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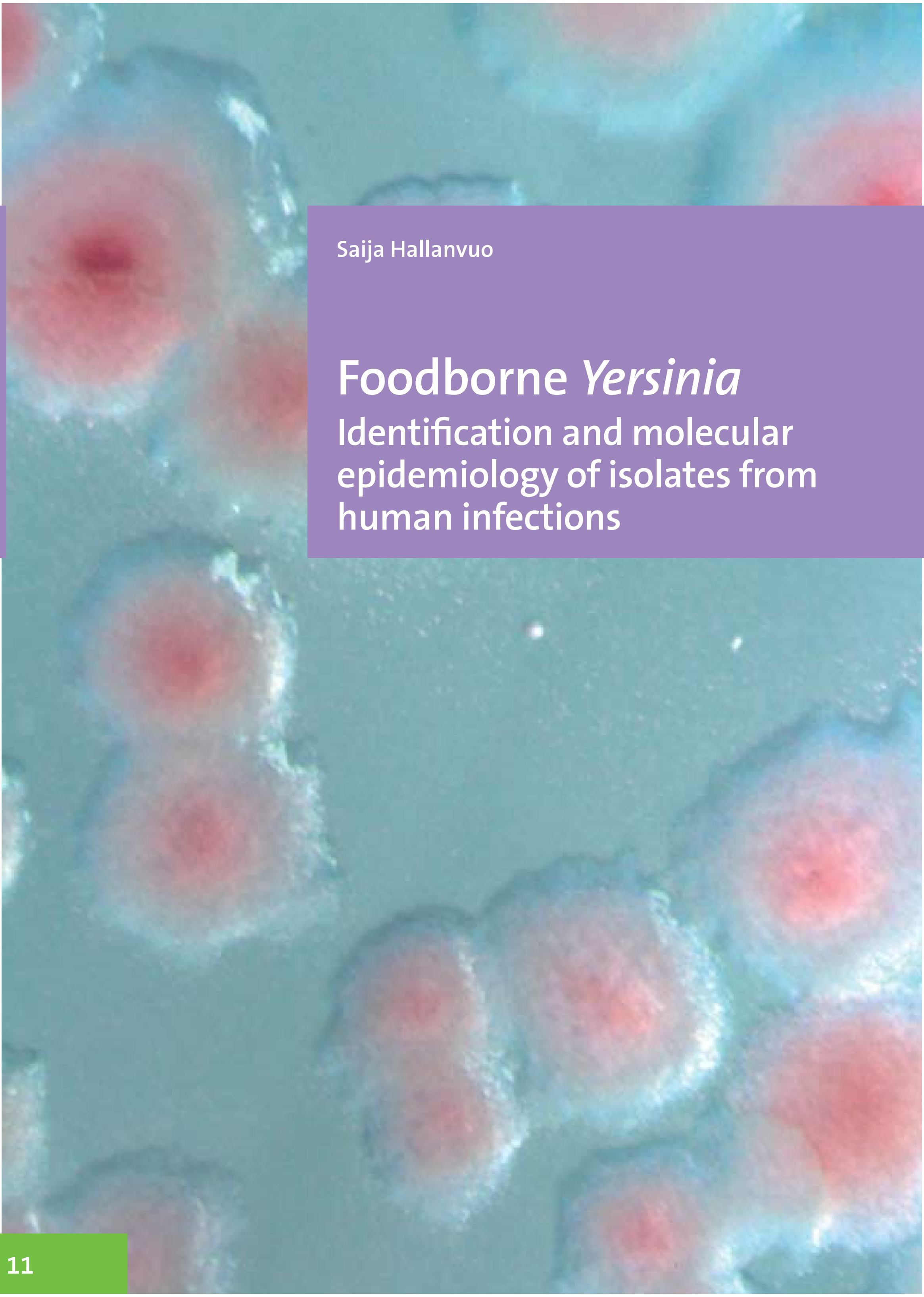

RESEARCH

Saija Hallanvuo

Foodborne *Yersinia*

Identification and molecular epidemiology of isolates from human infections

11

Saija Hallanvuo

Foodborne *Yersinia*

Identification and Molecular Epidemiology of Isolates from Human Infections

Academic dissertation

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Department of Infectious Disease Surveillance and Control
National Institute for Health and Welfare (THL),
Helsinki, Finland

and

Department of Applied Chemistry and Microbiology
Faculty of Agriculture and Forestry
University of Helsinki, Finland

RESEARCH 11

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Cover photo: Y. pseudotuberculosis (strain of human origin; two small round colonies below the title) surrounded by colonies of *Y. entercolitica* -like species and other *Enterobacteriaceae* on CIN agar (picture taken through a stereomicroscope, magnified 63 times).

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Supervised by
Anja Siitonen, Ph.D, Professor
Gastrointestinal Infections Unit
Department of Infectious Disease Surveillance and Control
National Institute for Health and Welfare (THL), Helsinki, Finland

Mikael Skurnik, Ph.D, Professor
Infection Biology Research Program
Department of Bacteriology and Immunology
Haartman Institute, University of Helsinki, Finland

Reviewed by
Johanna Björkroth, D.V.M., Ph.D, Professor
Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine, University of Helsinki, Finland

Elisabeth Carniel, M.D., Ph.D,
Yersinia Research Unit
National Reference Laboratory and WHO Collaborating Center for *Yersinia*
Institut Pasteur, Paris, France

Opponent
Sinikka Pelkonen, D.V.M., Ph.D, Professor
Veterinary Bacteriology Research Unit, Research Department
Finnish Food Safety Authority Evira, Kuopio, Finland

To my family

Abstract

Saija Hallanvuo. Foodborne *Yersinia* – Identification and Molecular Epidemiology of Isolates from Human Infections. National Institute for Health and Welfare. Research 11. Helsinki, Finland 2009. pp. 169. ISBN 978-952-245-065-4

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are among the major enteropathogenic bacteria causing infections in humans in many industrialized countries. In Finland, *Y. pseudotuberculosis* has caused 10 outbreaks among humans during 1997–2008. Some of these outbreaks have been very extensive involving over 400 cases; mainly children attending schools and day-care. *Y. enterocolitica*, on the contrary, has caused mainly a large number of sporadic human infections in Finland.

Y. pseudotuberculosis is widespread in nature, causing infections in a variety of domestic and wild animals. Foodborne transmission of human infections has long been suspected, however, attempts to trace the pathogen have been unsuccessful before this study that epidemiologically linked *Y. pseudotuberculosis* to a specific food item. Furthermore, due to modern food distribution systems, foodborne outbreaks usually involve many geographically separate infection clusters difficult to identify as part of the same outbreak.

Among pathogenic *Y. enterocolitica*, the global predominance of one genetically homogeneous type (bioserotype 4/O:3) is a challenge to the development of genetic typing methods discriminatory enough for epidemiological purposes, for example, for tracing back to the sources of infections. Furthermore, the diagnostics of *Y. enterocolitica* infections is hampered because clinical laboratories easily misidentify some other members of the *Yersinia* species (*Y. enterocolitica*–like species) as *Y. enterocolitica*. This results in misleading information on the prevalence and clinical significance of various *Yersinia* isolates.

The aim of this study was to develop and optimize molecular typing methods to be used in epidemiological investigations of *Y. enterocolitica* and *Y. pseudotuberculosis*, particularly in active surveillance and outbreak investigations of *Y. pseudotuberculosis* isolates. The aim was also to develop a simplified set of phenotypic tests that could be used in routine diagnostic laboratories for the correct identification of *Y. enterocolitica* and *Y. enterocolitica*–like species.

A PFGE method designed here for typing of *Y. pseudotuberculosis* was efficient in linking the geographically dispersed and apparently unrelated *Y. pseudotuberculosis* infections as parts of the same outbreak. It proved to be useful in active laboratory-based surveillance of *Y. pseudotuberculosis* outbreaks. Throughout the study period, information about the diversity of genotypes among outbreak and non-outbreak related strains of human origin was obtained. Also, to our knowledge, this was the first study to epidemiologically link a *Y. pseudotuberculosis* outbreak of human illnesses to a specific food item, iceberg lettuce.

A novel epidemiological typing method based on the use of a repeated genomic region (YeO:3RS) as a probe was developed for the detection and differentiation between strains of *Y. enterocolitica* subspecies *palearctica*. This method was able to increase the discrimination in a set of 106 previously PFGE typed Finnish *Y. enterocolitica* bioserotype 4/O:3 strains among which two main PFGE genotypes had prevailed. The developed simplified method was a more reliable tool than the commercially available biochemical test kits for differentiation between *Y. enterocolitica* and *Y. enterocolitica* – like species. In Finland, the methods developed for *Y. enterocolitica* and *Y. pseudotuberculosis* have been used to improve the identification protocols and in subsequent outbreak investigations.

Keywords: *Y. pseudotuberculosis*, *Y. enterocolitica*, epidemiology, identification, foodborne pathogen, molecular typing

Abstract in Finnish

Saija Hallanvuo. Foodborne *Yersinia* –Identification and Molecular Epidemiology of Isolates from Human Infections. Terveyden ja hyvinvoinnin laitos. Tutkimus 11. Helsinki 2009. 169 sivua. ISBN 978-952-245-065-4

Y. enterocolitica ja *Y. pseudotuberculosis* ovat merkittäviä elintarvikevälitteisiä suolistoperäisiä taudinaiheuttajia sekä Suomessa että muissa teollistuneissa maissa. Suomessa on vuosien 1997–2008 aikana todettu 10 pääasiassa kouluruokailuun liittynyttä *Y. pseudotuberculosis* epidemiaa. Laajamittaisimmissa epidemioissa on sairastunut kerralla yli 400 henkilöä, valtaosa koululaisia tai päiväkotilapsia. *Y. enterocolitica* aiheuttaa Suomessa lähinnä yksittäisiä infektioita, joita kuitenkin vuositasolla esiintyy enemmän kuin *Y. pseudotuberculosis* infektioita. Bakteerit aiheuttavat suolistotulehduksen jonka oireina esiintyy kuumetta, vatsakipuja ja ripulia. Vatsakipu voi muistuttaa umpilisäkkeen tulehdusta ja johtaa aiheettomaan leikkaukseen. Infektion sairastamisen jälkitautina voi esiintyä reaktiivista niveltulehdusta ja iho-oireista kyhmyruusua.

Y. pseudotuberculosis on yleinen ympäristössä ja sen lähteenä toimivat monet eläimet, etenkin jyrsijät, peurat, jänikset ja linnut. Tartunta saadaan usein saastuneen elintarvikkeen tai veden välityksellä. Bakteerin alkuperää ei kuitenkaan ennen tätä tutkimusta ole pystytty jäljittämään epidemiologisen tutkimuksen avulla tiettyyn elintarvikkeeseen. Nykyaikaiset elintarvikkeiden jakeluketjut asettavat lisäksi haasteita elintarvike-epidemioiden selvitystyölle; epidemiat koostuvat usein pienistä, maantieteellisesti hajanaisista tautikeskittymistä, jotka on vaikea havaita osaksi samaa epidemiaa.

Maailmanlaajuisesti levinnein muoto *Y. enterocolitica* -bakteerista (bioserotyyppi 4/O:3) on perimältään hyvin yhdenmukainen. Tämä asettaa haasteita riittävän erottelukykyisen genotyypitysmenetelmän löytämiseksi kyseisen bakteerin tartuntareittien selvittelyyn. Lisäksi *Y. enterocolitica*n erottaminen muista sen kaltaisista *Yersinia*-suvun bakteereista on usein vaikeaa kliinistä diagnostiikkaa tekevissä laboratorioissa. Virheelliset tunnistukset vääristävät tietoja *Y. enterocolitica*n ja sen kaltaisten bakteerien esiintyvyydestä ja vaikeuttavat bakteerien kliinisen merkitsevyyden arvioimista.

Tässä tutkimuksessa kehitettiin molekyylibiologisia tyypitysmenetelmiä *Y. enterocolitica* ja *Y. pseudotuberculosis* -bakteerien tartuntareittien jäljitykseen. Kehitetyistä menetelmistä PFGE-genotyypitysmenetelmää sovellettiin käytäntöön tutkimuksen aikana ja myös sen jälkeen esiintyneiden *Y. pseudotuberculosis* epidemioiden selvityksessä. Menetelmä todettiin tehokkaaksi laboratoriopohjaisen epidemiaseurannan apuvälineeksi, jonka avulla tunnistettiin maantieteellisesti hajanaiset tautikeskittymät osaksi samaa epidemiaa. Monitahoisten epidemiologisten tutkimusten avulla pystyttiin ensimmäistä kertaa tunnistamaan *Y. pseudotuberculosis* epidemiaa välittänyt elintarvike, jäävuorisalaatti. Tutkimusjakson aikana kerättiin myös tietoa genotyyppien jakautumisesta epidemioihin liittyvien ja epidemioiden ulkopuolisten kantojen osalta.

Y. enterocolitica subspecies *palearctica* -bakteerille kehitettiin uusi epidemiologinen tyypitysmenetelmä, joka perustuu alalajille tyypillisen toistuvan sekvenssin (YeO:3RS) käyttöön tunnistimena tyypityksessä. Tämä menetelmä lisäsi PFGE-menetelmän erottelukykyä, kun sen avulla tyypitettiin 106 aikaisemmin PFGE-tyypitettyä suomalaista bioserotyypin 4/O:3 kantaa. Työssä kehitettiin lisäksi monia kaupallisia biokemiallisia tunnistustestisarjoja luotettavampi, mutta silti yksinkertainen tapa havaita *Y. enterocolitica*n kaltaiset bakteerit. Tutkimuksessa kehitettyjä menetelmiä voidaan hyödyntää elintarvikevälitteisten *Yersinia*-suvun bakteerien tarkemmassa tunnistamisessa, tartuntareittien selvittelyssä ja sitä kautta epidemioiden ennaltaehkäisyssä.

Avainsanat: *Y. pseudotuberculosis*, *Y. enterocolitica*, epidemiologia, tunnistus, elintarvikevälitteinen patogeeni, tyypitys

Abstract in Swedish

Saija Hallanvuo. Foodborne *Yersinia* – Identification and Molecular Epidemiology of Isolates from Human Infections. Institutet för hälsa och välfärd. Forskning 11. Helsingfors 2009. 169 sidor. ISBN 978-952-245-065-4

Y. enterocolitica och *Y. pseudotuberculosis* är bland de viktigaste enteropatogena bakterierna som orsakar infektion hos människor i många industriländer. I Finland har *Y. pseudotuberculosis* gett upphov till 10 utbrott mellan åren 1997–2008. I de mest omfattande av dessa har vid ett och samma tillfälle över 400 personer insjuknat, främst skolelever eller barn på daghem. *Y. enterocolitica* å andra sidan ger upphov till ett stort antal enstaka fall i Finland. Bakterierna orsakar tarminfektion med feber, magsmärtor och diarré som symtom. Magsmärtorna kan påminna om appendicit och leder därför ibland till onödig operation. Som följdsjukdomar kan reaktiv artrit och knölros förekomma.

Y. pseudotuberculosis finns allmänt i naturen och orsakar infektioner hos en rad olika både tama och vilda djur. Livsmedelsburen smittöverföring till människor har länge misstänkts men inte förrän i denna studie har man lyckats visa den epidemiologiska kopplingen mellan *Y. pseudotuberculosis* och ett specifikt livsmedel. De moderna distributionskedjorna för livsmedel innebär dessutom en extra utmaning i utredningsarbetet; utbrotten resulterar ofta i små, geografiskt spridda lokala klustrar av fall som är svåra att identifiera som delar av ett och samma utbrott.

Den globalt mest utbredda formen av *Y. enterocolitica* bakterien (bioserotyp 4/O:3) har en mycket homogen genuppsättning. Detta ställer speciella krav att utveckla tillräckligt diskriminerande genetiska typningsmetoder som gör det möjligt att i epidemiologiskt syfte spåra bakteriens smittvägar. Ytterligare en försvårande omständighet är att laboratorier som utför klinisk diagnostik ofta har svårt att särskilja arten *Y. enterocolitica* från andra medlemmar av *Yersinia* släktet – de så kallade *Y. enterocolitica* – liknande arterna. Detta identifieringsfel förvanskar uppgifterna om prevalensen och den kliniska signifikansen av olika *Y. enterocolitica* -isolat.

Målet med denna studie har varit att utveckla och optimera molekylära typningsmetoder som verktyg vid epidemiologiska utredningar avseende *Y. enterocolitica* och *Y. pseudotuberculosis*. Målet har också varit att utveckla en förenklad uppsättning av fenotypiska tester för användning i rutindiagnostiska laboratorier för att korrekt kunna identifiera *Y. enterocolitica* och *Y. enterocolitica* – liknande arter.

