DIAGNOSTICS AND EPIDEMIOLOGY OF *YERSINIA PSEUDOTUBERCULOSIS*

Taina Niskanen

Department of Food and Environmental Hygiene

Faculty of Veterinary Medicine

University of Helsinki, Finland

Helsinki 2010

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ACADEMIC DISSERTATION

Supervising Professor

Professor Hannu Korkeala, DVM, PhD, MSocSc
Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Supervised by

Professor Hannu Korkeala, DVM, PhD, MSocSc
Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Maria Fredriksson-Ahomaa, DVM, PhD
Institute of Food Hygiene and Technology
Faculty of Veterinary Medicine
Ludwig-Maximilians-Universität Munich
Germany

Reviewed by

Professor Dr. Lieven De Zutter
Faculty of Veterinary Medicine
Ghent University, Belgium

Professor Stan Fenwick, BVMS, PhD
Faculty of Health Sciences
Murdoch University, Australia

Opponent

Docent Kaisa Granfors, PhD
National Institute for Health and Welfare (THL)
Turku, Finland

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Acknowledgements

I am indebted to both of supervisors. I am deeply grateful to Professor Hannu Korkeala, Head of the Department of Food and Environmental Hygiene, for the opportunity to conduct my research in the inspiring atmosphere the department. Thank you for motivation, guidance and constructive criticism during this project. Maria Fredriksson-Ahomaa, DVM, PhD, is warmly thanked for generous help, practical advice and a tireless and positive attitude.

I thank Professor Mikael Skurnik, Head of the *Yersinia* Research Laboratory of the Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, for the honour of working in his research group and for his kind guidance in the world of microbiology and *yersinia*.

Professor Dr. Lieven De Zutter and Professor Stan Fenwick are acknowledged for reviewing the thesis and Carol Ann Pelli for editing the language of my manuscripts and summary.

A special thanks is owed to Professor Johanna Björkroth for enthusiastic support and advice, fruitful discussions and lasting friendship. Warm thanks are due to all my co-authors in original publications, especially Saija Hallanvuo, Maija Hatakka, Riikka Laukkanen, Anna Murros, Björn Olsen, Anja Siitonen and Jonas Waldenström, for their invaluable contributions. The excellent technical staff of the deparment, especially Sirkku Ekström, Raija Keijama, Maria Stark, Kirsi Ristkari, Heimo Tasanen, and our secretary Johanna Seppälä is acknowledged.

I owe my sincere thanks to the many people who have helped me during this project. I am particularly grateful to my colleagues and fellow researchers at the Department of Food and Environmental Hygiene and at the *Yersinia* Research Laboratory. Thank you all for your help and for sharing your knowledge and bringing joy into the working days. I have been privileged to work with exceptional and unforgettable people.

Finally, my heartfelt thanks are owed to my friends, relatives and family. I am grateful to my brother Markku, sister-in-law Katri and their wonderful children Ilpo and Vilma, and my mother Kaisu and father Tarmo for their support during my studies and their steadfast belief that I will finish this project.

Abbreviations

Ail	attachment invasion locus
ATCC	American type culture collection
CCUG	culture collection, University of Göteborg, Sweden
cfu	colony forming unit
CIN	cefsulodin-irgasan-novobiocin
CIP	culture collection of Institute Pasteur, France
CR-MOX	Congo red-magnesium oxalate agar
DSM	German Collection of Microorganisms and Cell Culture
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESP	EDTA-sodium lauroyl sarcosine buffer with proteinase
ERIC	enterobacterial repetitive intergenic consensus sequences
EVI	Elintarvikevirasto, Finnish Food Authority; name changed 1.5.2006 to Finnish Food Safety Authority, Evira
HPI	high-pathogenicity island
Inv	invasin
IS	insertion sequence
kDa	kiloDalton = 1.660×10^{-21} g
KOH	potassium hydroxide
KTL	Kansanterveyslaitos, National Public Health Institute (NPHI); name changed 1.1.2009 to National Institute of Health and Welfare (THL)
LcrV	V antigen
LPS	lipopolysaccharide
MAC	MacConkey
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MRB	modified Rappaport broth
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pH6-Ag	pH6 antigen
PIV	tris-NaCl buffer
PMB	buffered peptone saline supplemented with mannitol and bile salts
PsaA	*Y. pseudotuberculosis* adhesin
pYV	plasmid for *Yersinia* virulence
PYZ	pyrazinamidase
RAPD	random amplified polymorphic DNA

REAP	restriction endonuclease analysis of virulence plasmids
RovA	regulator of virulence A
SMAC	sorbitol-MacConkey
TBE	tris-Borate with EDTA
TE	tris-EDTA
TSB	trypticase soya broth
T3SS	Type III secretion system
UPGMA	unweighted pair-group method with arithmetic averages
VirF/LcrF	virulence regulon transcriptional activator
YadA	*Yersinia* adhesin A
Yops	*Yersinia* outer membrane proteins
YPM	*Y. pseudotuberculosis*-derived mitogen
Ysc	Yop secretion system

Abstract

The sources and vehicles of *Yersinia pseudotuberculosis* infections remain obscure. No specific foods have been reported as a source of human yersiniosis, but indirect evidence has indicated that *Y. pseudotuberculosis* is a potential foodborne pathogen. The aim of this project was to investigate pigs, wild birds, and the environment as possible reservoirs of *Y. pseudotuberculosis* and to examine a *Y. pseudotuberculosis* O:3 outbreak and its source. The isolates were characterized with phenotypic and genotypic methods to obtain further information on the epidemiology of *Y. pseudotuberculosis*.

Isolation of low numbers of *Y. pseudotuberculosis* can be difficult in clinically healthy animals and in food and environmental samples. When different culture methods, including direct plating, overnight enrichment, selective enrichment and cold enrichment with and without alkali treatment, were compared in their ability to isolate *Y. pseudotuberculosis*, the most productive isolation method was cold enrichment. Using two weeks' cold enrichment with alkali treatment, the highest isolation rate was obtained from all samples of animal, food and environmental origin.

The prevalence of *virF*-positive *Y. pseudotuberculosis* was investigated in tonsils of pigs at slaughter. The mean prevalence among fattening pigs and sows was 4% and 0, respectively. To evaluate the distribution of *virF*-positive *Y. pseudotuberculosis* in pigs at farm level, the positive animals were traced back to their rearing farm. The prevalence of *virF*-positive *Y. pseudotuberculosis* was investigated in feces of pigs of different ages and in the pig house environment. At positive farms, a high prevalence of *Y. pseudotuberculosis,* from 3% to 14%, in pooled fecal samples was recovered. All *Y. pseudotuberculosis*-positive findings at both slaughterhouses and farms were from fattening pigs only; piglets, sows and boars were negative. One pig pen floor sample was also *Y. pseudotuberculosis*-positive. All *Y. pseudotuberculosis* isolates belonged to bioserotype 2/O:3.

Migratory birds representing 57 different bird species were studied for *virF*-positive *Y. pseudotuberculosis* during spring and autumn migration. *virF*-positive *Y. pseudotuberculosis* of bioserotype 1/O:2 was recovered from two species of thrushes during spring migration. The mean prevalence of *virF*-positive *Y. pseudotuberculosis* in wild birds was 1%.

Y. pseudotuberculosis O:3 was confirmed to be the cause of a widespread foodborne outbreak through domestically produced iceberg lettuce. Environmental sampling was carried out on lettuce farms implicated by trace-back investigation. Farms were inspected for sanitary conditions and water quality. *virF*-positive *Y. pseudotuberculosis* bioserotype 1/O:2 was found in iceberg lettuce. Although the exact mechanism of contamination of lettuce remained unknown, several factors predisposing to *Y. pseudotuberculosis* contamination of lettuce were found on the farms, including a large population of roe

deers, a potential reservoir of *Y. pseudotuberculosis*, and free access of wild animals to unfenced lettuce fields and to irrigation water sources.

Pulsed-field gel electrophoresis (PFGE) is an efficient method for subtyping of *Y. pseudotuberculosis* isolates of different serotypes and origin, but also within the same serotype. *Y. pseudotuberculosis* isolates of different sources showed high genetic diversity, while *Y. pseudotuberculosis* isolates of bioserotype 2/O:3 found in pigs showed limited genetic diversity. PFGE was also used to compare *Y. pseudotuberculosis* isolates from patients and from food and environmental sources in the outbreak investigation.

Identification of environmental *Y. pseudotuberculosis* isolates was found to be incorrect based solely on biochemical tests. When an isolate identified as *Y. pseudotuberculosis* by API 20E cannot be serotyped to known *Y. pseudotuberculosis* serotypes, the species identification and pathogenicity potential of the isolate should be determined. Polymerase chain reaction (PCR) targeting specific genes, such as the *inv* gene in the chromosome and the *virF* gene on the virulence plasmid of *Y. pseudotuberculosis,* is one rapid method for molecular identification and characterization of isolates.

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I   Niskanen T, M Fredriksson-Ahomaa and H Korkeala, 2002. *Yersinia pseudotuberculosis* with limited genetic diversity is a common finding in tonsils of fattening pigs. J Food Prot. 65: 540-545.

II   Niskanen T, R Laukkanen, M Fredriksson-Ahomaa and H Korkeala, 2008. Distribution of *virF/lcrF*-positive *Yersinia pseudotuberculosis* serotype O:3 at farm level. Zoonoses Public Health 55: 214-221.

III   Niskanen T, J Waldenström, M Fredriksson-Ahomaa, B Olsen and H Korkeala, 2003. *VirF*-positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* found in migratory birds in Sweden. Appl Environ Microbiol. 69: 4670-4675.

IV   Nuorti JP, T Niskanen, S Hallanvuo, J Mikkola, E Kela, M Hatakka, M Fredriksson-Ahomaa, O Lyytikäinen, A Siitonen, H Korkeala and P Ruutu, 2004. A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. J Infect Dis. 189: 766-774.

V   Niskanen T, R Laukkanen, A Murros, J Björkroth, M Skurnik, H Korkeala and M Fredriksson-Ahomaa, 2009. Characterisation of non-pathogenic *Yersinia pseudotuberculosis*-like strains isolated from food and environmental samples. Int J Food Microbiol. 129: 150-156.

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1. Introduction

Yersinia pseudotuberculosis was first isolated over 120 years ago, in 1883, from tuberculosis-like (pseudotuberculosis) lesions in guinea pigs (Malassez and Vignal 1884). The bacterium received several names, including *Bacterium pseudotuberculosis rodentium,* Bacillus *pseudotuberculosis, Pasteurella pseudotuberculosis* and *Shigella pseudotuberculosis* (Mollaret 1965). The current name, *Y. pseudotuberculosis*, was established in 1974, when the bacterium was placed in the genus *Yersinia. Y. pseudotuberculosis* was the first species identified in this genus (Mollaret and Thal 1974), named for the French bacteriologist A.J.E. Yersin, the discoverer of the plague bacillus *Y. pestis.*

The genus *Yersinia* comprises three pathogenic species: *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* (Brubaker 1991). *Y. pseudotuberculosis* has mostly been considered as an animal pathogen causing infections in humans only occasionally. The host range is broad, including both wild and domestic animals. However, in culture-based detection surveys, *Y. pseudotuberculosis* has been isolated at low isolation rates from different animal and environmental sources. Its principal reservoir hosts are considered to be wild animals, especially rodents, and birds. Among domestic animals, clinically healthy pigs have sporadically been found to carry *Y. pseudotuberculosis*. In Finland, the occurrence of *Y. pseudotuberculosis* in wild and domestic animals or in the environment remains obscure.

Y. pseudotuberculosis has been presumed to be a possible foodborne pathogen, but the evidence for this is limited. The bacterium has seldom been isolated from foods. Most of the reported human infections have been sporadic, where the source of infection is difficult to trace. In a few large reported outbreaks in Canada, Finland, Japan and Russia, the source and origin of the infections have also gone undetermined. At the beginning of 2000, *Y. pseudotuberculosis* emerged as an outbreak-associated pathogen in Finland. More information about the reservoirs and transmission routes of this pathogen is required.

Difficulties associated with isolation of *Y. pseudotuberculosis* from naturally contaminated samples are the low number of *Y. pseudotuberculosis* present and the abundant background flora. More efficient culture methods are needed to isolate low levels of *Y. pseudotuberculosis* in food and environmental samples and in asymptomatic animal carriers. Identification of *Y. pseudotuberculosis* requires several biochemical tests and can be unreliable and time-consuming. The majority of *Y. pseudotuberculosis* strains are considered pathogenic, but the degree of pathogenicity varies (Fukushima et al. 2001). A rapid method for identification and confirmation of pathogenity of isolates is PCR targeting specific genes of the bacteria. The most commonly used target to confirm pathogenicity of *Y. pseudotuberculosis* isolates is *virF* on the virulence plasmid (Wren and Tabaqchali 1990).

2. Review of the literature

2.1. *Yersinia pseudotuberculosis*

2.1.1. Classification and characteristics

Y. pseudotuberculosis is a Gram-negative rod of diameter 0.5-0.8 μm and length 1-3 μm. The genus *Yersinia*, a member of the family *Enterobacteriaceae*, is presently composed of 14 species (Bercovieri and Mollaret 1984, Aleksic et al. 1987, Wauters et al. 1988, Sprague and Neubauer 2005, Merhej et al. 2008, Sprague et al. 2008). All *Yersinia* species are catalase-positive, oxidase-negative, facultatively anaerobic and non-spore-forming bacteria (Bercovieri and Mollaret 1984). The optimum growth temperature of *Yersinia* is 28-29°C, but they can grow at temperatures range from 4°C to 42°C (Bottone et al. 2005). As a psychotrophic organism some *Yersinia* strains can grow at temperatures as low as -5°C, although growth is very slow below 0°C (Bergann et al. 1995).

Three *Yersinia* species are pathogenic and can cause disease in humans and animals: *Y. pestis* and enteropathogenic *Y. pseudotuberculosis* and *Y. enterocolitica* (Brubaker 1991). *Y. pestis*, a causative agent of plague, is a clone that evolved from *Y. pseudotuberculosis* O:1b ca. 1 500-40 000 years ago (Achtman et al. 1999, Skurnik et al. 2009). These two species are genetically almost identical, showing 97% homology in genome sequences (Bercovier et al. 1980, Chain et al. 2004). However, they have profoundly different ecology and epidemiology. *Y. pestis* has a fleaborne transmission through the skin, while *Y. pseudotuberculosis* and *Y. enterocolitica* are food- and waterborne pathogens causing gastroenteritis in humans and animals by oral route (Cornelis 1998).

All *Yersinia* species, except *Y. pestis,* are urease-positive. *Y. pseudotuberculosis* can be differentiated from other urease-positive *Yersinia*, especially from enteropathogenic *Y. enterocolitica* on the basis of the Voges-Proskauer test and differing ability to ferment sorbitol, rhamnose, sucrose and melibiose. Unlike *Y. enterocolitica* strains, all *Y. pseudotuberculosis* strains are sucrose and sorbitol negative. *Y. pseudotuberculosis* gives a negative reaction in the Voges-Proskauer test independent of incubation temperature. *Y. pseudotuberculosis* is non-motile when incubated at 37°C, but motile with peritrichous flagella when grown below 30°C (Bercovieri and Mollaret 1984).

Y. pseudotuberculosis can be divided into 4 biotypes based on biochemical reactions (Tsubokura and Aleksic 1995), 15 O-serotypes (O:1-O:15) and 10 subtypes (O:1a-O:1c, O:2a-O:2c, O:4a-O:4b, O:5a-O:5b) based on variability in lipopolysaccharide (LPS) O-antigen profiles (Skurnik et al. 2000). These serotypes differ in their geographical distributions. All *Y. pseudotuberculosis* strains are considered potentially pathogenic.

However, differences are present in the virulence properties, which depend on several virulence factors. Serotypes O:1 and O:3 dominate in Europe, and O:4 and O:5 in the Far East (Fukushima et al. 2001). Based on comparison of *Y. pseudotuberculosis* serotypes and restriction endonuclease analysis of virulence plasmid (REAP), a geographical borderline of distribution of different *Y. pseudotuberculosis* clones may exist between Europe and Asia (Fukushima 1998). The main clinical manifestations of *Y. pseudotuberculosis* infections in the Far East are usually more diverse and severe than those in Europe (Yoshino et al. 1995, Fukushima et al. 1998). Serotypes O:6-O:14 have been isolated only from non-human sources, i.e. from animals and the environment, not from human infections (Fukushima et al. 1998, Fukushima et al. 2001, Skurnik et al. 2004).

2.1.2. Pathogenicity

Several virulence factors expressed both in the chromosome and on the plasmid of *Y. pseudotuberculosis* are needed for full virulence during infection (Table 1).

Table 1. Main chromosomally and plasmid-encoded virulence factors of *Yersinia pseudotuberculosis*.

Factor	Function	Reference
Chromosomal virulence factors		
Ail (attachment and invasion locus)	Serum resistance	Miller and Falkow 1988, Bliska and Falkow 1992
Inv (invasin)	Attachment and invasion, colonization of lymph nodes	Isberg and Leong 1990, Marra and Isberg 1997
HPI (high-pathogenicity island) production of Yersiniabactin (Ybt)	Iron capture	Carniel 2001
LPS (lipopolysaccharide) with lipid A and O-antigen (O side chain)	Colonization of host tissue, reduced biological activity, resistance to antimicrobial peptides	Porat et al. 1995, Skurnik et al. 1999, Mecsas et al. 2001
PsaA (pH6 antigen)	Fimbrial adhesins, antiphagocytic activity	Isberg 1989, Yang et al. 1996

Table 1. Continued.

Factor	Function	Reference
YPM (*Y. pseudotuberculosis* - derived mitogen)	Superantigenic toxins activation of T-lymphocytes	Abe et al. 1993 Uchiyma et al. 1993
Plasmid-encoded virulence factors		
pYV (plasmid for *Yersinia* virulence)	Survival and multiplication in lymphoid tissues	Gemski et al. 1980
YadA (*Yersinia* adhesin A)	Autoagglutination , adhesion to epithelial cells, invasion of host cells, binding to fibronectin, haemagglutination of erythrocytes	Skurnik et al. 1984, Kapperund et al. 1987, Yang and Isberg 1993, El Tahir and Skurnik 2001, Eitel and Dersch 2002, Heise and Dersch 2006
Yops (*Yersinia* outer membrane proteins)	Antiphagocytic activity, apoptosis, increase of virulence	Cornelis 1998, 2002, Navarro et al. 2005

Chromosomally encoded virulence factors of Y. pseudotuberculosis

The virulence factors expressed in the chromosome are affecting such functions as adhesion, invasion, serum resistance, iron uptake and superantigenic toxin production.

The *inv* encodes invasion (Inv), a 103-kDa outer membrane protein required for *Y. pseudotuberculosis* adherence to and invasion of intestinal epithelium cells (Isberg and Falkow 1985, Isberg et al. 1987, Marra and Isberg 1997). Inv has been shown to bind to host cell β_1 integrins, which are expressed on the surface of the M-cells of Peyer's patches on the intestine (Isberg and Leong 1990). This mechanism is important especially during the early stages of infection (Isberg and Leong 1990, Clark et al. 1998). For *Y. pseudotuberculosis* grown at ambient temperature, Inv is the primary invasive factor and contributes to adhesion of bacteria to cultured mammalian cells. Inv expression is regulated in response to a variety of environmental signals, such as temperature, growth phase, nutrients, osmolarity and pH. RovA, a transcriptional regulator is required for *inv* expression in *Y. pseudotuberculosis* (Nagel et al. 2001). Expression occurs maximally in stationary-phase bacteria, at 20-25°C and when pH is 7–8.5. At pH below 7 and at higher temperatures, the expression is reduced (Nagel et al. 2001).

Ail is a 17-kDa outer membrane protein encoded by *ail* in the chromosome of *Y. pseudotuberculosis*. Ail mediates more host-specific attachment of Y*ersinia* to eukaryotic

cell cultures than Inv (Miller and Falkow 1988). The functional Ail expressed by *Y. pseudotuberculosis* has, however, no adhesion or invasion activity on culture cells (Yang et al. 1996). Ail of *Y. pseudotuberculosis* has the capacity to promote resistance to complement-mediated killing (Bliska and Falkow 1992).

