hariharan *et al.*, 1995) and Japan (Maruyama, 1987; Fukushima *et al.*, 1990; Shiozawa *et al.*, 1991) have shown that pathogenic strains of *Y. enterocolitica* are common commensals of pigs worldwide, although regional differences in bioserotype

distribution are recorded. Despite the fact that human infections with *Y. enterocolitica* are reported mainly from temperate regions, pigs in sub-tropical and tropical regions also appear to carry the organism, possibly because many original populations were established with breeding stock from Europe and Canada (Ho and Koh, 1981; Chen *et al.*, 1983; Okoroafor *et al.*, 1988; Adesiyun and Krishnan, 1995). While early surveys of pigs attempted to isolate the organism from faeces and intestinal contents (Hurvell *et al.*, 1979; Christensen, 1980), later studies showed that the frequency of isolation of serotype O:3 from the tongue and palatine tonsils of pigs at slaughter was approximately ten times greater than that reported for other sites, with isolation rates of up to 56% being recorded (Pedersen, 1979; Wauters, 1979; Schiemann, 1980; de Boer *et al.*, 1986). The oral cavity of pigs is believed to be the natural reservoir of human pathogenic strains of *Y. enterocolitica* and removal of the head before processing, or excision of the tissues of the pharyngeal region, are recommended measures to reduce carcase contamination by the organism (Kapperud, 1991). Nevertheless, as the organism is also frequently isolated from faeces, a slaughtering procedure which prevents dissemination of yersiniae from the intestine to the carcass has also been recommended for the hygienic production of pork (Nesbakken and Borch, 1995).

In addition to abattoir surveys, epidemiological studies have also focused on pig farms. In 1980, Christensen surveyed a number of Danish farms and found that although *Y. enterocolitica* O:3 had a farm-wide distribution, with herds either constantly positive or negative during a one-year period, the prevalence varied widely between herds. Furthermore, his farm survey revealed that the *Yersinia*-positive farms were all of the open management type, where 6-8 week-old pigs were purchased from various sources, indicating that this was an important factor in the spread of the organism. In 1991, Andersen *et al.* further investigated the epidemiology of *Y. enterocolitica* O:3 in 99 Danish pig herds and found that 82% of herds were infected, with an overall prevalence of 25%. The 18% of herds which were negative for *Y. enterocolitica* were mostly represented by a limited number of pigs, however, no other herd-management factors were statistically associated with the presence of the organism. Fukushima *et al.* (1983) examined pigs from 5 Japanese farms for their *Y. enterocolitica* carrier status and found differences in the

seasonal isolation rates between them. In heavily infected herds, *Y. enterocolitica* was recovered in all months, whereas in other farms the organism was not recovered in summer. In addition, yersiniae were not recovered from breeding animals, only from the younger fattening stock. They also investigated the transmission of *Y. enterocolitica* on the farms and found that piglets were infected within 2-7 weeks of being moved to a pen which had been washed thoroughly after previously housing another batch of infected animals. These findings led them to suggest that piglets were probably infected with a relatively small number of organisms and that infected faeces and pen floors were the most important source of infection. In a further experimental study, Fukushima *et al.* (1984a) demonstrated that pigs infected with either serotype O:3 or O:5,27 were protected from challenge by the heterologous strain, indicating that competitive exclusion might result in farms being only infected with a single strain. Serological surveys have been used to determine the *Y. enterocolitica* status of pig herds in Germany (Bockemuhl and Roth, 1978), UK (Wrathall *et al.*, 1993; Spencer *et al.*, 1994) and China (Chen *et al.*, 1983), and could be useful for the initial screening of farms prior to cultural studies with their inherent sensitivity problems.

Surveys investigating the prevalence of *Y. enterocolitica* in slaughtered pigs and pig products have been hampered by overgrowth of the organism with other *Enterobacteriaceae* at 37°C incubation temperatures, and many enrichment methods have been used in an attempt to overcome this problem (Schiemann, 1979a; Van Pee and Stragier, 1979; Head *et al.*, 1982; Wauters *et al.*, 1988a; de Boer, 1992). Most of these methods, however, make use of the ability of *Y. enterocolitica* to outgrow competitive microflora at 4°C, in a technique known as cold-enrichment. Cold-enrichment was first used in 1963 by Paterson and Cook for the recovery of *Pasteurella (Yersinia) pseudotuberculosis* from animal faeces and it is still widely used today. In it's simplest form it involves incubation of a sample in phosphate buffered saline (PBS), pH 7.6, for three weeks, before plating onto solid media, although numerous combinations of selective and enrichment agents have since been included to achieve maximum isolation of pathogenic serotypes of the organism in a shorter period of time (Van Pee and Stragier, 1979; Wauters *et al.*, 1988; Schiemann, 1989). A two-step enrichment procedure was shown by

Nesbakken *et al.* (1985), to be superior to other methods for the recovery of pathogenic *Y. enterocolitica* serotypes from pigs and was the method of choice for this study.

The ability of *Y. enterocolitica* to survive and multiply at 4°C, the temperature used for preservation of chilled food, makes it of concern to the food industry (Hurvell, 1981; Palumbo, 1986; Kapperud, 1991; Schofield, 1992) and pathogenic strains of the organism, probably arising from carcase contamination at the time of slaughter, have been recovered from meats, particularly pork and pork products (Schiemann, 1980; Andersen, 1988; Fukushima *et al.*, 1990). In addition, a Danish study in 1987 recovered bioserotype 4/O:3 from the environment of 10% of pork butcher's shops, with a higher rate of contamination seen in small family butchers than in larger establishments (Christensen, 1987a). Nevertheless, despite the development of new enrichment media and protocols, and the frequent recovery of human pathogenic yersiniae from freshly slaughtered pigs and pig carcases, isolation from retail pork products has often been paradoxically low (Schiemann, 1980; Stern, 1981; Kapperud, 1991; de Boer, 1992). Nesbakken *et al.* (1991a), however, used a DNA hybridisation assay to detect *Y. enterocolitica* in naturally contaminated pork products and found that 60% contained virulent strains of the organism. Their results indicated that the occurrence of pathogenic *Y. enterocolitica* on Norwegian pork products was substantially higher than had been previously demonstrated by Nesbakken *et al.* (1985), and that pork represented an important potential source of human infection in Norway.

Despite strong epidemiological evidence for the connection between *Y. enterocolitica* infections in pigs and human yersiniosis (Tauxe *et al.*, 1987; Ostroff *et al.*, 1994), few cases have been reported where pigs or pork products were directly implicated as the source of infection (de Boer and Nouws, 1991), although circumstantial evidence exists for their involvement. In 1976, Sebes *et al.* reported on a case of *Y. enterocolitica* in a 65-year-old pig farmer, suggesting that his occupation may have resulted in his infection. In 1982, Aulisio *et al.* described a large outbreak of *Y. enterocolitica* O:13 involving consumption of infected pasteurised milk. On investigation, it was found that unsold milk was taken to a pig farm for feeding to the pigs and the milk crates stored near pig pens were shown to be contaminated with the same unusual serotype that was recovered from human patients,

indicating a possible porcine source for the organism. Walker and Grimes (1985), demonstrated that water runoff from a pig farm contained the same serotype of *Y. enterocolitica* that had been isolated from the pigs, and suggested that pigs could infect people indirectly via contaminated water. In 1987, a large epidemiological study was carried out by Tauxe *et al.* to determine the risk factors for human yersiniosis in Belgium. They found that *Y. enterocolitica* infection was strongly associated with the consumption of raw pork, a delicacy in Belgium, and that the incidence of infection was lowest among non-pork eating groups, including muslims. More evidence for the role of pigs in human yersiniosis came in 1988 from a Spanish study of antibiotic resistance patterns in *Y. enterocolitica* (Trallero *et al.*, 1988). Unusual resistance was demonstrated to chloramphenicol and streptomycin-sulphonamide in a cluster of human isolates and in strains recovered from pigs and pork products during the same period, supporting the hypothesis that in Spain pigs form the reservoir and source of many human *Y. enterocolitica* infections. In 1990, Joseph *et al.* reported details of a large outbreak of *Y. enterocolitica* infection that had occurred in a UK boarding school. A cohort study of the school population showed an independent association between gastrointestinal illness and contact with pigs on a small farm run by the pupils. Also in 1990, Lee *et al.* described another large outbreak of yersiniosis among infants in Atlanta, Georgia. The illnesses were strongly associated with the household preparation of chitterlings, which are the large intestines of pigs. Although none of the infants had direct contact with the raw chitterlings, in nearly all cases the persons cleaning the pig intestines were also caring for the infants. In 1991, Ichinohe *et al.* reported the first case of *Y. enterocolitica* bioserotype 1B/O:8 from Japan, in which the source was believed to be raw pork, although this was not confirmed. In 1994, a Norwegian case-control study to identify sources of infection for sporadic yersiniosis showed that people with *Y. enterocolitica* infection reported having eaten significantly more pork items in the two weeks prior to their illness than controls (Ostroff *et al.*, 1994). Patients were also more likely than controls to have stated a preference for eating raw or rare meat.

Evidence for the involvement of pigs as a source of infection for pork butchers and abattoir workers was gathered via a serological survey in Finland in 1991 (Merilahti-Palo *et al.*,

1991). Antibodies against *Y. enterocolitica* O:3 were observed more often in butchers handling pig throats and intestines than in the sera of healthy blood donors. In addition, 30-40% of the workers reported diarrhoea or abdominal pain in the 6 months preceding the study, although the symptoms did not correlate with the occurrence of antibodies. In another serological survey, Nesbakken *et al.* (1991b), examined sera from Norwegian slaughterhouse employees, veterinarians and military recruits for antibodies to *Y. enterocolitica* O:3. They found that the prevalence of antibodies was higher among workers involved in the slaughtering process than among those from other areas in the plants or office staff. However, a relatively high prevalence was also found in military recruits from urban centres and thus no firm conclusions could be drawn regarding pig contact as a risk factor for yersiniosis.

Although pigs are known to be asymptomatic carriers of pathogenic *Y. enterocolitica*, a report from China in 1987 described the isolation of serotypes O:3 and O:9 from diarrhoeic pigs belonging to four farms (Zheng, 1987). No other reports of clinical disease resulting from *Y. enterocolitica* infection have been recorded in pigs, however, experimental infections have been initiated on a few occasions. Robins-Browne *et al.* (1985), fed virulent serotype O:3 strains to neonatal gnotobiotic piglets and produced clinical illness, with intestinal lesions similar to those reported in people. The clinical response was dose-related, ranging from subclinical or mild illness in animals given 2×10^9 cfu, to death in those given 4×10^{10} cfu. In 1988, Schiemann studied the pathogenicity of *Y. enterocolitica* serotypes O:3, O:8, O:21 and O:13 in piglets by oral challenge of two litters, one caesarean-derived and colostrum-deprived and the other born naturally. Of eight caesarean-derived piglets, four died or were destroyed because of severe illness, however, the other four, and all the naturally-born piglets, showed no clinical symptoms of disease. In 1995, Shu *et al.* orally infected newborn, colostrum-deprived piglets with 3×10^{10} cfu of a human isolate of *Y. enterocolitica* O:3 in an attempt to develop an animal model for yersiniosis. Of 14 infected piglets, 11 became anorexic, 5 vomited and 13 developed diarrhoea. Damage to the mucosa was observed in the whole gastro-intestinal tract, but was more severe in the small intestine and caecum. It was hypothesised that hypoacidity in the newborn stomach may have produced favourable conditions for bacterial invasion, and a similar situation may have occurred in the pigs in China, leading to symptoms of gastroenteritis.

1.3.2 Dogs and cats

In 1973, circumstantial evidence was published that implicated sick dogs as the source of infection in a large inter-familial outbreak of *Y. enterocolitica* enteritis involving sixteen people, two of whom died (Gutman *et al.*, 1973). Five puppies from a litter of nine had died of a diarrhoeal illness the week before the first human case and the children of both families had cared for the puppies when they were ill. Unfortunately, surviving dogs were destroyed at the request of the families after the onset of the outbreak and without culture of their faeces. In another case in the USA in 1976, involving one child, faecal specimens were examined from three surviving puppies in a litter belonging to the family, in which eight animals had died of a wasting illness the month before the child became ill (Wilson *et al.*, 1976). *Yersinia enterocolitica* bioserotype 1B/O:20, was isolated from the animals, dried canine stool specimens and the sick child. No further reports of the involvement of dogs in human yersiniosis have been made.

Surveys carried out in Denmark and Japan have shown that dogs may carry strains of *Y. enterocolitica* potentially pathogenic for people, however, the prevalence in dogs was far lower than that commonly reported in pigs (Fukushima *et al.*, 1985a; Andersen, 1988). Danish workers isolated *Y. enterocolitica* bioserotype 4/O:3 from dogs on two occasions. Isolates were obtained from 1/40 (2.5%) dogs examined in 1976 (Pedersen, 1976) and from 2/115 (1.7%) dogs examined in 1979 (Pedersen and Winblad, 1979). In Japan in 1977, Kaneko *et al.* isolated *Y. enterocolitica* serotypes O:3, O:5,27 and O:9 from 23/451 (5.1%) dogs. No other reports of the carriage of serotype O:9 by dogs have been made. In 1978, Yanagawa *et al.* examined a further 704 dogs and recovered serotypes O:3 and O:5,27 from 40 (5.7%). Fukushima *et al.* (1984b), also recovered these serotypes from a number of dogs, with puppies found to be most frequently infected with O:3 and adults with O:5,27. In addition to these surveys, sporadic reports of the isolation of serotypes O:3 and O:5,27 from canine sources have been made from Italy (Tiscar *et al.*, 1992), Spain (Ferrer *et al.*, 1987) Brazil (Falcao, 1987) and England (Caugant *et al.*, 1989).

Although dogs appear mainly to be asymptomatic carriers of yersiniae, sporadic cases of enteritis have been recorded in Norway (Farstad *et al.*, 1976), USA (Papageorges *et al.*, 1983), Italy (Fantasia *et al.*, 1985) and Canada (Lynch, 1986), associated with *Y. enterocolitica* bioserotype 4/O:3 (the most commonly isolated strain from human infections worldwide) or bioserotype 2/O:5,27 (Lynch, 1986). In addition, biotype 1B, serotypes O:8 and O:20 have been associated with disease in dogs in the USA, as discussed previously (Gutman *et al.*, 1973; Wilson *et al.*, 1976). In all cases, the affected animals were either puppies or young dogs less than one year old.

Less frequently, sporadic reports of the isolation of pathogenic yersiniae from cats have been documented (Hurvell, 1981). In 1978, Yanagawa *et al.* examined the intestinal contents of 373 stray cats and found 11 (2.9%) to be carrying *Y. enterocolitica*. Of these isolates, only 3 belonged to a potentially pathogenic bioserotype, namely 4/O:3. This was in contrast to dogs examined in the same survey, where 56 out of 59 *Y. enterocolitica* strains isolated belonged to either bioserotype 2/O:5,27 or 4/O:3. Fukushima *et al.* (1985a), also examined dogs and cats for the presence of pathogenic yersiniae and although 6% of dogs carried bioserotypes 2/O:5,27, 3/O:3 and 4/O:3, cats were consistently negative. This led them to speculate that cats may be a less important source of infection than dogs.

1.3.3 Other animals

Early investigators were interested primarily in sources of *Y. pseudotuberculosis*, as this organism was responsible not only for human infections, but also for disease in a wide range of animal species, including farm animals, domestic pets, wild mammals and birds (Mair, 1965; Schiemann, 1989; Fukushima and Gomyoda, 1991). Despite a rising incidence of human infections in Europe and North America in the 1960's, interest in *Y. enterocolitica* in animals was initially stimulated by epizootics of bioserotype 3/O:1,2,3 infection in chinchillas in the USA, with the subsequent export of infected animals to Europe (Nilehn, 1967; Hubbert, 1972; Bottone, 1977; Schiemann, 1989), and by hare mortalities in Europe caused by bioserotype 5/O:2,3 (Mair, 1973). The organism was only recovered

sporadically from other animal species at this time, including pigs (Dickinson and Mocquot, 1961), and dogs (Nilehn, 1967).

While serotype O:1,2,3 was isolated occasionally from clinically affected people, it was rapidly superseded in importance by serotype O:3 infections in Europe and Canada, with an editorial in a Canadian journal in 1973 describing the "spectacular rise of *Yersinia enterocolitica*" (Anonymous, 1973). Despite this dramatic increase, little was known of the epidemiology of *Y. enterocolitica* in animals and more intensive investigations were carried out in many countries during the 1970's and 1980's in an attempt to identify potential reservoirs of human infection. These studies showed that *Y. enterocolitica* and *Y. enterocolitica*-like organisms could be recovered from a diverse range of animal species, although excluding pigs, dogs and cats, most animals carried strains that were not recognised as being human pathogens (Langford, 1972; Hacking and Sileo, 1974; Toma and Lafleur, 1974; Kapperud, 1975; Kapperud, 1977; Wooley *et al.*, 1980; Kapperud, 1981; Shayegani *et al.*, 1981; Adesiyun *et al.*, 1986; Mingrone and Fantasia, 1988).

Nevertheless, a closer look at some of the survey results reveals that occasional isolates of pathogenic *Y. enterocolitica* were in fact recovered. For example, serotype O:3 was recovered from a diarrhoeic cow in Brazil (Falcao, 1987), biotype 4 (O:3) from a bovine abortion in the UK (Brewer and Corbel, 1983), serotypes O:3 and O:9 from sheep in Russia (Kolos *et al.*, 1985), O:9 from abortions in Indian buffaloes (Das *et al.*, 1986), from raw goats' milk in the UK (Wale *et al.*, 1991) and from healthy cattle and goats in France (Reynaud *et al.*, 1993). Antibodies to serotype O:3 were also found in various domestic animal species in Nigeria (Adesiyun *et al.*, 1986). In addition, serotype O:5,27 (recognised as a human pathogen more frequently in North America and Japan than Europe) appeared to have an even wider host range than serotypes O:3 and O:9, including cattle (Brewer and Corbel, 1983; Kolos *et al.*, 1985), raccoon (Hacking and Sileo, 1974), camel and chinchilla (Toma and Lafleur, 1974), sheep (Brewer and Corbel, 1983) and unspecified wild animals and birds (Shayegani and Parsons, 1987). These results indicated that, while pigs and occasionally dogs and cats were probably the principal species harbouring serotypes potentially pathogenic for people, other animal species could well be involved in the epidemiology of human yersiniosis.

1.3.4   Infection of animals in New Zealand and Australia

Prior to 1985, human *Yersinia* infections were reported to be extremely uncommon in Australia and therefore little effort had been made to identify possible sources of the organism (Steele and McDermott, 1979; Tzipori and Robins-Browne, 1983; Mollee and Tilse, 1985). The first attempt to recover *Y. enterocolitica* from animals was in 1977, when Blackall examined intestinal contents and rectal swabs from a total of 360 pigs in Queensland (Blackall, 1977). Only *Y. pseudotuberculosis* serotype III was isolated, from 6 of the pigs. In 1979, following a survey of human faecal samples for yersiniae in South Australia, faeces and lymph nodes from 50 sheep and 50 pigs were examined for the organisms, but were all found to be negative (Steele and McDermott, 1979). A survey of milk products and the environment of a dairy farm appears to be the first indirect evidence of animal carriage of *Y. enterocolitica*, but all isolates were biotype 1A, usually considered to be non-pathogenic (Hughes, 1979; Hughes, 1980). Between 1988 and 1990 a number of cases of enteritis in cattle, sheep, goats, pigs and deer associated with *Y. pseudotuberculosis* infection were reported (Callinan *et al.*, 1988; Slee *et al.*, 1988; Jerrett *et al.*, 1990; Slee and Button, 1990b). However, it was 1990 before the first description of enteritis caused by *Y. enterocolitica* was documented in sheep and goats, involving bioserotype 5/O:2,3, a strain that has never been recovered from human sources (Slee and Button, 1990a). Further reports of disease in sheep, involving *Y. enterocolitica* O:2,3 and *Y. pseudotuberculosis* serotype III, were recorded in 1991, suggesting that these are important enteropathogens in this species (Philbey *et al.*, 1991). A study into the epidemiology of *Y. enterocolitica* and *Y. pseudotuberculosis* infections in sheep in 1992 found that inapparent infections were common, particularly in young animals, and that these provoked a marked serological response (Slee and Skilbeck, 1992; Robins-Browne *et al.*, 1993a). As clinical disease was not seen during the study period it was thought possible that the disease was only produced during periods of stress. In 1993, the first Australian isolation of *Y. enterocolitica* O:3 was made from pigs in South Australia, a state that had experienced a recent marked increase in human infections (Ormerod *et al.*, 1993). Pig and human isolates were shown to be very similar to each other using ribotyping and allozyme analysis, suggesting that pigs were a source of infection for people.

In New Zealand a similar situation existed in people, with a survey in 1979 failing to isolate *Y. enterocolitica* from faecal samples submitted to a hospital laboratory, leading the authors to conclude that the organism was an infrequent cause of enteritis in New Zealand (Watson *et al.*, 1979). In 1987, however, two letters to the New Zealand Medical Journal quoted figures showing that an apparent rise in incidence was being observed, particularly in the Auckland region (Ameratunga *et al.*, 1987; Jones and Bruns, 1987).

Yersinia infections in animals had been observed in New Zealand throughout the 1970's, coincidental with the development of the deer farming industry, although most disease was associated with *Y. pseudotuberculosis*. At Invermay Animal Health Laboratory, from 1979 to 1982, 59 cases of cervine yersiniosis were recorded, 57 due to *Y. pseudotuberculosis* and only 2 where *Y. enterocolitica* was involved (Henderson, 1983b). This prompted an attempt to isolate *Yersinia* from deer faeces and of 922 faecal samples examined, 176 were positive for *Y. enterocolitica* and 7 for *Y. pseudotuberculosis* (Henderson, 1984). As the *Y. enterocolitica* isolates were not serotyped the public health significance was not able to be determined, however, approximately 7% of strains were Hela cell invasive and were believed to be potentially pathogenic. The low prevalence of *Y. pseudotuberculosis* recovered in this study and in a subsequent national deer survey performed in 1981 (where only 5 out of 3810 faecal samples examined were positive) led to the assumption that clinically normal deer were either infrequent carriers of the organism, or that laboratory procedures lacked sensitivity (Henderson and Hemmingsen, 1983). Further surveys of deer faeces were carried out in 1984-1985, however, only the isolation of *Y. pseudotuberculosis* serotypes I, II and III was recorded, *Y. enterocolitica* being considered of minor importance (Hodges *et al.*, 1984a; Hodges *et al.*, 1984b; Mackintosh and Henderson, 1984; Hodges and Carman, 1985). Despite the regular isolation of yersiniae from sick and healthy deer, only environmental *Yersinia* species have been isolated from venison products (Bosi *et al.*, 1995).

While many of the early reports of yersiniosis were from deer, in 1984 a report of the recovery of *Y. pseudotuberculosis* serotypes I and III was made from 8 sheep that had died of enteritis in the Otago region between 1979-1982 (Hodges *et al.*, 1984a). Also in 1984,

an outbreak of diarrhoea in young sheep, affecting 30% of 450 animals, was diagnosed in Northland and *Y. enterocolitica* biotype 2, serotype 5 was isolated from 3 affected sheep. The disease appeared to be associated with nutritional stress in winter (McSporran *et al.*, 1984). The authors reported further isolations of *Y. enterocolitica* from sheep and goats in their region following this outbreak. This bioserotype combination was most likely to be O:5,27, as O:5 is associated with biotype 1A. This is therefore a potential human pathogen, the first recorded from an animal in New Zealand. A single isolate of O:5,27 was subsequently recovered in 1992 from roast lamb during a survey of ready-to-eat fleshfoods purchased from local retailers, the only isolate retrieved in the survey that was potentially pathogenic for people (Hudson *et al.*, 1992). A survey of the rectal contents of 66 lambs and 350 cattle at an abattoir in 1987 recovered 13 isolates of *Y. enterocolitica* from the lambs and none from the cattle (Bullians, 1987). Of the 13 isolates, 4 were biotype 5, 2 were serotype O:3 and the rest were untypable. Given the rather vague nature of the typing, the O:3 strains could have actually been O:2,3, the serotype associated with biotype 5, rather than the serotype responsible for human infections, but this is hypothetical and the author made the point that lamb meat could pose a public health hazard for people. A later survey, however, failed to isolate *Y. enterocolitica* from 100 bovine and 100 ovine carcases at two North Island abattoirs (Hudson and Mott, 1994).

Since 1979, goat farming in New Zealand has undergone a rapid expansion and *Yersinia* infections have been identified as a significant cause of enteritis and mortality. Between 1979-1982, 13 cases of *Y. pseudotuberculosis* infection were recorded at Invermay laboratory, involving serotypes I and III (Hodges *et al.*, 1984a). A study into goat mortalities in the southern North Island between 1985 and 1986 found that yersiniosis was responsible for 21 out of 324 goat deaths, with *Y. enterocolitica* biotype 5 recovered from all but one case. The other involved *Y. pseudotuberculosis* (Buddle *et al.*, 1988). Predisposing factors for yersiniosis included heavy rainfall, transportation, or nutritional problems. In another reported outbreak in Otago in 1987, most of a mob of 22 goat weanlings developed diarrhoea and 3 died (Orr *et al.*, 1987). *Y. enterocolitica* was isolated from the dead animals but no further typing was performed. Although pigs had been housed in the same shed a few weeks previously, yersiniae could not subsequently be recovered from the drains or bedding.

As a result of the importance of yersiniosis in goats, surveys were performed to ascertain the extent of *Yersinia* carriage in healthy animals. In one study at Invermay, faeces from 50 goat kids were sampled at 4 to 6 week intervals between 1987-1988 (Orr *et al.*, 1990). All kids remained healthy throughout the trial, however, 36% were culture positive on at least one occasion, with 32% excreting *Y. pseudotuberculosis* and 10% excreting *Y. enterocolitica*. The majority of subclinical infections occurred in autumn when kids were 5 to 7 months old. A prevalence study of 30 goat flocks carried out in the Manawatu in 1990 found that 60% of goats were infected with *Yersinia* species (Lanada, 1990). In a continuation of this study, three groups of animals, kids, hoggets and adults, were sampled monthly over a one-year period for yersiniae. *Yersinia pseudotuberculosis* and *Y. enterocolitica* bioserotypes 2/O:5,27 and 5/O:2,3 were recovered. The biotype 2 strains are potentially pathogenic for people so this was the first evidence that goats are possible reservoirs of human infection in New Zealand. The incidence of pathogenic strains was highest in winter and in the young kids.

As with other animals in New Zealand, cattle are also affected by yersiniosis, *Y. pseudotuberculosis* being the pathogen responsible in all reported cases. In the study mentioned previously from Invermay, 56 isolates of *Y. pseudotuberculosis* were made from diseased cattle between 1979-1982, 51 of which were serotype III (Hodges *et al.*, 1984a). From 1983 to 1984, a further 251 cases of *Y. pseudotuberculosis* were recorded in cattle (Hodges and Carman, 1985). This apparent increase prompted a survey of healthy cattle and *Y. pseudotuberculosis* was recovered from 134 out of 509 faecal samples (26.3%) and from 42 of 50 farms. Serotype III accounted for 93% of isolates. A survey of raw milk for *Y. enterocolitica* in 1987 did not succeed in isolating any pathogenic strains of the organism (Stone, 1987).

While the vast majority of *Y. enterocolitica* strains recovered from animals in New Zealand had previously been regarded as non-pathogenic for people, false-positive reactions occurred in cattle and deer tested as part of the *Brucella abortus* surveillance programme in 1992-1994 that were believed to be due to serological cross-reactions with *Y. enterocolitica* bioserotype 2/O:9 (MAF, 1993; MAF, 1994; Hilbink *et al.*, 1995).

Subsequent faecal culture recovered this bioserotype from a number of reactor animals. This was an interesting observation as this bioserotype had not been previously recovered from animals in New Zealand and had only been isolated from human infections since 1990.

1.4 MOLECULAR METHODS FOR DETECTION OF *Y. ENTEROCOLITICA*

In recent years, new methods using molecular techniques have altered both the means of rapidly diagnosing infectious diseases and the ability to perform epidemiological studies of microorganisms (Eisenstein, 1990). The principle behind these methods involves the identification of DNA nucleotide sequences unique to the bacterial pathogen of interest and then the use of either molecular probes and nucleic acid hybridisation (Tenover, 1988), or enzymatic amplification by the Polymerase Chain Reaction (PCR) (Persing, 1991), to recognise these sequences in either pure cultures, clinical specimens or other material.

While DNA probes for the detection of enteric pathogens have been widely used in research laboratories and for epidemiological surveys, the technology is still relatively complex and labour-intensive and has not been readily adopted for use by clinical laboratories (Echeverria *et al.*, 1985). The advent of the PCR in 1985 and it's commercialisation by Cetus Corporation (Mullis, 1990) appeared to offer certain advantages over DNA probes, such as speed, simplicity and increased sensitivity, and numerous investigations into it's potential for the detection of food-borne bacterial pathogens have been reported in the literature (Olsen *et al.*, 1995).

1.4.1 Targets for molecular detection systems

(i) *Yersinia* virulence plasmid

Virulent strains of *Yersinia*, including *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, have been shown to harbour a 40-48 megadalton plasmid (approximately 70 kilobases) that encodes for several essential virulence characteristics of the bacteria (Portnoy and

Martinez, 1985; Wartenberg *et al.*, 1988). Restriction maps have been made of the virulence plasmid carried by *Y. enterocolitica* and a high degree of homology has been shown between plasmids carried by different pathogenic serotypes, with some regions of the plasmid being highly conserved (Laroche *et al.*, 1984; Pulkkinen *et al.*, 1986). The discovery of the virulence plasmid offered the possibility that it's detection by a DNA probe could provide a rapid, specific method for identifying virulent strains of *Y. enterocolitica* in contaminated samples.

(ii)   *Yersinia* chromosomal virulence genes

In addition to plasmid-encoded virulence factors, a number of chromosomal genes important for virulence have also been recently identified and characterised, including the *inv* (invasin) gene that encodes for invasion in *Y. pseudotuberculosis* and *Y. enterocolitica*, the *ail* (attachment invasion locus) gene encoding for invasion by *Y. enterocolitica*, and the *yst* (*Yersinia* heat-stable enterotoxin) gene encoding for enterotoxin production in pathogenic strains of *Y. enterocolitica* (Miller *et al.*, 1988; Delor *et al.*, 1990). These genes have also been targeted in hybridisation assays to detect virulent yersiniae.

1.4.2   DNA probes for *Yersinia enterocolitica*

The first published report of the use of a plasmid-based probe to differentiate virulent and non-virulent strains of *Y. enterocolitica* was that of Hill *et al.* in 1983. Using ^{32}P-labelled *Bam*H1 restriction endonuclease fragments as probes in a colony hybridisation assay they detected pathogenic *Y. enterocolitica* in artificially contaminated foods without the need for enrichment or pure cultures. In 1986, Jagow and Hill assessed the sensitivity of the radio-labelled probe for the detection and enumeration of virulent *Y. enterocolitica* in a larger range of foodstuffs. They found that the detection limit was <100 colony forming units/gram (cfu/g) and that the efficiency of enumeration ranged from 66 to 100%, being influenced by the number of indigenous bacteria in the food samples, but not by the type of food. In 1988, they improved the efficiency of enumeration by introducing a KOH-NaCl pre-enrichment step to eliminate background interference from indigenous microflora and

showed that paper filters were cheaper, easier to use and as effective as nitrocellulose filters (Jagow and Hill, 1988). During this period, Gemski *et al.* (1987), also cloned plasmid DNA fragments and used them as gene probes for virulent *Yersinia*. They found that the most effective probe was a 4.5-kb DNA fragment from the conserved region of the plasmid that conferred calcium dependency on virulent yersiniae. Other plasmid fragments had a reduced sensitivity in colony blot hybridisations.

In 1989, Miliotis *et al.* developed a synthetic oligonucleotide probe to a 2.6-kb fragment of the virulence plasmid encoding for the production of conjunctivitis in guinea pigs, and found it to be highly specific and sensitive for virulent yersiniae. They used the radio-labelled probe successfully to screen *Yersinia*-infected, cultured food samples. Kapperud *et al.* (1990a), also developed a synthetic oligonucleotide probe, based on the *yop*A gene carried on the plasmid, and compared it with a cloned polynucleotide probe from the same region for the detection and enumeration of virulent *Y. enterocolitica*. Both probes were equally effective, permitting rapid and reliable identification of all pathogenic serogroups, without the need for enrichment or esoteric identification protocols. In 1991, Nesbakken *et al.* compared the use of a synthetic probe and a DNA-DNA hybridisation assay with two conventional isolation procedures for the detection of *Y. enterocolitica* serotype O:3 in naturally contaminated Norwegian pork products. Using the conventional methods they found that 18% of 50 pork samples were positive for the pathogenic serotype, whereas the probe identified 60% as positive, indicating that the occurrence of pathogenic *Y. enterocolitica* in Norwegian pork products was substantially higher than previously demonstrated (Nesbakken *et al.*, 1985).

Feng (1992), designed oligonucleotide probes to the *inv* and *ail* genes and found that the *inv*-probe was highly specific for *Y. pseudotuberculosis* whereas the *ail*-probe detected only pathogenic serotypes of *Y. enterocolitica*. Neither probe hybridised with non-pathogenic *Yersinia* species or strains, or any other bacteria tested. The finding that the *inv* probe only hybridised with *Y. pseudotuberculosis* was unexpected as the *inv* gene is also found in *Y. enterocolitica*, however, as the homology between the genes in the two species is 71%, the oligonucleotide chosen was believed to be from a *Y. pseudotuberculosis*-specific region of the gene.

Ibrahim *et al.* (1992b), used PCR to generate a 208bp digoxigenin-dUTP labelled probe to the enterotoxin gene of *Y. enterocolitica* and used the probe to screen 113 *Yersinia* strains for the presence of this gene. The probe clearly discriminated between pathogenic and non-pathogenic strains of *Y. enterocolitica*, but also hybridised with three strains of *Y. kristensenii*. Artificially inoculated faecal samples were used to check the specificity of the probe and the results showed that the probe detected only pathogenic *Y. enterocolitica* in the mixed cultures. This was the first time that a non-radioactive labelling system had been used for *Yersinia*-specific probes.

1.4.3   DNA probes for detection of *Y. enterocolitica* in clinical samples

As rapid and accurate methods were required to screen clinical samples for the presence of pathogenic *Y. enterocolitica*, the development of nucleic acid-based protocols for the detection of a number of foodborne pathogens provided the means to improve *Yersinia* diagnostics for clinical, food and research laboratories (Olsen *et al.*, 1995). Initially, radio labelled DNA probes were used successfully on artificially contaminated foods to identify and enumerate virulent *Y. enterocolitica*, despite a large background of indigenous bacteria in the samples (Jagow and Hill, 1986; Miliotis *et al.*, 1989; Kapperud *et al.*, 1990a). Later, Nesbakken *et al.* (1991), compared a DNA-DNA hybridisation assay using a radiolabelled, synthetic oligonucleotide probe with two conventional isolation procedures for the detection of *Y. enterocolitica* O:3 in naturally contaminated pork products. Hybridisation led to a 380% increase in detection rate and indicated that the occurrence of pathogenic *Y. enterocolitica* in Norwegian pork products was substantially higher than previously demonstrated. A non-radioactive, PCR-generated, *yst* probe was utilised to identify *Y. enterocolitica* in artificially inoculated faecal samples, and was shown to be highly specific and sensitive, detecting 10^2 organisms in only a few hours (Ibrahim *et al.*, 1992c). Non-radioactive, digoxigenin-labelled *ail* and *inv* probes were also used by Goverde *et al.* (1993), to investigate the presence of pathogenic *Y. enterocolitica* in naturally infected human faeces, pig organs and slaughterhouse swab samples. Results of their experiments showed that the sensitivity of colony hybridisation equalled, and in some cases improved on, conventional isolation methods.

1.4.4 PCR development for detection of *Yersinia enterocolitica*

PCR was first used to detect pathogenic *Y. enterocolitica* in 1990, with primers derived from the *vir*F gene carried on the virulence plasmid (Wren and Tabaqchali, 1990). While the reaction was highly specific, it lacked sensitivity, with false-negative results believed to be due to loss of the plasmid on prior subculture of the organisms, a recognised problem with *Y. enterocolitica* (Kapperud, 1991). Other workers have also targeted this gene in a PCR assay to differentiate pathogenic from non-pathogenic strains of *Y. enterocolitica*, however, the procedure involved the initial selection of small, calcium-dependent colonies from CIN agar that were known to be plasmid-bearing (Koeppel *et al.*, 1993). Another plasmid gene that has been used as the target for a PCR assay is *yad*A, which encodes an outer membrane protein of *Y. enterocolitica* (Kapperud *et al.*, 1993). While the authors were aware of the potential for false-negative results they did not experience any problems with the sensitivity of the reaction.

To avoid the problems associated with plasmid-loss, Fenwick and Murray (1991), designed a set of primers targeting the invasion-associated *ail* gene found only on the chromosome of pathogenic strains of *Y. enterocolitica* (Miller *et al.*,1989), and details of the development, evaluation and application of the PCR assay are reported later in this thesis (Chapters 6 and 7). In 1992, Kwaga *et al.* compared a PCR for the *ail* gene with digoxigenin-labelled polynucleotide probes derived from the *ail* and *yst* genes, for the detection of pathogenic *Y. enterocolitica*. They found that the *ail*-PCR and *ail*- probe gave identical results and were both highly specific, but that the *yst*-probe gave false-positive results with non-pathogenic yersiniae and six other species of *Enterobacteriaceae*. Ibrahim *et al.* (1992c), had also previously found strains of *Y. kristensenii* that hybridised with a *yst* probe and similar results were achieved when they used a PCR assay to detect the gene. By subsequently using a probe internal to the amplified fragment the problem was overcome, as it only hybridised with DNA amplified from *Y. enterocolitica*.

Another candidate chromosomal gene for a *Yersinia*-PCR assay is *inv*, which encodes for a gene product allowing pathogenic yersiniae to invade epithelial cells (Miller and Falkow,

1988). Unfortunately, DNA homologous to the *inv* locus is found in all *Y. enterocolitica* strains, although non-pathogenic isolates do not contain functional *inv* sequences (Pierson and Falkow, 1990). Rasmussen *et al.* (1994b), found that the unwanted detection of non-pathogenic strains could be circumvented by using a two-step PCR involving hot-start, annealing at 72°C and the addition of 1% dimethylsulphoxide, with only pathogenic *Y. enterocolitica* being amplified.

Nakajima *et al.* (1992), used a combination of primers against the *inv*, *ail* and *vir*F genes in a multiplex reaction to identify and differentiate pathogenic and non-pathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis*. Harnett *et al.* (1996), also developed a multiplex PCR assay to detect the presence of the *ail*, *yst* and *virF* genes simultaneously, quickly and accurately. In addition, amplification of the *virF* gene was achieved from strains of *Y. pseudotuberculosis*.

1.4.5 PCR detection of *Y. enterocolitica* in clinical samples

Nevertheless, despite dramatic reductions in the time required for the isolation and identification of virulent yersiniae, hybridisation assays were still relatively time-consuming and labour intensive and necessitated initial primary culture on laboratory media. To overcome these problems, attention shifted to the polymerase chain reaction, which had the potential to produce results in a matter of hours. Unfortunately, although this procedure proved highly specific and sensitive when used on pure bacterial cultures, problems arose in it's adaptation for use with clinical specimens. The principal problem being a decrease in sensitivity due to inhibition of the enzyme by non-specific components of many biological samples, often requiring lengthy DNA extraction protocols to be included in the assays (Rossen *et al.*, 1992).

PCR was first used on clinical samples by Feng *et al.* (1992), to directly identify *Y. enterocolitica* DNA in extracted blood, enabling as few as 500 bacteria to be detected in 100 µl of sample. In the same year, Nikkari *et al.* (1992), used a double round of amplifications with two pairs of primers, one pair internal to the other (nested PCR), to

examine synovial specimens from patients with *Y. enterocolitica* O:3-triggered reactive arthritis. All results were negative and culturing of the samples also failed to detect any bacteria.

Kapperud *et al.* (1993), using seeded food and water samples, compared two procedures for sample preparation prior to nested PCR. One was based on immunomagnetic separation (IMS) of the target bacteria from the sample, using magnetic particles coated with specific O:3 immunoglobulins, the other consisted of a series of centrifugation steps followed by proteinase treatment. Both methods were capable of detecting 10-30 colony forming units (cfu)/g of meat, however, when samples were enriched overnight in a non-selective medium the sensitivity was increased to 2 cfu/g, except for samples with a background flora of $>10^7$ cfu/g. Detection of the amplification products was by a colorimetric detection method, eliminating the need for electrophoresis and potentially lending itself to automation. In 1995, Dickinson *et al.* extracted DNA from food samples inoculated with *Y. enterocolitica* using a rapid lysis method with proteinase K. They detected 10^4 cfu/g in raw chicken, camembert cheese and coleslaw using a standard PCR assay. Also in 1995, Kaneko *et al.* compared 4 methods for the extraction of template DNA from artificially contaminated pork samples in PBS, prior to PCR. These included boiling of the sample, washing with TE then boiling, extraction by adaptation of a plasmid preparation protocol and extraction using silica particles. No amplification was shown with the first method, 10^3-10^4 cfu/ml were amplified using the second method and 10^1-10^2 cfu/ml using the latter two methods. They concluded, however, that template DNA was easiest to prepare using the fourth method, extraction with silica particles. Sandery *et al.* (1996), developed a two-step PCR procedure to examine water samples seeded with known dilutions of *Y. enterocolitica*. After a 48-hour enrichment, two rounds of amplification were carried out, the first a short run of 5 cycles, followed by a longer run of 25 cycles with a lower denaturation temperature of 87°C. A positive result was shown with 10/11 seeded samples containing 5-175 cfu/500ml, compared with 6/11 using conventional culture methods. The assay was also used to successfully detect pathogenic *Y. enterocolitica* in samples of natural water from four sites. Thisted Lambertz *et al.* (1996), developed a nested PCR assay for the detection of *Y. enterocolitica* in food and compared it with an official culture method established by the

Nordic Committee on Food Analysis (NMKL-117). They found that although the final results of both procedures were similar, the PCR offered the advantages of higher sensitivity and specificity.

In addition to the examination of food, use of the PCR for the analysis of faecal samples has also been documented by several workers. In 1992, Ibrahim *et al.* used crude DNA extracted from both artificially contaminated and naturally infected human stool samples in a PCR assay designed to detect the *yst* gene. They successfully identified positive samples seeded with as few as 10^2 cfu of *Y. enterocolitica*. Kapperud and Vardund (1995), extended the range of samples analysed by their previously described PCR assays to include faeces, however, due to the presence of inhibitory compounds, modifications to the original protocols were required to overcome the reduced sensitivity. For example, when faecal samples diluted 1:100 in PBS were examined directly following centrifugation and proteinase treatment, an inoculum of $>10^4$-10^5 cfu/g was required to obtain a positive result. If the samples were first enriched overnight in TSB, then diluted 1:8 in PBS prior to centrifugation, this figure was reduced to 10-30 cfu/g. The need for dilution was reduced to 1:2 when T4 gene 32 protein, a helix-destabilising protein, was added to the PCR mixture. The other method, using immunomagnetic separation, gave negative results without overnight enrichment, and only gave positive results when samples were enriched, diluted 1:16 and T4 gene 32 protein was added to the PCR mixture. Rasmussen *et al.* (1995), also used immunomagnetic separation prior to PCR (IMS-PCR) to concentrate *Y. enterocolitica* in seeded faecal samples and successfully detected as few as 200 cfu/g of faeces. However, IMS-PCR on naturally contaminated pig faecal samples detected only 2/15 culture-positive samples, with the high number of false-negative results being attributed to low numbers of cells in the original samples. They concluded that pre-enrichment of the samples was essential to eliminate these false-negative results. In a further attempt to solve the problem of PCR-inhibitory factors in faeces, Weynants *et al.* (1996), used a conventional DNA extraction procedure prior to amplification with encouraging results. Nevertheless, in addition to starting with a high number of cells in the sample (10^5 cfu/g), their procedure was relatively complex and showed no major improvement on previously documented methods. Harnett *et al.* (1996), compared

template prepared from seeded stools with or without DNA purification for use in a multiplex PCR and showed that purification was essential to overcome inhibition. Furthermore, of two methods used to purify the DNA, more consistent results were achieved using ion-exchange columns than phenol-chloroform extraction.

Studies to assess the prevalence of yersiniae in pigs tonsils have relied on a three-week cold-enrichment protocol, a time-consuming and insensitive method for detection of pathogenic strains of *Y. enterocolitica*. Rasmussen *et al.* (1995), examined swab samples from 195 pig tonsils using IMS-PCR in an attempt to reduce the time required for detection. Of 164 culture-positive samples, 60 were positive with IMS-PCR. Three culture-negative samples were also positive in the assay. Following 7-10d cold-enrichment 31/45 culture-positive and 5 culture-negative samples were detected by the IMS-PCR method. They concluded that IMS-PCR can be used to detect *Y. enterocolitica* O:3 after pre-enrichment, but direct detection needs further optimisation of the sample preparation procedures. In 1996, Thisted Lambertz *et al.* used nested PCR to examine 6 fresh tonsillar samples homogenised in PBS on a number of occasions between days 0-21 of cold-enrichment. They found that using nested PCR, 5/6 of the samples gave unambiguous positive results from day 0, the day of collection. Single PCR was sufficient for detection only after 8d of cold-enrichment. No special treatment of the samples was required before amplification and the PCR method was found to be superior to the NMKL standard protocol for the detection of *Y. enterocolitica* in tonsil tissue.

1.5   MOLECULAR TYPING OF *YERSINIA ENTEROCOLITICA*

Despite the widespread use of phenotypic typing techniques for the characterisation of *Y. enterocolitica* in clinical microbiology laboratories, the techniques share a common theoretical disadvantage in that they all depend on the stability of gene expression *in vitro*. In addition, the relatively few bioserotypes associated with human infections poses a problem for the epidemiological tracing of strains to identify potential sources of infection. For example, the most prevalent human pathogenic strain of *Y. enterocolitica* in New

Zealand and many other countries is bioserotype 4/O:3, which often constitutes up to 90% of all isolates, and thus the ability to subtype this strain is essential for identifying the routes of transmission among animals and from animals to people.

In recent years, investigations into the epidemiology of bacterial infections have utilised a wide variety of techniques derived from immunology, biochemistry and genetics, often referred to collectively as molecular epidemiology (Maslow *et al.*, 1993). The basic premise inherent in these typing systems is that epidemiologically related isolates are derived from the clonal expansion of a single precursor and, consequently, share characteristics that differ from those of epidemiologically unrelated isolates. With the development of highly sensitive molecular techniques, the ability to detect subtle strain variations has increased substantially. Epidemiological typing of bacterial strains is crucial in public health microbiology, as the data obtained can be used to monitor trends in the occurrence of pathogenic strains, or to identify potential sources of infection. Typing methods should fulfil three main criteria: discriminatory power (the ability to discriminate between unrelated isolates), reproducibility (results must be repeatable) and typeability (all isolates should be typeable by the method applied) (Olsen *et al.*, 1993). Unfortunately, no single typing method meets all these criteria, and many molecular techniques have been developed and applied for subtyping pathogenic bacteria, including *Y. enterocolitica*.

1.5.1 Plasmid analysis

Techniques for the molecular analysis of plasmids, including plasmid fingerprinting and restriction endonuclease digestion, have been used for many years in epidemiolgic investigations to identify epidemic strains of Gram-negative enteric bacteria (Farrar, 1983). Since the discovery of a plasmid in virulent strains of *Y. enterocolitica* by Zink *et al.* in 1980, molecular techniques have been used to subtype the organism. Heesemann *et al.* (1983), examined 115 strains of *Y. enterocolitica* for plasmids and found that human strains of serotypes O:3 and O:9 harboured plasmids of 46 and 44 Md respectively, which showed 90% DNA homology. Other workers have also shown that all pathogenic serotypes of *Y. enterocolitica* possess a single, similar-sized plasmid, thus simple plasmid isolation was not

considered sufficiently discriminatory for epidemiological purposes (Portnoy *et al.*, 1984; Bottone *et al.*, 1985). Nesbakken *et al.* (1987), used restriction endonuclease analysis of the plasmid (REAP) to compare the structural variability of the virulence plasmid from 129 *Y. enterocolitica* strains recovered from a variety of sources worldwide. They found that DNA fragment profiles varied not only between, but also within certain serotypes, and that plasmids from pig and human strains had identical restriction patterns. Nevertheless, plasmids from the commonest serotypes O:3 and O:9 were surprisingly homogeneous, having identical plasmid profiles, despite being obtained from many different regions. It was thus postulated that they came from clones of recent origin that had become distributed over wide geographic areas. Plasmids from serotypes O:5,27 and O:8 were more heterogeneous, representing a greater spectrum of evolutionary diversity. Similar results demonstrating the homogeneity of REAP patterns within serotypes O:3 and O:9 have also been reported by other groups (Wartenberg *et al.*, 1988; Kwaga and Iversen, 1993), which, together with the frequent loss of plasmids during laboratory subculture, raises questions about the usefulness of this technique for strain discrimination.

1.5.2 Restriction endonuclease analysis of the chromosome

Restriction endonuclease analysis of the chromosome (REAC), using frequent cutting restriction endonucleases, has been adapted for the typing of many bacteria of clinical and public health importance. However, while the technique circumvents the problems associated with plasmid loss, the complexity of the banding patterns produced has limited it's value as an epidemiological tool (Fukushima *et al.*, 1993). Kapperud *et al.* (1990b), compared REAC with phenotypic typing methods to study polymorphism among pathogenic *Y. enterocolitica* isolates from a number of serotypes. The method showed the greatest discriminatory power with strains of serotype O:8, but strains of serotypes O:3, O:5,27 and O:9 were relatively homogeneous, with only 1-2 patterns being identified in each. Comparison of the discriminatory power of six phenotypic and genotypic typing methods revealed that REAC ranked second, above H-antigen typing but below antibiogram typing (the use of antibiotic profiles to discriminate between strains). Both REAC and REAP patterns from pig and human isolates of *Y. enterocolitica* were found to be identical,

strongly supporting an epidemiological relationship between the two species. Fukushima *et al.* (1993), examined strains of serotype O:5,27 by conventional phenotypic methods and by the genotypic methods REAC and REAP. They found three distinct REAP patterns, correlated closely with the global distribution of the serotype. Strains producing each of the three REAP patterns were shown to have a single REAC pattern.

1.5.3   Ribotyping

The interpretation of complex REAC patterns may be enhanced by the use of specific DNA or RNA probes, the latter technique being referred to as ribotyping. With these techniques, genomic DNA is digested by restriction endonucleases and transferred by Southern blotting onto membranes for probing. The probes used hybridise to specific DNA sequences on the Southern blots, detecting restriction fragment length polymorphisms (RFLPs). Ribotyping has been used widely in epidemiological studies for the comparison of bacterial isolates and involves the application of universal ribosomal RNA probes to identify RNA genes, which are commonly repeated throughout the genome. In several studies the discriminatory ability of ribotyping has proven superior to phenotyping methods (Versalovic, 1993). Andersen and Saunders (1990), digested *Y. enterocolitica* genomic DNA with *Nci*I and probed the digests with a cloned DNA fragment of *Legionella pneumophila* that included the 16S and 23S rRNA genes. Using this method they identified different RFLP types within bioserotypes which were indistinguishable by other means. Analysis of 37 bioserotype 4/O:3 strains from pigs and people produced five different RFLP-types, however, bioserotypes 2/O:5,27 and 2/O:9 were more homogeneous, producing only one RFLP each with *Nci*I. Using additional enzymes, however, increased the discrimination of the method with serotypes O:8 and O:5,27. Within the bioserotype 4/O:3 isolates, evidence of geographical variation was seen, with different patterns recognised in isolates from Canada, Norway and Denmark. Blumberg *et al.* (1991), assessed 20 different restriction enzymes for their usefulness in ribotyping and found *Nci*I to give the best discrimination. Ribotyping clearly differentiated serotype O:3 from all other pathogenic serotypes and also distinguished four clones within 53 O:3 strains. Ribotypes I and II contained the majority of strains, including those recovered from pigs and people, and corresponded to phage

types 9b and 8 respectively. However, the authors concluded that a limited number of clones appeared to have disseminated within the USA and globally.

1.5.4 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE) is a technique whereby bacterial strains are characterised by the electrophoretic mobility of a large number of metabolic enzymes, detected by chromogenic reactions (Selander *et al.*, 1986). If a sufficient number of enzymes are included, isolates can be characterised by a pattern of electromorphs called an electrophoretic type (ET) or zymotype, which may be equated with the bacterial genotype (Dolina and Peduzzi, 1993). MEE has been used for both epidemiological and taxonomic studies of various bacterial genera, including *Yersinia*. In 1984, Goullet and Picard analysed esterases from 53 strains of *Y. enterocolitica* and showed that biotype 1 strains constituted 2 zymotypes, biotypes 2 and 3 one zymotype each and biotypes 4 and 5 shared a zymotype. They stated that variation in the mobility or number of bands within each biotype could represent an additional marker for epidemiological analysis. In 1988, they examined a further 192 *Yersinia* isolates by MEE and demonstrated that the enzyme data were sufficient to identify them to species level and, in addition, supported the phenotypic characterisation of yersiniae based on biochemical tests (Goullet and Picard, 1988). Caugant *et al.* (1989), also used MEE to assess allelic variation in the genome of 81 isolates of *Y. enterocolitica* from a number of countries worldwide. They distinguished 13 ETs, clustered into two groups, one which was widely distributed and contained the common human pathogenic serotypes O:3, O:5,27 and O:9, and the other, which was restricted to North America, containing serotype O:8. Despite the large number of ETs, little genetic diversity was seen among isolates of the same serogroup, reducing the value of the technique for epidemiological studies. Similar findings were reported by Picard-Pasquier *et al.* (1990), who showed that inter- and intra-species classification of *Yersinia* were comparable with MEE and ribotyping, but that there was limited diversity within each species with either technique. In contrast, Dolina and Peduzzi (1993), differentiated 16 ETs among *Y. enterocolitica* O:3 strains of phage type VIII isolated from Belgium and Switzerland.

1.5.5 PCR typing

In addition to it's use as a diagnostic test, the PCR has also been used recently as an epidemiological tool to differentiate "European" strains of *Y. enterocolitica* (including serotypes O:3, O:5,27 and O:9) from "American" strains (including serotype O:8), based on amplification of the enterotoxin (*yst*) gene (Ibrahim *et al.*, 1992a). Further differentiation of bacterial species has been accomplished with PCR-based fingerprinting techniques such as random amplified polymorphic DNA (RAPD) and inter-repeat-PCR (IR-PCR) (Matthews, 1993). RAPD is based on the random amplification of genomic DNA at low annealing temperatures with a single primer of arbitrary nucleotide sequence. IR-PCR, on the other hand, amplifies specific genomic regions known to be variable among different bacteria. Rasmussen *et al.* (1994a), used RAPD and three different primers to examine the relationship between 87 isolates of *Y. enterocolitica*. The primers were equally successful in separating the isolates into three distinct groups, pathogenic "American" strains, pathogenic "European" strains and non-pathogenic strains, supporting the presence of three distinct evolutionary lines within the species. Additionally, one of the primers also differentiated between serotypes O:3 and O:9, and O:5,27 and O:9, but not between O:3 and O:5,27. However, as O:5,27 is considered rare in Europe, the authors concluded that RAPD could provide a useful alternative to serotyping. Odinot *et al.* (1995), compared RAPD and IR-PCR for their ability to characterise 48 clinical isolates of *Y. enterocolitica*. IR-PCR, using enterobacterial repetitive intergenic consensus primers, resulted in poorly discriminated patterns and was not considered useful. RAPD, however, resulted in 13 and 14 different fingerprints using two primers, which, when combined, differentiated 22 genotypes. This compared well with biotyping and serotyping, which together differentiated 10 types. Most bioserotypes were associated with more than one genotype, for example, seven genotypes were recognised in bioserotype 4/O:3 strains, allowing the authors to conclude that the technique would be helpful in epidemiological studies.