En PFGE-genotypningsmetod utvecklades för typning av *Y. pseudotuberculosis*. Metoden visade sig vara ett effektivt redskap vid laboratoriebaserade utbrottsuppföljningar och gjorde det möjligt att sammanlänka geografiskt spridda humana fall av *Y. pseudotuberculosis* till ett och samma utbrott. Med hjälp av mångsidiga epidemiologiska undersökningar kunde man för första gången identifiera ett livsmedel (isbergssallad) som vektor vid överföringen av *Y. pseudotuberculosis* i samband med ett utbrott. Under avhandlingsarbetets gång samlades även data som visar den mångfald av genotyper som finns bland såväl utbrottsstammar som icke-utbrottsrelaterade stammar av humant ursprung.

En ny epidemiologisk typningsmetod som bygger på användning av en repeterad genomisk sekvens, YeO:3RS, utvecklades. Metoden användes för att påvisa och differentiera mellan stammar av *Y. enterocolitica* underart *palearctica*. Med denna metod förbättrades diskrimineringen i en uppsättning av 106 finska stammar av *Y. enterocolitica* bioserotyp 4/O:3 som med PFGE tidigare hade gett upphov till i huvudsak två rådande PFGE-genotyper. Den förenklade uppsättningen av fenotypiska tester som utvecklades visade sig vara en mer tillförlitlig metod än de kommersiellt tillgängliga biokemiska testkit för differentiering mellan stammar av *Y. enterocolitica* och *Y. enterocolitica*-liknande arter. Resultaten i denna studie kan utnyttjas för detaljerad identifiering av livsmedelsburna isolat av släktet *Yersinia*, vidare vid utredning av smittvägar och därigenom i arbetet med att förebygga utbrott.

Nyckelord: *Y. pseudotuberculosis*, *Y. enterocolitica*, epidemiologi, identifiering, typbestämning

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ORIGINAL PUBLICATIONS

List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I–IV). In addition, some unpublished data are presented.

I Hallanvuo, S., Skurnik, M., Asplund, K., Siitonen, A., 2002, Detection of a novel repeated sequence useful for epidemiological typing of pathogenic *Yersinia enterocolitica*. *Int. J. Med. Microbiol.* 292: 215–225.

II Hallanvuo, S., Peltola, J., Heiskanen, T., Siitonen, A., 2006, Simplified phenotypic scheme evaluated by 16S rRNA sequencing for differentiation between *Yersinia enterocolitica* and *Y. enterocolitica*-like species. *J. Clin. Microbiol.* 44: 1077–1080.

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

IV Jalava, K., Hallanvuo, S., Nakari, U.M., Ruutu, P., Kela, E., Heinasmaki, T., Siitonen, A., Nuorti, J.P., 2004, Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. *J. Clin. Microbiol.* 42: 2789–2791.

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Author's contribution

Publication I

Saija Hallanvuo set up the novel epidemiological typing method (YeO:3RS genotyping) for *Y. enterocolitica* and genotyped the strains used in the study. She designed the protocol for cell pretreatment and conducted the experiments for the location of the genotyping potential in the sequence used for typing. She interpreted the results and wrote the article.

Publication II

Saija Hallanvuo planned the experimental design and set up the biochemical and microscopic methods used in the study for the Enteric Bacteria Laboratory (EBL), National Public Health Institute (KTL). She was responsible for the biotyping and genotyping experiments. She interpreted the results, designed the phenotypic scheme described and wrote the article.

Publication III

Saija Hallanvuo designed the protocol for pulsed-field gel electrophoresis (PFGE) typing of *Y. pseudotuberculosis* strains including cell pretreatment, restriction analysis and running conditions. She carried out the genotyping studies of *Y. pseudotuberculosis* human strains and took part in the trace-back and environmental investigations. She interpreted the genotyping results and wrote these parts of the article together with the co-authors.

Publication IV

Saija Hallanvuo was responsible for PFGE typing procedures in the study. She interpreted the genotyping results and wrote these parts of the article together with the co-authors.

Abbreviations

Ail	Attachment invasion locus
AFLP	Amplified fragment length polymorphism
ATCC	American type culture collection
BT	Biotype
CIN	Cefsulodin-irgasan-novobiocin agar
CR-MOX	Congo-red magnesium oxalate agar
DI	Discrimination index
EBL	Enteric Bacteria Laboratory of KTL; name changed 1.1.2009 to: Gastrointestinal Infection Unit of the National Institute of Health and Welfare (THL)
ECDC	European Centre for Disease Control
EFSA	European Food Safety Authority
ERIC	Enterobacterial repetitive intergenic consensus (sequences)
FESLF	Far East scarlet-like fever
HLA B27	Human leukocyte antigen B27
HPI	High pathogenicity island
Inv	Invasin
ITC	Irgasan-ticarcillin-potassium chlorate broth
NCTC	National collection of type cultures
KTL	Kansanterveyslaitos, National Public Health Institute; name changed 1.1.2009 to: National Institute of Health and Welfare (THL)
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable number tandem repeat (analysis)
Myf	Mucoid *Yersinia* factor/fibrillae
NF-κB	Nuclear factor kappa B
orf	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPs	Peyer's patches
pil	Operon encoding a type IV pilus (located in YAPI)
PsaA	*Y. pseudotuberculosis* adhesin A
pYV	Plasmid for *Yersinia* virulence (also pLCR; plasmid for low calcium response, or pCD1: plasmid for calcium dependence)
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindromic (elements)

RovA	Regulator of virulence A
SodA	Superoxide dismutase A
SSDC	*Salmonella-Shigella*-deoxycholate calcium chloride agar
Type III secretion system	Contact dependent secretion system (T3SS)
VNTR	Variable number of tandem repeat regions
VP	Voges-Proskauer reaction (production of acetoin)
YadA	*Yersinia* adhesin A
YAPI	*Yersinia* pathogenicity island
YeO:3RS	*Y. enterocolitica* O:3 repeated sequence
Yops	*Yersinia* outer proteins
YplA	*Yersinia* phospholipase A
YPM	*Y. pseudotuberculosis* –derived mitogen
Ysa	*Yersinia* secretion apparatus
Ysc	Yop secretion (Ysc –Yop type III virulence apparatus)
Ysp	*Yersinia* secreted proteins (Ysa – Ysp type III secretion apparatus)
Yst	*Yersinia* heat-stable enterotoxin

INTRODUCTION

Foodborne diseases are an important and growing public health and economic problem in many countries. Many factors related to societal and environmental changes favour the spread of infectious diseases transmitted by food. These include an increase in the number of people with impaired host defences, changes in the way food is processed and distributed, and alterations in the habitats of animals and arthropods that transmit disease.

The genus *Yersinia* is composed of 14 species, of which only *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are known human pathogens. *Y. pseudotuberculosis* and some types of *Y. enterocolitica* are enteropathogenic members among *Yersinia* species. In many industrialized countries, enteropathogenic *Yersiniae* cause a significant proportion of foodborne enteric infections in humans. The infection, yersiniosis, typically involves gastroenterocolitis with non-specific diarrhoea and fever (*Y. enterocolitica*) or acute mesenteric lymphadenitis, "pseudoappendicitis", with little or no diarrhoea (*Y. pseudotuberculosis*). The post-infectious complications include reactive arthritis and erythema nodosum.

Y. enterocolitica is a heterogeneous group of organisms based on biochemical, antigenic and virulence properties. The strains can be divided into six biogroups, among which the biotypes 1B-5 have been related to pathogenicity and the biotype 1A is considered non-pathogenic. For full virulence, all pathogenic *Yersinia* need a 70-kb plasmid called pYV (*Yersinia* virulence plasmid). Within *Y. enterocolitica* and related species, there are at least 76 serotypes based on variability in the O-antigen structure. Bioserotype 4/O:3 predominates globally among strains of *Y. enterocolitica*, and swine serve as a major reservoir of this bioserotype (Kapperud, 1991; Smego *et al.*, 1999). Tracing back the sources of infections of this genetically homogenic bioserotype demands discriminatory genotyping methods.

Y. pseudotuberculosis is generally pathogenic to humans. *Y. pseudotuberculosis* strains can be divided into 21 different serotypes based on O-antigen structural variability (Bogdanovich *et al.*, 2003). In addition, division into pathogenicity types following certain pathogenicity factors has been established. *Y. pseudotuberculosis* is a well-known cause of illnesses in animals, for example in hares, rodents, deer and sheep. The organism is persistent in the environment and able to survive for long periods in environmental waters, well water and soil (Inoue *et al.*, 1988a; Jalava *et al*, 2006). Foodborne transmission of human infections has long been suspected, however, attempts to epidemiologically trace the pathogen to a specific food source before this study have been unsuccessful.

To collectively distinguish other members of *Yersinia* species from *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, the term "*Y. enterocolitica*–like bacteria" has been generally used. These bacteria lack the classical *Yersinia* virulence markers and, thus, have generally been regarded as non-pathogenic. *Y. enterocolitica*–like bacteria are ubiquitous; they have been isolated from healthy and sick humans and from a wide variety of environmental sources (Sulakvelidze, 2000). In a clinical laboratory, *Y. enterocolitica*–like strains can be easily misidentified as *Y. enterocolitica* if identification is based on a diagnostic kit like API 20 E and commercial serotyping antisera. As a result, misleading information on the prevalence and clinical significance of both *Y. enterocolitica* and *Y. enterocolitica*–like isolates is obtained.

Globally, fresh produce has increasingly been identified as a source of outbreaks of different foodborne pathogens. Furthermore, due to modern food distribution systems, foodborne outbreaks usually involve many geographically separate infection clusters difficult to identify as parts of the same outbreak. In Finland, fresh vegetables and vegetable products were the most common reported food group causing an infection outbreak in 2006 with this group accounting for 31% of all outbreaks that year. *Y. pseudotuberculosis* caused 10 outbreaks during 1997–2008 in Finland. In the majority of these outbreaks, epidemiological investigation has identified vegetables as the source.

This study was conducted to develop and set up typing methods for epidemiological investigations of foodborne pathogenic *Yersinia* isolates. The PFGE genotyping method developed here for *Y. pseudotuberculosis* has been used in active surveillance and investigations of subsequent outbreaks. A novel epidemiological YeO:3RS genotyping method developed for *Y. enterocolitica* subspecies *palearctica* was able to increase the discrimination obtained previously by PFGE in a set of bioserotype 4/O:3 strains. Phenotypic methods were set up in order to aid clinical diagnostic laboratories in distinguishing between *Y. enterocolitica* and *Y. enterocolitica*–like strains and in avoiding misidentifications.

REVIEW OF THE LITERATURE

1 The genus *Yersinia*

Yersiniae are Gram-negative, facultative anaerobic, non-spore-forming straight rods or coccobacilli, 0.5 to 0.8 by 1 to 3 μm in size. They are often more active biochemically at 25°C than 37°C; for example, most of them are motile at 25°C but non motile at 37°C. (Bottone *et al*, 2005). *Yersiniae* are widely distributed in nature and adapted, depending on the species, to specific animal hosts or to humans.

The genus *Yersinia* is composed of 14 species, of which only *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. pestis* and *Y. ruckeri* are known pathogens for humans and animals. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogenic organisms that share common modes of transmission mainly through food and water. They typically cause a self-limiting gastroenteritis, yersiniosis, restricted to the intestinal tract and the intestinal lymphoid system. *Y. pestis* causes zoonosis with very different symptoms compared to its enteropathogenic counterparts, and has been responsible for three human plague pandemics (Prentice and Rahalison, 2007). The life cycle of this pathogen is very complex and involves a mammalian reservoir (primarily rodents) and a flea vector. *Y. ruckeri* is the causative agent of enteric red mouth disease (ERM) in salmonid fish, responsible for significant economic losses in the fish farming industry (Fernandez *et al*, 2007; Tobback *et al.*, 2007). The taxonomic status of *Y. ruckeri* as *Yersinia*, however, is still compromised.

1.1 History

The history of the *Yersinia* species dates back to 1884, when *Y. pseudotuberculosis* was first isolated and described as the causative agent of tuberculosis-like lesions (pseudotuberculosis) in guinea pigs (Malassez and Vignal, 1884). The bacterium was characterized and named *Bacillus pseudotuberculosis* (Pfeiffer, 1889). Soon after, the French bacteriologist Alexandre Yersin first isolated *Y. pestis* in 1894 in Hong Kong, to where this notorious agent of bubonic plague had spread from mainland China (Yersin, 1894). Lehmann and Neumann (1896) described the bacterium and proposed the name *Bacterium pestis*. It was not until 1944, however, when the genus concept *Yersinia* was established by Van Loghem (1944) and the genus name was proposed to honour A.J.E. Yersin. The new genus was intended to include *Y. pestis* and *Y. pseudotuberculosis*, known at that time as *Pasteurella pestis* and *Pasteurella pseudotuberculosis*, because of the significant differences these two bacteria had compared to the type species of *Pasteurella* at that time. Finally, the third human pathogen of the species *Yersinia* was discovered in 1939 when Schleifstein and

Coleman described a previously unidentified group of strains which they thought resembled *Actinobacillus ligneri* and *Pasteurella pseudotuberculosis*, and which originated from facial lesions and intestines of humans exhibiting symptoms of enteritis (Schleifstein and Coleman, 1939). Later on, they proposed the name *Bacterium enterocoliticum* for this group of bacteria (Schleifstein and Coleman, 1943). In 1964, the present name *Y. enterocolitica* was assigned to this species by Frederiksen (1964). The first *Y. enterocolitica*–like species was introduced in 1978, when *Y. ruckeri* was described (Ewing *et al.*, 1978). The taxonomy of *Yersinia* moved significantly forward by DNA-DNA hybridisation studies conducted by Brenner *et al.* (1976). These led to the description of three new *Y. enterocolitica*–like species; *Y. intermedia* (Brenner *et al.*, 1980a), *Y. frederiksenii* (Ursing *et al.*, 1980) and *Y. kristensenii* (Bercovier *et al.*, 1980b). The same new technique also revealed the close taxonomic relationship of *Y. pseudotuberculosis* and *Y. pestis* (Bercovier *et al.*, 1980a) and led to the proposal to rename *Y. pestis* as *Y. pseudotuberculosis* subspecies *pestis* (Bercovier *et al.*, 1981). This was, however widely argued against and *Y. pestis* maintained its species status. Some years later, Bercovier *et al.* increased the number of species in the genus *Yersinia* by proposing the name *Y. aldovae* for *Y. enterocolitica*-like isolates originally recovered from aquatic ecosystems and called the *Y. enterocolitica* group X2 (Bercovier *et al.*, 1984). Shortly after in 1987, Aleksic *et al.* proposed a new species name, *Y. rohdei*, for a bacterium first isolated from human and dog faeces and surface waters (Aleksic *et al.*, 1987). The genus *Yersinia* was completed for the foreseeable future in 1988 when Wauters *et al.* reclassified former *Y. enterocolitica* biotype 3A and 3B strains into the new species *Y. mollaretii* and *Y. bercovieri*, respectively (Wauters *et al.*, 1988b. The rearrangements in the genus *Yersinia* continued after quite a long pause in 2000 when Neubauer *et al.* proposed the division of *Y. enterocolitica* into two subspecies, *Y. enterocolitica* subspecies *enterocolitica* and *Y. enterocolitica* subspecies *palearctica* based on DNA-DNA reassociation values and 16S rRNA gene sequences (Neubauer *et al.*, 2000a). This proposal logically agreed with the long known division of *Y. enterocolitica* into the "American" and "European" strains (Caugant *et al.*, 1989; Ibrahim *et al.*, 1993). The most recent additions to the genus *Yersinia* are *Y. aleksiciae*, *Y. massiliensis* and *Y. similis* (Sprague and Neubauer 2005; Merhej *et al.* 2008; Sprague *et al.* 2008). The species name *Y. aleksiciae* was proposed by Sprague and Neubauer (2005) for a group of strains isolated from diverse origins (human faeces, rats, moles, reindeer and pigs, and from dairy products) formerly classified as *Y. kristensenii* serotype O:16. *Y. massiliensis*, a bacterium first isolated from hospital water distribution systems and well water, is closely related to *Y. bercovieri*, *Y. mollaretii* and *Y. frederiksenii*, but is biochemically separable from these organisms (Merhej *et al.*, 2008). *Y. similis* comprises organisms first identified phenotypically as *Y. pseudotuberculosis* but which were assigned to their own species after more detailed molecular and biochemical studies. It seems that *Y. similis* is well adapted to the environment and does not cause disease. However, it may interfere with the classical field diagnostics of plague due to its distribution in the Far East and in areas where plague is still endemic (Sprague *et al.*, 2008).