Y. pseudotuberculosis adhesin PsaA or pH6 antigen (pH6-Ag) is a chromosomally encoded thermo-inducible adhesion factor produced only at temperatures above 36°C (Isberg 1989). The cluster of *psa* genes is required for the synthesis of pH6 antigen, a surface polymer with fimbria-like structures composed of Psa. The PsaA is expressed at the highest levels during growth at 37° and at a pH below 6 (Isberg 1989). It has binding activity to cultured mammalian cells in *Y. pseudotuberculosis* strains lacking the other adhesion factors. The PsaA also promotes agglutination of erythrocytes (Yang et al. 1996).

Differences between low-pathogenicity and high-pathogenicity strains of *Y. pseudotuberculosis* depend on the presence of the high-pathogenicity island (HPI) and *Yersinia pseudotuberculosis*-derived mitogen (YPM) in the chromosome (Fukushima et al. 2001). HPI is a large 36-kb chromosomal DNA fragment found in highly pathogenic *Y. pseudotuberculosis* strains. HPI carries the virulence genes involved in siderophore-mediated iron acquisition, i.e. the yersiniabactin biosynthetic gene cluster. Yersiniabactin-mediated iron uptake is thought to be the main function of the HPI (Carniel 2001). Presence and size of the HPI are correlated with serotype. Complete HPI is present only in *Y. pseudotuberculosis* serotype O:1 and in some clinical strains of serotypes O:3 and O:5 in Europe and in the Far East, respectively. Most *Y. pseudotuberculosis* strains of serotypes O:3 in Europe have an incomplete HPI (truncated R-HPI) (Fukushima et al. 2001). HPI has been demonstrated to horizontally transfer between *Y. pseudotuberculosis* serotype O:1 strains, but not to *Y. pseudotuberculosis* strains of serotypes O:2 and O:4. Transfer of HPI was observable only at low temperatures, most efficiently at 4°C when iron and nutrient availability was limited (Lesic and Carniel 2005). Iron uptake is an essential requirement for all bacterial growth and dissemination. Presence of the HPI and yersiniabactin production has been shown to correlate with the level of virulence of the *Yersinae* (Carniel et al. 1987, Fukushima et al. 2001).

Y. pseudotuberculosis produces a superantigenic toxin termed YPM (Abe et al. 1993, Miyoshi-Akiyama et al. 1995). YPM is supposed to be a major pathogenic exotoxin in systemic *Y. pseudotuberculosis* infection. Three YMP variants (YPMa, YPMb and YPMc) have been detected, encoded by *ypmA, ypmB* and *ypmC* genes in the chromosome, respectively (Ramamurthy et al. 1997, Carnoy and Simonet 1999). The variant YPMa has been confirmed experimentally to have toxicity properties in systemic but not in gastroenteric infection in mice (Carnoy et al. 2000). YMPa selectively activates human T-cells (Ramamurthy et al. 1997, Carnoy and Simonet 1999). YMPa mediates activation of the immune system and clinical symptoms of Kawasaki disease and Izumi fever (Uchiyma et al. 1993, Fukushima et al. 2001). The *ypmA* is absent in strains belonging to serotypes

O:1a, O:1b, O:2b and O:3 from Europe, but is present in almost all clinical strains belonging to serotypes O:1b, O:2, O:3, O:4 and O:5 from the Far East (Yoshino et al. 1995, Fukushima et al. 2001). While YPMb has never been detected in clinical *Y. pseudotuberculosis* strains, it has been found in isolates from animals and the environment in Japan. YPMc has been observed in some clinical strains of serotype O:3 in Europe and Japan, and thus, has been classified as a European low-pathogenicity type (Fukushima et al. 2001).

Lipopolysaccharide (LPS) is the major component of the outer membrane of *Y. pseudotuberculosis*. It has three main structural components: lipid A, oligosaccharide core and O-antigen polysaccharide. The lipid A and O-antigen parts play a role in virulence of *Y. pseudotuberculosis;* the role of the core region remains unknown. The lipid A biosynthesis genes of *Y. pseudotuberculosis* are scattered around the genome, whereas the O-antigen biosynthesis genes form a distinct cluster between the *hemH* and *gsk* genes (Skurnik 1999). The number of genes varies in each serotype of *Y. pseudotuberculosis* (Bogdanovich et al. 2003). Lipid A is believed to be an endotoxin, with a role in sepsis and septic shock (Brubaker 1972). O-antigen is required for colonization of host tissues in the early stage of infection (Skurnik et al. 1999). Strains not producing O-antigen (rough strains) were unable to colonize Peyer's patches or efficiently invade epithelial cells (Mecsas et al. 2001). Complete LPS has an important role in resistance against cationic antimicrobial peptides (Skurnik et al. 1999). The LPS of *Y. pseudotuberculosis* participates in the resistance of complement-mediated lysis at 37°C (Porat et al. 1995). The O-antigen of *Y. pseudotuberculosis* is highly variable and forms the basis of serotyping of the strains (Tsubokura and Aleksic 1995, Bogdanovich et al. 2003).

Plasmid-encoded virulence factors

The 70-kb virulence plasmid termed pYV (plasmid for *Yersinia* virulence) is found in all pathogenic *Yersinia* spp. and is essential for full virulence of *Y. pseudotuberculosis* (Gemski et al. 1980). The virulence plasmid encodes several virulence factors, e.g. YadA (*Yersinia* adhesion A), an adhesion protein and Yops (*Yersinia* outer membrane proteins), enabling *Y. pseudotuberculosis* to survive and multiply in lymphoid tissues (Cornelis 1998, Navarro 2005, Heisel and Dersch 2006) (Table 1).

The YadA, encoded by *yadA*, is a major outer membrane protein associated with binding of *Y. pseudotuberculosis* (Yang and Isberg 1993). It promotes tight adhesion to and high-efficiency uptake into human cells, independently of invasin (Eitel and Dersch 2002). Like Inv, YadA binds to β1 integrins in host cells (Heisel and Dersch 2006). YadA production is regulated by temperature and bacterial growth phase and is expressed only at 37°C (Kapperud et al. 1985, Eitel and Dersch 2002). Relative to the other two adhesins of *Y. pseudotuberculosis* (pH6-Ag and Inv) encoded by the chromosome, transcriptional

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activation of yadA was highest under plasma growth conditions (Rosso et al. 2008). The YadA protein of *Y. pseudotuberculosis* is a multifunctional virulence factor causing bacterial aggregation and inducing agglutination of erythrocytes (Skurnik et al. 1984, Kapperud et al. 1987, El Tahir and Skurnik 2001) (Table 1).

The pYV encodes the secretion of *Yersinia* outer membrane proteins (Yops) and the *Yersinia* type III secretion system (T3SS) apparatus termed Ysc (the Ysc-Yop TTS system) (Cornelis 2002). The Yops, encoded by *yop* genes, are involved in preventing phagocytosis, promoting cytotoxicity and activating the immune response to establish infection. The following five Yops are detected in all pathogenic *Yersinia* spp.; YopE, YopH, YopM, YopO/YpkA and YopP/YopJ, and are intracellular effectors (Navarro et al. 2005). The Ysc translocation apparatus at the bacterial surface is composed of 25 proteins (Yops and LcrV). LcrV also termed V antigen is distinguished historically from the other Yops (Brubaker 1991b). The proper operation of the secretion system also requires small individual chaperones called Syc proteins. In contact with eukaryotic cells, the Ysc apparatus translocates the Yop-effector proteins directly into the target cells across the plasma membrane (Cornelis 2002).

Growth of plasmid-carrying *Y. pseudotuberculosis* strains is temperature- and calcium-regulated; this is also referred to as a low calcium response (Cornelis et al. 1998). At a low calcium concentration and a temperature of 37°C, growth of plasmid-bearing strains is restricted and production of Yops is increased. Expression of the Yops virulon system (Cornelis et al. 1989) and YadA (Skurnik and Toivanen 1992) is controlled by *virF* localized on the pYV. *virF* is a transcriptional activator of Yops and YadA. The Yop regulon is both temperature- and calcium-regulated, unlike the YadA regulon, which is only temperature-regulated (Skurnik and Toivanen 1992).

2.2. Isolation and identification of *Y. pseudotuberculosis*

2.2.1. Isolation

Isolation of *Y. pseudotuberculosis* is hindered by a high background flora of sample material, especially in food and environmental samples, which can easily overgrow the slow-growing *Y. pseudotuberculosis* bacteria present in lower numbers. No standardized reference methods are available for isolation of *Y. pseudotuberculosis* from food, environmental or clinical samples. Several enrichment and plating methods developed mostly for isolation of *Y. enterocolitica* have also been described as suitable for the recovery of *Y. pseudotuberculosis* in different samples. However, only limited information is available about the recovery of *Y. pseudotuberculosis* from environmental and food

samples. The presence of *Y. pseudotuberculosis* in low numbers in these samples makes the isolation demanding. The culture methods most commonly used for isolating *Y. pseudotuberculosis* include i) direct plating, ii) direct plating after potassium hydroxide (KOH) treatment (Fukushima 1985, Fukushima 1992, Fukushima et al. 1994a), iii) KOH treatment after 1-2 days' incubation at 26°C in phosphate-buffered saline (PBS) (Aulisio et al. 1980), iv) PBS incubated at cold temperatures (4°C) for 1-3 weeks (Oberhofer and Podgore 1980, Fukushima et al. 1984, Inoue et al. 1988b, Schiemann and Wauters 1992) and v) PBS incubated at cold temperatures for 1-3 weeks followed by KOH treatment (Aulisio et al. 1980, Schiemann and Wauters 1992). PBS supplemented with peptone and mannitol (Knisely et al. 1964, Fukushima et al. 1984) or with lysed blood (Fukushima et al. 1984, Inoue et al. 1988a) has proven useful for isolation of *Y. pseudotuberculosis.* To inhibit the growth of Gram-positive bacteria, bile salts have been added to the PBS broth (Mehlman et al. 1978). Selective enrichment broth, e.g. modified Rappaport broth (MRB) and irgasan-ticarcillin-potassium chlorate (ITC), developed for isolation of *Y. enterocolitica* O:3 is unsuitable for isolation of *Y. pseudotuberculosis* (Van Noyen et al. 1995, Laukkanen et al. 2008).

During clinical infections, *Y. pseudotuberculosis* often dominates in bacterial flora and may be isolated by direct plating (de Barcellos and de Castro, 1981, Fukushima et al. 1985b, Chiesa et al. 1993). In acute disease of animals, isolation of *Y. pseudotuberculosis* was more efficient when sampling was based on necropsy material rather than faecal material (Cork et al. 1999). *Yersinia* spp., including *Y. pseudotuberculosis,* is more tolerant of alkali conditions than most other bacteria. Post-enrichment alkali treatment with KOH has been used to selectively reduce the level of background flora, making selection of *Y. pseudotuberculosis* easier (Aulisio et al. 1980). Direct KOH treatment without prior enrichment of samples rendered the recovery of *Y. pseudotuberculosis* in artificially contaminated pork and water samples faster, thus being a promising alternative when the numbers of *Y. pseudotuberculosis* are sufficiently high and the background flora is low (Fukushima 1985, Fukushima 1987, Fukushima 1992, Fukushima et al. 1995).

As a psychrotrophic bacterium, *Y. pseudotuberculosis* multiplies in cold temperatures. Cold enrichment is a method frequently employed to isolate *Y. pseudotuberculosis* from clinically healthy animals and environmental and food samples (Bisset 1981, Inoue et al. 1988b, Fukushima et al. 1990a, Inoue et al. 1991, Schiemann and Wauters 1992, Chiesa et al. 1993, FDA 2007, Laukkanen et al. 2008). Cold enrichment has often been reported to increase the recovery of *Y. pseudotuberculosis* also in clinical samples other than diarrhoea when the number of *Y. pseudotuberculosis* is small (Paterson and Cook 1963, Oberhofer and Podgore 1980, Tertti et al. 1984, Nakano et al. 1989, Chiesa et al. 1993, Kontiainen et al. 1994, Van Noyen et al. 1995). During cold enrichment, other psychrotrophic bacteria and also apathogenic *Yersinia* spp. multiply in the samples (Kontiainen et al. 1994). A disadvantage of cold enrichment is the long incubation period

up to three weeks which make this method impractical for outbreak investigations and quality control of foods.

Cefsulodin-irgasan-novobiocin (CIN) agar designed for *Y. enterocolitica* is the most commonly used isolation medium also for *Y. pseudotuberculosis* from clinical specimens and samples from animals and the environment (Schiemann 1979). However, CIN agar can inhibit the growth of some *Y. pseudotuberculosis* strains (Fukushima and Gomyoda 1986, Bosi et al. 1994). Cefsulodin in CIN agar may inhibit the growth of *Y. pseudotuberculosis* especially at higher concentrations (Fukushima 1992). Other media, such as MacConkey (MAC) and MacConkey with 1% sorbitol (SMAC), have been proposed for detection of *Y. pseudotuberculosis,* especially in food samples, despite low selectivity (Fukushima and Gomyoda 1986, Shiozawa et al. 1991, FDA 2007). On CIN agar, *Y. pseudotuberculosis* grows as typical, small, deep-red colonies surrounded by a sharp transparent zone ("bull's eye" appearance), which makes the isolation of *Y. pseudotuberculosis* colonies easier than from other suggested agar media. Incubation at 32-35°C for 24-48 h has been proposed (Falcao et al. 1979, Fukushima et al. 1985b, Chiesa et al. 1993). Incubation of agars at 37°C for 24 h and further incubation at room temperature were described as efficient for slow-growing *Y. pseudotuberculosis* strains (Van Noyen et al. 1995).

2.2.2. Identification

Phenotypical methods

The key biochemical tests used to identify *Y. pseudotuberculosis* in the genus *Yersinia* are urease activity and fermentation of sorbitol, sucrose, xylose, rhamnose and melibiose. Esculin and Voges-Proskauer tests can be used as additional tests to differentiate *Y. pseudotuberculosis* strains from other *Yersinia* spp. including sucrose-negative, pathogenic *Y. enterocolitica* strains (Bercovieri and Mollaret 1984, Fredriksson-Ahomaa et al. 2002, FDA 2007). The commercial identification test API 20E has been shown to be accurate in identifying *Y. pseudotuberculosis* in the genus *Yersinia*. This system has a positive identification rate of 90% when incubated at temperatures between 25°C and 30°C, instead of 37°C (Neubauer et al. 1998).

Polymerase chain reaction (PCR)

For more rapid and reliable identification of *Y. pseudotuberculosis* isolates, DNA-based methods, such as PCR, have been developed for pure culture. The majority of the PCR assays are based on amplification of the chromosomally encoded gene regions, i.e. *inv, ail*

and *wzz* (Nakajima et al. 1992, Bogdanovich et al. 2003, Thisted Lambertz and Danielsson-Tham 2005). The most commonly used target for identification of *Y. pseudotuberculosis* isolates is *inv* (Nakajima et al. 1992, Kageyama et al. 2002, Thoerner et al. 2003). Using species-specific regions of *ail* in the chromosome as a target allows differentiation between *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* isolates (Thisted Lambertz and Danielsson-Tham 2005). *Wzz* is a specific target of *Y. pseudotuberculosis*, but in positive cases the presence of *Y. pestis* must be excluded (Bogdanovich et al. 2003). A single PCR method based on differences within the *yopT*-coding region on the virulence plasmid could differentiate *Y. pseudotuberculosis* isolates from *Y. pestis* and enteropathogenic *Y. enterocolitica* isolates (Arnold et al. 2001).

2.2.3. Confirmation of pathogenicity

All *Y. pseudotuberculosis* serotypes are considered potentially pathogenic, but the pathogenicity varies between isolates. All pathogenic *Y. pseudotuberculosis* strains carry a virulence plasmid (pYV). This plasmid is needed to cause *Y. pseudotuberculosis* infections and clinical symptoms of yersiniosis in humans and animals. The pYV of *Y. pseudotuberculosis* is unstable and can be lost during the isolation in serial culture transfers (Schiemann 1989, Fantasia et al. 1991).

Phenotypical methods

A number of phenotypic characteristics associated with virulence can be used to evaluate the pathogenicity of *Y. pseudotuberculosis,* e.g. autoagglutination at 35-37°C (Laird and Cavannaugh 1980), calcium dependence and Congo-red uptake measured as growth restriction with small orange colonies on Congo-red magnesium oxalate (CR-MOX) agar (Bercovier and Mollaret 1984, Riley and Toma 1989). All of these characteristics are associated with the presence of the pYV (Shiozawa et al. 1988, Fantasia et al. 1991, Tsubokura and Aleksic 1995, Martins et al. 1998). The pyrazinamidase activity (PYZ) test is included in the biotyping scheme of *Y. enterocolitica* to differentiate between non-pathogenic and potentially pathogenic isolates (Kandolo and Wauters 1985, Farmer et al. 1992), with reactions being negative in pathogenic isolates. This test is chromosomally mediated and cannot be used to confirm the presence of pYV, merely to confirm the ability of isolates to harbour the pYV. Pathogenic *Y. pseudotuberculosis* isolates have been shown to be negative for pyrazinamidase (Aleksic et al. 1995) or to have varied reactions (Martins et al. 1998), indicating that this test is not an efficient indicator of potential pathogenicity of *Y. pseudotuberculosis* isolates.

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Some PCR methods have been developed to verify the pathogenicity of *Y. pseudotuberculosis* for pure culture. PCR is much faster and more reliable in confirming pathogenicity of the isolates than phenotypic methods. The most commonly used target is *virF* on the pYV (Wren and Tabaqchali 1990, Nakajima et al. 1992, Kaneko et al. 1995, Martins et al. 1998, Kageyama et al. 2002, Thoerner et al. 2003, Thisted Lambertz and Danielsson-Tham 2005, Kechagia et al. 2007, Martins et al. 2007). Another possible target gene on the virulence plasmid is *yadA* (Thoerner et al. 2003, Kechagia et al. 2007). Based on the amplification of chromosomal virulence genes present in HPI (IS*100, psn, ybtE, irp1, irp2, ybtP-ybtQ, ybtX-ybtS*), a multiplex PCR has been developed to evaluate the pathogenicity potential of *Y. pseudotuberculosis* isolates together with *virF* (Martins et al. 2007). Complete and truncated HPI is present only in *Y. pseudotuberculosis* serotypes O:1 and O:3, respectively, and this PCR method gives additional information about HPI virulence genes present in the chromosome of these two serotypes.

2.3. Detection of *Y. pseudotuberculosis* with PCR

PCR has several advantages in detection of *Y. pseudotuberculosis* relative to conventional culture methods. Especially in infections and outbreaks, rapid, specific and sensitive methods are needed for screening of clinical, food and environmental samples. Food samples found negative by the culture method in *Y. pseudotuberculosis* outbreaks could be positive with PCR (Thisted Lambertz et al. 2008).

Only a few PCR methods have been developed to detect *Y. pseudotuberculosis* directly from food, water or clinical samples. Detection limits of the conventional PCR methods have been comparatively high. The detection of two multiplex PCR assays for *Y. pseudotuberculosis* in food and water samples has been based on amplification of *inv* in the chromosome in combination with plasmid-encoded virulence gene *virF* (Nakajima et al. 1992, Kaneko et al. 1995). The detection limit for *Y. pseudotuberculosis* was more than 10^3-10^4 cfu/ml for autoclaved and river water samples (Nakajima et al. 1992). A three-week cold enrichment was needed for pork samples with a detection limit 10^1-10^2 cfu/ml of *Y. pseudotuberculosis* in Kaneko et al. (1995). For detection of *Y. pseudotuberculosis* in clinical samples, *inv*, 16S and 23S rRNA genes have been used as targets (Trebesius et al. 1998, Tomioka et al. 2005, Kot et al. 2007). Trebesius et al. (1998) detected *Y. pseudotuberculosis* in faeces and tissue samples based on amplification of the 16S and 23S rRNA regions with in situ hybridization. A nested multiplex PCR microarray assay based on amplification of 16S rDNA, labelling, hybridization and image analysis of the PCR product had a detection limit of 50 cfu/ml of *Y. pseudotuberculosis* in blood samples

(Tomioka et al. 2005). A multiplex PCR assay was developed for detection and differentiation of *Y. pseudotuberculosis* and *Y. enterocolitica* in pig tonsils. This assay is based on the amplification of *inv* in the chromosome of *Y. pseudotuberculosis* (Kot et al. 2007).