1.5.6 Pulsed-field gel electrophoresis

Although developed more than ten years ago, pulsed-field gel electrophoresis (PFGE), a variation of REAC that possesses greater resolving power for strain analysis, has only recently been used successfully for the subtyping of a number of bacterial species (Harsono *et al.*, 1993; Sader *et al.*, 1993; Thong *et al.*, 1995). The procedure involves the digestion of chromosomal DNA within an agarose matrix by rare-cutting restriction endonucleases, resulting in a small number of large fragments, ranging from 10-800kb in length (Allardet-Servent *et al.*, 1989). As conventional electrophoresis cannot resolve such large fragments, PFGE initially utilised perpendicularly oriented, non-uniform, alternately pulsed electric fields to separate them, by forcing the DNA molecules to reorient periodically from one electric field direction to another (Schwartz and Cantor, 1984). The technique was limited by the non-uniformity of the electric field, but this was overcome by Chu *et al.* (1986), who applied a contour-clamped homogeneous electric field (CHEF) that alternated between two orientations. The electric field was generated by multiple electrodes arranged along a polygonal contour and clamped to predetermined electric potentials. Directional switching of the electric field caused molecules to change direction in the gel, the time taken being dependent on the fragment length.

PFGE was first used to study the chromosomal DNA of pathogenic yersiniae by Iteman *et al.* (1991). They found that the restriction profiles produced by enzymes with rare target sequences were species and serotype specific within the genus *Yersinia*, and concluded that the technique was a discriminatory tool which could be useful in epidemiological studies. Further assessment of the technique for subtyping yersiniae was not performed until 1994, when four papers on the subject were published. Buchrieser *et al.* (1994a), used the enzymes *Xba*1 and *Not*1 to analyse 27 strains of *Y. enterocolitica* from Austria, including serotypes O:3, O:5,27 and O:9. Their findings showed that PFGE was highly discriminatory, being able to distinguish not only between species and serotypes, but also within serotypes. Seven different restriction patterns were seen within 18 strains of bioserotype 4/O:3 with the enzyme *Not*1, four with the enzyme *Xba*1, giving a total of nine different profiles or pulsotypes. Saken *et al.* (1994), analysed a further 109 strains of *Y.*

enterocolitica from various sources and countries of origin, including 54 strains of serotype O:3, 18 of serotype O:9 and 15 of serotype O:5,27. Restriction fragments >100kb, generated using *Not*1, were clearly resolved and compared for discrimination of banding patterns. Three subtypes (showing minor deviations in one to three restriction fragments) were seen in bioserotype 4/O:3 strains from Finland, Norway, Canada, France, Japan and Germany. Human and pig strains from the same country shared common subtypes, indicating a common epidemiological link.

Similar studies using PFGE were also carried out by Buchrieser *et al.* (1994b), and Najdenski *et al.* (1994), who each examined 60 strains of *Y. enterocolitica* belonging to various pathogenic bioserotypes. Buchrieser *et al.* (1994b), using the enzymes *Not*1 and *Xba*1, generated 28 and 25 pulsotypes respectively, which when combined gave 34 unique genomic groups. Eleven, ten and three pulsotypes respectively, were recognised in bioserotypes 4/O:3, 2/O:9 and 2/O:5,27 strains using *Not*1, considerably greater discrimination within these strains than had been seen previously. Najdenski *et al.* (1994), using only *Not*1 digestion, discriminated even further within these bioserotypes, differentiating 11, 12 and 18 pulsotypes respectively within serotypes O:3, O:9 and O:5,27. Within bioserotype 4/O:3, 50% of all isolates recovered from different countries belonged to one pulsotype (4a), prompting the authors to speculate that this corresponded to the geographical spread of a single clone. Interestingly, no correlation with phage type was seen as both phage types VIII and IXa were found within this pulsotype. This apparent genetic homogeneity was also reported by Shayegani *et al.* (1995), who analysed 6 strains of O:3 from different regions of the USA using 4 different enzymes, including *Not*I, and found them all to have identical patterns. In contrast, a similar number of O:8 strains were all different indicating significant genetic diversity. Najdenski *et al.* (1995), investigated the stability of the *Y. enterocolitica* genome using PFGE and observed two profiles in different colonies derived from one strain of bioserotype 1A/O:5. More recently, Iteman *et al.* (1996), compared the efficiency of PFGE, REAP and ribotyping for typing and subtyping strains of *Y. enterocolitica*, and concluded that while REAP and ribotyping were valuable alternatives to bioserotype determination, PFGE was the most suitable technique for epidemiological tracing.

1.6 AIMS OF THE THESIS

(i)   To investigate aspects of the epidemiology of *Yersinia enterocolitica* infections in domestic animals and people in New Zealand.

(ii)   To investigate the carriage and transmission of *Y. enterocolitica* in experimentally infected dogs.

(iii)   To develop a rapid molecular diagnostic test for the identification of pathogenic strains of *Y. enterocolitica*.

(iv)   To adapt and utilise the rapid molecular diagnostic test for the detection of pathogenic strains of *Y. enterocolitica* in clinical samples.

(v)   To investigate the molecular epidemiology of *Y. enterocolitica* in animals and people in New Zealand, using strains of the organism recovered during the study and pulsed-field gel electrophoresis.

CHAPTER 2

2. SURVEY OF HUMAN *YERSINIA* INFECTIONS IN NEW ZEALAND, 1988-1995

2.1 INTRODUCTION

Within the past thirty years infections caused by *Yersinia enterocolitica* and *Y. pseudotuberculosis* have been reported with increasing frequency from many parts of the world (Schiemann, 1989). *Yersinia enterocolitica*, in particular, has become recognised as an important human pathogen, and in several countries it is nearly as common as *Salmonella* and *Campylobacter* as a cause of acute gastroenteritis (Fenwick and McCarthy, 1995). Typically, infections manifest as acute or chronic enteritis, with diarrhoea, abdominal pain and fever being the most frequently recorded symptoms. Less commonly, *Y. enterocolitica* has been implicated in cases of mesenteric lymphadenitis and terminal ileitis, septicaemia and pharyngitis. In addition, associated sporadic secondary immunological complications such as reactive arthritis, Reiter's syndrome and erythema nodosum have been reported (Bottone, 1981). In contrast, the symptoms of *Y. pseudotuberculosis* infection rarely include diarrhoea, most patients presenting with fever, abdominal pain, nausea and vomiting (Schiemann, 1989).

Despite the description of a few cases of *Yersinia* infection in the New Zealand literature, details of the epidemiology of such infections were lacking and this provided the impetus for the study described in this chapter. The aims of the study were deliberately broad, as the intention was to gather information on the incidence of the disease and the prevalence of particular strains of *Yersinia* in New Zealand, the age and sex distribution of cases, the clinical symptoms and duration of infection and any other relevant epidemiological features of the disease. In addition, the development of an extensive culture collection was essential in order to fulfil one of the other main objectives of this thesis, namely comparison of human and animal isolates of *Yersinia* to investigate the role, if any, of domestic animals as reservoirs of infection for people.

2.2   MATERIALS AND METHODS

2.2.1   Source of bacterial isolates

A collaborative study was initially set up with Diagnostic Laboratory, Auckland (ADL) in January 1988 for the purpose of gathering epidemiological data on *Yersinia* infections in New Zealand. The majority of isolates of *Yersinia* recovered by ADL were sent to the Department of Veterinary Pathology and Public Health, Massey University (DVPPH) for further characterisation. The methods used by ADL for the primary isolation of the organism from stools were described by McCarthy and Fenwick in 1991. From 1994 until the end of the study in December 1995 isolates of *Y. enterocolitica* bioserotype 4/O:3 were not sent to DVPPH but were characterised at ADL using a standardised methodology and the relevant data was sent along with the atypical strains.

During 1988 a further four laboratories joined the study and by 1991, following the inclusion of DVPPH in the Centre for Disease Control's (CDC) list of laboratories offering specialist diagnostic services, the number of participating laboratories had reached 21. By the end of the study in December 1995 a total of 43 laboratories had sent isolates to DVPPH for further typing, resulting in data on 2737 *Yersinia* strains. As the contribution of bacterial isolates and data to the study was entirely voluntary, only a few laboratories participated throughout the entire period 1988-1995. With the exception of ADL, however, (see above), all other isolates from participating laboratories were received and characterised at DVPPH. A list of all laboratories involved in the study is included in Appendix 1.

2.2.2   Confirmation of identity, biotyping, serotyping and virulence testing

All isolates were received as cultures on agar slopes or plates. On receipt each isolate was subcultured on blood agar to assess purity and a single colony was inoculated into tryptone water for biochemical testing. Each isolate was initially subjected to a full range of biochemical tests, however, an abbreviated scheme was later introduced that allowed the

confirmation of species and biotyping of *Y. enterocolitica* to be performed together. The tests used were as follows: motility at 25 and 37°C; indole production; urea and aesculin hydrolysis; ornithine decarboxylation and fermentation of the sugars rhamnose, sucrose, trehalose, xylose and salicin. A further series of tests was applied when the results were ambiguous, e.g. fermentation of melibiose, raffinose and α-methyl glucoside for rhamnose-positive organisms. Details of all tests used are supplied in Appendix 2.

Isolates confirmed as either *Y. enterocolitica* or *Y. pseudotuberculosis* were serotyped using commercial O-antisera (Denka-Seiken, Japan). Following completion of typing, all isolates were stored at -20 and -70°C in 15% glycerol broth.

During the study of porcine strains of *Yersinia,* a number of virulence tests were compared for their ability to identify potentially pathogenic yersiniae (see Chapter 3, Section 3.2.5). The simplest tests for prediction of virulence or non-virulence were aesculin hydrolysis, salicin fermentation, pyrazinamidase activity and calcium dependence at 37°C. As the first two tests were part of the biochemical screening procedure detailed above, the addition of a magnesium oxalate plate and pyrazinamidase slope to the identification protocol allowed the potential virulence of strains to be rapidly determined. After two years, however, these latter tests were discontinued and were only used when unusual isolates were being examined.

2.2.3 Data collection, storage and analysis

In return for the confirmation of identity and typing service provided, participating laboratories and clinicians were requested to complete a brief questionnaire (see Appendix 4 for a sample questionnaire) including the following patient details: age, sex, presence or absence of diarrhoea, abdominal pain and fever, duration of symptoms and type of sample provided.

All relevant data were entered into Panacea database (PAN Livestock Services Ltd., Department of Agriculture, University of Reading, Berkshire, England) and reviewed at the end of the study period. It must be stressed that although the data resulted in a

comprehensive overview of yersiniosis in New Zealand, the informal structure of the study meant that no inferential statistics were able to be utilised and descriptive analysis only was performed.

2.3 RESULTS

2.3.1 Species of *Yersinia* isolated

Table 2.1 shows the species of *Yersinia* recovered during the study from male and female patients. Of the 2737 isolates, 2654 (96.97%) were *Y. enterocolitica*, 58 (2.12%) were *Y. frederiksenii*, 12 (0.44%) were *Y. pseudotuberculosis*, 8 (0.29%) were *Y. kristensenii*, 4 (0.15%) were *Y. intermedia* and 1 (0.03%) was *Y. rohdei*. More isolates were recovered from males (54.2%) than from females (45.8%).

Table 2.1. Species of *Yersinia* isolated from males and females, 1988-1995

Species	Male	Female	Total
Y. enterocolitica (Ye)	1444	1210	2654
Y. frederiksenii (Yf)	24	34	58
Y. pseudotuberculosis (Yp)	9	3	12
Y. kristensenii (Yk)	5	3	8
Y. intermedia (Yi)	2	2	4
Y. rohdei (Yr)	-	1	1
Total	1484	1253	2737

2.3.2 Laboratory isolations of *Yersinia* species

A total of 43 laboratories from throughout New Zealand contributed data to the study during the period 1988-1995. The laboratories were coded for ease of data entry (1-43) and the complete list of names is included in the Appendix. Table 2.2 shows the distribution of *Yersinia* spp. between the various laboratories.

Of the 43 laboratories, only 11 submitted more than 25 isolates for typing during the study period and between them they contributed 94% of all isolates. These were Diagnostic Laboratory, Auckland (Lab. code 1, 62.2%), Ashburton Hospital (Lab. code 2, 1.7%), Christchurch Hospital (Lab. code 3, 4.1%), Hawera Hospital (Lab. code 4, 1.1%), Palmerston North Medical Laboratory (Lab. code 9, 2.6%), Valley Diagnostic Laboratory (Lab. code 11, 5.0%), Wellington Medical Laboratory (Lab. code 12, 6.6%), Whakatane Hospital (Lab. code 14, 1.4%), Royston Laboratory, Hastings (Lab. code 15, 6.7%), Wanganui Diagnostic Laboratory (Lab. code 16, 1.5%) and Auckland Children's Hospital (Lab. code 29, 1.1%).

Table 2.2.   Laboratory isolations of *Yersinia* spp.

Lab. code	Ye	Yf	Yp	Yk	Yi	Yr	Total
1	1671	23	1	4	1	1	1701
2*	42	2	2	1	-	-	47
3*	77	25	4	2	3	-	111
4*	29	-	-	-	-	-	29
5*	3	1	-	-	-	-	4
6	21	-	-	-	-	-	21
7*	3	-	-	-	-	-	3
8*	9	-	-	-	-	-	9
9	70	1	-	-	-	-	71
10*	4	-	1	-	-	-	5
11	136	-	-	-	-	-	136
12	182	-	-	-	-	-	182
13*	18	-	-	-	-	-	18
14*	35	2	-	1	-	-	38
15	181	2	-	-	-	-	183

Table 2.2 (cont). Laboratory isolations of *Yersinia* spp.

Lab. code	Ye	Yf	Yp	Yk	Yi	Yr	Total
16	42	-	-	-	-	-	42
17*	4	-	1	-	-	-	5
18*	4	-	-	-	-	-	4
19*	1	-	-	-	-	-	1
20	12	-	-	-	-	-	12
21	1	-	-	-	-	-	1
22*	12	-	-	-	-	-	12
23	7	-	1	-	-	-	8
24	3	-	-	-	-	-	3
25*	4	-	-	-	-	-	4
26*	3	-	-	-	-	-	3
27*	4	-	-	-	-	-	4
28	10	-	-	-	-	-	10
29*	28	-	1	-	-	-	29
30	1	-	-	-	-	-	1
31	7	1	-	-	-	-	8
32	6	-	-	-	-	-	6
33*	5	-	-	-	-	-	5
34	2	-	-	-	-	-	2
35	5	-	-	-	-	-	5
36*	1	-	1	-	-	-	2
37*	1	-	-	-	-	-	1
38*	1	-	-	-	-	-	1
39	4	-	-	-	-	-	4
40*	1	-	-	-	-	-	1

Table 2.2 (cont). **Laboratory isolations of *Yersinia* spp.**

Lab. code	Ye	Yf	Yp	Yk	Yi	Yr	Total
41*	1	-	-	-	-	-	1
42	1	1	-	-	-	-	2
43*	2	-	-	-	-	-	2
Total	2654	58	12	8	4	1	2737

Ye = *Y. enterocolitica*; Yp = *Y. pseudotuberculosis*; Yf = *Y. frederiksenii*; Yk = *Y. kristensenii*;

Yi = *Y. intermedia*; Yr = *Y. rohdei*; * = hospital microbiology laboratories, all others are private community laboratories

2.3.3 Biotypes and serotypes of *Y. enterocolitica* recovered

Table 2.3 shows the biotypes and serotypes of *Y. enterocolitica* recovered during the study. Due to the unavailability of commercial antisera the majority of biotype 1A isolates were not able to be typed in New Zealand, however, a few human and porcine isolates were sent for typing to the Japanese *Yersinia* reference laboratory at Tottori University. Of the biotype 1A isolates typed in Japan, the most common serotypes identified were O:5, O:6,30, O:6,31, O:7,8 and O:8. Biotype 2 contained two serotypes, O:5,27 and O:9; biotype 3 contained only the one serotype, O:1,2,3; biotype 4 also only contained the one serotype, O:3.

The most common bioserotype combination was 4/O:3 (83.3%), followed by 2/O:9 (8.5%), 1A (5.3%) and 2/O:5,27 (2.8%). No isolates of biotype 1B or biotype 5 were found. Twelve unusual *Yersinia* isolates recovered during the study have been included in the figures for biotype 1A as they were initially thought to belong in this group. These isolates are discussed further in Chapter 4 (4.3.6).

Table 2.3.   **Biotypes and serotypes of *Y. enterocolitica* recovered, 1988-1995**

Serotype	Biotype				
	1A	**2**	**3**	**4**	**Total**
Untypable	95	-	-	-	95
O:1/2/3	-	-	3	-	3
O:3	-	-	-	2212	2212
O:5	5	-	-	-	5
O:5/27	-	74	-	-	74
O:6/30	15	-	-	-	15
O:6/31	10	-	-	-	10
O:7/8	6	-	-	-	6
O:8	6	-	-	-	6
O:9	-	225	-	-	225
O:12	1	-	-	-	1
O:13/7	1	-	-	-	1
O:14	1	-	-	-	1
Total	140	299	3	2212	2654

2.3.4   Bioserotypes of *Y. enterocolitica* recovered from selected laboratories

Eleven laboratories contributed the majority of *Yersinia* isolates (94%) during the study.
Table 2.4 shows the bioserotypes of *Y. enterocolitica* recovered from each of these
laboratories.   Diagnostic Laboratory, Auckland had the highest number of isolations of all
bioserotypes, except 1A. However, the distribution of the bioserotypes isolated from each
laboratory varied considerably.

The combined isolation rate of biotype 1A strains from the 11 laboratories over the study
period was 4.3% (108 isolates).   Seven laboratories had an isolation rate higher than this,
with Christchurch Hospital's being considerably higher, 41.6%.   Other laboratories with a

higher rate were Ashburton Hospital (14.3%), Royston Laboratory, Hastings (9.9%), Whakatane Hospital (8.6%), Valley Diagnostic Laboratory (7.4%), Auckland Children's Hospital (7.1%) and Wanganui Diagnostic Laboratory (4.8%).

The combined isolation rate of bioserotype 2/O:5,27 strains was 2.6% (65 isolates). Five laboratories had higher rates, the highest being Auckland Children's Hospital (10.8%). The other laboratories were Hawera Hospital (6.9%), Whakatane Hospital (5.7%), Wellington Medical Laboratory (5.5%) and Ashburton Hospital (4.8%),. Five laboratories had an isolation rate of less than 1%, with Christchurch Hospital, Palmerston North Medical Laboratory and Wanganui Diagnostic Laboratory failing to recover this strain at any time.

The combined isolation rate of bioserotype 2/O:9 strains was 8.4% (210 isolates), the second most common bioserotype combination after 4/O:3. Seven laboratories had higher isolation rates, the highest being Wanganui Diagnostic Laboratory (16.7%). The other laboratories were Hawera Hospital (13.8%), Palmerston North Medical Laboratory (12.8%), Whakatane Hospital (11.4%), Ashburton Hospital (9.5%), Royston Laboratory, Hastings (9.4%) and Diagnostic Laboratory, Auckland (8.9%).

Bioserotype 3/O:1,2,3 strains were rarely recovered, only three isolates being cultured, from Diagnostic Laboratory, Auckland, Christchurch Hospital and Ashburton Hospital.

The combined isolation rate of bioserotype 4/O:3 strains was 84.5% (2107 isolates). Only three laboratories had a higher rate, Wellington Medical Laboratory (89.6%), Diagnostic Laboratory, Auckland and Valley Diagnostic Laboratory (86.8%). The lowest isolation rate was Christchurch Hospital (54.5%). Other laboratories with lower isolation rates of this bioserotype were Palmerston North Medical Laboratory (82.9%), Royston Laboratory, Hastings (80.1%), Whakatane Hospital (74.3%), Hawera Hospital (75.9%), Auckland Children's Hospital (75.0%) and Ashburton Hospital (69.0%).

Table 2.4.   **Bioserotypes of *Y. enterocolitica* from selected laboratories**

Lab. code	1A	2/O:5,27	2/O:9	3/O:1,2,3	4/O:3	Total
1	27 (1.6)[#]	44 (2.6)	149 (8.9)	1 (0.1)	1450 (86.8)	1671
2	6 (14.3)	2 (4.8)	4 (9.5)	1 (2.4)	29 (69.0)	42
3	32 (41.6)	-	2 (2.6)	1 (1.3)	42 (54.5)	77
4	1 (3.4)	2 (6.9)	4 (13.8)	-	22 (75.9)	29
9	3 (4.3)	-	9 (12.8)	-	58 (82.9)	70
11	10 (7.4)	1 (0.7)	7 (5.1)	-	118 (86.8)	136
12	4 (2.2)	10 (5.5)	5 (2.7)	-	163 (89.6)	182
14	3 (8.6)	2 (5.7)	4 (11.4)	-	26 (74.3)	35
15	18 (9.9)	1 (0.6)	17 (9.4)	-	145 (80.1)	181
16	2 (4.8)	-	7 (16.7)	-	33 (78.6)	42
29	2 (7.1)	3 (10.8)	2 (7.1)	-	21 (75)	28
Total	108	65	210	3	2107	2493
% Total	4.3	2.7	8.4	0.1	84.5	

[#] - nos. in parentheses are % of the total isolates from that particular laboratory

2.3.5   Annual isolation of *Y. enterocolitica*, all laboratories

Table 2.5 shows the annual isolations of bioserotypes of Y. enterocolitica over the study period. Biotype 1A was isolated every year, with the isolation rate ranging from 5-34 isolates per annum. The mean annual isolation rate of biotype 1A was 17.5 isolates per annum and in only two years has this rate been considerably higher, 1993 (34 isolates) and 1995 (24 isolates). Bioserotype 2/O:9 was not isolated from any laboratory prior to 1991. Since then, the annual isolation rate of this bioserotype has increased every year, from 11 isolates in 1991 to 118 in 1995. Bioserotype 2/O:5,27 was isolated every year in small numbers with the mean annual isolation rate being 9.25 isolates per year. No trend is obvious for this bioserotype with isolation rates ranging from 8-11 per year throughout the study. Bioserotype 3/O:1,2,3 was only isolated once in 1989 and twice in 1990.

Bioserotype 4/O:3 was the most frequently isolated bioserotype throughout the eight years of the study, ranging from 121 to 480 isolates per year. Isolations increased from 1988 to 1991 and then fluctuated until the end of the study, with the highest number of isolations recorded in 1994 (480 isolates). The mean annual isolation rate was 276.5 isolations per year and this was exceeded in 1991 (380 isolates), 1993 (318 isolates), 1994 (480 isolates) and 1995 (313 isolates).

The isolation rate of bioserotype 4/O:3 over the eight years was 83.3% (2212 of 2654 isolates). While annual isolation rates varied from 80-94% until 1994, a decrease occurred in 1995, to 67.6%. This coincided with an increase in the isolation rate of bioserotype 2/O:9 from approximately 8% in the period 1992-1994 to 25.5% in 1995.

Table 2.5.   **Annual isolation of bioserotypes of *Y. enterocolitica*, 1988-1995**

Year	1A	2/O:9	2/O:5,27	3/O:1,2,3	4/O:3	Total
1988	5	-	10	-	121	136
1989	19	-	7	1	180	207
1990	15	-	11	2	200	228
1991	7	11	8	-	380	406
1992	18	22	11	-	220	271
1993	34	32	11	-	318	395
1994	18	42	8	-	480	548
1995	24	118	8	-	313	463
Total	140	225	74	3	2212	2654
%	5.3	8.5	2.8	0.1	83.3	100

Figure 2.1 shows the annual isolation of bioserotypes of *Y. enterocolitica* as percentages of the total isolations in each year.

Figure 2.1. Annual isolation of *Y. enterocolitica*, all laboratories
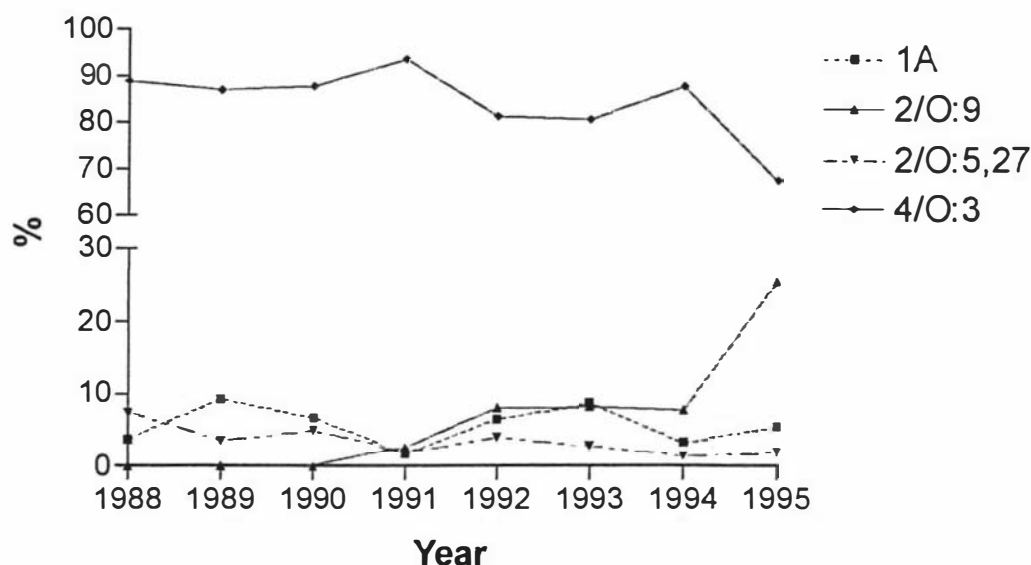
2.3.6 Annual isolation of *Y. enterocolitica*, selected laboratories

Only a few laboratories consistently supplied isolates and data for this study. Diagnostic Laboratory, Auckland collaborated for the entire period, Royston Laboratory, Hastings, from 1991 to 1995. Tables 2.6 and 2.7 show the annual isolation of different bioserotypes of *Y. enterocolitica* from Auckland and Hastings and illustrate differences in isolation between a large urban laboratory (Auckland) and a small laboratory covering both urban and rural communities (Hastings).

Diagnostic Laboratory, Auckland contributed 63% of all *Y. enterocolitica* isolates to the study and, apart from a small drop in 1992, the annual isolation rate of *Y. enterocolitica* increased gradually from 1988 (118 isolates) to 1994 (442 isolates) and then decreased in 1995 (308 isolates). The isolation rate of biotype 1A (1.6%) was less than a third that of the total rate (5.3%), that of bioserotype 2/O:5,27 (2.6%) was slightly lower than the total rate (2.8%), however, the isolation rates of bioserotypes 2/O:9 (8.9%) and 4/O:3 (86.8%) were slightly higher than the overall rates (8.5%, 83.3%). In agreement with the overall figures, the annual isolation of bioserotype 4/O:3 as a proportion of the total also decreased, from over 90% of the total number of isolates in 1988 to 72% in 1995, coincident with an increase in isolation of bioserotype 2/O:9 strains.

Table 2.6.   Annual isolation of bioserotypes of *Y. enterocolitica* from Auckland

Year	1A	2/O:9	2/O:5,27	3/O:1,2,3	4/O:3	Total
1988	-	-	9	-	109	118
1989	3	-	5	1	140	149
1990	4	-	9	-	144	157
1991	2	6	2	-	174	184
1992	8	14	6	-	102	130
1993	4	21	6	-	152	183
1994	4	27	4	-	407	442
1995	2	81	3	-	222	308
Total	27	149	44	1	1450	1671
%	1.6	8.9	2.6	0.1	86.8	100

Table 2.7.   Annual isolation of bioserotypes of *Y. enterocolitica* from Hawke's Bay

Year	Ye?	1A	2/O:9	2/O:5,27	4/O:3	Total
1991	-	-	-	-	26	26
1992	-	-	2	-	32	34
1993	12	1	1	-	40	54
1994	-	4	4	1	15	24
1995	-	1	10	-	32	43
Total	12	6	17	1	145	181
%	6.6	3.3	9.4	0.6	80.1	100

Ye? - Unusual yersiniae

In Hawke's Bay the isolation rates of biotype 1 A and bioserotypes 2/O:5,27 and 4/O:3 were all lower than the total isolation rates for these strains and the latter two strains also had a lower isolation rate than that recorded for Auckland. However, the isolation rate of

bioserotype 2/O:9 strains was higher than the total isolation rate and the Auckland isolation rate. In addition, a cluster of untypable yersiniae was recovered in one month in 1993 and these were not isolated by any other laboratory. These strains are discussed in more detail in Chapter 4 (4.3.6).

2.3.7 Cumulative monthly isolations of *Y. enterocolitica*, 1988-1995

Figure 2.2 shows the cumulative monthly isolations recorded during the period 1988 to 1995. The highest number of isolates was recorded in March (278 isolates), however, a similar figure was also recorded in February (277 isolates). The lowest number of isolates was recorded in July (147 isolates). Seasonal totals were Summer (December - February), 743 isolates; Autumn (March - May), 655 isolates; Winter (June - August), 590 isolates; Spring (September - November), 666 isolates.

Figure 2.2. **Monthly isolations of *Y. enterocolitica***
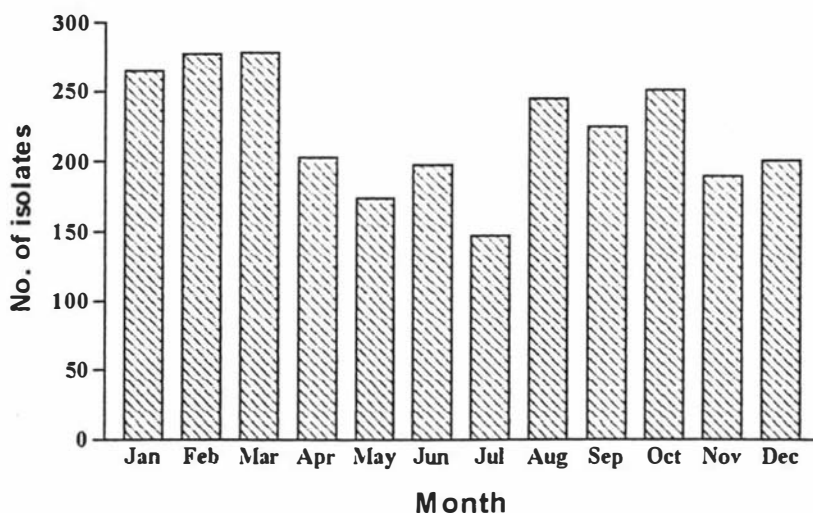
2.3.8 Age and sex distribution of *Y. enterocolitica*

Table 2.8 shows the age and sex distribution of all patients from whom *Y. enterocolitica* was isolated. Data were grouped in four-year age bands for analysis and a bimodal distribution was observed (Figure 2.3). The highest peak occurred in the 0-4 year age band (504 isolates, 19%) and a secondary peak was seen in 25-29 year olds (298 isolates, 11.2%). A cluster of cases was seen in young adults between the ages of 20-34 (803 isolates, 30.2%).

Males outnumbered females overall (1444:1210) and in all but three of the age bands (0-4, 75-79, 90+). The youngest patient was one month and the oldest was 98 years. Forty four percent of patients in the 0-5 age band were aged one year and under.

Figure 2.3. **Bimodal age distribution of *Y. enterocolitica* isolations**
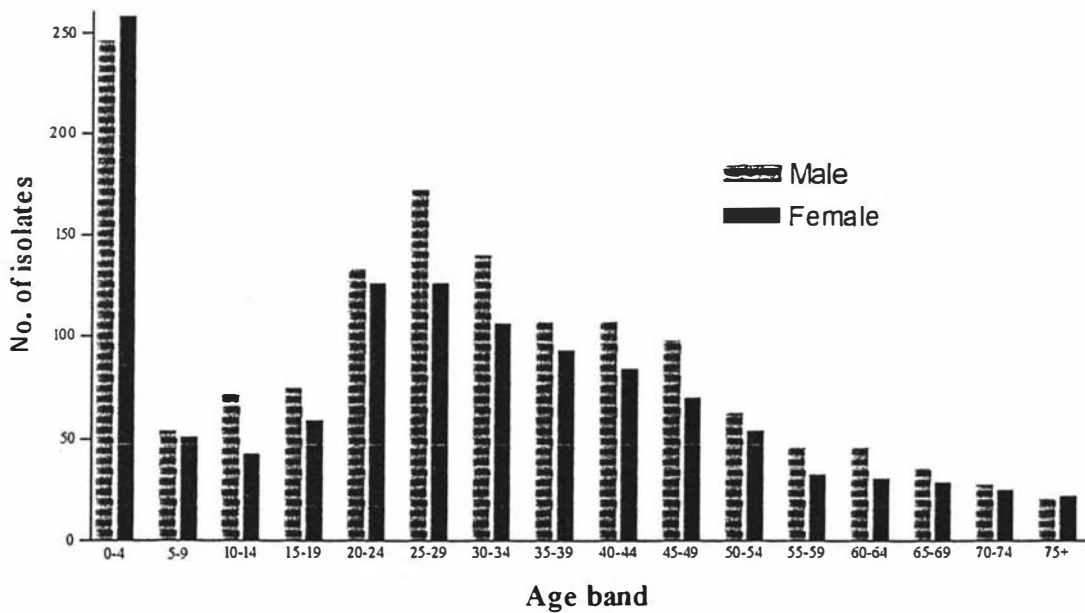
Table 2.8.   **Age and sex distribution of** *Y. enterocolitica* **isolations**

Age band	Male	Female	Total	%
0-4	246	258	504	19.0
5-9	54	51	105	4.0
10-14	72	43	115	4.3
15-19	75	59	134	5.0
20-24	133	126	259	9.8
25-29	172	126	298	11.2
30-34	140	106	246	9.3
35-39	107	93	200	7.5
40-44	107	84	191	7.2
45-49	98	70	168	6.3
50-54	63	54	117	4.4
55-59	46	33	79	3.0
60-64	46	31	77	2.9
65-69	36	29	65	2.4
70-74	28	25	53	2.0
75-79	6	9	15	0.6
80-84	9	6	15	0.6
85-89	6	5	11	0.4
90+	-	2	2	0.1
Total	1444	1210	2654	100

Table 2.9 shows the age distribution within the bioserotypes of *Y. enterocolitica*. The highest number of isolations for all bioserotypes occurred in the 0-4 age band. A bimodal age distribution is also apparent for all bioserotypes except 2/O:5,27 in which there is no secondary peak. Bioserotype 3/O:1,2,3 isolates were recovered rarely and therefore no pattern could be ascertained. A higher proportion of biotype 1A isolates were recovered from patients over 75 years than other bioserotypes (13 isolates out of 140, 9.3%). In

contrast, only 0.9% of the total bioserotype 4/O:3 strains, 4.0% of bioserotype 2/O:5,27 strains and 2.7% of bioserotype 2/O:9 strains were recovered from this age group.

Table 2.9. Distribution of bioserotypes of *Y. enterocolitica* between age bands

Age band	1A	2/O:5,27	2/O:9	3/O:1,2,3	4/O:3
0-4	24	26	44		410
5-9	6	4	6	1	89
10-14	5	3	10		97
15-19	3	2	6		123
20-24	13	6	19		221
25-29	15	6	19		258
30-34	7	5	26	2	208
35-39	11	4	20		165
40-44	8	3	15		165
45-49	6	3	20		139
50-54	3	1	14		99
55-59	6	3	6		64
60-64	7	4	6		60
65-69	8		3		54
70-74	5	4	5		39
75+	13	3	6		21
Total	140	74	225	3	2212

2.3.9 Age and sex distribution of *Y. pseudotuberculosis*

Table 2.10 shows details of the 12 isolates of *Y. pseudotuberculosis* received from participating laboratories during the study. Isolations were most frequent from patients in the age bands 5-9 (25%) and 10-14 (25%). With one exception, all patients were aged between 0 and 24 years of age. Of the 12 isolates, 10 were from males and 2 from females.

One of the isolates was from a case of pneumonia in a diabetic adult, 7 were from cases of mesenteric lymphadenitis and the remaining 4 were from cases of gastroenteritis.

Table 2.10. *Y. pseudotuberculosis* **patients, epidemiological data**

Year	Lab. code	Age	Sex	Specimen	Diagnosis	Serotype
1989	2	10	M	L. node	Mes. lymphadenitis	II
1989	3	3m	M	Faeces	Gastroenteritis	II
1990	3	5	F	L. node	Mes. lymphadenitis	II
1990	10	15	M	L. node	Mes. lymphadenitis	I
1990	3	24	M	Faeces	Gastroenteritis	II
1991	17	13	M	L. node	Mes. lymphadenitis	II
1993	1	11	F	Faeces	Gastroenteritis	I
1994	3	6	M	L. node	Mes. lymphadenitis	II
1994	36	54	M	Blood	Pneumonia	II
1995	2	1	M	Faeces	Gastroenteritis	II
1995	23	7	M	L. node	Mes. lymphadenitis	II
1995	29	22	M	L. node	Mes. lymphadenitis	II

Mes. lymphadenitis = mesenteric lymphadenitis; L. node = lymph node

2.3.10 Clinical symptoms of *Yersinia* infection

Clinical details were supplied for 1350 patients (49.3%). Of these, 80.3% had diarrhoea, 61.2% had abdominal pain, 9.3% had fever and 2% showed none of these symptoms. Table 2.11 shows the association between diarrhoea and abdominal pain. 36.8% of patients reported diarrhoea alone, 43.5% had diarrhoea and abdominal pain. 17.7% of patients reported abdominal pain alone.

Table 2.11.   **Association between diarrhoea and abdominal pain**

Symptoms	Abdominal pain	No abdominal pain	Total
Diarrhoea	587 (43.5%)	497 (36.8%)	1084 (80.3%)
No diarrhoea	239 (17.7%)	27 (2.0%)	266 (19.7%)
Total	826 (61.2%)	524 (38.8%)	1350

2.3.11 Extra-intestinal infections

Table 2.12 shows the types of specimen from which yersiniae were recovered and the proportion of each specimen submitted. The majority of specimens submitted were faeces (98.5%). All other sample types were submitted only to hospital laboratories. Blood samples accounted for 1.1% of all specimens. The majority of blood samples were from severely ill, septicaemic patients, some of whom died. However, *Y. enterocolitica* was also recovered from two blood samples from healthy blood donors. Lymph node specimens accounted for 0.3% of isolates and were all mesenteric lymph nodes removed at the time of appendectomy. The pus specimen was from a septicaemic patient diagnosed with liver abscesses at surgery. Of the two wound swabs submitted, one was from an appendicectomy wound the other from a draining sinus associated with osteomyelitis. Table 2.13 gives further details of patients from whom extra-intestinal samples were submitted.

Table 2.12.   **Distribution of total *Yersinia* isolates by specimen type**

Specimen	Male	Female	No. of isolates
Faeces	1455	1240	2695
Blood	20	10	30
Pus	1	-	1
Lymph node	7	2	9
Wound swab	1	1	2
Total	1484	1253	2737

Table 2.13. Details of extra-intestinal *Yersinia* infections

Specimen	Age	Sex	Bioserotype	Lab. code
Blood	2	M	4/O:3	18
Blood	10	F	4/O:3	7
Blood	15	M	4/O:3	27
Blood	19	M	2/O:9	8
Blood	20	F	4/O:3	13
Blood	21	M	4/O:3	29
Blood	23	F	4/O:3	3
Blood*	25	M	4/O:3	3
Blood	30	M	2/O:9	29
Blood*	35	M	4/O:3	8
Blood	54	M	Yp II	36
Blood	58	M	4/O:3	3
Blood	59	M	4/O:3	15
Blood	61	M	4/O:3	5
Blood	62	F	4/O:3	29
Blood	63	M	4/O:3	5
Blood	64	M	4/O:3	29
Blood	65	F	4/O:3	18
Blood	67	M	2/O:9	13
Blood	71	M	4/O:3	13
Blood	72	M	2/O:5,27	4
Blood	76	M	2/O:5,27	2
Blood	75	F	4/O:3	3
Blood	77	F	4/O:3	13
Blood	80	M	4/O:3	36
Blood	83	F	4/O:3	10
Blood	84	M	4/O:3	3

Table 2.13 (cont).   Details of extra-intestinal *Yersinia* infections

Specimen	Age	Sex	Bioserotype	Lab. code
Blood	84	M	4/O:3	8
Blood	85	F	4/O:3	3
Blood	90	F	4/O:3	13
Pus	71	M	4/O:3	10
L.node	5	F	Yp II	3
L. node	6	M	Yp II	3
L. node	7	M	Yp II	23
L. node	10	M	Yp II	2
L. node	13	M	Yp II	17
L. node	15	M	4/O:3	3
L. node	15	M	Yp I	10
L. node	22	F	4/O:3	3
L. node	22	M	Yp II	29
Wound	15	F	4/O:3	2
Wound	73	M	2/O:5,27	4

(* = healthy blood donor)

Twenty eight out of the 42 extra-intestinal infections were caused by *Y. enterocolitica* bioserotype 4/O:3 (66.7%), 8 were caused by *Y. pseudotuberculosis* (19.0%) and 3 each by *Y. enterocolitica* bioserotypes 2/O:5,27 and 2/O:9 (7.1% each). Thirteen of the isolates were from patients over the age of 70 (30.9%). Only 2 (4.8%) were from patients in the 0-5 age band.

2.3.12 Duration of infection

Table 2.14 shows details of the duration of symptoms in 839 patients. Approximately 75% had symptoms for 2 weeks or less. However, 5% had symptoms of enteric infection for more than nine weeks, including 5 patients who reported intermittent diarrhoea and abdominal pain for 3-10 months.

Table 2.14. Duration of infection

Duration	Male	Female	Total
0-1w	203	178	381 (45.4%)
>1-2w	125	119	244 (29.1%)
>2-3w	44	30	74 (8.8%)
>3-4w	33	30	63 (7.5%)
>4-5w	2	3	5 (0.6%)
>5-6w	3	6	9 (1.1%)
>6-7w	1	-	1 (0.1%)
>7-8w	10	9	19 (2.3%)
>8-9w	-	1	1 (0.1%)
>9w	19	23	42 (5.0%)
Total	440	399	839 (100%)

2.4   DISCUSSION

Despite the description of a number of sporadic cases of infection with *Y. enterocolitica* and *Y. pseudotuberculosis*, little information was available in New Zealand on the epidemiological features of the most commonly recognised enteric syndrome when this present study was initiated. The objectives of the present study sought to define some of the relevant factors in order to gain an understanding of the role of these pathogens in this

country. Data gathered on the crude isolation rates of enteric pathogens at Diagnostic Laboratory, Auckland, one of the laboratories involved in the study, between 1987 and 1989, showed that while the annual isolation rates have fluctuated, the total isolation rates have remained relatively static. Within this group, *Y. enterocolitica* has consistently been the third most common enteric pathogen isolated after *Campylobacter* and *Salmonella* (McCarthy and Fenwick, 1991).

2.4.1 Isolation of *Yersinia* species

A number of species of *Yersinia* were identified during the study, the majority of which had been isolated from patients showing symptoms of enteric infection. The predominant species was *Y. enterocolitica*, approximately 97% of isolates belonging to this species, and this is in accord with similar studies carried out in Belgium (Van Noyen *et al.*, 1987a) and Canada (Noble *et al.*, 1987). Although only accounting for 2% of isolations, *Y. frederiksenii* was the second most common species recovered and this was also the case in the overseas surveys mentioned above. The remaining 1% of isolates consisted of *Y. kristensenii*, *Y. intermedia*, *Y. pseudotuberculosis* and *Y. rohdei*. With the exception of the latter species, these strains were also recovered in low numbers in Belgium and Canada.

While *Y. enterocolitica* and *Y. pseudotuberculosis* are both well-recognised enteric pathogens, the other species isolated in this study are all usually considered to be environmental in origin and non-pathogenic (Bottone, 1981). However, they were recovered from patients showing symptoms of enteric infection in the absence of any other recognised pathogen and their presence should be treated with caution. In fact, several reports of the involvement of environmental species of *Yersinia* in enteric infections have been recorded, including *Y. frederiksenii*, *Y. intermedia* and *Y. kristensenii*, and although they lack the virulence plasmid and associated virulence factors they are thought to be capable of causing disease under certain circumstances (Bottone and Robin, 1977; Bottone, 1978; Dabernat *et al.*, 1979; Butler *et al.*, 1984; Scholey and Freeman, 1984). In fact, Kay *et al.* (1983), stated that it was not possible to say that all strains of *Y. enterocolitica* are pathogenic for people and that *Y. intermedia*, *Y. kristensenii* and *Y. frederiksenii* are

non-pathogenic. Despite the absence of plasmid-encoded virulence factors, some authors have suggested that other factors not associated with the plasmid may play a role in the pathogenesis of human disease (Noble *et al.*, 1987). In 1991, Robins-Browne *et al.* described a strain of *Y. kristensenii* that caused a fatal infection in iron-loaded mice and suggested that some strains can kill mice by a mechanism not previously recognised in yersiniae, such as perhaps a novel secreted toxin.

The recovery of the newly described species *Y. rohdei* on one occasion during this study was the first isolation of this species in New Zealand. Overseas workers have isolated it from the faeces of healthy people, dogs and water, although as it does not carry a virulence plasmid it's significance as a potential human pathogen remains uncertain (Aleksic *et al.*, 1987).

2.4.2 Distribution of *Yersinia* species

The 43 laboratories participating in this study covered all regions of the country, allowing an assessment of the regional distribution of *Yersinia* species to be made. *Yersinia enterocolitica*, the most frequently isolated species, was recovered by all laboratories. As the other species of *Yersinia* had a relatively low isolation rate, it is difficult to identify any regional differences, nevertheless, it can be seen that, with the exception of *Y. rohdei*, all species were recovered from both the North and South Islands. Two observations can be made, firstly, all but one isolate of *Y. pseudotuberculosis* were recovered from hospital laboratories rather than community laboratories. This possibly reflects the severity of infection with this species, with a greater probability of hospitalisation. Secondly, with the exception of Diagnostic Laboratory, Auckland, the majority of environmental species of *Yersinia* were isolated from hospital laboratories. For example, 30 isolates of *Y. frederiksenii* were recovered from hospital laboratories, 28 from community laboratories, including 23 from Diagnostic Laboratory. One laboratory in particular, Christchurch Hospital, had a very high isolation rate of environmental species, including 25/58 isolates of *Y. frederiksenii* (43%), 2/8 *Y. kristensenii* (25%) and 3/4 *Y. intermedia* (75%). These findings are difficult to explain. It is conceivable that hospital laboratories are more

rigorous in their examination of stool samples, or that the lowered resistance of hospitalised patients predisposes them to infection with these less-pathogenic strains. In addition, the involvement of environmental strains of *Yersinia* in nosocomial infections has been documented previously (Bottone, 1978; Dabernat *et al.*, 1979).

2.4.3 Bioserotypes of *Y. enterocolitica* recovered

Bioserotype 4/O:3 was the commonest bioserotype combination recovered in the present study, accounting for 83% of isolates. This reflects the global situation, with bioserotype 4/O:3 being the most prevalent pathogenic strain in all countries where yersiniosis is endemic (Lee *et al.*, 1991). Subdivision of this bioserotype has historically been by phage typing, with certain phage types (PT) associated with specific regions, e. g. PT VIII with Europe and Japan, PT IXa with South Africa and PT IXb with Canada (Mair and Fox, 1986). Five New Zealand strains of bioserotype 4/O:3 were phage typed together with Australian strains at the Pasteur Institute, Paris, as part of a collaborative study, and were found to belong to PT IXb, the Canadian phage type (Pham *et al.*, 1995). As the phage types have been shown to be specific for certain regions, this suggests that the original source of reservoir animals for New Zealand, i.e. pigs, was likely to have been Canada or Australia, however, this has not been possible to corroborate.

The second most common bioserotype combination isolated in this study was bioserotype 2/O:9 (8.5%), despite this strain not being recognised prior to 1990 in animals or people in New Zealand. The global distribution of this bioserotype is uneven as it is common in Europe (Vandepitte and Wauters, 1979; de Groote *et al.*, 1982), less common in Japan (Fukushima *et al.*, 1985b) and is rarely reported from Canada (Swaminathan *et al.*, 1982). In the UK, 2/O:9 strains comprised 44% of all pathogenic strains typed in 1988 (Prentice *et al.*, 1991). No reports of infection with this strain have been recorded in Australia. Thus, the origin of bioserotype 2/O:9 strains in New Zealand is unclear and may lie in importation of the strain by infected people or reservoir animals.

Serotype O:5,27 is found in biotypes 1, 2 and 3 in different parts of the world, however, only strains belonging to biotypes 2 and 3 are considered pathogenic (Mair and Fox, 1986). In this study, strains of serotype O:5,27 belonged to biotype 2, characterised by a delayed positive indole reaction. Once again, this bioserotype combination appears to have an uneven geographical distribution, being relatively common in North America (Bissett, 1979), less common in Japan (Fukushima *et al.*, 1985b) and rare or absent in Europe (Van Noyen *et al.*, 1987a). While bioserotype 2/O:5,27 has not been reported from Australia, an isolate of bioserotype 3/O:5,27 was recovered from a case of fatal Gram-negative shock in 1986 (Elrick, 1988).

Only three isolates of bioserotype 3/O:1,2,3 were recovered during this study. This pathogenic bioserotype has been uncommonly associated with disease in many countries, including USA (Bissett, 1979) and Australia (Steele and McDermott, 1979).

Biotype 1A of *Y. enterocolitica* comprises a heterogeneous group of strains containing numerous different serotypes (Mair and Fox, 1986). These strains are usually considered environmental and non-pathogenic, as they are cultured more frequently from the stools of asymptomatic than symptomatic people and fail to induce a consistent immune response (Cover and Aber, 1989). However, there are numerous reports in the literature documenting their isolation from cases of enteric disease and their pathogenic potential has been debated at length (Greenwood and Hooper, 1987). Although there are many serotypes associated with this biotype, certain strains appear to predominate in reports of sporadic human infection, including O:5, O:6,30, O:6,31 and O:7,8, and these may have virulence factors that have not yet been identified (Marymont *et al.*, 1982; Ratnam *et al.*, 1982; McIntyre and Nnochiri, 1986; Noble *et al.*, 1987; Simmonds *et al.*, 1987). Morris *et al.*(1991), carried out a cohort study in Chilean children and found a group of biotype 1A strains, including O:6 and O:7,8, to be significantly associated with diarrhoea. They suggested that these strains have virulence mechanisms other than cell invasion and that our understanding of the nature of *Yersinia* pathogenesis is incomplete. Robins-Browne *et al.* (1993), further investigated these isolates and found that one of seven non-invasive biotype 1A strains, belonging to serotype O:6, produced a novel enterotoxin resembling that

produced by pathogenic strains of *Y. enterocolitica*. While the biotype 1A isolates recovered in the present study have not all been fully characterised, 36 out of 45 (80%) that were serotyped belonged to serotypes O:5, O:6,30, O:6,31 and O:7,8, the ones most frequently associated with enteric disease. As all biotype 1A strains were recovered from the stools of patients with gastroenteritis, in the absence of any other pathogens their involvement in the disease process cannot be ruled out. Further evidence for the pathogenic potential of bioserotype 1A/O:6,30 was demonstrated by Corbel *et al.* (1992), who recovered the strain from an aborted ovine foetus and produced abortion experimentally in a further four ewes following intravenous inoculation.

In July 1993, a cluster of atypical *Yersinia* strains was recovered from twelve patients in the Hawke's Bay region with symptoms of gastroenteritis, indistinguishable from those seen with yersiniosis attributable to bioserotype 4/O:3. Details of the cases were unobtainable, however, the isolates were identical to others recovered from domestic animals in the Manawatu region (see Chapter 4, section 4.3.6). The temporal association of the human infections may have possibly been a result of the contamination of either a common food source or a water supply by animal faeces. A collaborative project aimed at characterising these strains has shown that they belong to two new serotypes, O:77 and O:78, tentatively allocated to a new biotype of *Y. enterocolitica*, as yet undefined (Fenwick *et al*, 1996). The human strains isolated in the outbreak in Hawke's Bay all belonged to serotype O:78, which was also recovered from a sheep and a goat. The strains did not carry the *Yersinia* virulence plasmid and further work is being carried out to identify other potential virulence factors.

2.4.4 Distribution of bioserotypes of *Y. enterocolitica*

Regional distribution of *Y. enterocolitica* bioserotypes is widely recognised in many countries (Simmonds *et al.*, 1987; Cover and Aber, 1989). In the UK, for example, isolation rates are considerably higher in England and Wales than in Scotland and Ireland (Prentice *et al.*, 1991). Similarly, in Australia, yersiniosis is relatively common in Queensland and New South Wales but uncommon in Victoria and South Australia

(Marriott, 1987). However, in this study, all bioserotypes were recovered from both hospital and community laboratories throughout the North and South Islands but their distribution varied widely between laboratories. Eleven laboratories, consisting of six community and five hospital laboratories, contributed 94% of all study isolates and their isolation rates of *Y. enterocolitica* strains were compared. Results showed that overall, hospital laboratories had a lower isolation rate of 4/O:3 strains (66%) than community laboratories (86%) and a higher isolation rate of biotype 1A environmental strains (21% compared to 3%). The figures may be skewed by the exceptionally high isolation rate of biotype 1A strains at Christchurch hospital. Nevertheless, the observation is in agreement with the higher isolation rate of environmental species that was also noted for other hospital laboratories. The reasons for the differences remain speculative and similar reasons to those discussed for the distribution of environmental *Yersinia* species (section 2.4.2) could have contributed to the contrasting isolation rates. Nosocomial infections with biotype 1A strains have been recorded previously in Canada (Ratnam *et al.*, 1982), Hong Kong (Seto and Lau, 1984) and the UK (McIntyre and Nnochiri, 1986). Ratnam *et al.* (1982), described a nosocomial outbreak in Canada, involving biotype 1A, serotype O:5, and noted that the patients were either very young or old, predisposing them to infection with a relatively innocuous agent due to their age and physical state. A nosocomial outbreak of septicaemia involving biotype 1A (three strains were O:17 and one was untypable) was also described in a Hong Kong hospital by Seto and Lau in 1984. The three patients infected with serotype O:17 were all children in the same ward, however, the source of infection was not discovered. All four patients had an underlying illness, two with metastatic neoplasms and two with burns, emphasising the potential role of these less-pathogenic strains in immunocompromised patients. Another possibility could be that hospitalised patients are more likely to have been treated with antibiotics. An association of *Y. frederiksenii* with antibiotic-associated diarrhoea has been suggested previously (Scholey and Freeman, 1984).

