Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
Finland

Molecular epidemiology of *yadA*-positive *Yersinia enterocolitica*

Maria Fredriksson-Ahomaa

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Auditorium Maximum, Hämeentie 57, Helsinki, on June 8th 2001, at 12 noon.

Helsinki 2001

Yliopistopaino
Helsinki 2001
ISBN 952-91-3465-7
ISBN 951-45-9988-8 (pdf)

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ACKNOWLEDGEMENTS

This study was carried out at the Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki during 1995-1999.

I am deeply grateful to:

My supervisor Professor Hannu Korkeala, DVM, PhD, the head of the Department of Food and Environmental Hygiene, who introduced me the world of science, who supported and guided, and who encouraged me to complete this work with his incredible optimism.

Professor Marja-Liisa Hänninen, DVM, PhD, who was always willing to share her vast knowledge with me. As a mother of three children, she understood that it was not always easy to combine work and family life.

Docent Kaisa Granfors, PhD and Senior Lecturer Stan Fenwick, DVM, PhD for reviewing my thesis, and Carol Pelli, HonBSc, for editing the English of my manuscripts and summary.

All the people at our Department, especially Johanna Björkroth, Sebastian Hielm, Eija Hyytiä-Trees and Katri Jalava, who guided me in the field of molecular biology; Tiina Autio, who inspired me to talk about genes during our numerous lunches; Ulrike Lyhs, who saw me through the difficulties with her sophisticated humour; Sirkku Ekström, who contributed enormously by typing thousands of *Yersinia* isolates; and Johanna Seppälä, who helped me with all manner of practical things.

My husband Pekka and children Kaarin and Eerik, who still love me after this most selfish of projects.

ABBREVIATIONS

Ail, attachment invasion locus
BOS, bile-oxalate-sorbose broth
CIN, cefsulodin-irgasan-novobiocin agar plate
DI, discrimination index
ESP, EDTA-sodium lauroyl sarcosine buffer with proteinase-K
Inv, invasin
ITC, irgasan-ticarcillin-potassium chlorate broth
LPS, lipopolysaccharide
MAC, MacConkey agar plate
MRB, modified Rappaport broth
PCR, polymerase chain reaction
PFGE, pulsed-field gel electrophoresis
PBS, phosphate-buffer saline broth
PBSSB, phosphate-buffer saline broth with sorbitol and bile salts
PIV, Tris-NaCl buffer
PYZ, pyrazinamidase
pYV, plasmid for *Yersinia* virulence
RAPD, randomly amplified polymorphic DNA
REAC, restriction endonuclease analysis of the chromosome
REAP, restriction endonuclease analysis of the plasmid
SEL, selenite broth
SSDC, salmonella-shigella-sodium deoxycholate-calcium chloride agar plate
TAE, Tris-Acetate with EDTA
TBE, Tris-Borate with EDTA
TE, Tris-EDTA buffer
TSB, tryptic soya broth
YadA, *Yersinia* adhesin A
YER, yeast extract-rosebengal broth
Yop, *Yersinia* outer membrane protein
Ysc, Yop secretion
Yst, *Yersinia* heat stable enterotoxin

ABSTRACT

While the epidemiology of pathogenic *Yersinia enterocolitica* remains obscure, some indirect evidence indicates that foods, particularly pork products, are important sources of human infections. However, considerable difficulties are associated with the isolation of *Y. enterocolitica* from foods. Most methods require time-consuming enrichment steps and are unable to differentiate pathogenic isolates from non-pathogenic ones. The purpose of this work was to study the prevalence of *yadA*-positive *Y. enterocolitica* in pigs, foods and the environment using the polymerase chain reaction (PCR) and culture methods, and to characterise the isolates with phenotypic and genotypic methods to obtain further information on the epidemiology of pathogenic *Y. enterocolitica.*

The prevalence of *yadA*-positive *Y. enterocolitica* was studied in pig tonsils, tongues and minced meat, on carcasses and offals, and in the pig slaughterhouse environment with the PCR and culture methods. The mean prevalence of *yadA*-positive *Y. enterocolitica* was 37% in pig tonsils when samples from 9 slaughterhouses were studied. This microbe was recovered from all slaughterhouses, with prevalence varying from 13 to 45%. The prevalence of *yadA*-positive *Y. enterocolitica* was 21% on pig carcasses. The highest detection rates of 38, 86 and 63% were obtained for livers, kidneys and hearts, respectively. *Y. enterocolitica* harbouring the *yadA* was also frequently detected in different environmental sites of the pig slaughterhouse. At retail level, the highest prevalence of 98% was obtained for pig tongues, with a detection rate of 25% for minced meat samples.

The prevalence of *yadA*-positive *Y. enterocolitica* was higher with the PCR than with the culture method. However, some false-negative results were obtained when tonsils and tongues were examined. Although food samples are known to contain inhibitory substances for PCR, thus far no method is available to overcome this problem. The nested-PCR method used targeted the *yadA* gene located on the virulence plasmid. YadA, encoded by the *yadA* gene, is an outer membrane protein, which is essential for pathogenesis.

Bioserotype 4/O:3 was the only *yadA*-positive type isolated. It is a common bioserotype isolated in human infections globally, including Finland. Most of the isolates of bioserotype 4/O:3 harboured the *yadA*, which was detected rapidly and conveniently with PCR. *Y. enterocolitica* was isolated with the culture method, including overnight enrichment, selective enrichment and cold enrichment. The majority of the *yadA*-positive isolates were recovered after selective enrichment. All isolates of

bioserotype 4/O:3 were characterised with pulsed-field gel electrophoresis (PFGE). This method was shown to be efficient for characterisation of isolates belonging to bioserotype 4/O:3 with a high discriminatory power when *Not*I, *Apa*I and *Xho*I enzymes were used. Ribotyping with *Hind*II, *Eco*RI, *Sal*I, *Bgl*I and *Nci*I enzymes was also tested but using this method the isolates of bioserotype 4/O:3 could not efficiently be subtyped.

Distribution of different genotypes of *Y. enterocolitica* 4/O:3 isolates recovered from pig slaughterhouses was studied with PFGE. The most common genotypes found in the pig tonsils were widely distributed amongst the slaughterhouses. These genotypes were also found on pig offals (livers, kidneys, hearts, ears) and carcasses, and in the environment. Of these strains, 64% were indistinguishable from tonsil strains when characterised with *Not*I, *Apa*I and *Xho*I enzymes, supporting the hypothesis that pig tonsils are a major source of contamination in pig slaughterhouses. Several genotypes obtained from the tonsils were shown to be identical with genotypes found on edible offals (tongues, hearts, kidneys) at retail level, demonstrating a possible transmission route from slaughterhouses to retail shops.

Sources of human sporadic *Y. enterocolitica* 4/O:3 infections were studied by comparison of 212 human and 334 non-human strains. When characterised with *Not*I, *Apa*I and *Xho*I enzymes, 80% of human strains were indistinguishable from strains of pig origin, indicating that the main source of human infections is pigs. In all, 71% of the human strains were indistinguishable from strains isolated from pig tongues, livers, kidneys and hearts, suggesting that pig offals contaminated with *Y. enterocolitica* 4/O:3 are an important transmission vehicle of this bacterium from pigs to man. To reduce human infections, removal of the pig head, containing the highly contaminated tonsils and tongue should be made mandatory.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to in the text by Roman numerals I to V:

I. Fredriksson-Ahomaa, M., Björkroth, J., Hielm, S. and Korkeala, H. 2000. Prevalence and characterization of pathogenic *Yersinia enterocolitica* in pig tonsils from different slaughterhouses. Food Microbiol. 17: 93-101.

II. Fredriksson-Ahomaa, M., Korte, T. and Korkeala, H. 2000. Contamination of carcasses, offals, and the environment with *yadA*-positive *Yersinia enterocolitica* in a pig slaughterhouse. J. Food Prot. 63: 31-35.

III. Fredriksson-Ahomaa, M., Hielm, S. and Korkeala, H. 1999. High prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tongues and minced meat at retail level in Finland. J. Food Prot. 62: 123-127.

IV. Fredriksson-Ahomaa, M., Autio, T. and Korkeala, H. 1999. Efficient subtyping of *Yersinia enterocolitica* bioserotype 4/O:3 with pulsed-field gel electrophoresis. Lett. Appl. Microbiol. 29: 308-312.

V. Fredriksson-Ahomaa, M., Hallanvuo, S., Korte, T., Siitonen, A. and Korkeala, H. 2001. Correspondence of genotypes of sporadic *Yersinia enterocolitica* bioserotype 4/O:3 strains from human and porcine sources. Epidemiol. Infect. In press.

1. INTRODUCTION

The genus *Yersinia* comprises three pathogenic species: *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis* (Bercovier and Mollaret 1984). *Y. enterocolitica* is highly heterogeneous and can be divided into several bioserotypes, only a few of which are known to associate with human disease (Robins-Browne 1997). *Y. enterocolitica* bioserotype 4/O:3 is the most common cause of human yersiniosis globally (Bottone 1999), including Finland (Kontiainen et al. 1994). Annual incidence rates of reported *Y. enterocolitica* infections in Finland have varied from 564 to 873 cases per 5 million persons during 1995-1999 (Anonymous 2000). The infectious rate may, however, be much higher since only the most serious cases are registered. Gastro-intestinal yersiniosis is the most frequently encountered form of *Y. enterocolitica*, occurring commonly in infants and young children (Bottone 1997). Sometimes post-infections, more specifically extra-intestinal sequelae, such as reactive arthritis and erythema nodosum, will occur in elderly children and adults. *Yersinia*-triggered reactive arthritis often occurs in Nordic countries, where HLA-B27 and bioserotype 4/O:3 are especially prevalent (Sievers et al. 1972; Bottone 1999).

The epidemiology of *Y. enterocolitica* infections is complex and poorly understood. Most cases of yersiniosis occur sporadically without an apparent source (Kapperud 1991; Bottone 1999; Smego et al. 1999). *Yersinia* is thought to be a significant foodborne pathogen, even though pathogenic isolates have seldom been recovered from foods (de Boer 1995). While pigs have been shown to be a major reservoir for human pathogenic strains of bioserotype 4/O:3 (Andersen et al. 1991; Kapperud 1991; de Boer 1995), the transmission route from pigs to humans remains unproven. Indirect evidence suggests that food, particularly pork, is an important link between the swine reservoir and human infections. In case-control studies, a correlation has been demonstrated between the consumption of raw or under-cooked pork and the prevalence of yersiniosis (Tauxe et al. 1987; Ostroff et al. 1994; Satterthwaite et al. 1999). However, no cases of human yersiniosis have yet been reported where pork products were clearly identified as the source of infection.

Difficulties associated with the isolation of *Y. enterocolitica* from foods stem from the high number of background flora in the food samples. Direct isolation, even on selective media, is seldom successful and time-consuming enrichment steps are needed. No single procedure is currently available which will recover all pathogenic serotypes (de Boer 1992). The low isolation rates of pathogenic *Y. enterocolitica* in food samples may be due to limited sensitivity of culture methods (Nesbakken et al. 1991a). Using DNA-based methods, including PCR and DNA colony

hybridisation, foodborne pathogens can be detected more rapidly and with greater sensitivity and specificity (Jagow and Hill 1986; Hill 1996).

Although *Y. enterocolitica* is a ubiquitous micro-organism, the majority of isolates recovered from foods are non-pathogenic, and thus, it is important to determine the pathogenic significance of isolates (Kapperud 1991; de Boer 1995). This can be done with several phenotypic tests, but these are time-consuming and not always reliable (Kwaga and Iversen 1992). PCR and DNA colony hybridisation assays have been used to verify the pathogenicity of *Y. enterocolitica* isolates rapidly and with specificity (Kapperud et al. 1990a; Wren and Tabaqchali 1990; Bhaduri et al. 1997). These methods are based on specific segments of the virulence plasmid that have known virulence functions such as *yadA* and *virF* genes. The virulence plasmid is essential for *Y. enterocolitica* to survive and multiply in lymphoid tissues (Cornelis et al. 1998).

Until recently, the relatedness of *Y. enterocolitica* isolates has been determined solely by testing for phenotypic markers using bio- and serotyping. Nevertheless, genotyping of *Y. enterocolitica* has made great strides in the last decades, and several different DNA-based methods have been used to characterise *Y. enterocolitica* strains (Nesbakken et al. 1987; Andersen and Saunders 1990; Kapperud et al. 1990b; Blumberg et al. 1991; Iteman et al. 1991). However, the high similarity between strains and the predominating genotypes of bioserotype 4/O:3 have limited the benefit of these methods in epidemiological studies. Thus many factors relating to the epidemiology of *Y. enterocolitica*, such as the sources and transmission routes of *Yersinia* infections, remain obscure.

2. REVIEW OF THE LITERATURE

2.1. Yersinia enterocolitica

2.1.1. Classification

The species name *Yersinia enterocolitica* was proposed by Frederiksen (1964). *Y. enterocolitica* is included in the genus *Yersinia*, which is classified into the family *Enterobacteriaceae*, a group of gram-negative, oxidase-negative and facultatively anaerobic bacteria. All bacteria belonging to the genus *Yersinia* are catalase-positive, non-spore-forming rods or coccobacilli of 0.5-0.8 x 1-3 μm in size (Bercovier and Mollaret 1984). This genus presently consists of 11 species, three of which can cause disease in humans and animals: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Bercovier and Mollaret 1984; Bercovier et al. 1984b; Aleksic et al. 1987; Wauters et al. 1988b). They are invasive pathogenic bacteria, which have a common capacity to resist non-specific immune response and are lymphotrophic (Cornelis et al. 1998). These three pathogenic species differ considerably in invasiveness; while *Y. enterocolitica* and *Y. pseudotuberculosis* can cross the gastro-intestinal mucosa to infect underlying tissue, *Y. pestis* is injected into the body by an insect bite, and thus, does not have to penetrate any body surface on its own (Cornelis et al. 1998).

Strains belonging to *Y. enterocolitica* are urease-positive and can be differentiated from other *Yersinia* strains with a positive result for fermentation of sucrose, and negative reactions for rhamnose and melibiose fermentation (Bercovier et al. 1980). *Y. enterocolitica* is more active biochemically at 25°C than at 37°C, giving, for example, a positive Voges-Proskauer test only at the lower temperature (Bercovier and Mollaret 1984). Most of the strains are motile at 25°C but non-motile at 37°C, except strains belonging to biotype 4, which display a rather weak motility at any temperature (Niléhn 1969b). *Y. enterocolitica* is heterogeneous in its biochemical and antigenic properties (Bercovier et al. 1980). Most *Y. enterocolitica* strains associating with human disease belong to the following bioserotypes: 1B/O:8; 2/O:5,27; 2/O:9; 3/O:3 and 4/O:3. These bioserotypes have been shown to have different geographical distributions. Strains that are largely responsible for human yersiniosis in Europe, Japan, Canada and USA belong to bioserotype 4/O:3 (Bottone 1999). Bioserotype 3/O:3 has been recovered in Japan (Fukushima et al. 1984c) and China (Zheng and Xie 1996), bioserotype 2/O:9 mostly in Europe, and bioserotype 2/O:5,27 is more widely distributed. Strains of bioserotype 1B/O:8 are mostly limited to the USA, but have sporadically appeared in France, Italy and Japan as well (Ostroff 1995). Biotype 1A is considered to be non-pathogenic, however, isolates of this biotype have constituted a sizeable fraction of isolates

from patients with gastro-enteritis (Burnens et al. 1996). Neubauer et al. (2000a,b) have demonstrated based on the different DNA-DNA hybridisation values and the 16S rRNA gene sequences that *Y. enterocolitica* should be divided into two subspecies, with one subspecies consisting of strains of biotype 1B, and the other of the remaining strains.

2.1.2. Pathogenicity

Plasmid-encoded virulence factors

All fully virulent *Y. enterocolitica* strains carry an approximately 70-kb plasmid (Vesikari et al. 1981; Heesemann et al. 1983; Skurnik et al. 1983), termed pYV (plasmid for *Yersinia* virulence), which is required for full expression of virulence (Portnoy and Martinez 1985). Virulence plasmids of pathogenic *Yersinia* are closely related to each other, sharing functional similarities and a high degree of DNA homology (Heesemann et al. 1983). The presence of pYV enables *Y. enterocolitica* to survive and multiply in lymphoid tissues of their host (Cornelis et al. 1998). This pYV codes for an outer membrane protein YadA (*Yersinia* adhesin A), a set of secreted proteins called Yops (*Yersinia* outer membrane protein), and their secretion apparatus called Ysc (Yop secretion).

The *yadA* codes for the major outer membrane protein YadA (Lachica et al. 1984; Skurnik and Wolf-Watz 1989), which forms a fibrillar matrix on the surface of *Y. enterocolitica* (Kapperud et al. 1987) and is only expressed at 37°C (Portnoy and Martinez 1985). YadA plays a protective role in *Y. enterocolitica*, with several different functions (Table 1).

Table 1. Role of YadA protein in the virulence of *Yersinia enterocolitica.*

Functions of YadA protein	Reference
Serum resistance	Heesemann et al. 1983
Surface hydrophobicity	Lachica and Zink 1984
Autoagglutination	Skurnik et al. 1984
Adhesion to epithelial cells	Heesemann and Grüter 1987
Expression of fibrils on the surface	Kapperud et al. 1987
Haemagglutination	Kapperud et al. 1987
Binding to intestinal brush border membranes	Paerregaard et al. 1991
Resistance to killing by polymorphonuclear leukocytes	Ruckdeschel et al. 1996

One major role of YadA is to protect *Y. enterocolitica* against killing by polymorphonuclear leukocytes. Although the mechanism is unknown, YadA has been suggested to act by binding to eukaryotic cells, and in doing so, allow delivery of the Yops, thus preventing phagocytosis (Ruckdeschel et al. 1996).