In recent years, real-time PCR methods have been developed for detection of *Y. pseudotuberculosis* in food and clinical samples with a low detection limit (Thisted Lambertz et al. 2008, Matero et al. 2009). Real-time PCR based on a species-specific region of *ail* enabled detection and identification of *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* in multiplex PCR. The lowest detection limit of *Y. pseudotuberculosis* for minced meat and grated carrots was 28-280 cfu/10g (Thisted Lambertz et al. 2008). A real-time PCR assay published earlier by Fukushima et al. (2003) differentiated *Y. pseudotuberculosis* and *Y. enterocolitica* in stool samples with 15 other foodborne pathogens based on the species-specific region of *yadA*. However, the detection level of *Y. pseudotuberculosis* was more than 10^5 cfu/g. Since stools of food poisoning patients usually harbour 10^6 or more *Y. pseudotuberculosis* per gram (Fukushima et al. 1984), this method was deemed an appropriate diagnostic tool for stool samples of patients with acute gastroenteritis (Fukushima et al. 2003). A new published real-time multiplex PCR assay targeting the *wzz* in *Y. pseudotuberculosis* and *Y. pestis* has been validated in clinical patient samples (Matero et al. 2009). The lower limit of the detection is 10-100 cfu/g. Sputum or faecal samples had no inhibitory effect on the assay, and it can be used for screening of clinical patient samples in infections and outbreaks for these two pathogenic *Yersinia* spp. (Matero et al. 2009).

2.4. Characterization of *Y. pseudotuberculosis*

2.4.1. Phenotyping

Serotyping

The most common typing method for *Y. pseudotuberculosis* is serotyping. A simplified antigenic scheme for serotyping of *Y. pseudotuberculosis* was proposed by Tsubokura and Aleksic (1995). *Y. pseudotuberculosis* can be divided into 15 O-serotypes (O:1-O:15) and 10 subtypes (O:1a-O:1c, O:2a-O:2c, O:4a-O:4b, O:5a-O:5b) based on variability in the lipopolysaccharide (LPS) O side chain (O-antigen) (Tsubokura and Aleksic 1995, Bogdanovich et al. 2003). Slide agglutination test with commercial antisera is available for serotypes of *Y. pseudotuberculosis* O:1-O:6. The multiplex PCR assay based on the

detection of LPS genes (*fcl, prt, manB, abe, wbyL, wbyH, ddhAB, wbyK, wzx*) allows distinction of all known *Y. pseudotuberculosis* sero- and subserotypes (Bogdanovich et al. 2003). Serotypes differ in their geographical distributions. Serotypes O:1 and O:3 are predominant in human infections in Europe and Canada, and O:4 and O:5 in human infections in the Far East (Fukushima et al. 2001). The relationship between pathogenicity and serotype is poorly understood (Nagano et al. 1997a). All serotypes are considered potentially pathogenic. However, serotypes O:1-O:5 have been isolated in humans with clinical infections, and serotypes O:6-O:14 only from non-human sources in Asia (Nagano et al. 1997a, Fukushima et al. 1998, Fukushima et al. 2001, Skurnik et al. 2004).

Biotyping

Biotyping of *Y. pseudotuberculosis* has been used in a limited number of studies (Aleksic et al. 1995, Tsubokura and Aleksic 1995, Martins et al. 1998, Martins et al. 2007). The biotyping scheme proposed by Tsubokura and Aleksic (1995) is based on the reactions for raffinose, melibiose and citrate (Table 2).

Table 2. Biochemical tests used for biotyping of *Yersinia pseudotuberculosis* (according to Tsubokura and Aleksic 1995).

Test	Biotype			
	1	2	3	4
Melibiose	+	-	-	+
Raffinose	-	-	-	+
Simmons citrate	-	-	+	-

With these reactions, *Y. pseudotuberculosis* is divided into four different biotypes (1-4). Since biotyping is not widely used to subtype *Y. pseudotuberculosis* strains, scant information exists about the different bioserotype combinations or their prevalences. In Germany, the majority (90%) of the *Y. pseudotuberculosis* strains isolated from humans and animals belong to biotypes 1 and 2 (Aleksic et al. 1995). In Brazil, biotype 2 has been most commonly (98%) isolated in non-human sources such as swine, cattle and buffalo (Martins et al. 1998, Martins et al. 2007).

2.4.2. Genotyping

Restriction endonuclease analysis of plasmids (REAP)

Restriction endonuclease analysis of the *Yersinia* virulence plasmid has frequently been used for molecular typing of pYV-positive *Y. pseudotuberculosis* isolates (Fantasia et al. 1991, Kaneko et al. 1991, Fukushima et al. 1994b). Isolated bacterial plasmid DNA is digested and separated electrophoretically by number and size. REAP has been used on a large number of isolates, but has a low discriminatory power. Spontaneous loss of the virulence plasmid is possible during isolation of *Y. pseudotuberculosis* isolates, limiting the applicability of the method in epidemiological investigations. Analyses of 289 and 687 *Y. pseudotuberculosis* strains belonging to serotypes O:1-O:7 and O:10 revealed 35 and 16 digestion patterns with *Bam*HI enzyme, respectively (Kaneko et al. 1991, Fukushima et al. 1994b). Kaneko et al. (1991) found serogroup-specific patterns. In the study of Fukushima et al. (1994), identical REAP patterns were obtained from *Y. pseudotuberculosis* strains of different serotypes and strains from both human and non-human sources. *Y. pseudotuberculosis* strains had different geographical distributions. The *Y. pseudotuberculosis* strains belonging to serotypes O:la, O:lb, O:2b and O:3 from Europe and Australasia had identical REAP profiles, but these were rarely prevalent in strains from Eastern Asia and North America. The REAP patterns of serotype O:lb, O:3, O:4a and O:4b strains from Russia and Japan were identical. The serotypes showing the same restriction patterns with *Bam*HI were further restricted with a combination of four different restriction enzymes. The 687 *Y. pseudotuberculosis* strains then revealed 29 different restriction patterns. Three of them were similar in more than one serotype isolated in Japan (Fukushima et al. 1994b).

Ribotyping

Ribotyping is a hybridization method targeting the ribosomal genes that has been used to differentiate various *Yersinia* species and to characterizate *Y. pseudotuberculosis* strains (Picard-Pasquier et al. 1990, Grif et al. 2003, Voskressenskaya et al. 2005, Ruppitsch et al. 2007). In ribotyping, restriction enzymes are used to digest the whole genomic DNA into smaller fragments. The fragments are separated by gel electrophoresis and transferred onto membrane by Southern blotting (Southern 1975). Labelled universal probe targeting of ribosomal 16S or 23S rRNA encoding genes is used for hybridization. The label of the probe is visualized to show the hybridized fragments as banding patterns termed ribotypes.

Ribotyping allows subtyping of *Y. pseudotuberculosis* strains of the same serotype (Voskressenskaya et al. 2003, 2005, Martins et al. 2007). However, it has a low discriminatory power with limited variation and number of bands within *Y.*

pseudotuberculosis strains. Among 68 *Y. pseudotuberculosis* strains isolated in animals in Brasil, only four ribotypes were identified (Martins et al. 2007). No clear relationship has been found between the ribotype and the serotype or the animal or geographical origin of the strains. *Y. pseudotuberculosis* strains from different continents have been found to cluster together and sometimes to have identical ribotypes. Ribotyping is easy to interpret, but it has limited use in outbreaks investigations (Voskressenskaya et al. 2003, 2005). It could be a more suitable tool for long-term epidemiology revealing information about global dissemination of strains. Ribotyping does not clearly differentiate between *Y. pseudotuberculosis* and *Y. pestis* strains, but it could be used to differentiate *Y. pseudotuberculosis* from other *Yersinia* spp. (Picard-Pasquier et al. 1990, Grif et al. 2003, Voskressenskaya et al. 2005, Ruppitsch et al. 2007).

Pulsed-field gel electrophoresis (PFGE)

PFGE has been shown to have the highest discriminatory power for subtyping of *Y. pseudotuberculosis* strains (Iteman et al. 1995, Odaert et al. 1996). In PFGE, the chromosomal DNA is cleaved by rare cutting enzymes. To protect the DNA from mechanical breakage, the bacterial suspension is mixed with melted agarose before the cell lyses. Slices of agarose plugs containing purified and digested DNA are inserted into the wells of an agarose gel. The DNA fragments are separated by the electrical field (Arbeit et al. 1990). The orientation of the electrical field alternates at predetermined intervals (pulsed-field electrophoresis). The isolate are closely related when the difference in restriction profiles is around two to three fragments, possibly related when it is four to six fragments and unrelated when the difference is seven or more fragments (Tenover et al. 1995, Barrett et al. 2006).

Iteman et al. (1995) found high polymorphism of the *Y. pseudotuberculosis* strains. From 30 *Y. pseudotuberculosis* strains of serotypes O:1, O:2 and O:3, altogether 27 pulsotypes were observed. PFGE efficiently subtyped strains belonging to the same serotype or obtained from the same host species or country. PFGE is highly reproducible and competitive relative to many other genotyping methods established for *Y. pseudotuberculosis* (Odaert et al. 1996, Olive and Bean 1999, Savelkoul et al. 1999, Kardos et al. 2007). However, the numerous restriction patterns and complexity of the profiles can make comparison of large numbers of *Y. pseudotuberculosis* strains difficult (Odaert et al. 1996). Standardized PFGE protocols and pattern analysis by computer-assisted techniques to compare strains between laboratories are not yet available for *Y. pseudotuberculosis*. In Finland, PFGE has successfully been used to compare the epidemiologically related *Y. pseudotuberculosis* isolates from patients and to determine the source of contamination in recent outbreak investigations. Serologically grouped isolates were further subtyped by PFGE. Indistinguishable PFGE patterns of *Y.*

pseudotuberculosis have been found in humans, vegetables and wild animals (Jalava et al. 2004, Jalava et al. 2006, Kangas et al. 2008, Rimhanen-Finne et al. 2008).

Other molecular typing methods used for Y. pseudotuberculosis

Other molecular typing methods used to characterize *Y. pseudotuberculosis* strains include multilocus enzyme electrophoresis (MLEE) (Goullet and Picard 1984, Goullet and Picard 1988, Dolina and Peduzzi 1993), molecular typing of insertion sequence (IS) elements, present in multiple copies in the genome (Odaert et al. 1996) and PCR-based methods; such as random amplified polymorphic DNA typing (RAPD) (Makino et al. 1994, Kageyama et al. 2002) and ERIC-PCR (Kim et al. 2003, Kardos et al. 2007). In MLEE, the differentiation is based on allelic variations at an individual gene locus. Strains of *Y pseudotuberculosis* obtained from animal (cattle) were genetically closely related to strains isolated from human clinical material by MLEE (Dolina and Peduzzi 1993). The analytical performance of IS200- like element fingerprinting was found to be almost equal to PFGE and better than ribotyping (Odaert et al. 1996). RAPD was considered a useful tool for subtyping of *Y. pseudotuberculosis* strains in Izumi fever outbreak investigations (Makino et al. 1994) and for studying the source of infection in breeding monkeys (Kageyama et al. 2002). However, these studies were applied to a limited number of strains, isolated from the same geographical area during the same outbreak (Makino et al. 1994), or failed to differentiate isolates of the same serotype (Kageyama et al. 2002). Enterobacterial repetitive intergenic (ERIC) sequences, also known as intergenic repeat units, are large 126 bp elements containing a highly conserved central inverted repeat located in extragenic regions. ERIC-PCR has been applied for *Y. pseudotuberculosis* (Kim et al. 2003). ERIC-PCR was used as a tool to type of *Y. pseudotuberculosis* strains in outbreak investigations among poultry (Kardos et al. 2007).

Multilocus sequence typing (MLST) is better suitable for the investigation of bacterial phylogeny and the evolution of lineages than for typing of strains in outbreaks. When different species of the genus *Yersinia* were examined by MLST *Y. pseudotuberculosis* was genetically more homogeneous with *Y. aldovae, Y. bercovieri, Y. intermedia, Y. pestis*, Y. *rohdei*, and *Y. ruckeri* than with *Y. enterocolitica, Y. frederiksenii, Y. kristensenii,* and *Y. mollaretii*. The MLST data supported the idea that *Y. pestis* and *Y. pseudotuberculosis* are two lineages within the same species rather than two distinct species. MLST was better suited for determining genetic relatedness among *Yersiniae* than was 16S RNA analysis (Kotetishvili et al. 2005)

2.5. Reservoirs of *Y. pseudotuberculosis*

2.5.1. Environment

Soil and water

Y. pseudotuberculosis has occasionally been isolated from environmental samples, e.g. soil and freshwater samples such as river, well and mountain stream water (Inoue et al. 1988b, Fukushima1989a, Tsubokura et al. 1989, Inoue et al. 1991, Fukushima 1992, Fukushima et al. 1994). In a *Y. pseudotuberculosis* outbreak investigation in Finland, serotype O:1 was found in soil samples that contained carrot residue on a production farm (Jalava et al. 2006). In Japan, *Y. pseudotuberculosis* was isolated from soil samples originating from dried-up puddles and a sandbox at concentrations of 10^2-10^3 cfu/g. The bacterium could be recovered on days 17 and 27 after a human infection, but not on day 37, indicating that *Y. pseudotuberculosis* does not survive long in soil (Fukushima 1989a). Experiments carried out in natural and sterile soil at 25°C showed that the number of *Y. pseudotuberculosis* is controlled mostly by the biotic components (protozoans) of ecosystems. *Y. pseudotuberculosis* survived for up to half a year in non-sterile silt or marshy soil, but was not detectable after 5-9 days in sterile soil (Litvin et al. 1990).

In Japan, Fukushima (1992) found *Y. pseudotuberculosis* to be widely distributed in river waters, especially in winter. The isolation rate was higher during the cooler months from September to May (27%) than during the warmer period from June to August (0.8%). *Y. pseudotuberculosis* has been shown to survive for several months in surface waters (Fukushima et al. 1988, Inoue et al. 1988a, Sato and Komazawa 1991, Fukushima 1992).

Y. pseudotuberculosis detected in water samples has belonged to the serotypes O:1b, O:2b, O:2c, O:3, O:4a, O:4b, O:5a and O:5b (Inoue et al. 1988a, Tsubokura et al. 1989a, Fukushima 1992) and the serotypes O:6, O:7, O:10, O:11 and O:12 (Fukushima et al.1994, Yoshino et al. 1995, Fukushima et al. 2001); however, the latter serotypes have not been reported in connection with human infections. From soil, serotypes O:1 and O:3 have been recovered (Fukushima 1989a, Fukushima et al. 1994, Jalava et al. 2006). Serotypes found in soil and water samples have also been prevalent in wild animals (raccoon dogs, mice and moles) in the same area (Tsubokura et al. 1989, Fukushima et al. 1990a, Fukushima and Gomyoda 1991).

Pig house environment

Pigs are regular carriers of *Y. pseudotuberculosis,* having a worldwide distribution, but the isolation rates have been low. In Japan, Fukushima et al. (1990) examined the prevalence of *Y. pseudotuberculosis* in 1200 pigs from 96 farms. Of the farms and in the pigs, 16% and 3%, respectively, were positive. In a survey in Finland, different production types and capacities were compared by Laukkanen et al. (2008). A total of 8% of the 358 fattening pigs on 15 farms were *Y. pseudotuberculosis*-positive. A higher prevalence was found in organic production, where 60% of the farms and 19% of the fattening pigs were *Y. pseudotuberculosis*-positive, compared with 30% and 3% in conventional production, respectively.

The primary source of *Y. pseudotuberculosis* and transmission routes at farm level are not well known. Rodents and birds may have a role in spreading and maintaining *Y. pseudotuberculosis* contamination on farms. The prevalence of *Y. pseudotuberculosis* increased in production systems where pigs have contact with the outside environment, e.g. in organic production (Laukkanen et al. 2008). Other farm-specific factors, such as high production capacity, large group size, pests and pet animals, also increased the prevalence of *Y. pseudotuberculosis* in conventional production (Laukkanen et al. 2008). *Y. pseudotuberculosis* has occasionally been implicated in diarrhoea of young pigs (8-18 weeks), alone or together with other pathogens (de Barcellos and de Castro 1981, Harper et al. 1990, Neef and Lysons 1994, Thomson et al. 1998).

Serotypes O:3 and O:1b were the most commonly (67%) isolated serotypes in pigs on farms in Japan, following by serotypes O:4b, O:2b and O:2c (Fukushima et al. 1989b, Fukushima et al. 1990). In Finland, only serotype O:3 has been isolated from pigs (Laukkanen et al. 2008).

Slaughterhouse environment

In slaughterhouses, *Y. pseudotuberculosis* has sporadically been detected from pig tonsils, caecal content, mesenteric and cervical lymph nodes, skin, carcasses and pluck sets and equipment used in the slaughter process (Fukushima et al. 1989b, Laukkanen et al. 2008). The carrier pigs are the most important contamination source at slaughterhouses, but cross-contamination between faecal matter, tonsils, pluck sets and carcasses is also possible during the slaughter process. As asymptomatic carriers, *Y. pseudotuberculosis*-positive pigs cannot be identified in the slaughter process; only strict slaughter hygiene can reduce the contamination.

Cross-contamination was shown by Fukushima et al. (1989b) between the caecal content, oral cavity, skin and carcasses of slaughter pigs. The same serotypes of *Y. pseudotuberculosis* isolated from carcasses were detected in tonsils, caecal content and

skin, indicating contamination in piggeries, lairage or during the slaughter process. When individual pigs were followed in the production chain from farm to slaughterhouse by Laukkanen et al. (2008), identical *Y. pseudotuberculosis* genotypes were recovered in live animals at the farm and on pluck sets (including tongue, tonsils, oesophagus, trachea, heart, lungs, diaphragm, liver and kidneys), and on carcasses at the slaughterhouse. The high prevalence of *Y. pseudotuberculosis* in pigs at the farm predisposed to further contamination of carcasses and pluck sets at the slaughterhouse. The same *Y. pseudotuberculosis* genotype found in pigs in either faeces at the farm or intestinal content or tonsils during slaughter was isolated from carcasses of the same animals. Moreover, possible cross-contamination was shown between *Y. pseudotuberculosis*-positive pluck sets and carcasses.

2.5.2. Animals

Wild animals

Wild animals are considered the main reservoirs for *Y. pseudotuberculosis*. The host range is broad and the bacterium has sporadically been isolated from numerous species of wild mammals and birds around the world. *Y. pseudotuberculosis* has most often been recovered from rodents (mice, rats and moles) (Kaneko et al. 1979, Mackintosh and Henderson 1984, Tsubokura et al. 1989, Fukushima et al. 1990a, Inoue et al. 1991, Zheng et al. 1995, Pocock et al. 2001), hares and rabbits (Mackintosh and Henderson 1984, Tsubokura et al. 1989, Inoue et al. 1991, Aleksic et al. 1995, Zheng et al. 1995), deers (Hodges et al. 1984b, Toma 1986, Fukushima and Gomyoda 1991, Aleksic et al. 1995, Aschfalk et al. 2008) and wild birds (Mackintosh and Henderson 1984, Toma 1986).

Among wild animals, subclinical infections and asymptomatic carriage of *Y. pseudotuberculosis* are common (Mair 1979, Tsubokura et al. 1989, Aschfalk et al. 2008). The highest prevalences of *Y. pseudotuberculosis* have been detected in rodents: 8% in rats in China (Zheng et al. 1995), 9% in rats in New Zealand (Mackintosh and Henderson 1984) and 15% in moles in Japan (Fukushima et al. 1990). Frölich et al. (2003) reported antibodies against pathogenic *Yersinia* spp. in 55% of European brown hares in Germany. *Y. pseudotuberculosis* has been isolated also from several other wild animal species, including both carnivores and herbivores, e.g. raccoon dogs, martens, foxes, wild cats, river otters, boars, mouflons and buffaloes (Inoue et al. 1991, Fukushima and Gomyoda 1991, Suzuki et al. 1995, Martins 1998, Nikolova et al. 2001). In the mountainous areas in Japan, the predators of small mammals, namely foxes, martens and raccoon dogs, have harboured *Y. pseudotuberculosis* at the highest rates (12%, 26% and 42%, respectively) (Inoue et al. 1991).