1.2 Classification and taxonomy

Yersinia is a genus in the family of *Enterobacteriaceae*, belonging to the *Gammaproteobacteria* class of phylum *Proteobacteria* (Figure 1). The placement of the genus *Yersinia* within the *Enterobacteriaceae* is supported by biochemical and DNA-DNA reassociation studies. Based on latter studies, the taxon *Yersinia* can be considered very homogenous (Bottone *et al.*, 2005), and it forms a coherent cluster within the *Gammaproteobacteria* when 16S rRNA gene sequences are also compared (Ibrahim *et al.*, 1993).

The 16S rRNA gene sequence similarities of *Yersinia* strains range from 96.9-99.8%. The closest relative to *Yersinia* within the *Gammaproteobacteria* is *Hafnia alvei* (Ibrahim *et al.*, 1993). The mol% G + C content within the genus *Yersinia* is consistent with that of *Enterobacteriaeceae* and ranges from 46 to 50. With the DNA relatedness of 40% or higher, all of the *Yersinia* species are more closely related to each other than to any other *Enterobacteriaceae* species (Bottone *et al.*, 2005; Brenner *et al.*, 1980b). Nevertheless, *Y. ruckeri* is an exception by being at most only 38% related to other *Yersinia* species, and having biochemical properties more similar to some other *Enterobacteriaceae* than to *Yersinia*. Because of its DNA mol%

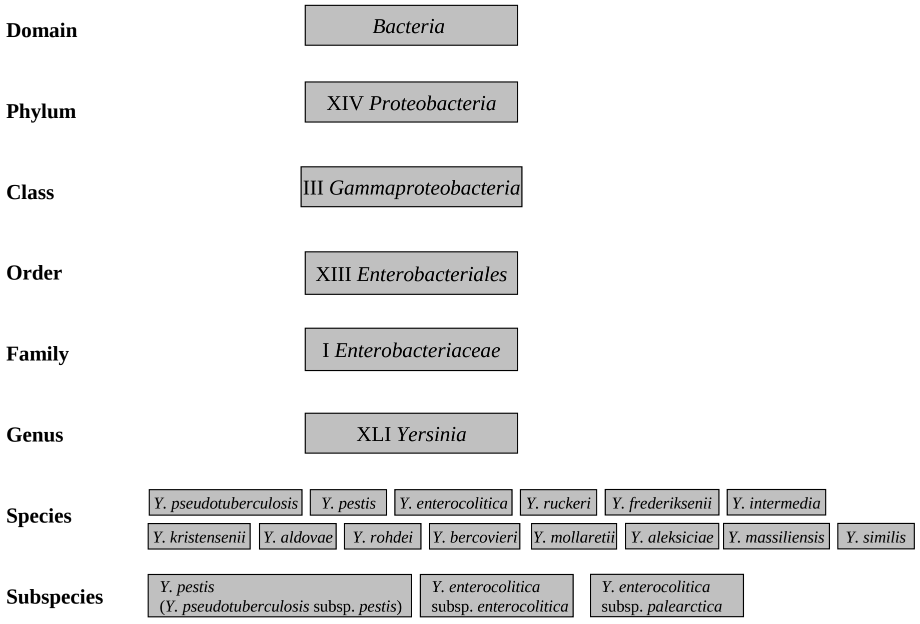

Figure 1. Taxonomy of Yersinia species

G+C closer to *Yersiniae* than to other *Enterobacteriaceae*, *Y. ruckeri* has maintained its controversial status as *Yersinia*.

There are still certain taxonomic problems among *Y. enterocolitica* –like species. For example, *Y. frederiksenii* is composed of at least three (Ursing *et al.*, 1980) or four (Ursing and Aleksic, 1995) genomospecies that appear to deserve a species status. This clustering has also been revealed by multilocus enzyme electrophoresis (MLEE) and by sequencing a segment of *gyr*B (Demarta *et al.*, 2004), a housekeeping gene that encodes the B subunit protein of DNA gyrase. However, because of the lack of phenotypic tests able to differentiate these groups from *Y. frederiksenii*, they have remained within this species. Recent studies revealed 100% sequence identity between 16S rRNA gene sequences of species *Y. massiliensis* and an atypical *Yersinia* isolate that had been previously tentatively assigned to *Y. frederiksenii* genomospecies 2 (Ibrahim *et al.*, 1997b; Merhej *et al.*, 2008). More detailed studies will elucidate if this representative of genomospecies 2 actually belongs to the newly named species *Y. massiliensis*, thus perhaps clarifying the complicated taxonomy of *Y. frederiksenii*. Accordingly, it has been noted, that *Y. kristensenii* strains with various MLEE clusters and 16S rRNA gene types exist (Goullet and Picard 1988; Caugant *et al.* 1989; Dolina and Peduzzi 1993; Neubauer *et al.* 2000b). It was not until recently, however, that some of these isolates originally phenotyped as *Y. kristensenii*, but displaying a different 16S rRNA gene sequence type, could be assigned to their own species, *Y. aleksiciae* (Sprague and Neubauer, 2005).

Y. pestis is considered a subspecies of *Y. pseudotuberculosis* and has only recently (within the last 1,500–40,000 years) evolved from *Y. pseudotuberculosis* (Achtman, 2004a; Skurnik *et al.*, 2000). By contrast, *Y. pseudotuberculosis* and *Y. enterocolitica* lineages separated between 0.4 and 1.9 million years ago (Achtman *et al.*, 1999). The evolution of *Y. pestis* from *Y. pseudotuberculosis* seems to have involved a combination of lateral gene transfer and gene inactivation (Achtman, 2004b; Chain *et al.*, 2004). These two organisms are identical in DNA-DNA reassociation studies and by 16S rRNA gene sequences (Bercovier *et al.* 1980b; Trebesius *et al.* 1998). However, *Y. pestis* and *Y. pseudotuberculosis* have maintained their separate species status and are currently reported separately due to the significant difference in the public health importance of these two organisms.

Recently, the use of an updated polyphasic approach in taxonomy exploiting a combination of data obtained by, for example, sequencing housekeeping genes and microarrays in addition to 16S rRNA gene analysis and DNA-DNA reassociation studies, has been encouraged in order to improve the data analysis in bacterial taxonomy (Vandamme *et al.* 1996; Stackebrandt *et al.* 2002). In the near future, this will probably lead to revaluation and redefining of the taxonomy of *Yersinia* as well. The examples of the new information gathered by using this approach include the description of two new *Yersinia* species, *Y. massiliensis* and *Y. similis*, and the provisional evidence of the existence of three *Y. enterocolitica* subgroups (Howard *et al.*, 2006).

2 General characteristics of *Yersinia* species

Y. enterocolitica and *Y. pseudotuberculosis* both display anaerogenic fermentation of glucose and other carbohydrates, produce urease, are motile at 25°C but not 37°C, and lack oxidase, phenylalanine deaminase, lysine decarboxylase and arginine dihydrolase activities. Additionally, *Y. enterocolitica* consists of sucrose and D-sorbitol positive, and L-rhamnose and melibiose negative strains. *Y. pseudotuberculosis*, on the other hand, is easily differentiated from *Y. enterocolitica* by negative reactions for sucrose and D-sorbitol, and positive reaction for L-rhamnose. (Bercovier *et al.* 1980a; Bottone 1997). Some of the biochemical characteristics of *Yersinia* species (see section 6) are temperature dependent (cellobiose and raffinose fermentation, ornithine decarboxylase, ONPG (*o*-nitrophenyl-ß-D-galactopyranoside) hydrolysis, indole production, and the Voges-Proskauer reaction) and are more constantly expressed at 28°C than at 37°C (Bottone *et al.*, 2005).

The optimum growth temperature for *Yersiniae* is 28°C-29°C with the range of 4°C to 42°C (Bottone *et al.*, 2005). Members of the genus *Yersinia* are psychrotrophic organisms and some strains of *Y. enterocolitica* can grow at temperatures as low as -5°C, although growth is very slow below 0°C (Bergann *et al.*, 1995). Cold-adapted organisms like *Y. enterocolitica* and *Y. pseudotuberculosis* must alter the composition of lipids and change the protein contents in the cell membrane in order to maintain essential functions like nutrient uptake, ion pumping and electron transport (Nagamachi *et al.* 1991; Goverde *et al.* 1994; Salamah and Ali 1995; Graumann and Marahiel 1996). The cold adaptation of *Y. enterocolitica* involves, for example, upregulation of specialized cold shock proteins (Goverde *et al.*, 1998; Neuhaus *et al.*, 1999) and genes encoding environmental sensors and regulators (Bresolin *et al.*, 2006b). *Y. enterocolitica* survives in frozen food for extended periods and even withstands repeated freezing and thawing. On the other hand, *Yersinia* is destroyed quite rapidly by heat; it survives only 18 s at 72°C in milk (Toora *et al.*, 1992). *Yersinia* species can grow in a pH range of 4.0–10.0 and tolerate an NaCl concentration of up to 5%. *Y. pseudotuberculosis* and *Y. pestis*, however, tolerate a pH range of 5.0–9.6 and an NaCl concentration of up to 3.5%. The optimum pH for all species is 7.2–7.4 (Bottone *et al.*, 2005).

Y. enterocolitica is a heterogenous species based on biochemical, antigenic and virulence properties (Bottone, 1997). Based on biochemical properties, *Y. enterocolitica* is divided into six biotypes (Wauters *et al.*, 1988b Wauters *et al.*, 1987). In *Y. enterocolitica* and related species, at least 76 serotypes based on variability in the O-antigen structure has been described (Wauters *et al.*, 1991). According to the division suggested by Neubauer *et al.* (2000a) *Y. enterocolitica* subsp. *palearctica* includes strains of European origin belonging to bioserotypes 4/O:3, 2/O:9, 2 or 3/O:5,27, 1A/O:7,8, 1A/O:6,30, and 1A/O:5. *Y. enterocolitica* subsp. *enterocolitica* includes strains of American origin belonging to bioserotypes 1B/O:8, 1B/O:13, 1B/O:18, 1B/O:20, 1B/O:21 and 1A/O:7,8. Only certain bioserotypes have been associated with human disease; most commonly 4/O:3, 2/O:9, 2/O:5,27, 3/O:3, and 1B/O:8, and less frequently 3/O:5,27 and other biotype 1B serotypes than O:8. During the past decades, the previously known geographic division of bioserotypes has been balanced out, making the bioserotype 4/O:3 the most common throughout the world, followed by bioserotypes 2/O:9 and 2/O:5,27 (Bottone, 1999). Bioserotype 3/O:3 has been responsible for yersiniosis in Japan and China (Fukushima *et al.*, 2001; Zheng and Xie, 1996). Bioserotype 1B/O:8, originally designated as "American" bioserotype, has appeared in France, Italy, Japan and recently in Germany (Ichinohe *et al.* 1991; Ostroff 1995; Bockemühl *et al.* 2002). The virulence of different pathogenic bioserotypes varies, for example, bioserotype 1B/O:8 is lethal to mice in contrast to bioserotypes 4/O:3 and 2/O:9, which are lethal only after iron is made available to the bacteria by pretreatment of mice with desferroxamine or iron (Robins-Browne and Prpic, 1985). The frequency of post-infection sequelae also varies depending on the serotype of the infecting strain. *Y. enterocolitica* biotype 1A is a heterogenic group of strains representing different serotypes and occupying a wide range of environmental niches. This biotype has traditionally been designated as non-pathogenic. However, according to recent suggestions, there may be a pathogenic "clinical" subgroup among these bacteria which cannot be readily identified because they lack the well-known virulence determinants of classical pathogenic bioserotypes (Tennant *et al.*, 2003). Biotype 1A can be regarded as non-pathogenic until there is evidence of the mechanisms of the suggested pathogenicity and the group of suggested pathogens can be identified.

Unlike many variations among *Y. enterocolitica*, *Y. pseudotuberculosis* is generally pathogenic to humans. Some isolates of animal and environmental origin belonging to serotypes O:6, O:7, O:9, O:10, O:11 and O:12 are considered non-pathogenic (Fukushima *et al.*, 2001; Nagano *et al.*, 1997b). However, *Y. pseudotuberculosis* cannot be straightforwardly divided into pathogenic and non-pathogenic strains according to serotype. Instead, division into pathogenicity types has been established among *Y. pseudotuberculosis* (Fukushima *et al.*, 2001). This division follows certain pathogenicity factors, and the strains representing the same serotype can be found in several pathogenicity types, even in the non-pathogenic group. Geographical division of these pathogenicity types has been demonstrated, and is responsible for the different clinical manifestations of *Y. pseudotuberculosis* noted in Far East and Western countries (Fukushima *et al.*, 2001). Although serotypes O:1 to O:5 have been isolated in both Europe and the Far East, it seems that serotypes O:1 and O:3 are more prevalent in Europe. Serotypes O:1b and O:3 have been the most common in Canada (Toma, 1986). In Japan, on the contrary, the majority of strains of human origin belong to serotype O:4b and the most often encountered serotypes are O:4b, O:3, O:5a, and O:5b (Tsubokura *et al.*, 1989).

The term "*Y. enterocolitica*–like bacteria" has generally been used for the species *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. rohdei*, *Y. ruckeri*, and *Y. aldovae* to collectively distinguish them from *Y. pestis*, *Y.*

pseudotuberculosis, and *Y. enterocolitica*. Among the recently described *Yersinia* species, at least *Y. aleksiciae* and *Y. massiliensis* could also be grouped with *Y. enterocolitica*–like species. *Y. enterocolitica*–like bacteria lack the classical *Yersinia* virulence markers and thus have generally been regarded as non-pathogenic. *Y. enterocolitica*–like bacteria have been isolated from healthy and sick humans, and from almost any possible environmental source imaginable (Sulakvelidze, 2000).

3 *Yersinia* infections in humans

3.1 Occurrence of *Y. enterocolitica* and *Y. pseudotuberculosis* infection

Yersiniosis is more common in countries with temperate climates rather than in tropical or subtropical regions. Bioserotype 4/O:3 predominates globally among *Y. enterocolitica*, and swine serve as a major reservoir of this bioserotype (Fredriksson-Ahomaa *et al.*, 2001a; Janda and Abbott, 2006; Kapperud, 1991; Smego *et al.*, 1999). In fact, data collected in Japan from imported pork, beef and fowl samples suggests that 4/O:3 strains have disseminated globally by means of imported domestic livestock, especially pigs, from European pig-producing countries to the U.S. and eventually to Japan (Fukushima *et al.*, 1997). Consequently, in areas of the world where pork consumption is restricted (i.e., the Middle East), incidence of yersiniosis is very low. The source of O:8 infections is more ambiguous, but data suggest that rodents may serve as a reservoir of this serotype (Hayashidani *et al.*, 1995; Janda and Abbott, 2006). *Y. enterocolitica* is the third most commonly reported cause of enteric zoonosis in Europe. In 2006, 8,979 confirmed cases of yersiniosis were reported in the EU with a decrease in incidence from 2.6 to 2.1 cases per 100,000 inhabitants from 2005. In Finland, yersiniosis is more commonly encountered than domestic salmonellosis. Most of the clinical isolates of *Y. enterocolitica* belong to biotype 1A (Sihvonen *et al.*, 2009; Sihvonen *et al.*, 2007). The incidence rate of *Y. enterocolitica* infections per 100,000 inhabitants has varied from 8 to 17 infections from 1995 to 2007 (Anonymous, 2008b).