The *yop* genes located on the pYV code for at least 14 Yops (Cheng and Schneewind 1999), which were originally described as *Yersinia* outer membrane proteins because they were detected in the outer membrane fraction of bacterial extracts (Portnoy et al. 1981). Today, they are considered secreted proteins (Michelis et al. 1990), which imbue *Y. enterocolitica* with the capacity to resist non-specific immune response (Cornelis 1998). Yops protect *Yersinia* from the macrophage by destroying its phagocytic and signalling capacities, and finally, inducing apoptosis. With the type III secretion system (Ysc), extracellularly located *Yersinia* that are in close contact with the eukaryotic cell deliver toxic bacterial proteins (Yops) into the cytosol of the target cell (Cheng and Schneewind 1999; Tardy et al. 1999). Genes specifying the type III machinery (*ysc*) are also located on the pYV. The *yop* and *ysc* genes are temperature- and calcium-regulated, being expressed maximally at 37°C in response to the presence of a low calcium concentration (Cornelis 1998). All *Yersinia* strains carrying the virulence plasmid exhibit a phenotype known as low-calcium response because it manifests only when pYV-bearing strains are grown at 37°C in media containing a low concentration of Ca^{2+} (Portnoy et al. 1984; Heesemann et al. 1986). This growth restriction phenomenon is associated with the massive production of Yops (Cornelis et al. 1998).

Chromosome-encoded virulence factors

Chromosome-encoded factors are also needed for pathogenicity. Virulence functions have demonstrated to be transferable with the virulence plasmid only to the plasmid-cured strains derived from virulent parenteral strains (Heesemann and Laufs 1983; Heesemann et al. 1984).

Adherence to and invasion of epithelial layers require at least two chromosomal genes, *inv* (invasion) and *ail* (attachment invasion locus) (Miller and Falkow 1988). The *inv* codes for Inv, an outer membrane protein, which appears to play a vital role in promoting entry into epithelial cells of the ileum during the initial stage of infection (Pepe et al. 1995). This gene is found in all *Yersinia* spp., however, non-pathogenic strains lack functional *inv* homologous sequences (Pierson and Falkow 1990). Although the Inv protein is maximally synthesised at temperatures below 28°C, under acidic conditions, the Inv protein is equally well produced at 37°C (Pepe et al. 1995). The *ail*, in turn, codes for the surface protein Ail, which is produced at 37°C (Miller et al. 1990). In contrast

to the *inv*, the *ail* is only found in *Y. enterocolitica* bioserotypes associated with disease (Miller et al. 1989).

The heat-stable enterotoxin (Yst) of *Y. enterocolitica* is chromosomally mediated (Delor et al. 1990). This enterotoxin is produced by most clinical isolates and is detectable in broth culture supernatant by the infant mouse test (Pai and Mors 1978). The role of enterotoxin in the pathogenesis of *Y. enterocolitica* infection is unclear. Non-pathogenic strains of *Y. enterocolitica* and strains of related species have been found to produce Yst using the infant mouse model (Kwaga and Iversen 1992), and the *yst* gene has been detected in strains of biotype 1A, *Y. kristensenii* and *Y. intermedia* (Delor et al. 1990; Kwaga et al. 1992). Absence of enterotoxin production *in vitro* at temperatures exceeding 30°C suggests that this toxin is not produced in the intestinal lumen. However, it has been demonstrated with isogenic Yst+ and Yst- strains in young rabbits that, at least in this model, Yst was responsible for diarrhoea (Cornelis 1994).

Lipopolysaccharide (LPS) is a major surface component of the outer membrane of gram-negative bacteria. In *Yersinia*, the genes directing the biosynthesis of LPS are chromosomally located. LPS is a complex molecule composed of three main parts: lipid A, oligosaccharide core and O-side chain (O-antigen). LPS of *Y. enterocolitica* O:3 has a unique structure in which the outer core forms a branch (Skurnik and Zhang 1996). The lipid A part is believed to be responsible for endotoxin activity and to play a central role in sepsis and septic shock due to gram-negative bacteria. Skurnik et al. (1999) have suggested that the outer core provides resistance against defence mechanisms, most probably those involving bactericidal peptides. Serotypes of *Y. enterocolitica* are mainly determined by the variability of O-antigen (Wauters et al. 1991). While the O-antigen is required for full virulence, its role has yet to be clarified (Skurnik and Zhang 1996). A total absence of O-antigen in *Y. enterocolitica* O:3 has been shown to reduce virulence in the infected mouse model (Skurnik et al. 1996).

Urease is produced by all clinical isolates of *Y. enterocolitica* and is encoded by the urease gene complex (*ure*) on the chromosome (de Koning-Ward et al. 1994). This enzyme hydrolyses urea to form carbonic acid and ammonia, leading to an increase in pH. Urease activity may contribute to the virulence of *Y. enterocolitica* by conferring acid tolerance and thereby enhancing bacterial survival in the stomach and other acidic environments (de Koning-Ward and Robins-Browne 1995). The decrease in virulence after intragastric inoculation of *Y. enterocolitica* O:3 urease-negative mutant indicates that the main role of urease is during the initial stage of the bacterial infection, when the bacteria reach the stomach (Gripenberg-Lerche et al. 2000).

Iron is an essential micronutrient for almost all bacteria, including *Y. enterocolitica.* A variety of alternative pathways have been elucidated for the uptake and utilisation of iron by *Yersinia* (Koornhof et al. 1999). To capture iron, strains of bioserotype 1B/O:8 synthesise an iron-chelating molecule, designated yersiniabactin. The yersiniabactin biosynthesis and transport genes are clustered within a region of the chromosome referred to as high-pathogenic islands (Rakin et al. 1999). The less virulent strains of other bioserotypes, including bioserotype 4/O:3, are able to bind and internalise a number of exogenously produced siderophores such as ferrioxamine and ferrichrome (Koornhof et al. 1999).

Virulence in animal models

Strains that carry the virulence plasmid can be divided into two groups based on relative virulence in animal models (Schiemann 1989). The first group consists of serotypes O:8, O:13 and O:21. These strains produce lethal infections in adult mice or gerbils with low doses by the intraperitoneal route or higher oral doses, and are capable of inducing conjunctivitis in guinea pigs (Aulisio et al. 1983). The second group comprises serotypes O:3, O:5,27 and O:9. These strains will produce fatal infection in suckling mice, but not in adult mice, unless the animals are overloaded with iron or have received an iron chelator (Bakour et al. 1985). Strains in this second group are not lethal for gerbils and induce only a mild and transitory conjunctivitis in guinea pigs (Aulisio et al. 1983). Strains of both groups will colonise the intestine of mice and be excreted for extended periods following oral infection (Bakour et al. 1985).

2.1.3. Factors affecting growth

Y. enterocolitica, as a psychrotrophic bacterium, has the ability to replicate at temperatures between 0 and 44°C. The doubling time at the optimum growth temperature (approximately 28 to 30°C) is around 34 min (Schiemann 1989). Although *Y. enterocolitica* can grow at temperatures as low as 0°C, the organism grows much more slowly as temperatures drop below 5°C (Goverde et al. 1994; Harrison et al. 2000). It has been shown that the number of *Y. enterocolitica* on pork can reach log 9 cfu per cm^2 after 5 days at 10°C (Nissen et al. 2001). Goverde et al. (1994) demonstrated that pYV-positive strains grow slower than pYV-negative ones at 30-35°C and 1-10°C. *Yersinia* withstands freezing and can survive in frozen foods for extended periods even after repeated freezing and

thawing, but it is susceptible to heat and is destroyed by pasteurisation at 71.8°C for 18 s (Toora et al. 1992).

Y. enterocolitica is able to grow over a pH range from approximately 4 to 10, with an optimum pH of around 7.6 (Robins-Browne 1997). *Yersinia* can survive alkaline conditions better than other gram-negative bacteria (Aulisio et al. 1980). However, since few foods have an alkaline pH, this high pH tolerance is relatively unimportant. The bacterium's tolerance of acidic conditions, on the other hand, is of great significance. Survival of the high acidity of some foods and the passage through the stomach suggests that *Y. enterocolitica* is relatively acid-resistant. Although the mechanism of acid tolerance is unknown, it may be due to the activity of urease, which catabolises urea to release ammonia, which in turn elevates the cytoplasmic pH (de Koning-Ward and Robins-Browne 1995). Tolerance of *Y. enterocolitica* to acid depends on the acidulent used, the environmental temperature, the composition of the medium, and the growth phase of the bacteria (Brocklehurst and Lund 1990). Acetic acid has been shown to be a more effective inhibitor than either lactic or citric acid (Brocklehurst and Lund 1990).

Y. enterocolitica is a facultatively anaerobic bacterium that can grow in anaerobic conditions. This bacterium can also grow well in modified atmospheres at 8°C (Harrison et al. 2000), but with higher levels of CO_2, the length of lag phase will increase and growth will be slower (Pin et al. 2000). *Y. enterocolitica* has been shown to grow well on meat when packaged in vacuum or in modified atmosphere and stored at 5°C (Doherty et al. 1995, Bodnaruk and Draughon 1998), even in the presence of high background flora (Barakat and Harris 1999; Bredholt et al. 1999). Nissen et al. (2001) demonstrated that *Y. enterocolitica* can grow well on both decontaminated and untreated pork when packaged in vacuum and stored at 10°C. However, the growth of serotype O:3 in raw minced meat has been found to be inhibited by natural microflora of the meat in some studies (Fukushima and Gomyoda 1986; Kleinlein and Untermann 1990).

Y. enterocolitica can tolerate salt (NaCl) at concentrations of up to 5% (Stern et al. 1980, Robins-Browne 1997). The inhibition caused by NaCl is strongly dependent on storage temperature. Brine concentration of 4.5% inhibits growth of *Y. enterocolitica* completely at 2°C and only partly at 5°C (Nielsen and Zeuthen 1985). *Y. enterocolitica* can tolerate both sodium nitrate and nitrite of up to 20 mg/ml for 48 h *in vitro* (de Giusti and de Vito 1992). However, a nitrite concentration of only 80 mg/kg has been reported to inhibit the growth of *Y. enterocolitica* in fermented sausages (Asplund et al. 1993).

2.2. Isolation and identification of pathogenic *Y. enterocolitica* from foods and environmental samples

2.2.1. Isolation

The source of *Y. enterocolitica* may markedly affect the methods of isolation. To find pathogenic isolates from food and environmental sources is generally more difficult than to find them from stools of infected individuals. During acute gastro-enteritis or with organ abscesses, pathogenic *Y. enterocolitica* is often the dominant bacteria and can easily be isolated by direct plating on conventional enteric media (Ahvonen 1972a). Because of the high number of background flora and the low number of pathogenic strains of *Yersinia* in food and environmental samples, direct isolation even on selective media is seldom successful. To increase the number of *Yersinia* strains in these samples, enrichment in liquid media prior to isolation on solid media is required (de Boer 1992). Several different methods are available for isolation of *Y. enterocolitica* from food and environmental samples (Table 2).

Table 2. Isolation methods of *Yersinia enterocolitica* most commonly used for food samples.

Pre-enrichment	Selective enrichment	Selective agar plate	Serotypes recovered	Reference
PBSSB[a]: 4°C, 3-4 weeks		MAC[b]: 25°C, 48 h CIN[c]: 30°C, 24 h	All All	Mehlman et al. 1978, NCFA 1996
PBS[d]/ PBSSB: 25°C, 1-3 days		CIN: 30°C, 24 h	All	Doyle and Hugdahl 1983, ISO 1994
	SEL[e]: 22°C, 3 days	MAC: 25°C, 48 h	O:3, O:8	Lee et al. 1980
PBSSB: 4°C, 8 days	MRB[f]: 22°C, 4 days	CIN: 30°C, 24 h	O:3, O:9	Schiemann 1982, NCFA 1996
YER[g]: 4°C, 9 days	BOS[h]: 22°C, 5 days	CIN: 30°C, 24 h	O:3, O:8	Schiemann 1982
TSB[i]: 22°C, 1 day	BOS: 22°C, 7 days	CIN: 30°C, 24 h	O:3, O:8	Schiemann 1983a
	ITC[j]: 25°C, 2 days	SSDC[k]: 30°C, 24 h	O:3	Wauters et al. 1988a, ISO 1994

[a] PBSSB, phosphate-buffer saline broth with sorbitol and bile salts.
[b] MAC, MacConkey agar plate.
[c] CIN, cefsulodin-irgasan-novobiocin agar plate.
[d] PBS, phosphate-buffer saline broth.
[e] SEL, selenite broth.
[f] MRB, modified Rappaport broth.
[g] YER, yeast extract-rosebengal broth.
[h] BOS, bile-oxalate-sorbose broth.
[i] TSB, tryptic soya broth.
[j] ITC, irgasan-ticarcillin-potassium chlorate broth.
[k] SSDC, salmonella-shigella-sodium deoxycholate-calcium chloride agar plate.

Cold enrichment

The psychrotrophic nature of *Y. enterocolitica* is unusual among enteric bacteria, and consequently, enrichment in different solutions at 4°C for prolonged periods has been used for isolation of *Yersinia* spp. (Eiss 1975). Cold enrichment in phosphate-buffered solution (PBS) has been widely used for clinical, food and environmental samples (Oosterom 1979; Pai et al. 1979; Kontiainen et al. 1994; Funk et al. 1998; Letellier et al. 1999). To increase sensitivity, sorbitol (1%) and bile salts (0.15%) have been added to PBS. This PBSSB has frequently been used in isolation methods, especially for foods (Mehlman et al. 1978; Schiemann 1982; Harmon et al. 1983; Logue et al. 1996; NCFA 1996). In addition, nutritionally richer media, such as tryptic soya broth (TSB), have been reported to yield better results, particularly when food and environmental samples are studied (Van Pee and Stragier 1979).

One major disadvantage encountered with cold enrichment is the long incubation period, typically 21 days, which is unacceptable for quality assurance of foods. Doyle and Hugdahl (1983) have shown that incubation in PBS for 1-3 days at 25°C is as efficient as enrichment at 4°C for some weeks. Another problem with cold enrichment is the presence of other psychrotrophic bacteria in foods, which also multiply during the enrichment. By treating cold enrichments with potassium hydroxide (KOH), the background flora can be reduced, making selection of yersinia colonies easier (Schiemann 1983b). This alkali treatment was developed by Aulisio et al. (1980) after they observed that *Yersinia* spp. are more tolerant of alkali solutions than many other gram-negative bacteria.

Selective enrichment

Several selective media for isolation of *Y. enterocolitica* at higher temperatures have been developed (Wauters 1973; Lee et al. 1980; Schiemann 1982; Wauters et al. 1988a; Toora et al. 1994), with different antimicrobial agents being used as selective supplements in these media. Wauters (1973) formulated a modified Rappaport broth (MRB) containing magnesium chloride, malachite green and carbenicillin, in which the sample was incubated at 25°C for 2-4 days. Later, Wauters et al. (1988a) developed an enrichment broth derived from the modified Rappaport base, supplemented with irgasan, ticarcillin and potassium chlorate (ITC). Both media have been shown to be efficient for recovery of strains of bioserotype 4/O:3, but inhibitory for strains of bioserotype 2/O:5,27 and 1B/O:8 (Oosterom 1979; Wauters et al. 1988a; Kwaga et al. 1990; De Boer and Nouws 1991). Schiemann (1982) developed a bile-oxalate-sorbose (BOS) medium for the isolation

of *Y. enterocolitica*, particularly for strains belonging to bioserotype 1B/O:8. Pre-enrichment in low-selectivity medium prior to selective enrichment in MRB (Harmon et al. 1983; NCFA 1996) or BOS (Schiemann 1983a; Walker and Gilmour 1986; Wauters et al. 1988a; Cox and Bailey 1990) has also been used for isolation of *Y. enterocolitica* from foods.

Selective agar plates

Several different selective agar plating media have been used for isolation of *Y. enterocolitica.* Initially, plating media, such as MacConkey (MAC) agar, deoxycholate citrate (DC) agar and *Salmonella-Shigella* (SS) agar, developed for other enteropathogens were used (Niléhn 1969a). On these media, *Y. enterocolitica* strains grow well but slowly and are easily overgrown by other enteric bacteria because of the low selectivity. Of the traditional enteric media, the most widely used is MAC agar (Doyle and Hugdahl 1983; Fukushima 1985; Sierra et al. 1995; Bhaduri et al. 1997). Both modifying existing enteric media and development of entirely new media have achieved improvements in selectivity. SS-agar was made more selective for *Y. enterocolitica* by addition of sodium deoxycholate and $CaCl_2$ (Wauters 1973; Wauters et al. 1988a). Used in combination with ITC enrichment, recovery of strains of bioserotype 4/O:3 is good (Wauters et al. 1988a). This agar is widely used because of its high selectivity and commercial availability (ISO 1994). However, differentiation of *Yersinia* from competing organisms, such as *Morganella, Proteus, Serratia* and *Aeromonas,* can be difficult. Cefsulodin-irgasan-novobiocin (CIN) agar is one of the media developed for isolation of *Y. enterocolitica* (Schiemann 1979). In several comparative studies, CIN agar was found to be the most selective plating medium for *Yersinia* spp. (Head et al. 1982; Harmon et al. 1983; Schiemann 1983a; Walker and Gilmour 1986; Cox and Bailey 1990). Organisms capable of fermenting mannitol, like *Yersinia*, produce red “bull's eye” colonies on CIN agar. Only *Citrobacter freundii, Enterobacter agglomerans* and species of *Aeromonas* and *Klebsiella* produce similar colony morphology (Devenish and Schiemann 1981; Harmon et al. 1983). Two other selective agars, BABY4 (Bercovier et al. 1984a) and VYE (Fukushima 1987), have been developed for isolation of *Y. enterocolitica* strains, but CIN agar is the most generally accepted because of its high selectivity and the high confirmation rate of presumptive isolates. Moreover, the commercial availability of this medium makes it convenient to use.

2.2.2. Identification

Devenish and Schiemann (1981) determined the minimum number of biochemical tests required for identifying *Yersinia* amongst bacteria growing and presenting similar colony morphology on CIN agar; two tests, Kligler iron and Christensen's urea tests, were sufficient. *Y. enterocolitica* can be identified with biochemical tests such as fermentation of sucrose, rhamnose and melibiose (Schiemann 1989). Commercial rapid identification tests provide suitable alternatives to the conventional tube tests (Cox and Mercuri 1978; Manafi and Holzhammer 1994; Varettas et al. 1995; Neubauer et al. 1998; Linde et al. 1999). The API 20E system, widely used for identification of presumptive *Yersinia* isolates, has been shown to be accurate in identifying of *Y. enterocolitica* (Archer et al. 1987; Sharma et al. 1990; Neubauer et al. 1998). This kit system has a positive identification rate of 93% for *Y. enterocolitica* incubated at 28°C instead of 37°C (Archer et al. 1987). In the study by Sharma et al. (1990), identification of *Y. enterocolitica* biotypes 3, 4 and 5 was excellent, with a positive predictive value of 99% when the strips were incubated at 28°C for 18-24 h. All pathogenic *Y. enterocolitica* strains were correctly identified with API 20E by Neubauer et al. (1998). *Y. enterocolitica* isolates have also been identified with PCR targeting the 16S rRNA gene combined with sequencing (Neubauer et al. 2000a).