Although wild animals have commonly been reported to be asymptomatic carriers of *Y. pseudotuberculosis*, some species of zoo and farmed animals seem to be more susceptible to clinical *Y. pseudotuberculosis* infection. Of zoo animals, monkeys especially will often develop a sudden fatal infection of *Y. pseudotuberculosis*, and several outbreaks among different monkey species have been described in zoos around the world (Rosenberg et al. 1980, MacArthur and Wood 1983, Taffs and Dunn 1983, Bielli et al. 1999, Kageyama et al. 2002, Iwata et al. 2008). The most likely source of *Y. pseudotuberculosis* infection in monkeys was suspected to be wild mice or rats around the outdoor cages (Kageyama et al. 2002). *Y. pseudotuberculosis* infections also pose a serious problem for deer farming; the bacterium has caused severe and large outbreaks of diarrhoeal illness and deaths (Mackintosh and Henderson 1984, Jerret et al. 1990, Welsh et al. 1992, Sanford 1995, Zhang et al. 2008). *Y. pseudotuberculosis* has been reported to be one of the most common infections among farmed deer in Australia. Yersiniosis among deers occurred more often during the cooler months, with younger age groups most affected (Jerret et al. 1990).

The *Y. pseudotuberculosis* serotypes reported in human infections are O:1-O:5 (Fukushima et al. 2001). The same serotypes have been isolated in a wide variety of wild animals worldwide, including Europe (Aleksic et al. 1995, Nikolova et al. 2001), North America (Toma 1986), Japan (Fukushima et al. 1990a, Fukushima and Gomyoda 1991) and China (Zheng et al. 1995). In Europe and North America, serotypes O:1 and O:3 dominate in human infections and in wild animals (rodents, hares and deers) (Toma 1986, Aleksic et al. 1995). In the UK, house mice were found to carry human pathogenic *Y. pseudotuberculosis* O:1a (Pocock et al. 2001). In Bulgaria, several wild animals studied (jackal, mouflon, fox, polecat and marten) were *Y. pseudotuberculosis* serotype O:3-positive (Nikolova et al. 2001). In the Far East, the most common *Y. pseudotuberculosis* serotypes detected in wild animals (rodents, hares, deers and raccoon dogs) have been O:1b and O:4b (Fukushima and Gomyoda 1991, Inoue et al. 1991, Suzuki et al. 1995, Zheng et al. 1995), with these also being the predominant serotypes in human infections. Rodents, especially mice and rats, are supposed to be an important reservoir and a source of human infections in Japan (Fukushima et al. 1990a). In wild animals (rabbits, hares, raccoon dogs, mice, moles and monkeys), also *Y. pseudotuberculosis* serotypes O:6-O:15, which have not been reported in human infections, have been detected (Fukushima et al. 1990a, Zheng et al. 1995, Fukushima et al. 2001, Iwata et al. 2008).

Wild birds

Birds are recognized as an important reservoir for *Y. pseudotuberculosis* (Hubbert 1972, Mair 1973*)*. Several wild bird species have been found to carry *Y. pseudotuberculosis* regularly, but at low isolation rates. This bacterium has sporadically been isolated in

apparently healthy passerine birds and psittaciformes (Mackintosh and Henderson 1984, Hamasaki et al. 1989, Fukushima and Gomyoda 1991, Inoue et al. 1991, Cork et al. 1995). Birds have a high mobility, and especially migrating birds can efficiently disseminate *Y. pseudotuberculosis* over geographically large areas along their migratory routes (Hubbert 1972, Kaneuchi 1989, Fukushima and Gomyoda 1991, Cork et al. 1995). The prevalence of *Y. pseudotuberculosis* in the surveys varied from 0.1% to 4.6% in healthy birds, the highest prevalence of 5.4% found in ducks in the study conducted by Mackintosh and Henderson (1984).

Birds are also reported to be relatively susceptible to infection with *Y. pseudotuberculosis*. It is a frequently observed pathogen among zoo birds and domestic fowl in both outbreaks and sporadic cases causing sudden deaths in aviaries (Kageruga et al. 1976, Harcourt-Brown 1978, Wallner-Pendleton and Cooper 1983, Parsons 1991, Cork et al. 1999). The susceptibility of wild birds to *Y. pseudotuberculosis* infections is not well known. Possibly, they are less affected and only develop acute clinical illness under stressful conditions, such as cold weather or migration (Mackintosh and Henderson 1984).

Pathogenic *Y. pseudotuberculosis* serotypes O:1 and O:2 are the most commonly isolated serotypes in diseased wild and captive birds (Kageruga et al. 1976, Borst et al. 1977, Mackintosh and Henderson 1984, Tsubokura et al. 1984, Toma 1986, Parsons 1991, Aleksic et al. 1995, Cork et al. 1999). In Japan, *Y. pseudotuberculosis* serotypes O:3 and O:4b were more frequently isolated in apparently healthy migratory and city-living birds (Hamasaki et al. 1989, Inoue et al. 1991, Otsuka et al. 1994).

Domestic animals

Y. pseudotuberculosis has sporadically been isolated from domestic animals. The most commonly reported findings have been from ruminants (cattle and sheep), swine and pets (cats and dogs) (Fukushima et al. 1985a, Hodges and Carman 1985, Bullians 1986, Toma 1986, Tsubokura et al. 1989, Inoue et al. 1991, Slee and Skilbeck 1992, Aleksic et al. 1995). These animals are mostly healthy carriers, but they may come ill and excrete the bacteria due to stress during cold weather, parasite infection or transportation (Jerret et al. 1990, Slee and Skilbeck 1992). Outbreaks of severe *Y. pseudotuberculosis* gastroenteritis have been reported, especially in young animals, e.g. in cats, calves, sheep, goats and pigs (Obwolo and Gruffydd-Jones 1977, Hodges et al. 1984a, Slee et al. 1988, Slee and Button 1990).

The most common serotypes isolated from domestic animals in Europe, North America, Australia and New Zealand have been O:1 and O:3, followed by serotype O:2 (Hodges et al. 1984a, Toma 1986, Aleksic et al. 1995), and in Japan O:1 and O:4, followed by O:2, O:3 and O:5 (Fukushima et al. 1985a, Tsubokura et al. 1989, Fukushima et al. 2001).

Pigs

Several investigations show that pigs could be an important asymptomatic carrier and a reservoir for *Y. pseudotuberculosis*. This pathogen has a worldwide distribution in pig populations and has sporadically been isolated from tonsillar and faecal samples of clinically healthy pigs (Zen-Yoji et al. 1974, Toma and Deidrick 1975, Tsubokura et al. 1976, Narucha and Westendoorp 1977, Weber and Knapp 1981, Fukushima et al. 1989b, 1990b, Tsubokura et al. 1989, Shiozawa et al. 1991, Chiesa et al. 1993, Aleksic et al. 1995, Kechagia et al. 2007, Laukkanen et al. 2008, Ortiz Martinez et al. 2009) (Table 3).

Table 3. Distribution of serotypes O:1 to O:5 of *Yersinia pseudotuberculosis* isolated from tonsils, faeces, skin and carcasses of pig and pork.

Source	Country	No. of samples	No. of strains (%)	Serotype								Reference
				1a	1b	2b	2c	3	4b	5a	5b	
Tonsils	Netherlands	163	7 (4.3)	UN[1]								Narucha and Westendoorp 1977
	Germany	480	28 (5.8)	15[2]		13[3]						Weber and Knapp 1981
	Japan	140	2 (1.4)						1	1		Shiozawa et al. 1991
	Italy	217	1 (0.5)			1						Chiesa et al. 1993
	Greece	455	3 (0.7)	UN								Kechagia et al. 2007
	Finland	350	34 (10)					34				Laukkanen et al. 2008
	Estonia	151	2 (1.3)					2				Ortiz Martinez et al. 2009
	Latvia	109	5 (4.6)					5				Ortiz Martinez et al. 2009
	Russia	197	13 (6.6)					13				Ortiz Martinez et al. 2009
Oral swabs	Japan	1200	52 (4.3)		8	4	7	11	22			Fukushima et al. 1989b
Faeces	Japan	1796	41 (2.3)	2[2]			38					Zen-Yoji et al. 1974
	Canada	544	14 (2.6)					14				Toma and Deidrick 1975
	Japan	2041	28 (1.4)		5			19	4			Tsubokura et al. 1976
	Netherlands	163	3 (1.8)	UN								Narucha and Westendoorp 1977
	Japan	1200	34 (2.8)		8	1	2	16	6			Fukushima et al. 1989b
	Japan	1200	33 (2.8)		8	1	2	16	6			Fukushima et al. 1990b
	Japan	140	7 (5.0)					7				Shiozawa et al. 1991
	Japan	585	12 (2.1)			x[4]		x	x		x	Inoue et al. 1991
	Italy	513	3 (0.6)					7				Chiesa et al. 1993
	Finland	358	24 (7)					24				Laukkanen et al. 2008
Pig	Japan	UN	71		8	1		51	11		1	Tsubokura et al. 1984
	Canada	UN	30		1			29				Toma 1986

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Table 3. Continued

Source	Country	No. of samples	No. of strains (%)	Serotype								Reference
				1a	1b	2b	2c	3	4b	5a	5b	
	Japan	UN	195		28	7	10	107	40	2	1	Tsubokura et al. 1989
	Germany	UN	3	1				3				Aleksic et al. 1995
Skin	Japan	550	8 (1.5)					8				Fukushima et al. 1989b
Pluck set	Finland	354	6 (2)					6				Laukkanen et al. 2008
Carcass	Japan	1200	3 (0.3)				1	1	1			Fukushima et al. 1989
	Finland	359	10 (3)					10				Laukkanen et al. 2008
Pork	Japan	66	1 (1.5)						1			Fukushima 1985
	Japan	UN	1						1			Tsubokura et al. 1989
	Japan/ Canada	250	1 (0.4)						1			Fukushima et al. 1997

[1] Unknown.
[2] Serotype O:1, not subtyped.
[3] Serotype O:2, not subtyped.
[4] Number of isolates not reported.

The prevalence of *Y. pseudotuberculosis* has varied in different studies, from 0.5% to 10% in tonsillar samples, and from 1.4% to 7% in faecal samples (Table 3). *Y. pseudotuberculosis* has been more common in tonsils than in faeces of pigs when studied at the same time (Narucka and Westendoorp 1977, Fukushima et al. 1989b, Laukkanen et al. 2008).

In earlier surveys, there has been a preponderance of serotype O:3 strains among pigs in all countries, but especially in Western Europe, North America and Australasia (Fukushima et al. 2001) (Table 3). Serotype O:3 is the only serotype isolated from asymptomatic pigs at slaughter in Finland (Laukkanen et al. 2008) and in Eastern Europe (Ortiz Martínez et al. 2009). Serotypes O:1b and O:4b have been reported among pigs, especially in Japan and Korea (Fukushima et al. 2001) (Table 3).

2.5.3. Food and drinking water

Although *Y. pseudotuberculosis* is considered a possible foodborne pathogen, it has rarely been isolated from foods (Fukushima et al. 1987, Schiemann 1989, Chiesa et al. 1993, Greenwood 1995). An association between human infections and pork has been suggested (Tsubokura et al. 1982, Fukushima et al. 1985b, Fukushima et al. 1994). *Y. pseudotuberculosis*-positive findings on pig carcasses and offal at the slaughterhouse (Fukushima et al. 1989, Laukkanen et al. 2008) can be a potential source of further contamination at processing plants and at retail. *Y. pseudotuberculosis* serotype O:4b was isolated from pork imported from Canada in a meat processing plant in a study in Japan (Fukushima et al. 1997) (Table 3). Only a few surveys have been conducted to investigate the occurrence of *Y. pseudotuberculosis* in meat products at retail level. In Japan, *Y.*

pseudotuberculosis O:4b was isolated from 2% of pork tongue and 1.5% of ground pork samples, while beef and chicken samples were negative (Fukushima 1985, Shiozawa et al. 1987, Tsubokura et al. 1989, Fukushima et al. 1997).

Food products have seldom been examined for *Y. pseudotuberculosis,* except in outbreak investigations. Homogenized milk was suggested to be a possible outbreak source in Canada (Nowgesic et al. 1999, Press et al. 2001). A single brand of homogenized milk was associated with illness and tested positive for *Y. pseudotuberculosis* serotype O:1b at the milk plant. Fresh produce (Tertti et al. 1984) and vegetable juice (Inoue et al. 1984) were suspected as potential sources of *Y. pseudotuberculosis* infection on the basis of descriptive data in Finland and Japan, respectively. *Y. pseudotuberculosis* has been isolated from vegetables in Finland (Jalava et al. 2006, Kangas et al. 2008, Rimhanen-Finne et al. 2008) and Russia (Fukushima et al. 1994, Fukushima et al. 2001) in foodborne outbreaks. *Y. pseudotuberculosis* serotype O:1 has been found on carrots, (Kangas et al. 2008, Rimhanen-Finne et al. 2008) and in carrot residues on peeling and washing equipment of a production farm in Finland (Jalava et al. 2006).

Untreated fresh water has been widely investigated and shown to be a reservoir of *Y. pseudotuberculosis* in Japan (Fukushima 1989a, Tsubokura et al. 1989, Fukushima 1992, Fukushima et al. 1994). Drinking contaminated surface water has been reported to be an source of sporadic *Y. pseudotuberculosis* serotype O:4b and O:5b infections (Fukushima et al. 1988, Sato and Komazawa 1991, Sunaharu et al. 2000) in Japan and Korea (Han et al. 2003). The *Y. pseudotuberculosis* serotypes O:4b and O:5b were detected in mountain stream and well waters during these infections. A community water outbreak was caused by *Y. pseudotuberculosis* serotype O:4b in a mountainous area in Japan (Inoue et al. 1988a).

2.6. Human yersiniosis

Y. pseudotuberculosis and *Y. enterocolitica* can cause an enteric infection called yersiniosis. *Y. pseudotuberculosis* infection is mainly an acute gastroenteritis with fever associated with ingestion of contaminated food or water. Infections have been reported at low incidences on all continents (Fukushima et al. 2001, EFSA 2007). Most of the infections are sporadic, but outbreaks have also been reported in some countries (Table 4).

2.6.1. Occurrence

Human infections of *Y. pseudotuberculosis* have primarily been reported in the northern hemisphere in Europe, Russia, Japan and North America, with most of them being sporadic (Toma 1986, Sato et al. 1987, Tsubokura et al. 1989, Sato 1991, Aleksic et al. 1995, Van Noyen et al. 1995, Sunahara et al. 2000, Hallanvuo et al. 2003, Smirnova et al. 2004). Before the mid-1990s, the source of *Y pseudotuberculosis* outbreaks reported mainly in Japan were associated with drinking contaminated water (Fukushima 1988, Inoue et al. 1988a, Tsubokura et al. 1989) and contact with infected animals (Fukushima 1991, Fukushima et al. 1994). Between 1997 and 2000 in Finland and in Russia, *Y. pseudotuberculosis* has caused large foodborne outbreaks almost annually and is considered an emerging foodborne pathogen (Hallanvuo et al. 2003, Jalava et al. 2004, Rimhanen-Finne et al. 2008, ProMED 1999, 2005, 2007, 2008) (Table 4).

Table 4. Reported foodborne and waterborne outbreaks of *Yersinia pseudotuberculosis* from 1981 to 2008.

Country	Year	Suspected or confirmed source	Serotype	Confirmed	No. of cases	Reference
Canada	1998	Pasteurized milk	O:1b	NO[1]	73	Nowgesic et al. 1999, Press et al. 2001
Finland	1981	Fresh produce	O:2b, O:3	NO	19	Tertti et al. 1984
	1987	Fresh produce	O:1a	NO	34	Tertti et al. 1989
	1997	Fresh produce	O:3	NO	35	Pedoby et al. 1997
	1998	Vegetables	O:3	NO	60	Hallanvuo et al. 2003
	1999	Vegetables	O:3	NO	31	Hallanvuo et al. 2003
	2001	Iceberg lettuce	O:1b, O:3	PFGE[2]	123	Jalava et al. 2004
	2003	Carrots	O:1b	PFGE	111	Jalava et al. 2006
	2004	Carrots	O:1b	PFGE	58	Takkinen et al. 2004, Kangas et al. 2008
	2006	Carrots	O:1b	PFGE	42	Evira 2007
	2006	Carrots	O:1b	PFGE	427	Rimhanen-Finne et al. 2008
	2008	Carrots	O:1	PFGE	50	NPHI 2008a
Japan	1977	Unknown	O:5b	NO	57	Tsubokura et al. 1989
	1977	Unknown	O:1b	NO	82	Tsubokura et al. 1989
	1979-1989	Water	O:1b, O:2a, O:2b, O:2c, O:3, O:4a, O:4b, O:5a, O:5b	NO	290	Sato and Komazawa 1991
	1981	Vegetables juice	O:5a	NO	188	Inoue et al. 1984
	1982	Food	O:5a	NO	67	Inoue et al. 1988a
	1982	Water	O:2c, O:4b	NO	260	Inoue et al. 1988a

Table 4. Continued.

Country	Year	Suspected or confirmed source	Serotype	Confirmed	No. of cases	Reference
	1984	Water	O:4b	NO	12	Inoue et al. 1988a
	1984	Barbecued foods	O:5a (two outbreaks)	NO	39	Nakano et al. 1989
Japan	1984	Unknown	O:3	NO	63	Tsubokura et al. 1989
	1985	Unknown	O:4b (two outbreaks)	NO	68	Tsubokura et al. 1989
	1986	Unknown	O:4b	NO	549	Tsubokura et al. 1989
	1991	Food	O:5a	REAP[3]	732	Toyokakawa et al. 1993
Mongolia	1986	Vegetables	O:1	NO	114	Markov et al. 1989
Russia	1999	NR[4]	NR	NR	145	ProMED:1990405.0551[5]
	2005	Cabbage, onion	NR	NR	33	ProMED: 20050202.0359
	2005	Cabbage, onion	NR	NR	42	ProMED: 20050427.1169
	2005	Cabbage, onion	NR	NR	18	ProMED: 20050427.1169
	2005	Cabbage, onion	NR	NR	9	ProMED: 20050427.1169
	2005	Vegetables	NR	NR	13	ProMED: 2005031.3178
	2005	Vegetables	NR	NR	24	ProMED: 20050216.3617
	2007	Unknown	NR	NR	11	ProMED: 20070501.1412
	2007	Unknown	NR	NR	16	ProMED: 20070803.2511
	2007	Unknown	NR	NR	30	ProMED: 20070803.2511
	2007	Vegetables	NR	NR	121	ProMED: 20071001.3240
	2008	Unknown	NR	NR	141	ProMED: 20080718.2184

[1] Genotyping method not used.
[2] Pulsed-field gel electrophoresis.
[3] Restriction endonuclease analysis of virulence plasmids.
[4] Not reported.
[5] ProMED (www.promedmail.org) archive number.

In France, a sudden increase in human *Y. pseudotuberculosis* infections was reported in 2004-2005 (Vincent et al. 2008). The incidence of sporadic *Y. pseudotuberculosis* infections is probably underestimated since the bacterium is not routinely studied in stool samples, especially in gastroenteritis with mild diarrhoeic symptoms. In human yersiniosis, *Y. pseudotuberculosis* serotypes differ in their geographical distribution; in Canada, serotypes O:1b and O:3 (Toma 1986), in Europe serotypes O:1a, O:1b and O:3 and in the Far East serotypes O:4b and O:5b are the most common in clinical samples (Fukushima et al. 2001).