Due to the inconsistency of surveillance systems in different countries, comparison of the incidences of *Yersinia* infections between different countries is only suggestive and susceptible to bias. For example, some of the countries report the incidence of *Yersinia* spp. (consisting mainly of *Y. enterocolitica* but containing also *Y. enterocolitica*–like and other *Yersinia* species), some report only *Y. enterocolitica* cases. In the latter cases, the prevalence of pathogenic *Y. enterocolitica* can also be overrepresented due to the inclusion of non-pathogenic biotype 1A strains. Currently, no method is available for identification of the potential clinically significant strains suggested among harmless, environmental strains of biotype 1A

(Tennant *et al.*, 2003). In 2006, the highest incidences of *Y. enterocolitica* infection in Europe were reported in Finland (15.1), Lithuania (12.1), Germany (6.3), and Sweden (6.2). Although the decrease in incidence was pronounced in Germany, this country still accounted for more than half (57.5%) of all infections reported in Europe. In addition, incidence rates above 1.0 has been reported in the Czech Republic (5.2), Denmark (4.0), Latvia (4.0), Slovenia (4.0), Estonia (3.5), Belgium (2.5), Norway (1.9), Austria (1.9), Slovakia (1.5), and Luxemburg (1.1) (EFSA, 2007a). According to the European Centre for Disease Control (ECDC, 2007), the trend of yersiniosis in Europe has been relatively stable between 1995 and 2004, although clear peaks were noticed in 1998 and 2002. The incidence of yersiniosis in Europe is highest among children under five years of age. In the U.S., Japan and Australia, which represent examples of non-European countries where data is readily available through open international sources, *Y. enterocolitica* incidence rates below 1.0 have been reported (Rocourt, 2003). Among the non-European countries 2001, the incidence rate in New-Zealand (11.5) was most similar to Finland (14.0) (Rocourt, 2003). In many countries, the actual incidence of yersiniosis is much higher than reported. Recently, it was estimated there were 2 200 gastroenteritis cases due to *Yersinia* spp. in an average year in Australia (Hall *et al.*, 2005) and studies conducted, for example, in England and Austria have revealed unreported subclinical or milder infections (Tomaso *et al.*, 2006; Wheeler *et al.*, 1999).

Surveillance data of *Y. pseudotuberculosis* is of limited availability throughout the world, but most likely the incidence of this infection is only a small fraction compared to *Y. enterocolitica* in most parts of the world. In the U.S., *Y. pseudotuberculosis* has been considered a serious and potentially emerging infection. Four of the 11 reported cases during 1996–2004 were diagnosed in 2003 (Long *et al.*, 2006). In Europe, many countries do not report *Y. pseudotuberculosis* separately from *Yersinia* spp., thus leaving the prevalence of this bacterium an enigma. Among EU member states in 2005, however, *Y. pseudotuberculosis* cases in humans were reported, in addition to Finland, by France (28 cases), Lithuania (6), the United Kingdom (4) and Spain (1) (EFSA, 2006). Recently, it was reported that *Y. pseudotuberculosis* has been rarely encountered in Spain with no apparent epidemiological relationship (Serra *et al.*, 2005). The situation in Finland, on the other hand, is unique among EU countries. The annual number of *Y. pseudotuberculosis* infections in Finland has been affected by outbreaks that have been recurring since 1997. Recently, the number of culture confirmed infections has followed the number of infections related to outbreaks (Figure 2). The number of *Y. pseudotuberculosis* cases in 2007 (confirmed by culture and antibody) dropped back to a low of 56 cases (representing an average year without outbreaks) from a high of 252 in 2006 (a year with two reported outbreaks), thus making the incidence of infection 1.0 per 100,000 inhabitants (Anonymous, 2008b). The situation most similar to that in Finland is probably in neighbouring Russia, where outbreaks of infection have also been described. Recently, the incidence of *Y. pseudotuberculosis* in Russia is believed to have risen due to rodent vectors, the numbers of which are increasing in city areas (Anonymous, 2006). In the city of Novosibirsk in Siberia, for example, the yersiniosis rate in 2004 was 29.35 infected per 100,000 inhabitants, a figure unexpectedly high compared to other countries and to the Russian average 3.96 per 100,000 (Anonymous, 2005c). A further increase in *Y. pseudotuberculosis* in Novosibirsk was noted in 2005 and 2006 (Anonymous, 2006). In Japan, many recurring outbreaks of *Y. pseudotuberculosis* have been described in the past (Tsubokura *et al.*, 1989). Recently, however, these outbreaks apparently have disappeared and the incidence of yersiniosis in Japan seems to be low (Rocourt, 2003).

Seasonal variation has been noted throughout the world with a tendency of *Yersinia* infections to occur during winter months in colder climates (Smego *et al.*, 1999). In the United States, the *Y. enterocolitica* infections have shown to accumulate between November and February. In Belgium, the infections have increased above average from August to October and decrease from February to June (Janda and Abbott, 2006). Overall in Europe, *Yersinia* follows an almost uniform seasonal distribution with a few more reported cases in the summer and early autumn months (EFSA, 2007a). In Japan, human infection by *Y. pseudotuberculosis* is most common in winter and spring (Vincent *et al.*, 2007). The seasonal variation of *Y. pseudotuberculosis* infections has not been pronounced in Finland, probably because outbreaks of infections have been observed year round.

3.2 Clinical features of *Y. enterocolitica* and *Y. pseudotuberculosis* infection

Yersiniae cause a variety of intestinal and extraintestinal illnesses ranging from pseudoappendicular syndromes to septicaemia, pharyngitis and infections of the joints and bones (arthritis and osteomyelitis). Typical forms of *Y. enterocolitica* and *Y. pseudotuberculosis* infections include gastroenterocolitis with non-specific diarrhoea and fever. Some patients, especially older children and adolescents, exhibit signs of a more invasive gastrointestinal disease such as acute mesenteric lymphadenitis, “pseudoappendicitis”, often with associated terminal ileitis with little or no diarrhoea. The most common post-infectious sequelae include reactive polyarthritis, erythema nodosum or, rarely, erythema multiforme (Bottone, 1997; Hartland and Robins-Browne, 1998; Smego *et al.*, 1999).

Antimicrobial therapy is not usually considered if enterocolitis is uncomplicated, but in severe systemic forms antimicrobials are useful. Most strains of *Y. enterocolitica* are inherently resistant to penicillins and aminopenicillins (including amoxicillin), carbenicillin (as well as ticarcillin) and narrow-spectrum cephalosporins (Janda and Abbott, 2006). The best results for treating *Y. pseudotuberculosis* infection have been obtained by using quinolones,

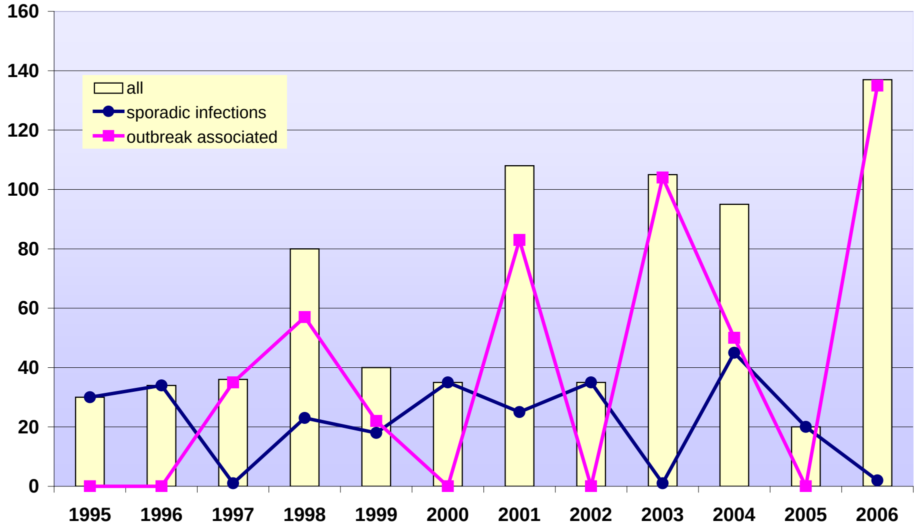

Figure 2. Microbiologically confirmed *Y. pseudotuberculosis* infections in Finland (according to the infectious diseases register of KTL, Finland). The term 'sporadic infections' refers to individual cases that are not linked to other known cases of illness. Outbreak associated infections have derived from a common source, as revealed by epidemiological investigation.

followed by doxycycline and gentamicin, in an invasive mouse model (Lemaitre *et al.*, 1991).

As for all pathogens, the outcome of exposure to pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* depends on a number of host factors including pre-existing immunity and the ability to elicit an immune response. Consequently, the incidence and severity of the infection and possibility of systemic infection is much higher in some groups of the population including children under five years of age (especially neonates <3 months of age), immunocompromised people (patients undergoing cancer chemotherapy, organ transplant, patients with metabolic or liver diseases, and the elderly (Gerba *et al.*, 1996; Janda and Abbott, 2006). In 2006, the age groups 0–4 and 5–14 years represented 32% and 20% of all reported *Y. enterocolitica* cases in Europe, respectively (EFSA, 2007a). The incubation period of *Y. enterocolitica* infection ranges from 1 to 11 days with symptoms typically persisting for 5 to 14 days and occasionally lasting for several months (Cover and Aber, 1989). Diarrhoea can vary in its severity from a few loose stools a day to a fulminate enterocolitis with ulcerative lesions involving the gastrointestinal tract (Cornelis *et al.*, 1987). In one study, the duration of infection was about 1 week in 22% and 2 to 4 weeks in 55% of the children studied. Additionally, 23% of the children had 3 to 4 periods of diarrhoea for 2 to 12 months (Hoogkamp-Korstanje and Stolk-Engelaar, 1995). Among the predisposing conditions increasing the risk for *Y. enterocolitica* bacteremia are iron overloaded conditions resulting, for example, from metabolic disorders hemochromatosis, hemosiderosis or beta-thalassemia (Adamkiewicz *et al.*, 1998; Janda and Abbott, 2006). There is a known association between deferoxamine, an iron chelator used to treat iron overload conditions, and *Yersinia* septicaemia. The iron is provided in utilizable form for *Y. enterocolitica* when deferoxamine complexes with iron *in vivo* (Robins-Browne, 1987). However, severe bacteremic forms are rare, and gastrointestinal yersiniosis is the most frequent outcome of *Y. enterocolitica* infection.

Y. pseudotuberculosis induces a wide variety of clinical symptoms in humans including fever, diarrhoea, scarlatiniform rash or erythema nodosum, vomiting and arthritis. The symptoms may be similar to *Y. enterocolitica* infection, but some differences also exist. Mesenteric lymphadenitis, presenting with fever, right lower quadrant abdominal pain imitating appendicitis, and leukocytosis is the most frequent clinical presentation of *Y. pseudotuberculosis* infections (Jalava *et al.*, 2006; Smego *et al.*, 1999; Tertti *et al.*, 1989). The "pseudoappendicitis" syndrome leads too often to unnecessary surgery revealing a usually normal appendix. Diarrhoea seems to be less frequent; in a study of *Y. pseudotuberculosis* O:1 outbreak, only 21% of the 40 case patients had diarrhoea compared to 93% and 83% with fever and abdominal pain, respectively (Jalava *et al.*, 2006). Similarly, in an outbreak of serotype O:3 infection, only four of the 19 case patients had diarrhoea (Tertti *et al.*, 1984). *Y. pseudotuberculosis* infections occur most frequently in older children, adolescents and young adults, especially males (Janda and Abbott, 2006; Smego *et al.*, 1999; Vincent *et al.*, 2007). Rarer complications of *Y. pseudotuberculosis* infection include hemolytic uremic syndrome (HUS) (Davenport and Finn, 1988). The incubation period of *Y. pseudotuberculosis* infection is somewhat longer than in *Y. enterocolitica* infection; the median incubation period is 8 days with the range of 2 to 25 days (Inoue *et al.*, 1988a; Jalava *et al.*, 2006; Sato and Komazawa, 1991). The median duration of illness in *Y. pseudotuberculosis* infection has been reported to be 18 days with the range from 1 to 37 days (Jalava *et al.*, 2006).

On the Pacific coast of Russia, the clinical picture of *Y. pseudotuberculosis* has sometimes been more severe and involved erythematous skin rash, conjunctivitis, skin desquamation, exanthema (scarlatinoid rash), strawberry tongue and toxic shock syndrome. This disease has been termed Far East scarlet-like fever (FESLF), or scarlatinoid fever because of the clinical similarities to scarlet fever caused by group A streptococci (Eppinger *et al.*, 2007). It has been known in Japan since the late 1920s as Izumi fever, and was first detected in Far East Russia in 1959-1960 (Vincent *et al.*, 2007). The disease is now also commonly found in Western Russia (Saint Petersburg region), and the strains capable of initiating the disease might now be spreading further west among wildlife (EFSA, 2007b; Eppinger *et al.*, 2007). Gastrointestinal *Y. pseudotuberculosis* infection has also been linked to Kawasaki syndrome, an acute multisystem vasculitis of young children, which is the leading cause of acquired heart disease in children living in industrialized countries (Vincent *et al.*, 2007). The clinical picture of Kawasaki disease resembles that of scarlatinoid fever, although the infectious etiology of this disease is unknown. It seems likely that, together with largely unknown host predisposing factors, superantigens produced by *Y. pseudotuberculosis*, streptococci, and staphylococci play an important role in the onset of Kawasaki disease (Matsubara and Fukaya 2007; Vincent *et al.* 2007).

The minimal infective dose for *Yersinia* infection in humans has not been determined. Ingestion of 3.5×10^9 *Y. enterocolitica* cells by a volunteer has been reported to result in a diarrhoea that began on the same day as the ingestion. However, in most clinical settings, an inoculum this large is presumably not ingested (Cover and Aber, 1989; Szita *et al.*, 1973). Most likely many strain dependent factors, not just the initial ingested dose of bacteria, are more important in the clinical outcome and onset of *Yersinia* infection. Among already identified associations is the bioserotype of the infecting strain, for example, bioserotype 1B/O:8 strains are capable of more invasive and destructive infection than, for example bioserotype 4/O:3 strains (Bottone, 1997). Most significant, however, is probably the overall balance between the host and the invading strain, that is, the interplay between the effectiveness of early host defence mechanisms and the ability of bacteria to survive these mechanisms and rapidly upregulate virulence determinants against the host immune system.

The most common post-infectious sequelae of yersiniosis are reactive arthritis and erythema nodosum occurring usually a few weeks after the acute phase. Reiter's syndrome (concurrent presentation of arthritis, conjunctivitis, and urethritis), uveitis, glomerulonephritis and myocarditis have also been reported (Aho *et al.*, 1974; Bottone, 1997). The most well-known enteric bacteria capable of triggering reactive arthritis are *Y. enterocolitica*, *Y. pseudotuberculosis*, different serovars of *Salmonella enterica*, *Campylobacter jejuni* and *Shigella flexneri* (Hannu *et al.*, 2006). A high rate (>50% or even 92% in age group <15 years) of post-infectious sequelae including erythema nodosum, arthritis, iritis and nephritis has been noted during gastrointestinal *Y. pseudotuberculosis* infections (Jalava *et al.*, 2006; Tertti *et al.*, 1984). It has been concluded from the literature of *Y. pseudotuberculosis* infections that the occurrence of reactive arthritis varies according to the differences in the arthritogenic potential of different *Y. pseudotuberculosis* serotypes, differences in case ascertainment and definitions used for *Y. pseudotuberculosis* infection in the outbreaks, as well as different definitions of reactive arthritis (Hannu *et al.*, 2003). Post-infectious reactive arthritis is associated with HLA (Human leukocyte antigen) B27 tissue type positive individuals (Aho *et al.*, 1973) whereas erythema nodosum is not (Tertti *et al.*, 1989). Although culturable bacteria are not usually isolated in affected joints, bacterial antigens are found within inflammatory cells (Granfors *et al.*, 1989). Among *Y. enterocolitica*, development of reactive arthritis is most often associated to bioserotype 4/O:3, and phage type VIII infections (Bottone, 1997).

Y. enterocolitica and *Y. pseudotuberculosis* O-antigen and LPS structures have a putative role in the pathogenesis of reactive arthritis (Skurnik and Bengoechea, 2003). Another factor involved includes a 19 kDa protein identified as urease ß subunit (Mertz *et al.*, 1991; Skurnik *et al.*, 1993) and, in the case of *Y. enterocolitica* bioserotype 1B/O:8 and *Y. pseudotuberculosis*, probably the superantigen activity produced by these bacteria (Simonet, 1999).

3.3 *Yersinia* virulence factors

Plasmid-encoded virulence factors

For full virulence, all pathogenic *Yersinia* need a 70-kb plasmid called pYV (for *Yersinia* virulence plasmid) (Gemski *et al.*, 1980b; Zink *et al.*, 1980). It was initially discovered that pathogenic strains were Ca^{2+}-dependent when grown at 37°C, a property that could be lost along with virulence and only later understood to be uniformly virulence plasmid encoded. Many chromosomal and pYV-encoded virulence factors contribute to the virulence of *Y. enterocolitica* and *Y. pseudotuberculosis* (Table 1, p. 35–36) and many of them are also common with *Y. pestis*. The pYV encodes type III secretion system, the effector Yops and outer membrane protein YadA, described below. In addition to pYV, *Y. pseudotuberculosis* has been known to harbour plasmids of various sizes, of which a large 153 kb plasmid (pYpsIP31758.1, also termed pVM82) is phylogenetically unrelated to all currently reported *Yersinia* plasmids and is associated to pathogenicity (Eppinger *et al.*, 2007; Gintsburg *et al.*, 1988). It encodes an *icm/dot* type IVB secretion system that could be involved in the unique host immune system response leading to typical clinical presentations of Far East scarlet-like fever (Eppinger *et al.*, 2007).