2.2.3. Confirmation of pathogenicity

Y. enterocolitica is a ubiquitous micro-organism and, although the majority of isolates recovered from non-human sources are non-pathogenic, thus having no clinical significance, it is important to assess the pathogenicity of isolates (Kapperud 1991).

Animal tests

The pathogenicity of *Y. enterocolitica* can been studied by animal tests such as the guinea pig conjunctivitis model (Sereny test) (Sereny 1955), suckling mouse assay, mouse intraperitoneal challenge, and mouse diarrhoea and splenic infection following oral challenge (Aulisio et al. 1983; Bakour et al. 1985). However, because animal testing tends to be costly and is subject to increasing public opposition, it has largely been replaced by *in vitro* tests.

Phenotypic tests

A number of phenotypic characteristics associated with the virulence plasmid have been described (Gemski et al. 1980; Heesemann et al. 1983; Lachica and Zink 1984; Skurnik et al. 1984; Skurnik 1985). Calcium dependence, measured by growth restriction on magnesium oxalate agar (Gemski et al. 1980; Bhaduri et al. 1990), autoagglutination at 35-37°C (Skurnik et al. 1984), uptake of Congo red (Prpic et al. 1983; Riley and Toma 1989) and crystal violet (Bhaduri et al. 1987) are the most popular indirect markers for identifying pathogenic strains of *Y. enterocolitica*. The pyrazinamidase (PYZ) test (Kandolo and Wauters 1985) and the tissue culture invasiveness assay (Lee et al. 1977) are proven indicators of potentially pathogenic isolates (Noble et al. 1987; Miller et al. 1989; Farmer III et al. 1992). However, both of these tests measures functions that are chromosomally mediated, and thus, cannot replace pathogenicity tests, since they are only correlated with the ability of the strain to harbour the plasmid, and not to the presence of the plasmid itself. No single phenotypic virulence-associated characteristic has been shown to be a reliable indicator of pathogenicity (Noble et al. 1987; Kwaga and Iversen 1992).

Genotypic tests

Because animal tests are less desirable and phenotypic tests are time-consuming and not always reliable, a number of rapid and specific DNA hybridisation tests for identifying pathogenic bacteria have been developed (Hill and Keasler 1991). These methods are based on specific segments of DNA that have known virulence functions. Several DNA colony hybridisation assays have been used to verify the pathogenicity of *Y. enterocolitica* isolates (Miller et al. 1989; Robins-Browne et al. 1989; Delor et al. 1990; Kapperud et al. 1990a; Ibrahim et al. 1992a).

Pathogenicity of bacteria can be determined rapidly with polymerase chain reaction (PCR). In this method, DNA sequences are specifically amplified with oligonucleotide primers to give over 10^6 - fold amplification of the selected region within a few hours (Saiki et al. 1988). In addition to speed, amplification of the target DNA with PCR offers maximum sensitivity and specificity (Kwaga et al. 1992). Numerous PCR methods (Wren and Tabaqchali 1990; Fenwick and Murray 1991; Nakajima et al. 1992; Rasmussen et al. 1994b; Ibrahim et al. 1997) have been developed to confirm pathogenicity of *Y. enterocolitica* isolates.

Genotypic markers for pathogenicity of *Y. enterocolitica* include both plasmid and chromosomal loci. When the full pathogenicity of *Yersinia* is being determined, the plasmid-encoded virulence

determinant, as the target for PCR or colony hybridisation, must be selected (Kapperud et al. 1990a; Wren and Tabaqchali 1990; 1993, Bhaduri et al. 1997). The diagnostic value of the primers or probes that target plasmid-encoded sequences has been questioned because accidental loss of the plasmid during isolation yields false-negative results (Fenwick and Murray 1991; Blais and Philippe 1995).

2.3. Detection of pathogenic *Y. enterocolitica* in foods and environmental samples with DNA-based methods

The majority of *Y. enterocolitica* isolates recovered from food and environmental samples are non-pathogenic with culture methods. Several investigations have been undertaken to develop rapid and reliable methods for detection of pathogenic *Yersinia* directly from natural samples such as clinical, food and environmental samples.

DNA colony hybridisation

Several DNA colony hybridisation assays with gene probes targeting virulence-related DNA sequences of *Y. enterocolitica* have also been developed for biological samples (Jagow and Hill 1986; Miliotis et al. 1989; Nesbakken et al. 1991a; Goverde et al. 1993; Durisin et al. 1997; Weagant et al. 1999). Colony hybridisation does not require isolation of pure cultures, and it enables rapid detection and enumeration of all pathogenic bioserotypes. High background flora does, however, reduce the efficiency of hybridisation because target cells grow insufficiently in the presence of competing microflora (Durisin et al. 1997). Despite this, Nesbakken et al. (1991a) found that the prevalence of pathogenic *Y. enterocolitica* in Norwegian pork products was substantially higher with the colony hybridisation method than with the culture method.

Polymerase chain reaction (PCR)

Using PCR, pathogenic bacteria can be detected from natural samples rapidly and with high specificity and sensitivity (Candrian 1995; Olsen et al. 1995; Hill 1996; Scheu et al. 1998). Several methods have been developed to detect *Y. enterocolitica* in clinical, food and environmental samples (Table 3).

Table 3. PCR methods developed for detection of *Yersinia enterocolitica* in clinical, food and environmental samples.

Sample	Gene region	Sample preparation prior to PCR	Detection system	Detection limit	Reference
Blood	*virF , ail*	Pre-enrichment + proteinase K treatment	PCR, agarose gel	50 cfu/ml	Feng et al. 1992
Faeces	*yst*	DNA purification	PCR, agarose gel	10^3 cfu/g	Ibrahim et al. 1992b
Food, water	*yadA*	Pre-enrichment + IMS[a] + proteinase K treatment	Nested PCR, agarose gel/ colorimetric detection	2 cfu/g	Kapperud et al. 1993
Faeces, tonsils	*inv*	Pre-enrichment + IMS + proteinase K treatment	PCR, agarose gel/ fluorescent detection	40-400 cfu/g	Rasmussen et al. 1995
Water	*ail*	Pre-enrichment + DNA purification	A two-step PCR, polyacrylamide gel	60 cfu/ml	Sandery et al. 1996
Tonsils	*virF, ail*	Pre-enrichment + NaOH treatment	Nested PCR, agarose gel	Natural samples	Thisted Lambertz et al. 1996
Faeces	*virF, ail, yst*	DNA purification	Multiplex PCR, agarose gel	5-10 cfu/ml	Harnett et al. 1996
Food	*yst*	Pre-enrichment + TritonX-100 treatment	PCR, agarose gel	40 cfu/g	Wang et al. 1997
Food	*virF, ail*	DNA purification	Multiplex PCR, agarose gel	10^2 cfu/g	Nilsson et al. 1998
Tissue, faeces	16S rRNA	DNA purification	Seminested PCR, fluorescent hybridisation	10^2 cfu/ml	Trebesius et al. 1998
Water, sewage	*yadA*	Pre-enrichment + proteinase K treatment	Nested PCR, agarose gel	8-17 cfu/100ml	Waage et al. 1999
Milk	*yst*	DNA purification	Multiplex and seminested PCR	10-240 cfu/ml	Özbas et al. 2000
Food, faeces	*ail*	Pre-enrichment + DNA purification	TaqMan assay (Fluorogenic PCR)	< 1 cfu/g	Jourdan et al. 2000
Blood	16S rRNA	DNA purification	TaqMan assay	6 cfu/200µl	Sen 2000
Food	*yst*	Pre-enrichment + DNA purification	TaqMan assay	10^3 cfu/g	Vishnubhatla et al. 2000

[a] IMS, immunomagnetic separation.

PCR have some disadvantages (Harris and Griffiths 1992), one of the most serious being the high sensitivity of the technique. Small concentrations of contaminating DNA may result from cross-contamination, reagents or accumulation of PCR products in the laboratory by repeated amplification of the same target sequences. To minimise contamination, laboratories must take specific precautions such as the use of disposable material, separate sets of pipettes only for PCR and analysis of amplification products in an area separate from that where reagents and samples are prepared. Another drawback of PCR is its inability to distinguish between viable and non-viable cells. However, this problem can be overcome with a short pre-enrichment step before PCR is carried out. A further disadvantage is that many materials, such as food, faeces and blood, contain substances inhibitory to PCR (Rossen et al. 1992; Lantz et al. 1994). Removal of such inhibitors is important. However, sample preparation must remain fairly simple. The short enrichment culture procedure without DNA isolation is one of the best approaches because it is easy to perform and gives a high sensitivity. In addition, enrichment culture procedures are helpful in distinguishing live cells from dead cells (Lantz et al. 1994). However, Lantz et al. (1999) have demonstrated that high concentrations of target bacteria will inhibit PCR when an enrichment step has been used. The presence of large amounts of other bacteria has also been shown to inhibit the PCR reaction (Rossen at al. 1992). Inhibition caused by high bacterial concentration can be avoided by a 100-fold dilution of the enrichment culture (Lantz et al. 1999).

2.4. Characterisation of *Y. enterocolitica*

In epidemiological studies, differentiation of species into types is necessary to ascertain the prevalence of pathogenic types in a particular region as well as to identify reservoirs of infection, transmission vehicles and routes. To differentiate *Y. enterocolitica* strains, both phenotyping and genotyping has been used.

2.4.1. Phenotyping

Biotyping

Biotyping has been extensively used because *Y. enterocolitica* comprises a biochemically heterogenous group of bacteria (Bercovier et al. 1980). The biotyping scheme proposed by Wauters et al. (1987) has been widely adopted and is based on the following reactions: tween-esterase activity, indole production, acid from salicin, trehalose and xylose, nitrate reduction, ß-

galactosidase (ONPG) activity, Voges-Proskauer reaction, proline peptidase activity, esculin hydrolysis and pyrazinamidase activity. With these reactions *Y. enterocolitica* is divided into six different biotypes: 1A, 1B, 2-5. Biotype 1A consists of non-pathogenic strains, and biotype 1B and 2-5 include strains that are associated with disease in man and animals. The most widespread strains of *Y. enterocolitica* belong to biotype 4.

Serotyping

Strains of *Y. enterocolitica* can also be subdivided on the basis of serotypes, with this being the most commonly used typing method for *Yersinia*. Serotyping is mostly based on LPS surface O antigen, and more seldom on H (flagellar) or K (fimbriae) antigens. Since the initial description of Winblad (1967) of eight O antigens, the list has been extended to 76 (Wauters et al. 1991). Aléksic and Bockemühl (1984) have proposed a revised and simplified typing scheme, which includes 20 antigenic factors for *Y. enterocolitica* alone. Serotype O:3 is most frequently isolated from humans globally. Other serotypes obtained from humans include serotype O:9 and O:5,27, particularly in Europe, and serotype O:8 in the USA. However, several O antigens, including O:3, O:8 and O:9, have been found in both pathogenic and non-pathogenic strains (Aleksic 1995). An accurate biochemical characterisation is needed before or after serological typing to allow for correct assessment of the relevance of strains especially from foods and the environment, since related species and biotype 1A strains are widely distributed in these samples (Wauters et al. 1991; Hoorfar and Holmvig 1999).

Phage typing

Two schemes (Swedish and French) are used for phage typing of *Y. enterocolitica* (Schiemann 1989). Of these, the French scheme has been used more often and recognises 12 phage types: I-X (including IXa-c). The Swedish scheme recognises seven phages (A1, A2, B1, B2, C32, C61, E1) and is used less frequently. Neither of these schemes has produced a large number of distinct epidemiological types because many strains fall into the same phage types. Strains of bioserotype 4/O:3 and phage type VIII predominate in Europe and Japan (Kapperud 1991), whereas phage type IXb has been isolated in Canada (Toma and Deidrick 1975) and in the USA (Doyle et al. 1981). Baker and Farmer III (1982) have developed a set of 24 phages, which offers a marked improvement for differentiation. Because of the need to maintain stocks of biologically active phages and control strains, phage typing is available at only a few laboratories.

2.4.2. Genotyping

Restriction endonuclease analysis of the plasmid (REAP)

Plasmid analysis, the first bacterial typing tool, has been used for differentiating bacterial strains (Farber 1996). Plasmids are isolated from each isolate and then separated electrophoretically in an agarose gel to determine their number and size. Pathogenic strains of *Y. enterocolitica* contain only one virulence plasmid of about 70 kb (Vesikari et al. 1981; Heesemann et al. 1983; Skurnik et al. 1983). To increase discriminatory power, the isolated plasmid is cut with different frequent-cutting restriction enzymes (Heesemann et al. 1983; Nesbakken et al. 1987; Kwaga and Iversen 1993). Restriction endonuclease analysis of the plasmid (REAP) yields specific patterns for each bioserotype. However, within bioserotype 4/O:3, the diversity of the REAP patterns is limited (Table 4).

Table 4: Different typing methods using restriction enzymes for characterisation of *Yersinia enterocolitica* bioserotype 4/O:3.

Typing method	Enzymes used	No. of strains	No. of types	Dominating types (No. of strains)	Reference
Restriction endonuclease analysis of the plasmid	*Eco*RI, *Bam*HI, *Hind*III, *Xba*I	18	2	I (6) , II (12)	Pulkkinen et al. 1986
	*Eco*RI, *Bam*HI	89	1		Nesbakken et al. 1987
	*Eco*RI, *Bam*HI	30	3	I (27)	Kapperud et al. 1990b
	*Eco*RI, *Bam*HI, *Hind*III	18	2	I (17)	Kwaga and Iversen 1993
	*Eco*RI, *Bam*HI	9	1		Iteman et al. 1996
	*Eco*RI, *Bam*HI	15	1		Fukushima et al. 1997
Restriction endonuclease analysis of the chromosome	*Hae*III	30	2	I (29)	Kapperud et al. 1990b
Ribotyping	*Ava*I, *Nci*I	37	5	I (23), II (9)	Andersen and Saunders 1990
	*Nci*I	53	4	I (33), II (18)	Blumberg et al. 1991
	*Eco*RI, *Eco*RV	20	1		Iteman et al. 1996
	*Hind*III, *Bgl*I, *Sal*I, *Nci*I	77	11	I (37)	Mendoza et al. 1996
	*Hind*III, *Bgl*I	56	11	I (39)	Lobato et al. 1998
Pulsed-field gel electrophoresis	*Not*I, *Xba*I	28	15	I (12)	Buchrieser et al. 1994
	*Not*I, *Xba*I, *Spe*I	20	11	I (10)	Najdenski et al. 1994
	*Not*I	51	3	I (36), III (14)	Saken et al. 1994
	*Not*I, *Xba*I	106	24	I (38), II (38)	Asplund et al. 1998

Restriction endonuclease analysis of the chromosome (REAC)

Chromosomal DNA restriction analysis was the first of the chromosomal DNA-based typing schemes. In this method, endonucleases with relatively frequent restriction sites are used to cut the DNA, thereby generating hundreds of fragments ranging from 0.5 to 50 kb in size (Maslow et al. 1993). A major limitation of this technique is the difficulty in interpreting complex profiles, which consist of hundreds of bands that may be unresolved and overlapping. Kapperud et al. (1990b) have used REAC to study polymorphism in restriction fragment patterns among *Y. enterocolitica* isolates belonging to different bioserotypes. A total of 22 distinct REAC patterns were distinguished among the 72 *Yersinia* strains examined, and the patterns varied clearly between bioserotypes. Some variation occurred among strains within the same bioserotype, but strains of bioserotype 4/O:3 were homogeneous (Table 4).

Ribotyping

To avoid problems associated with complex REAC patterns, probes, which hybridise to specific DNA sequences, are used. Ribotyping refers to the use of nucleic acid probes to recognise ribosomal genes, which are present in all bacteria (Farber 1996). In practice, chromosomal DNA is isolated and a frequent-cutting enzyme is used to cut the DNA into small fragments. Fragments are separated by electrophoresis through an agarose gel. The separated DNA fragments are transferred from the agarose to either a nitrocellulose or nylon membrane by Southern blotting (Southern 1975). Probing is usually done with labelled probes containing *E. coli* 23S, 16S and 5S rRNA sequences. After probing, fragments containing a ribosomal gene will be highlighted, creating a fingerprint pattern containing approximately 1 to 15 bands that can be compared easily among isolates.

Ribotyping has been used to characterise *Y. enterocolitica* isolates in several studies (Andersen and Saunders 1990; Blumberg et al. 1991; Iteman et al. 1996; Mendoza et al. 1996; Lobato et al. 1998; Fukushima et al. 1998). A close relationship has been found between the ribotypes and bioserotypes of *Y. enterocolitica* isolates. Although variation between ribotypes exists among isolates belonging to the same bioserotype, genetic diversity is limited among isolates of bioserotype 4/O:3 (Table 4).

Pulsed-field gel electrophoresis (PFGE) typing

Pulsed-field gel electrophoresis is a variation of agarose gel electrophoresis that permits analysis of large fragments of bacterial DNA. For PFGE, bacterial isolates grown either in broth or on solid media are combined with molten agarose and poured into small moulds. The embedded bacteria are then subjected to *in situ* detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs, containing the whole genome, are inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of large molecular length DNA fragments, ranging from 10 kb to 800 kb. PFGE provides a highly reproducible restriction profile, which typically shows distinct, well-resolved fragments representing the entire bacterial genome in a single gel (Logonne 1993). Because of the high discriminatory power, and good intra- and interlaboratory reproducibility, PFGE is still one of the best methods available when compared with the newer typing methods (Olive and Bean 1999).

A number of studies have been conducted to characterise *Y. enterocolitica* with PFGE (Iteman et al. 1991; Buchrieser et al. 1994; Najdenski et al. 1994; Saken et al. 1994; Hosaka et al. 1997). Iteman et al. (1996) compared PFGE with ribotyping and REAP, and found PFGE to be the most suitable technique for epidemiological tracing of *Y. enterocolitica*. PFGE allows subtyping of strains belonging to same bioserotype (Buchrieser et al. 1994; Najdenski et al. 1994; Saken et al. 1994). Najdenski et al. (1994) showed that the pulsotype resembles the biotype more closely than the serotype and that the genome of *Y. enterocolitica* is stable *in vitro*. The global homogeneity of the pulsotypes among strains of bioserotype 4O:3 has been shown to be high (Najdenski et al. 1994; Saken et al. 1994; Asplund et al. 1998). Although strains of bioserotype 4/O:3 can be subdivided into several pulsotypes, most strains fall into one or two dominating pulsotypes, decreasing the discriminatory power of PFGE (Table 4).