In Finland, yersiniosis is the third most common foodborne enteric infection after salmonellosis and campylobacteriosis. Most of the sporadic cases of yersiniosis are caused by *Y. enterocolitica* (NPHI 2008b). The annual incidence rate of *Y. pseudotuberculosis* was 0.6-5 cases per 100 000 inhabitants in 1995-2008 (NPHI 2005, 2006, 2007, 2008b). A continuous trend of increasing numbers of sporadic infections from 1995 to 2008 can be seen (Figure 1). In the last ten years, the annual number of sporadic infections has increased from around 30 to 80. Up to 220 culture and/or serologically confirmed cases have been reported in one outbreak (Table 4, Figure 1).
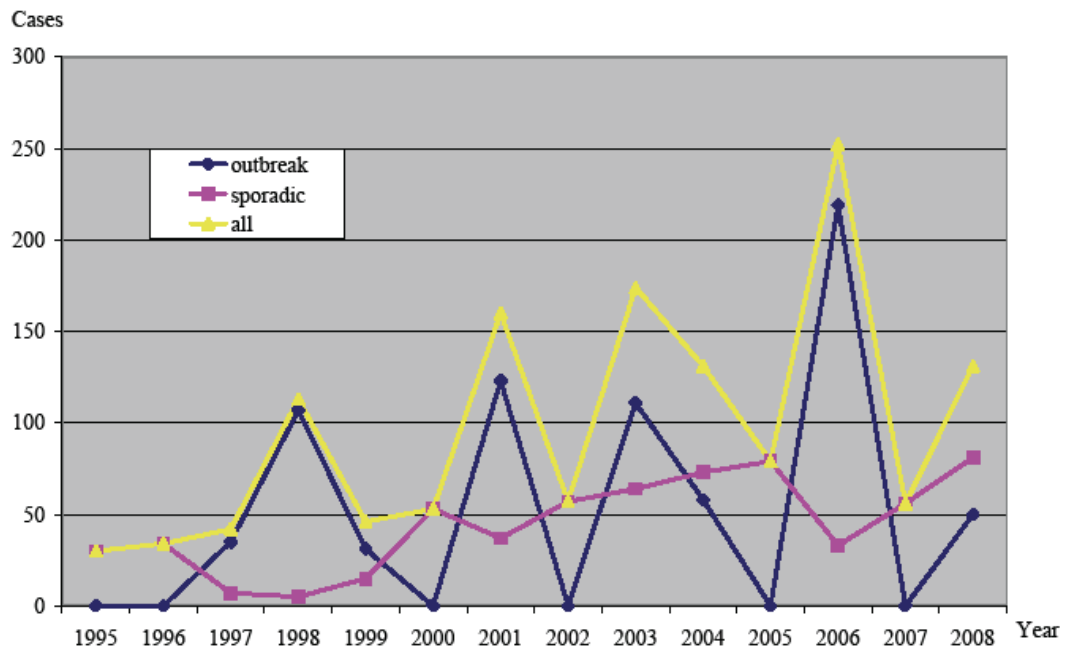
Figure 1. Confirmed *Yersinia pseudotuberculosis* infections reported in Finland in 1995-2008 (according to the National Infectious Diseases Register of NPHI, Finland).

2.6.2. Clinical symptoms

The clinical disease caused by *Y. pseudotuberculosis* is usually characterized by fever and acute abdominal pain. The right lower quadrant abdominal pain caused by mesenteric lymphadenitis can be confused with acute appendicitis (Tertti et al. 1984, Attwood et al. 1987). This may lead to an unnecessary appendectomy, especially in children, who more often get acute symptoms (Jalava et al. 2006, Rimhanen-Finne et al. 2008). Abdominal pain is a predominant symptom of *Y. pseudotuberculosis* infection, while diarrhoea is less common (Tertti et al. 1989). The gastroenteritis symptoms are usually mild and self-limiting, but severe bloody diarrhoea with intestinal necrosis and septicaemia, usually in patients with such underlying disorders as diabetes, hepatic cirrhosis or iron overload, can occur (Ljunberg et al. 1995, Vincent et al. 2008).

Postinfectious systemic complications, such as erythema nodosum and reactive arthritis, are relatively common (Tertti et al. 1984, Tertti et al. 1989, Hannu et al. 2003, Jalava et al. 2006). After a *Y. pseudotuberculosis* O:1 outbreak in Finland, 55% of affected

patients developed erythema nodosum (Jalava et al. 2006). In a follow-up study of *Y. pseudotuberculosis* O:3 outbreak, reactive arthritis was more common in adults than children (Hannu et al. 2003). HLA-B27 antigen was associated with the appearance of reactive arthritis. The incidence rate of reactive arthritis was 12-21% after a *Y. pseudotuberculosis* O:3 outbreak (Tertti et al. 1984, Hannu et al. 2003), but only 1-3% after a *Y. pseudotuberculosis* O:1 outbreak (Tertti et al. 1989, Jalava et al. 2006).

The highest rate of *Y. pseudotuberculosis* infections has been found in children aged 8-15 years and 1-3 years in Europe and the Far East, respectively (Tsubokura et al. 1989). In Finland, the highest incidence has been among schoolchildren aged 7-18 years (Huovinen et al. 2006). The incubation period for acute gastrointestinal symptoms has been shown to be 4-18 (median 8) days and the duration of illness 1-3 weeks (Jalava et al. 2006, Rimhanen-Finne et al. 2008). In post-infectious complications, the incubation period is about 3 weeks (median 19 days). Post-infectious symptoms can extend from one week to several months (Tertti et al. 1989, Jalava et al. 2006). The infectious dose of *Y. pseudotuberculosis* is unknown. The level of *Y. pseudotuberculosis* in outbreak samples was estimated to be 1×10^3 cfu/g (Jalava et al. 2006)

Although the main clinical manifestation of *Y. pseudotuberculosis* infections in Europe is mostly gastroenteritis, in the Far East (Japan, Far-Eastern Russia and Korea) the clinical disease commonly includes a variety of systemic symptoms, e.g. rash, desquamation, erythema nodosum, erythema multiforme and reactive arthritis (Sato et al. 1983, Inoue et al. 1984, Fukushima et al. 1985b, 1998, 2001). Also nephritis and acute renal failure have been reported (Kobayashi et al. 2000). The Far-Eastern scarlatine-like fever is a cutaneous manifestation of yersiniosis, including erythema nodosum and erythema multiforme. *Y. pseudotuberculosis* has been reported to cause outbreaks and sporadic systemic infections, namely Izumi fever and Kawasaki syndrome (mucocutaneous lymph node syndrome), which are important illnesses of children in Japan (Sato et al. 1983, Baba et al. 1991).

In most cases, the gastrointestinal infection is self-limiting and no antimicrobial therapy is needed. No relationship was found between antimicrobial use and post-infectious symptoms when sulpha, amoxicillin or erythromycin was used to treat acute symptoms of a *Y. pseudotuberculosis* O:1b outbreak in Canada (Press et al. 2001). In more severe infections, including systemic infection, bacteraemia and infections of patients with compromised immune systems, antimicrobials may be useful. Ofloxacin and doxycycline, which have good intracellular diffusion, are effective *in vivo* in treating systemic *Y. pseudotuberculosis* infections in mice (Lemaitre et al. 1991).

2.6.3. Pathogenesis

Y. pseudotuberculosis is a highly invasive pathogen of epithelial cells that binds and crosses the intestinal epithelium in the terminal ileum. The bacterium initially infects the Peyer's patches by translocating across M-cells on the intestinal mucosa (Marra and Isberg 1997). Attachment to the intestinal mucosa and uptake into M-cells requires adhesin and invasin proteins. Chromosomally encoded Inv protein has been demonstrated to be the most efficient factor promoting cell entry of *Y. pseudotuberculosis*. Binding of Inv occurs especially during the early stage of infection (Isberg and Falkow 1985, Yang and Isberg 1993, Marra and Isberg 1997). YadA of *Y. pseudotuberculosis* also have adhesion and invasin activity; it has shown to promote tight adhesion to and high efficiency uptake into human cells, independently of invasin (Eitel and Dersch 2002, Rosso et al. 2008). Receptors for Inv and YadA are members of the β_1 integrin family of cell adhesion molecules in M-cells (Isberg and Leong 1990, Heisel and Dersch 2006). The expression of invasin factors is thermally regulated; Inv is expressed maximally at 20-25°C, whereas the outer membrane protein YadA is expressed only at 37°C (Nagel et al. 2001, Eitel and Dersch 2002). O-antigen is required for colonization of host tissues (Skurnik et al. 1999). Strains not producing O-antigen (rough) were unable to colonize Peyer's patches or efficiently invade epithelial cells (Mecsas et al. 2001). Ail protein of *Y. pseudotuberculosis* has no adhesion or invasion capacity, but it promotes resistance to complement-mediated killing (Bliska and Falkow 1992).

After colonization of Peyer's patches and local lymphoid follicles, *Y. pseudotuberculosis* can spread in the lymphatic system and to other tissues, such as the kidney and liver, where they remain exclusively extracellular adherent and replicate outside the host cell (Simonet et al. 1990). Colonization can result in destruction and abscess formation in Peyer's patches and lymphadenitis in mesenteric lymph nodes, causing abdominal pain and inflammatory response (Tertti et al. 1989, Sakellaris et al. 2004). The ability to survive in lymphoid and other tissues is associated with the presence of pYV. Plasmid mediates the expression of outer membrane proteins YadA and Yops, which play a major role in yersinial virulence (Cornelis 2002). The involvement of YadA and Yops is multifactorial; they have been associated with preventing phagocytosis, promoting cytotoxicity and activating the immune response to establish infection (El Tahir and Skurnik 2001, Navarro et al. 2005).

The yersinia superantigen YMPa is mainly responsible for the systemic, severe clinical symptoms caused by the Far East type and the difference in clinical manifestation of *Y. pseudotuberculosis* infection between the Far East and Western countries. YMPa mediates activation of the immune system by direct activation of human T-cells (Uchiyma et al. 1993, Abe et al. 1997). The *ypm*A in the chromosome of *Y. pseudotuberculosis* responsible for the production of YMPa is frequently present in all of the dominating

clinical strains of *Y. pseudotuberculosis* belonging to serotypes O:1-O:5 in the Far East. In the European serotypes O:1 and O:3, which dominate in human infections, YMPa has not been detected (Yoshino et al. 1995, Fukushima et al. 2001).

Post-infectious clinical symptoms, such as arthritis and erythema nodosum, are relatively common after acute *Y. pseudotuberculosis* infection (Tertti et al. 1989, Hannu et al. 2003, Jalava et al. 2006). Primary infection may pass with very mild symptoms or may even be symptomless, and its severity is not at all related to the severity of the later reactive arthritis. Human leukocyte antigen, HLA-B27 had an association to appearance of reactive arthritis (Hannu et al. 2003, 2006). HLA-B27-positive individuals often have more severe arthritis symptoms and a greater tendency for chronicity than those who are negative (Leirisalo-Repo and Suoranta 1988, Yli-Kerttula et al. 1997). The pathogenesis is not fully understood, and the joint fluid is mostly defined as sterile. Bacterial antigens, including lipopolysaccharide, may be present intra- or extracellularly, but how they travel from mucosal tissue into joints remains unclear (Viitanen et al. 1991).

2.6.4. *Y. pseudotuberculosis* outbreaks

Most of the *Y. pseudotuberculosis* cases reported have been sporadic; however, several food- and waterborne outbreaks have occurred since the 1980s in Finland, Japan and Russia (Table 4). In recent years, *Y. pseudotuberculosis* has emerged as an outbreak-associated pathogen in Finland and Russia (Table 4).

Two outbreaks were investigated in Finland during the 1980s whose aetiology remains unknown (Tertti et al. 1984, 1989). While the source of the infection was suspected to be fresh produce, this was not confirmed. From 1997 to 2008, nine *Y. pseudotuberculosis* outbreaks have occurred in Finland (Table 4). In all nine outbreaks, the source of the infection was suspected or confirmed to be domestic fresh produce. The latest five outbreaks have been caused by consumption of contaminated carrots (Takkinen et al. 2004, Jalava et al. 2006, Evira 2007, Rimhanen-Finne et al. 2008, NPHI 2008a). The contaminated carrots were of domestic origin and stored at cold temperatures (1-2°C) for several months prior to consumption.

In Japan, use of unchlorinated water has been an important source of outbreaks in the 1980s (Inoue et al. 1988a). The majority of *Y. pseudotuberculosis* outbreaks have been among children eating lunch at schools or day-cares in Finland (Tertti et al. 1989, EVI 1999, Hallanvuo et al. 2003, Jalava et al. 2004, 2006, Evira 2007, Rimhanen-Finne et al. 2008, NPHI 2008a), Japan (Inoue et al. 1984, Tsubokura et al. 1989) and Russia (ProMED 1999, 2005, 2007, Smirnova et al. 2004).

Four outbreaks of *Y. pseudotuberculosis* serotype O:3 and six outbreaks of serotype O:1 have been reported in Finland in 1997-2001 and in 2001-2008, respectively. In the

2001 outbreak, serotypes O:1 and O:3 were simultaneously involved. Serotypes O:4b and O:5a have dominated in both food- and waterborne outbreaks in the Far East (Table 4).

2.7. Transmission routes of *Y. pseudotuberculosis* infections

Y. pseudotuberculosis occurs in the environment and has been isolated from a wide variety of wild and domestic animals (Fukushima 1989a 1990a, 1990b, 1991, Inoue et al. 1988b, 1991, Tsubokura et al. 1989, Nikolova et al. 2001). *Y. pseudotuberculosis* is considered a possible foodborne bacterium acquired fecal-orally through contaminated food or water, but the evidence for this is limited. Direct contact with an infected animal or an environmental source is another potential cause of human infection (Vincent et al. 2008). However, the transmission routes remain mostly unclear (Fukushima et al. 1994b, 2001, Vincent et al. 2008).

The *Y. pseudotuberculosis* serotypes found in human infections have also been isolated from production animals, especially pigs (Table 3). Pigs might have a role as a source in human infections, although this link has not yet been confirmed. Indistinguishable serotypes (Fukushima et al. 1989b, 1990b) and genotypes (Fukushima et al. 1994b, Laukkanen et al. 2008) of *Y. pseudotuberculosis* strains isolated from pigs and carcasses at the same slaughterhouse indicate that transmission of *Y. pseudotuberculosis* from pigs to carcasses during the slaughter process can occur. A high contamination level of *Y. pseudotuberculosis* in live pigs predisposed the carcasses to contamination (Laukkanen et al. 2008). A positive finding of *Y. pseudotuberculosis* in pork at processing and retail levels suggested the transmission of *Y. pseudotuberculosis* from contaminated carcasses to pork during further processing (Fukushima 1985, Fukushima et al. 1997). Based on the serotyping (Tsubokura et al. 1982, 1984, Fukushima et al. 1985b) and genotyping results (Fukushima et al. 1994b) of *Y. pseudotuberculosis* isolates from humans, pigs and pork, contaminated pork has been proposed as a possible source of *Y. pseudotuberculosis* human infections in Japan.

Contaminated fresh produce has been suspected to have a role as a vehicle of *Y. pseudotuberculosis* outbreaks (Table 4). However, in most of the outbreaks, the source of infection has not been confirmed. In two outbreaks in Russia, identical REAP patterns of *Y. pseudotuberculosis* have been shown among isolates from patients and vegetables (cabbage, carrot, onion, beets), kitchen tables and utensils, and rodents (Fukushima et al. 1994b, 1998). In Finland, fresh produce recently linked to *Y. pseudotuberculosis* outbreaks includes domestic carrots (Table 4). In microbiological and trace-back investigations, the outbreak strain of *Y. pseudotuberculosis* serotype O:1b was isolated from carrots, soil contaminated by carrot residues in storage, and washing and peeling equipment on a production farm. The exact mechanism of initial contamination of carrots could not be

clarified, but the roles of wild animals, contaminated soil and irrigation water have been discussed (Jalava et al. 2006, Evira 2007, Rimhanen-Finne et al. 2008). In a recent study, carrots had a potential association with contamination by a shrew (*Sorex araneus*) (Kangas et al. 2008). All carrot outbreaks occurred in the late spring only, after eating cold-stored carrots, which were peeled and grated before consumption. Possibly, the initial contamination level in fresh carrots is low, but prolonged cold storage over the winter (up to 10 months at 1-2°C) may favour the growth of psychrotrophic *Y. pseudotuberculosis* (Rimhanen-Finne et al. 2008). It remains unclear whether the environment (soil and irrigation water), hygiene conditions during harvesting, packing, storage and shipping or further processing at vegetable processing plants and storage in kitchens also have a role in the contamination and multiplication of *Y. pseudotuberculosis*. The growth of *Y. pseudotuberculosis* has not been studied in grated carrots, but in sliced cabbage and radish, *Y. pseudotuberculosis* was shown to multiply from 10- to 1000-fold in 2-7 days at 5°C (Ohsone et al. 1999).

Drinking unchlorinated water from wells, springs and streams has been linked to several *Y. pseudotuberculosis* outbreaks and infections in Japan and Korea (Fukushima et al. 1988, Inoue et al. 1988a, Sato and Komazawa 1991, Sunaharu et al. 2000, Han et al. 2003). Wild animals have regularly been found to harbour the same serotypes of *Y. pseudotuberculosis* found in water and humans (Fukushima et al. 1990a, 2001, Fukushima and Gomyoda 1991, Inoue et al. 1991). Untreated water contaminated with faeces of wild animals has been considered an important transmission route in these areas.

Transmission has also been reported through direct contact with an infected animal or its excrement in a family outbreaks (Randall and Mair 1962, Fukushima et al. 1989a). Family pets, such as dogs and cats, are potential reservoirs for *Y. pseudotuberculosis* (Fukushima et al. 1985a). Based on genotyping findings of isolates from pets and infected people, clinically healthy cats and dogs are suggested to be a direct source of human infections in Japan (Fukushima et al. 1994).

3. Aims of the study

This study was conducted to investigate *virF*-positive *Y. pseudotuberculosis* in domestic and wild animals and in the environment using different isolation and identification methods. Specific aims were as follows:

1. to compare different isolation methods of *Y. pseudotuberculosis* (I-IV),

2. to determine the prevalence of *virF*-positive *Y. pseudotuberculosis* in pig tonsils and faeces and in the pig house environment (I, II),

3. to determine the prevalence of *virF*-positive *Y. pseudotuberculosis* in wild birds and and on farms producing iceberg lettuce (III, IV),

4. to evaluate the genetic diversity of isolated *Y. pseudotuberculosis* strains by PFGE (I-IV),

5. to investigate and trace the source of a *Y. pseudotuberculosis* outbreak (IV),

6. to characterize *Y. pseudotuberculosis*-like strains (V).

4. Materials and methods

4.1. Sampling (I-IV)

Tonsil samples (n=425) were collected from 7 different slaughterhouses in various parts of Finland from June 1999 to March 2000 in Study I (Table 5).

Table 5. Samples investigated in Studies I-IV.

Source	Sample	No. of samples	Study
Fattening pig	Tonsils	210	I
	Faeces	107[1]	II
Sow	Tonsils	215	I
	Faeces	11[1]	II
Piglet	Faeces	37[1]	II
Boar	Faeces	6	II
Cat	Faeces	3	II
Dog	Faeces	3	II
Rabbit	Faeces	1	II
Bird	Faeces	468	III
Deer	Faeces	12	IV
Farm	Floor	37[2]	II
	Trough	41[2]	II
	Air	38	II
Environment	Soil	73	IV
	Sludge	21	IV
	Compost	4	IV
	Water	39	IV
	Water pipe	22	IV
Food	Iceberg lettuce	128	IV

[1] Pooled faecal samples, each sample containing faeces from five animals.
[2] Pooled envirnonmental samples, each sample containing five sampling pads.

In Study II, 8 farms from 5 slaughterhouses that were *Y. pseudotuberculosis* positive in Study I were investigated. The 8 farms located in southwestern and western parts of Finland were visited 6-10 months later. Altogether 155 pooled (piglets under 8 weeks, fattening pigs of 2-6 months and sows) and 13 individual faecal samples (boars, cats, dogs and a rabbit) and 78 pooled pig house environmental samples (pen floors and trough surfaces) and 38 air samples were collected in 2000 (Table 5). Faecal samples from five pigs were pooled together and transported in 10 ml of cold PMB (phosphate buffered

saline according Nordic Committee on Food Analysis [NCFA 1996]. Faecal material from pigs was collected using sterile dry cotton wool swabs, and premoistened cotton wool pads were used for environmental sampling. Four to six cefsulodin-irgasan-novobiocin (CIN, Yersinia Selective Agar Base, Oxoid, Basingstoke, UK) agar media, left uncovered for 5 min, were used to collect air samples from each pig house.

In Study III, 468 faecal samples, representing 57 species of passerine birds, were analysed. Birds were captured on two different islands in the Baltic Sea in southeast Sweden: the Ottenby Bird Observatory on the southernmost point of Öland Island (spring and autumn migration from March to November 2000) and the coastal shore of the southern tip of Gotland Island (spring migration in April 2000). Different approaches were used to obtain faecal samples, depending on the size of the bird: 1) small birds were put into a box with a clean sheet of paper at the bottom, 2) large birds (> 250 g) were sampled by using sterile dry cotton swabs and 3) samples from barnacle geese were taken by collecting fresh droppings with sterile cotton swabs. Faecal samples were placed in a charcoal transport medium (Transwab, BioDisc, Solna, Sweden) and transported to the laboratory at 4-8°C within 2-5 days.