Type III secretion system encoded by pYV is widespread among pathogenic Gram-negative bacteria and designed to counteract the multiple signalling responses in the infected host cell (Grosdent *et al.* 2002; Mota and Cornelis 2005; Viboud and Bliska 2005). The type III secretion system consists of an Ysc injectisome made of 29 Ysc proteins. The effector proteins called Yops (for *Yersinia* outer (secreted) proteins) are translocated into the host cytosol through a channel formed by the proteins YopB, YopD and LcrV (Cornelis, 2002b). The Ysc injectisome ends up with a needle made of YscF, the length of which is controlled by YscP. Secretion of some effector proteins through the injectisome also requires specific chaperones called Syc proteins. Four of the Yop effectors, YopE, YopH, YopT and YpkA (YopO in *Y. enterocolitica*) disturb the host cell cytoskeleton dynamics and contribute to the strong resistance of *Yersinia* to phagocytosis by macrophages. The targets of YopE, YopT, and YpkA (YopO) are Rho GTPases, small GTP-binding proteins that regulate a diverse range of cellular functions including regulation of the actin cytoskeleton and gene expression. YopH is a highly active protein tyrosine phosphatase that antagonizes several signalling pathways associated with phagocytosis of bacteria by host cells (Fällman *et al.*, 1997). YopJ (YopP in *Y. enterocolitica*) blocks the MAPK and NF-κB signalling pathways of host cells. Activation of NF-κB is central in the onset of inflammation and these events reduce the recruitment of neutrophils to the site of infection. In addition, YopJ/YopP induces macrophage apoptotic death. Like the other Yop effectors, YopM is delivered to the host cell, but based on current knowledge it seems that YopM does not encode an enzymatic activity. It travels to the nucleus of the target cell, but it is not yet known how this localization is related to its function. Along with other Yops, it is nevertheless an important *Yersinia* virulence factor (Cornelis, 2002b; Viboud and Bliska, 2005). Transcription of many pYV genes, including all the *yop* genes, *sycE*, *yadA*, and the *virC* operon, is dependent on the VirF/LcrF transcriptional activator (Cornelis *et al.*, 1998).

The outer membrane protein YadA (*Yersinia* adhesin A) is an important factor for the enteric route of infection in *Y. enterocolitica* and presents as a fibrillar surface matrix extending from the outer membrane. YadA promotes adhesive and invasive abilities by binding collagen, laminin and fibronectin and serves as an important colonization factor in addition to other roles in *Y. enterocolitica* virulence (El Tahir and Skurnik, 2001). In addition, YadA is a major serum resistance factor which can protect the bacteria against the complement mediated killing by binding complement mediating factors H and C4b (Biedzka-Sarek *et al.*, 2008; Biedzka-Sarek *et al.*, 2005; Kirjavainen *et al.*, 2008). However, for *Y. pseudotuberculosis* virulence, YadA seems to be dispensable, and in *Y. pestis*, YadA is not functional (Rosqvist *et al.*, 1988; Skurnik and Wolf-Watz, 1989). Nevertheless, a recent study has revealed YadB and C proteins in *Y. pseudotuberculosis* and *Y. pestis* that act as adhesins. The main function of these novel proteins is still under investigation and, according to the authors, it could be related to the more highly disseminatory character of these organisms compared to *Y. enterocolitica* (Forman *et al.*, 2008).

In addition to virulence associated genes, the virulence plasmid of low-pathogenic bioserotypes of *Y. enterocolitica* (for example bioserotype 4/O:3) is unusual for also containing resistance genes to arsenite and arsenate (Neyt *et al.*, 1997). Arsenical anti-spirochetal treatments were used for pigs in the past and arsenic resistance may have given some strains of *Y. enterocolitica* a survival advantage in those conditions and may have played a role in the global spread of these strains.

Chromosomally encoded virulence factors

Although many different virulence factors for surviving and multiplying in host are encoded by pYV, pathogenic *Yersiniae* need also chromosomally encoded virulence factors for full virulence (Table 1) (Revell and Miller, 2001). Virulence factors important for *Y. enterocolitica* in adherence to and invasion of epithelial cells of the host include *inv* (invasion) and *ail* (attachment invasion locus) (Miller and Falkow, 1988). Invasin encoded by the *Y. enterocolitica inv* gene is a 92-kDa (103-kDa in

Y. pseudotuberculosis) outer membrane protein and serves as a primary invasion factor in tissue culture models. A functional *inv* gene is present on the chromosome of *Y. pseudotuberculosis* and *Y. enterocolitica*, while *Y. pestis* has a disrupted form of *inv* (Revell and Miller, 2001). RovA, a transcriptional regulator is required for *inv* expression in *Y. enterocolitica* and *Y. pseudotuberculosis.* Mutation in *rovA* had a more severe impact on virulence than loss of *inv* alone (Revell and Miller, 2000). RovA seems to be important for the oral infection route and probably required in early events of infection that occur in the Peyer's patches (PPs) (Dube *et al.*, 2003). The surface protein Ail (*ail*–encoded) promotes tissue culture adherence and cell line–specific invasion. During the infection, it most probably serves as an attachment and secondary invasion factor. It contributes in resistance to killing by human serum (Bliska and Falkow, 1992; Pierson and Falkow, 1993) and it is present in *Y. enterocolitica* serotypes commonly associated with disease, as well as in *Y. pseudotuberculosis* and *Y. pestis* (Miller *et al.*, 1989; Parkhill *et al.*, 2001; Yang *et al.*, 1996). However, it seems that *ail* has a different role in the virulence of *Y. pseudotuberculosis*; the functional Ail protein has no adhesive activity in *Y. pseudotuberculosis* although it does promote serum resistance (Yang *et al.*, 1996).

Lipopolysaccharide (LPS) is an important surface component of Gram-negative bacteria. It consists of hydrophobic lipidA which is integrated in the outer leaflet of bacterial outer membrane and is responsible for the endotoxin activity. The most sensitive mechanism by which animals detect Gram-negative bacteria is recognizing lipid A (Munford, 2008). LPS further consists of the hydrophilic polysaccharide chain (core oligosaccharide and O-antigen) extending out from the bacterial surface. *Y. enterocolitica* O:3 LPS has a unique branched chain structure where O-antigen and outer core oligosaccharides are linked to different parts of the inner core. The outer core has an indirect role in resistance to killing by normal serum. It is required for full virulence of *Y. enterocolitica* O:3 and it most probably provides the bacteria with resistance to cationic bactericidal peptides (Skurnik *et al.*, 1999). *Y. enterocolitica* and related species are divided into at least 76 different serotypes based on structural variation of antigenic sugar residues present in the O-polysaccharides (O-antigen) (Wauters *et al.*, 1991). O-antigen is an essential virulence factor of *Y. enterocolitica* (Al-Hendy *et al.*, 1992; Zhang *et al.*, 1997) and among other possible functions, plays a role in the serum resistance of *Y. enterocolitica* O:3 along with YadA, Ail and outer core oligosaccharide (Biedzka-Sarek *et al.*, 2005). O-antigen also plays a role in the virulence of *Y. pseudotuberculosis* (Mecsas *et al.*, 2001), but not of *Y. pestis*, which does not express O-antigen at all. O-antigen, as an outermost part of the *Y. enterocolitica* membrane, plays a critical role in the bacterial interaction with the environment. The results of Bengoechea *et al* (2004) suggested that in *Y. enterocolitica* the absence of O-antigen either directly or indirectly acts as a regulatory signal affecting at least the expression of various outer membrane component genes, such as *flhDC*, *yplA*, *inv* or *ail*. Thus, changes in the O-antigen affect bacterial virulence and also expression of other virulence

Table 1. Major virulence factors of the enteropathogenic *Yersinia* species and *Y. enterocolitica*–like strains

Virulence marker	**Reference(s)**	**Phenotype/Function**	**Presence in pathogenic bioserotypes of *Y. ent.***	**Presence in *Y. pseudot.***	**Plays a role in *Y. enterocolitica* and *Y. pseudotuberculosis* virulence?**	**Presence in *Y. enterocolitica*-like species[1]**
Plasmid (pYV) encoded						
Yersinia outer membrane proteins (Yops)	Cornelis 1998	Confers resistance to phagocytic activity of the host cell, distrupts host signalling mechanisms (etc.)	+	+	Yes	Not found (including invasive strains). Some invasive strains carry large plasmids distinct from pYV. Their role in the invasion process or in resistance to phagocytosis observed in some *Y. enterocolitica* -like species has not been demonstrated
Yersinia adhesin (YadA)	El Tahir and Skurnik 2001	Adhesion factor that binds to extracellular matrix. Serum resistance and invasion factor.	+	+	Plays a role in virulence of *Y. enterocolitica*	
Chromosomally encoded						
Invasin (*inv*-encoded)	Isberg and Falkow 1985, Miller and Falkow 1988	Protein that attaches to specific ß1 integrin subset receptors located on mucosal cells; hence directly initiating the entry process via M cells	+	+	Yes	Found in several *Y. enterocolitica* -like species, but may be nonfunctional. The *inv*- negative *Y. bercovieri* strains can still invade Caco2 cells
RovA (*rovA*-encoded, regulator of virulence)	Revell and Miller 2000	Regulates expression of the invasin (*inv*). Mutation in *rovA* has a more severe impact on virulence than loss of *inv* alone	+	+	Yes	Not known
Ail (attachment invasion locus) (*ail*-encoded)	Miller *et al*. 1989, Yang *et al*. 1996	Encodes a protein (17 kDa) that promotes resistance to complement mediated killing (*Y. ent.* and *Y. pseudot.*). Serves as an attachment and secondary invasion factor (*Y. ent.*).	+	+	Yes	Not found
YSTs	Delor and Cornelis 1992	*Y. enterocolitica* heat-stable enterotoxins.Yst is a potential mediator of the diarrhoea observed in infants infected with *Y. enterocolitica*	+	-	Plays a role in *Y. enterocolitica* associated diarrhoea in young rabbit	Some species (e.g., *Y. bercovieri*, *Y. mollaretii*) produce heat-stable enterotoxins distinct from YSTs.
YPMs (*Y. pseudotuberculosis*-derived-mitogen)	Carnoy *et al*. 2006	Superantigenic toxins of 14,5 kDa that bind to antigen presenting cells (to major histocompatibility class II molecules) and specifially recognize the variable region of the ß chain (Vß) of T cell receptors. These interactions release large amounts of inflammatory cytokines, which can cause toxic shock and tissue damages.	-	+/-[2]	Plays a role in virulence of some strains of *Y. pseudotuberculosis*	Not found

Virulence marker	Reference(s)	Phenotype/Function	Presence in pathogenic bioserotypes of *Y. ent.*	Presence in *Y. pseudot.*	Plays a role in *Y. enterocolitica* and *Y. pseudotuberculosis* virulence?	Presence in *Y. enterocolitica*-like species[1]
Chromosomally encoded						
HPI (high-pathogenicity island)	Carniel 2001	Yersiniabactin siderophore production (iron uptake), present in high-pathogenic *Yersinia* species	+/-[3]	+/-[4]	Plays a role in virulence of some strains of *Y. enterocolitica* and *Y. pseudotuberculosis*	Not found
YAPI (pathogenicity island carrying *pil* operon)	Collyn *et al*. 2002, 2005	Encodes Type IV pilus which contributes to the pathogenicity of *Y. pseudotuberculosis* most likely by facilitating colonization of the host intestinal mucosa. Associated to the presence of *ypm* genes	+/-[5]	+/-[2]	Plays a role in virulence of some strains of *Y. enterocolitica* and *Y. pseudotuberculosis*	Not known
LPS O-antigen and outer core	Al-Hendy *et al.* 1992, Zhang *et al*. 1997, Skurnik and Bengoechea 2003	Interaction with the environment, plays an important role in effective colonization of host tissues, in resistance to complement-mediated killing and in resistance to cationic antimicrobial peptides	+	+	Yes	+
Urease (*ure*-encoded)	de Koning-Ward and Robins-Browne 1995, Gripenberg-Lerche *et al*. 2000	Helps the bacteria to survive in the acidic environment of the stomach before entering the small intestine. The urease activity leads to release of ammonia and thus to elevation of cytoplasmic pH	+	+	Plays a role in virulence of *Y. enterocolitica* but is not essential for the virulence of *Y. pseudotuberculosis*	+
Myf fibrillae (*myf*-encoded) (in *Y. enterocolitica*), pH 6 antigen (*psa*-encoded) (in *Y. pseudotuberculosis*)	Iriarte *et al*. 1993, Lindler *et al*. 1993, Yang *et al*. 1996	Proteic polymer functioning as fimbrial adhesins, antiphagocytic	+	+	The role in virulence not demonstrated (*myf* is associated with pathogenic bioserotypes of *Y. enterocolitica*)	Not known

+, factor present; -, factor absent; +/-, factor present in some strains.

[1] Data concerning *Y. enterocolitica* -like species and Yops, YadA, Inv, Ail, YSTs, and urease according to Sulakvelidze (2000).

[2] Not present in all strains. The prevalence is higher among *Y. pseudotuberculosis* strains of Far East origin.

[3] Present in *Y. enterocolitica* biotype 1B strains.

[4] Present in *Y. pseudotuberculosis* serotype O:1 (and O:3) strains.

[5] Homologous sequences have been detected in sequenced genome of bioserotype 1B/O:8 (Collyn *et al*. 2004)

factors. It seems that O-antigen is needed during the first hours of infection whereas the outer core is required for prolonged survival of the bacteria in PPs and for invasion of deeper tissues like liver and spleen (Skurnik *et al.*, 1999).

In addition to the Ysc type III secretion system encoded by the virulence plasmid pYV and important for systemic infections, chromosomal loci encoding an additional type III secretion system called Ysa (for *Yersinia* secretion apparatus) have recently been identified in all three highly virulent species of *Yersinia* (Haller *et al.*, 2000; Parkhill *et al.*, 2001). The presence of this Ysa-Ysp system in *Y. enterocolitica* is restricted to the biotype 1B (Foultier *et al.*, 2002; Foultier *et al.*, 2003), and it is important for the *Y. enterocolitica* survival during the gastrointestinal phase of infection (Venecia and Young, 2005; Young, 2007). Venecia and Young (2005) demonstrated that the Ysa type III systems enhance colonization of the terminal ileum and the cecum early during infection. The Ysa type III secetion system (T3SS) appears to deliver a collection of ten, and potentially eleven, effectors depending upon the strain examined, into the host cell (Matsumoto and Young, 2009). Three of these effectors, YopE, YopN and YopJ/P are shared with Ysc T3SS. Thus, Ysa T3SS plays a role in virulence although the exact functions of its effectors in the pathogenesis of *Y. enterocolitica* still remain to be elucidated. Recently, the presence also of type VI (T6SS) and plasmid-borne T4SS has been suggested in *Y. pseudotuberculosis* based on genomic analysis (Bingle *et al.*, 2008; Eppinger *et al.*, 2007) In addition to the described and potential secretion systems, the basal body of the motive organelle flagellum is capable of functioning as a type III secretion system in *Yersinia* (Young *et al.*, 1999). The structure of the basal body is similar to the type III secretion apparatus and it traverses from the cytoplasm to the outside of the cell (Kubori *et al.*, 1998). The flagellar type III secretion system usually exports the flagellum subunits for assembly to the outer surface of the cell, but it additionally also transports proteins not related to motility to the extracellular milieu. One of the flagellar secreted outer proteins (Fops) is the *yplA*-encoded phospholipase A (YplA) implicated in *Y. enterocolitica* virulence. An *yplA* mutant strain showed reduced inflammation in the mouse infection model (Schmiel *et al.*, 1998). Therefore, it has been suggested that the flagellar system may be a general mechanism for the transport of proteins that influence bacterial-host interactions (Young *et al.*, 1999).