Differences in the isolation rates of the other pathogenic bioserotypes, 2/O:5,27, 3/O:1,2,3 and 2/O:9 were also seen between laboratories, but no obvious patterns could be discerned. Bioserotype 2/O:5,27 strains were recovered from all but three of the aforementioned eleven laboratories, including both hospital and community laboratories. The comparative

isolation rates were 4.2% and 2.5% for hospital and community laboratories respectively. As this bioserotype is only sporadically isolated in most countries there are few reports available that document any differences in the epidemiology of infections. Bottone (1983), noted a marked increase in bacteraemic episodes due to serotype O:5,27 strains in New York, however, this was not a finding in the present study. Bioserotype 2/O:9 strains were recovered from all laboratories and isolation rates from hospital and community laboratories were very similar, 7.6% and 8.5% respectively. This bioserotype is commonly reported in Europe and no specific interaction with aged or hospitalised patients has been noted (Verhaegen *et al.*, 1991).

2.4.5   Annual incidence of *Y. enterocolitica*, 1988-1995

During the eight years of this study, a steady rise in the number of isolations of *Y. enterocolitica* was observed. This is consistent with the results of similar studies from other countries, including Belgium (Verhaegen *et al.*, 1991), USA (Bottone, 1983; Bissett *et al.*, 1990) and UK (Prentice *et al.*, 1991). In addition, a survey carried out in Belgium between 1963 and 1978 observed the occurrence of biannual peaks in the latter part of their study (de Groote *et al.*, 1982). Although biannual peaks are not specifically recognised in New Zealand, a decrease in the number of isolations was recognised in 1992, followed by a rise in 1993, and 1995. As in the other studies, there is no obvious explanation for the increased annual isolations, however, as the number of laboratories in the present study increased steadily over the eight-year period it is tempting to speculate that improved isolation methods and an increased awareness of the presence of the organism could be the reasons. Evidence to support the latter explanation is provided by a closer look at the two laboratories in Auckland and Hawke's Bay that consistently sent isolates and data during the study. These laboratories both used similar isolation methods for *Yersinia* and neither altered their protocols during the study. Despite the difference in the total number of isolations, it is obvious that both laboratories showed a steady increase in the annual number of isolations and this may have been due to an increased awareness of the problem by clinicians in their respective regions.

An interesting comparison can be made between the highest annual isolation rates in New Zealand and in other countries where yersiniosis is endemic. In New Zealand, the highest rate occurred in 1994, 548 isolations or 157 per million. Figures from the UK, Belgium and Australia show their highest rates as 3 per million, 298 per million and 28 per million respectively. These figures show that although Belgium has the highest rate in the world, the isolation rate in New Zealand is dramatically higher than in other Western countries. In fact, figures from Belgium show a steady decrease in the number of isolations between 1986 and 1989, due to precautions taken during the slaughter of pigs and campaigns to increase the awareness of the population (Verhaegen *et al.*, 1991), but until such initiatives have been taken in New Zealand a similar downward trend is not anticipated.

Although an increase in the total *Y. enterocolitica* isolations occurred during the study, a closer look at the different bioserotypes shows that this increase was not uniform. For example, the overall annual isolation of both biotype 1A and bioserotype 2/O:5,27 strains remained fairly constant throughout the eight years. A different picture is seen when the annual isolations of bioserotypes 4/O:3 and 2/O:9 are examined. A four-fold increase in both bioserotypes can be seen up until 1994, however, while the number of isolates of 4/O:3 dropped by 25% in 1995, isolates of 2/O:9 trebled, totalling 25% of the isolates in that year. This changing pattern of bioserotype distribution corresponds closely to that seen in Europe, with the proportion of 2/O:9 strains increasing from 0 to 44% in UK within 5 years (Prentice *et al.*, 1991) and from 7 to 23% in Belgium (Verhaegen *et al.*, 1991). The increase in 2/O:9 in Belgium, however, was a lot slower, taking 18 years, and following a peak in 1981 has since dropped back to the original level. Similar trends have also been seen in the USA, although with different bioserotypes, bioserotype IB/O:8 predominating until 1981, following which bioserotype 4/O:3 began to take over as the dominant strain (Bissett *et al.*, 1990).

Reasons for these changes in bioserotype distribution are not easily explained, although while discussing the trends of *Y. enterocolitica* isolates in New York, Bottone (1983), suggested that the emergence of strains of a particular serogroup in areas where none had existed previously indicated an ecological shift towards a new reservoir or vector. In UK

and the USA increases in 2/O:9 and 4/O:3 respectively have parallelled increases of these bioserotypes in pigs (Bottone, 1983; Prentice *et al.*, 1991), however, in New Zealand, 2/O:9 strains have never been isolated from this animal species. Nevertheless, as 2/O:9 isolations from domestic animals other than pigs have increased since 1991, it is probable that this bioserotype is a recent introduction to New Zealand and that it's low host specificity has allowed it to spread rapidly throughout the country. Fluctuations in the isolation of 4/O:3 strains, found commonly in New Zealand pigs, are more difficult to explain, but may have been influenced by factors such as changes in pork consumption or changes in the processing and marketing of pork products.

2.4.6 Seasonality of *Y. enterocolitica* isolations

The highest number of isolations during the eight years of the study was made in the summer months of December to February (743), although the peak incidence was seen in March, the first month of autumn. This was followed by a gradual decline through autumn and winter, with the lowest incidence being recorded in July, and an increase in spring. This is in contrast to the situation in many other parts of the world, where infections peak in the autumn and winter months (Swaminathan *et al.*, 1982; Cover and Aber, 1989). It is also at odds with figures published in 1995 from Diagnostic Laboratory, Auckland, showing that the mean isolation rate between 1988 and 1993 was higher in autumn and winter (Fenwick and McCarthy, 1995). Nevertheless, when figures for 1994 and 1995 from this laboratory are included, the seasonal distribution conforms to the overall figures recorded in this study, with the peak number of isolations being made in the summer months. The reasons for this are unclear. However, as both salmonellosis and campylobacteriosis also have summer peaks in New Zealand (Anonymous, 1992a; Anonymous, 1992b), it appears that certain undefined epidemiological factors involved in food-borne disease in this country, in particular with respect to yersiniosis, appear to differ from other countries.

2.4.7 Age and sex distribution of *Y. enterocolitica*

The age and sex distribution of patients infected with *Y. enterocolitica* show some similarities to infections with *Salmonella* and *Campylobacter* in New Zealand. Thus, infections appear to be more common in males than females and there is a distinct bimodal age distribution, with peaks in the 0-4 age group and in young adults between the ages of 20-34 (Anonymous, 1992b; Fraser, 1993). Within the 0-4 age group in the present study, infections were most common in infants between the ages of 1-3 (76%). A similar pattern of age and sex distribution of *Y. enterocolitica* infections is recognised in many countries where yersiniosis is endemic, for example, Japan (Fukushima *et al.*, 1985) and the USA (Lee *et al.*, 1991). Nevertheless, differences in the age distribution do exist in some countries, in the UK for instance, the majority of isolates are from patients over 20 (Prentice *et al.*, 1991) and in Belgium, *Y. enterocolitica* infection is primarily a disease of childhood, with 80% of infections in children 10 years and under (Verhaegen *et al.*, 1991).

The peak in children under 4 years probably reflects greater opportunities for faecal-oral transmission in this age group, although an increase in the numbers of stool cultures from young children may also introduce an element of bias to the figures. In the UK, a demographic survey of enteric infections showed that when a similar peak in young children was adjusted for the percentage of faecal specimens within each age group, infants had the lowest isolation rate and young adults the highest (Skirrow, 1987). As such information was not available, this remains conjectural in this study. However, the results of surveys from other countries have shown that enteritis due to *Y. enterocolitica* is more common in infants and young children (Metchock *et al.*, 1991), supporting the findings in the present study. Another hypothesis for the increased risk of infection in children is that they are more susceptible than adults. This was apparent during a large outbreak of *Y. enterocolitica* infection in the USA in 1984, where the age-specific attack rate was highest for children aged 0-9 years. Although it is also possible that the children had had an increased exposure to the source of infection, pasteurised milk, this was not apparent from the epidemiological survey performed (Tacket *et al.*, 1984).

The secondary peak observed in young adults has also been shown to occur in *Salmonella* and *Campylobacter* infections in this country and it has been suggested that it might be due to either increased contact with infants or to greater patronage of takeaway food establishments (Fenwick and McCarthy, 1995). Support for the theory that person-to-person spread within families contributes to the secondary peak can be found in a study carried out in Canada by Marks *et al.* (1980). They studied the spread of infection within 57 families following notification of an index case and found that transmission occurred in 27 families and approximately one-third of infected contacts developed diarrhoea. Several family outbreaks of infection have also been documented, including one in the USA where infection in a child was followed by sequential infection in a sibling and the child's mother (Martin *et al.*, 1982). Although such information was not sought during the present study, on three occasions the occurrence of infections in family members were recorded by collaborating doctors.

The predominance of males over females is not apparent in the 0-4 age group, in contrast to older children and adults in this study, indicating that *Yersinia* infections may not be sex-related *per se*, but that other as yet unknown variables such as a difference in eating habits or lifestyle may predispose to the disease.

When the distribution of the bioserotypes of *Y. enterocolitica* is examined, it is apparent that the highest number of isolates of all bioserotypes occurs in the 0-4 age group and that a bimodal distribution is also common to all bioserotypes except 2/O:5,27. The reason for the lack of a secondary peak of infection with bioserotype 2/O:5,27 is unknown, but may lie in the relatively small number of isolates of this strain recovered. A closer look at the distribution of biotype 1A strains shows that a small tertiary peak exists in patients over 75 years and that a higher proportion of these strains were isolated from this group than the other, more pathogenic, bioserotypes. A similar pattern has been noted in Belgium where *Yersinia* infections other than 4/O:3 and 2/O:9 are characterised by the absence of a typical age, sex and seasonal pattern (Verhaegen *et al.*, 1991). This may reflect an increased susceptibility to these less-pathogenic strains in older people or that their increased likelihood of hospitalisation leads to a greater risk of nosocomial infections, or a

combination of both factors. Evidence to support this is found in the description of a nosocomial outbreak of bioserotype 1A/O:5 infections in a hospital in Canada, where the patients involved in the outbreak were either very young or old, the authors suggesting that they were predisposed to infection with this innocuous strain by their age and physical state (Ratnam *et al.*, 1982). The description of another case of nosocomial infection with bioserotype 1A/O:6,30 in an 81-year-old diabetic patient in the UK adds credibility to the theory that older patients, whose health is otherwise compromised, have an increased risk of infection with environmental yersiniae (McIntyre and Nnochiri, 1986).

2.4.8   Age, sex and symptoms of patients with *Y. pseudotuberculosis* infections

Y. pseudotuberculosis was an uncommon cause of infection during the present study, being isolated on only 12 occasions. However, the relative severity of the disease caused by this organism prompts a closer look at some of it's features. The rarity of infections caused by this organism, in spite of widespread infection of farm animals in New Zealand (Hodges *et al.*, 1984a), is difficult to account for, nevertheless, as abdominal pain is reported to be the predominant symptom and diarrhoea is believed to be uncommon, under diagnosis may be considerable (Tertti *et al.*, 1989). In addition, during the study of the clinical manifestations of *Y. pseudotuberculosis* in Finnish children carried out by Tertti *et al.* in 1989, many infections were found to be subclinical and individual strains were thought to have differing capacities to cause post-infection complications. Of the 12 cases reported in this study, all but one were from hospitalised patients, 7 of whom were operated on for suspected appendicitis. Of the other 5, 4 had classical, but severe, gastroenteritis and one had pneumonia. All patients with the exception of the one with pneumonia were either children or young adults, and males outnumbered females 5:1.

The association with mesenteric lymphadenitis and appendectomy has been reported on many occasions, including a review of 70 cases of *Y. pseudotuberculosis* infection in England where all but one were diagnosed as having appendicitis before surgery (El Maraghi and Mair, 1979). A further study carried out in Ireland also found that *Y. pseudotuberculosis* was five times more likely to be associated with this syndrome than *Y.*

enterocolitica (Attwood *et al.*, 1987). This is in agreement with the present study where only 2/9 cases of mesenteric lymphadenitis involved *Y. enterocolitica*. The age and sex distribution of the New Zealand cases of *Y. pseudotuberculosis* is identical to that reported in a review by Bottone in 1981. He stated that the acute abdominal syndrome usually affects male children and young adults between the ages of 2-24. The reasons for this are not understood.

From the 12 cases of *Y. pseudotuberculosis* reported in the present study, serotype II was the predominant strain isolated (10 cases), while serotype I was only recovered on two occasions. Both serotypes I and II are recognised worldwide as human pathogens, with serotype I reported to account for over 90% of human infections in Europe (Bottone, 1981). Serotypes I, II and III have all been isolated from cattle and deer in New Zealand, however, I and III are more common than II (Hodges *et al.*, 1984a). Serotypes I and II have been recovered from cage birds, with serotype II being the most common strain isolated (Henderson, 1983a). In addition, cats have also been found to be infected with serotype II (see Chapter 4), possibly from eating infected birds. Thus, the isolation of serotype II from the majority of human cases may indicate that infections with *Y. pseudotuberculosis* are not principally food-borne in New Zealand but are associated with close contact with infected cats and birds. The suggestion that the epidemiology of infections with *Y. enterocolitica* and *Y. pseudotuberculosis* are different has also been proposed by Schiemann (1989), who noted that evidence for food-borne spread of *Y. pseudotuberculosis* is lacking, with only one outbreak of infection with this organism recorded and the source was unknown.

2.4.9 Clinical symptoms of infection with *Yersinia* spp.

The majority of patients from whom clinical details were available presented with the classical symptoms of gastroenteritis, namely diarrhoea, with or without abdominal pain. This is the most common syndrome reported in many studies from other countries (Snyder *et al.*, 1982; Vantrappen *et al.*, 1982; Bottone *et al.*, 1987). No association was observed between symptoms and bioserotype or species isolated, even where strains did not belong

to the principal pathogenic serotypes of *Y. enterocolitica*. Simmonds *et al.* (1987), also noted similar symptoms in 123 patients, many of whom were infected with *Y. enterocolitica* biotype 1A or *Y. frederiksenii*. On the other hand, de Groote *et al.* (1982) reported that symptoms of gastroenteritis were only recognised when *Y. enterocolitica* bioserotypes 4/O:3 and 2/O:9 were involved, and that biotype 1A strains were more often found in patients with atypical complaints or in individuals without overt disease. The occurrence of fever, which was reported in 10% of New Zealand patients, varies widely in other studies, from 10% (Simmonds *et al.*, 1987) to 43% (Snyder *et al.*, 1982).

The other major syndrome associated with *Yersinia* infections is the pseudo-appendicular syndrome, where patients present with abdominal pain alone, without diarrhoea. Studies from Belgium have reported this syndrome in 10-20% of patients (Vandepitte and Wauters, 1979; Vantrappen *et al.*, 1982), and the situation appears to be the same in New Zealand, with 18% of patients presenting only with abdominal pain. Ten patients out of the 239 in this group (4%) underwent surgery for suspected appendicitis in accordance with a survey in Belgium where 4% of patients also suffered unnecessary surgery (Vandepitte and Wauters, 1979).

While details of other symptoms were not specifically requested in this study, vomiting, bloody stools, pharyngitis and reactive arthritis were all recorded on a few occasions. In contrast, vomiting and bloody diarrhoea were reported to occur frequently in two studies in the USA, both involving *Y. enterocolitica* 4/O:3 (Bottone *et al.*, 1987; Lee *et al.*, 1991). Pharyngitis due to *Y. enterocolitica* infection has been reported on a number of occasions to have preceded the development of the more common enteric symptoms of diarrhoea and abdominal pain, but is probably often overlooked or even misdiagnosed as either streptococcal or viral pharyngitis (Tacket *et al.*, 1983; Rose *et al.*, 1987; Lello and Lennon, 1992). This affinity for the pharyngeal region is also demonstrated in pigs as *Y. enterocolitica* is regularly recovered from the oropharynx and tonsils (Andersen *et al.*, 1991) and it is interesting to speculate whether such tonsillar carriage occurs following human infection with the organism, and whether recovered carriers can act as a potential source of infection for those in close contact. A minority of patients (2%) presented only

with symptoms of general malaise, a manifestation of the disease also occasionally reported overseas (Snyder *et al.*, 1982).

Diagnostically, the predominant specimen submitted for culture was a stool sample, reflecting the fact that most patients presented with symptoms of uncomplicated gastrointestinal infection. Nevertheless, 1.5% of all specimens were from cases of extra-intestinal infection, including mesenteric lymphadenitis, septicaemia, liver abscesses and wound infections. All *Yersinia* isolates from extra-intestinal infections came from hospitalised patients. Cases of mesenteric lymphadenitis, diagnosed at surgery, all occurred in children and young adults between the ages of 5 and 24, with 7 out of 10 involving *Y. pseudotuberculosis* and the other three involving *Y. enterocolitica* bioserotype 4/O:3. This is in agreement with a study from Ireland where *Y. pseudotuberculosis* was five times more common than *Y. enterocolitica* (Attwood *et al.*, 1987), but is in contrast to studies in Scandinavia where *Y. enterocolitica* bioserotypes 4/O:3 and 2/O:9 were more frequently recovered from cases of acute surgical abdominal disease (Jepsen *et al.*, 1976; Saebo, 1983). In all but one case, mesenteric lymph nodes were the specimen submitted for culture. The other specimen was pus from an appendicectomy wound post-surgery.

Sepsis caused by *Yersinia* spp. is extremely uncommon, however, it is associated with very high mortality, often in older patients with other underlying diseases such as liver disease, tumours or diabetes (Bouza *et al.*, 1980; Foberg *et al.*, 1986). In this study, 28 patients were diagnosed with *Yersinia* septicaemia, with 20 (62%) being over 50 years old and 11 (40%) over 70. The majority of cases involved *Y. enterocolitica* 4/O:3, but bioserotypes 2/O:5,27, 2/O:9 and *Y. pseudotuberculosis* were also recovered. No environmental yersiniae were recovered from extra-intestinal infections. Of the 8 patients under 50, no details were available for two, one had lymphoma, one was a haemophiliac and one was diagnosed post-splenectomy. The remaining 3 were all recipients of *Yersinia*-infected blood and 2 died. Transfusion-related septicaemia has been reported previously in New Zealand (Ulyatt *et al.*, 1991; Wilkinson *et al.*, 1991) and overseas (Jacobs *et al.*, 1989) and asymptomatic bacteraemia in the donor is recognised as the source of contamination. To support this, 2 isolates of *Y. enterocolitica* recovered from donor blood involved in post-

transfusion septicaemia were shown to be the same bioserotype as those recovered from the respective patients. Details of the 20 older patients in this study show that one had pneumonia and lung abscesses, two had tumours, one had liver disease, one had heart disease and one had urinary tract infection. The other 14 had no history of underlying disease.

Metastatic abscesses, often in the liver, and soft tissue infections, are also reported as infrequent sequelae following *Yersinia* infections (Rabson *et al.*, 1972; Bottone *et al.*, 1987). In this study, only 3 infections fell into this category, two of them in patients over 70, one with hepatic abscesses and the other with osteomyelitis and a draining sinus. The third (younger) patient had an infected wound following appendectomy, as described above.

2.4.10 Duration of infection

In this study, the duration of infection was in most cases less than 2 weeks, in agreement with studies from other countries (Snyder *et al.*, 1982; Simmonds *et al.*, 1987). Nevertheless, chronic *Yersinia* infections are well-documented to last for up to several months (Cover and Aber, 1989) and this was borne out in the patients investigated in New Zealand, with 5% reporting that symptoms had lasted for 2-10 months. No differences were noted in duration of illness by age group, bioserotype or *Yersinia* spp. Hoogkamp-Korstanje *et al.* (1988), described ten patients with chronic *Y. enterocolitica* infections, where the organisms persisted in the body for several years. The patients ranged in age from 6 to 73 and suffered from periodic arthritis, lymphadenitis, ileitis, proctitis and hepatitis. Saebo and Lassen (1992), also investigated patients with chronic *Yersinia* infection and found that this condition exerted a substantial impact on long-term survival. No such follow-up studies have been performed in New Zealand, however, further investigation of chronic infections in this country is warranted.

CHAPTER 3

3. INVESTIGATION OF THE PREVALENCE OF YERSINIAE IN PIGS AND PIG PRODUCTS

3.1 INTRODUCTION

Yersinia enterocolitica is considered to be a foodborne pathogen and foods of animal origin appear to constitute the principal vehicle of human infection (Morris and Feeley, 1976; Kapperud, 1991; de Boer, 1992). The animal kingdom is, therefore, widely regarded as a major reservoir of human pathogenic yersiniae and a number of animal species have been investigated as possible sources of infection (Swaminathan *et al.*, 1982). The results of studies from many parts of the world have shown that while other domestic and wild animals can occasionally carry strains of *Y. enterocolitica* potentially pathogenic for people, such strains are more commonly isolated from pigs, implying that this animal constitutes the most important reservoir for human infections (Mair, 1973; Asakawa *et al.*, 1979; Shayegani *et al.*, 1981; Zen-Yoji, 1981; Christensen, 1987b; Adesiyun *et al.*, 1992).

Prior to this study, *Y. enterocolitica* had never been isolated from pigs in New Zealand, although human infections had been described sporadically since 1979 (Fallon, 1979). In Australia, two studies, in 1977 (Blackall, 1977) and 1979 (Steele and McDermott, 1979), failed to isolate *Y. enterocolitica* from the gastrointestinal tract of pigs. In a later survey, Krautil (1988) also failed to recover the organism from the surface of pig carcases in Victoria, however, bioserotype 4/O:3 was isolated from 13 of 22 pig tonsils sampled using cold-enrichment. This was the first evidence of this common human pathogenic strain in Australian pigs, although it had been recorded in people since 1979 (Steele and McDermott, 1979). Subsequently, Ormerod *et al.* (1993) reported the isolation of *Y. enterocolitica* bioserotype 4/O:3 from pigs in South Australia. Allozyme analysis and ribotyping were

used to compare the porcine isolates with recently recovered human strains and no differences were seen.

As the incidence of human infection with *Y. enterocolitica* bioserotype 4/O:3 appeared to be increasing in New Zealand, investigation of the role of pigs as a source of infection for people in this country was considered to be a priority. The aims of this part of the project, therefore, were three-fold: firstly, to estimate the prevalence of pathogenic yersiniae in slaughtered pigs; secondly, to examine retail pork products for the presence of pathogenic yersiniae; thirdly, to compare any *Yersinia* isolates recovered from pigs with human isolates in a range of virulence assays.

3.2   MATERIALS AND METHODS

3.2.1   Samples

Samples were collected from 200 bacon-weight pigs over a 12-month period at a local pig abattoir, Kiwi Bacon, Longburn. Tonsillar samples were collected monthly, 15-20 animals being sampled at each visit. Both palatine tonsils were excised from each animal using sterile scissors and forceps and were subsequently placed into individual plastic bags on ice for transport back to the laboratory.

For the second part of this investigation, 5 local supermarkets were visited on 7 occasions at 3-weekly intervals and samples of chilled pork mince and diced pork were purchased at each time.

All samples were processed within 4 hours of being obtained.

3.2.2 Initial processing

Both tonsils were cut up into small pieces using sterile scissors and forceps, the pieces were

put into plastic bags with 225mls of M/15 phosphate buffered saline, pH 7.6, (PBS) and were homogenised for 5 minutes in a Colworth Stomacher (A. J. Seward, London, England) (Mair and Fox, 1986).

Similarly, the pork samples were also cut up into small pieces and 25g portions were placed in a plastic bag with 225mls PBS before homogenising for 5 minutes as described above.

3.2.3 Isolation methods

All samples were subjected to a two-step enrichment procedure as described by Nesbakken and Kapperud, 1985. The homogenates in PBS were incubated at 4°C for 8 days after which 0.1ml of each was transferred to 10mls of Modified Rappaport Broth (MRB). The PBS was then incubated for a further 13 days at 4°C while the MRB was incubated at room temperature for 4 days. Following the two enrichment steps, aliquots of each broth culture were subcultured onto CIN, MacConkey and LSU (Lactose-Sucrose-Urea agar) and incubated at 27°C for 24-48 hours. Plates were examined and colonies suspected of being *Yersinia* spp. were subjected to a screening procedure, described below. For further details of the tests and media used see Appendix 2.

A slight variation to the original protocol was used for the pork samples, the initial enrichment being in PBS, supplemented with 1% sorbitol and 0.15% bile salts, as suggested by Nesbakken *et al.*, 1985. Following the two enrichments, the homogenates were plated onto CIN alone as this had been the most sensitive plating medium in the previous survey.

3.2.4 Screening and identification protocols

Colonies (2-3) showing the phenotypic characteristics typical of yersiniae were picked off each plate, inoculated into tryptone water (TW) and incubated at 27°C overnight. The TW was used to inoculate Triple Sugar Iron agar (TSI), Lysine Iron Agar (LIA) and Urea slopes. These were incubated at 27°C for 24 hours and cultures showing reactions typical

of *Yersinia* spp. were retained for a full range of identifying tests (adapted from - Bottone, 1977; Farmer *et al.*, 1985; Mair and Fox, 1986; Schiemann, 1989). For details of the tests and media used see Appendix 2.

Cultures identified as *Y. enterocolitica* were biotyped according to Wauters *et al.*, 1987 and serotyped using commercial antisera (Denka-Seiken, Japan). Cultures identified as *Y. pseudotuberculosis* were serotyped using commercial antisera (Denka-Seiken, Japan). All cultures were stored at -70°C in 15% glycerol broth according to the method of Park (1976), until required for further testing.

3.2.5 *In-vitro* virulence testing

All strains of *Yersinia enterocolitica* isolated from pig tonsils (biotypes 1A, 2 and 4) and representative isolates of all other *Yersinia* strains recovered (*Y. pseudotuberculosis* II and III, *Y. kristensenii* and *Y. intermedia*) were subjected to the following *in-vitro* virulence assays -

(i) Autoagglutination test

The autoagglutination test was carried out using modified minimum essential Eagle medium (MEM, Flow Laboratories, U. K) with 10% bovine foetal serum as described by Schiemann and Fleming (1981).

Two sterile tubes containing 2mls of the medium were inoculated with a single colony of each *Yersinia* strain grown on trypticase soy agar (TSA) plates for 48 hours at 25°C. One of the tubes was incubated at 25°C, the other at 37°C, for 18 hours.

Positive strains showed a clear medium and clumping at the bottom and sides of the tube incubated at 37°C, which remained clumped when the tubes were shaken. A uniform turbidity with some settling at the bottom of the tube but no evidence of clumping was seen in the tube incubated at 25°C. Negative strains exhibited the uniform growth without clumping at both temperatures.

(ii)   Calcium dependency at 37°C

Virulence plasmid-bearing *Yersinia* spp. grown at 37°C have a nutritional requirement for calcium expressed by pinpoint growth on a medium with a low concentration of calcium (Mair and Fox, 1986). Calcium dependency was first shown by Higuchi and Smith (1961) in *Y. pestis* grown on Magnesium Oxalate agar (MOX) and this agar was adapted as a virulence test for *Y. enterocolitica* by Gemski *et al.* (1980).

Single colonies from the test strains of *Yersinia* were streaked onto MOX and incubated for 18-24 hours at 37°C. Calcium dependent strains were seen as pinpoint colonies on the plates, often together with large colonies, whereas non-calcium dependent strains grew as large colonies only.

(iii)   Combined test for calcium dependency and congo red binding (CRMOX)

The binding of congo red by virulent, plasmid-bearing *Y. pestis* was described by Surgalla and Beesley (1969), and was adapted as a virulence test for *Y. enterocolitica* by Prpic *et al.* (1983). For simplicity, Riley and Toma (1989) formulated congo red-magnesium oxalate agar (CRMOX), combining the tests for congo red binding and calcium dependency in one agar plate.

Single colonies from the test strains of *Yersinia* were streaked onto CRMOX agar plates and the plates were incubated for 18-24 hours at 37°C. CRMOX-positive strains produced small, red colonies and larger, pale colonies. CRMOX-negative strains produced only large, pale colonies.

(iv)   Growth on VYE agar

VYE agar is a selective agar medium for the isolation of virulent *Y. enterocolitica* developed by Fukushima (1987). The medium contains aesculin to differentiate avirulent strains that hydrolyse aesculin from virulent strains that do not. Single colonies from each

of the test strains were streaked on the plates and these were incubated at 27°C for 18-24 hours.

Virulent serotypes of *Y. enterocolitica* formed red colonies while other environmental *Yersinia* formed dark red colonies surrounded by a dark zone as a result of aesculin hydrolysis.

(v)   Crystal violet binding assay

The ability of virulent *Y. enterocolitica* to bind crystal violet was first reported by Bhaduri *et al*. (1987), who found that the characteristic correlated fully with the presence of the virulence plasmid. The method was later evaluated successfully by Robins-Browne *et al*. (1989).

Single colonies from each of the test strains of *Yersinia* were streaked onto Brain Heart Infusion agar plates (BHI) and were incubated at 37°C for 18-24 hours. Plates were then flooded with 8mls of an 85μg/ml solution of crystal violet (Difco Laboratories, USA) for 4-5 minutes and decanted. Virulent strains of *Yersinia* bind the dye giving dark violet colonies, whereas avirulent strains are white.

(vi)   Pyrazinamidase test

The pyrazinamidase test (PYZ) was used successfully by Kandolo and Wauters (1985) to differentiate virulent from environmental strains of *Yersinia*. Pyrazinamidase activity was shown to be detectable only in environmental strains and unlike most other virulence tests is not reliant on the presence of a virulence plasmid. The tris-maleate buffer described in the method was found to be inhibitory and the concentration was reduced from 0.2M to 0.02M.

Pyrazinamidase test agar slants were inoculated with overnight broth cultures (in TW) of each of the test strains and were incubated for 48 hours at 27°C. 1ml of freshly prepared

aqueous ferrous ammonium sulphate solution was then poured over the slant and left for 15 minutes. Pink-brown colouration on the slant indicated the presence of pyrazinoic acid and PYZ+ strains. Slants of PYZ- strains remained colourless.

(vii)   Aesculin hydrolysis/salicin fermentation

The ability to hydrolyse aesculin and ferment salicin have been shown to be associated with avirulent, environmental strains of *Yersinia* (Schiemann *et al.*, 1981; Riley and Toma, 1989) and like the PYZ test do not appear to be reliant on the presence of a virulence plasmid.

Overnight broth cultures of each of the test strains were inoculated onto aesculin slopes and into salicin tubes. Both tests were incubated at 27°C for 18-24 hours. Positive tests showing blackening of the aesculin slopes and an indicator change to pink in the salicin tubes were indicative of avirulent, environmental strains of *Yersinia*.

3.3   RESULTS

3.3.1   Farm distribution of yersiniae

200 tonsillar samples were cultured from 22 farms, all of which were situated in the provinces of Manawatu, Horowhenua and Wairarapa in the lower half of the North Island. Table 1 shows the species and strains of *Yersinia* isolated from each farm. Only 3 farms were negative for *Yersinia* spp., however, only 1-3 animals were sampled from each of these farms. Of the others, 10 farms were positive for one strain only (*Y. enterocolitica* biotype 1A, 2 farms; bioserotype 2/O:5,27, 4 farms; bioserotype 4/O:3, 1 farm; *Y. pseudotuberculosis*, 3 farms), while the remaining 9 farms had one or more strains isolated.

3.3.2 *Yersinia* spp. isolation rates

No difference in the recovery rates was noted between the 2 enrichment methods used. Both CIN and LSU agar plates allowed the recovery of an equal number of strains, however, the presence of contaminating microflora was greater on LSU plates.

Of the 200 pigs sampled, 122 (61%) were positive for *Yersinia* spp. Of these, 57 (28.5%) were positive for *Y. enterocolitica* and 64 (32%) were positive for *Y. pseudotuberculosis*. Other species of *Yersinia* were recovered from only 3 animals (1.5%) and these included *Y. intermedia* and *Y. kristensenii*. Two animals had 2 strains of *Yersinia* isolated simultaneously, one had *Y. pseudotuberculosis* and *Y. enterocolitica* biotype 1A, the other had *Y. enterocolitica* bioserotype 2/O:5,27 and *Y. intermedia*.

Bioserotyping of the *Y. enterocolitica* strains recovered showed that 25 pigs carried bioserotype 4/O:3 (12.5% of pigs sampled, 43.9% of total *Y. enterocolitica* recovered); 23 pigs carried bioserotype 2/O:5,27 (11.5% of pigs sampled, 40.3% of total *Y. enterocolitica* recovered); 9 pigs carried biotype 1A strains (4.5% of pigs sampled, 15.8% of total *Y. enterocolitica* recovered). The biotype 1A strains belonged to a number of serotypes: O:5 (2), O:6,30 (4), O:7,8 (1) and O:15 (2). Table 3.1 shows the distribution of the pathogenic bioserotypes isolated.

Of the 64 *Y. pseudotuberculosis* strains isolated, 7 (10.9%) belonged to serogroup IIb and 57 (89.1%) to serogroup III. Table 3.1 shows the distribution of the serotypes isolated.

Table 3.1.   Farm distribution of *Yersinia* species and strains

Farm No.	No. tested	*Yersinia* species and bioserotypes isolated							% pigs positive
		Ye1	Ye2	Ye4	YpII	YpIII	Yi	Yk	
1	5					5			100
2	1					1			100
3	16	2	1		1	10			87.5
4	16		7			2			56.3
5	26		1			12			50
6	13	1				7			61.5
7	15			9		3			80
8	14	3*	1#	1	3*		1#	1	57.1
9	17			·15					88.2
10	2	1							50
11	1		1						100
12	1								0
13	13		1						7.7
14	19		9			8			89.5
15	3								0
16	4	1							25
17	1		1						100
18	1								0
19	17	1				1			11.8
20	1		1						100
21	1					1			100
22	13				3	7		1	84.6
Total	200	9	23	25	7	57	1	2	
%	100	4.5	11.5	12.5	3.5	28.5	0.5	1	62

Ye1 = *Y. enterocolitica*, biotype 1A; Ye2 = *Y. enterocolitica*, bioserotype 2/O:5,27; Ye4 = *Y. enterocolitica*, bioserotype 4/O:3; YpII = *Y. pseudotuberculosis* serogroup II; YpIII = *Y. pseudotuberculosis* serogroup III; Yi = *Y. intermedia*; Yk = *Y. kristensenii*; *# = simultaneous recovery of isolates from one pig.

3.3.3 *Yersinia* isolations from pork products

Table 3.2 shows the *Yersinia* spp. isolated from diced pork and pork mince on 7 occasions, from 5 local supermarkets. All but 3 of the isolates were environmental strains of *Y. enterocolitica* biotype 1A, *Y. intermedia* or *Y. kristensenii*. Three isolates belonged to *Y. enterocolitica* biotype 3 but were untypable with the available antisera. As they were salicin, aesculin and pyrazinamidase negative they are potentially virulent, however, they do not belong to any recognised human pathogenic serotype and are being further investigated in collaboration with a *Yersinia* reference laboratory in Belgium. Of the 70 products tested, 26 (37.1%) were positive. Of these, 12 were diced pork (46.2%), 14 were pork mince (53.8%). On two occasions no *Yersinia* spp. were isolated from any product tested, nevertheless, pork products from all supermarkets were positive on at least one occasion (range 3-7).

Table 3.2. *Yersinia* strains isolated from pork products

Product	Sample number							Total +ve
	1	2	3	4	5	6	7	
1D	Ye3	-	-	-	-	-	-	1
1M	Yel/Yi	Yi	-	Yel	-	-	Yk	4
2D	Yel/Yi	-	Yel	Yel/Yk	-	-	-	3
2M	Yel	Yi	-	Yi	-	-	-	3
3D	Yk	-	-	-	-	-	-	1
3M	Yi	Yel/Yi	-	-	-	-	-	2
4D	Yel	-	-	Yel	-	-	Yk	3
4M	Yi	Yi	Yel	Yel/Yk	-	-	-	4
5D	Yi	Ye3	Ye3	Yel	-	-	-	4
5M	-	Yi	-	-	-	-	-	1
TOTAL	9	6	3	6	0	0	2	26

D = Diced pork; M = Pork mince; Yel = *Y. enterocolitica*, biotype 1A;

Ye3 = *Y. enterocolitica*, biotype 3; Yi = *Y. intermedia*; Yk = *Y. kristensenii*

3.3.4 Virulence testing of *Yersinia* spp. isolated from pigs' tonsils

Table 3.3 shows the results of the virulence tests performed on the porcine isolates of *Yersinia enterocolitica* and representative strains of the other *Yersinia* spp. isolated. The majority of the potentially pathogenic strains, including *Y. enterocolitica* bioserotypes 2/O:5,27 and 4/O:3 and *Y. pseudotuberculosis* serogroups II and III, were positive in the CRMOX assay, autoagglutinated at 37°C only, absorbed crystal violet and had predominantly small colonies on MOX agar. Nevertheless, a proportion of *Y. enterocolitica* bioserotype 4/O:3 strains (20%) behaved atypically and were negative in these four assays.

Results were more consistent on the VYE test agar, where *Y. enterocolitica* bioserotype 2/O:5,27 and 4 /O:3 strains were aesculin negative, however, *Y. pseudotuberculosis* strains were positive for this characteristic. All potentially pathogenic yersiniae were negative for pyrazinamidase production, aesculin hydrolysis and salicin fermentation.

Table 3.3. Virulence test results for the porcine *Yersinia* isolates

Strain	No. tested	CRMOX		Autoagglutination		VYE agar		CV absorp.	MOX small cols.	PYZ
		small cols.	CR uptake	25°C	37°C	growth	aesc. hydrol.			
Ye4	25	+/-	+/-	-	+/-	+	-	+/-	+/-	-
Ye2	23	+	+	-	+	+	-	+	+	-
Ye1	9	-	-	-	-	+	+	-	-	+
YpII	7	+	+	-	+	+	+	+	+	-
YpIII	5	+	+	-	+	+	+	+	+	-
Yk	2	-	-	-	-	+	+/-	-	-	+
Yi	1	-	-	-	-	+	+	-	-	+

+ = positive in the test; - = negative in the test; +/- = strain variation in the test; small cols. = pinpoint growth; CR uptake = absorption of congo red; aesc. hydrol. = aesculin hydrolysis; CV absorp. = crystal violet absorption by colonies.

All other *Yersinia* strains, including *Y. enterocolitica* biotype 1A, *Y. kristensenii* and *Y. intermedia*, were negative in the CRMOX assay, did not autoagglutinate at 37°C, did not take up crystal violet, grew as large colonies only on MOX agar and were pyrazinamidase, aesculin and salicin positive. Apart from one strain of *Y. kristensenii*, all were also aesculin positive on VYE agar.

3.4   DISCUSSION

3.4.1   Isolation of yersiniae from pigs' tonsils and significance for human infections

Surveys conducted in many countries have demonstrated that potentially pathogenic strains of *Y. enterocolitica* can be readily isolated from pigs, in particular from the oral cavity. These include Denmark (Christensen, 1980; Andersen *et al.*, 1991), Norway (Nesbakken and Kapperud, 1985), Finland (Asplund *et al.*, 1990; Merilahti-Palo *et al.*, 1991), Holland (de Boer *et al.*, 1986; de Boer and Nouws, 1991), Belgium (Wauters, 1979), Canada (Toma and Deidrick, 1975; Schiemann and Fleming, 1981), USA (Hanna *et al.*, 1980; Doyle *et al.*, 1981; Harmon *et al.*, 1984; Kotula and Sharar, 1993), Spain (Trallero *et al.*, 1988), UK (Hunter *et al.*, 1983), Trinidad (Adesiyun and Krishnan, 1995), Japan (Fukushima *et al.*, 1990) and Nigeria (Okoroafor *et al.*, 1988). Furthermore, the bioserotypes isolated from pigs in these regions invariably reflect the predominant strains isolated locally from people (Wauters, 1979; Schiemann and Fleming, 1981; de Boer and Nouws, 1991). While not incontrovertible, the evidence nevertheless appears to point strongly to the role of pigs as reservoirs of human pathogenic *Y. enterocolitica*. Despite the increasing incidence of human *Y. enterocolitica* infections in New Zealand, however, pigs had not been identified as a possible source of infection prior to this study. In Australia, two studies have examined pigs for the presence of *Y. enterocolitica*, the first, undertaken in Queensland in 1977 failed to isolate the organism (Blackall, 1977), but in 1993 strains of serotype O:3 were isolated from pigs in South Australia following a marked increase in the incidence of human infections with the corresponding strain (Ormerod *et al.*, 1993).

The prevalence of pathogenic strains of *Y. enterocolitica* recovered from the oral cavity of pigs in other countries ranges from 0% in the UK (Hunter *et al.*, 1983) to 62.5% in Belgium (Wauters, 1979). The prevalence of 24% in this study is slightly lower than that recorded in Norway (31.7%, Nesbakken and Kapperud, 1985) and Denmark (30%, Pedersen, 1979; 26%, Christensen, 1980; 25%, Andersen *et al.*, 1991), both of which are countries where human yersiniosis is common and considered to be a major public health problem. The reported incidence of 2000 human cases per year in Denmark (Andersen, 1988) suggests that a similar situation probably exists in New Zealand and that the disease in this country is seriously under-reported at the present time (Fenwick and McCarthy, 1995). For comparison, *Salmonella* infections in New Zealand have remained stable at approximately 1500 cases per year from 1988-1993 (Fraser, 1993).

The prevalence of *Y. enterocolitica* bioserotype 4/O:3 from pigs in this study was lower than that recorded in Norway and Denmark, where it is the predominant strain, and in some cases the only strain, recovered. Nevertheless, it's recovery identifies pigs as the probable source of infection for people in New Zealand, in agreement with overseas studies (Swaminathan *et al.*, 1982). A later survey carried out at the same abattoir found this bioserotype in 34% of pigs examined (De Allie, 1994) and the reasons for the lower prevalence in the present study are unclear, although it could reflect an uneven farm distribution in this region, as has been reported in Denmark (Christensen, 1980). Another possibility is that this strain had only recently been introduced into New Zealand pigs, which would be consistent with the low number of human cases recorded prior to this study and the subsequent steady increase.

Anomalies exist, however, in the epidemiology of the other potentially pathogenic *Yersinia* strains isolated in this study. For instance, *Y. enterocolitica* bioserotype 2/O:5,27 constitutes only a small proportion of the human cases of yersiniosis in this country (Fenwick and McCarthy, 1995) but was recovered at a relatively high rate (11.5%) and from more farms than bioserotype 4/O:3 (9 out of 22, 40.9%, as compared with 3 out of 22, 13.6%). While most other countries also record a low incidence of human infection with this bioserotype, the recovery rate from pigs is also low (Swaminathan *et al.*, 1982;

de Boer and Nouws, 1991), the exception being Alberta, Canada, where bioserotype 2/O:5,27 is isolated from 26% of human cases and 24% of pigs in the region (Schiemann and Fleming, 1981).

Another observation from this study is the high prevalence of *Y. pseudotuberculosis* observed in pigs sampled compared with the extremely low rate of isolation from people, less than one case being recorded annually in New Zealand. This may, however, reflect the nature of the disease, as diarrhoea is rarely recorded and stool cultures are frequently negative (Schiemann, 1989). The principal syndrome associated with *Y. pseudotuberculosis* infection is one of abdominal pain resembling appendicitis and the pathogen may be under diagnosed in this country. Serotype IIb has been recovered from human infections in this country and overseas but serotype III has not, although it has been recorded in Japan (Tsubokura *et al.*, 1984) and Finland (Merilahti-Palo *et al.*, 1991). Many surveys carried out overseas have shown that pigs regularly carry *Y. pseudotuberculosis*, in particular serotype III (Toma and Deidrick, 1975; Mair *et al.*, 1979; Tsubokura *et al.*, 1984) and some workers have suggested that porcine strains of this serotype are avirulent for people (Mair *et al.*, 1979). Mair *et al.* (1979), examined 37 strains of serotype III from pigs and found them to be avirulent for guinea pigs, in contrast to other serotypes which killed these animals within 10 days. The only phenotypic difference between the pathogenic and non-pathogenic serotypes was that serotype III strains did not ferment melibiose. Japanese workers also examined a number of porcine serotype III strains and found that some fermented melibiose and were virulent for mice, whereas others did not ferment the sugar and were avirulent (Tsubokura *et al.*, 1984). Unfortunately the issue is clouded as they also recovered a melibiose non-fermenting serotype III strain from a diseased person, leading them to observe that further work is needed to clarify the role of such strains in human yersiniosis. In Finland, serotype III strains were isolated from pigs and antibodies to this serotype were recovered from abattoir workers from the same slaughterhouse (Merilahti-Palo *et al.*, 1991). However, despite symptoms of diarrhoea and abdominal pain being reported by the butchers they did not correlate with the occurrence of antibodies. All *Y. pseudotuberculosis* III strains recovered from pigs in this study failed to ferment both melibiose and rhamnose, in contrast to the serotype II strains recovered which fermented both sugars.

The other *Yersinia* strains isolated in this study are usually regarded as non-pathogenic, however, *Y. enterocolitica* bioserotype 1A/O:6,30 is frequently isolated from diarrhoeic human stools and this has led some workers to conclude that it may be a pathogen under certain circumstances, despite the lack of any recognised virulence factors (Schiemann, 1979a; Morris *et al.*, 1991). This bioserotype has also been recovered from human stool samples in this country and in the absence of any other recognised pathogens being simultaneously recovered, dismissal of it's pathogenic potential must be tempered with caution.

The absence of *Y. enterocolitica* bioserotype 2/O:9 in pigs was not considered unusual, as prior to this study it had not been recovered from human sources. Reports from Canada and the USA also recorded the apparent absence of this strain (Schiemann and Fleming, 1981; Swaminathan *et al.*, 1982). In 1990, human isolates were first recovered in New Zealand, nevertheless, a second survey of pigs at the same abattoir still failed to isolate the strain (De Allie, 1994). As pigs killed at this abattoir all originate from the southern half of the North Island, this may reflect a regional distribution of the serotype and further studies of other pig populations should be carried out to confirm it's absence in this animal species.

3.4.2 Farm distribution of *Yersinia* spp.

Of the 22 farms from which pigs were sampled during the survey, 19 (86.4%) were positive for yersiniae, and 17 (77.3%) were positive for the potentially pathogenic strains of the organism. Results from the 5 herds that were negative for potentially pathogenic yersiniae should, nevertheless, be interpreted with caution, as they were all small herds and only low numbers of animals (1-4) were sampled from each. The findings in this study, however, are in agreement with Andersen *et al.* (1991), who isolated *Y. enterocolitica* from 25% of pigs belonging to 82% of herds, with negative results being recorded from small herds of ten pigs or less. Other authors have also reported a variable distribution of the organism between herds and have suggested that the "open management type" where pigs are bought from markets or other farms are more likely to be positive, making this an important factor in the epidemiology of the organism (Christensen, 1980; Maruyama, 1987).

Another observation was the apparent exclusivity of a single pathogenic strain of *Y. enterocolitica* on individual farms, with only one out of eleven farms positive for both serotypes O:3 and O:5,27. The same was not true of *Y. pseudotuberculosis* as 6 farms were positive for both pathogenic species. Remarkably, in a survey carried out 4 years later at the same abattoir the distribution of the specific bioserotypes of *Y. enterocolitica* between the farms remained unchanged (De Allie, 1994), suggesting that the presence of one pathogenic *Y. enterocolitica* bioserotype on a farm may preclude the presence of another. Similar observations have been made in Japan and Fukushima *et al.* (1990) concluded that most farms in an area were contaminated with a single serotype of *Y. enterocolitica* only. They also showed that when more than one serotype or species was found in pigs at slaughter the probability was that the non-endemic strains for a particular farm had been obtained in the lairage at the slaughterhouse. Evidence to support the exclusion of non-endemic strains was put forward by Fukushima *et al.* (1984a), who infected and subsequently challenged pigs with serotypes O:3 and O:5,27 and found significant cross-protection between the strains. This led them to suggest that infection with *Y. enterocolitica* O:3 might act as an efficient barrier against colonisation by other virulent serotypes in pigs. This hypothesis was supported by the work of Fukushima and Tsubokura (1985), who found that the introduction and establishment of a new serotype on a farm resulted in the eventual disappearance of the original widespread pathogenic strain. No reasons, however, were given as to how the displacement occurred initially.

3.4.3 Isolation of yersiniae from pork products

Contamination of pig carcases and meat with *Yersinia* from the head region and faeces during the slaughter process is believed to be the way in which virulent *Y. enterocolitica* enter the food chain and studies overseas have shown that such contamination does occur (Andersen, 1988; Andersen *et al.*, 1991). Thus, it would be expected that in regions with a high prevalence of pathogenic *Yersinia* in the local pig population, pork products would harbour similar serotypes. However, apart from three potentially pathogenic *Y. enterocolitica* biotype 3 strains, all other isolates recovered in this survey of pork mince and pork pieces were of strains usually referred to as non-pathogenic and environmental. Given

the relatively high prevalence of pathogenic *Yersinia* found in local pigs this was a surprising result.

Nevertheless, the results were in agreement with a number of studies overseas which have shown that despite a high prevalence in pigs, meat products are usually found to be contaminated with avirulent strains (Lee, 1977; Hanna *et al.*, 1979; Greenwood and Hooper, 1985; Kapperud, 1991) and evidence for food-borne transmission of *Y. enterocolitica* via pig meat is still largely circumstantial (Tauxe *et al.*, 1987). A Norwegian study surveyed 152 samples of retail pork products and found 27% positive for yersiniae, the majority being *Y. enterocolitica* biotype 1A (Nesbakken *et al.*, 1985). Only one pathogenic strain of bioserotype 4/O:3 was isolated. A similar study of pork products in Holland isolated pathogenic *Yersinia* from only 1% of 400 samples (de Boer and Nouws, 1991). These workers suggested that contamination of carcases may not occur as frequently as had been previously reported.

One reason given for the low isolation rate of pathogenic yersiniae from meat products is that the isolation methods used are not sufficiently sensitive to identify low numbers of such strains against a large background microflora. Studies have been carried out that have demonstrated the inhibition of *Y. enterocolitica* 4/O:3 by non-pathogenic strains of *Yersinia* and other contaminating organisms (Fukushima, 1986b; Fukushima and Gomyoda, 1986). Nevertheless, the studies also showed that despite the inhibition, 4/O:3 strains survived in the meat products and could still be a source of human infection. It is well known that cold enrichment in PBS alone results in the recovery of large numbers of non-pathogenic strains and hence many new enrichment procedures have been developed to improve the recovery of pathogenic serotypes (Kapperud, 1991; de Boer, 1992). One method reported from Belgium was very sensitive for the isolation of bioserotype 4/O:3 from pork products but other serotypes appeared to be inhibited and it was concluded that no single method could allow optimum recovery of all strains (Wauters *et al.*, 1988a). In recent years, the development of molecular techniques such as genetic probes has dramatically increased the sensitivity of pathogen detection in meat products. Nesbakken *et al.* (1991a), found that when an oligonucleotide probe was used, the prevalence of pathogenic yersiniae in

Norwegian pork products was substantially higher than previously demonstrated using cold enrichment techniques.

3.4.4 Virulence testing of porcine isolates of *Yersinia*

In-vitro tests have been used for many years to predict the virulence of *Yersinia* spp. (Schiemann *et al.*, 1981; Kay *et al.*, 1983; Kwaga and Iversen, 1992). Many of these were designed to detect properties associated with the 42- megadalton virulence plasmid unique to pathogenic yersiniae, including autoagglutination, calcium dependency and the uptake of dyes. One problem associated with these tests is the potential loss of the plasmid during laboratory subculture with a concomitant loss of the encoded properties. Other tests therefore have been designed to detect chromosomally-encoded properties which are inherently more stable, such as the ability to produce pyrazinamidase, to hydrolyse aesculin and to ferment salicin.

A range of virulence tests were performed in this study on the *Yersinia* strains isolated in the abattoir, firstly, to assess the potential pathogenicity of the organisms and secondly, to evaluate the tests, many of which had only recently been described. Overall the results of the tests showed quite clearly that strains of *Y. enterocolitica* bioserotypes 2/O:5,27 and 4/O:3 and *Y. pseudotuberculosis* recovered from pig tonsils were phenotypically indistinguishable from similar strains recovered from people and were, therefore, potentially pathogenic for people. Nevertheless, a few atypical results were recognised with bioserotypes considered as pathogenic, which could have led to errors in the prediction of potential virulence had only one test been used.

Tests for phenotypic characteristics conferred by the plasmid were designed to obviate the need to perform lengthy plasmid extraction procedures and thus were relatively simple to perform. The combination of tests for calcium dependency and congo red uptake in one agar plate (CRMOX) was not found to offer any advantages over the more traditional magnesium oxalate agar (MOX) that recognised calcium dependence alone, as tiny colonies representing virulent strains could be easily recognised on both media. On the CRMOX

plates, a variation in the uptake of congo red by virulent *Yersinia* strains, particularly bioserotype 4/O:3 was seen, and they often looked pale in comparison to bioserotype 2/O:5,27 strains. The crystal violet uptake test also enabled virulent strains to be identified easily but was a test with the potential to cross-contaminate the workplace and surrounding cultures. The autoagglutination test was relatively simple to perform (although not as easy as streaking a plate), however, it was a reliable indicator of plasmid-bearing strains.

The problem alluded to earlier was that 5 out of the 25 *Y. enterocolitica* bioserotype 4/O:3 strains examined were negative in the tests for the presence of a plasmid, indicating plasmid loss. The virulence plasmid is well-recognised as being unstable during laboratory manipulation, especially if the bacteria are exposed to a temperature of 37°C or to prolonged storage (Miller *et al.*, 1989). It appears, therefore, that storage of these strains at -70°C for some months before virulence testing may have caused plasmid loss, interestingly only in bioserotype 4/O:3 strains. Whether the plasmid in strains of *Y. enterocolitica* bioserotype 2/O:5,27 and *Y. pseudotuberculosis* is inherently more stable is not known.

Chromosomally-encoded properties associated with virulence have been identified and include presence of the *ail* gene (Miller *et al.*, 1989), negative reaction in the pyrazinamidase test (Kandolo and Wauters, 1985), and in *Y. enterocolitica* the failure to hydrolyse aesculin or to ferment salicin (Riley and Toma, 1989). Unfortunately, detection of the *ail* gene is complicated, involving either tissue culture to detect cell invasion or molecular methods inappropriate for a diagnostic laboratory at the present time. In addition, some researchers have found that pathogenic strains of *Y. enterocolitica* can occasionally give positive reactions in the biochemical tests described above (Farmer *et al.*, 1992). This has led them to state that no individual virulence-associated characteristic was a reliable single indicator of virulence, implying that more than one test should be performed. The results of the pyrazinamidase, aesculin and salicin tests on the porcine strains isolated in this study all correlated well with the predicted virulence associated with the bioserotype. In addition, all were straightforward and easy to read. The VYE test agar identified aesculin-positive, avirulent strains and differentiated them from potentially

virulent strains, offering advantages over the traditional CIN agar, particularly as some *Yersinia* strains are reported to be inhibited on CIN (Fukushima, 1987).

In summary, the bioserotype of *Y. enterocolitica* is very well correlated with potential virulence and with the use of MOX agar and the PYZ test for confirmation was sufficient to identify pathogenic strains among the porcine isolates. Those which had lost their plasmids were MOX-negative, but the bioserotype and the negative PYZ test indicated that this might have occurred. This simple protocol for estimation of virulence is similar to that suggested by Riley and Toma (1989), with MOX being preferred to CRMOX for the reasons discussed above.