In Study IV, a total of 299 samples, including samples from iceberg lettuce (n=128) and environmental samples from soil (n=73), sludge (n=21), compost (n=4), irrigation water (n=39) and surface samples from the inside of irrigation water pipes (n=22), and samples from deers (n=12), were collected from four farms on the Ahvenanmaa Islands (Table 5). These four farms were implicated in the trace-back investigation after the *Y. pseudotuberculosis* outbreak. The environmental sampling was conducted from May to November 1999, and again a year later from June to October 2000. Farms were inspected for sanitary conditions and water quality, and the farmers were interviewed about lettuce growing, irrigation and harvesting handling practices during distribution. Samples from roe deer (*Capreolus capreolus*) were obtained from hunters on the Ahvenanmaa Islands during the 2000 hunting season. Human stool specimens from patients with acute gastroenteritis were routinely tested for *Yersinia* in addition to *Salmonella, Shigella* and *Campylobacter* spp. in local clinical microbiology laboratories. A *Y. pseudotuberculosis* case was defined as a culture-confirmed *Y. pseudotuberculosis* O:3 infection from 15 October to 6 November 1998 in laboratory-based surveillance.

4.2. Strains of *Yersinia* spp. (V)

Three *Y. pseudotuberculosis*-like strains were isolated from iceberg lettuce, water and soil samples in Study IV. The strains were initially identified as *Y. pseudotuberculosis* by the API 20E system. However, as these strains could not be serotyped by serotype O:1-O:6-specific antisera, they were further characterized together with 17 other sucrose-negative

Yersinia strains. These strains belonged to 5 *Yersinia* species, including *Y. aldovae* (reference strain CIP 103162), *Y. aleksiciae* (reference strain DSM 14987), *Y. kristensenii* (reference strains CIP 80.30 and ATCC 33639), *Y. pseudotuberculosis* (reference strains CCGU 5855, CIP 55.86, CIP 55.87 and CIP A119, 4 pig strains from Study I, a bird strain from Study III, a lettuce strain from Study IV, a bird strain isolated in Finland in 1998) and *Y. enterocolitica* (2 pig strains isolated in Finland in 1995).

4.3. Isolation of *Y. pseudotuberculosis* (I-IV)

4.3.1. Sample preparation (I-IV)

One 10-g sample of pig tonsil tissue was homogenized in 90 ml of trypticase soya broth (TSB, Difco, Detroit, MI, USA) and another 10-g sample was homogenized in 90 ml of PMB (buffered peptone saline supplemented with 1% mannitol and 0.15% bile salts) for 1 min in a stomacher blender in Study I. In Study II, from tubes containing fecal material in 10 ml of PMB, 100-μl samples were subcultured on CIN agar plates, and the tubes were then placed in the cold (4°C) for cold enrichment. The pooled cotton wool pads moistened with 10 ml of PMB used for environmental sampling were transferred to 90 ml of PMB. In Study III, 5 ml of PMB enrichment broth was added to each charcoal transport medium before direct plating on CIN agar media. In Study IV, a 10-g sample of soil, sludge, compost, and deer faeces and lymph nodes were homogenized in 90 ml of TSB and PMB. The whole iceberg lettuce head was homogenized with PMB in a dilution ratio of 1:10. The cotton wool pads moistened with 10 ml of PMB used for sampling inside surfaces of irrigation water pipes were transferred to 90 ml of TSB and PMB. Water samples of 500 ml were filtered through a 0.45-μm membrane (Millipore, Bedford, MA, USA) and placed into 90 ml of TSB and PMB enrichment broth.

4.3.2. Isolation methods (I-IV)

Several culture methods were compared to find the most productive isolation method for *Y. pseudotuberculosis* in animal, food and environmental samples. The following methods were used: (1) direct plating of 100 μl of homogenate onto CIN agar (I-IV), (2) overnight enrichment in TSB (I, IV) (3) selective enrichment in modified Rappaport broth (MRB, Merck, Darmstadt, Germany) (I, IV) and (4) cold enrichment in PMB (I-IV).

In overnight enrichment, TSB homogenate was incubated at 22°C for 16-18 h before streaking a 100-μl sample onto a CIN agar plate. For selective enrichment, 100 μl of

overnight TSB enrichment broth was inoculated into a 10 ml of MRB and incubated for 3 days at 25°C. In cold enrichment, the PMB homogenates were incubated at 4°C for 7, 14 and 21 days. Alkali treatment, where 0.5 ml of the sample was mixed with 4.5 ml of 0.25% KOH solution for 20 s before being streaked onto CIN agar, was used after 14 days of cold enrichment (I-IV).

CIN agar was used to isolate *Y. pseudotuberculosis* after every enrichment step in Studies I-IV. Sorbitol MacConkey agar plates (SMAC, Difco, Detroit, MI, USA) were used for cultivation of samples after 14 days of cold enrichment followed by KOH treatment (II). Inoculated CIN and SMAC agar plates were incubated at 30°C for 18-20 h and then further at 22°C for 24 h. One to five suspect small (<1 mm) colonies with typical "bull's eye" appearance (dark red centres surrounded by an outer transparent zone) on CIN agar and small, colourless colonies on SMAC agar were streaked onto blood agar plates for pure culture.

4.4. Identification of *Y. pseudotuberculosis* (I-V)

4.4.1. Identification with biochemical reactions (I-V)

One colony from pure culture on the blood agar was inoculated onto a urea agar slant (Difco) and incubated for 1 day at 30°C. Isolates showing urea hydrolysis were further identified using an API 20E system (BioMérieux, Marcy l'Etoile, France) with incubation at 25°C for 18-20 h (I-V). *Y. pseudotuberculosis* isolates were identified on the basis of Voges-Proskauer and citrate tests and acid production reactions of sorbitol, melibiose, sucrose and rhamnose. Due to the temperature dependence of the Voges-Proskauer test, the API 20E system was incubated at 25°C instead of 37°C. The strains were identified using the database provided by the manufacturer (APILAB V4.0, BioMeriéux). Sucrose-negative *Yersinia* strains were further tested for xylose, trehalose and salicin fermentation, esculin hydrolysis and tween esterase (I-III, V), performed according to the International Organization for Standardization (ISO 2003).

4.4.2. PCR analysis (II, V)

For molecular identification of *Y. pseudotuberculosis* isolates within the genus, *inv*-gene on the chromosome of *Y. pseudotuberculosis* was detected by PCR. The PCR assay targeting a 295-bp DNA fragment of the *inv*-gene was used according to Nakajima et al. (1992), with the modifications described in Studies II and V. Briefly, 3-5 colonies from

pure culture were suspended in 100 µl of water and boiled for 10 min to release DNA. The lysate was shortly centrifuged and 2 µl of the supernatant was used as a template in the PCR. The 50-µl reaction mixture contained 1 U of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland), 200 µM of each deoxynucleotide triphosphate (dNTP mix, Finnzymes) and 0.3 µM of each primer (Pharmacia Biotech, Vantaa, Finland). The amplification protocol was the following: initial denaturation at 94°C for 1 min, 29 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 70°C for 2 min, followed by a final extension at 70°C for 5 min.

The oligonucleotide primers based on the sequences of the *inv* (Table 6) according to Nakajima et al. (1992) were used for PCR, performed in a 16-well PTC-150 thermal cycler (MJ Research, Watertown, MA, USA). The size of the amplified PCR product was visualized in 0.8% agarose gel (SeaKem Gold, FMC Bioproducts, Rockland, Maine, USA) relative to DNA molecular weight marker VI (Boehringer Mannheim GmbH, Mannheim, Germany).

Table 6. Primers used in Studies I-IV.

Target gene		Sequence (5'->3')	Study	Reference
Single PCR				
inv	F[1]	TAAGGGTACTATCGCGGCGGA	II, V	Nakajima et al. 1992
	R[2]	CGTGAAATTAACCGTCACACT		
virF	F	TCATGGCAGAACAGCAGTCAG	I-III, V	Wren and Tabaqchali 1990
	R	ACTCATCTTACCATTAAGAAG		
Multiplex PCR				Bogdanovich et al. 2003
gmd-fcl	F	TCAAGATCGCCATGAGAC	V	
	R	AGGTTCATTCGTTGGTTC		
ddhC-prt	F	CGCATAGAAGAGTTTGTTG	V	
	R	CTTTCGCCTGAAATTAGAC		
manB	F	GCGAGCCATAACCCAATAGAC	V	
	R	GCCACCCATCAAATTCCATAC		
abe	F	AGAATAGTTCTGACTGGAGGAAG	V	
	R	TCAGGAGCCATTACCTCATC		
wbyL	F	TTGGAGAAACAAACCTATCTGG	V	
	R	TTTGCATAAAAACGACATAGGC		
wbyH	F	CGTTATCCCAAAAAAGAGG	V	
	R	ATGGGAGACGCTTGTGATG		

Table 6. Continued.

Target gene		Sequence (5'->3')	Study	Reference
ddhA-B	F	TGTCGCCTAAAGTTATCG	V	
	R	CGAATATCACCGATTTCC		
wbyK	F	CCGATTACCAGATTTTGAC	V	
	R	CAAAATTCTTATAACCACCACG		
wzx	F	GAAATTCGCATGTAAAGCTATTG	V	
	R	GAACCTAGACTTACCACCCCCAAC		
Additional double PCRs				Bogdanovich et al. 2003
wzz-gsk	F	GAAAAATACAGCGAGCAG	V	
	R	GAYTTGCGYTTACCAGGAAATTTCATTG		
hemH-ddhD	F	CAATCCAATGAAGAGTCAG	V	
	R	CCCTATGACATAAAAACCC		
Long-range				Bogdanovich et al. 2003
hemH-gsk	F	TGGAAGAAATMAAAGARCAAAATGAGAG	V	
	R	GAYTTGCGYTTACCAGGAAATTTCATTG		

[1]Forward
[2]Reverse

4.5. Bio- and serotyping (I-V)

Subtyping of the confirmed *Y. pseudotuberculosis* isolates into 4 biotypes was based on reactions to citrate, melibiose and raffinose according to Tsubokura and Aleksic (1995) (Table 2).

   All isolates were inoculated on the slant of agar tubes, incubated at 25°C, and the reactions were read within 2 days. The isolates were serotyped with slide agglutination using commercial O:1-O:6 antisera (Denka Seiken, Tokyo, Japan) in all studies.

   In Study V, a multiplex PCR method according to Bogdanovich et al. (2003) was used for further serotyping of *Y. pseudotuberculosis*–like strains. The multiplex PCR assay allows distinction of all 21 known *Y. pseudotuberculosis* sero- and subserotypes in two PCR runs. Nine sets of oligonucleotide primers targeting the *Y. pseudotuberculosis* O-antigen gene cluster genes (*gmd-fcl, ddhC-prt, manB, abe, wbyL, wbyH, ddhA-B, wbyK, wzx)* between *hemH* and *gsk* were used in the first run. This allows distinction of 18 O-

genotypes. Three serotypes, O:7, O:9 and O:10, require additional double PCR with two sets of oligonucleotide primers based on the sequences of *wzz-gsk* and *hemH-ddhD* (Table 6).

4.6. Virulence tests (I-III, V)

4.6.1. Phenotypic methods (I-III, V)

Pathogenicity of all *Y. pseudotuberculosis* isolates was determined by testing for calcium dependence and Congo red absorption with Congo red-magnesium oxalate agar (CR-MOX) (Riley and Toma 1986) (I-III, V). The strains from blood agar were plated onto CR-MOX agar plates and incubated at 37°C for 24 h. Pathogenic isolates of *Y. pseudotuberculosis* carrying the pYV have an ability to absorb Congo red and show calcium-restricted growth at 37°C. On CR-MOX agar plates, pYV-bearing strains (CR-MOX-positive) grew as small (pinpoint) orange-red colonies. The CR-MOX-negative isolates grow as large colourless colonies.

Pyrazinamidase activity was tested by the method of Kandolo and Wauters (1985) and performed according to ISO (2003) (I-III, V). The strains were inoculated on the slant of pyrazinamidase agar tubes, which were incubated at 25°C for 48 h. One millilitre of 1% freshly prepared ferrous ammonium sulphate solution was then flooded onto the slant. Non-pathogenic strains show a pyrazinamidase-positive reaction, manifesting as a red colour after 1-5 min. Pathogenic strains giving a negative reaction do not change the colour of the agar.

4.6.2. Detection of *virF* gene by PCR (I-III, V)

The pathogenicity of the *Y. pseudotuberculosis* isolates was studied with a PCR targeting the *virF* gene on the virulence plasmid by following the modified protocol of Nakajima et al. (1992). The modifications of the methods are described in Section 4.5.2. The sequences of the oligonucleotide primers were based on the sequences of the *virF* according to Wren and Tabaqchali (1990) (Table 6). Primes were used at concentrations of 0.3 μM in a total volume of 50 μl of the reaction mixture. The size of the amplified product was 591 bp visualized in 0.8% agarose gel.

4.7. PFGE analysis (I-V)

4.7.1. DNA preparation (I-V)

DNA preparation and isolation was done according to Björkroth et al (1996), with the modifications described by Fredriksson-Ahomaa et al. (1999) (I-III, V). In brief, a single colony grown on blood agar was inoculated into 5 ml of 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, MA, USA). The plugs were lysed for 3 h in 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 DNA isolation was completed with a single wash at 50°C with 2-h ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 µg/ml proteinase K) (I) and 1-h ESP (proteinase K was replaced with 100 µg/ml pronase) (II, III, V). The plugs were placed in fresh ESP solution and stored at 4°C. Before digestion, the plugs were washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA). Proteinase K/pronase was inactivated with 1 nM Pefablock SC (AEBSF, Roche, Mannheim, Germany), and the plugs were further washed two times with TE buffer.

In Study IV, bacterial cells grown overnight on nutrient agar plates at 37°C were suspended in 1,200 µl of TEN (0.1 M Tris-HCl, 0.15 M NaCl, 0.1 M EDTA [pH 7.5]) to an optical density at 600 nm of 0.100–0.150. Plug preparation and restriction digestion was done according to Lukinmaa et al. (1999). An additional overnight incubation with lysozyme and RNase (20 mg/ml and 0.02 mg/ml, respectively, in EC buffer [6 mM Tris-HCl, 0.1 M EDTA, 1M NaCl, 0.5% Brij 58, 0.2% Na-deoxycholate, 0.5% lauroylsarcosine, all w/v]) at 37°C, prior to proteinase K incubation (0.15 mg/ml in ES buffer [0.5 M EDTA, 1% *N*-lauroylsarcosine]) at 57°C was found to be necessary.

4.7.2. Macrorestriction analysis (I-V)

Three restriction enzymes *Not*I, *Xba*I and *Spe*I were chosen to test the cleavage of chromosomal DNA of *Y. pseudotuberculosis* isolates. Restriction endonuclease digestion was performed according to the manufacturer's instructions. DNA was digested with 6 U of *Not*I (New England Biolabs) (I-III, V), 3 U of *Spe*I (New England Biolabs) (I-III) and 4 U of *Xba*I restriction enzymes (New England Biolabs) (I, II). In Study IV, 10 U of

restriction enzymes *Spe*I, and *Not*I (Roche) were used. Electrophoresis was performed on 1 % SeaKem ME agarose gels (FMC BioProducts) in a 0.5 × TBE buffer (Amresco, Solon, OH, USA) at 12°C at 200 V with a Gene Navigator system (Pharmacia, Uppsala, Sweden) (I-III, V). Interpolation protocols ramping from 1 to 18 s for 20 h for *Not*I (I-III, V) and 1 to 15 s for 18 h for *Spe*I (I-III) and *Xba*I (I, II) were used. In Study IV, electrophoresis was run in 0.5 × TBE buffer on 1.2% agarose gel (Pronadisa D-5, Hispanlab, Madrid, Spain) with CHEF Mapper system (Bio-Rad Laboratories, Richmond, CA, USA) in running conditions of 7 to 15 s, 30 h, 6 V/cm, 120°, 14 °C.

Gels were stained for 30 min in 1 litre of running buffer containing 50 µl of ethidium bromide (10 mg/ml) after electrophoresis. The gels were destained in pure running buffer until an appropriate contrast was obtained. PFGE marker (New England Biolabs) was used for fragment size determination. The gels were photographed using 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 larger than 50 kb was observed.

4.8. Ribotyping (V)

For ribotyping, DNA was extracted according to Pitcher et al. (1989), as modified by Björkroth and Korkeala (1996). DNA of 8 µg was cleaved with *Hind*III and *Eco*RI (New England Biolabs) according to the manufacturer's instructions. Agarose gel electrophoresis, Southern blotting (Vacugene, Pharmacia, Uppsala, Sweden) and hybridization were performed according to Björkroth and Korkeala (1996). The cDNA probe was reverse-transcribed (AMV-RT, Promega, Madison, WI, USA) from *Escherichia coli* 16S and 23S rRNA (Blumberg et al. 1991). Detection of the digoxigenin-labelled fragments (ribopatterns) was performed as recommended by Roche Molecular Biochemicals.

The ribopatterns were scanned (ScanJet 4c/T Scanner, Hewlett-Packard, Boise, ID, USA) to BioNumerics software (version 4.5, Applied Maths, Sint-Martens-Latem, Belgium) as tiff files. The patterns were normalized based on the mobility of the standards, and a similarity matrix was created. The similarity between all pairs was expressed by Dice coefficient correlation, and UPGMA (unweighted pair-group method using arithmetic averages) clustering was used for the construction of the dendrogram.

4.9. Characterization of the O-antigen gene cluster (V)

4.9.1. Long-range PCR (V)

DNA for long-range PCR was isolated using the CTAB procedure (Ausubel et al. 1987). The O-antigen region between *hemH* and *gsk* was amplified in all three sucrose-negative *Y. pseudotuberculosis*–like strains by using the Expand^{TM} Long Template PCR System (Roche), with some modifications. In brief, mastermix 1 included dNTP (final concentration 500 μM), primers (500 nM), genomic DNA as template (400 ng), additional MgCl$_2$ (2.5 mM) and water, and mastermix 2 included PCR buffer 3 (10 x concentrate), DNA polymerases (2.5 units) and water. The final concentration of MgCl$_2$ was 2.6 mM. The long-range PCR amplification protocol applied was the following: (i) initial denaturation at 94°C for 2 min, (ii) 10 cycles of 92°C for 15 s for denaturation, of 62°C for 15 s for annealing and of 68°C for 12 min for elongation, (iii) 20 cycles of 92°C for 15 s for denaturation, of 62°C for 15 sec for annealing and of 68°C for 12 min plus 20 s for elongation for each cycle and (iv) final extension at 68°C for 7 min. The long-range PCR primers used in this study were previously designed and described by Bogdanovich et al. (2003) (Table 6).

The long-range PCR product of a 14-kb *hemH-gsk* region on the O-antigen cluster was restricted by *Eco*RV according to the manufacturer's instructions. One strain (Å125KOH2) was chosen for further typing in Study V since all three strains had indistinguishable restriction patterns for the PCR-amplified *hemH-gsk* fragment.

4.9.2. Nucleotide sequencing and sequence analysis (V)

After the long-range PCR, the *hemH-gsk* PCR product was restricted using *Sau*3AI and *Taq*I enzymes (Promega) according to the manufacturer's instructions. The *Sau*3AI and *Taq*I libraries of the Å125 KOH2 were constructed into pUC19. The libraries were screened by blue/white selection, and plasmids from white colonies were isolated for sequencing analysis. Plasmid DNA was isolated using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany).

Sequencing reactions using vector-specific primers were performed in ABI373A and ABI377 automatic sequencers using the AmpliTaq FS dye terminator kit or Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The obtained sequences were assembled into contigs using the Staden Package (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK), and the sequences were used to design new primers for primer walking to close the gaps between the contigs. The NCBI and EBI databases were searched using

the Blast programs. The 13970-bp sequence data were annotated and submitted to the European Bioinformatics Institute under accession number AJ871364.