The enzyme urease plays a major role in many bacteria, including *Y. enterocolitica* in response to acidity of the stomach. Urease is necessary for the survival and pathogenesis of *Y. enterocolitica*. Urease catalyzes the hydrolysis of urea to ammonia and carbamic acid and the latter spontaneously hydrolyzes to form carbonic acid and an additional molecule of ammonia. This results in an elevation of the cytoplasmic pH in *Yersinia* cells and enhances the survival of bacteria (de Koning-Ward and Robins-Browne, 1997). Another major function of urease is to utilize urea as a source of nitrogen necessary for growth in urease containing bacteria. It has been speculated that the structural unit UreB of *Y. enterocolitica* urease has a role as an arthritogenic factor; intra-articular injection of this polypeptide into preimmunized rats induced arthritis (Mertz *et al.*, 1991; Skurnik *et al.*, 1993). However, Gripenberg-Lerche *et al.* (2000) showed that *Y. enterocolitica* O:8 urease ß subunit does not play a role in the induction of arthritis after bacterial i.v. injection of rats. Unlike the role of urease in *Y. enterocolitica*, urease is not essential for *Y. pseudotuberculosis* virulence in mice (Riot *et al.*, 1997). According to the authors, this might be explained by the fact that *Y. pseudotuberculosis* requires a urea concentration of at least 20 mM, a concentration 15-fold higher than normally present in gastric secretions of mammals, in order to tolerate acidity (pH 2), whereas *Y. enterocolitica* tolerates acidity at urea concentrations of 0.3 mM.

The heat-stable enterotoxin Yst of *Y. enterocolitica* is encoded by the *yst* gene and resembles the heat-stable (ST) enterotoxin of enterotoxigenic *E. coli* (ETEC) (Delor *et al.*, 1990). *Y. enterocolitica* has YstA, YstB, and YstC variants. YstA is present mainly in pathogenic bioserotypes of *Y. enterocolitica* and may contribute to the pathogenesis of diarrhoea associated with acute yersiniosis (Delor and Cornelis, 1992). YstB seems to be associated to biotype 1A strains; more than 80% of these strains carry the *ystB* gene (Tennant *et al.*, 2003). YstC has the largest molecular size and the highest toxicity among STs (Yoshino *et al.*, 1995b) and has not been detected among *Y. enterocolitica* biotype 1A strains (Grant *et al.*, 1998). The role of Yst enterotoxin as a virulence factor inducing diarrhoea at body temperatures was doubted because of the observation that the enterotoxin gene is transcribed only at temperatures below 30°C. However, Mikulskis *et al.* (1994) reported that *ystA* transcription could be induced at 37°C by increasing osmolarity and pH to the values normally present in the ileum lumen. Furthermore, based on the experimental data from a young rabbit model, Delor and Cornelis (1992) postulated that YstA could be an important factor in diarrhoea in young children infected with *Y. enterocolitica*.

Some strains of *Y. pseudotuberculosis* produce superantigen toxins, *Y. pseudotuberculosis*–derived mitogens (YPMs), which contribute to the systemic illnesses associated with this organism (Abe *et al.*, 1993; Uchiyama *et al.*, 1993). YPM toxins are proteins that induce uncontrolled host immune system activation by stimulating the proliferation of polyclonal T lymphocytes (Abe *et al.*, 1993; Carnoy *et al.*, 2000). Superantigenic toxins are produced by a variety of Gram-positive bacteria and some viruses. *Y. pseudotuberculosis* is the only known Gram-negative bacterium capable of synthesizing superantigenic toxins (Carnoy *et al.*, 2006). There are three variants of YPM encoded by *ypmA*, *ypmB* and *ypmC* in *Y. pseudotuberculosis* (Carnoy *et al.*, 2006; Ramamurthy *et al.*, 1997). YPMa and YPMb share an 83% amino acid similarity. YPMc, on the other hand, differs from YPMa by only one amino acid, leaving only a single nucleotide difference for distinguishing between *ypmC* and *ypmA* genes by sequencing. The most frequent allele is *ypmA*, since it is found in 83% of superantigenic isolates, whereas *ypmB* and *ypmC* are present in 5% and 12% of these isolates, respectively (Carnoy *et* *al.*, 2006; Fukushima *et al.*, 2001) The systemic expression of YPMa has been linked to scarlatinoid fever syndrome, the more severe clinical manifestation of *Y. pseudotuberculosis*. The presence of the *ypmA* gene in *Y. pseudotuberculosis* is associated predominantly with strains of Far East Asian origin and also to the differences in the clinical manifestations observed between the Far East and Europe (Yoshino *et al.*, 1995a).

Almost all *Y. pseudotuberculosis* O:1 strains of European origin, *Y. enterocolitica* biotype 1B, and *Y. pestis* harbour the HPI (high-pathogenicity island), a cluster of genes intended for iron uptake (Carniel, 2001). Iron is an essential growth factor for nearly all bacteria (Finkelstein *et al.*, 1983). Iron is readily available in many environments and culture media, but in mammalian tissues, however, iron is tightly bound to carrier proteins such as transferrin and lactoferrin. Many bacteria release low-molecular-weight iron chelators known as siderophores in order to obtain iron in iron depleted environments. The *Yersinia* HPI includes genes involved in synthesis of the siderophore yersiniabactin, the presence of which is important for the systemic dissemination of bacteria in the host. Without available iron, the low-pathogenic *Y. enterocolitica* strains (biotype 4 and 2) usually cause moderate intestinal symptoms, whereas when iron is made available, these strains are able to multiply in the host and cause systemic infections (Carniel, 2001; Robins-Browne and Prpic, 1985). In *Y. pseudotuberculosis*, the presence of the HPI is associated with serotype O:1; all O:1a strains and 84% of O:1b strains originating from Western continents (Europe, Australasia, America) harbour a complete HPI (Fukushima *et al.*, 2001). Occasionally, the complete HPI can be detected in strains of serotypes O:3, O:5a, O:5b, O:13, O:14, and untypeable strains of *Y. pseudotuberculosis.* An incomplete HPI with a truncation in the right-hand part (R-HPI) seems to be specific for serotype O:3; R-HPI is detected in 57% of O:3 strains, but not in other serotypes. In *Y. enterocolitica*, the presence of the HPI is restricted to biotype 1B strains and it has not been detected in low-pathogenic or non-pathogenic strains of *Yersinia* (De Almeida *et al.*, 1993).

In addition to the HPI, some *Y. pseudotuberculosis* strains harbour a unique pathogenicity island, YAPI, which carries a *pil* operon encoding a type IV pilus. Type IV pili present as large bundles of pili aggregates on the surface of the bacteria and contribute to the pathogenicity of *Y. pseudotuberculosis* most likely by facilitating colonization of the host intestinal mucosa (Collyn *et al.*, 2002). Like HPI, YAPI is not uniformly distributed within the species. In contrast to the HPI, however, YAPI seems to be spread across the species independently of the O serotype and seems to be associated with the presence of the superantigen-encoding *ypm* genes (Collyn *et al.*, 2005; Collyn *et al.*, 2002). YAPI is not present in *Y. pestis,* whereas in sequenced genome of *Y. enterocolitica* bioserotype 1B/O:8, homologous pathogenicity island sequences have been detected (Collyn *et al.*, 2004).

Myf (for mucoid *Yersinia* factor/fibrillae) is a pilus-like adhesion factor the production of which is temperature and pH regulated. The 21-kDa major subunit of this antigen is encoded by *myfA*, whereas the transport and assembly of pilin subunits at the bacterial cell surface is encoded by *myfB* and *myfC*. The counterpart of MyfA in *Y. pseudotuberculosis* is the adhesin PsaA. The cluster of *psa* genes is required for the synthesis and assembly of pH6 antigen, a pilus-like surface structure responsible for thermoinducible binding and hemagglutination (Yang *et al.*, 1996). Although *psaA* has been associated with the virulence of *Y. pestis* (Lindler *et al.*, 1990) and the presence of Myf is restricted to pathogenic serotypes of *Y. enterocolitica* (Iriarte *et al.*, 1993), according to Revel and Miller (2001) neither of these two loci have been tested for their role in the virulence of *Y. pseudotuberculosis* or *Y. enterocolitica*, respectively.

Among the more recently identified chromosomal virulence factors of *Y. enterocolitica* is SodA, the Mn-cofactored superoxide dismutase. It has a potential role in protecting bacteria from reactive oxygen during the inflammatory process and thus conferring to a decrease in susceptibility to killing by neutrophils *in vitro* (Revell and Miller, 2001; Roggenkamp *et al.*, 1997).

In addition, many new virulence factors are continually being discovered. To identify virulence genes, the *in vivo* expression technology (IVET) and signature-tagged mutagenesis (STM) techniques have been used for human pathogenic *Yersinia* species (Darwin, 2005). IVET identifies bacterial genes that are expressed during an animal infection. However, to verify their role in virulence, null mutants of the identified genes must be tested in a mouse infection model. STM is a direct screen of null mutants that can identify mutants with either severe or subtle virulence defects in an animal model of infection. With this technique, for example, O-antigen biosynthesis genes and a chromosomal *pspC* gene, a phage shock protein homologue of *E. coli*, has been identified to be essential for *Y. enterocolitica* virulence (Darwin and Miller, 1999). Because of the different standpoints of these techniques (reviewed by Darwin, 2005), however, they usually identify different genes. Furthermore, because of the differences in animal infection models, some of the major known virulence factors of pathogenic *Yersinia* are often not identified with these techniques.

The pathogenicity of *Y. pestis*, *Y. pseudotuberculosis*, and certain biotypes of *Y. enterocolitica* is well established and many of the virulence factors of these species are thoroughly studied. On the other hand, *Y. enterocolitica* biotype 1A and *Y. enterocolitica*–like strains lack the known virulence determinants of classical pathogenic species and are, therefore, generally regarded as non-pathogenic. In addition, these bacteria are abundant in the environment and frequently isolated from asymptomatic patients. Tennant *et al.* (2003), however, have suggested that *Y. enterocolitica* biotype 1A harbours a "clinical" subgroup that is pathogenic via novel virulence mechanisms. Suggested candidates for pathogenicity factors of biotype 1A include heat-stable enterotoxin (YstB) and fimbrial adhesins (Table 1) along with genes homologous to insecticidal toxin complex genes (TC) (Tennant *et al.*, 2003; Tennant *et al.*, 2005). The toxin homologues *tcbA*, *tcaC*, and *tccC* were shown to be significantly more prevalent among clinical biotype 1A strains than among other *Yersinia* strains. The constructed toxin homologue mutants were attenuated in the ability to colonize the gastrointestinal tracts of perorally infected mice (Tennant *et al.*, 2005). Another study also suggests that the low-temperature induced insecticidal toxin genes *tcaA* and *tcaB* have a role in virulence outside mammalian hosts, possibly in invertebrates (Bresolin *et al.*, 2006a). Enterotoxin production has been detected in *Y. intermedia*, *Y. kristensenii*, *Y. frederiksenii*, *Y. bercovieri* and *Y. mollaretii*, although these enterotoxins diverged from the classical Yst enterotoxin of pathogenic *Y. enterocolitica* (Sulakvelidze, 2000). At least some strains of *Y. kristensenii* have been shown to be virulent for mice pretreated with iron dextran, although only upon intraperitoneal infection (Robins-Browne *et al.*, 1991). The study of Robins-Browne *et al.* (1991), however, found no correlation between the ability of *Y. kristensenii* strains to produce enterotoxin and their virulence for iron stressed mouse. The mechanism of the pathogenicity observed remains unknown. The pathogenic potential of *Y. enterocolitica*–like species is far from clear and remains to be elucidated by subsequent investigations.

3.4 Pathogenesis of *Y. enterocolitica* and *Y. pseudotuberculosis* infection

Y. enterocolitica and *Y. pseudotuberculosis* infections in humans are usually acquired by fecal-oral spread via ingestion of contaminated food products or water. Additional transmission routes include direct person-to-person spread and animal-to-human contact. In rare cases, yersiniosis can be acquired through blood transfusion since *Y. enterocolitica* is one of the most common contaminants of blood products (Vonberg and Gastmeier, 2007).

Soon after entering the host with ingested food or water, pathogenic *Yersinia* cells adapt to a temperature shift and prepare for host immune responses. After reaching the stomach, bacteria must survive the gastric acid barrier (pH 1–2), the first important defence mechanism of the host against infectious diseases transmitted by the oral route (Martinsen *et al.*, 2005; Tennant *et al.*, 2008). Ureolytic bacteria metabolize urea molecules to CO_2 and ammonia with the help of the urease enzyme. The release of ammonia elevates the cytoplasmic pH in the bacterial cells, and is thought to enhance the survival and colonization of ureolytic *Yersinia in vivo*. *Yersinia* urease most likely senses a decrease in cytoplasmic pH when cells are intact. In whole cells, the urease activity optimum is as low as pH 1.5, which explains the rapid response of bacteria to changes in pH (Young *et al.*, 1996). Some of the *Y. enterocolitica* initial virulence mechanisms are shown *in vitro* to be well adapted to the acidic environmental pH prevailing at this phase of infection, possibly favouring invasion also *in vivo*. The production of the Myf fibrillar adhesion factor, for example, requires an acidic pH and, like Yops and YadA, it is only produced at 37°C. Judging by the similarities to the enterotoxigenic *E. coli* fimbrial system CS3, it has been suggested that Myf could promote the adhesion of *Y. enterocolitica* to enterocytes and thus allow the action of Yst enterotoxin (Iriarte and Cornelis, 1995; Iriarte *et al.*, 1993). Just as Myf, Invasin, the invasion factor encoded by *inv* is produced *in vitro* at 37°C in acidic conditions although the production is otherwise reduced at this temperature (Pepe *et al.*, 1994). Invasin is expressed *in vitro* maximally after growth at 28°C, and as the temperature shifts to 37°C, the second adhesion and invasion factor Ail is maximally expressed. *Yersinia* is famous for its ability to switch on the virulence machinery upon entry to the host temperature and to downregulate this machinery, and even switch to the cold-adaptation process, when exiting to the environment or staying in food before entering the next host and temperature adaptation cycle.

The surviving enteropathogenic *Yersinia* have the potential to make their way to the small intestine to reach the terminal part of the ileum and the cecum, the area rich in lymphoid tissue called Peyer's patches (PPs). It was long thought that *Yersinia* cells first have to invade the intestinal mucosa and colonize the underlying PPs, in order to use the PPs as a gateway for entry into the deeper tissue. However, Barnes *et al.* (2006) recently challenged this theory by showing that systemic disease resulted from *Y. pseudotuberculosis* cells that spread by some site other than the intestinal lymphatic tissue. There are probably several possible routes for translocation across the intestinal epithelium. According to the study by Barnes *et al.* (2006) it appears that *Y. pseudotuberculosis* colonizes the intestinal niche, which possesses several entry portals for dissemination to systemic infection sites. Furthermore, it was shown that successful colonization of liver and spleen required preliminary replication in the intestines, but was independent of preliminary replication in the PPs and mesenteric lymph nodes.

Invasin is required in the early stages of infection; it promotes efficient entry into the PPs through interactions with β1 integrins that are expressed on the apical surface of M cells overlying Peyer's patches (Isberg and Leong, 1990; Pepe and Miller, 1993; Revell and Miller, 2001). The function of the M cells is to collect antigens from the intestinal lumen and present them to lymphocytes and macrophages. The β1 integrin receptors are abundant on the lumenal side of M cells but not on the lumenal side of enterocytes (Clark *et al.*, 1998) which targets the *Yersinia* invasion process specifically to M cells. Although important as antigen samplers, M cells therefore also represent a weak point of the intestinal epithelial barrier. Regarding the role of invasin in *Y. enterocolitica* infection, a recent study showed that dissemination from the small intestine to the spleen can have at least two routes; invasin dependent and invasin independent (Handley *et al.*, 2005).

A secondary invasion factor, YadA, seems to be required for persistence of *Y. enterocolitica* in PPs (Pepe *et al.*, 1995). *Y. enterocolitica* requires functional YadA to multiply extracellularly and form large microcolonies and clusters in lymphatic tissue (El Tahir and Skurnik, 2001) Expression of YadA is initiated soon after the temperature shifts to 37°C and is controlled by the temperature-dependent activator virF/LcrF (Bolin *et al.*, 1982; Skurnik and Toivanen, 1992). In addition to functioning in adhesion and invasion processes, the virulence associated surface proteins YadA and Ail are serum resistance factors which can individually, or in concert, protect *Y. enterocolitica* against the complement mediated killing (Biedzka-Sarek *et al.*, 2005). Furthermore, it has been speculated that for the efficient colonization of host cells, the *Y. enterocolitica* membrane components act together and are coordinated as a regulatory network where, for example, the absence of O-antigen could be a regulatory signal for expression of other membrane components (Skurnik and Bengoechea, 2003). By contact with *Yersinia* cells, it is postulated that host macrophages, neutrophils and dendritic cells (DCs), the preferred targets of *Yersinia* Yops, receive the "Yop-injection". This injection transports the effector proteins, Yops, into the cytosol of the host cell to counteract multiple signalling responses in the infected host cell. Injected Yops disrupt the dynamics of the host cell cytoskeleton, incapacitate phagocytosis, and turn down the production of proinflammatory cytokines, thus favouring the survival of *Yersinia* (Cornelis, 2002a; Heesemann *et al.*, 2006).