CHAPTER 4

4. DOMESTIC ANIMALS AS POTENTIAL SOURCES OF HUMAN *YERSINIA* INFECTION

4.1 INTRODUCTION

In the last eight years, human *Yersinia* infections have been shown to be widespread in New Zealand, however, the evidence pointing to animals other than pigs and dogs as reservoirs of human pathogenic *Y. enterocolitica* is ill-defined, largely due to a lack of specific typing of recovered isolates in the past. Therefore, a decision was made to reassess the situation, in an effort to elucidate the role of domestic animals in the epidemiology of human yersiniosis in this country.

A collaborative project was initiated with the Ministry of Agriculture and Fisheries (MAF) in 1992, to investigate the bioserotypes of *Y. enterocolitica* affecting domestic animals in New Zealand. All *Yersinia* isolates recovered from specimens submitted to MAF Regional Animal Health Laboratories were sent to the DVPPH for further characterisation. In addition, *Yersinia* isolates collected by the diagnostic laboratory in the DVPPH prior to this date, and stored at -70°C, were subjected to the same range of tests.

4.2 MATERIALS AND METHODS

4.2.1 *Yersinia* isolates

Between 1988 and 1995, 377 isolates of *Yersinia* spp. that had been recovered from animals were obtained from diagnostic laboratories around New Zealand for confirmation of

identity, biotyping, serotyping and virulence testing. While many of the isolates were from clinical cases submitted to the laboratories, a few originated from healthy animals surveyed in response to outbreaks of disease or for other purposes, such as *Brucella* surveillance. Yersiniae were isolated from a number of animal species including alpaca, cattle, sheep, goats, deer, dogs, cats, pigs, horses, primates and birds.

The six laboratories which participated in the study were the Regional Animal Health Laboratories in Auckland, Hamilton (Ruakura), Palmerston North (Batchelar), Christchurch (Lincoln) and Dunedin (Invermay) and the Microbiology Laboratory in the Department of Veterinary Pathology and Public Health, Massey University, Palmerston North.

4.2.2 Confirmation of identity

Isolates were usually only designated as *Yersinia* spp. when received and were all subjected to a full range of biochemical tests in order to confirm their identity. These are described in Appendix 2. As all participating laboratories used different isolation and identification procedures for yersiniae it was considered essential to use standardised methods for their confirmation, prior to further typing.

4.2.3 Biotyping, serotyping and virulence testing

Yersinia spp. other than *Y. enterocolitica* and *Y. pseudotuberculosis* were not typed further as they were considered to be environmental and non-pathogenic. *Yersinia pseudotuberculosis* isolates were serotyped using commercial antisera to serogroups I, II and III (Denka-Seiken, Japan). *Y. enterocolitica* isolates were first biotyped according to Wauters *et al.* (1987) and then serotyped using commercial antisera (Denka-Seiken, Japan). Once typing was completed, all isolates were frozen at -70°C in glycerol broth as described previously in Chapter 3.

Virulence testing was performed using the following tests - autoagglutination, calcium dependency at 37°C, aesculin hydrolysis, salicin fermentation and the presence of the enzyme pyrazinamidase. These tests had been found to provide the best correlation with virulence during an extensive study of porcine and human isolates described previously.

4.3   RESULTS

4.3.1   *Yersinia* species isolated

During the course of the study, 377 *Yersinia* isolates were received from contributing laboratories (see Table 4.1 for details). Strains of *Yersinia* potentially pathogenic for people, including *Y. enterocolitica* bioserotypes 2/O:5,27, 2/O:9, 3/O:1,2,3, 4/O:3 and *Y. pseudotuberculosis*, made up 33.7% of the total. Non-pathogenic, environmental strains, which included *Y. enterocolitica* biotype 1A, *Y. frederiksenii*, *Y. kristensenii* and *Y. intermedia*, accounted for 20.2% and *Y. enterocolitica* bioserotype 5/O:2,3 strains, which are only recognised as animal pathogens, 46.1% of the total isolates. All of the human and animal pathogenic strains gave positive results in the virulence tests used.

4.3.2   Distribution of *Yersinia* between animal species

Yersiniae were widely distributed between animal species as shown in Table 4.1. The majority of isolates came from cattle (117, 31%), followed by sheep (89, 23.6%), goats (59, 15.7%), deer (45,11.9%) and dogs (27, 7.2%). The remainder of the strains were recovered from birds (14, 3.7%), alpaca (11, 2.9%), horses (5, 1.3%), cats (4, 1.1%), pigs (4, 1.1%) and monkeys (2, 0.5%).

4.3.3   Association of *Yersinia* spp. with disease in animals

All of the *Y. enterocolitica* biotype 5 strains and 26 (93%) of the *Y. pseudotuberculosis* strains were associated with clinical disease in the species from which they were isolated. The principal manifestation of disease in animals was enteritis and the mortality rate was relatively high. Of the other 99 pathogenic *Y. enterocolitica* strains recovered (bioserotypes 2/O:5,27, 2/O:9, 3/O:1,2,3 and 4/O:3), 45 (45.5%) were from diseased animals. Clinical disease in sheep and goats was almost exclusively caused by infection with *Y. enterocolitica* bioserotype 5/O:2,3, whereas in cattle this strain accounted for approximately 50% of the clinically affected animals. *Yersinia enterocolitica* bioserotype

2/O:5,27 was isolated from a wide range of diseased animals, including cattle, sheep, goats, deer, dogs, cats, pigs and horses. Other bioserotypes involved in disease were 2/O:9 in sheep, dogs and cats, 3/O:1,2,3 in sheep and 4/O:3 in dogs.

4.3.4 Laboratory isolations

Table 4.2 shows details of the pathogenic yersiniae isolated from individual laboratories during the period 1988 to 1995. The largest number of strains were received from Massey University, however, this does not reflect a higher incidence of infection in animals attended to by the Faculty of Clinical Sciences, because other laboratories did not start forwarding isolates until 1992. In addition, the small number of isolates received from Auckland Animal Health Laboratory probably reflect it's urban location. Bioserotypes 5/O:2,3, 2/O:5,27 and 2/O:9 were recovered from all regions apart from Auckland which had no isolations of 2/O:9. The highest number of bioserotype 2/O:9 isolates recovered was from Ruakura in the Waikato region. *Yersinia pseudotuberculosis* strains were mainly received from Massey University, however, as the focus of the survey was on *Y. enterocolitica*, other laboratories may have recovered them but not sent them for typing. Two additional bioserotypes, 3/O:1,2,3 and 4/O:3, were only recovered from one and two laboratories, respectively.

Table 4.1.   Distribution of yersiniae between animal species

Animal	1A	2/O:9	2/O:5,27	3/O:1,2,3	4/O:3	5/O:2,3	Yps	Yf	Yi/Yk	Total
Cattle	7	25	21			34	17	8	5	117
Sheep	4	3	1	2		77	1	1		89
Goats			3			55			1	59
Deer	12	8	9			5		10	1	45
Alpaca	3	1	4			3				11
Dogs	5	4	9		4		2	3		27
Cats		1	2				1			4
Pigs	3		1							4
Horses	3		1					1		5
Monkeys								2		2
Birds	5						7	2		14
Total	42	42	51	2	4	174	28	27	7	377
%	11.1	11.1	13.5	0.5	1.1	46.2	7.4	7.2	1.9	

1A - *Y. enterocolitica* biotype 1A; 2/O:9 - *Y. enterocolitica* bioserotype 2/O:9; 2/O:5,27 - *Y. enterocolitica* bioserotype 2/O:5,27;

3/O:1,2,3 - *Y. enterocolitica* bioserotype 3/O:1,2,3; 4/O:3 - *Y. enterocolitica* bioserotype 4/O:3; 5/O:2,3 - *Y. enterocolitica* bioserotype 5/O:2,3;

Yps - *Y. pseudotuberculosis*; Yf - *Y. frederiksenii*; Yi - *Y. intermedia*; Yk - *Y. kristensenii*

Table 4.2. Pathogenic yersiniae recovered from participating laboratories

	Ye O:1,2,3	Ye O:2,3	Ye O:3	Ye O:5,27	Ye O:9	Yps	Total
Auckland		1		2			3
Ruakura		32		14	18		64
Batchelar		40		5	4		49
Lincoln		15	2	5	5	1	28
Invermay	2	51		8	6		67
Massey		35	2	17	9	27	90
Total	2	174	4	51	42	28	301

Ye O:1,2,3 - *Y. enterocolitica* bioserotype 3/O:1,2,3; Ye O:2,3 - *Y. enterocolitica* bioserotype 5/O:2,3; Ye O:3 - *Y. enterocolitica* bioserotype 4/O:3; Ye O:5,27 - *Y. enterocolitica* bioserotype 2/O:5,27; Ye O:9; *Y. enterocolitica* bioserotype 2/O:9; Yps - *Y. pseudotuberculosis*

4.3.5 Annual isolation of *Yersinia enterocolitica*

Table 4.3 shows the figures for annual isolation of pathogenic strains of *Y. enterocolitica*. As mentioned previously, isolates recovered between 1988 and 1991 were mainly from Massey University diagnostic laboratory and the principal strain isolated overall was the animal pathogenic bioserotype 5/O:2,3, from sheep and goats. During this period, however, two isolates of bioserotype 4/O:3 were recovered from canine infections, the first in 1989, the other in 1991. This common human pathogenic strain was only found on two other occasions, in 1995, also from dogs, in the Lincoln laboratory region. The first two isolates of another human pathogenic bioserotype, 2/O:5,27, were also made, from a goat in 1989 and a horse in 1990. The equine isolate came from Lincoln. This serotype was subsequently isolated each year from a number of animal species. The first isolations of bioserotype 2/O:9 were made in 1992, thereafter this strain was recovered annually. Bioserotype 3/O:1,2,3 was only recovered from sheep, on two occasions, in 1993 and 1994, from Invermay. The majority of *Yersinia* isolations were made in 1993 (92, 33.7%) including the highest number of strains potentially pathogenic for people. Bioserotype 5/O:2,3 was recovered from ruminants in all years.

Table 4.3.   Annual isolation of *Y. enterocolitica*

Year	O:1,2,3	O:2,3	O:3	O:5,27	O:9	Total	%
1988-91		30	2	3		35	12.8
1992		14		11	8	33	12.1
1993	1	52		26	13	92	33.7
1994	1	59		10	16	86	31.5
1995		19	2	1	5	27	9.9
Total	2	174	4	51	42	273	
%	0.7	63.7	1.5	18.7	15.4		100

% = percentage of the total number of isolates

4.3.6 Unusual *Yersinia* isolates recovered from animals

During the course of this investigation 36 *Yersinia* strains were recovered from ruminants and people that did not correspond biochemically to any previously published species (Fenwick *et al.*, 1996). Table 4.4 shows details of the strains isolated. The principal distinguishing tests of the initial isolates (designated Phenotype 1) were a negative ornithine reaction, positive reactions in the sugars melibiose and raffinose and a negative reaction in the rhamnose test (see Table 4.5 for comparison with other *Yersinia* species). The only ornithine-negative *Yersinia* species previously described in the literature was *Y. pseudotuberculosis*, however, this species is also usually rhamnose-positive, melibiose-negative and raffinose-negative. Subsequently, four very similar strains were recovered which were ornithine-positive and indole-negative (designated Phenotype 2). This second phenotype was differentiated from *Y. intermedia* on the basis of the indole and rhamnose tests and from indole-negative *Yersinia* with the Voges-Proskauer, raffinose and melibiose tests (see Appendix 2 for details of tests).

Twenty five strains were taken to the Microbiology Unit in the Faculty of Medicine, Catholic University of Louvain, Brussels, Belgium on sabbatical leave for further characterisation and virulence testing. Tests carried out there under the supervision of Professor Wauters confirmed that the strains belonged to a hitherto unrecognised *Yersinia*. Negative results in three tests used commonly to ascertain virulence, namely autoagglutination, calcium dependency and agglutination of serum raised against the Yad-A outer membrane protein (P1), indicated the absence of a virulence plasmid. Surprisingly, however, the strains were negative in the pyrazinamidase test, unlike all other avirulent yersiniae.

Additional characterisation of these strains has since been carried out by Professor Wauters in Belgium and Professor Ursing of Lund University, Malmo, Sweden. Professor Wauters identified the strains as belonging to two new *Yersinia* serotypes, O:77 and O:78. No relationship was apparent between serovars and phenotype. Professor Ursing performed deoxyribonucleic acid relatedness studies on the study strains and the results indicated that they formed a homogeneous group closely related to the type strain of *Y. enterocolitica*, (CCUG 11291).

Table 4.4.   **Distribution and characterisation of unusual *Yersinia* strains**

Strain	Year	Laboratory	Animal sp.	Phenotype	Serotype
A112	1988	Massey	Bovine	I	NT
A113	1988	Massey	Caprine	I	NT
B74	1988	Massey	Bovine	I	O:77
G17	1990	Massey	Caprine	I	O:78
G18	1990	Massey	Ovine	I	O:77
G19	1990	Massey	Ovine	I	O:78
J88	1991	Massey	Bovine	I	O:77
N94	1992	Massey	Caprine	I	O:77
P73	1992	Massey	Ovine	I	O:77
S90	1993	Massey	Cervine	I	O:77
U73	1993	Massey	Cervine	I	O:78
T1	1993	MLHB	Human	I	O:78
T2	1993	MLHB	Human	I	O:78
T3	1993	MLHB	Human	I	O:78
T4	1993	MLHB	Human	I	O:78
T7	1993	MLHB	Human	I	O:78
T8	1993	MLHB	Human	I	O:78
T9	1993	MLHB	Human	I	O:78
T10	1993	MLHB	Human	I	O:78
T11	1993	MLHB	Human	I	O:78
T12	1993	MLHB	Human	I	O:78
T13	1993	MLHB	Human	I	O:78
T14	1993	MLHB	Human	I	O:78
T70	1993	Massey	Bovine	II	O:77
V22	1994	Massey	Bovine	II	O:77
V26	1994	Invermay	Ovine	II	O:77

Table 4.4 (cont).   **Distribution and characterisation of unusual *Yersinia* strains**

Strain	Year	Laboratory	Animal sp.	Phenotype	Serotype
V27	1994	Invermay	Bovine	II	O:77
V39	1994	Massey	Cervine	I	NT
Y72	1995	Massey	Bovine	I	NT
Y73	1995	Massey	Bovine	I	NT
Y74	1995	Massey	Bovine	I	NT
Y75	1995	Massey	Bovine	I	NT
Y76	1995	Massey	Bovine	I	NT
Y96	1995	Massey	Bovine	I	NT
Y97	1995	Massey	Bovine	I	NT
Y142	1995	Massey	Bovine	I	NT

MLHB - Medical Laboratory, Hawke's Bay; O:77 and O:78 - new serotypes identified as a result of this study; NT - not tested

Table 4.5.   Comparison of the unusual study strains with other Yersinia species

Test	NB	YE	YK	YM	YB	YF	YI	YA	YR	YP
Ornithine	-/+	+	+	+	+	+	+	+	+	-
Voges-Proskauer	+	+/-	-	-	-	+	+	+	-	-
Indole	+/-	V	V	-	-	+	+	-	-	-
Aesculin (24hrs)	+	V	-	-	-	V	+	-	-	+
Lipase	+	V	V	-	-	+	+	V	-	-
Sucrose	+	+	-	+	+	+	+	-	+	-
Rhamnose	-	-	-	-	-	+	+	+	-	V
Raffinose	+	-	-	-	-	-	+	-	V	-
Melibiose	+	-	-	-	-	-	+	-	V	V
α-methyl glucoside	-	-	-	-	-	-	+	-	-	-

NB = new biotype; 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 negative; +/- = most strains positive

4.4 DISCUSSION

4.4.1 Animal carriage of human pathogenic strains of *Y. enterocolitica*

Yersiniosis is well-established as an important human foodborne disease and yet many surveys have shown that farm animals other than pigs appear to be infrequent carriers of strains of *Y. enterocolitica* potentially pathogenic for humans (Schiemann, 1989). For example, surveys carried out in the USA (Wooley *et al.*, 1980; Shayegani and Parsons, 1987), Scandinavia (Kapperud, 1975), Italy (Chiesa *et al.*, 1987) and the United Kingdom (Corbel and Cullen, 1970) recovered only environmental strains of *Y. enterocolitica* from a wide range of domestic and wild animal species. Nevertheless, on occasion one or two animals have been found to harbour bioserotypes associated with human disease, most commonly bioserotype 2/O:5,27. This bioserotype has been recovered from chinchillas, dogs, camels and racoons in Canada (Hacking and Sileo, 1974; Toma and Lafleur, 1974), sheep and cattle in the UK (Brewer and Corbel, 1983; Davey *et al.*, 1983), cattle in Japan and Russia (Inoue and Kurose, 1975; Kolos *et al.*, 1985), dogs in Japan (Fukushima *et al.*, 1993) and raw cow's milk in France (Vidon and Delmas, 1981). In addition, it has been recovered previously in New Zealand from sheep (McSporran *et al.*, 1984) and goats (Lanada, 1990). Thus it was not surprising to find that it was recoverable from a wide range of animals in New Zealand in this study, including all species of domestic animals examined. However, the number of species infected and the high rate of infection (13.5% of all *Yersinia* recovered) were both at variance with the very low recovery rates reported from other countries. Despite the widespread distribution of this pathogenic strain in New Zealand animals, however, human infections have remained low.

Y. enterocolitica bioserotype 2/O:9 has been isolated from pigs in Europe (Prentice *et al.*, 1991) but has only been infrequently isolated from other species of domestic animals, usually as a result of investigation of false-positive *Brucella abortus* reactions, reported in humans and animals since the 1960's (Mittal and Tizard, 1981). Animals other than pigs from which this serotype has been recovered include sheep (Kolos *et al.*, 1985), camels (Sunaga *et al.*, 1983), buffaloes (Das *et al.*, 1986), rats (Zen-Yoji *et al.*, 1974), dogs

(Kaneko *et al.*, 1977) cattle and goats (Reynaud *et al.*, 1993), although all isolations other than in the latter study from France were sporadic. In addition, serological evidence for infection has been shown in cattle and goats (Mittal and Tizard, 1980; Schoilew and Kosarow, 1987). Despite human infections with this bioserotype having been diagnosed in New Zealand since 1990, surveys of local pigs failed to recover this *Yersinia* strain, in contrast to studies overseas (De Allie, 1994). In fact, demonstration of this serotype in animals either culturally or serologically was not accomplished until 1992, when a number of deer in a consignment for export reacted in the *Brucella abortus* serum agglutination test (SAT) (Hilbink *et al.*, 1995). Subsequent culture of faecal samples from these deer and their contacts recovered *Y. enterocolitica* bioserotype 2/O:9 and acted as the stimulus for the present collaborative study with MAF. Results from the present study have shown that this strain is far from uncommon, with the prevalence of 11.1% of the total *Yersinia* isolates almost as high as that of bioserotype 2/O:5,27 (13.5%). Recovery was most frequent from cattle (59.5% of O:9 isolations) and deer (19%), however, this probably reflects the animals that were tested for *Brucella abortus*, and may not be the true situation. Other animals from which this bioserotype was isolated were sheep, dogs an alpaca and a cat, increasing the range of animals involved in it's epidemiology. The isolation of this bioserotype and 2/O:5,27 from farmed alpaca is the first record of *Yersinia* infection in this camelid species. Thus, in the absence of evidence for porcine infections with this serotype, it appears that cattle may be the principal reservoir for human infection in New Zealand, with a range of other domestic animals able to act as secondary sources.

Y. enterocolitica bioserotype 4/O:3 is the most commonly isolated human pathogenic strain in New Zealand (McCarthy and Fenwick, 1991) and overseas (Schiemann, 1989), however, it is also the strain with the narrowest host range, being recovered almost exclusively from pigs (Swaminathan *et al.*, 1982). Nonetheless, other animals have been identified as carriers on occasion, principally dogs (Pedersen, 1976; Fukushima *et al.*, 1984b), although cats have also been implicated (Toma and Lafleur, 1974; Yanagawa *et al.*, 1978). On rare occasions ruminants have been found to harbour O:3, including a single sheep in New Zealand (Bullians, 1987), three sheep in Russia (Kolos *et al.*, 1985) and one cow in Brazil (Falcao, 1987). Serological evidence of O:3 infection in cattle has been reported from Bulgaria

(Schoilew and Kosarow, 1987) and in numerous animals in Nigeria (Adesiyun *et al.*, 1986), however, without confirmation by culture these aberrant results are difficult to interpret. Recovery of bioserotype 4/O:3 from four unrelated dogs and from no other animal species during the present study is in accord with the epidemiological situation overseas and suggests that they may act as an additional, non-foodborne source of infection for people. In addition, the carriage of this bioserotype in New Zealand sheep, as reported by Bullians (1987), could not be substantiated and may, as mentioned earlier, have been a result of confusion with the more commonly isolated bioserotype 5/O:2,3. The discrepancy between the high carriage rate of bioserotype 4/O:3 by pigs described in Chapter 3 and the failure to isolate this strain in the present survey may be due to the asymptomatic nature of porcine infections and the sampling bias, whereby the majority of samples examined by the participating diagnostic laboratories were from diseased animals.

The least common strain of *Y. enterocolitica* recovered that was potentially pathogenic for people was bioserotype 3/O:1,2,3, isolated from two sheep. This strain has only been isolated from two cases of human infection in New Zealand and is also infrequently isolated overseas (Schiemann, 1989). Chinchilla are the only other animal associated with this bioserotype, in which species it appears to be a pathogen (Hubbert, 1972; Langford, 1972), however, as reports of outbreaks date back to the 1970's, the current status of this strain is unknown.

4.4.2 Association of *Y. enterocolitica* with animal disease

Yersiniosis in animals and birds has for many years been associated with *Y. pseudotuberculosis* infection and reports of disease caused by *Y. enterocolitica* have been infrequent (Mair, 1973). Nevertheless, as stated earlier, outbreaks of disease in chinchillas were recorded regularly in USA and Canada, as a result of infection with bioserotype 3/O:1,2,3 (Langford, 1972; Toma and Lafleur, 1974) and sporadic reports of infection with other serotypes have been noted in a number of animal species (Langford, 1972). It appears, therefore, that the strains of *Y. enterocolitica* pathogenic for people can, on occasion, cause disease in animals, although at a significantly lower frequency, as befits

their probable commensal relationship. Thus, bioserotype 4/O:3 has been linked to disease in dogs (Fantasia, *et al.*, 1985), cats (Toma and Lafleur, 1974) and on rare occasions, cattle, sheep and pigs (Yu *et al.*, 1983; Kolos *et al.*, 1985; Falcao, 1987); bioserotype 2/O:5,27 with disease in sheep (Brewer and Corbel, 1983; McSporran *et al.*, 1984) chinchillas, dogs and racoons (Toma and Lafleur, 1974) and bioserotype 2/O:9 with disease in sheep (Kolos *et al.*, 1985) and buffaloes (Das *et al.*, 1986). The most common symptom of disease in animals is enteritis, as it is in people, however, mastitis, abortion and sudden death have all been recorded.

The isolation of bioserotypes 2/O:5,27, 2/O:9 and 4/O:3 from dogs in this study confirms the longstanding relationship of *Y. enterocolitica* with canine infections and indicates that these organisms may in fact not be commensals in this species. Dogs may thus be equally as susceptible as people and may be at risk of infection from a common source, particularly if they are fed on household scraps. Three of the four isolates recovered came from cases of severe enteritis, two of which were associated with disease in litters of puppies. The other case presented as acute pharyngitis, a symptom commonly recognised in infected people. No reports of enteric disease were, however, recorded in the owners.

Sporadic infections caused by bioserotypes 2/O:5,27, 2/O:9 and 3/O:1,2,3 were also recorded from farmed animal species including cattle, sheep, goats and deer and the predominant clinical symptom was enteritis. Most of the cases were recorded in winter in young animals six- to twelve-months-old and were therefore probably associated with stresses such as poor feed, inclement weather and concurrent parasitism, similar to the predisposing factors reported for *Y. pseudotuberculosis* infection (Henderson, 1983a). The surprisingly high incidence of disease in animals caused by these *Y. enterocolitica* bioserotypes signifies either that a unique situation exists in New Zealand or that inappropriate laboratory techniques are being used for the diagnosis of enteric infections in animals overseas.

The most common bioserotype associated with disease in farmed ruminants, however, was 5/O:2,3, a cause of significant enteritis and mortality during the study period. This

bioserotype was described historically as an obscure pathogen that only caused mortality in hares in Europe (Mair, 1973), but in Norway in 1972, it was isolated from an outbreak of enteritis and deaths in winter-housed goats (Krogstad *et al.*, 1972; Krogstad, 1974; Krogstad, 1975). The organism was also linked serologically to human infections in the goat farmers but wasn't recovered from human faecal samples. Since that report, further cases have not been recorded in Europe, nevertheless, diagnosticians in New Zealand and Australia have recovered the strain frequently from disease in sheep and goats (Buddle *et al.*, 1988; Slee and Button, 1990a). The incidence of disease in other farmed animals is difficult to ascertain from the literature as *Y. enterocolitica* was rarely typed in older studies. However, this study has shown that all species of ruminants can be clinically affected by bioserotype 5/O:2,3 strains, including cattle, sheep, goats, deer and alpacas. Sheep, goats and cattle appear to be the most commonly affected animals. This strain of *Y. enterocolitica* has also been identified in clinically normal animals (Lanada, 1990; Slee and Skilbeck, 1992) and stresses similar to those described above are undoubtedly linked to disease. The pathogen appears to be specific for ruminants as it has never been recovered from any other animal species, and apart from the study in Norway (Krogstad *et al.*, 1972), further reports have not been made of it's association with human infections. Nonetheless, the presence of a virulence plasmid of a similar size to that found in other pathogenic *Y. enterocolitica* bioserotypes implies that it may indeed be a potential pathogen and it's zoonotic potential needs further investigation.

4.4.3   Regional distribution of pathogenic *Yersinia*

Studies overseas had shown clearly that regional differences existed in the distribution of pathogenic *Y. enterocolitica* (Swaminathan *et al.*, 1982) and the location of the laboratories participating in this study throughout the North and South Islands enabled regional comparisons of *Yersinia* isolations to be made. Not surprisingly, pathogenic strains of *Y. enterocolitica* were spread evenly over the whole country, reflecting the wide distribution of their associated host species. The principal discrepancy observed was in the number of isolates recovered from Massey University, including all but one of the *Y. pseudotuberculosis* strains, although as mentioned previously this reflects the period in

which laboratories were associated with the project and a bias towards *Y. enterocolitica* typing rather than a true regional distinction. In fact, if the figures for Massey are combined with the local Animal Health Laboratory, Batchelar, it would appear that yersiniosis is more of a problem in the Manawatu than in any other region, but again this inconsistency is probably artefactual. Similarly, the low number of isolates recovered at the Auckland laboratory may merely reflect it's urban location rather than being a true representation of the disease in the surrounding farm land.

4.4.4 Annual isolation of *Yersinia*

The highest annual incidence of *Yersinia* infection was seen in 1993 (33.7% of all isolates), followed closely by the incidence in 1994 (31.5% of all isolates). It is difficult to say whether this is a true reflection of the situation or whether, in fact, it demonstrates some bias in reporting from participating laboratories, however, it is interesting to note that human infections were also at their peak in these two years and perhaps climatic conditions assisted in an increased survival and dispersion of the organism.

4.4.5 Isolation of unusual *Yersinia* strains

Since the study began in 1988, a number of isolates of a hitherto undescribed *Yersinia* species were made that were biochemically quite distinctive. As they were commonly isolated from enrichment broths in small numbers and were negative in all virulence assays their significance was unknown and they were stored for future investigation. The majority of the 24 animal isolates have been from cattle (14, 58.3%), although they have also been recovered from other ruminants including sheep (4), goats (3) and deer (3). In 1993, however, a cluster of 12 human infections with this unusual strain was recognised in Hawke's Bay and this acted as an incentive for their further characterisation. A collaborative research project initiated with laboratories overseas has resulted in their tentative designation as a new biotype of *Y. enterocolitica*, incorporating two novel serotypes, O:77 and O:78. A recent survey of bovine faeces for enteric pathogens carried out locally has resulted in the isolation of further strains from healthy animals and has helped to confirm their commensal existence in this species. In addition, the first isolation

from pig faeces has recently been made, expanding the range of animal reservoirs beyond ruminants.

While the factors involved in the human infections were not identified it could be speculated that they were a result of contamination of a common water source by animal faeces. No further cases have been recorded, however, and this remains conjectural. A further question arises as to the potential pathogenicity of the strains as all the human cases suffered from enteritis characteristic of *Yersinia* infection and yet isolates appear non-pathogenic with *in-vitro* virulence tests. Other apparently non-pathogenic *Y. enterocolitica* have, nevertheless, been associated with disease in people (Morris *et al.*, 1991) and recent research into environmental *Y. enterocolitica* by Robins-Browne *et al.* (1993b) has shown the presence of a novel enterotoxin. Therefore, no conclusions can presently be made about their pathogenicity and further examination of these strains for as yet unrecognised virulence factors is indicated.

CHAPTER 5

5. DURATION OF CARRIAGE AND TRANSMISSION OF *YERSINIA ENTEROCOLITICA* BIOSEROTYPE 4/O:3 IN DOGS

5.1 INTRODUCTION

During the last three decades *Yersinia enterocolitica* has become recognised worldwide as an important human pathogen causing a number of clinical syndromes, of which acute and chronic enteritis are the most commonly recorded (Cover and Aber, 1989). It is considered to be principally a foodborne pathogen and studies have been undertaken to identify potential sources of human infection (Fukushima *et al.*, 1987). Although results from these studies have identified pigs as the major reservoir of human pathogenic strains of *Y. enterocolitica* (Andersen *et al.*, 1991), there is evidence to suggest that household pets, in particular dogs, can also carry such strains (Yanagawa *et al.*, 1978) and may in some instances be associated with human disease (Wilson *et al.*, 1976).

In 1989 and 1991, bioserotype 4/O:3 strains were isolated from two young dogs presented to the Small Animal Clinic of the Faculty of Veterinary Science, Massey University (Fenwick, unpublished). One dog had chronic enteritis and the other acute pharyngitis without signs of enteritis. As yersiniae had not been isolated from dogs in New Zealand previously, these cases prompted us to initiate a study to answer a number of questions regarding *Y. enterocolitica* infections in dogs, such as the persistence, site of infection, transmission and the clinical significance of the organism, so that the potential role of dogs as a reservoir of yersiniae for human infections could be assessed.

5.2   MATERIALS AND METHODS

5.2.1   Experimental animals

Fourteen 6-month-old Huntaway-cross dogs from 2 litters were divided into five separately housed groups.   Solid walls separated each group and measures to avoid cross-contamination included the use of clean boots and disinfectant footbaths (Virkon S, Antec International, Sudbury, England) at the entrance to each pen, provision of individual thermometers stored in disinfectant (Virkon S) for each group and the use of disposable plastic gloves for handling and taking samples from the dogs.   Pens were cleaned thoroughly each day by washing with clean water, though without disinfectant.   The dogs were fed a commercial dried dog feed once a day and drinking water was provided *ad libitum*.   The duration of the trial was limited to thirty days by the availability of the experimental dogs.

5.2.2   Experimental design

Group I. This group contained four dogs (1 to 4), all of which were inoculated experimentally at the beginning of the trial.

Group II.   Four dogs, of which two (5 and 6) were inoculated orally with *Y. enterocolitica*. Dogs 7 and 8 were left in contact with them for one week before being moved to Group III.

Group III.   This group initially contained two uninfected dogs (9 and 10).   One week after the trial started, the two dogs (7 and 8) from Group II were moved in with them for a further week.

Group IV.   This group initially comprised two uninfected dogs (11 and 12).   Two weeks after the trial started, the original two dogs (9 and 10) from Group III were moved in with them for the last 2 weeks of the trial.

Group V.   This group contained two dogs (13 and 14) which acted as uninfected controls for the duration of the trial.

5.2.3   Infection of dogs

All dogs in Group I and dogs 5 and 6 in Group II were inoculated orally with 5 ml of an overnight broth culture (Nutrient broth, Difco, Detroit, USA) containing 10^9 cfu/ml of *Y. enterocolitica* bioserotype 4/O:3, originally isolated from a human enteric infection. Virulence tests for yersiniae, including calcium dependency and auto-agglutination, were performed on the strain used, both before and after inoculation, to confirm the presence of a virulence plasmid.

5.2.4   Sampling protocol

Swabs of rectal faeces and the pharynx were taken from the dogs during the 5 weeks of the trial, as outlined in the scheme below.   Blood samples were taken pre-trial and weekly throughout the trial (5 samples per animal, total 70) and serum was stored at -20°C for serological examination.   Temperatures were recorded for all dogs daily and the animals were monitored for evidence of illness such as diarrhoea or inappetance.

Sampling scheme:

Pre-trial. Rectal swabs were collected from all fourteen dogs on three occasions during the week before the trial.

Groups I and II. Pharyngeal and rectal swabs were taken at 1-3 day intervals for 30 days after inoculation.

Groups III and IV. Pharyngeal and rectal samples were taken weekly from the start of the trial and at 1-3 day intervals following contact with infected or potentially infected dogs.

Group V. Pharyngeal and rectal swabs were taken at weekly intervals throughout the trial.

All swabs were cultured to detect the presence of yersiniae as outlined below.

5.2.5 Isolation of *Yersinia enterocolitica*

The media used for the isolation and identification of *Y. enterocolitica* are described in Appendix 2. Rectal swabs were placed in transport medium immediately after collection and were used to inoculate *Yersinia* selective agar (CIN - Difco, Detroit, USA) within two hours. The swabs were then agitated vigorously in 10 mls phosphate buffered peptone water (PBPW). The CIN agar plates were incubated for 24-48 hrs at 28°C and examined for typical bullseye colonies. These were identified, biotyped according to the revised scheme described by Wauters *et al.* (1987) and the serotype determined by slide-agglutination using commercial antisera (Eco-Bio, Woudstraat, Belgium). The PBPW bottles were incubated at 4°C for 3 weeks before subculture onto CIN agar. Yersiniae were then identified as before. Details of the identification protocols are described in Appendix 2.

Pharyngeal swabs were not cultured directly but were only inoculated into PBPW. This was subcultured on CIN agar after 3 weeks incubation at 4°C and isolates identified as above.

5.2.6 Enumeration of *Y. enterocolitica* in faeces

On three occasions during a period in which the dogs were actively excreting *Y. enterocolitica* (days 1-10), three faecal samples were collected for viable counts from the floor of the pen housing animals in Group I. Viable counts were performed as follows: Approximately 1g of faeces was added to 9 mls of distilled water (DW) and vortexed vigorously for one minute. Three serial dilutions of the suspension were made in DW and three CIN agar plates were inoculated with 0.1 ml of each dilution, which was then spread evenly over the surface of the plates using a glass rod. The plates were incubated at 28°C for 24 hours and the number of cfu counted. Viable counts were then calculated as cfu/gram.

5.2.7   Detection of antibody response in infected and in-contact dogs

The 70 serum samples collected from the dogs during the study period were taken to the Department of Microbiology and Infectious Diseases, Royal Children's Hospital, Melbourne, Australia for analysis in a recently developed ELISA for antibodies against *Yersinia* outer membrane proteins (Yops).   Yops used in the ELISA had been prepared previously as described by Robins-Browne *et al.*, (1993a) and for the assay were dissolved in carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) to a final concentration of 5µg/ml.   A preliminary assay was performed using sera from a known *Yersinia enterocolitica* O:3-positive dog to determine the optimum serum and conjugate dilutions for future assays.   Serum from dogs 13 and 14 were included as negative controls.

All wells in four 96-well Maxisorp Immuno plates (Nunc, Roskilde, Denmark) were inoculated with 100µl of Yops in coating buffer and the plate was left at 4°C overnight. Two of the plates were used for IgG determination and 2 for IgM.   The following day the antigen was removed by aspiration and the plates were washed four times in PBS/Tween 20 wash buffer. One hundred microlitres of each test serum, diluted 1 in 250 in PBS/Tween 20 plus 12 mg/ml sodium caseinate, were added to duplicate wells and incubated at 37°C for 1 hour. Sera were removed by aspiration and the plates were washed a further four times with wash buffer. One hundred microlitres of anti-dog IgG peroxidase conjugate (The Binding Site, Birmingham, England) diluted 1 in 1000 were added to each well of two plates and a similar volume and dilution of IgM conjugate was added to the other two plates. Plates were incubated at 37°C for one hour after which conjugate was removed by aspiration and a further four washes were performed.   One hundred microlitres of peroxidase substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA) were added to each well and the plates were left at room temperature until a blue colour developed (approximately thirty seconds). The reaction was stopped by the addition of 50µl of 2M sulphuric acid to each well and the plates were read in a Diagnostics Pasteur LP400 plate reader (Sanofi, Marnes-la-Coquette, France) at 450nm.   Results were expressed as the mean net OD of the duplicate wells after subtraction of the blank reading.

5.3   RESULTS

5.3.1   Clinical signs

Throughout the trial all dogs remained healthy, no clinical signs of infection were seen and all stools remained firm. Daily rectal temperatures of all dogs remained within the normal range (39 ± 0.5°C).

5.3.2   Pre-trial bacteriological culture of faeces

A number of *Yersinia* spp. were isolated from the dogs on three occasions during the week prior to the start of the trial (Table 5.1). These included *Y. pseudotuberculosis* serotype III (2 dogs), *Y. intermedia* (3 dogs), *Y. enterocolitica* biotype 1A (10 dogs), *Y. enterocolitica* bioserotype 2/O:9 (2 dogs) and *Y. enterocolitica* bioserotype 2/O:5,27 (6 dogs).   Only one dog (number 5) had no yersiniae isolated pre-trial.   No strains of *Y. enterocolitica* bioserotype 4/O:3 were isolated.

Eight dogs had more than one *Yersinia* species isolated during the pre-trial period, 5 of them having mixed infections on the first sampling occasion. Only one dog, number 1, had the same strain isolated from all three samples (bioserotype 2/O:5,27).

Table 5.1.   Isolation of *Yersinia* spp. from rectal swabs pre-trial

Dog Number	Sample 1	Sample 2	Sample 3
1	Ye 2/O:5,27	Ye 2/O:5,27	Ye 2/O:5,27
2	Ye 1A/Yi	Ye 2/O:5,27	Ye 2/O:5,27
3	-	Ye 2/O:5,27	-
4	Yp/Ye 1A	-	Ye 1A
5	-	-	-
6	Yp/Ye 1A	-	Ye 1A
7	-	-	Ye 1A
8	-	Ye 2/O:5,27	Ye 1A
9	-	-	Ye 2/O:9
10	Ye 1A	-	Ye 2/O:9
11	Yi/Ye 2/O:5,27	-	Ye 1A
12	-	-	Ye 1A
13	Yi	Ye 2/O:5,27	Ye 1A
14	Yi/Ye 1A	-	Ye 1A

Yp - *Y. pseudotuberculosis*; Ye - *Y. enterocolitica*; Yi - *Y. intermedia*

5.3.3   Duration of faecal shedding

The duration of faecal shedding by the dogs inoculated orally in Groups 1 and 2 (Nos. 1 - 6) varied (Table 5.2), ranging from 7-23 days (median 10.5 days).

Five of the six in-contact dogs (Nos. 7 - 12) excreted the organism following exposure to infected dogs (Table 5.2). One dog was positive on one occasion only (dog 9) and two were still positive at the end of the experiment (dog 11, 8 days shedding; dog 7, 26 days shedding).

In three dogs the organism was cultured for a longer period using cold enrichment than using direct culture. These were dog 1 (days 22-23), dog 7 (days 24-30), dog 10 (days 20-21) (Table 5.2).

At no time was the experimental organism detected in the faeces of the control animals (Nos. 13-14). In addition, dog 12 was culture negative throughout the trial.

The numbers of *Y. enterocolitica* in faeces collected from the floor of the pen housing Group I dogs were between 10^4 and 10^5 cfu/gm on each occasion.

5.3.4 Period from challenge to first isolation of yersiniae

Y. enterocolitica bioserotype 4/O:3 was isolated from the faeces of all six dogs on the day following experimental inoculation of the test organism. For the animals in-contact with the inoculated dogs, the time to the first faecal isolation of *Y. enterocolitica* was five days (dogs 7, 8 and 10), nine days (dog 11) and 12 days (dog 9). The median time from exposure to first isolation for in-contact dogs was 5 days.

5.3.5 Pharyngeal carriage

Y. enterocolitica bioserotype 4/O:3 was only isolated from the pharynx of two dogs orally inoculated at the beginning of the trial, dog 2 from days 2 to 7 and dog 3 on the first day only. All other pharyngeal samples were negative on culture.

Table 5.2. Duration of faecal shedding of *Y. enterocolitica* bioserotype 4/O:3

	Group I				Group II				Group III		Group IV		Group V	
Dog no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Days [δ]														
1-4	+	+	+	+	+	+	-	-	-	-	-	-	-	-
5-7	+	+	+	+	+	+	+*	+*	-	-	-	-	-	-
8-9	+	+	+	+	-	+	+	+	-	-	-	-	-	-
10-11	+	+	+	-	-	-	+	+	-	-	-	-	-	-
12	+	+	+	-	-	-	+	+	-	+	-	-	-	-
13-18	+	-	-	-	-	-	+	+	-**	+**	-	-	-	-
19	+	-	-	-	-	-	+	+	+	+	-	-	-	-
20-21	+	-	-	-	-	-	+	+	-	Φ	-	-	-	-
22	Φ	-	-	-	-	-	+	+	-	-	-	-	-	-
23	Φ	-	-	-	-	-	+	+	-	-	+	-	-	-
24-30	-	-	-	-	-	-	Φ	-	-	-	+	-	-	-
Total	23	12	12	9	7	9	26	19	1	10	8	0	0	0

δ - Day 1 is the first day after inoculation; + isolation by direct plating and cold enrichment; Φ isolation following cold enrichment only; - culture negative; * dogs transferred to Group III on day 7; ** dogs transferred to Group IV on day 14

5.3.6 Antibody response of infected and in-contact dogs to Yops

Table 5.3 shows the mean net OD's for IgG levels of all dogs before and during the trial. A strong serological response to the Yop antigens can be seen in all fourteen dogs pre-trial and in the four weeks post-infection. No distinction can be made between the control animals and those either infected or in-contact during the trial making it impossible to use these results to indicate sero-conversion following exposure to *Y. enterocolitica* 4/O:3. IgM levels were lower but the pattern was the same.

Table 5.3.   **Sequential IgG response to Yops in dog sera during the trial**

Dog No.	Pre-trial	Week 1	Week 2	Week 3	Week 4
1	1.94	1.91	1.86	1.83	1.75
2	1.8	1.66	1.94	1.76	1.66
3	1.81	1.6	1.76	1.71	1.66
4	1.9	1.65	1.8	1.81	1.59
5	1.97	1.77	1.71	1.37	1.23
6	1.81	1.8	1.7	1.78	1.66
7	1.7	1.54	1.31	1.11	1.52
8	1.86	1.75	1.3	1.17	1.07
9	1.91	1.79	1.88	1.8	1.57
10	1.94	1.85	1.93	1.88	1.74
11	1.64	1.78	1.88	1.91	1.7
12	1.91	1.85	1.87	1.71	1.64
13	1.96	1.9	1.82	1.69	1.66
14	1.81	1.56	1.25	1.11	0.81

5.4   DISCUSSION

5.4.1   Significance of *Yersinia* spp. isolated from dogs pre-trial

Prior to the start of the trial the experimental dogs were kept in kennels with access to a communal grassed enclosure for feeding and exercise. Three consecutive faecal samples were cultured from all the dogs and a range of *Yersinia* strains were isolated. The range of strains was similar to those recovered previously from farm animals in New Zealand (see Chapter 4) and may reflect environmental contamination by cattle, sheep and deer grazed in a paddock adjacent to the dog's run. This is supported by the transient nature of the carriage in all but one of the dogs. It is worth noting that our dogs were all 6-months of age at the time of the trial and sampling was carried out in winter. Fukushima *et al.* (1984b), also isolated a range of *Yersinia* strains from healthy dogs in Japan, more frequently in pups less than one year of age and during the colder months. Similarly, Kaneko *et al.* (1977), also noted that the highest prevalence of *Yersinia* carriage was in the 5- to 6-month-old age group.

Although some of the strains were considered environmental in origin (*Y. enterocolitica* biotype 1A and *Y. intermedia*), others were potential human pathogens (*Y. enterocolitica* bioserotypes 2/O:5,27 and 2/O:9 and *Y. pseudotuberculosis*). Human infections in New Zealand are usually associated with *Y. enterocolitica* bioserotype 4/ O:3, however, the other two potentially pathogenic bioserotypes isolated from the dogs have been recovered with increasing frequency from people (McCarthy and Fenwick, 1991). The detection of bioserotype 2/O:9 in two of the dogs was of interest since this strain has not been isolated from pigs in New Zealand and the source of human infection has not yet been identified. The only previous report of the isolation of this strain from dogs came from Japan in 1977, Kaneko *et al.* recovering it from 2 out of 451 healthy dogs (Kaneko *et al.*, 1977). Carriage of *Y. pseudotuberculosis* by dogs appears to be uncommon, however, Randall and Mair (1962) reported a family outbreak of *Pasteurella* (*Yersinia*) *pseudotuberculosis* infection associated with infection in a pet dog.

5.4.2 Clinical signs of infection in the study dogs

No clinical signs of disease were evident in our dogs and this is consistent with the findings in the majority of surveys in which *Yersinia* spp. were isolated only from apparently healthy subjects (Pedersen, 1976; Kaneko *et al.*, 1977; Yanagawa *et al.*, 1978; Pedersen and Winblad, 1979; Fukushima *et al.*, 1985a). Reports of clinical yersiniosis in dogs, which occurs sporadically, suggest that underlying stress factors may precipitate the development of disease, as they do in other animal species (Slee and Button, 1990a). A similar situation has been shown to occur with *Campylobacter* infections in dogs. Enteritis is occasionally diagnosed in young animals, although experimental infections with both canine and human isolates of *C. jejuni* have invariably failed to produce disease (Olson and Sandstedt, 1987; Macartney *et al.*, 1988; Boosinger and Dillon, 1992). Thus, while the organism has been recovered commonly from healthy dogs, it is not believed to be a primary canine pathogen but may exacerbate diseases caused by other organisms such as canine parvovirus (Newton *et al.*, 1988).

Nevertheless, other factors, such as the strain of *Y. enterocolitica* used to experimentally infect the dogs in this study, the route of infection or the age of the dogs, may have also contributed to the lack of clinical signs seen in this study. In the only other documented experimental infection of dogs, Adesiyun *et al.* (1990), administered *Y. enterocolitica* bioserotype 1B/O:8 to ten, 4-8 week-old puppies by three routes, oral (8), intraperitoneal (1) and intravenous (1). The puppies inoculated intravenously or intraperitoneally both died within 2-10 hours of infection, whereas 7 of the 8 orally infected puppies showed clinical signs of infection, including bloody diarrhoea, 2-3 weeks post-infection, but none died. The strain of *Y. enterocolitica* used had been isolated from a human patient in the USA and had been shown to cause diarrhoea in mice, a feature of biotype 1B strains that has been shown to be due to their increased virulence compared with strains of bioserotype 4/O:3 (Pearson *et al.*, 1979). Thus, in this case, either the experimental organism was more virulent or the younger age of the puppies made them more susceptible, or both. Since the completion of this study, *Y. enterocolitica* enteritis has been diagnosed in a further 9 dogs, involving bioserotypes 4/O:3 (4 cases), 2/O:5,27 (3 cases) and 2/O:9 (2 cases) (Fenwick, unpublished data). All of the animals were puppies a few weeks old, and in three cases involving

bioserotype 4/O:3, littermates were also reported to be infected. As the young animals were still suckling, it is possible that the stress of lactation brought about increased shedding of yersiniae by the dams, with subsequent infection of the puppies. Similar circumstances have been described previously with *C. jejuni* infections (Newton *et al.*, 1988). One of the animals affected with bioserotype 4/O:3 was also believed to be the source of infection for a 7-year-old girl in the same household who subsequently became ill with *Y. enterocolitica*, providing supporting evidence for the role of dogs in human yersiniosis.

5.4.3 Excretion of *Y. enterocolitica*

The duration of excretion is very important since long-term shedding increases the chance of dissemination of the organisms. Faecal shedding of *Y. enterocolitica* varied considerably between our experimental dogs, from 0-26 days, (median duration 9.5 days). It is not known whether this was continuous colonisation or reinfection from the environment, however, every effort was made to reduce environmental exposure by thorough daily cleaning of the kennels, although disinfectant was not used due to the presence of the dogs. Fantasia *et al.* (1993), monitored a kennel containing dogs from which *Y. enterocolitica* 4/O:3 had been isolated and found that the organism could persist for up to 20 months and could readily circulate between animals in the establishment via environmental contamination.

All pathogenic *Yersinia* spp. share common virulence-associated outer membrane proteins that induce a serological response (Vogel *et al.*, 1993), therefore the variation in faecal shedding could have been associated with the previous immune status of each of the dogs. This is supported by the finding that all dogs had high levels of IgG to Yops in pre-inoculation blood samples. However, no correlation was found between pre-trial carriage of potentially virulent yersiniae by the dogs and duration of shedding of the challenge organism. Similar findings were reported by Adesiyun *et al.* (1990), who found that orally infected dogs shed *Y. enterocolitica* for varying periods from 1 to 42 days post-exposure. Boosinger *et al.* (1992), found that dogs infected orally with *C. jejuni* also shed the organism in the faeces for up to 4 weeks and concluded that this enabled them to transmit the infection to other dogs and potentially to people.

5.4.4 Period from challenge to excretion of yersiniae

The period from challenge of the dogs to isolation of the organism from the faeces varied with the method of exposure. Animals that were orally inoculated all excreted the organism after 24 hours. This was consistent with the findings of Adesiyun *et al.* (1990). However, the first animals that were in-contact with the artificially infected dogs did not start excreting detectable numbers of organisms for 5 days. This period was extended even further with subsequent groups, the mean period from challenge to isolation for all dogs being 7.2 days. This suggests a progressively lower infectious dose for the in-contact dogs, although the numbers of *Y. enterocolitica* excreted in the faeces was not determined in dogs in Groups II to IV. Fukushima *et al.* (1984b), counted up to 10^7 cfu/g from the intestinal contents of asymptomatic dogs carrying *Y. enterocolitica* bioserotype 4/O:3. However, since the minimum infectious dose for *Y. enterocolitica* in dogs is not known the significance of these figures remains unclear. While studies on the recovery of *Y. enterocolitica* from dog faeces in public parks have not been performed, campylobacters have been isolated and have been shown to survive in faeces in parks for several weeks (Newton *et al.*, 1988). It is not unreasonable to expect, therefore, that *Yersinia* spp. could also survive well in a contaminated environment, acting as a source of infection for other dogs and perhaps infants.

5.4.5 Pharyngeal carriage of yersiniae

Pharyngeal carriage of *Y. enterocolitica* bioserotype 4/O:3 is common in pigs and the tonsils are believed to constitute the primary reservoir tissue (Kapperud, 1991), thus the possibility of a similar nidus in dogs was of interest. Pharyngeal carriage was detected, however, only one of two dogs (No. 2) carried the organism in the pharynx for an appreciable length of time (6 days). This could be due to the method of sampling employed, namely throat swabbing, because in pig surveys whole tonsils are usually removed, macerated and cold enriched for three weeks. Yersiniae are believed to colonise the tonsillar crypts in low numbers, therefore swabbing of the pharynx in live dogs may not be a particularly sensitive method of detection. Nevertheless, the finding of the organism in the pharynx of two dogs, one for nearly a week, suggests that colonisation of the throat is possible and that the licking action of dogs may transmit the organism, particularly to young children.

5.4.6   Antibody response to *Yersinia* infection

When the trial was designed it was expected that sera both from the dogs pre-trial and from the control animals during the trial would act as negative control sera for the serological assay. Unfortunately, a number of dogs were found to be infected with plasmid-bearing *Yersinia* strains before the trial started and this interfered with the attempt to use seroconversion in the infected animals to monitor the progression of infection. Robins-Browne *et al.* (1993a), who developed the Yop assay, used sera from lambs that had been bacteriologically negative for plasmid-bearing *Yersinia* since birth as negative controls to establish criteria for a positive titre (net OD value > 2 SD above the mean negative value) and for seroconversion (an increase in net OD of > 2SD). This was not possible in the present study and although the OD values calculated and the speed of colour development in the assay showed what was believed to be a strong serological response to Yops in all the dogs, without the benefit of negative control sera the results were inconclusive. Nevertheless, subsequent immunoblotting against Yops confirmed that the dog sera were uniformly positive (Bordun, personal communication) and with the inclusion of negative control sera, the ELISA used in this study could be a valuable epidemiological tool for screening large numbers of dogs for Yop-specific antibodies, to identify those that have been exposed to human pathogenic yersiniae.

5.4.7   Conclusion

In conclusion, the results of this study indicate that dogs may act as a possible source of *Y. enterocolitica* infection for humans because transmission occurs readily between dogs and faecal excretion of the organism can last for several weeks, leading to contamination of the environment. Stray dogs and dogs with diarrhoea may represent a greater threat, as they have been shown to excrete campylobacters more consistently and in greater numbers than in subclinically infected household dogs (Malik and Love, 1989). In addition, pharyngeal carriage has been shown to occur and thus the licking behaviour of pet dogs could conceivably transmit the organism. Infants and young children are probably most at risk of infection from canine sources as they are more likely to spend time on the ground and to come into contact with dog faeces. As *Y. enterocolitica* infections are most prevalent in

young children (Lee *et al.*, 1991), the possible role of dogs in the epidemiology of human yersiniosis should not be overlooked and may in fact warrant further attention. In addition, the public health implications of owning a dog should be widely publicised and a good example of such an approach comes from Denmark, where postage stamps showing the picking up and disposal of canine faeces have been used to promote public awareness of good dog hygiene (Locke, 1992)!

CHAPTER 6

6. DEVELOPMENT OF RAPID METHODS FOR THE IDENTIFICATION OF VIRULENT *YERSINIA ENTEROCOLITICA*

6.1 INTRODUCTION

The processing and evaluation of samples for bacterial enteric pathogens is one of the most labour-intensive and least cost-effective procedures performed in a diagnostic laboratory, involving isolation from other bacteria on selective media, testing of individual colonies in a variety of biochemical reactions and agglutination with specific antisera (Koplan *et al.*, 1980; Guerrant *et al.*, 1985). The isolation of *Yersinia enterocolitica* is particularly difficult because of the need for a lengthy cold-enrichment procedure and special selective media, taking anything from one to four weeks for final confirmation of identity (Kachoris *et al.*, 1988). In addition, the presence of many non-pathogenic strains of the organism necessitates further biochemical and serological characterisation in order to ascertain the potential virulence of isolates. Thus, reducing the time required to identify potentially pathogenic strains would help clinicians to quickly select the most efficacious therapy for patients and would reduce the overall laboratory costs associated with processing specimens in clinical laboratories.

Initially, the aim of this project was to construct a non-radioactive, plasmid-based probe that could be used in place of the time-consuming, routine laboratory procedures for identification of pathogenic *Y. enterocolitica* in clinical samples. This would then enable more rapid analysis of larger numbers of samples during epidemiological investigations and infection trials, such as those reported in previous chapters. Subsequent to development of the probe, a decision was made to design a PCR assay based on detection of the *ail* gene, in an effort to improve on the specificity and sensitivity obtained with the probe.