Randomly amplified polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed PCR, is a variation of the PCR technique employing a single short (typically 10 base pairs) primer that is not targeted to amplify any specific bacterial sequence. The primer hybridises at multiple random chromosomal locations and initiates DNA synthesis at low annealing temperatures. The resulting PCR products present a variety of different-sized DNA fragments that are visualised by agarose gel electrophoresis (Farber 1996). RAPD is a very simple and quick method, but its reproducibility is

low and standardisation of the technique is difficult (Olive and Bean 1999). Some studies have characterised *Y. enterocolitica* isolates with RAPD (Rasmussen et al. 1994a; Odinot et al. 1995; Leal et al. 1999). This method allows discrimination between *Y. enterocolitica* isolates belonging to different bioserotypes and, also in some cases, between isolates belonging to the same bioserotype (Odinot et al. 1995; Leal et al. 1999).

2.5. Prevalence of *Y. enterocolitica*

2.5.1. In animals

Animals have long been suspected of being reservoirs for *Y. enterocolitica,* and hence, sources of human infections. Numerous studies have been carried out to isolate *Y. enterocolitica* from a variety of animals (Hurvell 1981), including wild animals (Ahvonen et al. 1973; Kaneko et al. 1978; Kaneko and Hashimoto 1981; Kato et al. 1985; Shayegani et al. 1986; Kaneuchi et al. 1989; Iannibelli et al. 1991; Cork et al. 1995; Suzuki et al. 1995; Wuthe et al. 1995; Sulakvelidze et al. 1996) and farm animals (Ahvonen et al. 1973; Szita et al. 1980: Fukushima et al. 1983b; Christensen 1987b; Fantasia et al. 1993; Busato et al. 1999) (Table 5). However, most of the strains isolated from animal sources differ both biochemically and serologically from strains isolated from man with yersiniosis. Human pathogenic strains of *Y. enterocolitica* typically have only been isolated from slaughtered pigs. The highest prevalence of *Y. enterocolitica* belonging to bioserotypes associated with human yersiniosis has been obtained in pig tonsils, with bioserotype 4/O:3 being the most common. Experimental infection of pigs has shown that *Y. enterocolitica* remains longer (Nielsen et al. 1996; Thibodeau et al. 1999), and the number of isolates is higher (Shiozawa et al. 1991) in tonsils than in faeces.

Pet animals, such as cats and dogs, have been suspected of being reservoirs for human infections with *Y. enterocolitica,* because of their close contact with humans (Schiemann 1989). However, strains of *Y. enterocolitica* 4/O:3 have only occasionally been isolated from dogs and cats (Ahvonen et al. 1973; Yanagawa et al. 1978; Pedersen and Winblad 1979; Szita et al. 1980; Fukushima et al. 1984b; Fantasia et al. 1985; Christensen 1987b; Fredriksson-Ahomaa et al. 1999). These strains have mostly been isolated from apparently healthy dogs (Fukushima et al. 1984b; Fantasia et al. 1985; Fredriksson-Ahomaa et al. 1999). Dogs can asymptomatically carry *Y. enterocolitica* in the pharynx and excrete the organism in faeces for several weeks after infection (Fenwick et al. 1994).

Table 5. Prevalence of *Yersinia enterocolitica* belonging to pathogenic bioserotypes in pig oral cavity and faeces using culture methods.

Sample	No. of samples	Prevalence % of different pathogenic bioserotypes (No. of positive samples)					Country	Reference
		4/O:3	3/O:3	2/O:9	2/O:5,27	1B/O:8		
Faeces	544	3 (15[a])			1 (5[a])		Canada	Toma and Deidrick 1975
Throat	282	30 (84[a])					Denmark	Pedersen 1979
Faeces	399	4 (17[a])					Denmark	Pedersen and Winblad 1979
Tonsils	480	8 (36)		2 (9)			German	Weber and Knapp 1981a
Faeces	1206	3 (32)		0.1 (1)				Weber and Knapp 1981b
Faeces	1300	0.2 (2)					UK	Hunter and Huges 1983
Tonsils	461	32 (146)			0.2 (1)		Norway	Nesbakken and Kapperud 1985
Tonsils	40	10 (4)					Netherlands	De Boer et al. 1986
Tonsils	400	37 (149)					Denmark	Christensen 1987b
Faeces	110	5 (6)					Spain	Gurgui Ferrer et al. 1987
Faeces	1458	25 (360)					Denmark	Andersen 1988
Tonsils	54	61 (33)					Belgium	Wauters et al. 1988a
Throat	1200	4 (43)	4 (43)		0.1 (1)		Japan	Fukushima et al. 1989
Faeces	1200	4 (45)	4 (43)		0.1 (1)			
Faeces	200	13 (25)			1 (2)		Canada	Mafu et al. 1989
Tonsils	481	36 (175)					Finland	Asplund et al. 1990
Tonsils	120	26 (31[a])		1 (1[a])			Finland	Merilahti-Palo et al. 1991
Tonsils	86	38 (33)			3 (3)		Netherlands	De Boer and Nouws 1991
Faeces	100	16 (16)		1 (1)				
Tonsils	202	28 (57[a])			7 (15[a])		Canada	Hariharan et al. 1995
Tonsils	106	41 (43)		2 (2)			Italy	De Giusti et al. 1995
Faeces	510		7 (37)				China	Zheng and Xie 1996
Tonsils	100	35 (35)					Chile	Borie et al. 1997
Throat	3375	0.1 (4[a])			3 (96[a])		USA	Funk et al. 1998
Faeces	1420	17 (235[a])		0.6 (9[a])	2 (25[a])	0.1 (1[a])	Canada	Letellier et al. 1999
Tonsils	291	22 (63[a])		0.7 (2[a])	0.3 (1[a])		Canada	Thibodeau et al. 1999
Faeces	291	6 (17)						

[a] Only serotyping done.

2.5.2. At farm level

Y. enterocolitica of bioserotype 4/O:3 has a world-wide distribution in the pig population, but the prevalence does vary between herds in many countries. This herd-wise distribution has been demonstrated by culture methods in Denmark, Norway, Finland and Canada (Christensen 1980, 1987b; Nesbakken and Kapperud 1985; Asplund et al. 1990; Andersen et al. 1991; Letellier et al. 1999) and by serological tests in Denmark and Norway (Nielsen and Wegener 1997; Skjerve et al. 1998). By culture method, 18% (Andersen et al. 1991) to 64% (Asplund et al. 1990) of the herds have been negative for *Y. enterocolitica* 4/O:3. By contrast, serological investigations have shown that 70%-90% of the slaughter herds in Denmark and 63% in Norway are infected with serotype

O:3, and that nearly all finishing pigs in infected herds are seropositive (Nielsen and Wegener 1997; Skjerve et al. 1998).

The source and mode of *Y. enterocolitica* infection in pigs is still unclear. This organism has not been isolated from small breeding pigs, but rather from pigs that have been moved to the first or second fattening pens. These findings suggest that infected faeces and pen floors are likely the most important sources of infection (Fukushima et al. 1983a). More intensive farming and production systems have probably contributed to the high prevalence of pathogenic *Y. enterocolitica* in pigs. The prevalence of bioserotype 4/O:3 has been shown to be highest in large pig farms with open management, where small pigs have been purchased from various pig markets or pig producers (Christensen 1987b; Skjerve et al. 1998).

The presence of symptomless carriers together with the widespread occurrence of *Y. enterocolitica* 4/O:3 in herds render control of this bacterium at farm level difficult. Strict slaughter hygiene remains important in reducing contamination in slaughterhouses (Skjerve et al. 1998).

2.5.3. In pig slaughterhouses

Y. enterocolitica 4/O:3 is a common bacterium in slaughtered pigs (Table 5). Fukushima et al. (1990) have shown that *Y. enterocolitica* may be transferred horizontally from infected pigs to other pigs in the slaughterhouse. Pigs can become contaminated with *Yersinia* from faeces of infected pigs and from contaminated floors during transportation to and time spent in slaughterhouses.

Meat inspection procedures may not reveal the presence of *Y. enterocolitica*, since this infection is mostly present without any signs of illness or apparent macroscopic lesions. Swine slaughter is an open process with many opportunities for contamination of the carcass with *Y. enterocolitica*; hazard points are difficult, if not impossible, to eliminate (Borch et al. 1996). The most important contamination points are pig-related, such as the faeces and the pharynx (Borch et al. 1996; Nesbakken and Skjerve 1996).

Contamination of pig carcasses with *Y. enterocolitica* has been studied widely (Harmon et al. 1984; Andersen 1988; Nesbakken 1988; Fukushima et al. 1989; de Boer and Nouws 1991; Nesbakken et al. 1994; Saide-Albornoz et al. 1995). Nesbakken (1988) investigated the prevalence of *Y. enterocolitica* 4/O:3 on cut surfaces of pig carcasses and found the highest contamination rate on

the cranial incision, i.e. the pharyngeal-tonsillary region. Relatively lower prevalence was found on the circumanal incision. However, Andersen (1988) obtained the highest recovery rate on the surface of the medial hind limb, especially when the rectum was loosened manually.

The isolation rate from three sampling sites on the carcass surface varied greatly between manual and mechanical loosening of the rectum (Andersen 1988). Manual loosening caused the greatest occurrence of *Y. enterocolitica* on the carcass surface, whereas use of the bung cutter reduced the prevalence markedly, particularly when its use was supplemented with carefully enclosing the anus and rectum in a plastic bag (Andersen 1988). Nesbakken et al. (1994) have demonstrated that by sealing off the rectum with a plastic bag immediately after it has been freed the spread of *Y. enterocolitica* 4/O:3 to pig carcasses can be considerably reduced.

In some studies, pig slaughterhouse environment, equipment and workers have been investigated for the presence of *Y. enterocolitica* (Nesbakken 1988; Mafu et al. 1989; Fransen et al. 1996; Sammarco et al. 1997). Although no pathogenic yersinia was detected on equipment or workers, *Y. enterocolitica* 4/O:3 was recovered from the sludge (Fransen et al. 1996), and on the viscera table and floors (Nesbakken 1988). Isolates of bioserotypes 4/O:3 and 3/O:3 have also been sporadically recovered from rats in pig slaughterhouses (Kaneko et al. 1978; Zheng and Xie 1996).

2.5.4. In foods and drinking water

Food has often been suggested to be the main source of *Y. enterocolitica,* even though pathogenic isolates have seldom been recovered from food samples (de Boer 1995; Ostroff 1995). Raw pork products have been widely investigated because of the association between *Y. enterocolitica* 4/O:3 and pigs (Wauters 1979; Schiemann 1980; Harmon et al. 1984; Nesbakken et al. 1985; de Boer et al. 1986; Asplund et al. 1990; de Boer and Nouws 1991; Tsai and Chen 1991; de Giusti et al. 1995; Fukushima et al. 1997). The isolation rate of pathogenic bioserotypes of *Y. enterocolitica* has been low in raw pork, except for pig tongues; the most common type isolated being bioserotype 4/O:3 (Table 6). No pathogenic isolates of *Y. enterocolitica* were recovered from 104 pork samples studied in Finland (Asplund et al. 1990). The prevalence of bioserotype 4/O:3 has been exceptionally high in both pig tongues and minced meat in Belgium (Wauters et al. 1988a), where head meat including tonsils has been used for minced meat (Tauxe et al. 1987).

In a case-control study, *Y. enterocolitica* 4/O:3 infections have been associated with eating raw or undercooked pork in the two weeks before onset (Tauxe et al. 1987). In the USA, *Y. enterocolitica* 4/O:3 infections have been associated with the household preparation of chitterlings (intestines of pigs, which are a traditional holiday dish in the South), particularly among black children (Lee et al. 1990; Stoddard et al. 1994).

Table 6. Prevalence of pathogenic bioserotypes of *Yersinia enterocolitica* in raw pork using culture methods.

Sample	No. of samples	Prevalence % of different pathogenic bioserotypes (No. of positive samples)					Country	Reference
		4/O:3	3/O:3	2/O:9	2/O:5,27	1B/O:8		
Tongue	302	55 (165[a])		1 (3[a])			Belgium	Wauters 1979
	37	30 (11[a])					Canada	Schiemann 1980
	31	6 (2[a])				19 (6[a])	USA	Doyle et al. 1981
	47	55 (26)					Norway	Nesbakken 1985
	50	18 (9)	22 (11)				Japan	Shiozawa et al. 1987
	125	6 (8)					Spain	Gurgui Ferrer et al. 1987
	29	97 (28)					Belgium	Wauters et al. 1988a
	40	15 (6)		5 (2)			Netherlands	De Boer and Nouws 1991
	55	27 (14)					Germany	Karib and Seeger 1994
	86	2 (2)					Italy	De Giusti et al. 1995
Pork[b]	91	1 (1[a])			12 (10[c])	1 (1[a])	Canada	Schiemann 1980
	127	1 (1)					Norway	Nesbakken et al. 1985
	70	13 (9)	19 (13)	4 (3)			Japan	Shiozawa et al. 1987
	267	2 (6)					Denmark	Christensen 1987b
	50	24 (12)					Belgium	Wauters et al. 1988a
	400	1 (3)		0.3 (1)			Netherlands	De Boer and Nouws 1991
	67	1 (1)			12 (8[c])	4 (3)	China	Tsai and Chen 1991
	48	2 (1)		2 (1)			Germany	Karib and Seeger 1994
	40	5 (2)		3 (1)	10 (4)		Ireland	Logue et al. 1996
	1278	2 (26)	3 (38)		1 (14)		Japan	Fukushima et al. 1997
	300	2 (6)					Norway	Johannessen et al. 2000

[a] Only serotyping done.
[b] Other pork products, excluding tongues.
[c] Serotype O:5.

Beef, lamb, poultry and fish products have also been studied for *Y. enterocolitica* (Fukushima 1985; de Boer et al. 1986; Gurgui Ferrer et al. 1987; Cox et al. 1990; Ibrahim and MacRae 1991; Falcao 1991; Hudson et al. 1992; de Boer 1994; Toora et al. 1994; Sierra et al. 1995; Escudero et al. 1996; Khare et al. 1996; Logue et al. 1996; Velázquez et al. 1996; Fredriksson-Ahomaa et al. 2001b). Non-pathogenic strains have frequently been isolated from raw beef, lamb, poultry and fish (Cox et al. 1990; Ibrahim and MacRae 1991; Falcao 1991; de Boer 1994; Sierra et al. 1995; Khare et al. 1996; Velázquez et al. 1996). Bioserotype 4/O:3 has only been recovered a few times from beef samples and once from a poultry sample (Andersen et al. 1991; Logue et al. 1996; Fukushima et al.

1997). In these cases, cross-contamination from raw pork products to beef and chicken products has probably occurred during processing, packaging or handling since strains of *Y. enterocolitica* 4/O:3 have thus far never been recovered from cattle or poultry.

Milk (de Boer et al. 1986; Walker and Gilmour 1986; Christensen 1987b; Hughes 1987; Ibrahim and MacRae 1991; Larkin et al. 1991; Rea et al. 1992) and plant products (de Boer et al. 1986; Gurgui Ferrer et al. 1987; Tassinari et al. 1994; Toora et al. 1994; de Boer 1995; Beuchat 1996; Odumeru et al. 1997; Szabo et al. 2000; Fredriksson-Ahomaa et al. 2001b) have been extensively examined because outbreaks of yersiniosis have been linked to the consumption of these contaminated products (Cover and Aber 1989). While non-pathogenic *Y. enterocolitica* has frequently been recovered from raw milk and vegetables, pathogenic *Yersinia* has not been isolated from vegetables and only once from raw milk. In this case, pigs were the most likely source of the serotype O:8 contamination (Ackers et al. 2000).

Drinking water has been relatively widely investigated (Christensen 1980; Stengel 1986; Schiemann 1990; Gönül and Karapinar 1991; Brennhovd et al. 1992). In these studies, water has been shown to be a significant reservoir for non-pathogenic *Y. enterocolitica*. However, in epidemiological studies, untreated drinking water has been reported to be a risk factor for sporadic *Y. enterocolitica* infections in Norway (Ostroff et al. 1994; Saebø et al. 1994). Despite this, drinking water has only been identified as the source of infection in a few episodes (Cover and Aber 1989; Schiemann 1990). In two outbreaks involving a small number of individuals, serotype O:3 has been isolated from drinking water (Christensen 1979, Thompson and Gravel 1986). More recently, Karapinar and Gönül (1991) have demonstrated that *Y. enterocolitica* O:3 is able to grow in sterile spring water stored at 4°C.

2.5.5. At retail level

Several studies have been conducted to isolate pathogenic *Y. enterocolitica* from foods, especially from pork, at retail level (Wauters 1979; Schiemann 1980; Nesbakken et al. 1985; de Boer et al. 1986; Asplund et al. 1990; Tsai and Chen 1991; Khare et al. 1996; Logue et al. 1996; Fukushima et al. 1997; Szabo et al. 2000). However, *Y. enterocolitica* 4/O:3 isolates have seldom been recovered from foods, except for pig tongues (Wauters 1979; Schiemann 1980). Only a few studies have been carried out to find pathogenic *Y. enterocolitica* in the retail shop environments (Christensen 1987a; Hudson and Mott 1993). Christensen (1987a) found *Y. enterocolitica* 4/O:3 in 15 out of 159 shops

examined. The highest contamination rate occured in small-scale ‘family-type’ butcher shops, and the lowest in large-scale butcher shops and supermarkets. Cross-contamination from a contaminated environment to foods not undergoing heat treatment prior to consumption may occur. As a psychrotrophic organism, *Y. enterocolitica* can grow along the cold-chain, of which the cold-rooms in retail shops are an important part.