4.10. Epidemiological and traceback investigations (IV)

In October 1998, a marked increase in *Y. pseudotuberculosis* isolation was noted in clinical microbiology laboratories in southern Finland. A population-based case-control study was conducted by the National Public Health Institute (KTL) to determine factors associated with *Y. pseudotuberculosis* infections. From a total of 47 defined case-patients identified in laboratory-based surveillance, 38 were selected for epidemiological investigation. Two healthy controls for each case-patient (total 76 controls) were randomly selected from the general population and matched for age, sex and postal code. Trained personnel conducted telephone interviews between 2 and 17 December 1998 using a standard questionnaire. Participants were asked about illness, consumption of 16 different fresh produce and 10 meat products, untreated water, shopping locations and meals eaten outside the home. For case-patients, the questions referred to the 2 weeks before onset of illness, and for controls, the 2-week period before the interview. Mantel-Haenszel matched odds ratios (MORs) and 95% exact confidence intervals (CIs, for categorical variables), Kruskal-Wallis test (for continuous variables) and $\chi 2$ test (for trend) were applied by using Epi Info software (version 6.04, Centers for Disease Control and Prevention).

Four distinct clusters of case-patients were associated with lunch cafeterias. Menus and retail invoices of cafeterias were reviewed for iceberg lettuce purchased since lettuce had been implicated based on the case-control study. Three local distributors, which had sold iceberg lettuce to these cafeterias, were requested to provide bill of lading and shipping records. Four salad farms on the Ahvenanmaa Islands had been delivered iceberg lettuce by a single shipping company, which supplied the three distributors. Farms were visited for sample collection and inspected for sanitary conditions and water quality. Farmers were interviewed about lettuce growing, irrigation and harvesting practices during distribution.

5. Results

5.1. Isolation of *Y. pseudotuberculosis* (I-IV)

Cold enrichment for 14 days followed by alkali (KOH) treatment was the most productive isolation method for all samples when direct plating on selective CIN agar plates, overnight enrichment in TSB, selective enrichment in MRB and cold enrichment in PMB for 7, 14 and 21 days were compared (I-IV). A total of 1476 samples of animal and environmental origin, 81% of those positive, were recovered when this culture method was used (Table 7).

Table 7. Isolation of *Yersinia pseudotuberculosis* by different culture methods in Studies I-IV.

Origin of samples	Study	No. of samples	No. of positive samples	Method					
				No. of positive samples (%)					
				Direct plating	OE[1]	SE[2]	Cold enrichment[3]		
							7 days	14 days[4]	21 days
Tonsils	I	425	8	0	0	0	3 (38)	7 (88)	4 (50)
Faeces	II, III, IV	648	18	7 (39)	0	0	11 (61)	15 (83)	9 (50)
Soil	IV	73	1	0	0	0	0	1 (100)	0
Water	IV	39	1	0	0	0	0	1 (100)	0
Lettuce	IV	128	1	0	0	0	0	0	1 (100)
Other	II, IV	163	1	0	0	0	0	1 (100)	0
Total		1476	31	7 (16)	0	0	14 (45)	25 (81)	14 (45)

[1] OE, overnight enrichment in TSB at 22°C for 16-18 h.
[2] SE, selective enrichment in MRB at 25°C for 3 days.
[3] Cold enrichment in PMB at 4°C for 7, 14 and 21 days.
[4] KOH treatment in 0.25% solution for 20 s before plating.

Y. pseudotuberculosis was not isolated after overnight enrichment and selective enrichment in MRB (I, IV). Direct plating was shown to be useful for faecal samples, but not for other samples. A total of 39% of positive samples from the faeces of pigs and migratory birds were positive after direct plating compared with 0% from tonsils and environmental samples (Table 7). Two weeks' cold enrichment with alkali treatment yielded the highest number of positive samples from all samples (81%), and also when faecal samples were included (83%) (II, III). The *Y. pseudotuberculosis*-positive iceberg

lettuce sample (IV) was found after only three weeks (21 days) of cold enrichment. The small amount of *Y. pseudotuberculosis* in food samples makes it difficult to isolate without prolonged cold enrichment since it is easily overgrown by other bacteria. After 14 days of cold enrichment followed by alkali treatment, SMAC agar plates were comparable with CIN agar plates (II). From CIN and SMAC agar media, 13 and 11 of the 16 *Y. pseudotuberculosis*–positive faecal samples were recovered, respectively. One positive sample was found only on SMAC. However, SMAC agar media were easily overgrown, and *Y. pseudotuberculosis* colonies could not be distinguished from other bacteria growing as colourless colonies. Thus, *Y. pseudotuberculosis* isolates were more easily recovered from CIN agar plates.

5.2. Prevalence of *virF*-positive *Y. pseudotuberculosis* in pig tonsils and faeces and in the pig house environment (I, II)

The prevalence of *virF*-positive *Y. pseudotuberculosis* was investigated in pigs at slaughter and farm levels with a culture method (I, II). In Study I, the mean prevalence of *Y. pseudotuberculosis* in tonsils of fattening pigs at slaughter was 4% (8/210) (Table 8).

Table 8. Prevalence of *virF*-positive *Yersinia pseudotuberculosis* in tonsils and faeces of pigs in Finland.

Origin of samples	No. of samples	No. of slaughterhouses/ farms	No. of positive samples	Prevalence %	Bio-/ serotype
Tonsils	425	7			
Fattening pigs	210		8	4	2/0:3
Sows	215		0	0	ND[1]
Faeces	161	8			
Fattening pigs	107[2]		15[2]	3[3]-14[4]	2/O:3
Sows	11[2]		0	0	ND
Piglets	37[2]		0	0	ND
Boars	6		0	0	ND

[1]Not detected.
[2]Pooled faecal samples, each sample containing faeces from five animals.
[3]Minimum prevalence; only one positive animal in the pooled sample.
[4]Maximum prevalence; all five animals positive in the pooled sample.

In five of the seven positive slaughterhouses, the prevalence of *virF*-positive *Y. pseudotuberculosis* varied from 3% to 10% in tonsils of fattening pigs. All 215 sow tonsil samples tested negative for the bacterium (Table 8).

In Study II, *Y. pseudotuberculosis*-positive pigs in five slaughterhouses were traced back to eight different farms, which were visited 6-10 months after Study I. The distribution and persistence of *virF*-positive *Y. pseudotuberculosis* were investigated in pigs of different ages and in the pig house environment. Five of the eight farms had an indoor, one-site (farrow-to-finish) production system and three farms had an indoor, all-in, all-out production system. Four of the eight farms were found to be *Y. pseudotuberculosis*-positive in Study II. Three of the positive farms had a one-site production system, and one positive farm had an all-in, all-out production system. Prevalence of *Y. pseudotuberculosis* ranged from 5% to 26% on the positive farms with a one-site production system and from 1% to 5% on the positive farms with an all-in, all-out production system. Mean prevalence of *Y. pseudotuberculosis* in fattening pigs (15/107) varied from a minimum of 3% (only one positive animal in the pooled sample) to a maximum of 14% (all five animals positive in the pooled sample) (Table 8). Similar to Study I, all positive samples in Study II were recovered only from fattening pigs. Sows (n=11), piglets (n=37) and boars (n=6) were *Y. pseudotuberculosis*-negative on all 8 farms. The prevalence of *Y. pseudotuberculosis* in fattening pigs was significantly higher than in pigs of other age groups (χ^2, p<0.01). One pooled floor sample from 37 floor samples (3%) tested *Y. pseudotuberculosis*-positive. Other pooled environmental samples, comprising trough (n=41) and air (n=38) samples, were *Y. pseudotuberculosis*-negative. Air samples collected for 5 min on CIN agar media were overgrown after 20 h of incubation at 30°C, and the results could not be interpreted. All pet animals (n=7) studied on the farms were *Y. pseudotuberculosis*-negative.

All *Y. pseudotuberculosis* isolates from tonsils and faeces belonged to serotype O:3 and carried the *virF* gene on the virulence plasmid (I, II). Biotype 2 (I) and biotypes 2 and 3 (II) were involved, the latter in one isolate (Table 8).

5.3. Prevalence of *virF*-positive *Y. pseudotuberculosis* in wild birds (III)

The prevalence of *Y. pseudotuberculosis* was analysed in 468 samples, representing 57 species of birds during spring and autumn migration. The *virF*-positive *Y. pseudotuberculosis* was recovered from three individual birds representing two species of thrushes, the song thrush (*Turdus philomelos*) and the redwing (*Turdus iliacus*). The mean prevalence of *virF*-positive *Y. pseudotuberculosis* in migratory birds was 1% and in thrushes 8%. All *Y. pseudotuberculosis*-positive birds were caught during active spring

migration from apparently healthy individuals. All *Y. pseudotuberculosis* strains were of bioserotype 1/O:2.

5.4. Isolation of *virF* -positive *Y. pseudotuberculosis* from farms producing iceberg lettuce (IV)

The environmental sampling was conducted on four farms suspected of producing *Y. pseudotuberculosis*-contaminated iceberg lettuce. Farms were visited in 1999 and 2000 one and two years after the human outbreak of *Y. pseudotuberculosis* in 1998. By the time the trace-back was completed, no implicated iceberg lettuce was available and snow and ground frost made environmental sampling impossible.

Only one of 128 lettuce samples (1%) was positive for *Y. pseudotuberculosis*. This *virF*-positive *Y. pseudotuberculosis* belonged to bioserotype 1/O:2. All soil (n=73), sludge (n=21), compost (n=4), water (n=39) and waterpipe (n=22) samples were negative. Furthermore, all faecal samples of deer (n=12) were negative.

5.5. Genotyping of *Y. pseudotuberculosis* (I-IV)

All isolates were characterized with PFGE. The PFGE patterns of the *Y. pseudotuberculosis* strains from different sources obtained with *Not*I-digested DNA showed low similarity to each other (Figure 2).
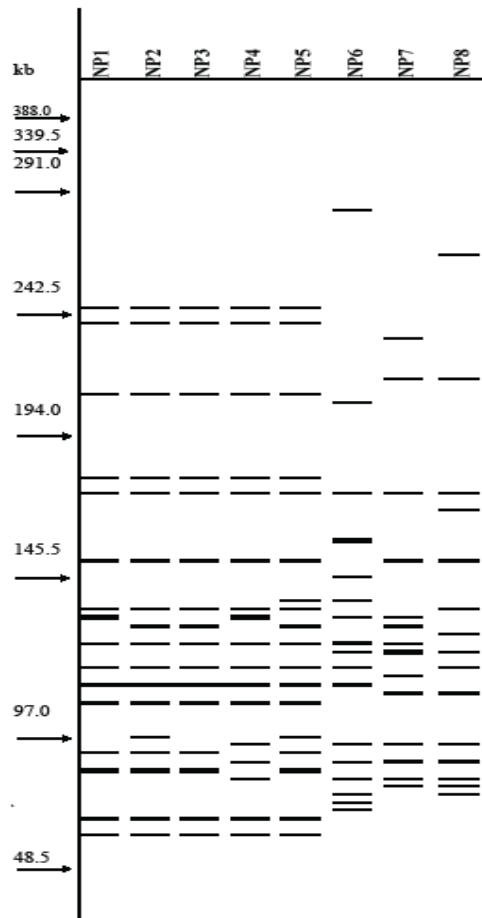
Figure 2. Different *Not*I profiles of *Yersinia pseudotuberculosis* isolates obtained from pigs (NP1-NP5), birds (NP6-NP7) and iceberg lettuce (NP8).

*Not*I enzyme clearly distinguished the isolates from pigs and birds, and from a lettuce sample (I-IV). However, the *Y. pseudotuberculosis* bioserotype 2/O:3 isolates from pigs showed small variations between each other. Five different PFGE patterns were obtained with *Not*I when 83 isolates of pig origin were characterized (I, II). When further digested with *Spe*I and *Xba*I enzymes, seven and two PFGE patterns were obtained from pig isolates, respectively (I, II). One- to six-band differences were observed with *Spe*I and *Not*I, and two-band differences with *Xba*I enzyme. A total of 15 genotypes (gI-XV) were identified by combining the various *Spe*I, *Not*I and *Xba*I digestion profiles of pig isolates (I, II).

Some genotypes obtained with *Spe*I, *Not*I and *Xba*I (genotypes gII-IV and gVI) previously detected in pig tonsils (I) were also present in pig faeces from the same farm several months later (II) (Table 9).

Table 9. Genotypes of *virF*-positive *Yersinia pseudotuberculosis* bioserotype 2/ O:3 isolates from fattening pigs at slaughterhouse and farm levels.

	Study I			Study II	
Slaughterhouse	Animal	Genotype (g)	Farm	Genotype (g)	
A	A26	I	8	ND[1]	
B	B3	II	7	II, XV	
C	C37	III, IV, V	3	IV, VII, XII	
	C40	III, IV	6	ND	
	C45	III	4	III, IV, XIII, XIV	
E	E23	I	5	ND	
G	G11	VI, VII, VIII	2	IV, VI, XII	
	G21	I, X, XI	1	ND	

[1]No positive samples detected.

The most common faecal genotype, gIV, was present in three of the four *Y. pseudotuberculosis*-positive farms, and six months earlier in the tonsils of pigs of one slaughterhouse. However, genotype gI, which was detected in 40% of the *Y. pseudotuberculosis*-positive pigs in slaughterhouses, was not found in faecal samples on farms. Four genotypes, gXII-XV, not present in tonsils of fattening pigs, were obtained from faecal samples. Several *Y. pseudotuberculosis*–positive pigs (4/8) were found to carry more than one (2-3) genotype in tonsils (I). From two to four different genotypes were found on the same farm (II). Overall, more genotypes were obtained from tonsils of pigs in slaughterhouses (n=11) than on faeces on farms (n=9). The *virF*-positive *Y. pseudotuberculosis* strain recovered from the pen floor of fattening pigs was the same genotype (gII) isolated from pigs on this farm.

Two isolates from migratory birds divided into two profiles by PFGE with *Not*I and *Spe*I enzymes (III) were clearly differentiated from each other and from the pig strains and the lettuce strain. The PFGE patterns of lettuce strains did not match the outbreak strain (IV).

5.6. Investigation and trace-back of the source of a *Y. pseudotuberculosis* outbreak (IV)

An outbreak caused by *Y. pseudotuberculosis* O:3 occurred in Finland between 15 October and 6 November 1998. Altogether 47 culture-confirmed cases were identified nationwide. In the epidemiological analysis, only iceberg lettuce was significantly (p<0.01) associated with infections, and consumption of other types of lettuce, fresh produce, water, pork or beef was not associated with illness. Infected persons had eaten in lunch cafeterias of two primary schools and two manufacturing plants. Iceberg lettuce was served in all four cafeterias. In each cafeteria, the trace-back could be narrowed down to a single batch of lettuce. On the basis of trace-back investigations, four farms were possible sources of the contaminated iceberg lettuce sold to the cafeterias.

On all four farms, two or three crops of iceberg lettuce were grown in open fields from May to October 1998. In the fields, untreated water from lakes and man-made ponds was supplied by rainwater, ditches and surface run-off. Animal manure was not used as fertilizer, and no domestic livestock was situated near the fields. Fields were unfenced and wildlife had free access to irrigation water sources and fields. On the Ahvenanmaa Islands, a large roe deer population exists, and large quantities of roe deer faeces were found all over the lettuce fields and around all irrigation water sources. At the farms, harvested lettuce heads had been packed unwashed into 5-kg cardboard boxes and loaded directly into trucks. There was no intermediate storage of the lettuce, and the lettuce was not handled or unpacked until delivery.

5.7. Characterization of *Y. pseudotuberculosis*-like isolates (V)

Three strains isolated from the environment were identified as *Y. pseudotuberculosis* with a likelihood of over 98% by the API 20E system (IV). However, all of the isolates were *inv*- and *virF*-negative with PCR, indicating non-pathogenicity. The environmental strains termed presumptive *Y. pseudotuberculosis*–like strains could not be serotyped with O:1-O:6 antisera or multiplex PCR assay, but belonged to biotype 2.

Using PFGE with *Not*I enzyme and ribotyping with *Eco*RI and *Hind*III enzymes, the *Y. pseudotuberculosis*-like isolates grouped genetically together, and could be differentiated from true *Y. pseudotuberculosis* strains and from strains belonging to other sucrose-negative *Yersinia* species. When the locus between the *hemH* and *gsk* genes carrying the O-antigen gene cluster in one *Y. pseudotuberculosis*-like strain (Å125KOH2) was characterized, it differed from those of known *Y. pseudotuberculosis* serotypes. Eleven genes between the *hemH* and *gsk* were identified and named *wzx, wbcH, wbcG, gmd, fcl, manC, manB, wbcO, wbcP, wbcQ and gne.* Comparison of the nucleotide sequence

showed that the outer core gene cluster of *Y. enterocolitica* O:3 was related most closely to the Å125 KOH2 gene cluster, sharing homologous flanking regions of 74% and 82% sequence identity. Except for the ca. 70% similarity of the *gmd-manB* block to those present in some of the 21 known *Y. pseudotuberculosis* serotypes (Bogdanovich et al. 2003), no similarities were detected. At protein level, homologues with other *Yersinia* spp. were showed to be present in WbcO (>50% identity with *Y. enterocolitica* and *Y. frederiksenii*), WbcP (>58% identity with *Y. enterocolitica* and *Y. frederiksenii*), WbcQ (85-89% identity in *Y. enterocolitica, Y. frederiksenii* and *Y. kristensenii*) and Gne (>85% identity in *Y. enterocolitica, Y. frederiksenii* and *Y. kristensenii*).

6. Discussion

6.1. Isolation of *Y. pseudotuberculosis* (I-IV)

The most efficient isolation method for *Y. pseudotuberculosis* was 14 days of cold enrichment in PMB followed by KOH treatment (I-IV). Using cold enrichment from one to three weeks according to NCFA (1996) for detection of *Y. enterocolitica,* the highest isolation rate of *Y. pseudotuberculosis* was obtained from all sample materials, i.e. tonsils and faeces of asymptomatic carriers and environmental and food samples. The psychrotrophic nature of *Y. pseudotuberculosis* favours its growth during prolonged cold enrichment and alkali treatment to reduce background flora. In our study, *Y. pseudotuberculosis* was not isolated after overnight enrichment in TSB or selective enrichment in MRB used in isolation of human pathogenic *Y. enterocolitica* (NCFA 1996). No standardized reference method exists for detection of *Y. pseudotuberculosis* in clinical, food or environmental samples, and the conventional isolation methods developed for *Y. enterocolitica* have been used to isolate *Y. pseudotuberculosis* with variable sensitivity. Enrichment in TSB or selective enrichment in MRB has been demonstrated to be unsuitable for isolation of *Y. pseudotuberculosis* (I). Direct plating was useful only for faecal samples (II, III), also in clinically healthy animals, but not for other samples with low levels of *Y. pseudotuberculosis* and high levels of background flora (I, IV). Cold enrichment for one to three weeks has most often been used in outbreak investigations (Tertti et al. 1984, Inoue et al. 1988, Nakano et al. 1989, Tertti 1989), and this method is recommended for isolation of *Y. pseudotuberculosis* from food, water and environmental samples by the FDA (2007). It is probably the most productive method available, but several weeks' enrichment is not ideal, especially in food- or waterborne outbreak investigations, when rapid detection of possible sources of infection is essential. Overall, the conventional isolation methods used to recover *Y. pseudotuberculosis* in samples other than clinical samples seem to be time-consuming and rather insensitive (I-IV).

  PCR targeting the *inv*-gene in the chromosome of *Y. pseudotuberculosis* was used to identify *Y. pseudotuberculosis* in pure samples according to Nakajima et al. (1992). The pathogenicity of *Y. pseudotuberculosis* isolates was studied with phenotypic and genotypic methods by targeting the *virF* on the plasmid by PCR according to Wren and Tabaqchali (1990) and Nakajima et al. (1992) (I-V). The phenotypic methods used to show the pathogenic potential of *Y. pseudotuberculosis* isolates, i.e. PYZ and CR-MOX, had variable results, in accordance with the study by Martins et al. (1998). PYZ reactions varied, indicating that this test is not an efficient indicator of the pathogenic potential of *Y. pseudotuberculosis* isolates. CR-MOX had total a correlation, so that all pathogenic *virF*-

positive isolates showed calcium dependence and Congo red absorption. However, PCR was a faster and more reliable method to detect isolates harbouring the virulence plamid, which is essential for full virulence of *Y. pseudotuberculosis*.