6.2   MATERIALS AND METHODS

A - DEVELOPMENT OF A DNA PROBE

6.2.1   Bacterial strains used for plasmid screening

Strains of *Y. enterocolitica* (n = 80) were examined in a mini-prep assay based on the method of Portnoy *et al*. (1981), to establish the presence of the virulence plasmid in New Zealand isolates of the organism. Strains used were of human or porcine origin, recovered during the surveys described previously (Chapters 2 and 3), and had been stored in 15% glycerol broth at -70°C after full phenotypic characterisation. Frozen isolates were resurrected onto blood agar and incubated for 24h at 27°C before plasmid isolation. Strains that were examined belonged to *Y. enterocolitica* bioserotype 4/O:3, bioserotype 2/O:5,27 and biotype 1A.

5.2.2   Plasmid screening assay

Yersinia strains were grown overnight in 5ml of L-broth at 30°C. A quantity of each culture (1.5ml) was aliquotted into microcentrifuge tubes (Eppendorf) and centrifuged for 5min at 12,000rpm. The supernatant was discarded and the cells were washed twice with 1ml TE (50mM tris hydrochloride (Tris): 10mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) and resuspended in 40µl TE pH 8.0. Cells were transferred to a fresh tube containing 600µl lysis buffer (TE plus 4% sodium dodecyl sulphate (SDS), pH 12.4) and were mixed rapidly by inversion before being incubated at 37°C for 20min. 130µl of 2M Tris, pH 7.0 was then added to each tube to reduce the pH to 8.0-8.5. DNA was precipitated by the addition of 160µl of 5M sodium chloride (NaCl) and the samples were chilled on ice for 1-2h before being centrifuged at 12,000rpm for 10min. Approximately 550µl supernatant was removed from each tube into fresh tubes containing an equal volume of cold isopropanol, and placed at -20°C for at least 30min. The DNA was precipitated by centrifugation at 12,000rpm for 5min at 4°C, the supernatant was discarded, 1ml of ice-cold ethanol was added and tubes were left on ice for 5min. The ethanol was decanted and the

tubes were dried under vacuum for 15min. Pellets were resuspended in 30μl TE pH 8.0 and 7μl of each sample was subjected to electrophoresis in a Tris borate buffered (TBE), 0.7% agarose gel for 1-1.5h at 100V. Gels were stained with ethidium bromide (EtBr) for 30min, visualised under UV light and photographed. DNA suspensions were frozen at -20°C until required.

6.2.3   Bulk preparation of plasmid DNA from a strain of *Y. enterocolitica* O:3

Following screening, a plasmid-bearing strain of *Y. enterocolitica* bioserotype 4/O:3 from a human enteric infection (freezer ref. C41) was chosen to prepare bulk plasmid DNA (pYV) for restriction endonuclease digestion and cloning into the vector pGEM-2 (Promega Corporation, Madison, Wisconsin, 53711-5399, USA). The method used for plasmid isolation was a modification of the method of Portnoy *et al.*(1981) used earlier for screening.

A single colony of C41 was inoculated into 5ml L-broth and incubated overnight at 30°C with shaking. From the resulting culture, 2.5ml was inoculated into 250ml L-broth and incubated overnight with shaking. The broth was poured into a sterile GSA rotor flask and spun in a Sorvall RC5C centrifuge for 10min at 9,000rpm and 4°C. The supernatant was decanted and the pellet resuspended in 250ml cold TE pH8.0 before being subjected to a further spin for 10min at 9,000rpm and 4°C. The pellet was resuspended in 4ml cold TE pH 8.0 and the resulting suspension was divided and each portion added to 30ml lysis buffer in sterile Falcon tubes (Becton Dickinson Labware, Lincoln Park, New Jersey, 07035, USA). The tubes were mixed immediately by inversion and incubated in a waterbath at 37°C for 30min. Approximately 1-2ml fresh 2M Tris pH7.0 was added to each tube to reduce the pH to 8-8.5. Sixteen millilitres of NaCl was added to each tube, mixed gently by inversion and chilled on ice for 1h. Tubes were then spun for 10min at 3,500rpm and 4°C. Supernatant from both tubes was pooled, spun again and the resulting supernatant decanted into two sterile SS34 centrifuge tubes (approximately 18ml in each). An equal volume of cold isopropanol was added to each tube and they were placed at -20°C for 2h. A 0.5ml aliquot of the supernatant was spun, washed, dried and electrophoresed through a 0.7% agarose minigel to check that the plasmid had been successfully isolated. The SS34

tubes were centrifuged for 30min at 10,000rpm and 4°C and the supernatant discarded. The pellets were washed with 70% ethanol, drained, vacuum dried for 20min and resuspended in 220µl TE pH 8.0 at 37°C. The contents of both tubes were pooled into a microcentrifuge tube, resulting in 400µl DNA suspension.

Tracking dye (20µl) was added and the total suspension was subjected to electrophoresis in a 0.7% agarose gel for 12.5h at 60V and a further 4h at 100V. After staining with EtBr for 30min, the gel was visualised under long-wave UV light and the plasmid band removed into dialysis tubing. The gel slice was electroeluted in 0.5xTBE at 50V for 15h, followed by 90V for 1.5h. The current was reversed for 1.5min before completion. Three millilitres of the plasmid suspension was removed from the tubing and spun in Centricon 30 microconcentrator tubes (Centricon Amicon Inc., Beverly, Massachusetts, 01915, USA) for 20min at 6,000rpm and 4°C, resulting in 53µl of suspension. A 5µl aliquot was run on a 0.7% agarose minigel and visualised.

The remaining 48µl of suspension was digested overnight at 37°C with 2µl (20U) *Bam*H1 restriction endonuclease (Boehringer Mannheim GmbH, Mannheim, Germany) in a total volume of 500µl digestion buffer. After the digestion, the DNA was divided into two microcentrifuge tubes and precipitated by the addition of 1/10 vol. 3M sodium acetate and 2.5 vols. cold 95% ethanol. At the same time 20µl (4µg) of pGEM-2 plasmid DNA prepared earlier was also digested with 2µl of *Bam*H1 at 37°C O/N. The resulting pGEM-2 digest was treated with 1µl alkaline phosphatase (Boehringer Mannheim, Cat. No. 713023) to prevent the ends from rejoining, the DNA was purified by phenol/chloroform extraction, and precipitated by sodium acetate/ethanol as before. Both digests (pYV and pGEM-2) were centrifuged at 12,000 rpm for 30min at 4°C, washed with 70% ethanol and vacuum dried. The pYV pellets were each resuspended in 10µl TE pH 7.5 and the pGEM-2 pellet in 50µl TE pH 7.5. The digests were stored at -20°C until required.

6.2.4   Ligation of pYV into the vector pGEM-2

The *Bam*H1-digested fragments of interest for use as a probe were located in the calcium-dependent region of the plasmid, in the range 4-5.2kb (Portnoy *et al.*, 1984), with a mean fragment size of 4.6kb. As pGEM-2 was 2.9kb, the ratio of pYV to pGEM-2 was required to be approximately 1:1.6 to achieve equimolar ends in the ligation reaction. In addition, the manufacturers of the DH5α maximum efficiency competent cells (Bethseda Research Laboratories Life Technologies Inc., Cat. No. 8258SA) advised that for efficient transformation the ligation reaction should be diluted 5-fold, and to avoid saturation of the cells the DNA concentration should be 1-10ng. Taking all these parameters into account, the 20μl ligation reaction included the following: 10.5μl pYV (210ng); 1.5μl pGEM-2 (120ng); 2μl 50mM DTT; 2μl 10mM ATP; 2μl 10x ligation buffer (1M Tris pH 7.5, 1M Magnesium chloride); 1μl T4 DNA ligase; 1μl distilled water   A control ligation was also set up without the pYV DNA, to test the efficiency of the alkaline phosphatase treatment. The ligations were incubated at 12°C for 16h and then stored at 4°C until required.

6.2.5   Transformation of the DH5α competent cells

One vial of competent cells was thawed on ice, and divided into 2x100μl aliquots. A 5μl aliquot of the ligation reaction was diluted 1 in 5 with TE 10:1 pH 7.5, to achieve an estimated final DNA concentration of 3.3ng/μl. A 2μl aliquot of the diluted DNA from each ligation was added to the competent cells, gently mixed and left on ice for 30min. The cells were then heat-shocked at 42°C for 45s and replaced on ice for 2min. The mixture was made up to 1ml with S.O.C. (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl$_2$, 10mM MgSO$_4$, 20mM glucose), giving a DNA concentration of 6.6x10^{-3} ng/μl, and was shaken for 1h at 225rpm and 37 C.   Serial volumes of transformed cells, from 1-200μl, were plated onto LB agar plates (test plates) containing 100μg ampicillin/ml and incubated overnight at 37°C. Colonies were counted and the transformation efficiency (CFU/μg) calculated. Bacterial growth was harvested from some of the test plates using sterile swabs, suspended in 5ml 65% glycerol broth (glycerol, 0.1M magnesium sulphate, 0.025M Tris pH 8.0) and 1ml aliquots were stored at -70°C until required.

6.2.6 Screening of transformants for the presence of plasmid inserts

The rapid boil method of Holmes and Quigley (1981) was used to isolate plasmid DNA from the transformants in order to select those carrying inserts in the required range (4-5kb). Five colonies were randomly selected from one of the test plates (samples 1-5) and one from the control plate (sample 6), each was inoculated into 5ml L-broth containing $100\mu g/ml$ ampicillin and the cultures were incubated at 37°C overnight with shaking. A 1.5ml aliquot of each culture was transferred to an eppendorf tube and centrifuged for 20s, the supernatant was removed and the pellet was resuspended in $350\mu l$ of HQ-STET (50mM Tris pH 8.0, 50mM EDTA, 8% sucrose, 5% Triton X-100). Freshly prepared lysozyme ($25\mu l$, 10mg/ml) was added to the tube and vortexed briefly before being left at room temperature for 5min. The tube was placed in boiling water for 45s and then centrifuged for 10min at room temperature. The pellet was removed with a sterile toothpick and the supernatant transferred to a fresh tube. An equal volume of cold isopropanol was added and the mixture was left at -20°C for 30min. After centrifugation for 5min at 4 C, the supernatant was removed, the pellet was washed with 95% ethanol, spun, drained and vacuum dried for 5min. Pellets were resuspended in $50\mu l$ TE 10:1 pH 8.0 and stored at 4°C. A $5\mu l$ aliquot of each DNA solution was digested with *Bam*H1 for 1h and $10\mu l$ of each digest ($2\mu l$ DNA) were electrophoresed through a 0.7% seaplaque gel, stained with EtBr and photographed. Lambda DNA 1kb ladder (Gibco BRL, Gaithersburg, Maryland, USA) was run on the gel as a reference marker. Seven hundred microlitres of each original broth was mixed with an equal volume of 65% glycerol in an eppendorf tube and stored at -70°C.

6.2.7 Purification of recombinant DNA for use as a probe

One of the samples (sample 5) had an insert of approximately 4-5kb and was chosen as the candidate for a probe. Cells were cultured from the frozen broth onto L-agar plus ampicillin ($100\mu g/ml$) and 10 individual colonies were inoculated into L-broth plus ampicillin for plasmid isolation by the rapid boil method described above. After plasmid isolation and digestion, aliquots of DNA were electrophoresed and photographed. Five of

the samples were pooled, giving a total of 145µl. This was treated with RNAse, extracted once with phenol-chloroform then once with chloroform and concentrated in a Centricon tube, giving a final volume of 55µl.

6.2.8 Non-radioactive labelling of the probe DNA

DNA was labelled using the Chemiprobe system (Orgenics Ltd., Yavne, Israel), a non-radioactive method whereby cytosine in the denatured DNA is sulphonated and, after probing, is visualised in a sandwich immune reaction using a specific monoclonal antibody to the sulphone groups, producing a chromogenic substrate.

The DNA extracted in section 6.2.7 was boiled for 7.5min and placed on ice. Modification solution A (27.5µl) was added, followed by modification solution B (6.9µl) and the resulting mixture was incubated at 25°C overnight. t-RNA (4.5µl) (Sigma chemicals, R4251) was added to the mixture, giving a final concentration of 100ng/µl. The DNA was precipitated with 2.5 volumes of cold 100% ethanol, dried, and resuspended in 120µl TE pH 7.5. Aliquots (40µl) of the labelled DNA were stored at -20°C until required.

In addition, 50µl of pGEM-2 DNA without the insert was also labelled as above and aliquots stored at -20°C for use in comparative experiments with the pYV probe.

6.2.9 Preparation of colony and DNA blots for probing

Colony blots:

(i) Thirty-three bacterial strains were grown in L-broth at 25 or 37°C overnight, including *Y. enterocolitica* biotype 1A, bioserotypes 2/O:5,27, 2/O:9 and 4/O:3, *Y. frederiksenii, Escherichia coli, Proteus mirabilis, Salmonella typhimurium* and *Klebsiella pneumoniae*. 1ml of each culture was pipetted into an eppendorf tube, centrifuged and the pellet washed with TE pH 7.5. Pellets were resuspended in 100µl TE pH 7.5 and 5µl of each isolate were spotted onto a nitrocellulose strip (12.5 x 7.5cm). The DNA was

denatured by soaking the nitrocellulose in denaturing solution (1M NaOH, 0.5M NaCl) for 4min and then in neutralising solution (1M Tris, 3M NaCl) for 6min. After a rinse in 2xSSC the strips were dried, baked for 3h at 70°C in a vacuum oven and stored in the dark with silica gel.

(ii)   Further colony blots were made using 10 known plasmid +ve and plasmid -ve strains of *Y. enterocolitica* (5 each of bioserotype 4/O:3 and biotype 1A) grown overnight on an L-agar plate at 25°C. Nitrocellulose filters (Schleicher and Schuell BA85, 82mm) were placed on the surface of the plate and a sterile glass spreader used to smooth out any air-bubbles. The filters were left in place for 2h, removed, and the DNA denatured, neutralised and fixed as described above.

DNA blots:

The following DNA samples were prepared and 1μl of each was dotted onto nylon blotting membrane (Hybond-M, Amersham), which was denatured and neutralised as described above. Fixing was by exposure to UV light for 2.5min.

(i)   Ten-fold dilutions (1000-0.5pg) of labelled salmon sperm DNA of known concentration, supplied with the kit as a control.

(ii)   DNA from plasmid +ve *Y. enterocolitica* strains (bioserotype 4/O:3) and plasmid -ve *Y. enterocolitica* strains (biotype 1A), isolated during the plasmid mini-preps. Labelled probe was used as the positive control and TE pH 7.5 as the negative control.

(iii)   Plasmid and chromosomal DNA isolated from 30 plasmid +ve (P+ve, bioserotype 4/O:3) and plasmid -ve (P-ve, biotype 1A) strains of *Y. enterocolitica* (see 6.2.2 and 6.2.14), and 1μl of each dotted together on the membrane. Controls used were as above.

6.2.10 Probing of nitrocellulose and nylon strips

Prepared filters (either nitrocellulose or nylon) were placed briefly in a container with 6xSSC before being sealed in a plastic bag with 5ml pre-hybridisation solution (0.2% Ficoll, 0.2% PVP, 0.2% BSA, 6xSSC) and incubated for 3h at 68°C with shaking. Labelled probe pYV-1 (40μl) was thawed, 8μl of herring sperm DNA was added, and the mixture was placed in boiling water for 5min, then onto ice. The filter was removed to a clean plastic bag with 2ml hybridisation solution (0.2% PVP, 0.2% Ficoll, 0.2% BSA, 1M NaCl, 50mM Na$_2$PO$_4$, 2mM EDTA, 0.5% SDS, distilled water). The probe was added to the hybridisation solution, and the bag was then sealed and incubated overnight at 68°C with shaking. The filter was removed from the bag and subjected to 3 low stringency washes (2xSSC, 0.1% SDS), one at room temperature for 15min and two at 68°C for 15min each, followed by two high stringency washes (0.2xSSC, 0.1% SDS) at 68°C for 5min each. The filter was allowed to dry for 15-20min.

Labelled pGEM-2 probe without the insert was also used to probe colony blots and DNA blots from P+ve and P-ve strains for comparison with the pYV probe.

6.2.11 Visualisation procedure

Each filter was placed in a clean plastic bag with 4ml blocking solution (made up from supplied solutions) and incubated at 25°C for 1h. Mouse monoclonal antibody against modified DNA (16μl) was then added to the bag, mixed, and incubated at 25°C for a further hour. The filter was removed from the bag, given 3 x 5min washes in 0.1M NaCl, 0.3% Brij, and placed in a clean bag with 4ml blocking solution. Alkaline phosphatase anti-mouse Ig conjugate (16μl) was added to the bag, mixed, and incubated at 25°C for 2h. A further three washes, 10min each, were performed and the filter removed to a fresh plastic bag. The colour reagent (16mg NBT glucose, 20μl BCIP, 4ml substrate buffer) was added to the bag, mixed, and the bag placed in the dark at 37°C for 3h.

B - DEVELOPMENT OF THE POLYMERASE CHAIN REACTION

6.2.12 Primer selection for the polymerase chain reaction

The *ail* locus is found uniquely in pathogenic strains of *Y. enterocolitica* (Miller *et al.*, 1989) and the publication of it's complete sequence in 1990 (Miller *et al.*, 1990) enabled a pair of primers to be designed that would allow amplification of a defined region by the PCR. A number of criteria were followed for the selection of appropriate primers in order to give efficient amplification. These were adapted from a primer selection algorithm published by Lowe *et al.* (1990) as follows:

(i)   The length of both primers should be between 18-22 nucleotides.

(ii)   If possible, primers should contain a GC-type sequence at their 3' end.

(iii)   Each primer should have a GC-type sequence content of between 45-55%.

(iv)   Primers should not contain more than four contiguous base pairs of homology to themselves or to each other, particularly at the 3' ends.

(v)   Both primers should have comparable melting temperatures.

(vi)   The amplification product should be less than 500bp.

Using these criteria as far as possible, 2 primers were chosen from the *ail* gene sequence that would produce an amplified product of 472bp (548-1019) (see Appendix 3)

Primer 1:   5' GTG TAC GCT GCG AGT GAA AG 3' (position 548-567)
                (Length 20 bases, Td 62°C, GC content 55%)

Primer 2:   5' ATC GAT ACC CTG CAC CAA GC 3' (position 1000-1019)
                (Length 20 bases, Td 62°C, GC content 55%)

A further set of oligonucleotide primers were designed using the same criteria, for use in the nested primer assay described in Chapter 7. These amplified a 359bp portion of the *ail* gene (576-934) (see Appendix 3)

Primer 3:   5' CTA TTG GTT ATG CGC AAA GC 3' (position 576-595)
(Length 20 bases, Td 58°C, GC content 45%)

Primer 4:   5' TGG AAG TGG GTT GAA TTG CA 3' (position 915-934)
(Length 20 bases, Td 58°C, GC content 45%)

The oligonucleotides were synthesised by the Centre for Gene Technology, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand and were reconstituted in sterile distilled water to give a 200µM solution of each primer. Primers were stored in aliquots of 50µl at -20°C until required and were diluted 1:10 before use.

6.2.13 PCR reaction components

Reactions were performed in either 50 or 100µl volumes, containing the following -

Addition order (µl)	50µl	100µl	Final concentration
Distilled water	26.5-30.5	58.5-62.5	-
10x reaction buffer	5	10	(1x)
dNTP's[1]	8	16	200µM each
Primer 1 (Ail A)[2]	2.5	5	1.0µM
Primer 2 (Ail B)	2.5	5	1.0µM
Taq DNA polymerase[3]	0.25	0.5	1.125-2.25U
Template	1-5	1-5	

1.   Deoxyribonucleoside triphosphates. A mixture of these (dATP, dCTP, dGTP, dTTP) was made containing 10mM of each in 1ml distilled water, final concentration 1.25mM. This was frozen at -20°C, thawed before use and 5 or 10µl added into the reaction as shown above, to give 200µM of each dNTP.

2. Primers were thawed before use and diluted 1:10 for addition to the reaction mix.

3. The enzyme was supplied at a concentration of 4.5U/µl (Promega Corporation) and was stored at -20°C until required.

Master mixes of all reagents were made and aliquotted into 0.5ml PCR reaction tubes (Perkin Elmer, Norwalk, Connecticut, 06859, USA) to avoid reagent losses. Sterile mineral oil (50µl) was added to each tube to reduce evaporation, and template was added through the mineral oil using aerosol barrier pipette tips (ART 20P - Molecular Bio-Products Inc., San Diego, California, 92121, USA). Tubes were kept on ice until template had been added and were then placed in the thermocycler for reactions to proceed. Separate rooms, equipment, reagents and disposables were used for making up reagent mixtures, adding template, running the PCR and visualising the products.

6.2.14 Templates for amplification

Initially, chromosomal DNA isolated from pathogenic and non-pathogenic *Y. enterocolitica* strains was used as the template in the PCR. Later, however, aliquots of overnight bacterial broth cultures were diluted 1:100 with distilled water and either boiled for 10min before addition, or were added direct to the reaction mixtures. Bacterial cells were used for the majority of assays in preference to DNA, as the principal aim of developing the PCR was to design a test for use directly with infectious material.

For DNA preparation, isolates were grown overnight at 25°C and 1ml was pelleted in an eppendorf tube. The pellet was resuspended in 100µl of 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0 (Solution 1) to which 0.4mg lysozyme had been added, and left at room temperature for 5min. Solution 2 (200µl) containing 0.1M NaCl, 0.1M Tris pH 8.5, 1%SDS was added and the suspension left on ice for a further 5min. Fresh Proteinase K 15mg/ml, 1µl, was added (final concentration 50µg/ml) and incubated at 37°C for 2h. 300µl phenol/ chloroform/ isoamyl alcohol (25:24:1) was then added, vortexed for 1min and centrifuged for 2min at room temperature. The supernatant was removed to a fresh

tube and the phenol/chloroform extraction was repeated. A 1/10 volume of 3M Sodium acetate and 2 volumes cold 95% ethanol were added to precipitate the DNA and the mixture was stored at -20°C overnight. Preparations were centrifuged for 10min at room temperature, the alcohol was decanted and the pellet was washed with 1ml 70% ethanol. After a 5min centrifugation, the alcohol was removed and a further wash step performed. Finally, the DNA was vacuum dried for 10min and resuspended in 200μl TE (10:1) plus RNAse. Aliquots (5μl) were electrophoresed in a 1% agarose gel at 90V for 30min, stained with EtBr and visualised. DNA was diluted 1:10 in distilled water and 5μl (= 0.5μl neat DNA) used as the template in the PCR. For negative controls, template was omitted and the volume of distilled water was increased accordingly. Once amplification was completed, aliquots of each reaction (usually 10μl) were electrophoresed in a 1% agarose gel at 90V for 30min. Gels were stained in EtBr and photographed.

6.2.15 Optimisation procedures

The sensitivity and specificity of the PCR are affected by magnesium concentration, denaturation, annealing and extension times, annealing temperature and the number of amplification cycles. Therefore, preliminary test reactions were carried out using Primers 3 and 4 initially to determine the optimum conditions.

(i)   Magnesium concentration

Magnesium concentrations in the reaction were increased by the addition of sterile $MgCl_2$ from 1.5mM to 9mM in increments of 1.5. Boiled *Y. enterocolitica* culture (5μl) was used as the template in each reaction. After amplification, aliquots of the reaction mixtures were electrophoresed in a 1% agarose gel and photographed.

(ii)   Denaturation, annealing and extension times and temperatures

A standard temperature of 94°C for 1min was chosen for denaturation. A 2min annealing step at 50°C was chosen initially, however, the time was reduced to 1min and the temperature increased to 55°C in order to enhance discrimination for the standard assay conditions. Primer extensions are typically performed at 72°C and 2min at this temperature was initially used for extension. The time was reduced to 1min for the standard assay conditions.

(iii)   Number of amplification cycles

The number of cycles can affect the yield of amplification product, with 30-35 cycles usually considered the optimum. Thirty cycles were chosen for the standard assay.

6.2.16 Standard PCR conditions

Amplifications were performed in 0.5ml PCR tubes in a programmable DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, Connecticut, USA). An initial 5min denaturation step was performed followed by 30 cycles of denaturation, annealing and extension. Different protocols were used for the two sets of primers as follows -

PCR steps	Primers 1/2	Primers 3/4
Denaturation	96°C/30s	94°C/1min
Annealing	60°C/1min	55°C/1min
Extension	72°C/1min	72°C/1min

After completion of the amplification cycles the tubes were cooled to 4°C to stop the reaction. Samples were stored at -20°C.

6.2.17 Sensitivity and specificity of the PCR

(i)   Sensitivity

The sensitivity of the PCR (defined as the lowest quantity of template detectable in the assay) was assessed using both extracted DNA and whole cells from a strain of *Y. enterocolitica* bioserotype 4/O:3 isolated from a human patient. Starting with a known quantity of DNA (2.5ng/μl), ten-fold dilutions were made from 0.25ng to 0.025pg and amplified in a standard PCR. Similarly, starting from a broth culture containing 10^8 cfu/ml, ten-fold dilutions were made from 10^{-1} to 10^{-7} and amplified in a standard PCR.

(ii)   Specificity

The specificity of the PCR (defined as the ability of the PCR to amplify only DNA from pathogenic *Y. enterocolitica*) was assessed using the following bacterial species and strains in a series of blind assays (n=120). Once results of the PCR were known, the identities of the target organisms were confirmed biochemically and serologically:

Y. enterocolitica biotype 1A (n=21), *Y. enterocolitica* bioserotype 2/O:5,27 (n=18), *Y. enterocolitica* bioserotype 2/O:9 (n=10), *Y. enterocolitica* bioserotype 3/O:1,2,3 (n=3), *Y. enterocolitica* bioserotype 4/O:3 (n=22), *Y. enterocolitica* bioserotype 5/O:2,3 (n=10), *Y. frederiksenii* (n=9), *Y. kristensenii* (n=2), *Y. intermedia* (n=4), *Y. pseudotuberculosis* (n=5), *Salmonella* spp. (n=2), *Pseudomonas* spp. (n=1), *Proteus* spp. (n=2), *Klebsiella* spp. (n=3), *E. coli* (n=4), *Enterobacter* spp. (n=1), *Streptococcus* spp. (n=1), *Listeria* spp. (n=1), *Rhodococcus* spp. (n=1).

6.2.18 Validation of PCR products

Two methods were used to validate the amplification product, restriction enzyme analysis and nucleotide sequencing.

(i)   Restriction enzyme analysis

The *ail*-gene sequence of *Y. enterocolitica* contained in GenBank was analysed using the GCG package (MapPlot and MapSort) and enzymes having only a single restriction site were identified. Of the 19 enzymes identified, only 2 were available commercially (*Taq*1 and *Mbo*II) and the enzyme *Mbo*II (BRL, 5241SA) was chosen as it gave larger fragments. DNA was amplified from pathogenic strains of *Y. enterocolitica* (bioserotypes 2/O:5,27, 2/O:9 and 4/O:3) using primers 3 and 4, and the product was precipitated with 5M ammonium acetate (1 vol) and 100% ethanol (2 vols), washed with 70% ethanol, vacuum dried and resuspended in 50µl TE (10:1). Digests were set up as follows: DNA 10µl, 10x restriction buffer (BRL React 1, 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$) 5µl, enzyme 2µl (16U), distilled water 33µl. Digests were incubated at 37°C overnight and 7µl of each reaction were electrophoresed in a 4% NuSieve gel (FMC Bioproducts, Rockland, ME, 04841, USA) at 80V for 2h, alongside 3µl of undigested product.

(ii)   Direct sequencing of PCR product

PCR product was generated from an overnight broth culture of an isolate of *Y. enterocolitica* bioserotype 4/O:3 from a human infection, using primers 1 and 2 in a standard 100µl reaction, without mineral oil. Following amplification, two tubes were pooled and the DNA purified using the Magic PCR Preps DNA purification system (Promega Corporation). To 100µl of direct purification buffer, 200µl of pooled amplified DNA was added and mixed. One millilitre of Magic PCR Preps resin was then added and vortexed briefly. The resin plus bound DNA was loaded into a mini-column using a 3ml disposable syringe and the column washed with 2ml column wash solution. The mini-column was attached to a microcentrifuge tube and centrifuged for 20s at 14,000 x g to dry the resin. 25µl of sterile distilled water was added to the column and left for 1min to elute the bound DNA. The column was transferred to a new microcentrifuge tube and centrifuged for 20s at 14000 x g. The purified DNA was stored at 4°C for use in the sequencing reaction.

Sequencing of the 472bp fragment was performed using a ssDNA sequencing kit (Sequenase version 2.0, United States Biochemical, Cleveland, Ohio, USA), based on the dideoxy chain termination method of Tabor and Richardson (1987). The method uses sequenase, a modified T7 DNA polymerase and ^{35}S dATP as the radiolabel source for the sequencing primer. The sequence was compared with other sequences held in the GenBank database, and was also compared with the *ail*-gene sequence in GenBank using the GCG package (BestFit).

6.3   RESULTS

A - DNA PROBE DEVELOPMENT AND TESTING

6.3.1   Plasmid screening assays

Eighty isolates of *Y. enterocolitica* recovered from pigs and people were examined for the presence of a virulence plasmid, including bioserotypes 4/O:3 (n=53) and 2/O:5,27 (n=17) and biotype 1A (n=10). Results of the assays can be seen in Table 6.1. Of the 70 pathogenic bioserotypes examined, 12 were negative in the assay (17%). None of the biotype 1A strains contained a plasmid in the 70kb size range.

Table 6.1.   Plasmid analysis of porcine and human strains of *Y. enterocolitica*

Bioserotype	Human	Pig	Total
4/O:3 P+ve	41	7	48
4/O:3 P-ve	4	1	5
2/O:5,27 P+ve	6	4	10
2/O:5,27 P-ve	5	2	7
1A P-ve	7	3	10
Total	63	17	80

P+ve = 70kb plasmid isolated; P-ve = no visible plasmid

6.3.2   Bulk preparation of plasmid DNA from *Y. enterocolitica* bioserotype 4/O:3

Following the plasmid isolation protocol, a minigel was run to check that plasmid DNA had been recovered. An aliquot of the plasmid solution (5μl) was electrophoresed through a 0.7% agarose gel alongside 5μl of pBR322 20ng/μl standard to estimate the amount of plasmid DNA recovered. The concentration was estimated as 10-20ng/μl.

Both pYV and pGEM-2 were digested with *Bam*H1 and aliquots of both digests were run alongside pBR322 standards (5-40ng DNA) to estimate DNA concentrations prior to ligation. From the resulting photograph, pYV was estimated at approximately 20ng/μl and pGEM-2 at 80ng/μl (Figure 6.1).

Figure 6.1.   Estimation of plasmid concentration
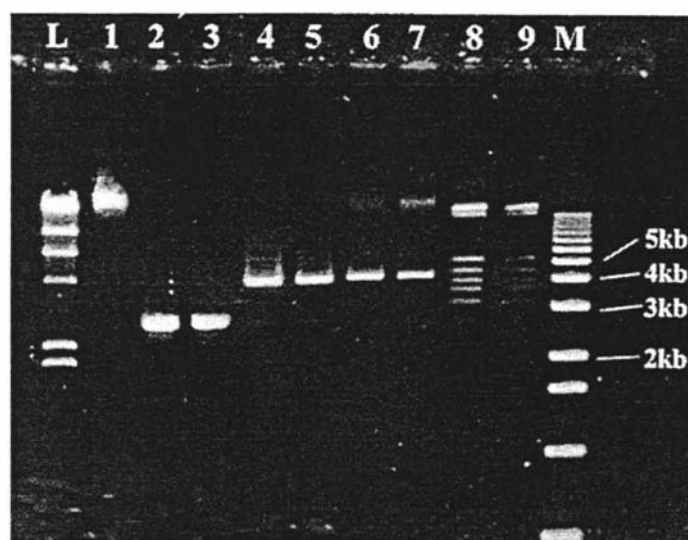
Lane no: L - λ DNA/ *Hind* III fragments (BRL 5612SA); 1 - *Y. enterocolitica* (Ye) 4/O:3 chromosomal DNA; 2 & 3 - pGEM-2 digested with *Bam*H1, 2 and 1 μl; 4 - pBR322 40ng standard; 5 - pBR322 20ng standard; 6 - pBR322 10ng standard; 7 - pBR322 5ng standard; 8 & 9 - *Yersinia* virulence plasmid (pYV) digested with *Bam*H1, 2 and 1 μl; M - molecular weight marker, 1kb DNA ladder (BRL 5615SB), (selected band sizes shown in base pairs)

6.3.3   Transformation of DH5α competent cells

Colony counts of transformed DH5α competent cells on the test and control plates are shown in Table 6.2. Ideally, no colonies should have grown on the control plates, however, the low numbers are probably due to pGEM-2 that had re-ligated because the alkaline phosphatase treatment was not 100% effective. From these figures, the transformation efficiency was calculated as 1.3×10^9 CFU/μg.

Table 6.2.   Colony counts of transformed cells

Plating volume	Control CFU	Test CFU
1μl	2	53
5μl	6	223
10μl	12	Many
50μl	97	Many
100μl	154	Many

6.3.4   Screening of transformants

Plasmid DNA was isolated from five separate colonies (samples 1 to 5) from one of the test plates and was subjected to digestion with the restriction endonuclease *Bam*H1, in order to identify clones containing an insert of approximately 4-5kb (containing the highly conserved calcium-dependent region). Figure 6.2 shows the results of the digestions, with transformants carrying inserts of 0.5-1kb (lanes 9 and 10) and 4-5kb (lanes 8, 11 and 12) visible on the gel. Lanes 2-6 show the corresponding uncut plasmids. Lanes 1 and 7 show uncut and cut control pGEM-2 without any inserts.

The 4.5kb fragment from sample 4 in lane 11 was chosen for labelling as a probe. Figure 6.3 shows the results of plasmid isolation and digestion from ten individual colonies of this clone. By comparison with *Bam*H1-digested pGEM-2 (80ng/μl), the concentration of plasmid DNA from the selected clone was estimated as 320ng/μl.

Lane no: L - λ DNA; 1 - undigested control pGEM-2; 2-6 - undigested plasmid DNA, samples 1 to 5; 7 - control pGEM-2 digested with *Bam* H1; 8-12 - plasmid DNA from samples 1 to 5 digested with *Bam* H1 **(lanes 8, 11 and 12 show an insert of approximately 4-5kb)**; M - molecular weight marker, 1kb DNA ladder (selected band sizes shown in bp)

Lane no: L - λ DNA; 1 - pGEM-2 control digested with *Bam* H1; 2 - blank; 3-12 - plasmid DNA from 10 colonies of sample 1 digested with *Bam* H1(4-5kb insert can be seen running behind the plasmid); M - molecular weight marker, 1kb ladder (selected band sizes shown in bp)

Figure 6.2.   Digestion of plasmid DNA from transformed DH5α cells

Figure 6.3.   Digestion of plasmid DNA from a selected clone containing a 4-5kb insert
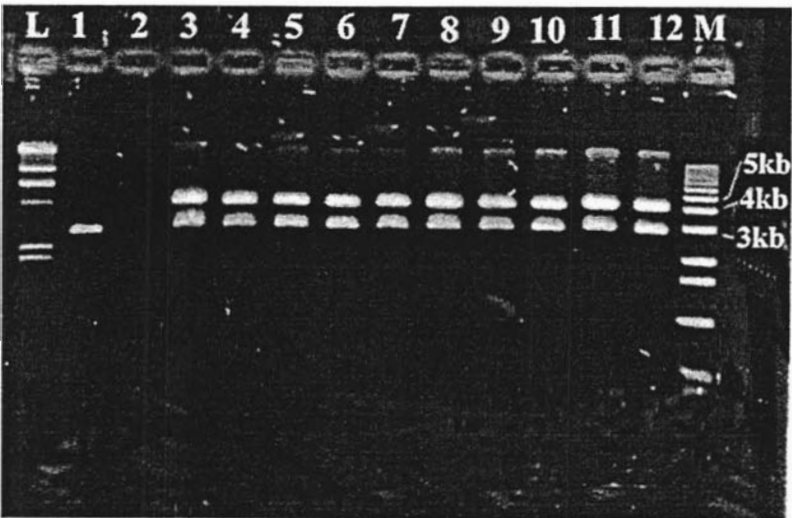
Five of the digests were pooled and the DNA concentrated and resuspended in 55μl TE 10:1 pH 8.0 for labelling with the Chemiprobe kit. One microlitre of the concentrated DNA was run on a gel alongside pBR322 20ng standards and the DNA was estimated to be approximately 400ng/μl.

6.3.5 Hybridisation of the pYV probe to DNA and colony blots

In an initial experiment, the probe (consisting of pGEM-2 + insert) was hybridised to DNA from 5 P+ve and 5 P-ve strains, using DNA isolated in the plasmid mini-preps, and it showed 100% specificity to the P+ve strains, as seen in Figure 6.4.

Figure 6.4. **Hybridisation of the pYV probe to DNA blots from pathogenic and non-pathogenic *Y. enterocolitica* strains**
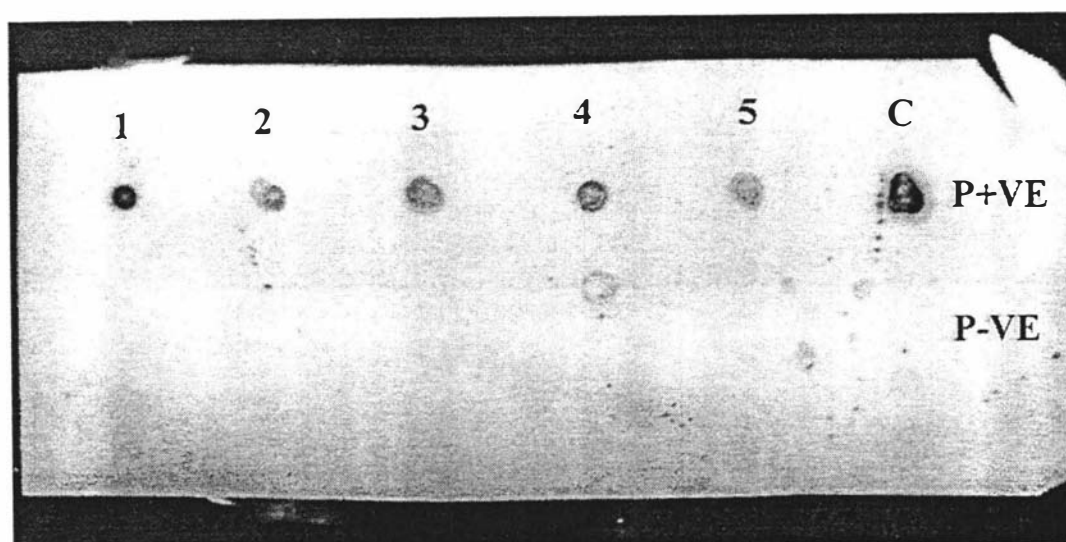
1-5 P +ve - plasmid DNA from strains of *Y. enterocolitica* 4/O:3; 1-5 P-ve - plasmid DNA from strains of *Y. enterocolitica* biotype 1A; C P+ve - control plasmid-positive DNA; P-ve - control plasmid-negative DNA

When the probe was used on colony blots of 33 bacterial strains on a nitrocellulose filter, including pathogenic and non-pathogenic *Y. enterocolitica* and other selected enterobacterial species, all 33 strains hybridised with the probe to varying degrees, suggesting that non-specific hybridisation was occurring.

In a second hybridisation, colony blots of 10 P+ve and P-ve *Y. enterocolitica* strains were probed with both pYV and pGEM-2 to try and ascertain if the vector was the cause of the non-specific background reaction. Hybridisation to all strains was seen with both probes.

To assess the specificity of the pYV probe against total DNA, hybridisation was performed against chromosomal and plasmid DNA from 30 P+ve and P-ve strains blotted together on the same nylon membrane. The result of this hybridisation showed a low specificity for pathogenic strains of the organism, with all 30 strains hybridising with the probe to varying degrees.

Due to the problems experienced with the probe, it became apparent that the limitations of the technique would make it's adaptation into a tool for epidemiological studies difficult. A decision was made to discontinue further work on development of the probe and instead to focus efforts on developing the PCR as an alternative rapid diagnostic method.

B - PCR DEVELOPMENT AND TESTING

6.3.6   Optimisation of the Polymerase Chain Reaction

Optimisation of the PCR was performed for a number of parameters, including magnesium concentration, annealing temperature and the number of amplification cycles. The optimum magnesium concentration was shown to be 1.5mM, the concentration in the commercial buffer supplied with the *Taq* polymerase. Increasing the concentration of magnesium from 1.5 to 9mM resulted in a decrease in amplification (Figure 6.5).

Figure 6.5.   Optimisation of magnesium concentration
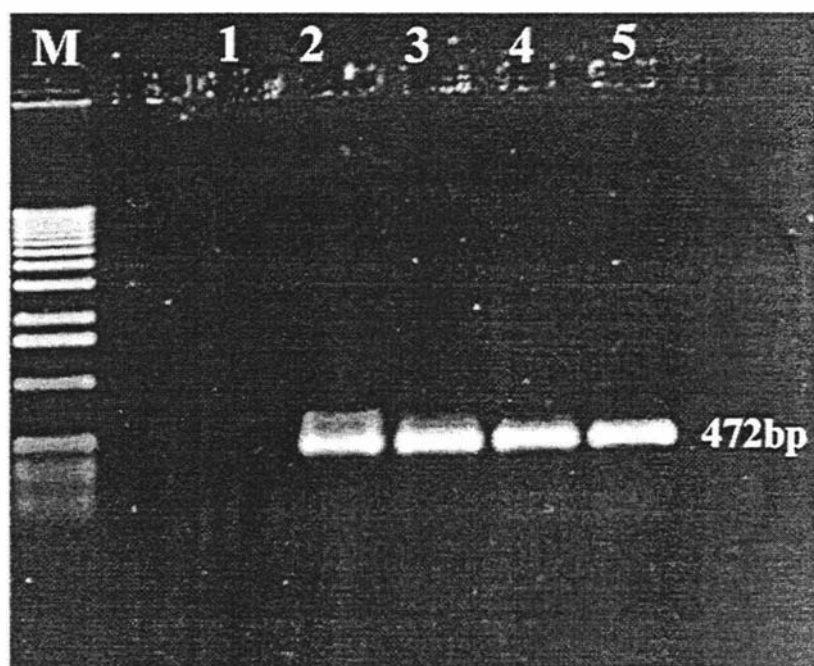
Lane no: M - molecular weight marker, 1kb ladder (size of amplification product shown in bp); 1 - *Y. intermedia*; 2 - Ye 4/O:3 + 1.5mM Mg (commercial buffer); 3 - Ye 4/O:3 + 3mM Mg; 4 - Ye 4/O:3 + 6mM Mg; 5 - Ye 4/O:3 + 9mM Mg; 6 - reagent negative control

Initial reactions using an annealing temperature of 50°C successfully amplified pathogenic strains of *Y. enterocolitica*, but non-specific amplification was seen with strains of *Y. enterocolitica* biotype 1A (Figure 6.6). This has been well-documented by other workers and is associated with low annealing temperatures and long extension times (Rychlick *et al.*, 1990; Steffan and Atlas, 1991). Raising the annealing temperature to 55°C and reducing the annealing time from 2min to 1min abolished all non-specific amplification. No significant increase in sensitivity was seen when the number of cycles was increased from 30 to 60 cycles and 30 cycles was chosen as the standard to reduce the time for completion of the PCR.

Figure 6.6. **Non-specific amplification of** *Y. enterocolitica* **biotype 1A at 50°C annealing temperature**

Lane no: M - molecular weight marker, 1kb DNA ladder (size of amplification product shown in base pairs); C - reagent negative control; 1 & 2 - *Y. enterocolitica* biotype 1A; 3 & 4 - *Y. enterocolitica* 4/O:3

6.3.7 Sensitivity of the PCR

Following optimisation of the PCR, the sensitivity of the assay was evaluated using dilutions of both purified DNA and bacterial cultures. Using purified DNA, the sensitivity of detection was 0.25pg of DNA (Figure 6.7). When boiled bacteria were diluted and added to the reaction, the detection limit was approximately 250 organisms (Figure 6.8).

Lane no: M - molecular weight marker, 1 kb DNA ladder (size of amplification product shown in base pairs); 1 - Ye 4/O:3 DNA, 2.5ng; 2 - Ye 4/O:3 DNA, 0.25ng; 3 - Ye 4/O:3 DNA, 25pg; 4 - Ye 4/O:3 DNA, 2.5pg; 5 - Ye 4/O:3 DNA, 0.25pg; 6 - Ye 4/O:3 DNA, 0.025pg; 7 - reagent negative control

Lane no: M - molecular weight marker, 1kb DNA ladder (size of amplification product shown in base pairs); 1- Ye 4/O:3, 2.5 organisms; 2 - Ye 4/O:3, 25 organisms; 3 - Ye 4/O:3, 250 organisms; 4 - Ye 4/O:3, 2500 organisms; 5 - Ye 1A, 25 organisms; 6 - Ye 1A, 250 organisms; 7 - Ye 1A, 2500 organisms; 8 - reagent negative control; 9 - *E. coli*

Figure 6.7.   Sensitivity of the PCR using purified DNA

472bp
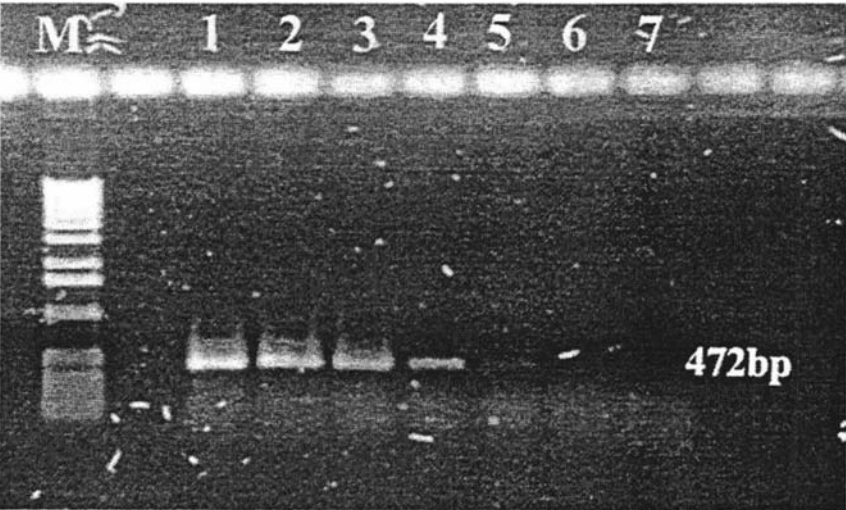
Figure 6.8.   Sensitivity of the PCR using boiled bacteria

359bp
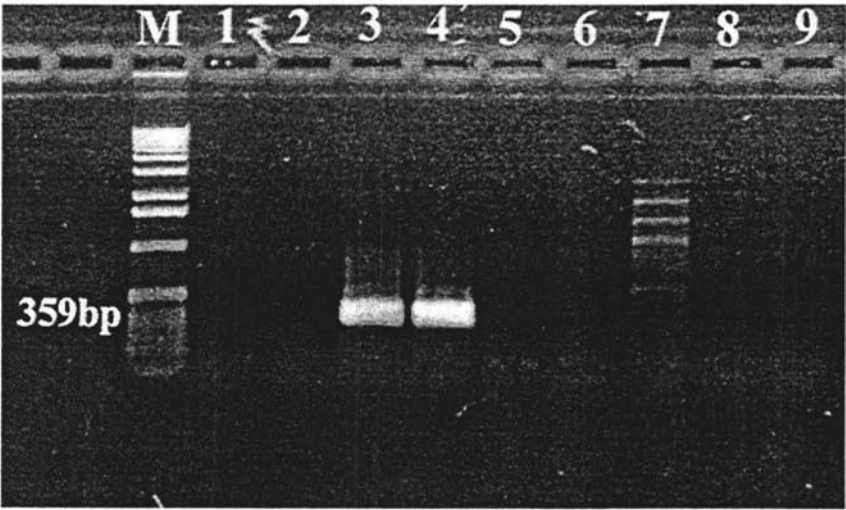
6.3.8 Specificity of the PCR

Using the optimised reaction conditions, the PCR was 100% specific for pathogenic strains of *Y. enterocolitica*, including bioserotypes 2/O:5,27; 2/O:9; 3/O:1,2,3; 4/O:3 and 5/O:2,3. All 63 isolates of these strains were positive in the assay, giving an amplified product with either or both sets of primers. The other animal pathogenic *Yersinia* species, *Y. pseudotuberculosis*, did not produce an amplification product. *Yersinia enterocolitica* biotype 1A strains and other environmental yersiniae, which do not contain the *ail* gene, were also all consistently negative in the PCR. Similarly, nine other bacterial genera commonly found in faeces were all negative in the assay. Figures 6.9-6.11 show the results of amplification of selected isolates.

Figure 6.9.   Specificity of the PCR (1)

Lane no: M - molecular weight marker, 1kb DNA ladder (size of amplification product shown in base pairs); 1 - reagent negative control; 2, 4, 7 - *Y. enterocolitica* biotype 1A; 3, 5, 6 - *Y. enterocolitica* 4/O:3; 8 - *Y. kristensenii*; 9 - *Y. enterocolitica* 3/O:1,2,3; 10 - *Y. enterocolitica* 5/O:2,3; 11 - *Y. intermedia*

Lane no: M - molecular weight marker, 1kb DNA ladder (size of amplification product shown in bp); 1 - reagent negative control; 2 & 9 - *Y. enterocolitica* 2/O:9; 3 & 10 - *Y. enterocolitica* 2/O:5,27; 4 - *Y. frederiksenii*; 5 & 6 - *Y. pseudotuberculosis*; 7 & 8 - *Y. enterocolitica* 1A

Lane no: M - molecular weight marker, 1kb ladder (size of amplification product shown in bp); 1- *Escherichia coli*; 2 - *Proteus mirabilis*; 3 - *Klebsiella pneumoniae*; 4 - *Y. pseudotuberculosis*; 5 - *Y. enterocolitica* 1A; 6 - *Enterobacter agglomerans*; 7 - *E. coli*; 8 - *Y. enterocolitica* 4/O:3; 9 - *K. oxytoca*; 10 - *Salmonella typhimurium*

Figure 6.10.   Specificity of the PCR (2)
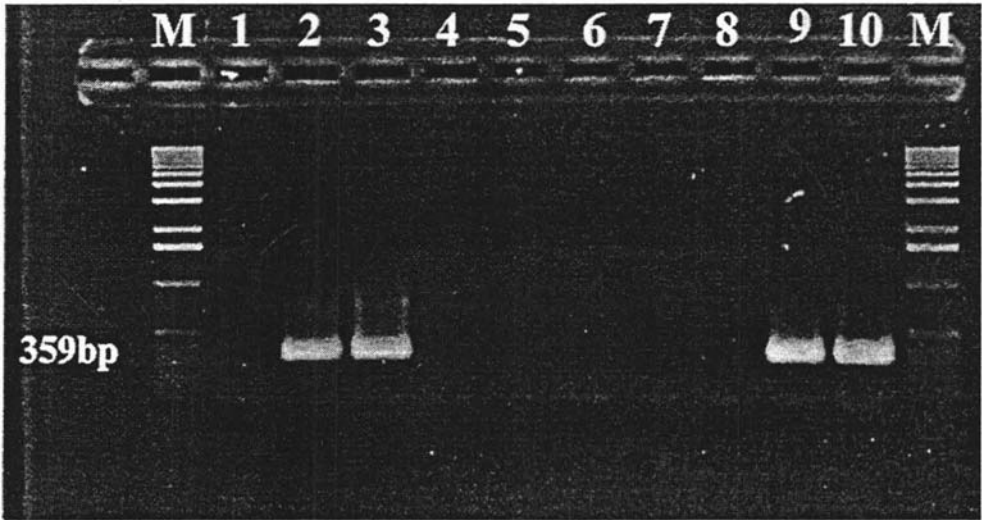
Figure 6.11.   Specificity of the PCR (3)
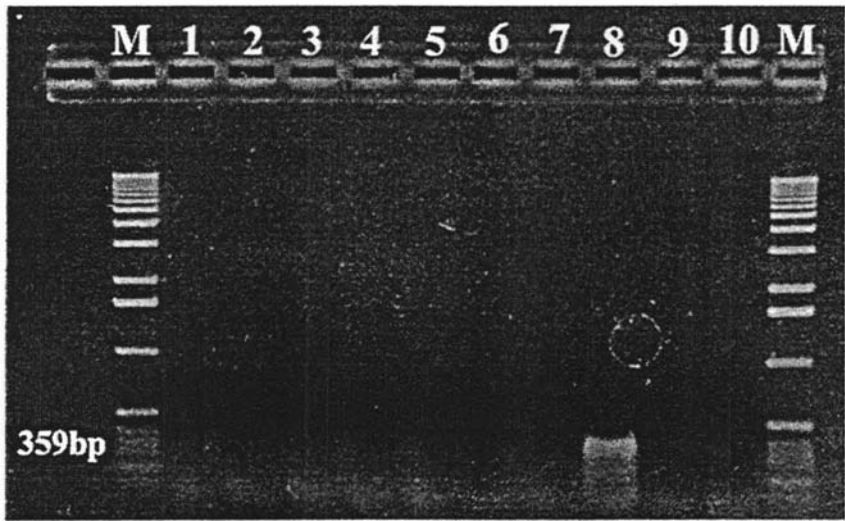
6.3.9 Validation of the PCR product

(i) Restriction endonuclease digestion

The sequence-specific restriction endonuclease *Mbo*II was derived from *Moraxella bovis* by Gelinas *et al.* in 1977. In 1980, the specific non-symmetrical sequence recognised by the enzyme was identified (Brown *et al.*, 1980), with the cleavage site 8 nucleotides to the right of the upper sequence and 7 nucleotides to the right of the lower sequence, as shown below. Based on this information, it was estimated that cleavage of the *ail*-gene product amplified by primers 3 and 4 would result in 2 fragments of 219 and 140bp.

$$5'......GAAGA(N)_8 \blacktriangledown ......3'$$
$$3......'CTTCT(N)_7 \blacktriangle ......5'$$
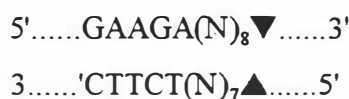
Figure 6.12. Results of the digestion of PCR products with *Mbo*II
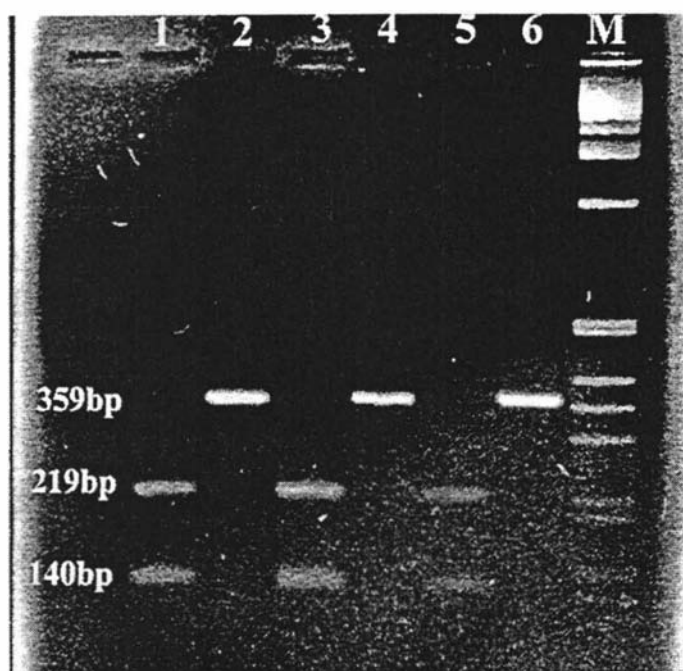
Lane no: 1 - Ye 4/O:3 amplification product digested with *Mbo*II; 2 - Ye 4/O:3 product undigested; 3 - Ye 2/O:5,27 product digested with *Mbo*II; 4 - Ye 2/O:5,27 product undigested; 5 - Ye 2/O:9 product digested with *Mbo*II; 6 - Ye 2/O:9 product undigested; M - molecular weight marker, 1kb DNA ladder (selected band sizes are shown in bp)

Figure 6.12 shows the results of the *Mbo*II digestion of amplification products from 3 *Y. enterocolitica* strains, including bioserotypes 2/O:5,27; 2/O:9 and 4/O:3. All 3 amplification products have been identically cleaved, giving 2 fragments in the expected size range, thus verifying the identity of the product.