2.5.6. In the environment

Most of the *Y. enterocolitica* isolates recovered from environmental samples, including fodder, soil, foliage, surface water, sewage water and sludge, have been non-pathogenic (Christensen 1980, 1987b; de Boer et al. 1986; Berzero et al. 1991; Cork et al. 1995; Fransen et al. 1996; Sulakvelidze et al. 1996). Strains of bioserotype 4/O:3 have occasionally been isolated from sewage water (Christensen 1987b), but not from other sources. Sandery et al. (1996) and Waage et al. (1999) have shown with PCR that pathogenic strains of *Y. enterocolitica* can exist in environmental waters. Terzieva and McFeters (1991) demonstrated that a significant portion of serotype O:3 isolates could persist in culturable state for weeks in surface water at 6 and 16°C. However, Chao et al. (1988) and Tashiro et al. (1991) have shown that *Y. enterocolitica*, including serotype O:3, cannot survive in natural river water over long periods, especially at 20°C.

2.5.7. In man

Y. enterocolitica has been isolated from humans on all continents (Bottone 1999). Bioserotype 4/O:3 is the most common type of *Y. enterocolitica* recovered from humans with diarrhoea (Bissett et al. 1990; Gonzalez Hevia et al. 1990; Bucci et al. 1991; Kontiainen et al. 1994; Munk Petersen et al. 1996; Stolk-Engelaar and Hoogkamp-Korstanje 1996). The highest incidence of enteritis caused by this type has been found in young children (Stolk-Engelaar and Hoogkamp-Korstanje 1996). However, Morris et al. (1991) have also isolated strains of bioserotype 4/O:3 at a high rate from asymptomatic children. Annual incidence rates of reported *Y. enterocolitica* infections in Finland have varied between 564 and 873 cases per 5 million persons during 1995-1999 (Anonymous 2000). The infection rate is probably much higher since only the most serious cases are registered. The prevalence of *Y. enterocolitica* O:3/O:9 specific antibodies was relatively high in Finland (19% and 31% by enzyme immunoassay and immunoblotting, respectively) and in Germany (33%, 43%) when healthy blood donors were studied (Mäki-Ikola et al. 1997). This may indicate a high amount of subclinical *Yersinia* infections in the healthy population.

2.6. Y. enterocolitica infections

2.6.1. In animals

Several reports have been presented on isolation of *Y. enterocolitica* strains from a variety of animals, but descriptions of observed clinical manifestations or patho-anatomical changes are sparse (Hurvell 1981; Schiemann 1989). Sporadic, small outbreaks of enteritis caused by *Y. enterocolitica* have been reported in chinchillas, hares, sheep and goats (Hurvell 1981; Slee and Skilbeck 1992; Gill 1996). However, both biochemical and serological patterns deviated from those of human strains.

Strains of bioserotype 4/O:3 have frequently been isolated from pigs (Table 3). In most cases, this bacterium has been isolated from seemingly healthy pigs with sampling carried out in connection with normal slaughter. However, samples have also been collected from animals with clinical symptoms (Hurvell 1981; Zheng 1987). Zheng (1987) recovered strains of serotype O:3 and O:9 from 48% of pigs, all of which had diarrhoea. Strains of bioserotype 4/O:3 have been isolated from dogs, especially puppies, and cats with diarrhoea, suggesting that this microbe may play a role in the infection (Ahvonen et al. 1973; Fantasia et al. 1993).

Pigs have been experimentally infected with bioserotype 4/O:3 in several studies (Fukushima et al. 1984a; Robins-Browne et al. 1985; Schiemann 1988; Shu et al. 1995; Nielsen et al. 1996; Najdenski et al. 1998; Thibodeau et al. 1999). The clinical response to infection in caesarean-derived piglets has ranged from a subclinical or mild illness to death after an incubation period of two days (Robins-Browne et al. 1985). Strains of *Y. enterocolitica* 4/O:3 have been shown to cause gastro-enteritis in new-born, colostrum-deprived piglets (Shu et al. 1995), whereas full-term colostrum-fed piglets seem to be quite resistant to infection (Schiemann 1988). In colostrum-fed piglets, colonisation was typically restricted to the throat and intestinal tract without development of serious illness (Schiemann 1988). Fattening pigs have been shown to excrete high numbers of *Y. enterocolitica* 4/O:3 in faeces for several weeks after infection mostly without any symptoms (Fukushima et al. 1984a; Nielsen et al. 1996). However, Thibodeau et al. (1999) demonstrated that the faecal shedding stops soon after ingestion of bacteria and only tonsillar infection occurs.

2.6.2. In humans

Y. enterocolitica can cause gastro-intestinal symptoms ranging from mild self-limited diarrhoea to acute mesenteric lymphadenitis evoking appendicitis (Ahvonen 1972b). Sometimes focal disease, such as pharyngitis, cellulitis, subcutaneous abscess, pneumonia and meningitis, may occur without gastro-intestinal illness (Tacket et al. 1983; Rose et al. 1987; Cover and Aber 1989; Bin-Sagheer et al. 1997). The incubation period of *Y. enterocolitica* enterocolitis ranges from 1 to 11 days. The minimal infective dose for humans has not been determined. Symptoms of enterocolitis typically persist for 5 to 14 days, but they may occasionally last for several months. The duration of the excretion of the organism in stool has been reported to range from 14 to 97 days (Cover and Aber 1989). The clinical manifestations of infection depend on factors such as the age and physiological state of the host and the pathogenic properties of the particular strain (Cover and Aber 1989).

Most commonly, *Y. enterocolitica* infections occur in young children (Stolk-Engelaar and Hoogkamp-Korstanje 1996). In patients under 5 years of age, yersiniosis presents as diarrhoea, often with low-grade fever and sometimes with abdominal pain (Hoogkamp-Korstanje and Stolk-Engelaar 1995). The symptoms can even be so faint and short-lived that yersiniosis is not diagnosed, despite faecal carriage (Olsovsky et al. 1975; van Ossel and Wauters 1990). In older children and young adults, acute yersiniosis can be present as a pseudo-appendicular syndrome, which is frequently confused with appendicitis (Stoddard et al. 1994). Sepsis is a rare complication of *Y. enterocolitica* infection, except in patients who have a predisposing underlying disease (Kellogg et al. 1995) or are in an iron-overloaded state (Cover and Aber 1989; Hopfner et al. 2001). Sepsis can also occur during blood transfusion (Mitchell and Brecher 1999). One source of *Y. enterocolitica* -contaminated red blood cell concentrate has been reported to be a blood donor with asymptomatic bacteremia (Strobel et al. 2000).

Normally, yersiniosis is a self-limited disease, but sometimes long-term sequelae, including reactive arthritis, erythema nodosum, uveitis, glomerulonephritis and myocarditis, will occur. Post-infection complications usually develop within one week to one month of initial infection, and these may be the only obvious clinical manifestation of *Yersinia* infection (Ahvonen 1972b; Sievers et al. 1972; Toivanen et al. 1985). Reactive arthritis and erythema nodosum are the most common complications (Ahvonen 1972b; Sievers et al. 1972; Leirisalo-Repo 1987). Reactive arthritis associated with urethritis and/or conjunctivitis is often termed Reiter's disease. *Yersinia*-triggered reactive arthritis (Aho et al. 1974; Leirisalo-Repo and Suoranta 1988), Reiter's disease (Aho et al. 1974) and uveitis (Careless et al. 1997) are strongly associated with the human leukocyte antigen

HLA-B27. *Yersinia*-triggered reactive arthritis often occurs in Nordic countries, where HLA-B27 and bioserotype 4/O:3 are especially prevalent (Sievers et al. 1972; Bottone 1999).

2.6.3. Pathogenesis

After ingestion of pathogenic *Y. enterocolitica*, this organism travels to the terminal ileum and binds to the intestinal epithelium. Attachment to the intestinal brush border is enhanced through the plasmid-encoded outer membrane protein YadA, which is optimally expressed at 37°C (Cornelis et al. 1998). The bacterium penetrates the intestinal mucosa through M cells, specialised cells involved in intestinal antigen uptake (Autenrieth and Firsching 1996; Vazguez-Torres and Fang 2000). Attachment and invasion of M cells are mediated by chromosomal determinants, Inv and Ail proteins, and plasmid determinant YadA (Miller and Falkow 1988; Vazguez-Torres and Fang 2000). After penetration of the intestinal epithelium, *Y. enterocolitica* colonises Peyer's patches, local lymphoid follicles, and can spread via the lymphatic system to other tissues (Bottone 1997). The ability to survive within the lymphoid follicles and other tissues is associated with the presence of virulence plasmid, which is essential for the pathogenesis of *Yersinia* (Visser et al. 1996). The bacteria provoke an inflammatory response that is responsible for the abdominal pain. The strains that cause pain severe enough to be confused with appendicitis are the ones that survive and multiply to the greatest extent in the Peyer's patches and thus evoke a more intense inflammatory response. Most infections are localised and self-limiting because the host's inflammatory response is usually able to eliminate the invaders (Bottone 1999).

Some patients develop reactive arthritis. The synovial fluid from affected joints of patients with *Yersinia*-induced arthritis is culture-negative but contains bacterial antigens, which suggests that only part of the microbe enters the joint (Granfors et al. 1989; Viitanen et al. 1991). How the antigens migrate from the mucosal tissue into the joints remains unknown, but mononuclear phagocytosis has been suggested to be responsible for the dissemination of bacterial antigens and the initiation of joint inflammation (Wuorela et al. 1999). Most individuals with post-infective reactive arthritis are positive for human leukocyte antigen HLA-B27 (Aho et al. 1974). In addition, HLA-B27 -positive individuals have more severe arthritic symptoms and a more prolonged course than patients who are HLA-B27 -negative. HLA-B27 has also been commonly found in patients with acute anterior uveitis, irrespective of the presence of underlying spondyloarthropathy (Careless et al. 1997). *Yersinia*-induced reactive arthritis is thought to be mediated by the immune system. Kirveskari et al. (1999) have shown that yersinia infection can down-regulate expression of major

histocompatibility complex class I molecules *in vivo* and that down-regulation is predominant in patients with the HLA-B27 genotype. However, the exact mechanism of the effect of HLA-B27 in the pathogenesis of reactive arthritis remains open.

2.7. Foodborne outbreaks

Foodborne outbreaks of *Y. enterocolitica* bioserotype 4/O:3 are uncommon, although it is theoretically possible for this bacterium to contaminate and then grow in many types of refrigerated foods (Kapperud 1991). In Japan and Czechoslovakia, some large outbreaks have been documented (Olsovsky et al. 1975; Kapperud 1991). In all cases, *Y. enterocolitica* serotype O:3 was the causative agent, but the source of infections went undetected. In the USA, one outbreak of serotype O:3, which was implicated with chitterlings (a dish made from pig intestine), has been reported (Lee et al. 1990). In addition, six major outbreaks of other serotypes have occurred in the USA; five of these were caused by serotype O:8. The outbreaks were associated with chocolate milk (Black et al. 1978), powdered milk and chow mein (Shayegani et al. 1983), tofu (Tacket et al. 1985), bean sprouts (Cover and Aber 1989) and pasteurised milk (Ackers et al. 2000). A rarer serotype, O:13, has caused an outbreak where pasteurised milk was the common source (Tacket et al. 1984). However, most *Y. enterocolitica* 4/O:3 infections are sporadic (Robins-Browne 1997). Due to the long incubation time and the predominance of cases going undiagnosed until 2-3 weeks after the infection as arthritic symptoms, the route of infection has failed to be traced in most cases.

2.8. Possible transmission routes of sporadic *Y. enterocolitica* infections

Pigs are considered to be the main source of human *Y. enterocolitica* 4/O:3 infections, even though a definite connection between isolates from pigs and human infections has still to be established. Elevated serum antibody concentrations have been found among people involved in swine breeding or pork production, suggesting a direct transmission of this bacterium from pigs to humans. In Finland, slaughterhouse workers and pig farmers were observed to have elevated antibody levels to *Y. enterocolitica* O:3 twice as frequently as grain- or berry farmers (Seuri and Granfors 1992) or randomly selected blood donors (Merilahti-Palo et al. 1991). Similar differences have also been discovered between people involved in swine slaughtering practices and office personnel in Norway (Nesbakken et al. 1991b). A close genetic relationship between pig isolates and human isolates has been shown by REAP (Nesbakken et al. 1987), REAC (Kapperud et al. 1990b), ribotyping (Andersen and Saunders 1990) and PFGE (Asplund et al. 1998).

Pet animals have also been suspected of being sources for human infections because of their close contact with humans (Schiemann 1989). However, direct transmission from pets to humans has yet to be proven.

The most common transmission route of pathogenic *Y. enterocolitica* is thought to be faecal-oral via contaminated food (Schiemann 1989; Smego et al. 1999), although pathogenic isolates have seldom been recovered from food samples (de Boer 1995; Ostroff 1995). *Y. enterocolitica* 4/O:3 infection has been associated with consumption of raw or undercooked pork and untreated water in case-control studies (Tauxe et al. 1987; Ostroff et al. 1994; Satterthwaite et al. 1999).

Direct person-to-person contact has not been demonstrated, but Lee et al. (1990) reported *Y. enterocolitica* O:3 infections in infants who were probably exposed to infection by their caretakers. Indirect person-to-person transmission has apparently occurred in several instances by transfusion of blood products (Mitchell and Brecher 1999). In these cases, the most likely source of *Yersinia* has been blood donors with subclinical bacteremia (Feng et al. 1992).

3. AIMS OF THE STUDY

The objectives of this thesis were to investigate the prevalence of pathogenic *Y. enterocolitica* in samples of pig origin and the distribution of pathogenic *Y. enterocolitica* in the slaughterhouse environment, and to characterise the recovered isolates to obtain further information on the epidemiology of this bacterium. The specific aims were as follows:

1. to determine the prevalence of *yadA*-positive *Y. enterocolitica* in pig tonsils, on carcasses and offals, and in the slaughterhouse environment (I, II),

2. to determine the prevalence of *yadA*-positive *Y. enterocolitica* in pig tongues and minced meat samples from retail shops (III),

3. to evaluate the use of the PCR method in the detection of *yadA*-positive *Y. enterocolitica* in pig tonsils, carcasses, pork and environmental samples (I-III),

4. to improve the use of the PFGE method in characterisation of *Y. enterocolitica* 4/O:3, and to compare this method with ribotyping (IV),

5. to study the distribution of different genotypes of *Y. enterocolitica* 4/O:3 strains isolated in pig slaughterhouses using PFGE (I, II, V), and

6. to study the sources of sporadic *Y. enterocolitica* 4/O:3 infections using PFGE (V).

4. MATERIALS AND METHODS

4.1. Samples (I-IV)

Tonsil samples (185) were collected in study I from nine slaughterhouses located in various parts of Finland during May and June 1995. In study II**,** 292 samples were collected from one pig slaughterhouse in December 1997 and January 1998. Of these 292 samples, 131 were surface samples from pig carcasses and pig offals, 89 were surface samples from different sites of the environment and 72 were air samples. In study III, 51 pig tongues and 255 minced meat samples (containing minced pork) were purchased from 40 retail shops in the Helsinki area during the period extending from February to July 1996. In study IV, 40 pig tongues were purchased from 7 retail shops in the Helsinki area in October and November of 1996.

Surface samples in study II were collected by swabbing the surface with a 7.5 x 7.5 cm gauze-covered cotton-wool pad moistened with 10 ml of TSB (trypticase soya broth, Difco, Detroit, MI, USA). The swabbed area consisted of the split surface of the cranial and abdominal incision and the shoulder from half of the carcass. The entire surface of the offal (heart, liver, kidney or ear) was swabbed, so that one sample contained five swabbed surfaces. The environmental surface samples from different sites (a brisket saw, a splitting saw, hooks, knives, knife sheaths, a refrigerator, floors, meat containers, handrails, meat-cutting tables, aprons, hands, a computer keyboard and the handle of a coffeemaker used by slaughterhouse workers) were taken by swabbing an area approximately 15 x 15 cm with a moistened 7.5 x 7.5 cm gauze-covered cotton-wool pad. Air samples in study II were collected using a sedimentation method where open cefsulodin-irgasan-novobiocin (CIN) agar plates (Yersinia Selective Agar Base, Oxoid, Basingstoke, UK) and MacConkey agar plates (Difco) were exposed for 4 h at nine different sites: bleeding, eviscerating, meat-inspection, offal-harvesting, weighing, trimming, head-cutting and chilling areas.

4.2. Strains of *Y. enterocolitica* 4/O:3 (V)

Strains used in study V were isolated from different sources in the period between 1995-99 (V, Table 1). The human strains were isolated in 1995-99 from patients with diarrhoea from different parts of Finland. Tonsil strains were isolated from slaughterhouses A to I in 1995 (I). Faeces strains were isolated from slaughterhouses D in 1997 and G in 1996. Carcass strains were recovered from slaughterhouses G and I in 1997, and from slaughterhouses J in 1997-98 (II) and H in 1998. Heart,

kidney and liver strains were recovered from slaughterhouses J in 1997-98 (II) and H in 1998, and from retail shops in the Porvoo area in 1998. Tongue and minced meat strains were recovered from retail shops in the Helsinki area in 1996 (III, IV) and in the Porvoo area in 1998. Strains from the slaughterhouse environment were isolated from different sources in slaughterhouses G in 1997, I and J (II) in 1997-98 and H in 1998. Pet strains were isolated from dog faeces in 1998-99 and cat faeces in 1998.

4.3. Sample preparation (I-IV)

In study I, a 10-g tonsil sample was homogenised in 90 ml TSB for 1 min in a Stomacher Lab Blender (Seward Medical, London, UK). In study II, a cotton-wool pad, premoistened with 10 ml of TSB, was transferred to 90 ml of TSB and shaken vigorously. In study III, a tissue sample of a pig tongue surface was obtained by cutting pieces from the upper surface of the tongue. A 25-g sample of the tongue tissue or a minced meat sample was homogenised in 225 ml TSB for 1 min in the stomacher blender. In study IV, from each tongue, nine subsamples intended for quantitative analysis were taken: 3x10 g, 3x1 g, 3x0.1 g, and placed in 90 ml (10-g sample) or in 10 ml (1-g and 0.1-g sample) of TSB. In all of these studies, samples were enriched in TSB at 22°C for 16-18 h, and following overnight enrichment, studied with both the PCR and culture methods.