6.2. Prevalence of *virF*-positive *Y. pseudotuberculosis* in pig tonsils and faeces and in the pig house environment (I, II)

Y. pseudotuberculosis carrying *virF* was common in clinically healthy pigs (I, II). When studied in tonsils of fattening pigs, the isolation rate varied from 0 to 10% between slaughterhouses, the mean prevalence being 4% (I). This is in accordance with earlier studies in Europe and Japan (Narucha and Westendoorp 1977, Weber and Knapp 1981, Fukushima et al. 1989b) and later in Finland (Laukkanen et al. 2008, Ortiz Martinez et al. 2009). The prevalence of *Y. pseudotuberculosis* varied between slaughterhouses and herds. This may be due to different occurrence of infected herds in different areas.

The positive pigs were traced back to the farm level, where *Y. pseudotuberculosis* was found several months or even one year later. This result shows that the bacteria can survive on farms for long periods between batches. The mean prevalence of *virF*-positive *Y. pseudotuberculosis* was 3-14% in faeces at positive farms. This was higher than findings in previous studies, where the *Y. pseudotuberculosis* recovery rate has been from 0.5% to 5% in faecal samples (Toma and Deidrick 1975, Fukushima et al. 1990b, Shiozawa et al. 1991, Chiesa et al. 1993). In our study, 14% was considered a maximum occurrence due to pooled sampling and preselected farms. Since *Y. pseudotuberculosis* has previously been found to be more common in tonsils than in faeces of pigs (Narucka and Westendoorp 1977, Fukushima et al. 1989), a pooled sampling was chosen in order to investigate a larger number of animals on farms. When investigating pigs of different ages at the slaughterhouse and farm levels, all positive animals were fattening pigs (2-6 months), whereas younger piglets (<2 months) and older animals (i.e. sows and boars) were negative (I, II). The reason for this may be that older animals develop a natural resistance to *Y. pseudotuberculosis*, and suckling piglets are protected by passive immunization (Tizard 2004, Nesbakken et al. 2006).

Two different production systems represented on the farms showed different *Y. pseudotuberculosis* prevalences. *Y. pseudotuberculosis* was more often and in higher quantities found on farms with a one-site production system (farrow-to-finish) than on those with a specialized all-in, all-out system. In both of these systems, pigs of all ages were kept indoors. Laukkanen et al. (2008) found associations between high prevalence of *Y. pseudotuberculosis* and different farm factors, the most important being organic production, where pigs had contact with the outside environment. In our study, on one farm with one-site production, pathogenic *virF*-positive *Y. pseudotuberculosis* was also

recovered from the immediate environment of fattening pigs (I). The isolated strains from a pen flood and from pigs on the same farm represented identical phenotypes and genotypes. This indicates that pigs may become infected not only when in close contact with each other, but also from a contaminated surrounding environment.

In Studies I and II, the only serotype found in pigs was O:3. Serotype O:3 has sporadically been isolated in clinically healthy pigs worldwide, especially in Canada, Germany, Greece and New Zealand (Fukushima et al. 2001), and is the only serotype found pigs in Finland (I, II, Laukkanen et al. 2008, Ortiz Martinez et al. 2009).

Except for one strain of biotype 3 (II), all isolated strains represented biotype 2 (I, II). Since biotyping is seldom used for characterization of *Y. pseudotuberculosis*, limited comparable data exists for different bioserotype combinations from different sources or countries. In other studies, bioserotype 2/O:3 has occasionally been reported in pigs and humans in Germany (Aleksic et al. 1995). In Brazil, 95% of *Y. pseudotuberculosis* strains isolated from animals (pigs, cattle, buffaloes) were bioserotype 2/O:3 (Martins et al. 1998, Martins et al. 2007). These result indicates that pigs could be an important reservoir of *Y. pseudotuberculosis* bioserotype 2/O:3. Serotype O:3 is the most common serotype, followed by serotype O:1, in human infections in Finland (Jalava et al., 2004, Nuorti et al., 2004, NPH I2005), and pigs could be a potential source of human *Y. pseudotuberculosis* infections.

6.3. Prevalence of *virF*-positive *Y. pseudotuberculosis* in wild birds and on farms producing iceberg lettuce (III, IV)

virF-positive *Y. pseudotuberculosis* 1/O:2 was isolated from migratory birds during the spring migration in April (III). The positive findings were from thrushes; two from song thrushes and one from a redwing. Individuals of these bird species spend the non-breeding season in Western Europe, from Spain and Portugal, through to France and extending to the British Isles. Thus, the findings of *Y. pseudotuberculosis* in spring give an estimate of the import of *Y. pseudotuberculosis* from the continental parts of northwest Europe and the British Isles. The low isolation rate is consistent with previous studies (Mackintosh and Henderson 1984, Hamasaki et al. 1989, Fukushima and Gomyoda 1991, Inoue et al. 1991, Cork et al. 1995), where the prevalence of *Y. pseudotuberculosis* has varied from 0.1% to 4.6% in healthy birds. Despite the low prevalence, the positive finding indicates than healthy birds are potential carriers and disseminators of *Y. pseudotuberculosis* over large areas during migration. No *Y. pseudotuberculosis* was isolated in autumn at the beginning of the migration, indicating the higher occurrence of bacteria during stressful conditions, such as a long migration and cold weather.

During the two-year investigation of iceberg lettuce and the growth environment on farms, *virF*-positive *Y. pseudotuberculosis* was isolated from an iceberg lettuce sample (IV). The isolated strain was of bioserotype 1/O:2. The presence of *Y. pseudotuberculosis* organisms in iceberg lettuce indicates that the contamination of lettuce occurred during growth on the farm before distribution. Isolated *Y. pseudotuberculosis* strains from birds and the environment were both of bioserotype 1/O:2. Serotype O:2 is one of the two most commonly isolated serotypes (along with serotype O:1) previously isolated in wild birds by Kageruga et al. (1976), Fukushima and Gomyoda (1991) and Cork et al. (1999). No information exists about the distribution of *Y. pseudotuberculosis* bioserotype 1/O:2 in different countries, except for one study in which bioserotype 1/O:2 was detected in humans in Japan and Germany (Tsubokura and Aleksic 1995). Although the recovered strains was not related to the outbreak strain in genotyping studies, wild birds may be an important reservoir of *Y. pseudotuberculosis* bioserotype 1/O:2. The presence of *Y. pseudotuberculosis* in wild birds and vegetables indicates that these sources cannot be excluded from epidemiological discussions about human yersiniosis.

6.4. Genotyping and molecular epidemiology of *Y. pseudotuberculosis* (I-V)

REAP, ribotyping and PFGE are the genotyping methods that have most commonly been used to characterize *Y. pseudotuberculosis* strains of different origin (Fukushima et al. 1994, Iteman et al. 1995, Voskressenskaya et al. 2005, Martins et al. 2007). PFGE has been shown to be the most efficient in subtyping of *Y. pseudotuberculosis* strains, also strains of the same serotype (Iteman et al. 1995). Three different enzymes, *Not*I, *Spe*I and *Xba*I, were used in our studies (I-V). *Spe*I and *Not*I proved to be suitable enzymes for characterizing *Y. pseudotuberculosis* isolates. With *Not*I and *Spe*I enzymes, more pattern variations were found than with *Xba*I. Isolates of the same serotype cleaved with *Xba*I enzyme were divided into only two groups. Iteman et al. (1995) have characterized *Y. pseudotuberculosis* isolates most efficiently with *Not*I enzyme; in our study, *Spe*I had better discriminatory power in strains of the same serotype (I, II). The differences between the PFGE patterns were clear, especially between strains of different serotypes and origins.

Y. pseudotuberculosis strains isolated from fattening pigs of different batches from different parts of the country over a two-year period represented bioserotype 2/O:3 and had limited pattern variation in PFGE (I, II). A combination of at least two enzymes (*Not*I and *Spe*I) was necessary for efficient characterization of the isolates. Fifteen different genotypes were identified by combining the digestion profiles of three enzymes (*Not*I, *Spe*I and *Xba*I). The same genotypes were found in slaughterhouses and on farms, but

with different frequencies. The most common faecal genotype, gIV, present in 75% of *Y. pseudotuberculosis*-positive farms, was isolated six months earlier in the tonsils of two pigs at one slaughterhouse. On all positive farms, at least one genotype found in tonsils at the slaughterhouse was also isolated at the farm level. This shows that certain *Y. pseudotuberculosis* strains can persist from one batch to another and in the housing environment in close contact with animals. Four of the eight pigs in Study I, were found to harbour several different genotypes in tonsils, and two to four different genotypes were obtained in one pooled faecal sample. This could indicate that the faeces in pooled samples were from more than one positive animal or that there were several infection sources during the rearing. In general, more different genotypes were found in tonsils of pigs in slaughterhouses than in faeces from a single farm. This could indicate a high diversity of *Y. pseudotuberculosis* in tonsils of pigs. *Yersinia* contamination could also occur between infected and uninfected herds, especially during transportation or in holding pens in the slaughterhouse, when mixing the animals (Fukushima et al. 1990). This could also explain the negative findings on half of the farms in Study II. Some genotypes were found on more than one farm, indicating they are geographically more widely distributed than others. Since the same pig can carry several *Y. pseudotuberculosis* strains, more than one isolate should be characterized during the isolation to obtain more information about genetic diversity.

The porcine genotypes differed clearly from genotypes of *Y. pseudotuberculosis* strains isolated from other sources of different serotypes. PFGE analysis with the *Not*I and *Spe*I enzymes divided the strains isolated from two species of birds into two genotypes. Both strains from the song thrushes clustered together and were different from the strain found in the redwing, indicating more than one source of infection. The pathogenic *Y. pseudotuberculosis* strains from birds and iceberg lettuce belonged to bioserotype 1/O:2. However, the genotype of the lettuce strain differed from the isolates from birds as well as from the isolates from pigs and the *Y. pseudotuberculosis* human outbreak strain, indicating a high genetic diversity of *Y. pseudotuberculosis* from different sources. The genotyping results showed that PFGE was a useful tool for comparing *Y. pseudotuberculosis* isolates of the same serotype (I-IV) and of different origins in an outbreak investigation (IV).

6.5. Investigation and trace-back of the source of a *Y. pseudotuberculosis* outbreak (IV)

Y. pseudotuberculosis O:3 caused a widespread outbreak, which was strongly associated with consumption of iceberg lettuce in an epidemiological investigation. Iceberg lettuce was produced domestically and traced back to farm level. This is the first reported *Y.*

pseudotuberculosis outbreak in which a specific food was implicated in an epidemiological study and traced back to the source of contamination. In previous *Y. pseudotuberculosis* outbreaks, contaminated food has been suspected as a source of infections (Inoue et al. 1984, Tertti et al. 1984, Inoue et al. 1988a, Nakano et al. 1989, Tertti et al. 1989, Tsubokura et al. 1989, Pebody et al. 1997, Nowgesic et al. 1999). The bacterium has been isolated in untreated natural water, which has been found to be a source of a *Y. pseudotuberculosis* outbreak in Japan (Inoue et al. 1988a). *Y. pseudotuberculosis* has rarely been isolated from foods, and in outbreaks no source or contamination route of specific food products has previously been confirmed. Controlled epidemiological investigations and genotyping of isolates have not been used in earlier *Y. pseudotuberculosis* outbreak investigations. Trace-backs of possible sources of infections and contamination routes have mainly been based on information about phenotypic characteristics of the *Y. pseudotuberculosis* isolates.

The exact mechanism for contamination of iceberg lettuce remains unknown, but contamination likely occurred on the farm. The Ahvenanmaa Islands had a large population of roe deers introduced in the 1960s. Wild animals had free access to unfenced irrigation water sources and lettuce fields. Large quantities of roe deer faeces were found around the irrigation water ponds and fields. *Y. pseudotuberculosis* has been isolated from many domestic and wild animals with a low isolation rate. Among deer, subclinical infections and asymptomatic carriage are common, making it a possible animal reservoir (Hodges et al. 1984b, Toma 1986, Fukushima and Gomyoda 1991, Aleksic et al. 1995, Aschfalk et al. 2008). *Y. pseudotuberculosis* can survive in surface water and soil (Inoue et al. 1988b, Fukushima 1989a, Tsubokura et al. 1989, Inoue et al. 1991, Fukushima 1992, Fukushima et al.1994). No domestic animals were situated in the vicinity of the fields, and animal manure was not used as a fertilizer. This indicates that contamination of lettuce may have resulted through direct faecal contamination from infected wild animals or via the irrigation water. Contamination might also have occurred from surface water runoff.

The *Y. pseudotuberculosis* isolated from iceberg lettuce was unrelated to the outbreak strains in PFGE typing. The presence of *virF*-positive *Y. pseudotuberculosis* in iceberg lettuce does, however, support the assumption that contamination with *Y. pseudotuberculosis* occurs during growth in open fields. Fresh produce may become contaminated during irrigation, harvesting, packing, shipping or processing. The large surface area of lettuce favours bacterial attachment, and the difficulty of thorough cleaning may make lettuce an efficient vehicle for infection. Transportation and storage of lettuce at low temperatures may be particularly favourable for survival and multiplication of psychrotrophic *Y. pseudotuberculosis*.

To prevent future *Y. pseudotuberculosis* outbreaks from lettuce, there is a need to implement farm-level measures. Clean or treated water should be used in spray irrigation.

To reduce *Y. pseudotuberculosis* contamination, access to fields and irrigation water sources by wild animals should be restricted.

6.6. Characterization of *Y. pseudotuberculosis*-like isolates (V)

When three presumptive *Y. pseudotuberculosis* isolates from soil, water and lettuce were further characterized, it was shown that identification of *Y. pseudotuberculosis* from environmental sources using solely biochemical reactions can be incorrect. The three strains were identified as *Y. pseudotuberculosis* with a likelihood of over 98% with the API 20E system, but the strains could not be serotyped by O:1-O:6-specific antisera. In previous studies, the API 20E system has been reported to be accurate in identifying *Y. pseudotuberculosis* in genus *Yersinia* with a positive identification rate of 90% when incubated at temperatures between 25°C and 30°C (Neubauer et al. 1998). Serotyping performed by slide agglutination is sometimes unable to type *Y. pseudotuberculosis* strains, especially rough ones. Rare serotypes of O:7-O:14 with different pathogenic potential have been isolated from non-human sources, i.e. from animals and the environment, in Japan (Fukushima et al. 2001). To identify all known *Y. pseudotuberculosis* serotypes, an O-antigen gene cluster-specific multiplex PCR was used. This assay has been shown to correlate with classical serotyping and be a useful tool for correcting misidentification of strains as *Y. pseudotuberculosis* (Bogdanovich et al. 2003).

The three presumptive *Y. pseudotuberculosis* strains were studied with PCR targeting the *virF* and *inv* genes. Being *virF*- and *inv*-negative strains, they were further characterized together with other sucrose-negative strains belonging to different *Yersinia* species. The PFGE and ribotyping patterns clearly differed from the patterns obtained for *Y. pseudotuberculosis* strains, and by ribotyping the three environmental strains clustered together. The characterization of the O-antigen gene cluster yielded a completely different result from that of *Y. pseudotuberculosis*. The three strains were confirmed not to be *Y. pseudotuberculosis* by using PCR, PFGE, ribotyping and characterization of O-antigen gene cluster and were tentatively named non-pathogenic *Y. pseudotuberculosis*-like strains.

7. Conclusions

1. In comparing the different isolation methods, 16%, 45%, 81% and 45% of the *Y. pseudotuberculosis* -positive samples were recovered after direct plating and cold enrichment of 7, 14 and 21 days, respectively. The most productive culture method for isolation of *Y. pseudotuberculosis* was a two-week cold enrichment followed by alkali treatment and plating of the samples onto CIN agar. Using this method, the highest isolation rate from all samples of animal and environmental origin was obtained. Direct plating was shown to be useful for faecal samples, but not for other samples.

2. The prevalence of *virF*-positive *Y. pseudotuberculosis* was 4% in tonsils of fattening pigs from 7 different slaughterhouses in various parts of Finland. All sows were negative at slaughter. The *Y. pseudotuberculosis*-positive pigs were traced back to farm level. The prevalence of *Y. pseudotuberculosis* in pooled faecal samples of fattening pigs varied from 3% to 14% at 4 positive farms. The 14% was considered a maximum occurrence due to pooled sampling and preselected farms. All *Y. pseudotuberculosis*-positive findings were from fattening pigs (2-6 months old), other age groups, i.e. piglets, sows and boars, were negative. *Y. pseudotuberculosis* was also isolated from pig pen floors. All *Y. pseudotuberculosis* isolates belonged to bioserotype 2/O:3. These findings indicate that clinically healthy fattening pigs are an important reservoir of *virF*-positive *Y. pseudotuberculosis* 2/O:3.

3. The *virF*-positive *Y. pseudotuberculosis* of bioserotype 1/O:2 was recovered from wild birds and iceberg lettuce. The mean prevalence of *Y. pseudotuberculosis* in both migratory birds and lettuce samples was 1%. In birds, *Y. pseudotuberculosis* was isolated from two species of thrushes, the prevalence being 8%. *Y. pseudotuberculosis* was isolated only during spring migration. Positive findings show that birds are one reservoir of *Y. pseudotuberculosis* 1/O:2. The *virF*-positive iceberg lettuce was recovered in a follow-up environmental study on a lettuce farm after a *Y. pseudotuberculosis* outbreak. Although unrelated to the outbreak strain, recovery of *Y. pseudotuberculosis* indicates that contamination of lettuce occurs during growth on the farm.

4. PFGE was shown to be an efficient method for subtyping of *Y. pseudotuberculosis* isolates. Pattern variations were clear but limited among strains of the same bioserotype and from the same origin. A combination of at least two enzymes should be used, especially when isolates of the same bioserotype are characterized. The combination of *Spe*I and *Not*I enzymes was shown to be suitable for subtyping *Y.*

pseudotuberculosis isolates. The limited pattern variation of pig strains of bioserotype 2/O:3 indicates genetic homogenicity among pig strains in Finland. On all positive farms, at least one genotype previously found in tonsils of pigs at the slaughterhouse was also isolated in pig faeces on farms. This indicates that *Y. pseudotuberculosis* can persist at farm level for several months between batches and be transported to the slaughterhouse in the tonsils and faeces of pigs. More different genotypes were found among strains from tonsils at the slaughterhouse than in faeces on farms, which could indicate higher diversity of *Y. pseudotuberculosis* strains in tonsils of pigs or contamination during transport and holding in the slaughterhouse. The genotypes of porcine strains of bioserotype 2/O:3 differed clearly from the genotypes of strains of bioserotype 1/O:2. The bird strains differed clearly from the lettuce strains even though they all belonged to bioserotype 1/O:2. Furthermore, the strains isolated from two species of birds were divided into two genotypes. This may indicate more than one source of infection. PFGE was shown to be a useful tool for comparing *Y. pseudotuberculosis* strains of different origin in outbreak investigations.

5. *Y. pseudotuberculosis* serotype O:3 was identified as a cause of human outbreak by 47 culture-confirmed cases nationwide. In epidemiological analysis, iceberg lettuce was associated with infections through domestically produced iceberg lettuce. Hygiene conditions on lettuce farms were studied to determine the origin of *Y. pseudotuberculosis* contamination. In lettuce fields, untreated water was used for spray irrigation. Fields were unfenced and wild animals had free access to irrigation water sources and fields. Large quantities of roe deer faeces were found all over lettuce fields and around the irrigation water sources. Although the exact mechanism of contamination of lettuce remains unknown, contamination likely resulted through direct faecal contamination from infected wild animals or through irrigation water.

6. The identification of environmental *Y. pseudotuberculosis* strains was incorrect based on the API 20E system or solely on biochemical tests. Using PFGE and ribotyping, the non-serotypeable isolates grouped genetically together and could be differentiated from true *Y. pseudotuberculosis* strains. Sequences of the O-antigen gene cluster were completely different from that of *Y. pseudotuberculosis*. Identification and pathogenicity of the strains were determined by targeting the *inv*-gene in the chromosome of *Y. pseudotuberculosis* and the *virF*-gene on the virulence plasmid by PCR. Combining the results from PCR, PFGE, ribotyping and characterization of O-antigen gene cluster, the three environmental strains were confirmed not to be *Y. pseudotuberculosis*. When a strain identified as *Y. pseudotuberculosis* by API 20E cannot be serotyped to known *Y. pseudotuberculosis* serotypes, species identification and pathogenicity of the isolate should be determined.

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