(ii) Sequencing of the PCR product

Sequencing of part of the purified amplification product from *Y. enterocolitica* bioserotype 4/O:3 was carried out and the PCR product was identical to the published sequence of the *ail* gene (Miller *et. al*, 1990).

6.4 DISCUSSION

Within the last seven years, *Y. enterocolitica* has become recognised as a frequent cause of gastroenteritis in New Zealand, however, attempts to elucidate the true prevalence of infection have been hampered by the difficulties involved in it's isolation and identification by standard bacteriological methods. There is, therefore, a need for a rapid and more specific method of diagnosing infections with the organism and the intention of this project was to design a specific DNA detection assay that could be used on clinical samples, or other material of animal origin, for the identification of pathogenic strains of *Y. enterocolitica*, .

6.4.1 Plasmid screening assays

The purpose of screening pathogenic and non-pathogenic *Y. enterocolitica* of human and porcine origin for the presence of the virulence plasmid was two-fold. Firstly, to compare similar bioserotypes from pigs and people in order to assess the potential of pigs as a reservoir of human pathogenic yersiniae in New Zealand, and secondly, to identify a plasmid-bearing strain for use in the development of a DNA probe assay. In the first part of the study, results showed that isolates of the pathogenic bioserotypes 4/O:3 and 2/O:5,27 recovered from pigs in an earlier survey carried a plasmid similar in size to the same

serotypes recovered from human enteric infections. Although such a comparison had not been performed in New Zealand previously, the results were not unexpected, as similar studies overseas had found plasmids from pig and human isolates to be identical in size and restriction fragment patterns (Kapperud *et al.*, 1985; Kwaga and Iversen, 1993). The results, however, confirmed that pigs or pig products were a potential source of infection for people in this country and justified the development of a rapid assay for further epidemiological studies. One point worth noting was the relatively high number of plasmid-negative strains of 4/O:3 and 2/O:5,27, particularly in those isolated from human infections. Plasmid loss is a well-documented characteristic of pathogenic yersiniae and is linked to repeated subculturing in the laboratory, particularly at 37°C (Hill *et al.*, 1983). As all human diagnostic laboratories culture faecal samples at this temperature, the negative results were not surprising. While pig isolates had not been cultured at temperatures above 30°C, the loss of plasmids is harder to explain, but may reflect excessive manipulation of the strains.

6.4.2   Development of a non-radioactive DNA probe

Following the plasmid screening assays, the protocol was scaled up and used to isolate a large quantity of plasmid DNA from one of the human P+ve strains. The objective was to digest the plasmid DNA with the restriction enzyme *Bam*H1 and identify fragments corresponding to the region of the plasmid responsible for the calcium dependent phenotype. This had previously been shown by Nesbakken *et al.* (1987), to be a highly conserved region in virulence plasmids from all pathogenic *Y. enterocolitica* serotypes. The maintenance of calcium dependency in virulent strains of widespread geographical origin probably reflects a strong selective pressure to conserve those genes responsible for expression of this property. Cloning of the plasmid fragments into a vector capable of transforming *E. coli* would allow the production of a sufficient quantity of recombinant DNA for labelling and use as a DNA probe. This approach had first been used by Hill *et al.* (1983), to produce probes for the identification of virulent *Y. enterocolitica* in food.

The techniques used to clone plasmid fragments into the vector pGEM-2 and transform DH5α competent cells were successful, resulting in a transformation efficiency similar to that described by the manufacturers of the cells. Screening of these transformants for the presence of recombinant plasmids revealed a number that contained inserts of the expected size range from the calcium-dependent region, 4.0, 4.4, 5.3kb (Pulkkinen *et al.*, 1986). One recombinant containing a 4-5kb insert was chosen for the probe, grown in bulk and labelled using a non-radioactive system. Whole pGEM-2 plasmids containing the inserts were labelled for use as the probe, a simple system that had been used previously for detection of enteric pathogens (Kennedy *et al.*, 1989).

6.4.3   Hybridisation assays for pathogenic *Y. enterocolitica* detection

The early hybridisation assays for *Y. enterocolitica* were performed using the radioactive isotope ^{32}P (Hill *et al.*, 1983). However, a non-radioactive hybridisation assay was chosen for this study as radioisotopes are not particularly suitable for use in a clinical laboratory due to their short half-lives and safety issues. Non-radioactive systems had been in use since 1981, with the development of the biotin-avidin labelling and detection protocol (Tenover, 1988). Chemical modification of DNA and antibody detection systems were introduced later, with a number of chemicals being used for labelling, including sodium bisulphite (Walker and Dougan, 1989).

Initially, colony blots of pathogenic and non-pathogenic *Y. enterocolitica* and other enteric organisms were hybridised with the labelled probe pYV in an effort to eliminate time-consuming DNA extraction procedures, and with a view to probing stool blots in the future. Probing of colony and stool blots had been used to good effect previously for the detection of enterotoxigenic *E. coli* (Vieira *et al.*, 1991). Unfortunately, the results were ambiguous, as non-specific background hybridisation resulted in a number of false-positives, despite the high stringency conditions used during hybridisation and washing. It was confirmed that pGEM-2 vector sequences were contributing to the non-specificity when pGEM-2 was shown to hybridize to both pathogenic and non-pathogenic *Y. enterocolitica*.

Problems with non-specific binding had been reported with biotin-avidin systems, particularly when crude samples had been used as the target, and had been attributed to the detection system rather than the labelled probe. The use of purified DNA, however, successfully eliminated much of the background reaction (Kennedy *et al.*, 1989). Non-specific reactions had also been seen when radioactive and non-radioactive probes had been used on stool blots, thought to be due to protein in faecal material (Tompkins and Krajden, 1986; Taylor and Hiratsuka, 1990). Therefore, it was decided to use DNA isolated previously during plasmid mini-preps as the target. This appeared to be successful, with 100% specificity for DNA from pathogenic strains of *Y. enterocolitica* being achieved. As the objective was eventually to be able to probe whole cells, however, a further hybridisation was performed using total DNA from 30 pathogenic and non-pathogenic strains of the organism. Again, hybridisation was seen, with all 30 strains hybridising with the probe to varying degrees i.e. specificity was very poor. Probing of the total DNA with labelled pGEM-2 gave a similar result. When DNA from plasmid mini-preps alone was probed with labelled pYV and pGEM-2, most of the false-positives were eliminated. Nevertheless, 2 P-ve strains of biotype 1A hybridised with both probes.

The sensitivity of the assay was not assessed due to the problems with specificity, however, all P+ve strains were detected by the pYV probe when plasmid DNA was used as the target. The nature of the non-specific reaction was unclear and may have been due to DNA homology between *Y. enterocolitica* DNA and sequences from pGEM-2. Walker and Dougan (1989), stated that the use of the entire insert plus vector as a probe requires some caution due to potential homologous vector sequences. Another possibility is that non-specific binding of the probe to bacterial cellular components precipitated during DNA extraction may have occurred. Kennedy *et al.* (1989), demonstrated this problem with a streptavidin-based protocol, and identified streptavidin-binding proteins in *E. coli*. The problem of background non-specificity in their assay was reduced by the use of different DNA extraction procedures. The fact that reduced background was seen in this study when DNA isolated by plasmid mini-preps was used, tends to lend credence to this theory. Obviously the next step was to purify and label the 4.4kb insert for use as a probe and this was done, unfortunately, the hybridisation reaction did not work and it was presumed that labelling of the probe had not been successful.

Superior protocols for non-radioactive probe development have recently become commercially available, e.g. digoxigenin labelling, and Goverde *et al.* (1993), used labelled *inv*- and *ail*-probes to successfully identify pathogenic *Y. enterocolitica* in clinical specimens and naturally contaminated pig samples (Goverde *et al.*, 1993). More recently, Fliss *et al.* (1995), developed a multiplex riboprobe system using a pool of RNA probes, targeting both chromosomal and plasmid-borne sequences, for the detection of virulent *Y. enterocolitica*. Thus, probe technology has advanced rapidly and now offers the possibility of safe and rapid diagnosis of *Yersinia* infections. However, repeat experiments, for example using fragments of the insert as probe, were not performed in this study as development of a PCR was thought to offer a better option for a rapid diagnostic assay at this stage.

6.4.4 Development of a PCR for the detection of pathogenic *Y. enterocolitica*

When this part of the study was embarked upon, the PCR had been used successfully to detect a number of bacterial pathogens, but had not been used previously for *Y. enterocolitica* (Wright and Wynford-Thomas, 1990). The identification of chromosomal *ail*-gene sequences only in pathogenic *Y. enterocolitica* (Miller *et al.*, 1989), together with the problems of plasmid loss encountered earlier, potentially allowed the development of a rapid, sensitive and specific assay for detection of pathogenic strains of the organism. Two sets of oligonucleotide primers were designed from the *ail*-gene sequence that successfully amplified a 472bp fragment (Primers 1 and 2) and a 359bp fragment (Primers 3 and 4) of the gene in the PCR. Initial problems were encountered with the sensitivity and specificity of the reactions and modifications to optimise the protocol were required. Following optimisation, different protocols were chosen for the two primer sets. Optimal conditions for Primers 1 and 2 were denaturation at 96°C for 30s, annealing at 60°C for 1min and extension at 72°C for 1min. The corresponding conditions for Primers 3 and 4 were 94°C for 1min, 55°C for 1 min and 72°C for 1 min. The best results were seen when these conditions were used for 30 cycles.

Earlier PCR protocols had all used extracted DNA as target in the assay and chromosomal DNA was initially used for optimisation of the *Yersinia* PCR. Once the sensitivity and specificity were acceptable, a decision was made to simplify the procedure by using boiled cultures as the template material, as the objective was to develop an assay for use on clinical material. This also worked well, although a slight reduction in sensitivity was observed. Finally, intact bacterial cells were used directly in the PCR, without prior boiling, and similar results to boiled bacteria were achieved. The initial five minute denaturation step before cycling proceeds appears to lyse the cells and denature the DNA sufficiently for amplification to occur. The use of whole cells in the PCR has since been published by other groups as a viable alternative to DNA extraction or boiling (Joshi *et al.*, 1991; Madico *et al.*, 1995). Direct amplification from whole cells offers the advantages of speed and convenience and should be especially useful in large epidemiologic studies when many strains must be typed. In addition, whole cell template reduces the hazards inherent in handling infectious organisms.

Following resolution by agarose gel electrophoresis, PCR-generated DNA fragments should, in general, be further validated by one of three methods - Southern blot analysis using hybridisation probes, restriction enzyme analysis or nucleotide sequencing (Hung and Siebert, 1991). In the present study, the latter two methods were both used to verify the product as having been amplified from the *ail*-gene, thus fulfilling the scientific criteria necessary for validation of the PCR assay.

6.4.5 Sensitivity and specificity of the PCR

Theoretically, the PCR technique is able to detect one single copy of target DNA. In practice, however, this detection limit is rarely reached due to non-specific inhibition of *Taq* polymerase by compounds co-precipitated with the sample DNA, either from the extraction reagents or from bacterial cellular debris (Rossen *et al.*, 1992). Olive *et al.* (1988), discussed the inhibitory effect of bacterial protein on the activity of the DNA polymerase and the need for DNA extraction to improve sensitivity. Initially, assessment of the sensitivity of the PCR was performed using dilutions of purified chromosomal DNA and the

detection limit of 0.25pg was comparable with other studies using PCR for bacterial detection, for example Kwang *et al.* (1996), used PCR for *Salmonella* detection and reported a sensitivity of 1 pg for the assay. Using boiled bacteria as the template, the lower limit of detection was approximately 250 organisms, probably due to slight inhibition by bacterial cellular debris or incomplete denaturation of the target cells. Nevertheless, this figure is consistent with similar reports on the use of PCR methodology for detection of *Y. enterocolitica* and other enteric organisms. Reported limits of detection using whole cells or boiled whole cells vary considerably, from 2 colony forming units (cfu) (Sandery *et al.*, 1996) to 100 cfu (Weynants *et al.*, 1996) for *Y. enterocolitica*; 300 cfu for *S. enteritidis* (Rahn *et al.*, 1992) and 2×10^5 cfu for enterotoxigenic *E. coli* (Woodward *et al.*, 1992).

In addition to correlating sensitivity with the limit of detection of the target it is also measured by the proportion of false-negative results, and reduced sensitivity is seen in circumstances where there is loss of the particular gene being amplified. For instance, Wren and Tabaqchali (1990), experienced a reduction in sensitivity with a PCR designed to detect *vir*F, a plasmid-associated *Y. enterocolitica* gene, due to plasmid loss during subculture. By targeting a chromosomal gene in the present study, this problem was overcome.

The specificity of the PCR is calculated from the number of false-positive reactions occurring, and commonly occurs when lower annealing temperatures are used or when complementary sequences to the target sequence are found in other organisms. Prior to optimisation of the PCR in this study, non-specific amplification products were seen with non-pathogenic strains of *Yersinia*, however, increasing the annealing temperature and decreasing the times of annealing and extension eliminated the problem. Using a number of strains of *Y. enterocolitica*, both pathogenic and non-pathogenic, and other bacteria, the PCR was shown to be 100% specific for pathogenic strains of *Y. enterocolitica*. This is in contrast to some other *Yersinia*-PCR assays where false-positive reactions occurred and additional manipulations had to be performed to achieve a satisfactory specificity. For example, Ibrahim *et al.* (1992a), used primers to amplify a fragment of the *yst* gene, which, although non-functional, does occur in some non-pathogenic yersiniae, with subsequent false-positive reactions. These were overcome by the use of an internal probe that

hybridised only with DNA amplified from pathogenic *Y. enterocolitica*, significantly increasing the time and effort required to produce a result. Similarly, Rasmussen *et al.* (1994b), used primers to amplify the *inv*-gene, which is also found in non-pathogenic yersiniae, and had to design a two-step assay to overcome the problem of false-positives. The PCR assay used in this study needed no additional steps to increase the specificity.

An additional observation was that amplification of isolates of pathogenic *Y. enterocolitica* from animal and human sources produced an identical amplification product, and while this does not prove that animals and their products are a source of infection for people it suggests that domestic animals have a role in the epidemiology of human yersiniosis. Interestingly, positive amplification of *ail*-gene sequences from strains of bioserotype 5/O:2,3 (see Figure 6.9), only ever recovered from animal infections, presented a number of intriguing possibilities. Firstly, that these strains could be pathogenic for people; secondly, that the *ail* gene is not an absolute marker for pathogenicity for people; thirdly, that other as yet unidentified genes are essential for the pathogenicity of *Y. enterocolitica* for people. Further research on the pathogenic potential of these strains is warranted.

The objective of the present study was to design a rapid assay for the detection of pathogenic *Y. enterocolitica* that could be used on clinical material, reducing the time required for diagnosis of infection by eliminating the need for cold-enrichment. This was successful, and in the next chapter the adaptations of the assay required for the examination of clinical material are reviewed and discussed.

CHAPTER 7

7. APPLICATION OF THE POLYMERASE CHAIN REACTION FOR THE DETECTION OF *Y. ENTEROCOLITICA* IN CLINICAL SAMPLES

7.1 INTRODUCTION

Attempts to elucidate the true prevalence of *Y. enterocolitica* infection and the source of human infections have been hampered by the difficulties involved in the isolation and identification of the organism by standard bacteriological methods. Isolation of the organism from faecal samples, foods and animal tissues is difficult due to it's slow growth on laboratory media and subsequent overgrowth by competing microorganisms. Many selective media and enrichment techniques have been devised to overcome these problems, with the most successful method to date involving cold-enrichment for up to three weeks followed by subculturing onto CIN selective agar and biochemical confirmation of identity (Van Noyen *et al.*, 1987b; Greenwood and Hooper, 1989). In addition to the time constraints of this protocol, it still suffers from a lack of sensitivity, particularly when small numbers of organisms are present in the samples to be tested, as is often the case with foods, faeces and abattoir specimens (Swaminathan *et al.*, 1982).

Following optimisation of the sensitivity and specificity of the PCR for amplification of the *ail* gene (Chapter 6), further experiments were designed to investigate it's ability to detect pathogenic *Y. enterocolitica* directly in a range of clinical specimens, including pig tissues and dog faeces. The principal aim of the experiments was to design a simple PCR assay that could be used to enhance future protocols for research into the epidemiology of human and animal yersiniosis. The PCR experiments described below were performed prior to most of the work detailed in the literature review on the use of the PCR for detection of pathogenic strains of *Y. enterocolitica* in clinical samples.

7.2   MATERIALS AND METHODS

7.2.1   Samples

(i)   Pig tissues   In a concurrent trial, new-born piglets were infected orally with *Y. enterocolitica* O:3 and were euthanased at regular intervals to examine a number of gut developmental parameters (Shu *et al.*, 1995).   At the time of euthanasia, tissue samples were removed from both infected and uninfected control animals for assay by the PCR.   These included tongues, tonsils and mediastinal lymph nodes. Tissues were cut into small pieces and were homogenised with 10ml PBS in a stomacher for 10min.   The homogenates were decanted into sterile universal bottles and were stored at -70°C until required.

(ii)   Pig faeces   During the above trial, faeces were also recovered from infected and uninfected control piglets.   One gram was added to 10ml PBS, mixed well and the suspension stored at -70°C until required.

(iii)   Dog faeces   In an earlier trial, where dogs were infected with *Y. enterocolitica* 4/O:3 (Chapter 5), faecal samples were taken at regular intervals and were mixed 1:10 with phosphate-buffered peptone water (PBPW) for cold-enrichment.   Following mixing, a 1ml aliquot of each suspension was removed and stored at -70°C until required.

7.2.2   Template preparation protocols for PCR

(i)   Tissue samples

Two methods were used for template preparation from tissue homogenates, DNA extraction and boiling.   Bacterial counts were made from each homogenate on CIN agar using 1 and 10µl calibrated loops, to enable estimates of the sensitivity of the PCR to be

made. In this context sensitivity was defined as the minimum number of colony forming units per given volume or mass of tissue that could be detected by the PCR.

- **DNA extraction**

Total DNA was extracted from tissue homogenates following a method described by Ausubel *et al.* (1987). Samples stored at -70°C were thawed and a 200µl aliquot removed to a sterile eppendorf tube. After the addition of 300µl extraction buffer (100mM NaCl; 10mM Tris pH 8.0; 25mM EDTA; 0.5% SDS; 0.1mg/ml proteinase K) the sample was mixed gently and incubated overnight at 50°C. The following day, the DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), sodium acetate added to 0.3M and the DNA precipitated with cold 100% ethanol. Following a wash with 70% ethanol, the pellet was dried and resuspended in 50µl sterile DW. Aliquots of 15µl were used as template in 100µl PCR reactions.

- **Boiling**

A 100µl aliquot of each homogenate was removed to a sterile eppendorf tube and boiled for 10min. Following boiling, the sample was centrifuged at 12,000rpm for 3min and stored on ice. Aliquots of 5µl were used as template in 50µl PCR reactions.

(ii) Faecal samples

Several methods were used to prepare template DNA from faecal samples in order to overcome PCR inhibition, including DNA extraction, harvesting of growth from agar plates and enrichment broths and boiling. Bacterial counts were made to enable estimation of the sensitivity of each method to be made.

- **DNA extraction**

A similar extraction protocol to that used for the tissue samples was used on 200µl aliquots

of the faecal suspensions. After the lysis steps, the DNA was either extracted by phenol/chloroform as before, or was purified using a DNA purification kit, following the manufacturer's instructions (Prep-A-Gene, Bio-Rad). Aliquots of 5µl were used as template in 50µl PCR reactions.

The Prep-A-Gene protocol was as follows: A 20µl aliquot of the DNA suspension was transferred to a fresh tube, 75µl of Prep-A-Gene binding buffer was added and the mixture was vortexed. After addition of 5µl of Prep-A-Gene matrix the combined mixture was vortexed again and incubated at room temperature for 10min with gentle shaking. The matrix was pelleted by centrifugation for 30s in a microcentrifuge and the supernatant was decanted. The pellet was rinsed twice by resuspending in 250µl of binding buffer and centrifuging for 30s as above. After removal of the supernatant, the pellet was washed three times with the wash solution provided. Following the last wash, the matrix was spun for 1min and all the supernatant was removed. The bound DNA was eluted by resuspending the pellet in 10µl of elution buffer and incubating the suspension at 37°C for 5min. After a 1min spin, the supernatant was removed to a fresh tube and the pellet was eluted again. The eluted DNA was combined (approximately 20µl), stored at 4°C and aliquots of 5µl were used as template in 100µl PCR reactions.

- **Primary plate cultures**

A loopful of each faecal suspension was plated onto blood, MacConkey and CIN agars and incubated overnight at 29°C. Following incubation, sterile cotton swabs were used to transfer bacterial growth from the plates into sterile DW. Aliquots of 50µl were boiled for 10min, centrifuged at 12,000 rpm for 1min and 5µl used as template in 50µl PCR reactions.

- **Enrichment broths**

Three different enrichment broths were used to culture the pig faecal suspensions; tryptone water, selenite and CIN. For dog faeces, CIN broth was used as it was found to give better amplification than other broths, possibly due to a higher background flora in the other

media. A loopful of each faecal suspension was inoculated into the sterile broths and incubated overnight at 29°C. Broth cultures were diluted 1:10 and 1:100 and 5μl aliquots of each dilution were used as template in 50μl PCR reactions.

- **Boiling**

Aliquots of each faecal suspension (200μl) were boiled for 10min, centrifuged at 12,000 rpm for 3min and 5μl of the supernatant was used as template in 100μl PCR reactions.

7.2.3 PCR protocols

(i) Standard assay

PCR reactions were carried out in either 50 or 100μl volumes as described in Chapter 6 (section 6.2.13). Thirty cycles of amplification were performed using either primers 1 and 2 (product of 472bp), or primers 3 and 4 (product of 359bp), visualised on a 1% agarose gel. Cycle parameters used for these primers are described in section 6.2.16.

(ii) Nested primer assay

In order to increase the sensitivity of the standard assay, nested primers were designed to amplify an internal fragment within the 472bp product, using the supernatant from boiled samples (5μl) as the template. In a preliminary experiment, 1μl aliquots of the standard assay were removed after every 5 cycles and subjected to a second round of amplification with primers 3 and 4, which amplified a 359bp fragment (section 6.2.12). Results of this experiment showed that at least 25 cycles of the standard assay had to be completed before a product was visible in the nested assay and it was decided to use two rounds of 30 cycles in the nested PCR protocol.

After completion of the standard assay using primers 1 and 2, reaction tubes were removed to a safety cabinet and 1μl aliquots of each were transferred to fresh tubes of reaction mix

containing primers 3 and 4. Special care was taken during transfer of the amplified product to prevent cross-contamination between tubes, including the use of separate pairs of gloves and aerosol barrier pipette tips between each sample and the transfer of an aliquot of the negative control from the first round of PCR to act as a negative control in the nested assay. After 30 cycles, products were visualised on a 1% agarose gel as before.

To estimate the sensitivity of the nested PCR, 20µl aliquots of dilutions of an overnight *Y. enterocolitica* O:3 broth culture were mixed with 80µl of supernatant from control negative pig tonsil homogenates, boiled, and 5µl of each dilution were subjected to 2 rounds of PCR using nested primers. Bacterial counts were made from each dilution following mixing.

7.2.4 Application of the nested PCR for the detection of *Y. enterocolitica* in infected pig tissues and dog faeces

(i) Pig tissues

Following preliminary experiments to optimise the nested PCR, a series of assays were performed using a range of tissues from experimentally infected and control pigs as template to assess the suitability of the test for future epidemiological studies. Tissue homogenates were thawed and template for the PCR prepared by the boiling method. At the same time, bacterial counts were made from the tissues to compare the sensitivity of the nested PCR with standard culture methods. A total of 32 samples from 16 piglets (4 control, 12 infected) were examined, including mesenteric lymph node (4), heart (1), tongue (4), tonsil (3), oral swabs (7), small intestine (5), large intestine (6) and rectal swabs (2).

(ii) Dog faeces

During the course of the dog infection trial described in Chapter 5, aliquots of dog faeces in PBS were frozen at -70°C before cold-enrichment. A total of 133 stored samples that had been shown at the time of the infection trial to be either culture-positive (64) or culture-

negative (69) were thawed and enriched overnight in CIN broth before being used as template in the PCR. Nested PCR assays were performed on all samples that were negative after the first round of PCR. Bacterial counts were carried out on all broth cultures for comparison with the PCR results.

7.3   RESULTS

7.3.1   Template preparation

(i)   Pig tissues

Table 7.1 shows the results of a single round of PCR following either DNA extraction or boiling for preparation of the template from selected tissues. Culture results for each of the tissues are also shown as either positive or negative for *Y. enterocolitica*, bacterial counts were not performed. False-negative results were seen with all samples where boiling was the method of template preparation and with one sample of tongue tissue where DNA extraction was performed. Figure 7.1 shows the results of PCR amplification of selected pig tissues post-DNA extraction.

Table 7.1. .   Single round PCR following different DNA extraction procedures

Preparation	CT^1	CT^2	IT^1	IT^2	IM	IM	IS	IB
DNA extraction	-	-	+	+/-	+	-	+	-
Boiling	-	-	-	-	-	-	-	-
Culture result	-	-	+	+	+	-	+	-

C - Uninfected animals; I - Infected animals; CT^1 - Control tonsil; CT^2 - Control tongue; IT^1 - Infected tonsil; IT^2 - Infected tongue; IM - Infected mesenteric lymph node; IS - Infected small intestine; IB - Infected blood; + = positive result; - = negative result; +/- = positive and negative results achieved with different samples

Figure 7.1.   Amplification of pig tissues following DNA extraction
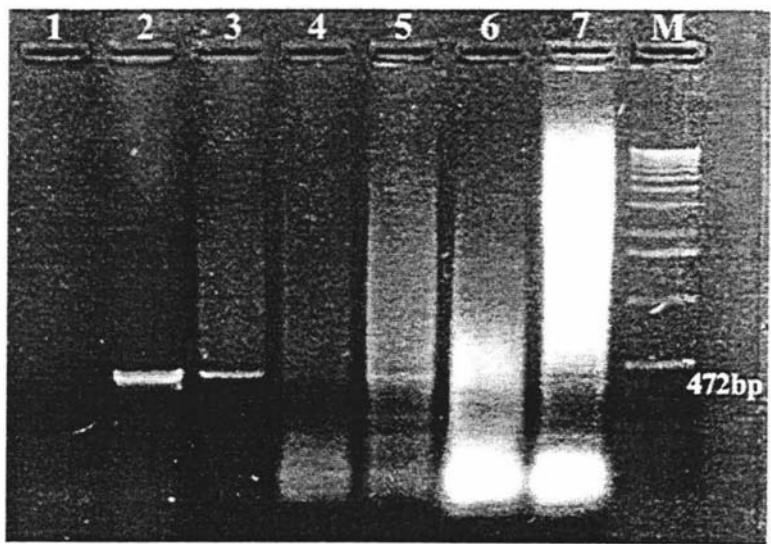
Lane no: 1 - reagent negative control; 2 - positive control. Ye 4/O:3 DNA; 3 - infected mesenteric lymph node; 4 - infected tongue; 5 - infected tonsil; 6 - control tongue; 7 - control tonsil; M - 1kb DNA ladder (size of amplification product shown in base pairs)

(ii)   Pig faeces

Table 7.2 shows the results of a single round of PCR using template prepared from faecal samples by different methods of DNA extraction.   The number of cfu per reaction was estimated from plate counts of the faecal suspensions pre-treatment. False-negative results were seen with both the DNA extraction/phenol-chloroform and boiling methods of template preparation but not with samples where DNA extraction was followed by Prep-A-Gene purification. Figure 7.2 shows the results of PCR amplification of pig faeces post-DNA extraction, followed by either phenol-chloroform or Prep-A-Gene purification. Table 7.3 shows the results of a single round of PCR following overnight growth of the sample on solid media or in enrichment broths. False-negative results were seen with two samples, one following selenite enrichment and the other after growth on MacConkey agar. Figure 7.3 shows the results of PCR after overnight enrichment in CIN broth. Figure 7.4 shows PCR amplification of colony sweeps from solid media after overnight growth of faecal samples.

Table 7.2.   Template preparation from pig faeces for PCR

Method of template preparation	cfu per reaction*	PCR result
DNA extraction/phenol chloroform	3.04×10^4	+
	4.2×10^4	+
	4.2×10^3	-
	4.2×10^2	-
DNA extraction/Prep-A-Gene	1.6×10^4	+
	5.4×10^4	+
	7.6×10^3	+
Boiling	4.1×10^4	+
	6.1×10^4	-

* - cfu per reaction estimated from plate counts of faecal suspensions pre-treatment

Figure 7.2.   PCR post-DNA extraction of pig faeces
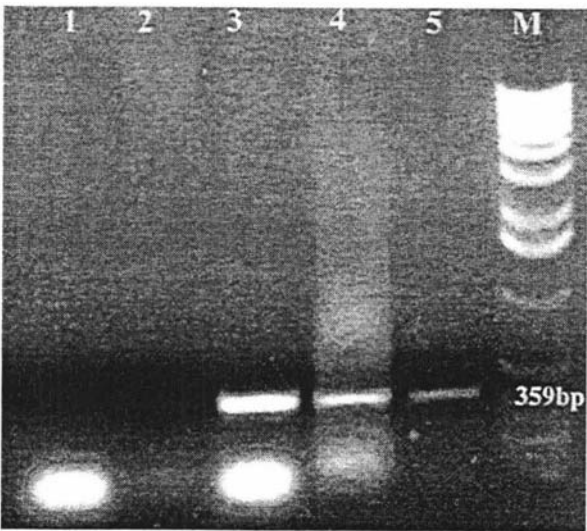
Lane no: 1 - Ye biotype 1A; 2 - reagent negative control; 3 - positive control, Ye 4/O:3 DNA;
4 - infected faeces post-DNA extraction, phenol-chloroform; 5 - infected faeces post-DNA
extraction, Prep-A-Gene; M - 1kb DNA ladder (size of amplification product shown in base pairs)

Table 7.3. **Results of PCR following overnight faecal enrichment**

Enrichment method	Culture result*	PCR result
Tryptone water broth	+	+
	-	-
	+	+
	+	+
	-	-
Selenite broth	+	-
	+	+
CIN broth	+	+
	+	+
CIN agar plate	-	-
	+	+
MacConkey agar plate	-	-
	+	-
Blood agar plate	+	+

* - Culture results were either positive or negative for *Y. enterocolitica* O:3, no bacterial counts were made

Lane no: 1 - reagent negative control; 2 - positive control Ye 4/O:3 DNA; 3 - infected faeces pre-enrichment; 4 - infected faeces post-enrichment; M - 1kb DNA ladder (size of amplification product shown in base pairs)

Lane no: 1 - reagent negative control; 2 - growth of Ye 4/O:3 off blood agar plate; 3 - growth of infected faeces off MacConkey agar; 4 - growth of infected faeces off CIN agar; 5 - growth of uninfected faeces off MacConkey agar; 6 - growth of uninfected faeces off CIN agar; M - 1kb DNA ladder (size of amplification product shown in base pairs)

Figure 7.3. PCR following overnight enrichment of pig faeces in CIN broth
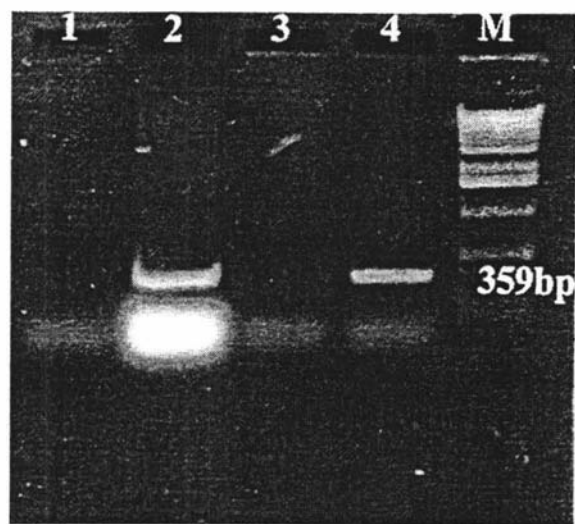
Figure 7.4. PCR following overnight growth of pig faeces on solid media
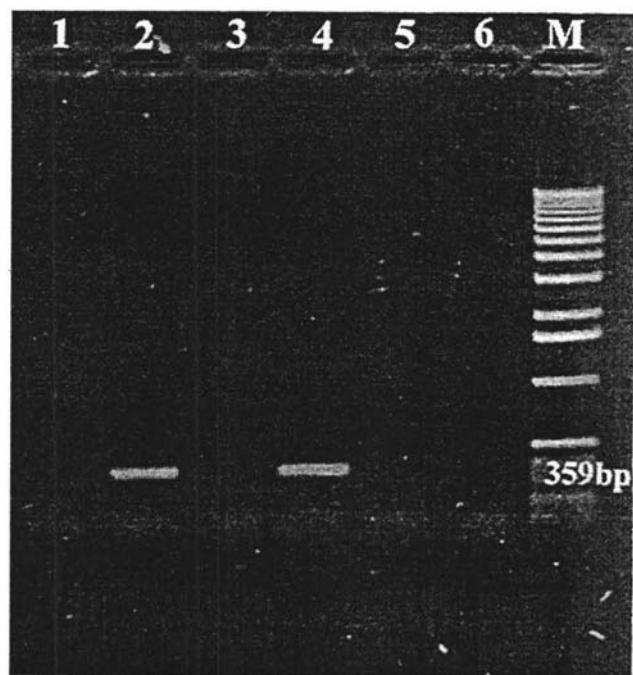
7.3.2   Nested PCR

In a preliminary experiment, template for the initial round of PCR was prepared from a number of pig tissue and faecal samples by the boiling method and was subjected to two rounds of amplification in a nested PCR assay. The results are shown in Table 7.4. While bacterial counts were not performed for every tissue, template from faecal samples for the first round of PCR was estimated to contain approximately 10^3-10^4 cfu per reaction and from tissue samples, 50-100 cfu per reaction. Figure 7.5 shows the results of a nested PCR assay performed on a selection of samples from infected and uninfected pigs.

Table 7.4.   **Nested PCR of tissues from infected and uninfected pigs**

Template	Culture	PCR 1	PCR 2
Infected faeces	+	+	+
Infected faeces	-	-	+
Infected faeces	-	-	-
Infected faeces	-	-	-
Control faeces	-	-	-
Infected tonsils	+	-	+
Infected tonsils	+	-	+
Infected tonsils	+	-	+
Infected tonsils	+	-	+
Control tonsils	-	-	-
Infected small intestine	+	-	+
Control small intestine	-	-	-

Infected - piglets had been experimentally infected with *Y. enterocolitica* bioserotype 4/O:3; Control - uninfected piglets, reared separately

Figure 7.5.   Nested PCR of selected pig tissues
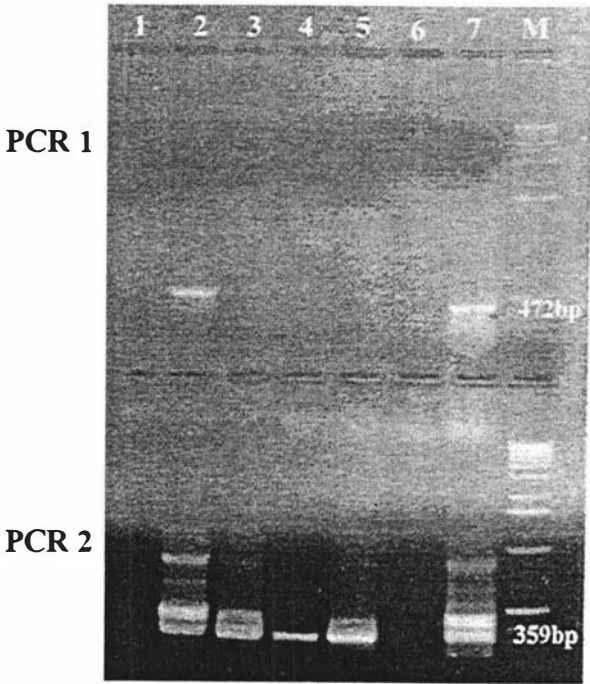
Lane no: 1 - reagent negative control; 2 - positive control, Ye 4/O:3 DNA; 3 - infected tonsil; 4 - infected tonsil; 5 - infected tonsil; 6 - control tonsil; 7 - infected faeces; M - 1kb DNA ladder (size of amplification products shown in base pairs)

Lane no: M - 1kb DNA ladder (size of amplification product shown in base pairs);
1 - tonsils plus 10^5 cfu/ml Ye 4/O:3; 2 - tonsils plus 10^4 cfu/ml Ye 4/O:3; 3 - tonsils plus 10^3 cfu/ml Ye 4/O:3; 4 - tonsils plus 10^2 cfu/ml Ye 4/O:3; 5 - tonsils plus 10 cfu/ml Ye 4/O:3; 6 - tonsils plus 1 cfu/ml Ye 4/O:3; 7 - control negative tonsils; 8 - control positive, Ye 4/O:3 DNA; 9 - reagent negative control

Lane no: M - 1kb DNA ladder (size of amplification product shown in base pairs);
1 - tonsils plus 10^5 cfu/ml Ye 4/O:3; 2 - tonsils plus 10^4 cfu/ml Ye 4/O:3; 3 - tonsils plus 10^3 cfu/ml Ye 4/O:3; 4 - tonsils plus 10^2 cfu/ml Ye 4/O:3; 5 - tonsils plus 10 cfu/ml Ye 4/O:3; 6 - tonsils plus 1 cfu/ml Ye 4/O:3; 7 - reagent negative control

Figure 7.6.   Nested PCR assay to assess lower limits of sensitivity

PCR 1
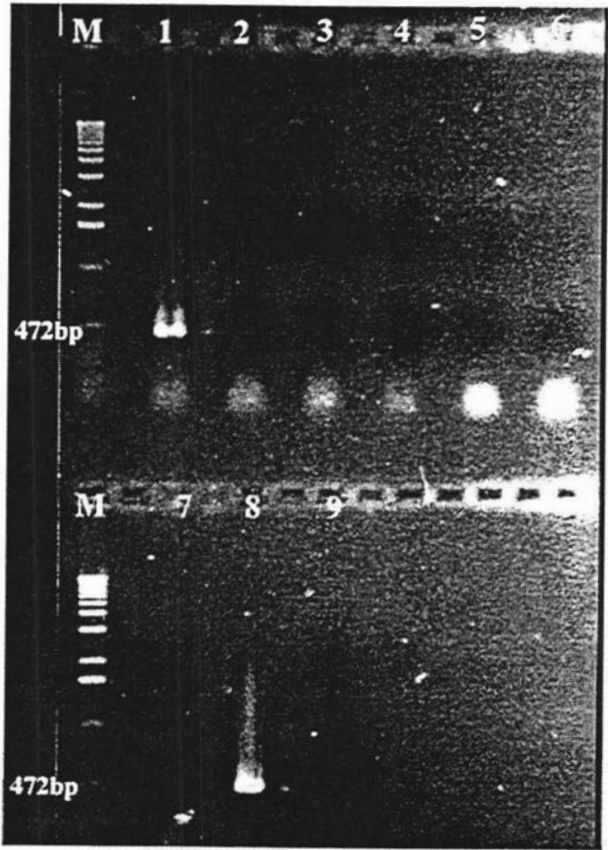
PCR 2
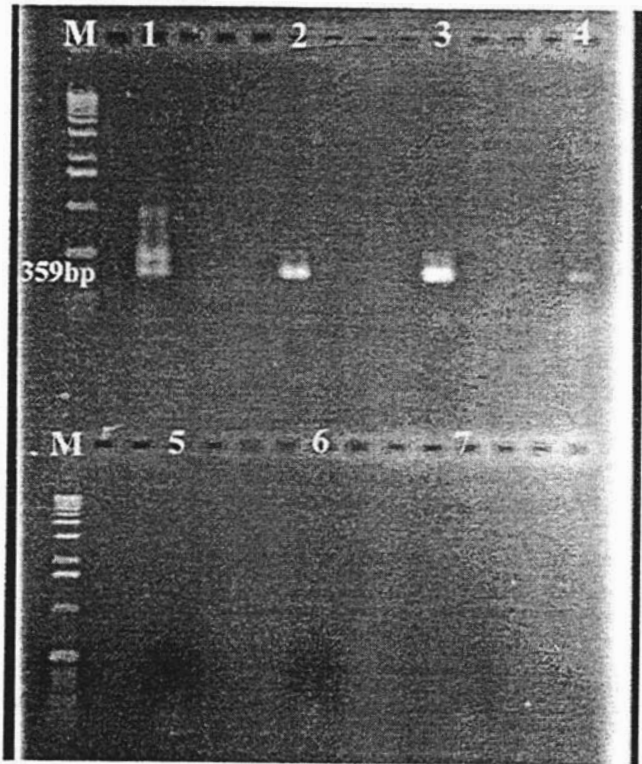
7.3.3 Sensitivity of the nested PCR assay

Table 7.5 shows the results of the nested PCR assay using pig tonsillar tissues artificially contaminated with serial dilutions of *Y. enterocolitica* bioserotype 4/O:3. The lower limit of sensitivity after the first round of amplification was approximately 10^5 cfu per reaction. After the second round of amplification this had been reduced to approximately 10^2 cfu per reaction. Figure 7.6 shows the products of the 2 rounds of amplification.

Table 7.5.   Sensitivity of the nested PCR assay

Template	cfu/reaction	PCR 1	PCR 2
Infected tonsils	10^5	+	+
	10^4	-	÷
	10^3	-	÷
	10^2	-	+
	10	-	-
	1	-	-
Control tonsils	-	-	-

7.3.4 Application of the nested PCR assay for detection of infected pig tissues

The results of the nested PCR assays using boiled pig tissue samples as the initial template are shown in Table 7.6. All 5 control samples were negative on both culture and PCR. A further 6 samples from experimentally infected animals were also negative on culture and PCR, including heart, oral swabs (3) and rectal swabs (2). Five samples were positive on both the initial and nested rounds of PCR. Estimates of the cfu per reaction in these samples were 5 (tongue), 150 (oral swab), 850 (large intestine), 1000 (small intestine) and 1500 (large intestine). Twelve samples were positive on nested PCR only, with estimated bacterial counts per initial reaction ranging from <5 cfu (oral swab, mediastinal lymph nodes) to 5000 cfu (large intestine). Four samples were positive on culture and negative in both rounds of PCR. These were tongue (2 samples, 20 and 45 cfu per reaction

respectively), oral swab (100 cfu per reaction) and small intestine (600 cfu per reaction). In contrast, three samples were negative on culture and positive on nested PCR (oral swab, mediastinal lymph nodes).

Table 7.6.   **Detection of *Y. enterocolitica* in infected pig tissues by nested PCR**

Pig I-D	Tissue	cfu/ml	cfu/reaction	PCR 1	PCR 2
I-1	MLN	2×10^3	10	-	+
I-3	MLN	$<1 \times 10^3$ *	<5	-	+
I-6	MLN	3.3×10^4	165	-	+
I-53	MLN	$<1 \times 10^3$ *	<5	-	+
I-1	S. intestine	2×10^5	1000	+	+
I-2	S. intestine	2.5×10^5	1250	-	+
I-3	S. intestine	1.2×10^5	600	-	-
I-6	S. intestine	2×10^5	1000	-	+
I-39	Heart	-	-	-	-
I-1	Tongue	1×10^3	5	+	+
I-6	Tongue	9×10^3	45	-	-
I-39	Tongue	4×10^3	20	-	-
I-3	Tonsils	1.6×10^4	80	-	+
I-39	Tonsils	1.4×10^4	70	-	+
I-1	L. intestine	1.7×10^5	850	+	+
I-2	L. intestine	1×10^6	5000	-	+
I-3	L. intestine	5×10^4	250	-	+
I-6	L. intestine	3×10^5	1500	+	+
I-42	Oral swab	2.2×10^4	110	-	+
I-43	Oral swab	$<1 \times 10^3$ *	<5	-	+
I-47	Oral swab	-	-	-	-

Table 7.6 cont.   **Detection of *Y. enterocolitica* in pig tissues by nested PCR**

Pig I-D	Tissue	cfu/ml	cfu/reaction	PCR1	PCR2
I-48	Oral swab	-	-	-	-
I-52	Oral swab	2×10^4	100	-	-
I-53	Oral swab	-	-	-	-
I-54	Oral swab	3×10^4	150	+	+
I-47	Rectal swab	-	-	-	-
I-48	Rectal swab	-	-	-	-
C-5	Tongue	-	-	-	-
C-40	Tonsils	-	- ·	-	-
C-41	L. intestine	-	-	-	-
C-46	L. intestine	-	-	-	-
C-46	S. intestine	-	-	-	-

MLN - mediastinal lymph node; S. intestine - small intestine; L. intestine - large intestine;
* - no colonies counted on plating using a 1µl calibrated loop; I - infected animals;
C - control animals

7.3.5   Application of the nested PCR for detection of infected dog faeces

Positive culture results were achieved with only 35 out of the 133 samples (compared to
64 positive at the time of collection), indicating that long-term storage at -70°C in the
absence of a cryoprotectant, such as glycerol, decreases the viability of the bacteria. Of
these 35, 33 were positive in the first round of PCR. Bacterial counts in these broths
ranged from 5×10^2 - 5×10^5 cfu/ml. The other two culture-positive samples had counts
of 6-7 x 10^2 cfu/ml and, although negative in the first round of PCR, were positive
following nested PCR. A further 9 samples were negative on culture but positive following
PCR, 6 after one round of amplification and the other 3 only after nested PCR. Seven of
these 9 samples had been culture-positive at the time of collection, the other 2 had been

culture-negative. Of the remaining 89 samples that were *Yersinia*-negative on culture, 13 grew other bacterial genera. All 89 were negative after two rounds of PCR. Table 7.7 shows the results of culture and the PCR assays. Figures 7.7 and 7.8 are photos of gels showing the results of a typical nested PCR assay.

Table 7.7.   Results of culture and PCR of stored dog faecal samples

Number of samples	Culture results	PCR 1	PCR 2
33	Ye +ve	+	ND
2	Ye +ve	-	+
6	Ye -ve*	+	+
3	Ye -ve=	-	+
89	Ye -ve**	-	-

ND - nested PCR not performed; * - all were positive at the time of collection; # - one out of the 3 was positive at the time of collection; ** - twenty were positive at the time of collection

Lane no: M - 1kb DNA ladder (size of amplification product shown in base pairs); 1-4 & 6 - faecal samples were *Yersinia*-negative but positive for other faecal bacteria; 5, 7, 8 - *Yersinia*-positive faeces (6 x 10^3, 2 x 10^4 and 4 x 10^4 cfu/ml respectively); 9 - positive control, Ye 4/O:3 DNA; 10 - reagent negative control

Lane no (correspond to Fig 7.7): M - 1kb DNA ladder (size of amplification product shown in base pairs); 1-4 & 6 - faecal samples were *Yersinia*-negative but positive for other faecal bacteria; 5, 7, 8 - *Yersinia*-positive faeces (6 x 10^3, 2 x 10^4 and 4 x 10^4 cfu/ml respectively); 9 - reagent negative control; positive control was omitted

Figure 7.7.   Faecal samples, first round of amplification
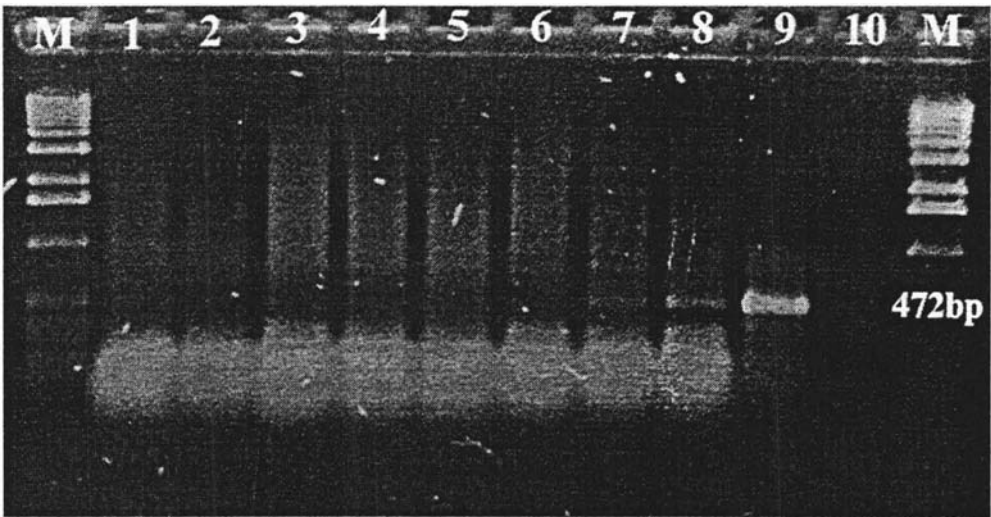
Figure 7.8.   Faecal samples, nested PCR
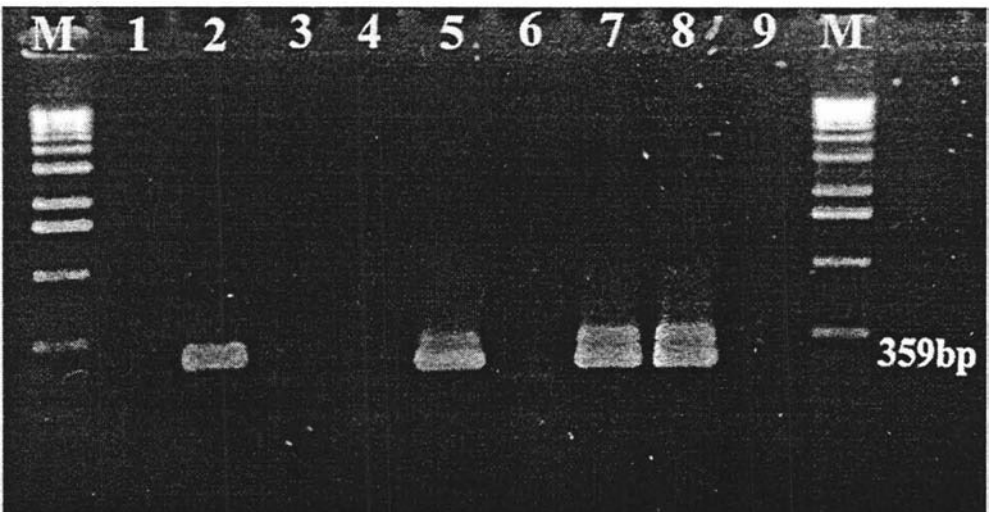
7.4   DISCUSSION

Although the PCR is, in principle, an extremely useful technique for the rapid detection and identification of pathogenic bacteria, problems have arisen, particularly with the sensitivity of the assay, when attempts have been made to adapt it for use directly with clinical samples in the diagnostic laboratory (Rossen *et al.*, 1992). Explanations offered for this are, firstly, that such samples are very varied and of complex composition, thus hindering the development of a simple, universal procedure that would allow the preparation of pure DNA without inhibitory substances. Secondly, that only very low numbers of pathogenic microorganisms are often present in clinical samples, for example, in tissues from asymptomatic carrier animals, such as pigs infected with *Y. enterocolitica*. The objectives of this study, therefore, were to compare various methods for the preparation of template from infected material for use in the PCR, and to develop simple, sensitive protocols for the analysis of stored samples from previous studies involving pigs and dogs.

7.4.1   Methods of template preparation

Two methods were used for the preparation of template DNA from a range of pig tissues, DNA extraction with phenol-chloroform and a simple boiling procedure. Results achieved with both methods showed that extraction of DNA, while time-consuming, resulted in a more sensitive assay, with all but one sample positive on both culture and PCR. The only negative result with the extraction method was with pig tongue, which may be attributable to the presence of mucus, shown previously to be inhibitory to the PCR in human sputum samples (Amicosante *et al.*, 1995). On the other hand, template prepared by homogenisation and boiling of samples appeared to inhibit the PCR in all instances, possibly because this crude lysate contained non-specific inhibitors that were only removed by extraction and purification of DNA.

In addition to the phenol-chloroform extraction and boiling methods used for tissue samples, a commercial DNA purification procedure, Prep-A-Gene, and a pre-enrichment step were also assessed for the preparation of template from faecal samples. Equivocal

results were achieved with the phenol-chloroform extraction and boiling methods, with 50% of samples assayed producing false-negative results. In contrast, when extracted DNA was purified using the Prep-A-Gene protocol, no false-negative results occurred. False-negatives were not unexpected with faecal samples as other researchers have found them to be inhibitory to the PCR (Challans *et al.*, 1994), and the use of immuno-magnetic separation, nested PCR and subsequent probing have all been used to overcome the problem (Ibrahim *et al.*, 1992c; Kapperud *et al.*, 1993; Rasmussen *et al.*, 1995). Further complicating the situation, however, is the fact that DNA extraction solutions may also be inhibitory to the PCR (Rossen *et al.*, 1992) and this may explain why a commercial DNA purification kit produced a "cleaner" template in this study. Weynants *et al.* (1996) also used a commercial DNA purification kit for the detection of *Y. enterocolitica* in faeces and achieved good sensitivity in the PCR with the resulting template.

One modification to the template preparation protocol that has achieved good results in other studies with a range of pathogens is the inclusion of a pre-enrichment step (Wernars *et al.*, 1991; Bej *et al.*, 1994). This has advantages in that it achieves a dilution of inhibitory compounds, and allows an increase in the concentration of viable target cells. In addition, it is a simple and inexpensive procedure that would fit easily into diagnostic laboratory methodology. The only disadvantage is the increased time required to produce a result. Pre-enrichment can be achieved in liquid broths or on solid media and examples of both were assessed in this study. PCR results following enrichment with some media, however, were shown to be better than with others. For example, enrichment in selenite broth and on MacConkey agar appeared to produce some inhibition, whereas no inhibition was experienced when tryptone water, CIN broth and CIN agar were used. Rossen *et al.* (1992) also found some culture media to be inhibitory, including those that incorporated bile salts, such as MacConkey agar. Although other workers had used non-selective enrichment media in *Y. enterocolitica* template preparation protocols, including trypticase soy broth (Kapperud *et al.*, 1993) and PBS (Rasmussen *et al.*, 1995), in this study better amplification signals were achieved after growth of the organism in a selective medium, CIN broth. One reason for this may be that other faster-growing enteric organisms in faecal samples may interfere with the PCR assay. Kapperud *et al.* (1993) achieved very good

results when a non-selective broth was used for enrichment, except in a small percentage of samples where a high background flora existed. While similar results in this study were achieved using faecal samples either enriched in CIN broth or grown on CIN agar, the use of broth cultures gave better amplification signals.