4.4. Detection of *yadA*-positive *Y. enterocolitica* in samples of pig origin and from the pig slaughterhouse environment with PCR (I-III)

The *yadA*-positive *Y. enterocolitica* was detected from TSB-homogenates with the nested-PCR method of Kapperud et al. (1993), with modifications as described in studies I and III. Briefly, 100 µl of TSB overnight enrichment (Fig. 1) was centrifuged and the pellet was resuspended in 50 µl of 1x PCR buffer (Finnzymes, Espoo, Finland) containing 0.2 mg of proteinase K (Finnzymes)/ml. After incubation at 37°C for 1 h, the suspension was boiled for 10 min. After centrifugation, 25 µl of the supernatant was used as a template in the first PCR step, and 2 µl of the first PCR product was used as template in the second PCR step. Two sets of oligonucleotide primers (Pharmacia Biotech, Vantaa, Finland), based on the nucleotide sequences of the *yadA* (I, Table 1; III, Table 1), were used for PCR amplification, which was performed in a 16-well PTC-150 thermal cycler (MJ Research, Watertown, MA, USA). The sample volume was 50 µl and contained 1 U of Dynazyme DNA polymerase (Finnzymes), 200 µM of each dNTP and 0.1 µM of each primer. Plasmid-positive

Y. enterocolitica 4/O:3 as positive control and water instead of template as negative control were included in each analysis. The size of the amplified second PCR product (about 530 bp) was determined in 0.7% agarose gel by comparison with the DNA molecular weight marker VI (Boehringer Mannheim, Mannheim, Germany) (III, Fig. 1).

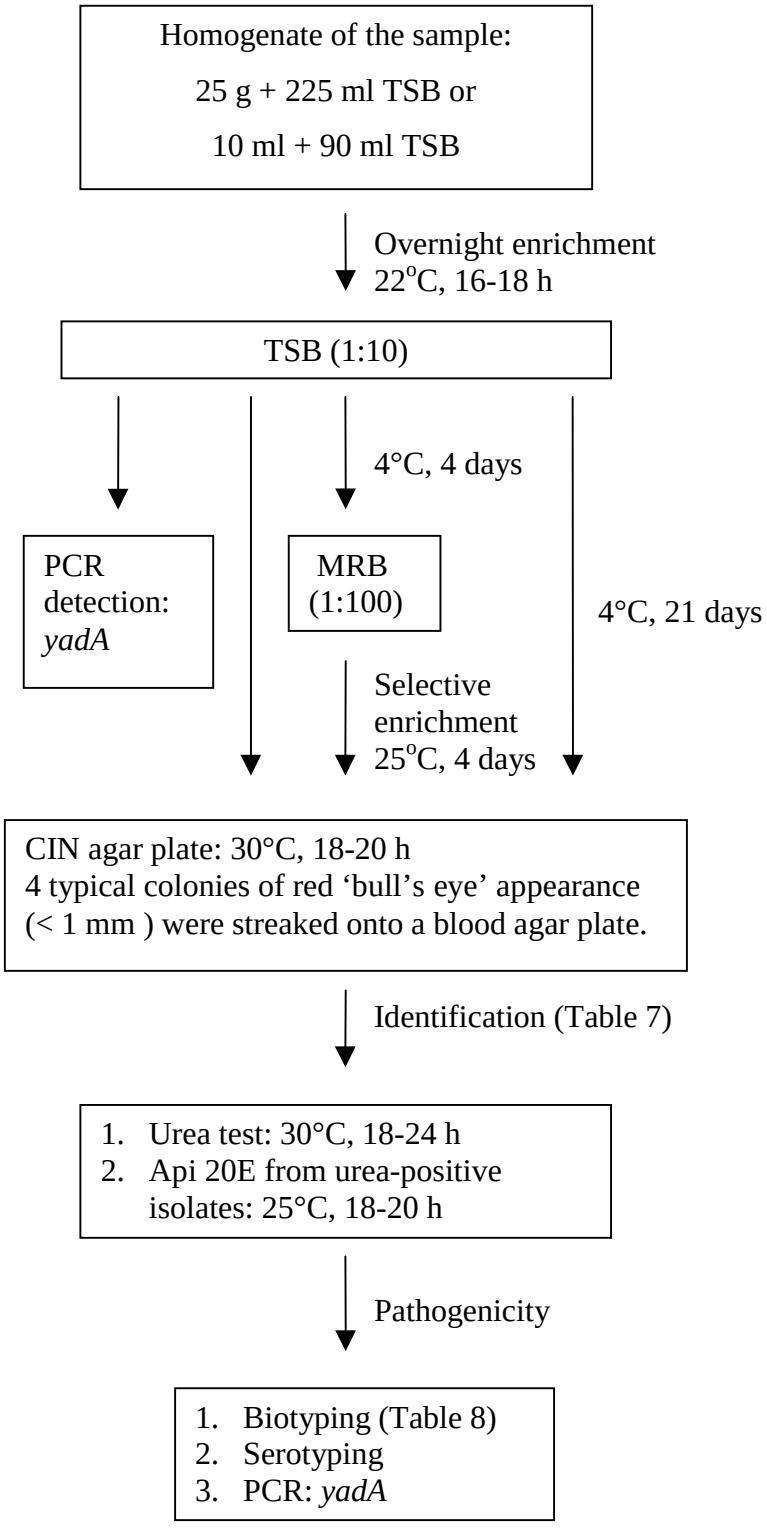

Figure 1. Detection and isolation of *yadA*-positive *Yersinia enterocolitica.*

4.5. Isolation of *Y. enterocolitica* from samples of pig origin and from the pig slaughterhouse environment (I-IV)

Y. enterocolitica was isolated with the culture method, which included overnight enrichment, selective enrichment and cold enrichment (Fig. 1). Every sample was initially enriched in TSB at 22°C for 16-18 h and then further enriched at 4°C for 4 days, after which 100 µl of this enrichment was inoculated into 10 ml of modified Rappaport broth (MRB) (Wauters 1973) and incubated at 25°C for 4 days. The TSB-homogenates were also enriched at 4°C for 21 days. Subculture on selective CIN agar plates (Oxoid) was done after every enrichment step according to the method of the Nordic Committee on Food Analysis (1996). One to four suspect colonies of typical ´bull´s eye´ appearance on the CIN agar plates were streaked onto blood agar plates to create a pure culture.

One colony from pure culture was inoculated onto a urea agar slant (Difco) and incubated for 1 day at 30°C. The isolates showing urea hydrolysis were further identified using the API 20E system (BioMérieux, Marcy l'Etoile, France) and incubated at 25°C for 18-20 h. *Y. enterocolitica* isolates were identified on the basis of Voges-Proskauer and citrate tests, and acid production reactions to sorbitol, melibiose, sucrose and rhamnose (Table 7). For the temperature-dependent Voges-Proskauer test, the API 20E system was incubated at 25°C instead of 37°C, which is the correct temperature according to the manufacturer's instructions.

Table 7. Biochemical differentiation of urea-positive *Yersinia* species after incubation at 25°C for 18-20 h.

Species	Reaction					
	Voges-Proskauer	Citrate	Sorbitol	Rhamnose	Sucrose	Melibiose
Y. enterocolitica	+/-	-	+	-	+	-
Y. pseudotuberculosis	-	-	-	+/-	-	+/-
Y. frederiksenii	+	+/-	+	+	+	-
Y. intermedia	+	+	+	+	+	+
Y. kristensenii	-	-	+	-	-	-
Y. aldovae	+	+/-	+	+	-	-
Y. rhodei	-	+/-	+	-	+	+/-
Y. mollaretii	-	-	+	-	+	-
Y. bercovieri	-	-	+	-	+	-

Y. enterocolitica was isolated from the air using sedimentation on CIN and MAC agar plates. The plates were incubated at 30°C for 18-20 h. Lactose-negative colonies on MAC agar plates were streaked onto CIN agar plates, and all colonies of typical appearance on these CIN agar plates were identified.

4.6. Bio- and serotyping of *Y. enterocolitica* isolates (I-V)

All *Y. enterocolitica* isolates were biotyped according to the revised scheme of Wauters et al. (1987). Subdivision into 6 biotypes (1A, 1B, 2, 3, 4 and 5) was based on the following reactions: pyrazinamidase activity, esculin hydrolysis, salicin acidification, tween-esterase activity, indole production, xylose acidification and nitrate reduction (Table 8).

Table 8. Biochemical tests used for biotyping *Yersinia enterocolitica.*

	Biotype reactions after incubation at 25°C for 48 h					
Test	1A	1B	2	3	4	5
Pyrazinamidase [a]	+	-	-	-	-	-
Esculin hydrolysis	+	-	-	-	-	-
Salicin (acid production)	+	-	-	-	-	-
Tween-esterase	+	+	-	-	-	-
Indole production	+	+	+	-	-	-
Xylose (acid production)	+	+	+	+	-	-
Nitrate reduction	+	+	+	+	+	+

[a] According to Kandolo and Wauters (1985).

In all studies, serotyping of *Y. enterocolitica* was carried out with slide agglutination using commercial antisera O:3, O:5, O:8 and O:9 (Denka Seiken, Tokyo, Japan).

4.7. Detection of *yadA* gene in *Y. enterocolitica* isolates with PCR (I-V)

The pathogenicity of *Y. enterocolitica* isolates was confirmed with PCR targeting the *yadA* on the pYV (Fig. 1). Four small (< 1 mm) colonies from pure culture were boiled in 100 µl of water for 10 min, and 2 µl of this boiled bacteria suspension was used as a template in PCR (Kapperud et al. 1993). PCR was carried out as described in Section 4.4.

4.8. Genotyping of *Y. enterocolitica* 4/O:3 (I, II, IV, V)

4.8.1. DNA isolation (I, II, IV, V)

DNA extraction was performed according to Maslow et al. (1993), with the modifications as described by Björkroth et al. (1996) (I, II) and Autio et al. (1999) (IV-V). Briefly, a single colony grown on blood agar was inoculated into 5 ml TSB and incubated overnight at room temperature. The cells in late log phase (18 h) were harvested from 2 ml of TSB. The cells were washed once in 5 ml of cold PIV (10 mM Tris [pH 7.5], 1 M NaCl) and then resuspended in 750 µl of cold PIV. Of this cell suspension 0.5 ml was mixed with an equal amount of 2% (w/v) low melting temperature agarose (InCert agarose; FMC Bioproducts, Rockland, ME, USA) and cast in GelSyringe dispensers (New England Biolabs, Beverly, Mass., USA). The plugs were lysed for 3 h (IV-V) or overnight (I, II) in about 2.5 ml of lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, 20 µg/ml RNase, 1 mg/ml lysozyme) at 37°C with gentle shaking. The isolation was completed with a single 2-h (IV-V) or 2 overnight (I, II) ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 µg/ml proteinase K) washes at 50°C. The plugs were placed in fresh ESP solution and stored at 4°C until restriction enzyme digestion was performed.

4.8.2. Macrorestriction analysis with PFGE (I, II, IV, V)

Plugs were washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA) before proteinase K was inactivated with phenylmethylsulfonyl fluoride. Plugs were further washed two times with TE buffer before restriction endonuclease digestion was performed according to the manufacturer's instructions. In studies I and II, two rare-cutting restriction enzymes, *Not*I and *Xba*I (New England Biolabs), were used. In study IV, *Not*I, *Apa*I, *Xba*I, *Xho*I and *Spe*I were selected for restriction enzyme digestion after a pilot study where 35 enzymes (*Apa*I, *Asc*I, *Avr*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bss*HII, *Bst*EII, *Cla*I, *Ceu*I, *Eag*I, *Fse*I, *Kas*I, *Kpn*I, *Mlu*I, *Nae*I, *Nci*I, *Nhe*I, *Nru*I, *Pac*I, *Pme*I, *Pst*I, *Pvu*II, *Rsr*II, *Sac*I, *Sac*II, *Sal*I, *Sfi*I, *Sma*I, *Spe*I, *Ssp*I, *Xba*I, *Xho*I and *Xma*I) were tested. In study V, *Not*I, *Apa*I and *Xho*I were used. All samples were electrophoresed using a Gene Navigator system (Pharmacia, Uppsala, Sweden) with a hexagonal electrode through a 1% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts) in a 0.5 x TBE buffer (Amresco, Solon, OH, USA) at 12°C and 200 V. Switching times from 1 to 15 s over 18 h for *Xba*I, *Xho*I and *Asc*I, from 1 to 18 s over 20 h for *Not*I and *Spe*I, and from 1 to 20 s over 20 h for *Apa*I were used. Low-Range, Mid-Range I and Lambda

Ladder PFG markers (New England Biolabs) were used for fragment size determination. The gels were stained for 30 min in 1 litre of running buffer containing 50 µl of ethidium bromide (10 mg/ml) and destained in running buffer until appropriate contrast was obtained for standard photography and/or digital imaging with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA). The banding patterns were interpreted visually. Isolates were considered to be different when a one-band difference between fragments over 70 kb was observed.

4.8.3. Ribotyping (IV)

A 1-mm thick agarose-embedded slice of DNA was cleaved with *Hind*II, *Eco*RI, *Sal*I, *Bgl*I and *Nci*I as recommended by the manufacturer. The digested DNA was run in a 0.8% agarose gel overnight at 25 V in TAE buffer (Amresco). Digoxigenin (DIG)-labelled lamda *Hind*III digest (Molecular weight marker II: Boehringer Mannheim) was used as a size marker. The cDNA probe was prepared from Escherichia coli 16S and 23S rRNA (Boehringer Mannheim) by reverse transcription (Promega, Madison, WI, USA) (Blumberg et al. 1991). Southern transfer and hybridisation were performed according to Björkroth and Korkeala (1996).

4.8.4. Discrimination index (IV-V)

The discriminatory power of PFGE with the combinations of *Not*I, *Apa*I, *Xba*I, *Xho*I and *Spe*I enzymes in study IV, and *Not*I, *Apa*I and *Xho*I enzymes in study V was calculated with Simpson's index of diversity (Hunter and Gaston 1988). The index estimates the probability of two strains sampled from the test population being placed into different typing groups. The discrimination index (DI) is given by the following equation:

$$DI = 1 - 1 / N (N - 1) \sum_{j=1}^{s} n_j (n_j - 1),$$

where N is the total number of strains, s is the total number of types described , and n_j is the number of strains belonging to j^{th} type.

5. RESULTS

5.1. Prevalence of *yadA*-positive *Y. enterocolitica* in pig tonsils, on carcasses and offals, and in the slaughterhouse environment (I-II)

Prevalence of *yadA*-positive *Y. enterocolitica* was studied in samples from pig tonsils, carcasses, offals and the slaughterhouse environment (I, II) with both the PCR and culture methods (Table 9). The mean prevalence of *yadA*-positive *Y. enterocolitica* in pig tonsils was 37%. The *yadA*-positive *Y. enterocolitica* was detected in all nine slaughterhouses, with prevalence varying from 13% to 45% (I, Table 2). The prevalence of *yadA*-positive *Y. enterocolitica* was higher on hearts, livers and kidneys than on carcasses (Table 9).

Table 9. Prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tonsils, on carcasses and offals, and in the slaughterhouse environment in Finland.

Origin of samples	No. of samples	No. of PCR-positive samples	No. of culture-positive samples	No. of PCR- or culture-positive samples	Prevalence %
Tonsils	185	52	48	68	37
Carcasses	80	17	5	17	21
Ears	17	4	2	4	24
Livers	13	5	4	5	38
Kidneys	13	11	9	11	85
Hearts	8	5	4	5	63
Environment	89	12	4	12	13

Pathogenic yersinia was also detected in the slaughterhouse environment from different sites, including the brisket saw, the hook from which the pluck set (heart, lungs, oesophagus, trachea, diaphragm, liver, kidneys, and tongue with tonsils) hung, the knife used for evisceration, the floor in the eviscerating and the weighing area, the meat-cutting table, the aprons used by trimming workers, the computer keyboard used in the meat inspection area, the handle of the coffeemaker used by slaughterhouse workers, and the air in the bleeding area (II, Table 2).

5.2. Prevalence of *yadA*-positive *Y. enterocolitica* in pig tongues and minced meat at retail level (III)

Prevalence of pathogenic *Y. enterocolitica* was studied in pig tongues and minced meat samples from retail shops with both the PCR and culture methods (III). At the retail level, prevalence of *yadA*-positive *Y. enterocolitica* was extremely high in pig tongues (Table 10).

Table 10. Prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tongues and in minced meat at retail level in Finland.

Origin of samples	No. of samples	No. of PCR-positive samples	No. of culture-positive samples	No. of PCR- or culture-positive samples	Prevalence %
Tongues	51	47	40	50	98
Minced meat	255	63	4	63	25

5.3. Detection of *yadA*-positive *Y. enterocolitica* in samples of pig origin and from the pig slaughterhouse environment using PCR (I-III)

In studies I-III, all samples, except for air samples in study II, were investigated with nested-PCR from overnight enrichments (Fig. 1). After the second PCR step, a 530-bp product, which was recognised as a predominant band in agarose gel, was produced (III, Fig. 1). More positive samples were yielded by PCR than by the culture method, especially when minced meat samples were studied (Table 10). Some false-negative (culture-positive and PCR-negative) results were obtained when tonsils and tongues were studied (Table 11).

Table 11. Detection of *yadA*-positive *Yersinia enterocolitica* with the PCR and culture methods.

Samples		Culture-positive samples	Culture-negative samples
Tonsils	PCR-positive samples	32	20
(185)[a]	PCR-negative samples	16	117
Carcasses and offals	PCR-positive samples	24	18
(131)	PCR-negative samples	0	89
Environment	PCR-positive samples	4	8
(89)	PCR-negative samples	0	77
Tongues	PCR-positive samples	37	10
(51)	PCR-negative samples	3	1
Minced meat	PCR-positive samples	4	59
(255)	PCR-negative samples	0	192

[a] Total number of samples.

5.4. Isolation of *yadA*-positive *Y. enterocolitica* from samples of pig origin and from the pig slaughterhouse environment (I-III)

Y. enterocolitica was isolated with the culture method, which included overnight enrichment, selective enrichment and cold enrichment (Fig. 1). In all studies (I-III), selective enrichment was the most productive. Overnight and cold enrichments were useful only when tonsils were studied. Prevalence of *yadA*-positive *Y. enterocolitica* in tonsils and on edible offals (tongues, hearts, livers and kidneys) was high even with the culture method (Tables 9 and 10). However, prevalence was lower with the culture method than with PCR, especially when minced meat samples were studied (Table 10). Bioserotype 4/O:3 was the only pathogenic type found. The *yadA* was not detected in all isolates, but most isolates of bioserotype 4/O:3 were *yadA*-positive (Table 12).

Table 12. Detection of *yadA* in isolates of *Yersinia enterocolitica* 4/O:3 recovered from different sources.