In a mouse model within 24 h after oral inoculation, enteropathogenic *Yersinia* can be detected in the mesenteric lymph nodes, after which they appear in the liver and spleen 48-72 h after inoculation (Mecsas *et al.*, 2001; Pepe *et al.*, 1995) In otherwise healthy humans, however, the infection is usually self-limiting and restricted to the gastrointestinal tract and the regional lymph nodes. For example, a direct lymphatic link exists between the PPs and the mesenteric lymph nodes enabling the onset of mesenteric lymphadenitis when bacteria disseminate from PPs to these lymphatic sites. In rarer occasions in humans, the bacteria can disseminate into deeper tissues with more severe symptoms. For the systemic dissemination of *Yersinia* in the host, an important factor is the presence of available iron or the ability to produce iron capturing siderophore yersiniabactin (requiring a functional HPI). After gastrointestinal infection, some patients, especially HLA B27 tissue type positive, may develop post-infectious reactive arthritis (Aho *et al.*, 1973). Bacteria gain access to the circulation and might be transported, either in blood or in lymphatic cells, to the joint, where they enter synovial cells and replicate (Meyer-Bahlburg *et al.*, 2001). After several weeks, at the time of sampling and diagnosis of reactive arthritis, bacterial cells have eventually been killed, or are no longer being culturable. Meanwhile, the degradation of the cells has resulted in accumulation of arthritogenic material and, by mediation of the immune system, the onset of reactive arthritis. In self-limited arthritis, after several weeks or months, all bacterial products are eventually degraded by the host and the arthritis disappears.

4 Sources of *Yersinia* infections and transmission by food and water

Yersiniosis is the third most common bacterial gastroenteritis after campylobacteriosis and salmonellosis in Europe (EFSA, 2007a). *Y. enterocolitica* and *Y. pseudotuberculosis* cause zoonotic diseases, capable of being transmitted from infected animals to humans. Most cases occur sporadically without an apparent source. However, in the 1980s, *Y. enterocolitica* and *Y. pseudotuberculosis* emerged as important agents of foodborne gastroenteritis outbreaks in the USA and Japan, respectively (Bottone 1997; Vincent *et al.* 2007). Most of the *Yersinia* infections in Europe are reported to be domestically acquired (EFSA, 2007a) In Finland, the data concerning travel associated yersiniosis is not currently collectively available. In Norway, travel-related infections constituted 20% of all cases of yersiniosis in 1998. The infections were most commonly reported to have been acquired elsewhere in Europe.

Pathogenic *Y. enterocolitica* strains show two clearly different epidemiological patterns based on the bioserotype of the strain. One of these patterns is represented by high-pathogenicity group (containing pYV and HPI) biotype 1B organisms that are restricted to specific geographical locations and isolated mainly in North America followed by Japan, but rarely in Europe. These strains can be found in the environment (including water) and have caused several outbreaks. The other pattern is represented by moderate pathogenicity group (containing pYV but no HPI) biotype 2-5 organisms. These organisms are more global, especially bioserotype 4/O:3 which is most commonly isolated throughout the world. The main reservoir of these biotypes is animals (pigs and cattle) and they are rarely isolated in the environment. In contrast to the epidemiological pattern of biotype 1B strains, strains of biotypes 2-5 typically cause sporadic cases in humans.

4.1 *Y. enterocolitica* in animals

Pigs are considered the major reservoir for *Y. enterocolitica* infections in humans. Strains belonging to human pathogenic types are frequently isolated from the oral cavity, especially from the tonsils, submaxillar lymph nodes, intestines and faeces of pigs. During the slaughter process, carcasses may become contaminated if infected heads are disposed of improperly or the mechanisms of faecal contamination of the carcasses are not controlled (Andersen, 1988; Smego *et al.*, 1999). Other reservoirs of *Y. enterocolitica* infection include a variety of wild animals, birds and rodents, domestic animals like goats and cattle, and pet animals (Bottone, 1997; Janda and Abbott, 2006; Kaneko and Hashimoto, 1981; Kapperud, 1981; Kapperud and Olsvik, 1982; Sulakvelidze *et al.*, 1996). For example, *Y. enterocolitica* bioserotype

4/O:3 strains have been isolated from apparently healthy dogs and cats (Fenwick *et al.* 1994; Fredriksson-Ahomaa *et al.* 2001). Dogs have been found to excrete these organisms in their faeces for several weeks after infection and thus present an additional potential source of human infections especially in children (Fenwick *et al.*, 1994). Most of the *Y. enterocolitica* strains isolated from other animals are generally non-pathogenic.

4.2 *Y. enterocolitica* in food and drinking water

Some surveillance data outside the reported outbreak cases is available to evaluate the foodborne transmission rate of *Yersinia* infections. For example, the percentage of cases transmitted by food of the total number of cases for a given pathogen was recently estimated in the USA, UK and Australia using mainly epidemiological data (Mead *et al.* 1999; Adak *et al.* 2002; Hall *et al.* 2005). The data indicated foodborne transmission rates of 90 % (USA, England and Wales) and 75% (Australia) for *Y. enterocolitica* (*Yersinia* spp. in Australia), which supports the significance of the foodborne infection route of this pathogen. Consumption of pork products has been demonstrated to be associated with *Y. enterocolitica* infection (Lee *et al.*, 1990; Tauxe *et al.*, 1987) and the global spread of *Y. enterocolitica* among pigs is believed to have occurred in the 1970s (Tauxe, 1997; WHO, 1976). A prospective case-control study in Norway revealed a link between *Y. enterocolitica* infections and consumption of undercooked pork and sausage products and untreated water (Ostroff *et al.* 1994). Similar findings have also been made elsewhere and in Finland (Huovinen *et al.*, 2008). Pork products have been extensively studied and more sensitive methods, including PCR, have recently revealed high detection rates of pathogenic *Y. enterocolitica* in pig offal (67–83% of samples positive), raw pork meat and ground beef (10–47%) (Fredriksson-Ahomaa and Korkeala, 2003b). Moreover, pathogenic *Y. enterocolitica* has been detected in ready-to-eat food by PCR, including lettuce, tofu and vegetables (3–15% of samples positive), and in water (10%) (Fredriksson-Ahomaa and Korkeala, 2003b). By using real-time PCR for detection of *Y. enterocolitica* carrying virulence-associated *ail* gene (Thisted Lambertz *et al.*, 2008b), 16% of the 79 grated carrots and lettuce samples studied at the Environmental and Food Research Laboratory TavastLab (Hämeenlinna, Finland) in 2008 were positive (S. Hallanvuo; unpublished data). In addition, dairy products, seafood, lamb and chicken, for example, have been identified as sources of pathogenic *Y. enterocolitica* (Janda and Abbott, 2006).

4.3 Outbreaks of human *Y. enterocolitica* infections

Food and water borne outbreaks of *Y. enterocolitica* infection have been reported throughout the world (Table 2). In the 1970s and 1980s, many outbreaks of bioserotype 1B/O:8 were described in the U.S., after which this bioserotype seemed

Table 2. A selection of infection outbreaks caused by *Y. enterocolitica*

Country	**Year**	**Cases**	**Bio/serotype**	**Source/vector**	**Reference**
Japan	1972	198	O:3	Not identified	Zen-Yoji and Maruyama 1972
Japan	1973	189	O:3	Not identified	Asakawa *et al*. 1973
Japan	1973	544	O:3	Not identified	Asakawa *et al*. 1973
Finland	1973	7	O:9	Hospital patients	Toivanen *et al*. 1973
North Carolina, US	1973	16	O:8	Dog1)	Gutman *et al*. 1973
Czechoslovakia	1975	15	O:3	Not identified	Olsovský *et al*. 1975
Canada	1976	138	O:5,27	Non-pasteurized milk1)	deGrace *et al*. 1976, Kasatiya 1976
New York , US	1976	38	O:8	Chocolate milk	Black *et al*. 1978
Japan	1980	1,051	O:3	Milk	Maruyama 1987
New York, US	1981	239	O:8	Powdered milk, chow mein	Shayegani *et al*. 1983
Washington, US	1981	50	O:8	Tofu and untreated spring water used to wash tofu at plant	Tacket *et al*. 1985
Pennsylvania, US	1982	16	O:8	Bean sprouts immersed in contaminated well water	Aber *et al*. 1982
Southern US	1982	172	O:13a, 13b	Pasteurized milk (statistically associated)1)	Tacket *et al*. 1984, Toma *et al*. 1984
Finland	1982	26	O:3	Not identified (contaminated food eaten in canteen suspected)	Tuori and Valtonen 1983
Hungary	1983	8	O:3	Brawn	Marjai *et al*. 1987
Canada	1984	2	4/O:3	Well water	Thompson and Gravel 1986
Australia	1987-1988	11	O:3; O:6,30	Not identified	Butt *et al*. 1991
Georgia, US	1988	15	O:3; O:1,2,3	Handling of raw pork intestines (chitterlings)	Lee *et al*. 1990
Sweden	1988	61	O:3	Milk, cream1)	Alsterlund *et al*. 1995
Vermont, US	1995	10	O:8	Pasteurized milk1)	Ackers *et al*. 2000
India	1997	25	4/O:3	Water (used to dilute buttermilk consumed at a feast)	Abraham *et al*. 1997
Tennessee, US	2001-2002	12	4/O:3	Handling of raw pork intestines (chitterlings) (statistically associated)1)	Jones *et al*. 2003
Croatia-Italy (oil tanker)	2002	22	O:3	Not identified	Babic-Erceg *et al*. 2003
Finland	2003	12	4/O:3	Not identified (contaminated food eaten in workplace canteen suspected)	Anonymous 2004
Japan	2004	42	O:8	Salads (containing apples, cucumber, ham, potatoes, carrots and mayonnaise)	Sakai *et al*. 2005
Norway	2005	4	4/O:3	Homemade Christmas brawn	Tafjord Heier *et al*. 2007
Norway	2005-2006	11	2/O:9	Homemade Christmas brawn2)/pork chops	Grahek-Ogden *et al*. 2007, Stenstad *et al*. 2007

Adapted from Cover and Aber (1989) and Thisted Lambertz (2007)

1) Disease agent could not be isolated from suspected source

2) Suspected vehicle was positive in PCR testing but disease agent could not be isolated

to be replaced by bioserotype 4/O:3. Serotype O:8 recently re-emerged as an outbreak strain in Japan (Sakai *et al.*, 2005). Most of the outbreaks in the 2000s, however, have been due to bioserotype 4/O:3 and associated, for example, with the handling of raw pork intestines (chitterlings) in the U.S, or consumption of contaminated Christmas brawn in Norway (Jones, 2003; Tafjord Heier *et al.*, 2007). Recently, semi-cooked cocktail sausages were reported to be associated with a small yersiniosis outbreak in children in New Zealand; unfortunately, the serotype responsible was not stated (Anonymous, 2007c).

In addition, several hospital outbreaks of *Y. enterocolitica* have been described (McIntyre and Nnochiri, 1986; Ratnam *et al.*, 1982; Toivanen *et al.*, 1973). These outbreaks are usually believed to have occurred through common-source contamination (for example from food), rather than via person-to-person transmission (Bottone, 1997; Janda and Abbott, 2006). Additionally, *Y. enterocolitica* is a contaminant of blood products (Leclercq *et al.*, 2005). Blood transfusion with *Y. enterocolitica* and *Serratia* spp. contaminated blood products has resulted in the highest mortality rates among outbreaks related to contaminated substances in hospital settings (Vonberg and Gastmeier, 2007).

4.4 *Y. pseudotuberculosis* in the environment

Y. pseudotuberculosis has been isolated from various environmental water sources such as streams and springs, rivers and well water (Fukushima *et al.*, 1988; Han *et al.*, 2003; Inoue *et al.*, 1988b). During outbreak investigations, it has also been recovered from soil and carrot residues in carrot processing facilities (Fukushima *et al.*, 1989; Jalava *et al.*, 2006; Rimhanen-Finne *et al.*, 2008). *Y. pseudotuberculosis* can survive for long periods in environmental waters and well water (Inoue *et al.*, 1988a). It can also be persistent in soil; during outbreak investigations, *Y. pseudotuberculosis* serotype O:1b strain harbouring an indistinguishable genotype from the outbreak strain was found in soil samples taken from a carrot processing plant over two months after processing of the epidemiologically implicated carrots (Jalava *et al.*, 2006). Similarities in the seasonal distribution of environmental findings to human infections have been noticed. A one-year survey of fresh water from 40 rivers in Japan revealed that *Y. pseudotuberculosis* could only be isolated from November (51.7% of the rivers) to May (17.5%). Similar results were found in a survey of 1,712 wild micromammals in which *Y. pseudotuberculosis* was recovered only from November to June, with a peak in December-February (Vincent *et al.*, 2007).

4.5 *Y. pseudotuberculosis* in animals

Y. pseudotuberculosis is responsible for epizootics in numerous animal species, especially in rodents and birds. *Y. pseudotuberculosis* has been isolated on all continents from many animal species, including cattle, horses, deer, sheep, goats, swine, salmon, cats, dogs, monkeys, buffaloes, wild boars, hares, foxes, raccoon dogs, minks, rodents (wild mice, rats, moles, guinea pigs) and birds (Brenner *et al.*, 1976; Callinan *et al.*, 1988; Fukushima *et al.*, 2001; Fukushima *et al.*, 1984; Hamasaki *et al.*, 1989; Hayashidani *et al.*, 2002; Kaneko *et al.*, 1979; Niskanen *et al.*, 2003; Riet-Correa *et al.*, 1990; Slee and Skilbeck, 1992; Tsubokura *et al.*, 1989; Zheng *et al.*, 1995). Birds associated with *Y. pseudotuberculosis* infection include turkeys, ducks, chickens, parrots, pigeons, crows, swallows and various other migratory birds and wild fowl. Birds, rodents and pigs appear to be major reservoirs of infection in Europe, the United States and Japan. Pigs appear to be the predominant reservoir of infection, particularly in Japan (Janda and Abbott, 2006; Tsubokura *et al.*, 1989). Recent studies in Finland have also highlighted the role of pigs as a reservoir of human infections (Niskanen *et al.*, 2002; Niskanen *et al.*, 2008).

Of the afore-mentioned animals, wild fowl and rodents, as well as pigs, monkeys, goats, sheep, rabbits and guinea pigs have shown signs of disease (Janda and Abbott, 2006; Neef and Lysons, 1994; Philbey *et al.*, 1991; Tsubokura *et al.*, 1989). Animals typically suffer illnesses ranging from chronic diarrhoea and mesenteric adenitis to fatal episodes of septicaemia. *Y. pseudotuberculosis* has for long been recognized as an epizootic agent in zoos throughout the world (Baskin *et al.*, 1977; Bielli *et al.*, 1999; Iwata *et al.*, 2008; Parsons, 1991; Welsh *et al.*, 1992). Infections in susceptible animals quite commonly occur as outbreaks and have been described at least among farmed deer, horses, cattle, and goats (Callinan *et al.*, 1988; Czernomysy-Furowicz, 1997; Sanford, 1995; Seimiya *et al.*, 2005). On the other hand, many of these animals, such as pigs, are possible carriers without signs of symptoms (Niskanen *et al.*, 2002; Tsubokura *et al.*, 1989).

As indicated above, *Y. pseudotuberculosis* has been found in many different types of animal, such as carnivorous animals (such as martens), herbivorous animals (deer, hares, ducks) and omnivorous animals (raccoon dogs) in addition to isolation from soil and environmental waters. *Y. pseudotuberculosis* circulates in the environment and possible routes of animal infection include preying upon animals infected with *Y. pseudotuberculosis* and ingesting environmental substances contaminated with *Y. pseudotuberculosis* (Fukushima and Gomyoda, 1991). In case of pig farms, pest animals seem to have a substantial role in spreading and maintaining the *Y. pseudotuberculosis* contamination on the farm, from where *Y. pseudotuberculosis* can transmit to the slaughterhouse level and even to the pork production chain (Laukkanen *et al.*, 2008).