7.4.2   Nested PCR

In addition to improved DNA extraction protocols, another approach to overcoming the problem of PCR inhibition is the use of nested primers in the assay. This technique, which involves reamplification of an aliquot of the original amplification products with nested primers specific for sequences within the product, has been used successfully for the detection of low levels of pathogenic microorganisms in clinical samples where a single round of PCR was ineffective (Pierre *et al.*, 1991; Song *et al.*, 1993). Nested PCR assays have also been used for the detection of pathogenic *Y. enterocolitica* in meat (Kapperud *et al.*, 1993), pig tonsils (Thisted Lambertz *et al.*, 1996) and water (Sandery *et al.*, 1996). Thisted Lambertz *et al.* (1996), found that the use of nested PCR on cold-enrichment broths reduced the detection time from eight days to zero, i.e. on the day of tonsillar collection.

In order to justify the extra time required for two rounds of PCR, it was felt that the method of template preparation should be as simple as possible, therefore, boiling of tissue homogenates and faecal suspensions was the method of choice, despite the poor results achieved in earlier experiments following a single round of amplification. Encouraging results were obtained in a preliminary appraisal of the technique, with positive amplification after a second round of PCR often following a negative result in the first round. When compared to culture, both the sensitivity and specificity of the nested assay were 100% (i.e. no false-negatives or false-positives), and in one instance nested PCR appeared to prove more sensitive than culture, with a positive amplification on a culture-negative pig faecal sample. This could have been due to the presence of either very low numbers of bacteria (less than 1×10^3 CFU/ml will appear as no growth when a $1\,\mu l$ calibrated loop is used) or non-viable bacteria in the original sample. It could also have been a false-positive result due to contamination of the PCR reaction, however, as the negative controls in this and other

experiments all remained negative this was considered unlikely. In addition, other workers have reported results in which samples were both culture-negative and nested PCR-positive (Song *et al.*, 1993).

To assess the sensitivity of the nested primer assay, serial dilutions of *Y. enterocolitica* in pig tonsil homogenates were examined. After one round of amplification only 1×10^5 cfu per reaction could be detected, however, this was reduced to 1×10^2 cfu following a second round, a one thousand-fold increase in sensitivity. These findings supported the opinion that this approach would prove to be useful for the rapid detection of *Y. enterocolitica* in clinical samples, and it was decided to use it to analyse a number of stored samples from previous infection studies, in combination with standard culture methodology.

7.4.3 Application of the nested PCR assay for the detection of *Y. enterocolitica* in infected pig tissues

When the nested PCR assay was applied to the analysis of pig tissue samples that had been stored at -70°C following an infection trial, it was shown to be 100% specific, i.e. no false-positives occurred. False-negative results (when compared to culture) were seen with two tongue samples, an oral swab and a small intestine sample. Surprisingly, however, five samples that were positive in the first round of PCR also included intestinal samples (comprising tissue and contents), an oral swab and a tongue sample, all of which had been reported to be among the most inhibitory samples for the PCR by other researchers. In fact, the tongue sample was calculated to contain only 1×10^3 cfu per reaction, the lowest limit of sensitivity seen with the single round of PCR. Inhibition of the PCR by samples from the pharynx may be related to an excess of blood and mucus in this type of tissue. Similar inhibition of the PCR by oral swabs had been documented previously by Rasmussen *et al.* (1995), who suggested that it was due to blood and mucus collected on the swab at sampling. Faecal samples have been well-documented as the most difficult samples for DNA extraction and amplification and Monteiro *et al.* (1997) identified complex polysaccharides, possibly originating from vegetable material in the diet, as inhibitors in this type of sample. Despite this, the results of the current experiment indicate that while

inhibition of the PCR may be anticipated with certain categories of samples, it does not always occur, although the reasons for this are unclear.

Overall, the results following nested PCR in this experiment showed a significant improvement over a single round of amplification, with the number of positive samples detected increasing from 5 to 17. In addition, the nested PCR was shown to be highly sensitive, with 10 cfu or less in the initial reaction being detected on four occasions. Other workers have also found the nested PCR to be highly sensitive, with Sandery *et al.* (1996) showing it to be capable of detecting approximately 2 cells in an aliquot of template. However, three of these four nested PCR-positive samples, although from known infected animals, were negative on culture, a finding reported by other researchers using a nested primer assay (Song *et al.*, 1993) and although difficult to confirm, the PCR results were believed to be valid in this experiment.

7.4.4 Application of the nested PCR assay for the detection of *Y. enterocolitica* in infected dog faeces

Traditionally, the diagnosis of *Yersinia* infections has relied on either cold or selective enrichment and subsequent culture on selective media, a relatively time-consuming procedure that has also been shown to lead to an underestimation of the level of infection, particularly in prevalence studies (Nesbakken *et al.*, 1991a). Thus, PCR theoretically offers the advantages of decreasing the time involved to make a diagnosis and increasing the sensitivity of detection. Unfortunately, faeces has previously been shown to be inhibitory for the PCR and a number of adaptations have been used to circumvent the problem, including the use of pre-enrichment followed by nested PCR (Kapperud and Vardund, 1995). As faecal samples from the dog infection trial described in Chapter 5 had been frozen at -70°C, the opportunity was available to compare traditional culture techniques with a recently developed PCR-based procedure. From the results of preliminary experiments assessing methods of template preparation, it was decided that the combination of pre-enrichment and nested PCR would provide the highest level of sensitivity and specificity and would overcome the deficiencies identified in the previous method, where boiling was used to prepare template.

In contrast to the previous experiment with infected pig tissues, 33 out of 35 culture-positive faecal samples were positive on the first round of PCR (94.3%), emphasising the advantage of the pre-enrichment step as a means of increasing the quantity of template DNA, diluting inhibitory factors, or both. The remaining two culture-positive samples were detected following the second round of amplification, increasing the sensitivity of the complete assay to 100%. Bacterial counts on the enrichment broths from these two samples found them to be similar to those that were positive in the first round (approximately 6-700 cfu/ml) and the reason for the discrepancy is unclear. As 13 of the 89 culture-negative samples grew other bacterial genera, but were negative in the PCR, the specificity of the assay was also 100%. In addition, a further 9 culture-negative samples were shown to be positive in the PCR, 6 after only one round of amplification. All 9 samples had originated from known infected animals and although only 7 had been culture-positive when first collected some months previously, subsequent samples from the other 2 dogs were culture-positive. This implies that these two dogs were in the early stages of infection and accentuates the sensitivity of the nested PCR protocol for the detection of low numbers of bacteria.

A similar study, involving beagles experimentally infected with *Salmonella*, used pre-enrichment, one round of PCR and hybridisation, to detect faecal carriers of the organism. After 24h of enrichment, 25 out of 64 samples were positive by culture and 36 by PCR-hybridisation, comparable results to those achieved in the present study with a nested PCR assay (Stone *et al.*, 1995).

In conclusion, pre-enrichment and nested PCR is a rapid and highly sensitive technique for the detection of *Y. enterocolitica* in faecal samples, and has the potential for integration into current laboratory methodology once the technology becomes more accessible.

CHAPTER 8

8. TYPING OF ANIMAL AND HUMAN ISOLATES OF *Y. ENTEROCOLITICA* BY PULSED FIELD GEL ELECTROPHORESIS

8.1 INTRODUCTION

Bacterial pathogens, including *Y. enterocolitica*, have traditionally been identified and characterised by phenotypic methods, including cell morphology, biochemical reactions, serotyping and phage typing. Until 1980, *Y. enterocolitica* was regarded as a heterogeneous group classified as *Y. enterocolitica*-like organisms, however, in 1980 Bercovier *et al*. redefined the species as *Y. enterocolitica sensu stricto*, on the basis of biochemical and other phenotypic characteristics. Nilehn (1969), was the first bacteriologist to formally propose a biotyping scheme for the subdivision of *Y. enterocolitica* into five biotypes, and, although the criteria for inclusion in a biotype have been amended by successive workers (Bercovier *et al*., 1980a; Wauters *et al*., 1987), this method for subtyping the species is still widely used (Fukushima *et al*., 1984c). In conjunction with biotyping, serotyping of the O antigens of *Y. enterocolitica* has been the primary tool for the identification of clinically significant strains and epidemiological investigations (Buchrieser *et al*., 1994a). Serotyping was developed by Winblad (1967) and the scheme was later expanded by Wauters *et al*. (1971, 1972) and revised by Aleksic and Bockemuhl (1984).

Despite their usefulness, biotyping and serotyping have low discriminatory capabilities and a number of molecular techniques have been used in recent years to further define the species, including restriction endonuclease analysis of the plasmid (REAP: Nesbakken *et al*., 1987), restriction endonuclease analysis of the chromosome (REAC: Kapperud *et al*., 1990b), ribotyping (Andersen and Saunders, 1990), multilocus enzyme electrophoresis

(MEE: Goullet and Picard, 1984), polymerase chain reaction (PCR: Ibrahim *et al.*, 1992a), random amplified polymorphic DNA (RAPD: Rasmussen *et al.*, 1994a) and pulsed field gel electrophoresis (PFGE: Iteman *et al.*, 1991). Recently, Iteman *et al.* (1996), compared a number of these for their ability to subtype strains of *Y. enterocolitica* and concluded that PFGE was the most suitable for epidemiological tracing.

Prior to this study, only the phenotypic techniques biotyping and serotyping had been used to type *Y. enterocolitica* isolates in New Zealand (McCarthy and Fenwick, 1991). While these had provided useful preliminary information on the local distribution of strains, the information was limited by the apparent homogeneity of the organism, with the majority of isolates belonging to one bioserotype, 4/O:3. Detailed epidemiological information on characteristics of the organism such as clonality, subtype distribution or transmission from animals to people is considered essential before any attempts can be made to institute programmes aimed at controlling it's dissemination. Therefore, in order to investigate these and other features of *Y. enterocolitica*, a comprehensive study was initiated using a highly discriminatory method, PFGE.

8.2   MATERIALS AND METHODS

8.2.1   Description of study strains

A total of 602 strains were chosen from the culture collection at Massey University, representing different bioserotypes recovered from various animal species, including people (Table 8.1). When possible, isolates of each serotype were selected from geographically diverse regions of New Zealand (Tables 8.2 and 8.3). Type strains of each bioserotype from the Pasteur Institute in Paris were included in the study for comparison with New Zealand strains. Isolates were retrieved from the -70°C freezer onto blood agar plates and were incubated at 25°C overnight before being biotyped and serotyped for confirmation of identity, as described previously in Chapter 3.

Table 8.1. Source of strains used in the study

Type	Man	Pig	Dog	Cat	Cow	Deer	Sheep	Goat	Horse	Alpaca	Total
4/O:3	161	70*	6	-	-	-	-	-	-	-	237
2/O:5,27	54	68	6	2	16	7	1	7	1	4	166
2/O:9	158	-	2	1	23	7	4	-	-	1	196
3/O:1,2,3	1	-	-	-	-	-	2	-	-	-	3
Total	374	138	14	3	39	14	7	7	1	5	602

* - includes two strains isolated from pork mince

Table 8.2.   **Regional distribution of human strains of *Y. enterocolitica***

Region	4/O:3	2/O:5,27	2/O:9	Total
Northland	2	-	1	3
Auckland	20	35*	93	148
Waikato	5	1	3	9
Bay of Plenty	5	-	1	6
Rotorua	8	1	4	13
Gisborne	1	-	-	1
Taranaki	8	3	5	16
Wanganui	15	-	8	23
Hawke's Bay	21	1	13	35
Manawatu	21	-	12	33
Wellington	17	10	12	39
Nelson	1	-	-	1
Canterbury	32	3	5	40
West Coast	-	1	1	2
Otago	1	-	-	1
Southland	4	-	-	4
Total	161	55	158	374

* - includes one strain of bioserotype 3/O:1,2,3

Table 8.3.   **Regional distribution of animal strains of *Y. enterocolitica***

Region	4/O:3	2/O:5,27	2/O:9	3/O:1,2,3	Total
Auckland	2*	1	-	-	3
Waikato	-	13	17	-	30
Manawatu-B	-	1	4	-	5
Manawatu-M	72[#]	85[#]	6	-	163
Canterbury	2	4	5	-	11
Otago	-	8	6	2	16
Total	76	112	38	2	228

* - two isolates recovered from pork mince; [#] - includes porcine isolates from Manawatu and Wairarapa

Auckland - Auckland Animal Health Laboratory; Waikato - Ruakura Animal Health Laboratory; Manawatu-B - Batchelar Animal Health Laboratory; Manawatu-M - Massey University Department of Veterinary Pathology; Canterbury - Lincoln Animal Health Laboratory; Otago - Invermay Animal Health Laboratory

8.2.2   Preparation of genomic DNA from study strains

DNA from *Yersinia* strains was prepared in low-gelling point, pulse-field certified agarose (PFC-Bio-Rad) using a modification of the techniques described by Olsen *et al.* (1994) and Barrett *et al.* (1994).

Isolates of *Y. enterocolitica* were inoculated into 4ml Brain Heart Infusion broth (BHI) and incubated overnight at 25°C. A 100µl aliquot of each broth culture was pipetted into a sterile micro-centrifuge tube and centrifuged at 10,000 rpm for 4min. The supernatant was removed, the pellet resuspended in 150µl ice-cold Pett IV buffer (1M NaCl, 10mM Tris HCl pH 8.0, 10mM EDTA pH 8.0) and the suspension centrifuged at 10,000 rpm for a further 4min. The supernatant was removed and the pellet resuspended in 50µl cold Pett IV buffer and kept on ice until required.

Seventy five microlitres of molten 1% PFC agarose (10 mg ml^{-1} agarose in Pett IV buffer) was added to the suspension and mixed carefully by pipetting to avoid air-bubbles. The resultant agarose/buffer suspension was transferred quickly into a plug mould and left to solidify on ice for at least 10min. When solid, plugs were removed into fresh microcentrifuge tubes containing 1ml Urea-ESP buffer (6M Urea, 50mM Tris-HCl pH8.0, 50mM EDTA pH8.0, 1% sodium lauryl sarcosine, 0.2% sodium deoxycholate, 0.5 mg ml^{-1} Proteinase K) and incubated overnight in a waterbath at 56°C. Following overnight incubation, the plugs were washed 10 times in ice-cold TE pH8.0, for at least 30min each wash and stored in TE pH8.0 at 4°C until required.

8.2.3   Restriction endonuclease digestion of plug-incorporated DNA

Initial experiments with various infrequent cutting restriction endonucleases and enzyme concentrations had shown that the most appropriate enzyme for use with *Y. enterocolitica* was *Not* I (New England Biolabs) and that the optimum concentration was 15U of enzyme. Other enzymes that were assessed included *Xba* I, *Sma* I, *Spe* I, *Swa* I and *Xho* I. Criteria for assessing the enzymes were cutting ability, the number of bands produced (with a range of 20-30 being considered ideal), and the size range of the bands.

Approximately one quarter of each plug was cut off using a sterile scalpel blade, placed in 100µl of 1x restriction buffer (12µl 10x New England Biolabs Buffer 3, 1µl 100mg ml^{-1} Bovine Serum Albumin (BSA), 87µl sterile distilled water) and equilibrated for at least 45min on ice. Ten to fifteen plugs were processed at one time and a cocktail of the restriction buffer was made up depending on the number of plugs being digested and aliquotted accordingly. The restriction buffer was decanted and replaced with 80µl cutting buffer (8µl 10x restriction buffer, 0.8µl 100 mg ml^{-1} BSA, 15U restriction endonuclease, distilled water). Again, cocktails of the cutting buffer were made up depending on the number of plugs to be digested. Plugs and buffer were equilibrated for 45min before incubation overnight in a waterbath at 37°C. No significant deterioration of DNA was seen in plugs stored up to 6 months at 4°C.

8.2.4 Pulsed-field gel electrophoresis of digested DNA

The electrophoresis system used was a contour-clamped homogeneous electric field (CHEF) apparatus, including variable speed pump, gel chamber, power module and mini chiller (CHEF Mapper, Bio-Rad Laboratories, Richmond, California, USA). A number of preliminary experiments were performed to optimise the electrophoresis parameters for maximum resolution of the digested DNA fragments, resulting in two protocols that were used throughout the study (Table 8.4). Standard gel protocols were used for all isolates, long gel protocols were used for selected isolates, to achieve improved separation of specific regions of the banding patterns.

Once digestion was completed, plug slices were loaded into the wells of a 1% PFC agarose gel (800mg in 80ml 0.5x TBE buffer), which had been allowed to equilibrate in the electrophoresis chamber with the buffer (0.5x TBE) circulating, for at least 2h. In addition to the plugs, up to three molecular weight markers were also loaded in each gel:

(i)   Gibco BRL "High molecular weight" marker (Cat. no. 15618-010)
        Range 8.3-48.5 kb. 2.5µl + 5µl TE + 2.5µl dye in eppendorf, heated at 50°C for
        1min. Total amount loaded into gel.

(ii)   Gibco BRL "Mega Base II DNA Standard" (Cat. no. 15627-011)
        Range 48.5-1309.5 kb. 10µl + 10µl TE + 2.5µl dye in eppendorf, heated at 50°C
        for 1min. Total amount loaded into gel.

(iii)   Bio-Rad *Saccharomyces cerevisiae* chromosomes in agarose blocks
        Range 225-2200 kb. Slice removed from a block and loaded in the same way as
        plugs.

After electrophoresis, gels were stained in fresh aqueous ethidium bromide (80µl of 10mg ml^{-1} stock solution in 800ml DW) for 1h, then destained in distilled water overnight. Gels were examined under UV light and photographed.

Table 8.4.   Gel running parameters

Parameter	Standard gel	Long gel
Agarose type/concentration	1% PFC	1% PFC
Buffer type/concentration	0.5x TBE	0.5x TBE
Voltage gradient	6.0V cm^{-1}	6.0V cm^{-1}
Buffer temperature	14°C	12°C
Pulse angle	120°	120°
Initial switch time	3s	5s
Final switch time	25s	25s
Switch-time ramp	79%	79%
Run time	24h	29h

8.2.5   Interpretation of pulsed-field profiles

Gels were analysed visually for differences in banding patterns. The first isolate examined in each serotype was arbitrarily designated as Type 1 and all other isolates were compared with this "type strain". Bands were identified numerically from the highest molecular weight downwards, with positions fixed in relation to Type 1, double bands, recognised by variations in intensity, were counted as such. Bands were also described by their approximate size in kilobases relative to one of the molecular weight markers. Isolates were subsequently classified into Types and pulsotypes on the basis of their band differences to the Type 1 strain. Following the guidelines published by Tenover *et al.* (1995) on the interpretation of chromosomal DNA restriction patterns produced by PFGE, isolates were assigned to new Types when they differed by four or more bands from the Type 1 strain, and to pulsotypes when they differed by 1-3 bands from the Type strains.

To assess the stability of banding patterns, a number of isolates were examined on two or more occasions during the course of the study. In addition, as the presence or absence of the 70kb virulence plasmid was not determined before PFGE, DNA fragments at or below this size were considered to be potentially of plasmid origin. This was taken into account when assigning isolates to Types and pulsotypes.

8.3 RESULTS

8.3.1 Choice of restriction enzyme and gel-running parameters

All the enzymes tested successfully cut the agarose-incorporated DNA and provided some degree of discrimination between strains. However, the best discrimination was achieved with *Not*I, and this enzyme was chosen for the study on the basis of it's efficiency, cost, and the production of 35-45 bands between 20-370kb, allowing patterns to be easily interpreted. Also, many overseas studies of *Y. enterocolitica* using PFGE had employed this enzyme, making valid comparisons with overseas strains a possibility. Various enzyme concentrations were used in the initial phase of the project, with the best results being achieved with a concentration of 15-20 units per digest and an overnight digestion at 37°C.

The optimisation process also included a number of experiments to assess the effect of altering certain key variables on the separation of the DNA bands. Parameters that were shown to be important included DNA concentration, agarose concentration, switch times, switch-time ramp, run time and buffer temperature. Manipulation of these parameters resulted in two protocols (described in Materials and Methods) that allowed optimal resolution of different regions of the banding patterns. Nevertheless, differences were recognised in the ability of the protocols to resolve DNA from the three serotypes examined, with the best separation being achieved with serotype O:3 and the worst with O:5,27. The reasons for this are unclear but possibly involve differences in cell wall composition between strains, resulting in the plug preparation protocol used for O:3 strains being sub-optimal for strains of serotype O:9 and particularly for strains of serotype O:5,27. This assumption is based on an improvement in the resolution of all 3 serotypes when 6M Urea was included in the lysis buffer and an increased number of wash steps was adopted.

Despite these measures, the overall difference in resolution between the serotypes remained the same and identical protocols were used throughout the study.

Repeat analysis of a proportion of the study strains showed that banding patterns were almost invariably consistent, an important consideration when the subtyping of strains is to

be used for drawing epidemiological conclusions. On one occasion, the loss of bands approximately 70kb in size was assumed to be due to loss of the virulence plasmid, however, this was not confirmed.

8.3.2 PFGE analysis of strains of *Y. enterocolitica*, bioserotype 4/O:3

Two hundred and thirty seven strains were analysed and these were subdivided into two major Types, I and II, having four or more band differences (Table 8.5). Type I was further subdivided into 12 pulsotypes, designated I-I_L, having up to three band differences from the principal strain (pulsotype 1 was the same as Type 1). Type II was subdivided into six pulsotypes using similar criteria. The distribution of Types and pulsotypes and their hosts of origin are shown in Table 8.5. Bioserotype 4/O:3 strains were very homogeneous, with 3 pulsotypes (I, I_C and II) making up approximately 88% of all isolates. Pulsotype I_C may in fact be a plasmidless strain of Type I as it is characterised by missing bands at 48 and 28kb, although this was not verified (see Figure 8.2). Nine pulsotypes contained only one strain each. Figure 8.1 is a composite gel of selected Type I pulsotypes.

Table 8.5 shows that all but one of the canine isolates, the majority of the porcine isolates and a large proportion of the human isolates belonged to pulsotype I. One canine isolate belonged to pulsotype I_D. The remainder of the porcine isolates were shown to be either pulsotypes I_A or I_C. Other than these four pulsotypes, the only other non-human isolates were two from pork mince, recovered during public health investigations in Auckland. The canine isolates were recovered at Massey University (4) and Lincoln Animal Health Laboratory (2). The porcine isolates were recovered from tonsils during abattoir surveys carried out in 1989 and 1993, and originated from 4 farms, as shown in Table 8.6. Figure 8.2 shows a selection of isolates recovered from the four pig farms.

Table 8.5.   **Pulsotypes of** *Y. enterocolitica* **bioserotype 4/O:3**

Type	No.	%	People	Pigs	Dogs
Type I*	186	78.5	122	59	5
Pulsotype I_A	1	0.4	-	1	-
Pulsotype I_B	5	2.1	5	-	-
Pulsotype I_C	13	5.5	5	8	-
Pulsotype I_D	2	0.8	1	-	1
Pulsotype I_E	3	1.3	3	-	-
Pulsotype I_F	5	2.1	5	-	-
Pulsotype I_G	1	0.4	1	-	-
Pulsotype I_H	1	0.4	1	-	-
Pulsotype I_J	1	0.4	1	-	-
Pulsotype I_K	1	0.4	1	-	-
Pulsotype I_L	1	0.4	1	-	-
Type II*	9	3.8	8	1#	-
Pulsotype II_A	2	0.8	2	-	-
Pulsotype II_B	1	0.4	1	-	-
Pulsotype II_C	1	0.4	-	1#	-
Pulsotype II_D	3	1.3	3	-	-
Pulsotype II_E	1	0.4	1	-	-
Total	237	100	161	70	6

* - Type 1 is the same as pulsotype I, Type II is the same as pulsotype II; # - isolated from pork mince at Auckland Public Health Laboratory

Figure 8.1.   Representative Type I pulsotypes of bioserotype 4/O:3
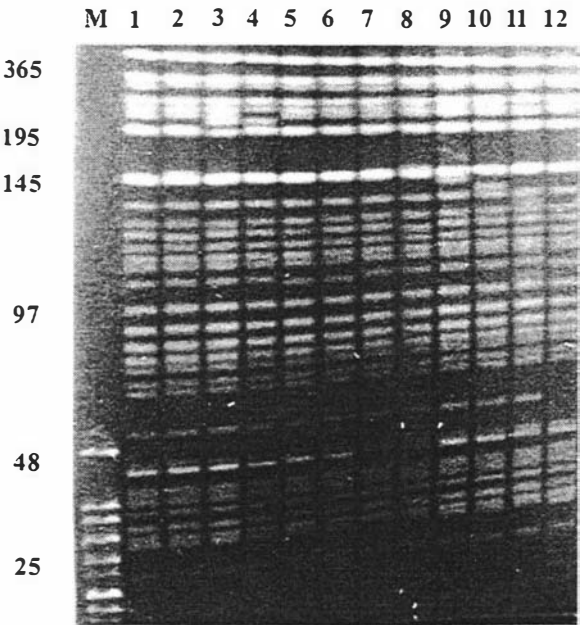
Lane no: M - BRL high molecular weight marker (Cat. no. 15618-010), (selected band sizes shown in kb); 1- Type I (pulsotype I), 2 - pulsotype (pt) I_J; 3 - pulsotype I_K; 4 - pulsotype I_A; 5 - pulsotype I_B (human); 6 - pulsotype I_B (porcine); 7 - pulsotype I_C; 8 - pulsotype I_D; 9 - pulsotype I_E; 10 - pulsotype I_L; 11 - pulsotype I_F; 12 - pulsotype I_G

Table 8.6.   Pulsotypes of *Y. enterocolitica* bioserotype 4/O:3 recovered from pig farms

Farm/Location	Pulsotype I	Pulsotype I_A	Pulsotype I_C	Total
A - Rongotea[1]	38	1	3	42
B - Carterton[2]	5	-	4	9
C - Carterton[2]	15	-	1	16
D - Opiki[3]	1	-	-	1

1 - Manawatu region; 2 - Wairarapa region; 3 - Horowhenua region

Figure 8.2. Porcine isolates of *Y. enterocolitica* bioserotype 4/O:3
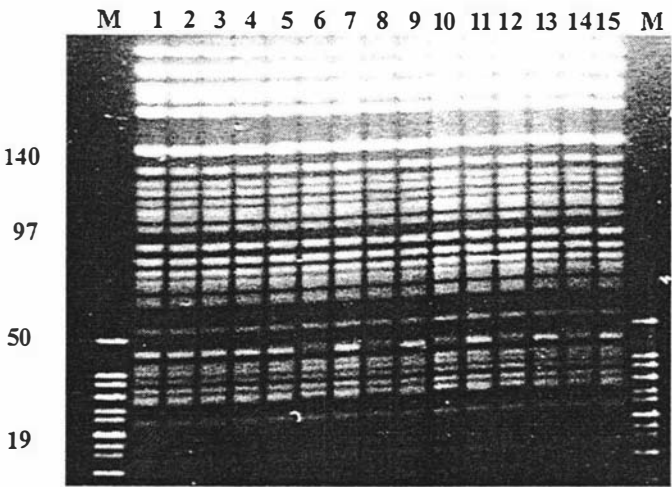
Lane no: M - BRL high molecular weight marker (selected band sizes are shown in kb); all isolates are pulsotype I except lanes 6, 8, 10, 12 and 14 which are pulsotype I_C (identical to I except that bands at 45 and 28kb are missing, may be due to loss of plasmid)

Figure 8.3. Strains of *Y. enterocolitica* bioserotype 4/O:3 recovered from pigs, dogs and people
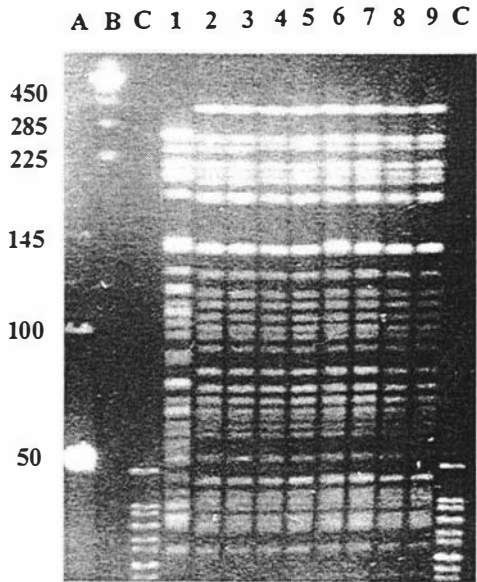
Lane no: A - BRL Megabase II DNA standard (Cat. no. 15627-011); B - Bio Rad *Saccharomyces cerevisiae* chromosomes; C - BRL high molecular weight marker; 1 - Ye 4/O:3 type culture (Pasteur Institute, Paris, Ye 134); 2 - pt I, blood donor isolate; 3 - pt I, contaminated blood bag; 4 - pt I, canine; 5 - pt I, canine; 6 - pt I_D, canine; 7 - pt I, canine; 8 - pt I, porcine; 9 pt I, porcine

Figure 8.3 shows a selection of isolates from pigs, dogs and people, all but one of which belong to pulsotype I. One of the canine isolates belongs to subtype I_D, which has an extra high molecular weight band at approximately 140kb. The two human isolates originated from a case of transfusion-related septicaemia, one from the donor and the other from the contaminated blood bag. A reference bioserotype 4/O:3 strain from the Pasteur Institute included on the gel contrasts with the distinctive appearance of the New Zealand 4/O:3 strains.

8.3.3 PFGE analysis of strains of *Y. enterocolitica*, bioserotype 2/O:5,27

One hundred and sixty seven strains were analysed and were subdivided equally into two major types (I and II) and 40 pulsotypes, using similar criteria to those used for 4/O:3 strains. Type I consisted of 15 pulsotypes, Type II consisted of 25 pulsotypes. Tables 8.7 and 8.8 show the distribution of pulsotypes and their hosts of origin. Bioserotype 2/O:5,27 strains were considerably more heterogeneous than bioserotype 4/O:3 strains, with 6 pulsotypes making up approximately 68% of the total number analysed (I, I_A, I_B, I_O, II, II_A). In addition, 24 pulsotypes contained only one strain each.

Table 8.7 shows that isolates belonging to Type I were recovered only from people, pigs and cattle. All but 7 of the porcine isolates shared pulsotypes with human isolates. In contrast, only 1 out of 3 cattle isolates shared a pulsotype with a human isolate. The majority of the porcine isolates (86%) belonged to only 3 pulsotypes, I, I_A and I_B. The human isolates, on the other hand, were distributed evenly across all pulsotypes. The porcine isolates were recovered from tonsils during abattoir surveys in 1989 and 1993.

Table 8.7.   Type I pulsotypes of *Y. enterocolitica* bioserotype 2/O:5,27

Pt	No.	%#	People	Pigs	Cattle
I	25	15	4	21	-
I_A	24	14.4	4	20	-
I_B	14	8.4	1	13	-
I_C	1	0.6	1*	-	-
I_D	1	0.6	1	-	-
I_E	3	1.8	1	1	1
I_F	1	0.6	1	-	-
I_G	3	1.8	2	1	-
I_H	1	0.6	1	-	-
I_J	1	0.6	1	-	-
I_K	1	0.6	1	-	-
I_L	1	0.6	-	-	1
I_M	1	0.6	-	-	1
I_N	2	1.2	2	-	-
I_O	7	4.2	-	7	-
Total	86	51.5	20	63	3

Pt - pulsotype; * - Reference strain from Pasteur Institute (Ye 134); # - % of total number of strains analysed

Table 8.8.   **Type II pulsotypes of** *Y. enterocolitica* **bioserotype 2/O:5,27**

Pt	No	%*	Man	Pig	Dog	Cow	Deer	Goat	Alpaca	Cat	Sheep	Horse
II	9	5.4	5	2	-	1	1	-	-	-	-	-
II$_A$	34	20.3	9	3	4	5	4	5	3	-	-	1
II$_B$	3	1.8	3	-	-	-	-	-	-	-	-	-
II$_C$	4	2.4	4	-	-	-	-	-	-	-	-	-
II$_D$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_E$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_F$	5	3.0	2	-	1	1	-	-	-	1	-	-
II$_G$	2	1.2	2	-	-	-	-	-	-	-	-	-
II$_H$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_J$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_K$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_L$	4	2.4	-	-	1	-	-	2	-	1	-	-
II$_M$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_N$	2	1.2	-	-	-	-	1	-	-	-	1	-
II$_O$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_P$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_R$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_S$	2	1.2	1	-	-	-	-	-	1	-	-	-
II$_T$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_U$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_V$	1	0.6	-	-	-	-	1	-	-	-	-	-
II$_W$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_X$	1	0.6	1	-	-	-	-	-	-	-	-	-
II$_Y$	1	0.6	-	-	-	1	-	-	-	-	-	-
II$_Z$	1	0.6	1	-	-	-	-	-	-	-	-	-
Total	81	48.5	35	5	6	13	7	7	4	2	1	1

Pt - pulsotype; * - % of total number of *Y.* strains analysed

Table 8.8 shows that Type II pulsotypes were apparently non-host-specific, and as well as human isolates, included the majority of isolates recovered from domestic animals. Nevertheless, a closer look at the distribution shows that some of the pulsotypes appear more host-specific than others. For example, sixteen of the pulsotypes contain human isolates, but only 4 of these also contain isolates recovered from other species (II, II_A, II_F, II_S). Of these, II_A was the most common and widely distributed, containing isolates from people, pigs, dogs, cattle, deer, goats, alpaca and a horse. Of the other 9 pulsotypes, 6 contained only single cattle isolates (II_K, II_M, II_T, II_U, II_W, II_Y), one contained only a single deer isolate (II_V), one contained dog, goat and cat isolates (II_L), one contained deer and sheep isolates (II_N). Figure 8.4 shows a selection of bioserotype 2/O:5,27 pulsotypes.

Figure 8.4. **Representative pulsotypes of *Y. enterocolitica* bioserotype 2/O:5,27**
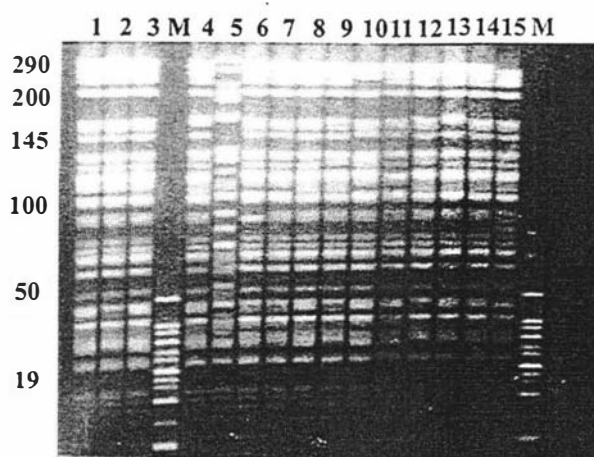
Lane no: M - BRL high molecular weight marker; 1 - Type I (pulsotype I); 2 - pulsotype I_B; 3 - pulsotype I; 4 - pulsotype II ; 5 - bioserotype 3/O:1,2,3; 6 - pulsotype I_D; 7 - pulsotype I_E; 8 - pulsotype II_A; 9 - pulsotype II_C; 10 - pulsotype I_N; 11 - pulsotype I_B; 12 - pulsotype II_C; 13 - pulsotype II_B; 14 - pulsotype II_A; 15 - pulsotype II_E

Table 8.9.   Pulsotypes of *Y. enterocolitica* bioserotype 2/O:5,27 recovered from pig farms

Farm/Location	I	I_A	I_B	I_E	I_G	I_O	II	II_A	Total
1-Featherston*	-	7	-	-	-	7	-	-	14
1-Featherston#	19	10	8	-	-	-	2	-	39
2-Rongotea*	-	-	-	-	1	-	-	-	1
2-Rongotea#	-	-	-	-	-	-	-	1	1
3-Foxton	-	1	4	-	-	-	-	-	5
4-Masterton	-	-	-	-	-	-	-	1	1
5-Opiki	-	-	-	1	-	-	-	-	1
6-Palmerston Nth.	2	2	1	-	-	-	-	-	5
Total	21	20	13	1	1	7	2	2	67

* - survey 1989; # - survey 1993

Table 8.9 shows the pulsotypes of bioserotype 2/O:5,27 recovered from each farm in the abattoir survey. Pulsotypes I, I_A, I_B and II_A were more widely distributed than other pulsotypes, pulsotype I and II_A being recovered from 2 farms, I_A and I_B from 3 farms each. The other 4 pulsotypes isolated from pigs were only recovered from 1 farm each. Pulsotype I_A was isolated from pigs supplied to the abattoir by Farm 1 in both 1989 and 1993. All other pulsotypes recovered from Farms 1 and 2 differed on the two occasions. Only one other porcine isolate was examined during the study, a pulsotype I_A strain recovered from a clinical case submitted to Lincoln Animal Health Laboratory.

8.3.4   PFGE analysis of strains of *Y. enterocolitica*, bioserotype 2/O:9

One hundred and ninety six strains were analysed and these were subdivided into two major Types (I and II). Type I was further subdivided into 19 pulsotypes. Type II contained only one isolate, recovered from a patient in Auckland, that showed a number of similarities to the reference strain from the Pasteur Institute. The distribution of Type I pulsotypes

between animal species is shown in Table 8.10. Bioserotype 2/O:9 was even more homogeneous than 4/O:3, with the principal pulsotype (pulsotype I) containing 84.2% of the strains analysed. Thirteen out of the nineteen pulsotypes in Type I consisted of only one isolate each. Figure 8.5 shows a representative selection of Type I pulsotypes.

Figure 8.5. **Representative pulsotypes of *Y. enterocolitica* bioserotype 2/O:9**
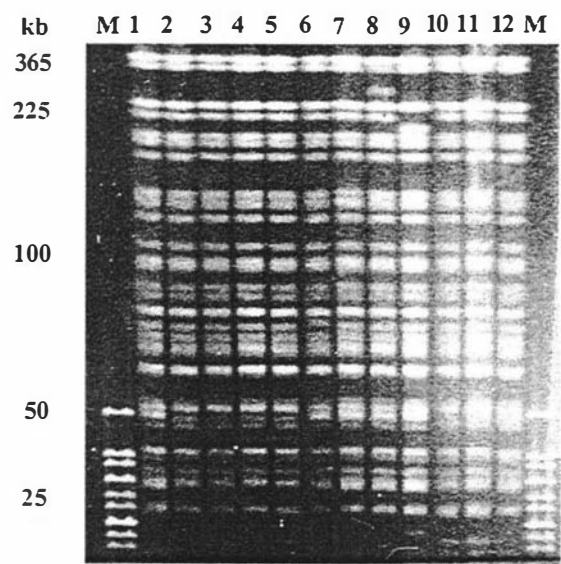
Lane no: M - BRL high molecular weight marker; 1-4 - pulsotype 1; 5 - pulsotype I_S; 6 - pulsotype I_J; 7 - pulsotype I; 8 - pulsotype I_M; 9 - pulsotype I_L; 10-11 - pulsotype I_A; 12 - pulsotype I

Table 8.10.   **Pulsotypes of** *Y. enterocolitica* **bioserotype 2/O:9**

Pt	No.	%*	Man	Cow	Deer	Sheep	Dog	Cat	Alpaca
I	165	84.2	134	21	5	4	-	1	-
I_A	6	3.1	6	-	-	-	-	-	-
I_B	1	0.5	1	-	-	-	-	-	-
I_C	3	1.5	3	-	-	-	-	-	-
I_D	1	0.5	1	-	-	-	-	-	-
I_E	2	1.0	-	-	2	-	-	-	-
I_F	3	1.5	2	-	-	-	1	-	-
I_G	1	0.5	1	-	-	-	-	-	-
I_H	1	0.5	-	-	-	-	-	-	1
I_J	1	0.5	1	-	-	-	-	-	-
I_K	1	0.5	-	-	-	-	1	-	-
I_L	1	0.5	1	-	-	-	-	-	-
I_M	1	0.5	-	1	-	-	-	-	-
I_N	1	0.5	1	-	-	-	-	-	-
I_O	1	0.5	-	1	-	-	-	-	-
I_P	3	1.5	3	-	-	-	-	-	-
I_Q	1	0.5	1	-	-	-	-	-	-
I_R	1	0.5	1	-	-	-	-	-	-
I_S	1	0.5	1	-	-	-	-	-	-
II	1	0.5	1	-	-	-	-	-	-
Total	196	100	158	23	7	4	2	1	1

Pt - pulsotype; * - % of total number of strains analysed

Table 8.10 shows that Type I isolates are clustered into one major pulsotype (pulsotype I), which appears to be non-host-specific, containing most of the human (84.8%) and animal (81.6%) strains. Of the remaining 18 Type I pulsotypes, only one other contains isolates

from more than one species (I_F, human and canine), 12 contain only human strains, 2 contain only bovine strains and 3 contain only cervine, canine or camelid strains.

8.3.5 Discrimination between bioserotypes of *Y. enterocolitica*

Figure 8.6 is a composite gel, comparing a spectrum of pulsotypes from the 3 principal bioserotypes, together with the relevant Pasteur Institute reference strains. All 3 bioserotypes, with one exception (2/O:9, pt II), are quite distinct, both from each other and from their respective reference strains.

Figure 8.6.   Comparison of pulsotypes between bioserotypes of *Y. enterocolitica*
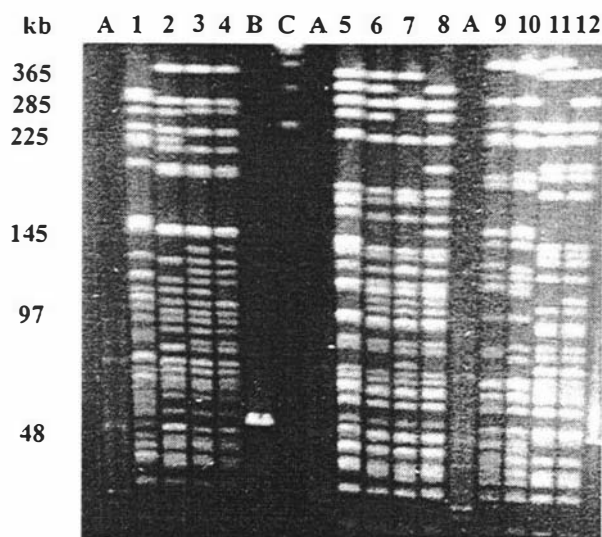
Lane no: A - BRL high molecular weight marker; B - BRL megabase II DNA standard; C - Bio Rad *Saccharomyces cerevisiae* chromosomes; 1 - Ye 4/O:3 type culture (Pasteur Institute Ye 134); 2 - Ye 4/O:3 pt I; 3 - Ye 4/O:3 pt II; 4 - Ye 4/O:3 pt II_B; 5 - Ye 2/O:5,27 type culture (Pasteur Institute Ye 885); 6 - Ye 2/O:5,27 pt II_A; 7 - Ye 2/O:5,27 pt II_M; 8 - Ye 2/O:5,27 pt II_V; 9 - Ye 2/O:9 type culture (Pasteur Institute Ye 383); 10 - Ye 2/O:9 pt II; 11 - Ye 2/O:9 pt I; 12 - Ye 2/O:9 pt I_R

The only other human pathogenic bioserotype recovered from people and animals in New Zealand was 3/O:1,2,3. Three strains were characterised, one human and two ovine. All 3 pulsotypes were quite different from each other and from the Pasteur Institute reference strain, as seen in Figure 8.7. Bioserotype 1B/O:8 is included for reference.

Figure 8.7. Pulsotypes of *Y. enterocolitica* bioserotype 3/O:1,2,3
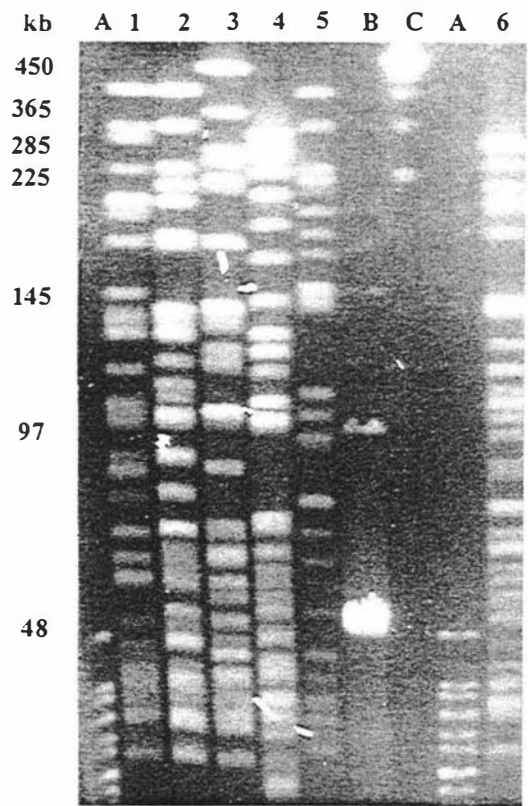
Lane no:A - BRL high molecular weight marker; B - BRL megabase II DNA standard; C - Bio Rad *Saccharomyces cerevisiae* chromosomes; 1 - Ye 3/O:1,2,3 type culture (Pasteur Institute Ye 135); 2 - L2 (human isolate); 3 - T35 (ovine isolate); 4 - V30 (ovine isolate); 5 - Ye 1B/O:8

8.3.6 Regional distribution of the most commonly isolated pulsotypes of *Y. enterocolitica* in New Zealand

Table 8.11 shows the distribution of the most common pulsotypes identified within each of the 3 principal bioserotypes, including isolates recovered from both animals and people. Of the 15 regions contributing bioserotype 4/O:3 strains, the main pulsotype, type I, was widespread, being recovered from 12. The other 2 common pulsotypes were only recovered from 4 regions each. By comparison, bioserotype 2/O:5,27 appeared to have a more regional distribution, as of the 10 regions recovering this strain, only 6, 2, 2, 2 and 4 regions respectively harboured the 5 main pulsotypes. This could be a result of the small

number of strains tested from some of the regions and a larger study might show a similar pattern to that seen with 4/O:3 and 2/O:9. The distribution of bioserotype 2/O:9 strains was similar to 4/O:3, with 12 out of 13 regions which had recovered this strain harbouring the main pulsotype, type I. The only other pulsotype of note, I_A, was recovered from only 3 regions.

Table 8.11.   Regional distribution of common *Y. enterocolitica* pulsotypes

Bioserotypes	Ye 4/O:3			Ye 2/O:5,27					Ye 2/O:9	
Region	I*	I_C	II	II_A	I	I_A	I_B	II	I	I_A
Northland	+	-	-						-	-
Auckland	+	+	+	+	-	+	+	+	+	+
Waikato	+	-	-	+	-	-	-	+	+	-
Bay of Plenty	+	-	-						+	-
Rotorua	+	-	-	-	-	-	-	-	÷	+
Gisborne	-	+	-							
Hawke's Bay	+	-	+	-	-	-	-	-	+	+
Taranaki	+	-	-	-	-	-	-	-	+	-
Wanganui	+	-	-						+	-
Manawatu	+	-	+	+	+	+	+	+	+	-
Wellington	+	-	-	+	+	-	-	+	+	-
Nelson	+	-	-							
Canterbury	+	+	-	+	-	-	-	-	+	-
Otago	-	-	-	+	-	-	-	-	+	-
Southland	-	+	+							
West Coast				-	-	-	-	-	+	-

* - Pulsotypes are tabulated in order of isolation frequency, left to right, within bioserotypes; shading indicates that no strains of these bioserotypes were examined from these regions

8.3.7 Annual isolation of the most common pulsotypes of *Y. enterocolitica*

Table 8.12 shows the years in which the most common pulsotypes of *Y. enterocolitica* were isolated. Pulsotype I of bioserotype 4/O:3 was isolated from 1988-1995, pulsotype I_C from 1993-1994 and pulsotype II from 1992-1995. Pulsotype II_A of bioserotype 2/O:5,27 was isolated from 1988-1994, pulsotypes I, I_A and I_B from 1988-1993 and pulsotype II from 1991-1994. Pulsotype I of bioserotype 2/O:9 was isolated from 1991-1995, I_A only in 1995.

Table 8.12. Annual isolation of the principal pulsotypes of *Y. enterocolitica*

Pulsotypes		1988	1989	1990	1991	1992	1993	1994	1995
O:3	I	+	+	+	+	+	+	+	+
	I_C	-	-	-	-	-	+	+	-
	II	-	-	-	-	+	-	+	+
O:5,27	II_A	+	+	+	+	+	+	+	-
	I	+	-	-	-	+	+	-	-
	I_A	+	-	+	-	+	+	-	-
	I_B	+	-	-	-	-	+	-	-
	II	-	-	-	+	+	+	+	-
O:9	I	-	-	-	+	+	+	+	+
	I_A	-	-	-	-	-	-	-	+

8.4   DISCUSSION

8.4.1   Methodology

For the purposes of epidemiological investigations, a highly discriminatory and reproducible typing system, able to differentiate between phenotypically similar bacterial isolates, is essential. Although a number of researchers have attempted to subdivide bioserotypes of *Y. enterocolitica* using molecular techniques other than PFGE, the relative homogeneity within each group has resulted in little differentiation between strains (Nesbakken *et al.*, 1987; Kapperud *et al.*, 1990b; Blumberg *et al.*, 1991). The aim of this study was to examine strains of pathogenic bioserotypes of *Y. enterocolitica* recovered from animals and people between 1988 and 1995 in New Zealand, a geographically isolated region, using pulsed-field gel electrophoresis. This technique appeared to have the potential to be highly discriminatory and suitable for epidemiological studies (Iteman *et al.*, 1991). Although other studies had also recently employed this technique, they had only examined small numbers of isolates within each bioserotype (3-54) and the isolates had commonly been derived from many countries (Iteman *et al.*, 1991; Buchrieser *et al.*, 1994a; Buchrieser *et al.*, 1994b; Najdenski *et al.*, 1994; Saken *et al.*, 1994).

The rare-cutting enzyme *Not*I was used for digestion of agarose-embedded chromosomal DNA because it was shown in preliminary experiments to produce sufficient bands to allow easy visual discrimination between strains and, in addition, other overseas studies had also identified it as the most appropriate enzyme for *Y. enterocolitica* typing (Buchrieser *et al.*, 1994a; Saken *et al.*, 1994). In this study, the enzyme provided reproducible results that were highly discriminatory, being able to differentiate clearly between bioserotypes and to enable subdivision of bioserotypes into numerous subtypes (pulsotypes). In general, the 3 major bioserotypes were each subdivided into 2 principal Types, with these being further divided into pulsotypes. While differences between pulsotypes were often limited to a single band, the patterns appeared to be relatively stable, being seen in individual pulsotypes recovered over a period of years. By examination of *Not*I patterns produced in other studies, and by comparison with reference strains from the Pasteur Institute, New Zealand strains appeared distinct from European, Japanese and North American strains.

8.4.2 Epidemiological observations on the typing of strains of *Y. enterocolitica*, bioserotype 4/O:3

The division of New Zealand bioserotype 4/O:3 isolates into 18 distinct pulsotypes was an improvement on previous PFGE studies using *Not*I, where up to 11 pulsotypes had been identified in collections of isolates from geographically widespread areas (Buchrieser *et al.*, 1994b; Najdenski *et al.*, 1994). Nevertheless, despite this subdivision, 4/O:3 strains were genetically homogeneous, with approximately 79% of isolates belonging to pulsotype I. As this strain was recovered in every year between 1988 and 1995, it is in all probability the original clone of 4/O:3 in the country and has remained relatively stable over a period of time. Other isolates, although easily distinguishable, are closely related to this original clone as they differ from it by only 1-5 bands, considered by Tenover *et al.* (1995) to be consistent with one to two independent genetic events such as simple insertions or deletions of DNA, or the gain or loss of restriction sites. These findings are in agreement with those of Buchrieser *et al.* (1994a), who found that 4/O:3 strains from Austria and Germany belonged to a limited number of clones. Type II strains, which made up only 7.2% of the total, were quite distinctive, differing from Type I strains by 4-5 bands, possibly indicating an early divergence from the parent clone. Type II strains were first identified in 1992 in this study and have been isolated in most years since then.

Bioserotype 4/O:3 isolates have long been recovered only from a limited number of animal species, with pigs, dogs, people and occasionally cats being the only animals from which they are recovered (Swaminathan *et al.*, 1982). Despite this, the association between carriage of this bioserotype by domestic animals and infections in people has been largely circumstantial. Results from this study show that the majority of porcine and canine isolates belong to pulsotype I and are indistinguishable from human isolates, implying a strong epidemiological relationship between the three species. Type II strains did not include any animal isolates and may be a human-adapted group. Alternatively, as all of the porcine isolates came from the lower North Island, Type II strains may be found in pig populations in other regions. The second hypothesis appears to be borne out by the recovery of Type II strains from two samples of pork mince, however, contamination from human or other

sources cannot be ruled out. Interestingly, on 3 of the 4 pig farms surveyed, two pulsotypes were apparently endemic, possibly indicating that animals had originally been purchased from the same source or that the farms were linked epidemiologically in other ways.

Analysis of the regional distribution of the 3 principal pulsotypes showed that the commonest strain, pulsotype I, was widely distributed over most areas of the country, including both the North and South Islands. Only 3 regions did not recover this strain, however, the limited number of isolates from these areas prevents any firm conclusions being made. The two other pulsotypes that were identified came from widely separated areas of the country and may have a more regional distribution, making them potential epidemiological markers for public health investigations. PFGE was able to support a link between isolates of bioserotype 4/O:3 from a patient with transfusion-related septicaemia and the donor blood believed to be responsible for the infection, as both isolates belonged to pulsotype I. However, as 79% of all bioserotype 4/O:3 isolates were of this pulsotype, it can only really be said that the results are consistent with the donor blood being responsible for the infection.

8.4.3 Epidemiological observations on the typing of strains of *Y. enterocolitica*, bioserotype 2/O:5,27

In contrast to bioserotype 4/O:3, strains of bioserotype 2/O:5,27 appeared to be genetically heterogeneous, with 2 major Types comprising 40 pulsotypes being recognised, and no pulsotype containing more than 20% of the isolates analysed. PFGE is thus more discriminatory than other molecular typing methods for this bioserotype as Fukushima *et al.* (1993) subdivided isolates into only 3 groups based on restriction endonuclease analysis of virulence plasmid and chromosomal DNA. The significance of this finding is not understood but could indicate that this bioserotype has been in the country for a longer period of time than the other two pathogenic bioserotypes. This is borne out by the fact that 4 of the 5 most common pulsotypes, belonging to both major Types, I and II, were isolated at the beginning of the study in 1988, and that the isolates analysed were equally distributed between the two Types. As very few isolates of this bioserotype have been

examined by PFGE in overseas studies, it is difficult to say whether or not this heterogeneity is a feature of New Zealand isolates. Another unusual feature of this bioserotype is that the reference strain from the Pasteur Institute, although unique, was more closely related to local strains (specifically pulsotype I) than the reference strains of the other two bioserotypes. Again, the reason for this is not easily explained, although it could be postulated that strains of this pulsotype are globally widespread, or that the heterogeneity in this bioserotype is maintained worldwide. As bioserotype 2/O:5,27 has been recovered from wild birds, migratory species could have been involved in their dispersal (Shayegani and Parsons, 1987).

In other countries where bioserotype 2/O:5,27 is recognised as a human pathogen, pigs have been found to be the most common reservoirs of the strain (Aleksic *et al.*, 1988; Kotula and Sharar, 1993) and New Zealand is no exception. Porcine isolates of the organism belonged to a wide range of pulsotypes (8 out of 40) which included all the commonest pulsotypes identified within this bioserotype. Of the 8 porcine-associated pulsotypes, all but one (I_O) also contained isolates recovered from human infections. Interestingly, pulsotype II_A, commonly isolated from a wide range of animals and people, was one of the less common pulsotypes isolated from pigs. As discussed earlier, pigs examined in this study originated only from the lower North Island, and investigation of pigs from other regions could well identify other porcine-adapted pulsotypes. The 3 most common pulsotypes associated with pigs, I, I_A and I_B, were each identified in animals from 2-3 farms. In addition, one of the 3 farms had 5 pulsotypes isolated from slaughtered pigs, one of which (I_A) was recovered in surveys carried out in 1988 and 1993, supplying strong evidence for the stability of the individual pulsotypes. The isolation of multiple strains from animals on one farm implies either a longstanding relationship or the purchase of animals from a variety of sources.