Origin of samples	No. of isolates		No. of *yadA*-positive isolates		No. of *yadA*-negative isolates	
Pig tonsils (I)	61	(61)[a]	48	(48)	13	(13)
Pig carcasses (II)	18	(5)	16	(5)	2	(1)
Pig offals (II)	69	(19)	61	(19)	8	(4)
Environment (II)	26	(7)	22	(6)	4	(1)
Minced meat[b] (III)	5	(5)	4	(4)	1	(1)
Pig tongues (III)	92	(42)	80	(40)	12	(9)
Pig tongues (IV)	128	(33)	114	(33)	14	(5)
Human strains (V)	334	(249)	310	(225)	24	(24)
Non-human strains (V)	212	(212)	195	(205)	7	(7)

[a] Number of samples.
[b] Containing pork.

Two out of four air samples from the bleeding area were positive for *yadA*-positive *Y. enterocolitica* on CIN agar plates when the sedimentation method was used. A total of eight isolates of bioserotype 4/O:3 were recovered, seven of which carried the *yadA*.

5.5. Genotyping of *Y. enterocolitica* 4/O:3 (I, II, IV, V)

PFGE with *Not*I and *Xba*I enzymes was used to characterise the isolates of *Y. enterocolitica* 4/O:3 in studies I and II. All isolates were easily typed with these enzymes. Interpretation of PFGE patterns was at times challenging because of the large amount of fragments very closely spaced together, but in general, the profiles were simple to interpret. A fragment of about 40 kb was observed in the PFGE patterns of *yadA*-positive isolates, but not in those of *yadA*-negative isolates produced by *Not*I enzyme (I, Fig. 1; IV, Fig. 1; V, Fig. 1). Although the profiles were not identical, most displayed only minor deviations (Fig. 2).

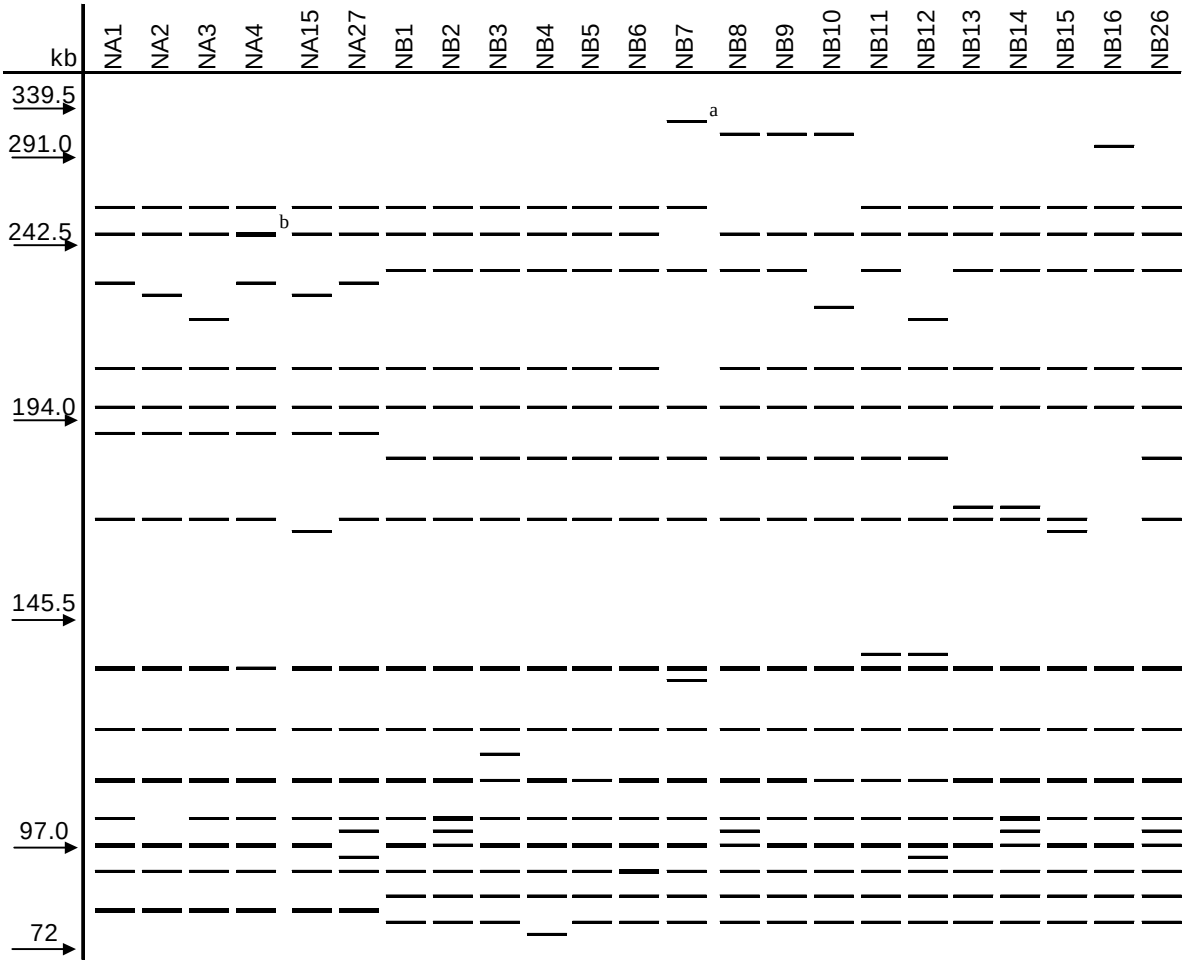

Figure 2. The 23 different *Not*I profiles of *Yersinia enterocolitica* 4/O:3 isolates obtained in studies I and II. [a] Single band. [b] Double band.

A total of 28 different genotypes (termed pulsotypes in studies I and II) for 61 tonsil isolates (I) and 9 genotypes for 113 isolates from a pig slaughterhouse (II) were obtained by combining the various *Not*I and *Xba*I profiles (Table 13). Two predominating genotypes, NA1/XA1 and NB1/XB1, were obtained.

Table 13. Genotypes obtained in studies I and II with *Not*I and *Xba*I enzymes.

Genotype		Pulsotype[a]	Total no. of strains	Sources (No. of samples)
*Not*I	*Xba*I			
NA1	XA1	1a	39	Tonsils (20), carcass (3), ear (2), liver (2), kidney (5), heart (2), brisket saw (1), knife (1), hook (1), air (2)
NA1	XA2	1b	1	Tonsils (1)
NA1	XA3	1c	1	Tonsils (1)
NA1	XA4	1d	2	Tonsils (2)
NA1	XA5	1e	1	Tonsils (1)
NA2	XA6	2f	1	Tonsils (1)
NA3	XA1	3a	1	Tonsils (1)
NA4	XA1	1a, 21a	5	Tonsils (1), liver (2), kidney (1), computer keyboard (1)
NA15	XA2	22b	1	Computer keyboard (1)
NA27	XA1	1a	1	Tonsils (1)
NB1	XB1	4g	16	Tonsils (12), kidney (1), computer keyboard (1), air (2)
NB2	XB2	5h	1	Tonsils (1)
NB3	XB1	6g	3	Tonsils (2), heart (1)
NB4	XB3	7i	1	Tonsils (1)
NB5	XB5	8k	1	Tonsils (1)
NB5	XB12	8s	4	Liver (1), kidney (3)
NB6	XB1	9g	1	Tonsils (1)
NB7	XB6	10l	1	Tonsils (1)
NB8	XB7	11m	1	Tonsils (1)
NB9	XB1	12g	1	Heart (1)
NB9	XB4	12j	1	Tonsils (1)
NB9	XB8	20n, 12r	3	Tonsils (1), carcass (2)
NB10	XB1	13g	1	Tonsils (1)
NB11	XB8	14n	1	Tonsils (1)
NB11	XB9	14o	1	Tonsils (1)
NB12	XB8	15n	1	Tonsils (1)
NB13	XB1	16g	1	Tonsils (2)
NB14	XB1	17g	1	Tonsils (1)
NB15	XB10	18p	1	Tonsils (1)
NB16	XB11	19q	3	Tonsils (1), kidney (2)
NB26	XB8	4n	1	Tonsils (1)

[a] Labelled in studies I and II.

In study IV, the efficiency of PFGE for differentiation of isolates of bioserotype 4/O:3 was evaluated. A total of 128 isolates recovered from 33 retail pig tongues were characterised with *Not*I enzyme. *Apa*I, *Xba*I, *Xho*I and *Spe*I enzymes were selected to further differentiate the isolates of the predominating *Not*I profiles, NA1 and NB1, because they gave the best resolution after a pilot study where 35 enzymes (*Apa*I, *Asc*I, *Avr*II, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bss*HII, *Bst*EII, *Cla*I, *Ceu*I, *Eag*I, *Fse*I, *Kas*I, *Kpn*I, *Mlu*I, *Nae*I, *Nci*I, *Nhe*I, *Nru*I, *Pac*I, *Pme*I, *Pst*I, *Pvu*II, *Rsr*II, *Sac*I, *Sac*II, *Sal*I, *Sfi*I, *Sma*I, *Spe*I, *Ssp*I, *Xba*I, *Xho*I and *Xma*I) were tested (IV, Table 1). The isolates were easily typed with these four enzymes. The discrimination index increased from 74% to 93%, and the number of different genotypes increased from 15 to 30 when the isolates of the predominant types with *Not*I were further characterised with *Apa*I and *Xho*I.

Two genomic groups, A and B, were obtained on the basis of a group-specific fragment in the restriction patterns of *Not*I, *Apa*I, *Xho*I, *Xba*I and *Spe*I enzymes (IV, Fig.1-3). To confirm this grouping, 15 isolates of different *Not*I profiles were characterised using ribotyping with 5 enzymes (*Eco*RI, *Nci*I, *Hind*III, *Bgl*I and *Sal*I) in study IV. No genomic groups were found with ribotyping. The ribotypes of isolates of different groups were identical with *Eco*RI and *Nci*I enzymes, and very similar to *Hind*III, *Bgl*I and *Sal*I enzymes, indicating that the genomic groups obtained with PFGE were genetically closely related.

5.6. Distribution of different genotypes of *Y. enterocolitica* 4/O:3 strains isolated from slaughterhouses (I, II, V)

The most common genotypes, NA1/XA1 and NB1/XB1, were found in 33% and 20% of the tonsils, respectively, and these genotypes were widely distributed amongst the slaughterhouses. Genotype NA1/XA1 was also found on pig carcasses, offals (ears, livers, kidneys, hearts), the brisket saw, the hook from which the pluck set (heart, lungs, oesophagus, trachea, diaphragm, liver, kidneys, and tongue with tonsils) hung, the knife used for evisceration and from the air (Table 13). Genotype NB1/XB1 was also recovered on kidneys, on the computer keyboard and in the air. The genotype NA4/XA1, which was also isolated from tonsils, was detected on livers, kidneys and the computer keyboard. Other types found in tonsils, NB3/XB1, NB9/XB8 and NB16/XB11, were recovered from hearts, carcasses and kidneys, respectively. More than two different genotypes were observed in samples from livers, kidneys, hearts and the computer keyboard.

In study V, 61 (64%) out of 95 strains found in pig slaughterhouses from carcasses, offals and the environment were indistinguishable from tonsil strains when characterised with *Not*I, *Apa*I and *Xho*I enzymes (Table 14).

Table 14. Distribution of different genotypes of tonsil strains in pig slaughterhouses.

PFGE patterns obtained by *Not*I, *Apa*I and *Xho*I	No. of tonsil samples	Sources contaminated with strains of indistiguishable genotypes from tonsil strains	
		Samples from pig origin (No. of samples)	Samples from environment (No. of samples)
NA1/AA1/HA3	2	Heart (1), liver (1), kidney (1)	Conveyor belt (1)
NA1/AA2/HA2	4	Heart (1)	
NA1/AA2/HA3	8	Carcass (19), heart (2), liver (3), kidney (4),	Brisket saw (1), sludge (1), air (1)
NA3/AA15/HA3	1	Heart (1)	
NA4/AA2/HA3	1	Carcass (1), heart (1), liver (1),	Computer keyboard (1)
NA27/AA18/HA2	1	Carcass (1)	
NB1/AB1/HB1	8	Carcass (1), kidney (3),	Computer keyboard (1), conveyor belt (1), air (1)
NB2/AB15/HB1	1	Carcass (1), heart (1), liver (4), kidney (1)	
NB9/AB14/HB1	1	Carcass (4)	
NB26/AB14/HB1	1	Heart (1)	

5.7. Sources of sporadic *Y. enterocolitica* 4/O:3 infections (V)

A total of 64 different PFGE profiles were obtained when 546 strains of *Y. enterocolitica* 4/O:3 were characterised with *Not*I enzyme. The discrimination index was 0.80. Altogether 194 (92%) out of 212 human strains were indistinguishable from 140 (86%) out of 163 strains from slaughterhouses, 140 (85%) out of 164 strains from retail outlets and all 7 strains from pet animals. These 481 strains belonging to 22 genotypes were further characterised with *Apa*I and *Xho*I enzymes. The number of different genotypes increased from 64 to 126, and the discrimination index rose from 0.80 to 0.94 when identical human and non-human strains with *Not*I were further characterised with *Apa*I and *Xho*I.

Genotypes commonly found in human *Y. enterocolitica* 4/O:3 infections were recovered from many sources of pig origin in slaughterhouses and retail shops. Altogether 114 (54%) out of 212 human strains were indistinguishable from tonsil strains when characterised with *Not*I, *Apa*I and *Xho*I

enzymes. A total of 75 (35%) and 110 (52%) strains found in human infections were indistinguishable from strains recovered in pig slaughterhouses on carcasses and edible offals (heart, liver, kidneys), respectively. In addition, 140 (66%) and 66 (31%) human strains were indistinguishable from strains from retail shops isolated on pig offals (tongues, kidneys and hearts) and in pork, respectively. Two common genotypes, NA1/AA2/HA3 and NB1/AB1/HB1, were also isolated from a cat and a dog, respectively. In all, 151 (71%) human strains were indistinguishable from strains isolated from pig tongues, hearts, livers and kidneys.

6. DISCUSSION

6.1. Prevalence of *yadA*-positive *Y. enterocolitica* in pig tonsils, on carcasses and offals, and in the slaughterhouse environment (I-II)

Y. enterocolitica harbouring the *yadA* was found to be common in Finnish pig tonsils and was detected in all 9 slaughterhouses. Ten years ago, 4 out of 7 slaughterhouses were negative for pathogenic *Y. enterocolitica* (Asplund et al. 1988). In our study, the prevalence varied significantly between slaughterhouses, which may be due to different prevalences of infected herds in different areas (I). Herd-wise distribution has been demonstrated by culture methods (Christensen 1980, 1987b; Nesbakken and Kapperud 1985) and by use of serological tests (Nielsen and Wegener 1997; Skjerve et al. 1998) in Denmark and Norway. Prevalence of *Y. enterocolitica* 4/O:3 has been shown to be highest in large pig farms, where piglets have been purchased from various pig markets or pig producers (Christensen 1987b; Skjerve et al. 1998). The prevalence of pathogenic yersinia in herds and between herds in Finland has yet to be examined.

The prevalence of *yadA*-positive *Y. enterocolitica* on pig carcasses was moderately high (21%) using PCR (II). The isolation rate of bioserotype 4/O:3 was only 6%, which is lower than in other Nordic countries. In the last studies performed in the Nordic countries before the use of the plastic bag technique, the isolation rate of bioserotype 4/O:3 was 17%, 12% and 8% in Denmark, Norway and Sweden, respectively (Andersen 1988, Nesbakken et al. 1994). The prevalence of *yadA*-positive *Y. enterocolitica* was high on livers, kidneys and hearts with both the PCR and culture methods. The high contamination rate may be due to cross-contamination from tonsils to offals during slaughter. The spread of yersinia from tonsils to edible offals is unavoidable when tonsils are removed along with the pluck set (heart, lungs, oesophagus, trachea, diaphragm, liver, kidneys and tongue) and hung together on a hook.

The *yadA*-positive *Y. enterocolitica* was detected from many different environmental sources in the pig slaughterhouse with PCR (II), indicating the vast contamination of the pig slaughterhouse environment with pathogenic yersinia. This is the first time, to our knowledge, that pathogenic yersinia has been detected in a slaughterhouse environment to this extent. *Y. enterocolitica* 4/O:3 was isolated from several sources: the brisket saw, the hook from which the pluck set hangs, the knife used for evisceration, the computer keyboard used in the meat-inspection area and the air in the bleeding area. In previous studies, *Y. enterocolitica* 4/O:3 has been isolated in pig

slaughterhouses only from floors and the viscera table (Nesbakken 1988), or from sludge samples (Fransen et al. 1996). As far as we are aware, this is the first time that pathogenic yersinia has been isolated from the air.

6.2. Prevalence of *yadA*-positive *Y. enterocolitica* in retail pig tongues and minced meat (III)

Prevalence of *yadA*-positive *Y. enterocolitica* at retail level was studied in pig tongues and minced meat. The prevalence in pig tongues was high with both PCR and culture methods. One reason for the contamination rate of 98% may be the slaughtering process, where the tonsils are removed in conjunction with the tongue. It is impossible to avoid the spread of pathogenic yersinia from the tonsils to the tongue when they hang together on a hook in the slaughterhouse. Cross-contamination from tongue to tongue may occur in the slaughterhouse and later at retail level.

The isolation rate of bioserotype 4/O:3 was 82%. While several studies have isolated *Y. enterocolitica* from pig tongues in slaughterhouses and at retail level, only in Belgium has the prevalence of bioserotype 4/O:3 in pig tongues been as high (Wauters et al. 1988a). It is possible that in some countries tongues have received scald treatment, which has decreased the amount of heat-sensitive bacteria, such as *Yersinia* in the tongues (Harmon et al. 1984). Scald treatment should be obligatory immediately after removal of the tongue to decrease further contamination in the slaughterhouse and at retail level.

The prevalence of *yadA*-positive *Y. enterocolitica* in minced meat was moderately high (25%) with PCR. In Norway, using the same PCR method as in our studies, pathogenic *Y. enterocolitica* was detected in 15% and 17% of pork samples in slaughterhouses and retail shops, respectively (Johannessen et al. 2000). The isolation rate of *Y. enterocolitica* 4/O:3 in minced meat was 2% in our study, which it is in accordance with previous studies (Nesbakken et al. 1985; Christensen 1987a; de Boer and Nouws 1991; Tsai and Chen 1991; Karib and Seeger 1994; Loguet et al. 1996; Fukushima et al. 1997; Johannessen et al. 2000). Belgium is an exception; there, bioserotype 4/O:3 was isolated from 24% of minced meat samples (Wauters et al. 1988a). One reason for this high prevalence might be the use of head meat and tonsillar tissue in minced meat (Tauxe et al. 1987).