Although many countries have reported the sporadic isolation of bioserotype 2/O:5,27 from animals other than pigs (Fukushima *et al.*, 1993), the range of animals that were found to be carrying this strain in New Zealand is unequalled. PFGE typing of these domestic animal isolates highlighted a number of interesting features. Firstly, all but 3 of the non-porcine

animal isolates were found in pulsotypes belonging to Type II. The 3 non-porcine isolates in Type I were all bovine in origin, only one of which was also recovered from a human infection. Thus, Type I strains may be host-specific for pigs and people, with only occasional spillover into other domestic animal hosts. Secondly, within Type II strains, pulsotype II_A had a particularly broad host range, containing isolates recovered from a diverse range of animal species, both ruminant and non-ruminant, as well as people. The apparent pathogenicity of this strain in domestic animals on occasion supports the hypothesis discussed earlier that bioserotype 2/O:5,27 strains have been in New Zealand a long time, with the evolution of a non-host-adapted subtype being a relatively recent event and of potential advantage to the organism. As the highest number of human infections were also associated with this pulsotype, it appears that a wider range of domestic animals can act as potential sources of this pulsotype for people than with other pulsotypes, and that it may be more pathogenic than other pulsotypes. Thirdly, as 19 of the 25 Type II pulsotypes were associated with a single species, 12 with people, 6 with cattle and one with deer, certain strains could be becoming more host-adapted in order to occupy particular niches, although due to the small numbers of pulsotypes involved this remains conjectural.

Examination of the distribution of the major pulsotypes shows that most strains are comparatively localised, with only II_A being identified throughout the North and South Islands, although this may be related to the low number of isolates examined from some areas. If this observation was confirmed by a larger study a regional distribution of pulsotypes would be useful in epidemiological investigations.

One feature of bioserotype 2/O:5,27 strains that remains puzzling is the low incidence of infection in people despite the wide range of potential sources of infection, both for foodborne transmission and direct contact. The incidence was never high and is now apparently decreasing. Further research is required to investigate this enigma.

8.4.4 Epidemiological observations on the typing of strains of *Y. enterocolitica*, bioserotype 2/O:9

The first isolation of bioserotype 2/O:9 from human infections in New Zealand was in 1991 and from animals was in 1992. Thus, the genetic homogeneity within this strain was not unexpected, with all but one isolate belonging to a single Type and 84% of isolates analysed belonging to pulsotype I, supporting the theory that this strain had been recently introduced. Nevertheless, despite the relative homogeneity, the occurrence of 19 distinct pulsotypes in Type I was surprising given the limited time-frame, suggesting the rapid development of genetic mutations and emergence of new clones. The majority of these pulsotypes (13/19), however, contained only one isolate and all were closely related to the Type I reference strain. In a comparative although smaller study, Buchreiser *et al.* (1994a) examined seven 2/O:9 isolates from Austria and found that they belonged to 5 different *Not*I pulsotypes. They stated that although there was a greater diversity in 2/O:9 strains than in 4/O:3 strains, the similarity among the strains was still high. As other researchers have also shown considerable heterogeneity among 2/O:9 strains (Najdenski *et al.*, 1994; Saken *et al.*, 1994) this might explain the number of pulsotypes seen in the present study.

The only other strain that was sufficiently different to be classified as a distinct Type (Type II) was a solitary isolate from a human infection in Auckland. As the pulsotype of this isolate was remarkably similar to that of the Pasteur Institute reference strain, it is interesting to speculate that the infection had been acquired overseas, perhaps in Europe. Whether spread of this strain has occurred is unknown at the present time. Continued surveillance using PFGE may show Type II's emergence in the future.

Although it cannot be stated with certainty that bioserotype 2/O:9 strains were recently introduced to New Zealand, the evidence from laboratory surveillance and this genetic study suggests that they were. Thus, the rapid spread of pulsotype I strains to a wide range of domestic animal species is a fascinating observation. Whether or not the bioserotype was first introduced in animals or people is not known, however, very few live animals have been introduced to New Zealand in the past 20 years, whereas approximately one million

people visit this country annually. There is, therefore, a distinct possibility that human sewage could have contaminated animal feed, such as pasture, resulting in introduction of the strain. Despite the fact that 13 of the 20 pulsotypes are only associated with human pathogenic strains, implying a longer-standing relationship, it may merely reflect the greater number of such strains examined. Of interest is that the first animal strains recovered, from deer, belonged to an unusual pulsotype (I_E) that has not been further identified. This could mean that the organism has been established in animals for some time but has not been isolated or fully characterised in Animal Health Laboratories. Despite this, the majority of animal strains belong to pulsotype I, with cattle, and to a lesser extent other ruminants, being a potentially significant source of infection for people via the food-borne route. The identification of a pulsotype I strain from a cat emphasises the potential role that pet animals might play in the epidemiology of yersiniosis. In addition, two isolates were recovered from dogs, belonging to subtype I_F (containing human strains) and I_K (single isolate) and as dogs were also found to harbour bioserotypes 4/O:3 and 2/O:5,27 strains their causal relationship with human infections is upheld. Five pulsotypes were associated only with animal isolates, from deer, dog, alpaca and cattle (2), and this may be linked with the development of host-specificity. The apparent lack of porcine 2/O:9 isolates in this study may, as stated earlier, be due to the limited areas from which pigs were surveyed, and pigs may harbour this strain in other parts of the country.

Despite bioserotype 2/O:9 only being recognised since 1991, the regional distribution of the most common pulsotype is almost countrywide. This rapid dissemination suggests a food-borne aetiology, possibly involving meat from a number of animal species. Other considerations are that there was a lack of immunity to the new strain, contributing to it's rising incidence, or that the strains are in some way more virulent than other *Y. enterocolitica* bioserotypes. It is difficult to ascertain where the bioserotype originally became established as the two earliest isolates identified were recovered one month apart in Wellington and Auckland. Of interest, however, is that the earliest isolate typed, from Wellington, was not pulsotype I but pulsotype I_G, a strain that has never been subsequently identified. This could imply that the original clone occurred earlier in Wellington, although this is only speculation. All other early isolates from this region belong to pulsotype I. The

next most common pulsotype, I_A, is less widely distributed, being recovered only from 3 regions in the upper North Island, nevertheless, as it was not isolated prior to 1995 it is likely to be a recently emerged clone.

CHAPTER 9

9. GENERAL DISCUSSION

Yersinia enterocolitica has been recognised as an important enteric pathogen in many countries for the past thirty years (Cover and Aber, 1989) however, little information other than isolated case reports was available on the incidence of the disease in people in New Zealand (McCarthy and Fenwick, 1991). Contact with the enteric reference unit at the National Health Institute (now the Communicable Disease Centre, CDC) for information on the status of *Y. enterocolitica* resulted in the following details - (i) Few isolates were received at the unit and only confirmation of the pathogen's identity was undertaken, no further characterisation was performed; (ii) A slight rise in the number of isolates received had been experienced, from 27 in the period 1981-1983 to 38 in 1987 (Diane Norris, personal communication, 1988). Thus, the situation in New Zealand in 1988 was vague and data on the incidence of the disease, or indeed any other features, was non-existent.

In order to investigate the current New Zealand situation, a collaborative survey was set up with Diagnostic Laboratory, Auckland (ADL) whereby all *Yersinia* isolates recovered at that laboratory would be sent to the Department of Veterinary Pathology and Public Health (DVPPH) for biotyping and serotyping. In addition, data on patients of a demographic and clinical nature such as age, sex, duration of infection and clinical symptoms, were sought from physicians treating cases of yersiniosis and were entered into a database for analysis. This collaboration has continued until the present time and a number of other medical laboratories have also been encouraged to send isolates and a similar range of patient data on a regular basis. The status of the typing service was formalised in 1992, when the DVPPH was included in a list of Reference Laboratories for specific pathogens, established by the CDC.

Information resulting from the survey has enabled a number of features of yersiniosis to be ascertained, including the principal serotypes prevalent in the country, the age distribution of human cases and the predominant clinical signs. Thus, it is now known that the principal human pathogenic bioserotypes in NZ are 2/O:5,27, 2/O:9 and 4/O:3, and that the situation in this country differs very little from other countries where yersiniosis is endemic (Swaminathan *et al.*, 1982). In addition, bioserotype 4/O:3, the predominant strain recovered in NZ (comprising approximately 90% of all isolates), is also the strain most commonly recovered in other countries (Bottone *et al.*, 1987).

Similarly, the age distribution of human cases in NZ corresponds to that reported from overseas, with the highest incidence recorded in children under 5 years old and a secondary peak recognised in young adults between the ages of 25-35 (Swaminathan *et al.*, 1982). This is in agreement with the patterns of infection seen with other enteric pathogens such as *Salmonella* and *Campylobacter* (Anonymous, 1992a; 1992b) and is believed to reflect a greater potential for faecal-oral spread in young people who have a less well-developed immune system. The secondary peak in the older group may reflect greater contact with pre-school children, a less sedentary lifestyle and a concomitant increase in the consumption of ready-to-eat food (Anonymous, 1992b).

The symptoms of yersiniosis were found to include diarrhoea and abdominal pain, often together, but commonly occurring alone. In a few cases, neither of these symptoms were reported, with patients presenting only with feelings of general malaise. This may have contributed to an underdiagnosis of infection as faecal samples are often only requested from patients with frank diarrhoea (McCarthy, personal communication).

Finally, once the project was underway and results were disseminated at conferences and meetings, other laboratories began to send isolates for typing and it became apparent that geographically, yersiniosis was widespread throughout the country and that only a lack of awareness of the organism and the appropriate media for it's detection had resulted in the low reported incidence and the dearth of information in the literature.

Once it had been established that *Y. enterocolitica* was a problem for people in NZ, the next question requiring an answer was the source of infection. Yersiniosis is a food-borne disease and many surveys have been performed overseas to identify animate or inanimate reservoirs and vehicles of infection. Results of the surveys have shown that although, on occasion, water and foodstuffs of non-animal origin can harbour yersiniae, the bioserotypes recovered are predominantly non-pathogenic and considered environmental in origin. These include *Y. enterocolitica* biotype 1A, a heterogeneous group of organisms, and other species of *Yersinia*, such as *Y. kristensenii*, *Y. intermedia* and *Y. frederiksenii* (Swaminathan *et al.*, 1982). Outbreaks of yersiniosis that were linked to consumption of contaminated water or milk were thought to be associated with initial contamination by animal faeces (Tacket *et al.*, 1984).

Surveys of numerous animal species were also performed in endemic regions such as Europe (Kapperud, 1981; Kapperud and Rosef, 1983), North America (Langford, 1972) and Japan (Fukushima and Gomyoda, 1991) and, while most reported strains distinct from those found in people, pigs, and occasionally dogs and cats, were shown to harbour the strains of *Y. enterocolitica* potentially pathogenic for people, namely bioserotypes 4/O:3, 2/O:5,27 and 2/O:9 (Kaneko et al., 1977; Narucka and Westendoorp, 1977). Extensive characterisation, both phenotypic and genotypic, of these strains found them indistinguishable from those recovered from human infections (Kapperud and Nesbakken, 1987). Subsequent research performed on pigs showed that in fact they were probably the principal reservoir of infection for people and that the organisms were commensals of the tonsils, the pharynx and the intestinal tracts of these animals, with clinical signs of disease rarely, if ever, being seen (Andersen, 1988). Dogs, on the other hand, occasionally showed clinical signs of yersiniosis after being infected with strains similar to those found in people (Wilson *et al.*, 1976) and their potential role as reservoirs was less well-defined. Infections in these species could in fact have come from contact with people or from a common food source.

Little was known about *Y. enterocolitica* infections in animals in NZ, as although surveys had been carried out in the mid-1970's, they had been aimed principally at identifying

sources of *Y. pseudotuberculosis*, an important cause of mortality in farmed deer (Hodges *et al.*, 1980; Anonymous, 1982). When *Y. enterocolitica* strains were identified in such surveys they were assumed to be non-pathogenic and, consequently, were not characterised further (Henderson, 1983b). The increase in goat farming in the mid-1980's, however, shed new light on the pathogenic potential of *Y. enterocolitica* in animals, as goats were found to be susceptible to yersiniosis under conditions of stress, such as following feed shortages or inclement weather (Buddle *et al.*, 1988). The bioserotype most commonly recovered was 5/O:2,3, a strain that has never been recovered from people.

A survey was therefore initiated to examine whether pigs in NZ carried human pathogenic yersiniae on their tonsils as they did in other countries. Following the sampling of 200 pigs from 22 farms at a local abattoir, 56% were found to be carrying strains that, phenotypically, at least, resembled those isolated from people; *Y. enterocolitica* bioserotypes 4/O:3 (12.5%), 2/O:5,27 (11.5%) and *Y. pseudotuberculosis* serotypes II and III (32%) (Zen-Yoji and Maruyama, 1972; Tertti *et al.*, 1984). In addition, it was noted that bioserotype 2/O:9 strains were not recovered and that affected farms appeared to harbour only one pathogenic *Yersinia* strain during the year of the study. The fact that bioserotype 2/O:9 strains were not recovered may merely reflect the infection status of pigs in the Manawatu region, and until more extensive studies are carried out it is difficult to state categorically that NZ pigs are only reservoirs of two of the three principal pathogenic bioserotypes. In 1994, a further study was carried out and slaughter pigs originating from the same farms as the present survey were found to be still carrying the same bioserotypes (De Allie, 1994). This may indicate that an element of exclusion exists between *Yersinia* strains, i.e. that the presence of one pathogenic bioserotype on a farm precludes colonisation with another. However, further research is necessary to establish whether other factors such as management practices are involved.

Earlier surveys of a number of other domestic animal species in NZ had identified *Y. enterocolitica* in the faeces of deer, sheep and cattle (Henderson, 1983a; Henderson, 1984; Bullians, 1987), but had not fully characterised these isolates, so some doubt remained as to whether such strains were potentially pathogenic for humans. Nevertheless, some

evidence for the carriage of pathogenic serotypes by animals other than pigs was shown at the beginning of this study, as strains recovered during a research project investigating *Yersinia* carriage in goats (Lanada, 1990) and from the diagnostic laboratory in the DVPPH were typed alongside human strains and were found to be *Y. enterocolitica* bioserotypes 2/O:5,27 (goats) and 4/O:3 (dogs). The isolates from dogs were from two cases referred to the Department of Clinical Sciences, Massey University, one involving a dog with acute enteritis and the other a dog with pharyngitis. These cases were particularly interesting as they were evidence of a reservoir other than pigs for this common human pathogenic bioserotype. Enteric disease in dogs involving this bioserotype had previously been reported from Norway (Farstad *et al.*, 1976) and Italy (Fantasia *et al.*, 1985). In addition, surveys in other countries had shown that dogs were occasionally infected asymptomatically with *Y. enterocolitica* 4/O:3 (Pedersen, 1976).

Following the demonstration of the carriage of pathogenic *Yersinia* spp. in animals other than pigs, and mindful of the previous NZ-based reports of surveys in other domestic animal species, where *Y. enterocolitica* was cultured but not typed, the assumption was made that in NZ there were many potential reservoirs of the organism, in contrast to the situation overseas, where only pigs were commonly implicated. To confirm this, from 1993 all MAF Animal Health Laboratories in the country were requested to submit any *Yersinia* spp. recovered from clinical specimens to the DVPPH for further typing, resulting in 377 isolates from various animal species being sent for analysis. The majority of these were from animals showing signs of enteric infection. A total of 127 isolates (33.7%) belonged to bioserotypes of *Y. enterocolitica* (99 isolates, 26.3% of total yersiniae) and *Y. pseudotuberculosis* (28 isolates, 7.4% of total yersiniae) considered to be potentially pathogenic for people.

These results supported the hypothesis that a wide range of domestic animals carried, or were clinically affected by, strains of *Y. enterocolitica* phenotypically identical to those recovered from human infections, and a difference in the ecology of the animal-associated strains was evident. Only four isolates were recovered from pigs, none of them bioserotype 4/O:3, indicating that pigs are rarely affected clinically by this strain, in agreement with

observations made overseas (Swaminathan *et al.*, 1982). Four isolates of bioserotype 4/O:3 were recovered from clinically affected dogs during the study, with one animal believed to have been the source of infection for a 7-year-old girl in the same household, supporting the belief that dogs could act as a reservoir for human infections (Yanagawa *et al.*, 1978, Trimnell and Adesiyun, 1988). Bioserotypes 2/O:5,27 and 2/O:9 on the other hand, appear to be widespread in the animal population, with 2/O:5,27 being recovered from all species of domestic animals in this country, including pigs and dogs. The principal animal species from which strains of 2/O:9 were recovered was cattle and as this bioserotype has not yet been recovered from pigs in NZ, cattle may be the hitherto unrecognised source of human infections. Surveys of cattle carried out recently in France (Reynaud *et al.*, 1993) and Belgium (Wauters, personal communication, 1996) supported this finding. It is also worth noting that 2/O:9 strains were also recovered from dogs and cats, suggesting that these animals could also have a role to play in the epidemiology of human yersiniosis in NZ.

Subsequent to this finding, an infection trial was set up to investigate the potential role of dogs in the epidemiology of human yersiniosis. The study examined the carriage and transmission of *Y. enterocolitica* bioserotype 4/O:3, following oral infection of fourteen 6-month-old cross-bred dogs with a strain recovered from a case of human yersiniosis in Auckland. Bacteriological examination of faecal and pharyngeal samples, using direct culture and cold-enrichment, showed that dogs can be readily infected by the oral route and can shed the organism for at least 23 days in their faeces. Subsequent transfer of infected dogs to pens containing uninfected animals showed that this bioserotype can also be easily transmitted between dogs. At no time did any of the dogs show clinical signs of infection, in contrast to infected dogs in the survey conducted with MAF. These findings suggest that dogs can carry *Yersinia* asymptomatically, and hence might act as a potential source of infection for people via handling or contamination of the environment.

In addition, pharyngeal carriage was exhibited in two dogs following oral infection, one of them for 6 days, indicating that the organism may potentially be transmitted by licking behaviour. This was not surprising, as pharyngeal carriage of *Yersinia* is well-recognised in pigs (Pedersen, 1979) and pharyngitis is documented in reports of human yersiniosis (Fenwick, 1992), suggesting that *Yersinia* spp. have a particular affinity for tonsillar tissue.

Infants and young children are probably most at risk of infection from canine sources as they are more likely to spend time on the ground and to come into contact with dog faeces. As *Y. enterocolitica* infections have been shown during the course of this study and others (Lee *et al.*, 1991) to be most prevalent in children under the age of five, the possible role of dogs in the epidemiology of human yersiniosis is worthy of further study.

Throughout the course of this study, one impediment has been the necessity to use cold-enrichment to obtain maximum recovery of yersiniae from canine and porcine clinical samples. This technique was developed by Paterson and Cook (1963), to enhance recovery of *Yersinia* spp. from animal sources during surveys, when only small numbers of the organism and overgrowth by competing microflora were expected. The optimum period for cold-enrichment of three weeks, coupled with 5-7 days for identification of the recovered bacteria, makes survey work very time-consuming. Reports in the literature of more rapid methods for the identification and characterisation of microorganisms, such as the use of DNA probes and the PCR indicated a way to markedly reduce the time required to investigate *Yersinia* infections in animals (Tenover, 1988; Harris and Griffiths, 1992).

The presence in pathogenic *Yersinia* of a virulence plasmid has been well documented and the plasmid has been extensively characterised (Pulkkinen *et al.*, 1986). Following plasmid isolation from a human strain of bioserotype 4/O:3 and restriction enzyme digestion, a specific fragment, corresponding to part of a highly-conserved region of the plasmid, was identified and purified for use as a diagnostic probe in a non-radioactive hybridisation assay. The choice of a non-radioactive assay was made as it was thought to be most applicable to future epidemiological studies in laboratories where access to radioactive isotopes was restricted. A dot blot assay successfully differentiated pathogenic and non-pathogenic *Y. enterocolitica* when DNA was used as the target for the probe, however, problems were encountered with the sensitivity and specificity of the reaction when attempts were made to probe colony blots. Thus, it was decided to discontinue this approach and to concentrate on the development of a PCR assay to improve the rate of detection.

One of the problems encountered with DNA probes used to identify carriage of the virulence plasmid by yersiniae is that the plasmid can be lost during laboratory culture,

resulting in false-negative reactions (Kapperud, 1991). A chromosomal virulence gene present in pathogenic, but not in non-pathogenic strains, was therefore considered to be a more reliable candidate for amplification by the PCR. The gene encoding for attachment and invasion of virulent yersiniae to cells in tissue culture (*ail* locus) was chosen for the development of a PCR assay.

Using the published sequence data (Miller *et al.*, 1990), primers to the *ail* gene were designed and tested for their ability, in the first instance, to differentiate pathogenic *Yersinia* from non-pathogenic strains and other microflora. The assay proved to be highly specific and sensitive when purified DNA was used as the template, with a detection limit of 0.25pg DNA, comparable to results achieved by other workers (Kwang *et al.*, 1996). The parameters were then optimised for amplification of the gene from boiled bacterial lysates, in an attempt to reduce the reliance on lengthy DNA isolation procedures. Although the sensitivity was reduced by at least ten-fold, it was still sufficiently sensitive to detect 10^2 - 10^3 cfu per ml of broth culture and was highly specific for virulent yersiniae. Validation of the PCR was achieved by restriction endonuclease digestion of the amplified product with an enzyme having one known restriction site in the gene, and by sequencing of the product.

The next step was adaptation of the assay for use directly on clinical samples, in order to fulfil the requirement for a test to obviate the lengthy cold-enrichment procedure used to date in *Yersinia* investigations (Kontiainen *et al.*, 1994). Reports of the use of the PCR for detection of enteric pathogens directly in clinical samples had been published previously, although not for *Yersinia* (Stoker, 1990). Problems were, however, widely reported of reduced sensitivity due to non-specific inhibitors in the reaction when certain clinical materials were used as the template, such as faeces, blood and mucus (Wright and Wynford-Thomas, 1990). Inhibition of the PCR was also encountered in this study, particularly when faecal samples and pharyngeal samples were used as template in the assay, and a number of strategies were devised to circumvent the problem.

Initially, DNA was extracted from artificially infected tissue and faecal samples and purified, using either phenol-chloroform extraction or a commercial DNA purification kit (Prep-A-Gene, Bio-Rad). Although total chromosomal DNA was visible on agarose gels it was

invariably negative for *Yersinia* when tested in the PCR unless high numbers of organisms were used to spike the samples. This reduced sensitivity was thought to be due to either co-extraction of inhibitory substances with the DNA or to DNA losses during the extraction process, resulting in levels of template below the limits of detection, or a combination of the two. Both of these explanations for poor sensitivity following extraction of DNA from samples have been documented elsewhere (Olsen *et al.*, 1995).

Two methods were eventually developed that increased the sensitivity of the PCR, although they both also increased the time required to complete the tests. Firstly, the application of a two-step amplification using nested primers gave significantly higher sensitivity than a single reaction and worked very well with tissue samples, including a number collected from piglets necropsied during a concurrent *Yersinia*-infection trial. These included tongue, tonsils and mesenteric lymph nodes. Unfortunately, it was only partially successful when faecal samples were used. The detection of *Yersinia* in infected faeces was only made possible through the inclusion of an enrichment step in a *Yersinia*-selective broth. The subsequent use of an aliquot of the broth in the amplification reaction resolved the problem and produced a simple, effective assay.

Faecal samples from the dog infection trial had been stored in PBS at -70°C for some months and it was decided to test the effectiveness of the PCR assay by comparing it in a blind trial with culture of the samples. Amplification of the samples was performed using the enrichment-PCR protocol and the results were found to compare favourably with the culture results. All samples positive on culture were positive in the PCR and a few negative on culture were also positive, correlating well with the previously observed pattern of infection in the dogs. Of interest was the fact that two dogs which were assumed to be *Yersinia*-negative at the time of sampling appeared to have in fact been harbouring very small numbers of the pathogen, indicating that they could have posed a risk for people in contact with them.

One problem with the PCR described above is the inability to detect either other pathogenic *Yersinia* spp., such as *Y. pseudotuberculosis*, or to identify specific bioserotypes of *Y. enterocolitica*. Recently, a multiplex PCR assay using a combination of primer sets to

amplify *ail*, *inv* and *vir*F genes, has been used successfully to detect both *Y. pseudotuberculosis* and *Y. enterocolitica* in mixed cultures (Nakajima *et al.*, 1992). In addition, Weynants *et al.* (1996), designed primers to amplify fragments of the *rfb*B and *rfb*C genes responsible for the biosynthesis of the O side chain of *Y. enterocolitica* O:3 in a multiplex assay with the 3 primer sets mentioned above, and was able to distinguish *Y. pseudotuberculosis* and *Y. enterocolitica* 4/O:3 from other pathogenic *Y. enterocolitica* bioserotypes. This has considerable implications for epidemiological investigations, for example on pig farms, and is a significant advance on the single primer pair assay assessed in this study.

During the course of this study a large reference collection of *Y. enterocolitica* strains had been accumulated and the final part of this research project was aimed at identifying whether strains recovered from animals in NZ were the same as those recovered from human infections. Although biotyping and serotyping are used extensively for the characterisation of *Y. enterocolitica*, the limited number of pathogenic bioserotypes limits their usefulness for epidemiological studies and pulsed field gel electrophoresis (PFGE), a more discriminatory molecular technique, was chosen to examine selected strains. Following optimisation of the PFGE protocols, the enzyme *Not* I was used to digest total DNA from 602 isolates of pathogenic bioserotypes of *Y. enterocolitica*, including 2/O:5,27, 2/O:9, 3/O:1,2,3 and 4/O:3. Isolates were chosen that represented a range of animals and people from different geographical regions of NZ. Band patterns produced after digestion and electrophoresis were photographed and comparisons made visually, with isolates being classified as Types (having 4 or more band differences) and subtypes (having up to 3 band differences from a Type strain), following guidelines published by Tenover *et al.*, (1995). The term pulsotype was used to refer to the Type-subtype combination. Visual comparison of pulsotypes suffered from a number of drawbacks, for example, it was both time-consuming and subjective. Computer programmes are now available for scanning and analysis of banding patterns and establishment of databases, and efforts will be made to acquire the capability for future analyses using PFGE.

Bioserotype 4/O:3 was divided into 2 Types (I and II), containing 12 and 6 pulsotypes respectively, but as approximately 80% of all isolates belonged to one pulsotype, Type I, subtype I, it was remarkably homogeneous. As this pulsotype was recovered in all years of the study, it is possible that it is the original clone in the country and has remained relatively stable since it's introduction. In addition to human isolates, included in this pulsotype were the majority of isolates recovered from pigs and all but one of the isolates recovered from dogs, emphasising the importance of these animal species in the epidemiology of human yersiniosis. The remaining 20% of isolates were made up of 17 different pulsotypes, a number of which contained only one representative strain. Although Type II strains did not include any porcine isolates, they did include 2 recovered from pork mince in the Auckland region, and pigs in this area might carry different clones of the bioserotype from those in the lower North Island, where the study isolates originated.

Bioserotype 2/O:5,27 was considerably more heterogeneous than 4/O:3, with two major Types and 40 pulsotypes, 24 of which contained only one isolate. Approximately 70% of the isolates were clustered into six pulsotypes, 4 of which included only human and porcine strains, indicating a degree of host-specificity. As the 4 most common pulsotypes within the 2 Types were recovered from pigs and people from the beginning of the study, it indicates a longstanding epidemiological relationship between the species. The 15 pulsotypes belonging to Type I contained only isolates from people, pigs and cattle, whereas those belonging to Type II originated from a wide range of animal species and people. The most heterogeneous pulsotype, II_A, was the most widely distributed, and included isolates recovered from people, pigs, dogs, cattle, deer, goats, alpacas and a horse. Many of the other pulsotypes were comparatively localised to regions and species. These results suggest that within the bioserotype there are some strains that are less host-specific than others, with pulsotypes of low host-specificity possibly being evolutionarily older clones that have adapted over time to a wide range of animal hosts, with easy, frequent transmission between them. Despite this wide range of potential reservoirs, human infections with this bioserotype have steadily decreased over the study period and it may be less virulent for people than the other two.

Bioserotype 2/O:9 was the most homogeneous of the 3 major human pathogenic strains, with all but one of the isolates belonging to Type 1. Within this Type there were 19 pulsotypes, however, 84% of the isolates belonged to pulsotype I, and 13 of the pulsotypes contained only one strain each. The degree of homogeneity, together with the apparent increase in isolations of this bioserotype from human cases since 1991, suggests that the strain is a relatively recent arrival in New Zealand. If this is the case, the wide host-range associated with the most common pulsotype (including people, cattle, deer, sheep and a cat) implies that it has a low host-specificity and that it is readily transmitted between different animal species. This has implications for human health and suggests that this strain may well supersede bioserotype 4/O:3 as the most commonly isolated strain in the future. The only isolate belonging to Type II originated from a human infection in the Auckland region and it's similarity to the Pasteur Institute type culture from Europe suggests that the person may have been infected overseas.

Although isolates from all areas of the country were examined in this study, no evidence for regional clustering of pulsotypes was seen and the most common pulsotypes in each bioserotype were found to be widespread. This distribution supports the case for a food-borne mode of transmission, involving meat from a number of animal species, depending on the bioserotype and it's host-specificity. Nevertheless, contact with dogs and cats may also constitute a risk for infection, particularly in young children, and care must be taken to include these animals when sources of infection are being investigated.

Thus, in conclusion, this study has shown that *Y. enterocolitica* is a common cause of gastroenteritis in people in New Zealand and that a number of domestic animals, including pigs and dogs, are capable of acting as reservoirs of infection for people. The development of a rapid method for detection of pathogenic yersiniae, adaptable for different samples, should help in the further investigation of the epidemiology of infections in these animals, with the ultimate aim of breaking the infection cycle and reducing the risk for people of contracting this disease.

LIST OF APPENDICES

Appendix 1.   List of participating medical laboratories

1. Diagnostic laboratory, Auckland
2. Ashburton hospital
3. Christchurch hospital
4. Hawera hospital
5. Lower Hutt hospital
6. Medical laboratory, Auckland
7. Princess Mary hospital, Auckland
8. Palmerston North hospital
9. Palmerston North medical laboratory
10. Tauranga hospital
11. Valley diagnostic laboratory, Lower Hutt
12. Wellington medical laboratory
13. Waikato hospital, Hamilton
14. Whakatane hospital
15. Royston laboratory, Hastings
16. Wanganui diagnostic laboratory
17. Auckland hospital
18. Wellington hospital
19. Middlemore hospital, Auckland
20. Pearson laboratory, Christchurch
21. Medical laboratory, Dunedin
22. Greymouth hospital
23. Southland hospital, Invercargill
24. New Plymouth medical laboratory
25. Taranaki Base hospital, New Plymouth
26. North Shore hospital, Auckland
27. Timaru hospital
28. Northland Pathology laboratory, Whangarei
29. Auckland Children's hospital
30. Nelson Public Health laboratory
31. Christchurch Public Health laboratory
32. Rotorua medical laboratory
34. Public Health laboratory, Dunedin
35. Hamilton medical laboratory
36. Dunedin hospital
37. Gisborne hospital
38. Nelson hospital
39. Auckland Public Health laboratory
40. Wairau hospital, Blenheim
41. Hastings hospital
42. Diagnostic laboratory, Nelson
43. Thames hospital

Appendix 2.   Isolation, identification and characterisation of *Y. enterocolitica*

2.1   Enrichment broths

(i)   M/15 Phosphate-buffered saline, pH 7.6 (Mair and Fox, 1986)

1.   Prepare Solution A by dissolving 9.07g of potassium dihydrogen phosphate (KH$_2$PO4) in 1 litre of distilled water.

2.   Prepare Solution B by dissolving 11.87g of disodium hydrogen phosphate (Na$_2$HPO$_4$.2H$_2$O) in 1 litre of distilled water.

3.   Mix 128ml of Solution A and 872ml of Solution B and add 9g of NaCl.

4.   Dispense 10ml amounts in universal bottles. Autoclave for 15min at 121°C.

(ii)   Modified PBS formulation

1.   PBS   1000ml

       Sorbitol   10g

       Bile salts   1.5g

2.   Mix well, adjust the pH to 7.6. Dispense 10ml amounts in universal bottles. Autoclave for 15min at 121°C.

(iii)   Modified Rappaport broth (Schiemann, 1982; Nesbakken and Kapperud, 1985)

1.   Solution A: Bacto tryptone   4g

                       Distilled water   400ml

       Mix well, autoclave for 15min at 121°C and cool to room temperature.

2.   Solution B: Na$_2$HPO$_4$.2H$_2$O   4.72g

                       Distilled water   400ml

       Mix well, autoclave for 15min at 121oC and cool to room temperature.

3.   Solution C: $MgCl_2.6H_2O$ 40g

                 Distilled water   100ml

    Mix and boil for a few minutes, cool to room temperature.

4.   Solution D: Malachite green   0.2g

                 Sterile distilled water   100ml

    Mix well, do not heat.

5.   To make 250ml MRB, the following amounts are mixed:

    155ml A + 40ml B + 53ml C + 1.6ml D

6.   Dispense 10ml amounts into universal bottles, store at 4°C.

(iv)   *Yersinia* selective broth

1.   Bacto yeast extract   2g
      Bacto peptone   17g
      Proteose peptone   3g
      Mannitol   20g
      Sodium deoxycholate   0.5g
      Sodium cholate   0.5g
      Sodium chloride   1g
      Sodium pyruvate   2g
      Magnesium sulphate, heptahydrate   10mg
      Bacto neutral red   30mg
      Bacto crystal violet   1mg
      Irgasan   4mg
      Distilled water   1000ml

2.   Mix well and heat to boiling, autoclave for 15min at 121°C, cool to 45-50°C.

3.   Aseptically add 10ml rehydrated Bacto Yersinia Antimicrobic Supplement CN (Difco).

4.   Mix thoroughly and dispense 10ml amounts into sterile universal bottles. Store at 4°C.

2.2 Plating media

(i) *Yersinia* selective agar (CIN - Difco; Schiemann, 1979b)

1. Suspend 59.5g *Yersinia* selective agar base in 1 litre distilled water and heat to boiling to dissolve completely.

2. Autoclave for 15min at 121°C and cool to 45-50°C.

3. Aseptically add 10ml rehydrated *Yersinia* antimicrobic supplement CN.

4. Mix thoroughly, avoiding the formation of air bubbles, and dispense 20ml into sterile 90-100mm Petri dishes. Store at 4°C for up to 2w.

(ii) MacConkey agar (Difco)

1. Suspend 50g Bacto MacConkey Agar in 1 litre distilled water and heat to boiling to dissolve completely.

2. Autoclave for 15min at 121°C, cool to 45-50°C and dispense in 15ml amounts in sterile 90-100mm Petri dishes. Store at 4°C for 2-3w.

(iii) Lactose sucrose urea agar (LSU - Juhlin and Ericson, 1961)

1. Solution 1:

Distilled water	850ml
Lactose	40g
Saccharose	40g
Beef extract (Difco)	5g
Proteose peptone No. 3 (Difco)	3g
Peptone	7g
Disodium hydrogen phosphate (Na$_2$HPO$_4$.2H$_2$O)	6.2g
Monopotassium dihydrogen phospate (KH$_2$PO$_4$)	4.34g
Water blue (Merck-Art 1279)	0.06g
Phenol red (Merk-Art 7241)	0.1g
1-Naphtholphthalein (Merck-Art 6246)	0.1g
Agar	15g

Heat the ingredients to dissolve, autoclave for 10min at 110°C and cool to 50°C.

2.   Solution 2:   Distilled water   53ml

	Distilled water	53ml
	Potassium tetrathionate	0.5g
	Urea	5g
	Sodium desoxycholate	2g
	Trisodium citrate	2g

Dissolve the ingredients in an 80°C water bath for 20min.

3.   Add Solution 1 to Solution 2 in the 80°C water bath.

4.   Mix well and dispense 20ml amounts into sterile 90-100mm Petri dishes. Store at 4°C for up to 2w.

2.3   Screening media

The following media were used to screen colonies off agar plates for the presence of *Yersinia* spp.

(i)   Tryptone water

Used for inoculation of screening media and for the indole test (see confirmatory tests 2.4)

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

2.   Dispense in 3ml amounts into bijoux bottles.

3.   Autoclave for 15min at 121°C and store at 4°C.

4.   Inoculate with one suspect *Yersinia* colony and incubate overnight at 25°C.

(ii)   Triple Sugar Iron Agar (TSI - Difco)

1.   To 1 litre of distilled water, add 65g TSIA and dissolve by heating.

2.   Dispense 6ml amounts into tubes and autoclave for 15min at 121°C.

3.   Allow the tubes to solidify in a slanting position so that a generous butt is formed.

4.   Inoculate with a straight wire by stabbing to the base of the butt and streaking the slant.

5.   Incubate overnight at 25°C with caps loosened.

(iii)   Lysine Iron Agar (LIA - Difco)

1.   To 1 litre of distilled water, add 34.5g of LIA and dissolve by heating.

2.   Proceed as above for TSI (steps 2-6).

(iv)   Urea hydrolysis

1.   To rehydrate, suspend 29g Bacto Urea Agar Base (Difco) in 100ml distilled water and mix thoroughly to dissolve completely.

2.   Filter sterilise, do not boil or autoclave, store at 4°C.

3.   Dissolve 15g Bacto Agar in 900ml distilled water by boiling and autoclave for 15min at 121°C.

4.   Allow to cool to 50-55°C and aseptically add 100ml of the filter-sterilised, concentrated Bacto Urea Agar Base to the cooled Bacto Agar.

5.   Mix thoroughly and dispense 3ml amounts into sterile bijoux bottles. Cool in a slanting position and store at 4°C.

6.   Inoculate slants with 1-2 drops of a bacterial culture grown overnight at 25°C in tryptone water. Incubate at 25°C for 48h.

2.4   Media and tests used for confirmation of identity and differentiation of *Yersinia* spp.

In addition to the above screening tests, a number of other biochemical media were used to differentiate *Y. enterocolitica* from other *Yersinia* spp.

(i)   Motility test (Bacto Motility Test Medium, Difco)

1.   Suspend 20g in 1 litre distilled water and heat to boiling to dissolve completely.

2.   Dispense 15ml into universal bottles and autoclave for 15min at 121°C, cool quickly.

3.   Inoculate 2 bottles from each broth culture by stabbing through the centre of the medium with a straight needle. Incubate one at 25°C and one at 37°C for 24h.

(ii)   Ornithine decarboxylation

1.   To 1 litre of distilled water add 10.5g Bacto Decarboxylase Broth Moeller (Difco) and dissolve by gentle heating.

2.   Dispense into 200ml bottles. To each bottle add 2g L-ornithine (Sigma).

3.   Adjust pH to 6.0 and dispense 3ml amounts into bijoux bottles. Autoclave for 15min at 121°C.

4.   Inoculate medium with 1-2 drops of an overnight bacterial culture, overlay with mineral oil and incubate at 25°C for 24h.

(iii)   Voges-Proskauer test

1.   Dissolve 17g Bacto MR-VP medium (Difco) in 1 litre distilled water.

2.   Dispense 3ml amounts into bijoux bottles and autoclave for 15min at 121°C.

3.   Inoculate medium with 1-2 drops of an overnight broth culture and incubate at 25°C for 24h.

4.   To 1ml of MR-VP broth add 0.6ml α-naphthol and 0.2ml 40% potassium hydroxide. Mix well and observe colour change.

(iv)   Citrate utilisation

1.   Suspend 24.2g Bacto Simmons Citrate Agar (Difco) in 1 litre distilled water and heat to boiling to dissolve completely.

2.   Autoclave for 15min at 121°C, dispense 3ml amounts into sterile bijoux bottles and cool in a slanting position. Store at 4°C until required.

3.   Inoculate with an overnight broth culture by streaking the surface of the slant with a straight needle. Incubate at 25°C for up to 5d.

(v)   Aesculin hydrolysis

1.   Polypeptone (Oxoid)   1g
         Aesculin   0.1g
         Ferric ammonium citrate   0.1g
         Agar   0.5g
         Distilled water   100ml

2.   Mix well and dissolve by boiling. Dispense 3ml amounts into bijoux bottles and autoclave for 15min at 121°C. Cool in a slanting position and store at 4°C.

3.   Inoculate medium with 1-2 drops of an overnight broth culture and incubate at 25°C for 24h.

(vi)   Carbohydrate fermentation

1.   Peptone (Difco)   10g
         Meat extract (Gibco)   3g
         Sodium chloride   5g
         Andrade's solution   10ml
         Distilled water   1000ml

2.   Dissolve by stirring, adjust pH to 7.2 and dispense into 200ml bottles.

3.   Autoclave for 15min at 121°C, cool and add 20ml Seitz filtered carbohydrate*.

4.   Dispense 3ml amounts aseptically into sterile bijoux bottles. Store at 4°C.

5.   Inoculate carbohydrates with 1-2 drops of an overnight broth culture and incubate at 25°C for up to 5d.

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

(vii) Indole test

1.   Inoculate tryptone water with one colony off an agar plate and incubate at 25°C for up to 3d.

2.   Add a few drops of Kovac's reagent to the broth and observe the colour change.

2.5   Biotyping of *Y. enterocolitica*

The biotyping scheme used in this study was adapted from Wauters *et al* (1987) and comprised a number of tests that have been described above. The following table shows the abbreviated scheme used.

Test/biotype	1A	2	3	4	5
Indole	+#	+*	-	-	-
Salicin	+	-	-	-	-
Aesculin	+	-	-	-	-
Xylose	+	+	+	-	+/-
Trehalose	+	+	+	+	-

\# = positive after 24h incubation; * = positive after 48-72h incubation; +/- = majority of strains positive, occasional strains negative

2.6   Serotyping of *Y. enterocolitica*

1.   Two sources of specific, cross-absorbed antisera were used -

        (i)   Denka Seiken, Japan - serotypes O:1,2,3; O:3; O:5,27; O:6,30; O:9.

        (ii)   Professor Wauters, Department of Microbiology, University of Louvain, Brussels, Belgium - serotypes O:2,3; O:3; O:5; O:5,27; O:7,8; O:9; O:27.

2.   Using an automatic pipette, 15-20µl of antiserum was dropped onto a clean glass slide. A loopful of bacteria from a blood agar purity plate was emulsified in the antiserum and the slide rocked gently for 1min. The presence of agglutination was indicative of a positive reaction.

3.   The following table shows the relationship between biotype and serotype, the combination being described as the bioserotype.

Relationship between biotype and serotype

Serotype	Biotype				
	1A	2	3	4	5
O:2,3	-	-	-	-	+
O:3	-	-	-	÷	-
O:5,27	-	+	+	-	-
O:6,30	+	-	-	-	-
O:7,8	+	-	-	-	-
O:9	-	+	-	-	-

2.7 Virulence testing of *Y. enterocolitica*

(i) Autoagglutination test (Laird and Cavanaugh, 1980)

1. Suspend 9.72g of Modified Minimum Essential Medium Eagle (Flow Laboratories, UK) in 1 litre distilled water and mix for 30min.

2. Add 1g sodium bicarbonate, mix and adjust the pH to 7.0-7.5.

3. Filter sterilise the solution and add 100ml foetal bovine serum (Gibco, Life Technologies, Auckland) to make a 10% solution. Dispense in 200ml amounts and store at 4°C.

4. To test for autoagglutination, dispense 2ml of the medium into a small tube and inoculate it with a single colony grown on trypticase soy agar. Incubate the tube at 37°C for 24h.

5. Autoagglutination-positive strains form a layer of irregularly-edged, flocculent growth at the bottom of the tube, while the medium remains clear. Autoagglutination-negative strains remain suspended in the medium, making a homogeneous, turbid suspension.

(ii)   Calcium dependence using Magnesium Oxalate (MOX) agar (Mair and Fox, 1986)

1. | Sodium oxalate | 2.68g |
    |---|---|
    | Magnesium chloride | 4.06g |
    | Columbia base agar (Difco) | 39g |
    | Distilled water | 1000ml |

2. Mix well, heat to boiling to dissolve completely and autoclave for 15min at 121°C.

3. Cool to 45-50°C and dispense 15ml amounts into sterile 90-100mm Petri dishes. Store at 4°C for up to 2w.

4. Inoculate a MOX plate with a colony of *Y. enterocolitica* grown at 25°C for 24h on blood agar and incubate at 37°C for 24h.

5. MOX-positive (plasmid-bearing) strains show pinpoint growth, often together with large colonies 0.5-2.0mm in diameter (indicating plasmid loss). MOX-negative strains show only the large colonies.

(iii)   Calcium dependence on Congo Red-Magnesium Oxalate (CRMOX) agar (Riley and Toma, 1989)

1. Mix 40g tryptic soy agar with 825ml distilled water and autoclave for 15min at 121°C. Cool to 55°C.

2. Add the following solutions: 80ml of 0.25M magnesium chloride; 80ml of 0.25M sodium oxalate; 10ml of 20% D-galactose; 5ml of 1% congo red. All these solutions are autoclaved for 15min at 121°C prior to addition, except the D-Galactose which should be filter-sterilised.

3. Mix thoroughly, dispense 15ml amounts into sterile 90-100mm Petri dishes and store at 4°C for up to 2w.

4. Inoculate a CR-MOX plate with a colony of *Y. enterocolitica* grown at 25°C for 24h on blood agar and incubate at 37°C for 24h. CRMOX-negative strains produce only large colourless colonies while CRMOX-positive strains produce both small, red colonies (plasmid-bearing) and large, colourless, plasmidless colonies.

(iv)   Growth on VYE agar (Fukushima, 1987)

1. | | |
|---|---|
| Bacto peptone | 17g |
| Proteose peptone | 3g |
| Sodium deoxycholate | 1g |
| Mannitol | 10g |
| Aesculin | 1g |
| Ferric citrate | 0.5g |
| Sodium chloride | 1g |
| Neutral red | 0.03g |
| Crystal violet | 0.001g |
| Agar | 13.5g |
| Distilled water | 1000ml |

Adjust to pH 7.4 with 1N sodium hydroxide and autoclave for 15min at 121°C. Cool to 45°C.

2.   Add the following supplements:

Irgasan (Ciba-Geigy)	0.004g
Cefsulodin (Takeda Chemicals)	0.004g
Oleandomycin (Sigma Chemicals)	0.01g
Josamycin (Wako Pure Chemicals)	0.02g

3.   Mix well and pour 15ml amounts into sterile 90-100mm Petri dishes. Store at 4°C.

4.   Inoculate the plate with a single colony of *Y. enterocolitica* grown at 25°C for 24h on blood agar, and streak to achieve single colonies. Incubate overnight at 25°C.

5.   Virulent *Y. enterocolitica* strains produce red colonies, environmental *Y. enterocolitica* strains produce dark colonies with a dark peripheral zone as a result of aesculin hydrolysis.

(v)   Pyrazinamidase test (adapted from Kandolo and Wauters, 1985)

1. | | |
|---|---|
| Tryptic soy agar (Difco) | 30g |
| Yeast extract (Difco) | 3g |
| Pyrazinecarboxamide (Merck) | 1g |
| Tris-maleate buffer, 0.02M | 1000ml |

2.   Dissolve by boiling and dispense 5ml amounts into screw-capped, 16 x 160mm polycarbonate tubes. Autoclave for 15min at 121°C and cool in a slanting position. Store at 4°C.

3.   Inoculate slants with 1-2 drops of a bacterial culture grown overnight in tryptone water at 25°C. After incubating for 48h, the presence of pyrazinoic acid is detected

by flooding the inoculated slant with 1ml of a freshly-prepared, 1% (w/v) ferrous ammonium sulphate (aqueous) solution.

4.   The results are read after 15min, with the formation of a brownish-pink colour on the slope indicative of a positive reaction. Pyrazinamidase-negative strains remain colourless.

(vi)   Salicin fermentation and aesculin hydrolysis

These tests are performed as described in section 2.4. Negative results in both tests are indicative of virulent strains of *Y. enterocolitica*.

(vii)   Crystal violet binding assay (Bhaduri *et al.*, 1987)

1.   Grow test strains overnight in tryptone water at 25°C, plate them onto duplicate Brain Heart Infusion Agar (Difco) plates and incubate them overnight at 25 and 37°C.

2.   Flood plates with 8ml of an 85μg/ml solution of crystal violet (Difco) for 2min and decant.

3.   Virulent, plasmid-bearing strains of *Y. enterocolitica* bind crystal violet at 37°C (dark violet colonies) but not at 25°C (white colonies). Environmental *Yersinia* spp. do not bind crystal violet at either temperature.

2.8   Storage of *Yersinia* strains (Park, 1976)

1.   Make up a 15% glycerol solution in tryptone water and dispense 3ml amounts into bijoux bottles. Autoclave for 15min at 121°C, cool and store at 4°C until required.

2.   Inoculate a blood agar plate with a single colony of *Y. enterocolitica* and incubate overnight at 25°C.

3.   Scrape the growth off the plate with a sterile cotton swab and suspend it in a bijoux bottle containing the glycerol broth.

4.   Using a sterile pasteur pipette, deposit 1-1.5ml of the suspension into 2 x 2ml screw-capped tubes (Wheaton vials, Millville, New Jersey, USA. Cat. No. 224881) and freeze immediately at -20 and -70°C.

Appendix 3. **Nucleotide sequence of the *ail* gene showing positions of primers used in the PCR and nested PCR (Chapters 6 and 7) - adapted from Miller *et al.*, (1990)**

5'ATGAAAAAGACATTACTAGCTAGTTCTCTAATAGCCTGTTTATCAATTGCG

(Primer 1)   (Primer 3)
TCTGTTAAT*GTGTACGCTGCGAGTGAAAG*TAGTATTT*CTATTGGTTATGCGCA*

*AAGC*CATGTAAAAGAAAATGGGTATACATTGGATAATGACCCTAAAGGTTTT

AACCTGAAGTACCGTTATGAACTCGATGATAACTGGGGAGTAATAGGTTCG

(*Mbo*II)
TTTGCTTATACTCATCAG*GGATAT*GATTTCTTCTATGGCAGTAATAAGTTTGG

TCATGGTGATGTTGATTACTATTCAGTAACAATGGGGCCATCTTTCCGCATC

AACGAATATGTTAGCCTTTATGGATTACTGGGGGCCGCTCATGGAAAGGTTA

AGGCATCTGTATTTGATGAATCAATCAGTGCAAGTAAGACGTCAATGGCATA

(Primer 4)
CGGGGCAGGGG*TGCAATTCAACCCACTTCCA*AATTTTGTCATTGACGCTTCA

(Primer 2)
TATGAATACTCCAAACTCGATAGCATAAAAGTTGGCACCTGGAT*GCTTGGTG*

*CAGGGTATCGAT*TC3'

External primers: Primer 1 (bases 548-567); Primer 2 (bases 1000-1019)

Nested primers: Primer 3 (bases 576-595); Primer 4 (915-934)

Restriction site: *Mbo*II, cuts between GGA---TAT (recognition site 3'-CTTCT(N)7↑-5'), giving 2 fragments, 140 and 219bp, when Primers 3 and 4 are used.

Appendix 4.   Questionnaire used to acquire data on human infections from medical laboratories during the survey described in Chapter 2

YERSINIA SURVEY	
LABORATORY	
DATE	PATIENT I.D.
AGE/D.O.B.	SEX
DIARRHOEA (Y/N)	
ABDOMINAL PAIN (Y/N)	
FEVER (Y/N)	
DURATION OF ILLNESS	
ANY OTHER COMMENTS	

FOR LABORATORY USE ONLY		
SPECIES	BIOTYPE	SEROTYPE
COMMENTS		

Appendix 5. Publications arising from this thesis

(i) McCarthy, M D; Fenwick, S G. Experiences with the diagnosis of *Yersinia enterocolitica* - an emerging pathogen in the Auckland area, 1987-1989. New Zealand Journal of Medical Laboratory Science. 1991; 45: 19-22.

(ii) Fenwick, S G; Murray, A M. Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction. Lancet. 1991; 337: 496-497.

(iii) Fenwick, S G. Pharyngitis and infections with *Yersinia enterocolitica*. New Zealand Medical Journal. 1992; 105: 112.

(iv) Fenwick, S G; Madie, P; Wilks, C R. Duration of carriage and transmission of *Yersinia enterocolitica* biotype 4, serotype O:3 in dogs. Epidemiology and Infection. 1994; 113: 471-477.

(v) Fenwick, S G; McCarthy M D. *Yersinia enterocolitica* is a common cause of gastroenteritis in Auckland. New Zealand Medical Journal. 1995; 108: 269-271.

(vi) Hilbink, F; Fenwick, S G; Thompson, E J; Penrose, M; Ross, G P. Non-specific seroreactions against *Brucella abortus* in ruminants in New Zealand and the presence of *Yersinia enterocolitica* O:9. New Zealand Veterinary Journal. 1995; 43: 175-178.

(vii) Wright, J; Fenwick, S G; McCarthy, M D. Yersiniosis: an emerging problem in New Zealand. New Zealand Public Health Report. 1995; 2: 65-66.

(viii) Fenwick, S G; Wauters, G; Ursing, J; Gwozdz, M. Unusual *Yersinia enterocolitica* strains recovered from domestic animals and people in New Zealand. FEMS Immunology and Medical Microbiology. 1996; 16: 241-245.

(ix) Fenwick, S G. Domestic animals as potential sources of human *Yersinia* infection. Surveillance. 1997; 24: 3-5.

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"This is the end,
Beautiful friend,
This is the end,
My only friend the end."

Jim Morrison.

ERRATA

Page/line no.	Alteration to text (in bold font)
ii/18	..subdivided into 18, **40** and **20** pulsotypes respectively..
5/5	..motile at **25°C**..
42/21	..in **epidemiologic**..
59/15	..% Total - 4.3%; **2.6%**; 8.4%; 0.1%; 84.5%..
66/6	..Age Band 5-9 - biotype 2/O:5,27 - **3** isolates..
66/11	..Age Band 30-34 - biotype 2/O:5,27 - **3** isolates..
86/20	..with 20 (**72%**) being over 50 years old..
120/5	..in New Zealand since **1991**..
121/15	..isolated from **three** cases of human infection..
136/20	..**(Fenwick and McCarthy, 1995)**..
136/21	..in two of the dogs **in 1992** was of interest..
143/12	..**6.2.2 Plasmid screening assay**..
147/14	..for 5min at **4°C**..
162/12	..Figure 6.4 legend - CP +ve - control plasmid positive DNA; **CP -ve**..
170/17	..**enterocolitica**...
195/7	..Table 7.7 legend - ** **twenty two** were positive..
200/20	..calculated to contain only **5** cfu per reaction..
217/2	..Table 8.7 legend - Pasteur Institute (**Ye 885**)..
219/13	..Figure 8.4 legend - lane 4 - **pulsotype II_B** ..
219/15	..Figure 8.4 legend - lane 11 - **pulsotype I_E** ..
228/11	..differ from it by only 1-**3** bands..
233/20	..only being recognised **in people** since 1991..
240/20	..shed the organism for at least **26** days in their faeces..
241/24	..when **plasmid** DNA was used as the target..