6.3. Detection of *yadA*-positive *Y. enterocolitica* in samples of pig origin and from the pig slaughterhouse environment using PCR (I-III)

The prevalence of pathogenic *Y. enterocolitica* was higher with PCR than with the culture method (I-III), indicating the higher sensitivity of the former for naturally contaminated samples. These results are comparable with the study in Norway, where pathogenic *Y. enterocolitica* was detected in 17% and 2% of pork samples with the PCR and culture methods, respectively (Johannessen et al. 2000). Thisted Lambertz et al. (1996) compared the capacity of these two methods to detect *Y. enterocolitica* in naturally contaminated pig tonsils and found the PCR method to be more rapid, sensitive and specific. However, Rasmussen et al. (1995) have shown that a pre-enrichment step is needed before PCR to increase sensitivity when naturally contaminated samples are studied. A pre-enrichment step in a non-selective medium overnight at room temperature was used in our studies prior to PCR to increase sensitivity and also to ensure viability of the target cells.

Some samples were PCR-negative but culture-positive when tonsils and tongues were studied (I, III). Failure of the PCR method to detect all culture-positive pork samples was also reported by Johannessen et al. (2000). One reason for the false-negative results may be the high level of pathogenic *Y. enterocolitica* in these samples. Lantz et al. (1999) have demonstrated that high concentrations of target bacteria will inhibit PCR reaction. The presence of large amounts of other bacteria has also been shown to inhibit the PCR (Rossen et al. 1992). Another reason for false-negative results is inhibitory factors present in food samples (Rossen et al. 1992; Lantz et al. 1994). An easy method for overcoming this problem has not yet been discovered (Scheu et al. 1998). We used a fairly simple sample preparation including a pre-enrichment step, two centrifugation steps and a proteinase-K treatment step according to Kapperud et al. (1993). A dilution step after the enrichment step may have decreased the number of false-negative results. In addition, it would be beneficial to include an internal standard for monitoring possible false-negative results caused by inhibitory substances in the food sample (Thisted Lambertz et al. 1998). The possibility that the plasmid may be lost occasionally during the overnight enrichment step can not be excluded. However, Kapperud et al. (1993) have shown that no false-negative results were obtained after overnight enrichment of food samples seeded with low numbers of plasmid-positive *Y. enterocolitica* cells.

The PCR method presented by Kapperud et al. (1993) was used in our studies because it was developed specifically for food samples and the sample preparation was sufficiently simple for examining larger numbers of food samples. The PCR method is a nested system. An important

advantage conferred by the two-step PCR detection procedure with two nested primer pairs is its high specificity (Kapperud et al. 1993). The four primers have been selected on the basis of sequence analysis of the *yadA* in *Yersinia* spp. (Skurnik and Wolf-Watz 1989), such that they only target the *yadA* of *Y. enterocolitica*. The nested PCR may also increase sensitivity, since the inhibitory substances are diluted during the second PCR step, where only 1/25 of the reaction mixture of the first step is transferred to the second reaction mixture. This method has been shown to be capable of detecting low numbers of pathogenic *Y. enterocolitica* also in water samples containing high numbers of background organisms (Waage et al. 1999). The virulence plasmid has been chosen as the method's target because it is required by *Y. enterocolitica* to induce disease (Cornelis et al. 1998).

6.4. Isolation of *yadA*-positive *Y. enterocolitica* from samples of pig origin and from the pig slaughterhouse environment (I-III)

The highest prevalence of *yadA*-positive *Y. enterocolitica* was obtained after selective enrichment. Even after selective enrichment, pathogenic isolates were overgrown by other bacteria on selective agar plates, especially when minced meat samples were examined. Ineffective isolation methods are the most important reason for low prevalence rates of pathogenic *Y. enterocolitica* in foods. In addition, the isolation of the bacterium from food and environmental samples is time-consuming. Thus, more rapid and sensitive isolation methods are needed.

All *Y. enterocolitica* isolates were biotyped and belonged either to biotype 1A or 4. All biotype 4 isolates were of serotype O:3, which is the most common type found in human infections world-wide (Bottone 1999). Other serotypes, such as O:5,27 and O:9, have sporadically been isolated from humans in Finland (Kontiainen et al. 1994). However, pigs do not appear to be the reservoir for these types in Finland.

The pathogenicity of *Y. enterocolitica* isolates was studied with PCR targeting the *yadA*. This method proved to be a rapid and convenient method for confirming pathogenicity. Due to the instability of the virulence plasmid at 37°C, the isolates were not exposed to temperatures above 30°C to avoid the possibility of losing the plasmid. Most of the isolates harboured the virulence plasmid, giving a positive result with PCR. However, the isolation rate of pathogenic *Y. enterocolitica* would have been overestimated if the pathogenicity of the isolates was not confirmed. In study I, the isolation rate of all bioserotype 4/O:3 isolates and only *yadA*-positive

isolates in pig tonsils was 33% and 26%, respectively. Prevalence has reported to decrease from 26 to 11% and from 32 to 15% in Finland and Norway, respectively, after pathogenicity of tonsil strains was verified using an autoagglutination test (Nesbakken and Kapperud 1985; Merilahti-Palo et al. 1991). The isolation rate of bioserotype 4/O:3 in pig tongues was 82% when both *yadA*-positive and *yadA*-negative isolates were counted, and 78% when only *yadA*-positive isolates were counted (III). In Norway, the prevalence of pathogenic *Y. enterocolitica* 4/O:3 in tongues decreased from 55% to 11% when only isolates with positive autoagglutination reaction were counted. Overall, comparison of different prevalence studies is difficult, particularly if pathogenicity is not confirmed.

6.5. Genotyping of *Y. enterocolitica* 4/O:3 (I, II, IV, V)

A wide variety of DNA-based techniques have been used in the study of the molecular epidemiology of *Y. enterocolitica*, with PFGE being one of the most suitable techniques for subtyping *Y. enterocolitica* 4/O:3 isolates. *Not*I and *Xba*I enzymes were used in our studies (I, II), since these enzymes have given good resolution for all *Y. enterocolitica* serotypes tested (Iteman et al. 1991; Buchrieser et al. 1994; Najdenski et al. 1994; Saken et al. 1994). The isolates were subdivided into several *Not*I and *Xba*I profiles, with the majority belonging to two predominant genotypes. Most of the PFGE patterns displayed only minor deviations, indicating a rather limited genetic variation among *Y. enterocolitica* 4/O:3 isolates, in accordance with Buchrieser et al. (1994).

Altogether 35 enzymes were tested in order to select endonucleases capable of subdividing the isolates belonging to the predominating *Not*I profiles (IV). We also tried to find an enzyme that would produce a smaller number of resolvable macrorestriction fragments suitable for isolate comparison. No ideal rare-cutting enzyme was found. *Apa*I, *Xho*I, *Xba*I and *Spe*I enzymes, producing the best banding patterns without smearing or partial digestion, were used for further testing. While these enzymes had the best resolution, the number of small fragments was high and many fragments were very closely spaced together, occasionally making interpretation challenging. *Apa*I and *Xho*I enzymes were shown to be the most efficient in differentiating between isolates with identical *Not*I profiles, thus increasing the discriminatory power of the PFGE method (IV).

All isolates of bioserotype 4/O:3 were easily typed with *Not*I, *Apa*I and *Xho*I enzymes, and no variations in PFGE patterns from the same isolate could be detected with these enzymes (IV, V).

The isolates were considered different when even a one-band difference was noted, because the genetic variation within *Y. enterocolitica* 4/O:3 has been demonstrated to be limited (Buchrieser et al. 1994; Saken at al. 1994; Asplund et al. 1998), the genotypes have shown to be stable *in vitro* (Najdenski et al. 1994), and the same band difference rule has been used to differentiate *Y. enterocolitica* isolates in previous PFGE studies (Buchrieser et al. 1994; Najdenski et al. 1994; Saken at al. 1994; Iteman et al. 1996; Asplund et al. 1998). The plasmid did not interfere with the PFGE patterns produced when only fragments exceeding 70 kb were used for isolate discrimination. However, a fragment of about 40 kb was observed in the PFGE patterns of *yadA*-positive isolates, but not in the PFGE patterns of *yadA*-negative isolates produced by *Not*I enzyme. Najdenski et al. (1994) have demonstrated that *Not*I cleaves the pYV into three fragments, the largest of which is about 48 kb. *Not*I enzyme was used as a screening enzyme because the banding patterns were clear with no smearing or partial digestion. In addition, the presence of the virulence plasmid could easily be confirmed from the *Not*I patterns, and no other enzyme that would produce a smaller number of resolvable macrorestriction fragments was found. It was also the enzyme that has been used in all previous studies (Iteman et al. 1991; Buchrieser et al. 1994; Najdenski et al. 1994; Saken et al. 1994; Iteman et al. 1996; Asplund et al. 1998).

6.6. Distribution of different genotypes of *Y. enterocolitica* 4/O:3 strains in pig slaughterhouses (I, II, V)

The most common genotypes, NA1/XA1 and NB1/XB1, which were found in pig tonsils, were widely distributed amongst the slaughterhouses and may have been derived from a common source (I). However, in each slaughterhouse, different genotypes were recognised and no predominant genotype was found in most of the slaughterhouses (I). The two prevailing genotypes found in pig tonsils were also found on pig offals (livers, kidneys, hearts, ears) and carcasses, and in the slaughterhouse environment, indicating that the tonsils are an important contamination source in the pig slaughterhouse (II). The spread of this bacterium from the tonsils to the liver, heart and kidneys is unavoidable when the tonsils are removed in conjunction with the pluck set (heart, lungs, oesophagus, trachea, diaphragm, liver, kidneys and tongue) and then hung on a hook. Several genotypes obtained from the tonsils were shown to be indistinguishable from the genotypes found in tongues (V). The tongue is easily contaminated when it hangs together with the tonsils.

The NA1/XA1 genotype was also found on carcasses, the hook from which the pluck set hung, the brisket saw and the knife used for evisceration. The highly contaminated tonsils will spread

pathogenic yersinia to carcasses, tools and machines during the slaughter process. Identical genotypes were found on the computer keyboard in the meat-inspection area and on livers and kidneys, which reinforces the assumption that meat inspectors spread pathogenic yersinia from offals with their hands. Two different genotypes were found in the air, and the same types were also recovered from other sites in the slaughterhouse, indicating that airborne contamination may occur during slaughter.

In study V, 61 (64%) out of 95 strains found in pig slaughterhouses from carcasses, offals and the environment were indistinguishable from the tonsil strains when characterised with *Not*I, *Apa*I and *Xho*I enzymes, lending further support to our hypothesis that tonsils are the main source of contamination in pig slaughterhouses. The isolation rate of *Y. enterocolitica* has been shown to be higher in tonsillary than in faecal samples (de Boer and Nouws 1991; Thibodeau et al. 1999). Likely reasons for this are that infected pigs carry *Y. enterocolitica* for a longer time (Nielsen et al. 1996, Thibodeau et al. 1999) and the number of *Y. enterocolitica* isolates is higher (Shiozawa et al. 1991) in tonsils than in faeces. However, faeces is another important contamination source of pathogenic *Y. enterocolitica* in pig slaughterhouses. Pathogenic yersinia will spread from the intestines to the carcass, mainly during the loosening of the rectum. The spread of *Y. enterocolitica* 4/O:3 from faeces to carcasses can be considerably reduced by sealing off the rectum with a plastic bag immediately after it has been freed (Andersen 1988, Nesbakken et al. 1994). This method is commonly used in Denmark, Norway and Sweden. (Borch et al. 1996).

Pathogenic yersinia will easily spread from contaminated tonsils and faeces to other sources. The meat-inspection procedure will not necessarily reveal the presence of *Y. enterocolitica* since this infection is mostly present without any signs of illness or apparent macroscopic lesions. Swine slaughter, being an open process, offers many opportunities for cross-contamination. The only way to prevent the spread of pathogenic yersinia from pig tonsils to other sources is to modify the European Union legislation (64/433/EEC), to mandate that the head, containing the tonsils and tongue be removed prior to evisceration and that inspection of the head, tonsils and tongue occur in a separate room. Moreover, to decrease the spread of pathogenic yersinia via faeces, the plastic bag technique is recommended.

6.7. Sources of sporadic *Y. enterocolitica* 4/O:3 infections (V)

The main source of sporadic human *Y. enterocolitica* 4/O:3 infections in Finland seems to be the pig. A total of 80% of human strains were indistinguishable from strains of pig origin when 212 human strains isolated from *Y. enterocolitica* 4/O:3 infections were compared with 334 non-human strains. In all, 39 genotypes found in human infections were found in different sources of pig origin. Pig offals contaminated with *Y. enterocolitica* 4/O:3 were revealed to be an important transmission vehicle of this bacterium from pigs to man, since 71% of the human strains were indistinguishable from strains isolated from pig tongues, livers, kidneys and hearts. Pathogenic *Y. enterocolitica* spreads from pig slaughterhouses to retail level via contaminated offals. Cross-contamination from offals to other sources occurs directly or indirectly via equipment and food handlers at retail shops and residential kitchens. As a psychrotrophic microbe, *Y. enterocolitica* is able to multiply along the cold-chain from the slaughterhouse to the home refrigerator.

Y. enterocolitica 4/O:3 infections have been associated with pork in case-control studies (Tauxe et al. 1987; Ostroff et al. 1994). Of strains found in sporadic infections, 35% and 31% were indistinguishable from strains recovered on pig carcasses and in pork, respectively, which strongly supports the association between yersiniosis and pork. Raw meat, particularly raw pork, is not eaten in Finland, except for occasional nibbling on raw minced pork while preparing pork dishes. Cross-contamination of cooked foods or foods not harbouring *Y. enterocolitica* is the more probable route of infection.

Pet animals may be another source of human infections, especially among young children. Two common genotypes found in human infections were found in a cat and a dog. These same genotypes were also present in pig tonsils, offals and pork. Pathogenic yersinia can be transmitted from pigs to dogs and cats via raw pork and offals (Fredriksson-Ahomaa et al. 2001a), which are often fed to pets, and then from pets to humans via contaminated faeces. Although transmission from pets to humans has yet to be proven, Fenwick et al. (1994) have shown that dogs can carry *Y. enterocolitica* bioserotype 4/O:3 asymptomatically and excrete this organism in the faeces for weeks.

7. CONCLUSIONS

1. The mean prevalence of *yadA*-positive *Y. enterocolitica* in pig tonsils was 37% when samples from 9 slaughterhouses located in various parts of Finland were examined with both the PCR and culture methods. In addition, 21% of pig carcasses were contaminated with *yadA*-positive *Y. enterocolitica*. This bacterium was detected on 38, 86 and 63% of livers, kidneys and hearts, respectively. This high prevalence on edible pig offals likely indicates extensive cross-contamination from tonsils to offals during slaughter. *Y. enterocolitica* carrying the *yadA* gene was detected from many different environmental sources in the pig slaughterhouse with PCR, confirming that the pig slaughterhouse environment is contaminated with pathogenic *Y. enterocolitica*. All *yadA*-positive isolates belonged to bioserotype 4/O:3.

2. The prevalence of *yadA*-positive *Y. enterocolitica* at retail level was studied in pig tongues and minced meat using both the PCR and culture methods. The prevalence of pathogenic *Y. enterocolitica* in pig tongues was high with both methods. One reason for the prevalence of 98% may be the slaughtering process, where the tongue is removed together with the tonsils. The prevalence of *yadA*-positive *Y. enterocolitica* in minced meat was moderately high (25%) with PCR. These results indicate that pig tongues and minced meat are important sources of pathogenic *Y. enterocolitica* at retail level. All *yadA*-positive isolates belonged to bioserotype 4/O:3.

3. The prevalence of pathogenic *Y. enterocolitica* was clearly higher with PCR than with the culture method, especially when minced meat samples were studied. In these samples, the isolation rate was particularly low because of the high background flora on CIN agar plates. Some tonsil and tongue samples were PCR-negative but culture-positive. False-negative results are a problem when food samples are studied because there is not yet any easy method for removing PCR-inhibitory substances from the sample. PCR proved to be a rapid, specific and convenient method to confirm pathogenicity of *Y. enterocolitica* 4/O:3 isolates.

4. The PFGE method was shown to be an efficient technique for characterisation of bioserotype 4/O:3 when isolates with the same PFGE pattern with *Not*I enzyme were further characterised with *Apa*I and *Xho*I. *Not*I was found to be a good screening enzyme, producing a clear banding pattern without smearing or partial digestion. The presence of the virulence plasmid is easily confirmed from the *Not*I patterns. *Y. enterocolitica* 4/O:3 isolates were divided into several

genotypes with *Not*I, but because of the two main types, isolates with identical *Not*I profiles were further characterised with *Apa*I and *Xho*I enzymes. All the ribotypes and most of the PFGE patterns displayed only minor deviations, indicating a limited genetic variation among the isolates.

5. Distribution of different genotypes of *Y. enterocolitica* 4/O:3 strains recovered from slaughterhouses was studied using PFGE. The most common genotypes obtained with *Not*I and *Xba*I enzymes, found in pig tonsils, were widely distributed amongst most of the slaughterhouses. The same genotypes were recovered from pig offals (livers, kidneys, hearts, ears), carcasses and the environment. When all strains isolated from slaughterhouses were further characterised with *Apa*I and *Xho*I enzymes, 64% of the strains isolated from carcasses, offals and the environment were indistinguishable from the tonsil strains, indicating that tonsils are a major contamination source in slaughterhouses.

6. The main source of sporadic human *Y. enterocolitica* 4/O:3 infections in Finland seems to be pigs. A total of 80% of 212 human strains were indistinguishable from strains of pig origin. Pig offals contaminated with pathogenic *Y. enterocolitica* 4/O:3 were revealed to be an important transmission vehicle of human sporadic infections since 71% of the human strains were indistinguishable from strains isolated from tongues, livers, kidneys and hearts. Pet animals may also serve as a source of human infections. Pathogenic yersinia can be transmitted from pigs to dogs and cats via raw pork and offals, and then from dogs and cats to humans via contaminated faeces.

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