4.6 *Y. pseudotuberculosis* in food and drinking water

As *Y. pseudotuberculosis* is found in a wide range of animal and environmental sources, it can occasionally contaminate drinking water and food. During outbreak investigations in Japan, *Y. pseudotuberculosis* has been isolated from untreated well and mountain stream water used for drinking water (Inoue *et al.*, 1988b; Tsubokura *et al.*, 1989). Very limited data is available for the isolation of *Y. pseudotuberculosis* in food (Greenwood, 1995), perhaps because this organism is not usually actively sought in food, and it can be easily overlooked in food samples with high background flora, and because the methods currently used for the detection of *Y. enterocolitica* in foods are not optimal for the recovery of *Y. pseudotuberculosis*. Nevertheless, *Y. pseudotuberculosis* has occasionally been isolated from pork in Japan (Fukushima *et al.*, 1997), homogenized milk in Canada (Nowgesic *et al.*, 1999), vegetables in Russia (Fukushima *et al.*, 2001), and iceberg lettuce (Nuorti *et al.*, 2004) and carrots (S. Hallanvuo, unpublished data) in Finland. With real-time PCR based detection of the *ail* gene, *Y. pseudotuberculosis* has also been detected in grated carrot samples implicated in outbreak investigations in Finland (Thisted Lambertz *et al.*, 2008a).

4.7 Outbreaks of human *Y. pseudotuberculosis* infections

In Finland, altogether 10 outbreaks of *Y. pseudotuberculosis* infection related to contaminated fresh produce have been reported since 1997 (Table 3). These outbreaks have involved serotypes O:3 and O:1 and have been associated with consumption of iceberg lettuce and grated carrots distributed by institutional kitchens. The molecular epidemiology of five oubreaks during 1997–2001 is described and discussed in more detail in the Results and Discussion sections. During outbreak investigations in 2003, 111 case patients were identified in the Kotka area and consumption of grated carrots was clearly associated with the illness. *Y. pseudotuberculosis* with pulsed-field gel electrophoresis (PFGE) pattern indistinguishable from that of the outbreak pattern S12N12 was isolated from 5 (42%) of 12 soil samples containing carrot residues (Jalava *et al.*, 2006). In 2004, *Y. pseudotuberculosis* serotype O:1 caused an outbreak among schoolchildren in the Haapavesi region (North-Western Finland) and PFGE type S12 was again involved (Anonymous, 2005a; Kangas *et al.*, 2008). During 2006, two different outbreaks of *Y. pseudotuberculosis* O:1 where domestic grated carrots were implicated was detected. A smaller outbreak in Nurmes (North-Eastern Finland) in May-June involved 56 patients and *Y. pseudotuberculosis* with PFGE pattern indistinguishable from that of the outbreak subtype S12N12 was again isolated from environmental and carrot samples taken from a carrot farmer's storage (Rimhanen-Finne *et al.*, 2006). Later in August-September, over 400 children from 23 schools and 5 day-care centres fell ill in Tuusula and Kerava municipalities in Southern Finland. Investigation revealed that the carrot distributor had a temporary shortage of fresh carrots and therefore poor quality carrots stored for over 10 months were grated, mixed with fresh ones, and distributed to schools. *Y. pseudotuberculosis* serotype O:1b genotype S12 was detected in patient samples, as well as in a carrot residue sample, and in three surface samples originating from a vegetable distributor's storage facility where the previous year's carrots had been stored (Rimhanen-Finne *et al.*, 2008). The 10th *Y. pseudotuberculosis* outbreak after 1997 took place in the Kajaani region. After April 2008, over 30 people had *Y. pseudotuberculosis* O:1 infections that were associated with the consumption of grated carrots. *Y. pseudotuberculosis* was isolated from environmental samples taken from the carrot distributor, and additionally detected in the implicated lot of grated carrots by real-time PCR (Anonymous, 2008a; Thisted Lambertz *et al.*, 2008a).

Just as in Finland, many recurring *Y. pseudotuberculosis* outbreaks affecting primarily children in day-care centres and schools have been reported in the former Soviet Union (Anonymous, 2005b, c, d, e, 2007a, b). As in Finland recently, serotype O:1b seems to be a common causative serotype of outbreaks among humans in Russia (Shubin, 1997). Contaminated vegetables and various rodents as vectors have been identified as a major factor for the spread of outbreaks and sporadic cases in Russia (Voskressenskaya *et al.*, 2003). In addition, many outbreaks of *Y. pseudotuberculosis* have been described in Japan (Table 3). The most common causative serotypes have been O:4b and O:5a. Serotype O:4b outbreaks have been associated with school lunches and drinking water. Drinking mountain stream water contaminated with wild animal faeces was suspected as the cause of one outbreak of serotype O:4b and O:2c infection. Serotype O:2c was detected in a one patient's stool and wild animal's faeces in a remote village area where the outbreak occurred (Inoue *et al.*, 1988a). Similarly, an outbreak of serotype O:4b in a remote mountain village in Japan was probably linked to drinking of untreated well water; serotype O:4b was isolated from both the patient's stool sample and the well water sample taken from the location of the outbreak (Inoue *et al.*, 1988a). Vegetable juice consumed at a primary school was suspected as the cause of a large outbreak of serotype O:5a in early 1980s (Inoue *et al.*, 1984). In two outbreaks of the same serotype, 39 people developed symptoms after eating at a barbecue restaurant. The specific vehicle of this outbreak was not found, although meat products, vegetables and rice were suspected (Nakano *et al.*, 1989). A large scale outbreak of serotype O:5a infection was associated with school catering, although the responsible food item could not be identified (Toyokawa *et al.*, 1993). Similarly, in a serotype O:5b outbreak of 67 people, mainly children and adolescents, became ill after eating lunch sandwiches served in an athletic event, although the food items were not available for demonstration of the source of this outbreak (Inoue *et al.*, 1988a). In addition to the afore-mentioned countries, at least England, Canada and Mongolia have experienced a *Y. pseudotuberculosis* outbreak.

Table 3. *Y. pseudotuberculosis* human infections related to disease outbreaks or to an environmental source

Country	Year	Cases	Serotype	Source/vector	Reference
England	1961	4	O:1a	Not identified (pet dog co-infected - possible for infection of family from dog)	Randall and Mair 1962
Japan	1977	57	O:5b	Not identified (outbreak in middle school)	Tsubokura *et al.* 1989
Japan	1977	82	O:1b	Not identified (outbreak in kindergarten, water suspected)	Tsubokura *et al.* 1989
Japan	1981	535	O:5a	Vegetable juice consumed at primary school (suspected)	Inoue *et al.* 1984
Finland	1981-1982	19	O:3; O:2b	Not identified (vegetables suspected)	Tertti *et al.* 1984
Japan	1982	67	O:5b	Lunch sandwiches eaten at athletic event (suspected)	Inoue *et al.* 1988
Japan	1982-1983	35	O:4b; O:2c	Mountain stream water contaminated with wild animal faeces (suspected)	Inoue *et al.* 1988
Japan	1982	1	O:4b	Infection contracted by rats via mountain stream water	Fukushima *et al.* 1988
Japan	1984	39	O:5a (two outbreaks)	Not identified (meat products, rice and vegetables eaten at a barbecue restaurant suspected)	Nakano *et al.* 1989
Japan	1984	63	O:3	Not identified (outbreak in elementary school and kindergarten)	Tsubokura *et al.* 1989
Japan	1984	11	O:4b	Well water	Inoue *et al.* 1988
Japan	1985	8	O:4b	Not identified (outbreak in elementary school and kindergarten)	Tsubokura *et al.* 1989
Japan	1985	60	O:4b	Not identified (outbreak in elementary school)	Tsubokura *et al.* 1989
Japan	1986	549	O:4b	School lunch (outbreak in elementary school)	Tsubokura *et al.* 1989
Mongolia	1986	114	O:1	Salad (vegetables and spring onions)	Markov 1989
Finland	1987	34	O:1a	Not identified	Tertti *et al.* 1989
Japan	1988	2	O:1b, O:3	Children infected when drinking water from garden puddles contaminated by cat faeces	Fukushima *et al.* 1989
Japan	1979-1989	290	O:1b, O:2a, O:2b, O:2c, O:3, O:4a, O:4b, O:5a, O:5b	Drinking untreated well water or mountain spring water	Sato and Komazawa 1991
Russia	1991	4		Fresh cabbage salad	Zaĭdenov *et al.* 1991
Japan	1991	732	O:5a	Not identified (associated with school catering)	Toyokawa *et al.* 1993
Finland	1997	35	O:3	Not identified (associated with school catering)	Hallanvuo *et al.* 2003
Canada	1998	74	O:1b	Homogenized milk	Nowgesic *et al.* 1999, Press *et al.* 2001
Japan	1998	3	O:5b	Well water (heavy rain causing overflow of river and contamination of well water)	Sunahara *et al.* 2000
Finland	1998	53	O:3	Not identified (associated with school catering)	Hallanvuo *et al.* 2003

Table continues

Country	**Year**	**Cases**	**Serotype**	**Source/vector**	**Reference**
Finland	1998	47	O:3	Iceberg lettuce contaminated at field level (several geographically separate clusters of infection)	Nuorti *et al.* 2004
Finland	1999	31	O:3	Not identified (iceberg lettuce suspected)	Hallanvuo *et al.* 2003
Finland	2001	89	O:1b; O:3	Not identified (eating outside home strongly associated, iceberg lettuce suspected, many clusters, including schoolchildren)	Jalava *et al.* 2004
Spain	2001	3	O:1	Not identified (common source suspected)	Serra *et al.* 2005
Finland	2003	111	O:1b	Grated carrots contaminated at farm level and distributed by an institutional kitchen (school or day care children)	Jalava *et al.* 2006
Korea	2001	1	O:4b	Drinking of untreated mountain spring water	Han *et al.* 2003
Finland	2004	53	O:1b	Grated carrots	Rimhanen-Finne *et al.* 2006, Kangas *et al.* 2008
France	2004-2005	27	O:1	Sudden increase of genetically diverse isolates (rodent vector suspected)	Vincent *et al.* 2008
Finland	2006	42	O:1b	Grated carrots (distributed by an institutional kitchen mostly to school or day care children)	Rimhanen-Finne *et al.* 2006
Finland	2006	402	O:1b	Grated carrots contaminated during prolonged storage (distributed by an institutional kitchen to school or day care children)	Rimhanen-Finne *et al.* 2008
Finland	2008	~30	O:1	Grated carrots contaminated during prolonged storage	Anonymous 2008a

5 Isolation of foodborne pathogenic *Yersiniae*

Based on the large number of pathogens present, isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* from diarrhoeal stool samples is usually easier than isolation from other sources, for example, from food where the pathogen is present in low numbers. For example, a faecal sample can contain 10^6–10^9 *Yersinia* cells/g in the acute phase of infection. In clinical samples, a selective medium is often needed for culturing *Yersinia* especially from non-sterile sites, even though these species grow on most routine media including blood, chocolate and MacConkey agars. On media, such as MacConkey agar, which incorporates lactose fermentation as an indicator, *Y. enterocolitica* colonies are colorless. Due to fermentation of sucrose and xylose, many clinically useful isolation agars, for example xylose-lysine-deoxycholate (XLD) agar, offer no advantage in the differentation of *Yersinia* species from the microbial population of normal stools (Bottone, 1997). One of the widely used selective media, originally developed for more efficient isolation of *Y. enterocolitica* from pork products, is *Salmonella-Shigella*-deoxycholate calcium chloride agar (SSDC) improved by Wauters *et al*. (Wauters, 1973; 1988a). Furthermore, Cefsulodin-Irgasan-Novobiocin (CIN) agar has been found to provide better recovery rates than, for example, MacConcey or *Salmonella-Shigella*-agar in the isolation of *Y. enterocolitica* (Head *et al*., 1982). CIN agar specifically designed for *Yersinia* (Schiemann, 1979), inhibits the growth of many other organisms of the family *Enterobacteriaceae* to the advantage of the more slowly growing *Yersinia* species. *Y. enterocolitica* forms distinctive colonies with a deep red centre (bull's eye) with a sharp border surrounded by a translucent zone on CIN agar. Some of the competing *Enterobacteriaceae* species including *Citrobacter*, *Enterobacter, Aeromonas, Serratia and Klebsiella* able to grow on CIN agar produce colonies larger than *Yersinia* but similar in appearance (Devenish and Schiemann, 1981; Harmon *et al*., 1983; Head *et al*., 1982), and thus create a possible source of error when a limited number of presumptive colonies is picked for identification. In order to discriminate some non-pathogenic *Yersinia* species from esculin non-hydrolysing pathogenic *Y. enterocolitica*, Fukushima (1987) developed a new agar medium called VYE (virulent *Y. enterocolitica* agar). Similarly to CIN agar, *Yersinia* strains form red colonies (mannitol fermentation) on VYE agar, but environmental *Yersinia* organisms (*Y. enterocolitica* BT 1A, *Y. intermedia*, and *Y. frederiksenii*) are further differentiated by a dark peripheral zone as a result of esculin hydrolysis. The drawbacks of VYE agar are the tendency of the dark zone resulting from esculin hydrolysis to mask the target colonies, and its inability to differentiate between pathogenic *Y. enterocolitica* and *Y. kristensenii*, a common potentially non-significant co-isolate of many different sample types. Furthermore, VYE agar is not suitable for the recovery of *Y. pseudotuberculosis*. Although inhibition of some *Y. bercovieri* and *Y. pseudotuberculosis* strains on CIN agar has been detected (Fukushima and Gomyoda, 1986), CIN agar remains the agar of choice also for the isolation of *Y. pseudotuberculosis* and *Y. enterocolitica* –like strains in the absence of better alternatives. *Y. pseudotuberculosis* strains form red pin-point colonies (under 1 mm in diameter) on CIN agar after 24 to 48 h incubation. The form of the colony varies from a less irregular to a star like appearance (depending on serotype) and the colonies have a raised, irregular, red centre ("fried egg" appearance) viewed with a stereomicroscope. The translucent zone surrounding the red centre is usually narrow or almost missing. Recently, a selective agar medium, termed BIN, was developed for the isolation of *Y. pestis* (Ber *et al*., 2003). This agar also supports the growth of the target organism's close relative, the equally slow growing *Y. pseudotuberculosis*. Another promising innovation in the field of *Yersinia* selective agars is the new chromogenic agar (YeCM) for the isolation of potentially virulent *Y. enterocolitica* (Weagant, 2008).

Cold enrichment at 4°C in buffer or broth for several days to weeks before isolation on a plate has also been used for the recovery of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* from clinical samples (Greenwood *et al.* 1975; Pai *et al.* 1979; Van Noyen *et al.* 1981). Among others, cold enrichment in phosphate-buffered saline (PBS) or phosphate-buffered saline with sorbitol and bile salts (PSB) has been used for *Y. enterocolitica* (reviewed by Fredriksson-Ahomaa and Korkeala, 2003a). Isolation by cold enrichment seems to be a matter of controversy; it seems that cold enrichment has no advantage over direct plating in acute *Y. enterocolitica* serotype O:3 and O:9 enteritis (Van Noyen *et al.*, 1981), but in some instances it can increase the sensitivity of the detection of pathogenic *Y. enterocolitica* (Eiss, 1975; Kontiainen *et al.*, 1994; Pai *et al.*, 1979). Furthermore, cold enrichment has been shown to increase the recovery of *Y. enterocolitica*-like strains (Van Noyen *et al.*, 1981), and *Y. enterocolitica* biotype 1A strains (Van Noyen *et al.*, 1980; Weissfeld and Sonnenwirth, 1980) which have questionable pathogenicity for humans or are non-pathogens widely distributed in nature (Sulakvelidze, 2000; Tennant *et al.*, 2003). In a recent study by Sihvonen *et al.* (2009), 25% of the strains belonging to pathogenic bioserotypes of *Y. enterocolitica* were only detected after cold enrichment of the clinical stool samples. However, cold enrichment also increased the number of isolates representing biotype 1A and *Y. enterocolitica* –like strains.

Isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* from food and environmental samples is challenging. The small number of pathogenic bacteria usually present and the high background microbial population capable of growing more rapidly than pathogenic *Yersinia* hampers detection methods regardless of the sample type. The current widely used culturing methods suffer from a lack of sensitivity and are likely to lead to underestimating of the actual prevalence of pathogenic *Yersinia* in foods. Several different enrichment methods have been described for the recovery of *Y. enterocolitica* from foods that usually exploit enrichment in one or two non-selective or selective broths (Fredriksson-Ahomaa and Korkeala, 2003a). In the International Standard Organization method ISO 10273:2003 mostly used for food samples in Europe, *Y. enterocolitica* is enriched in irgasan-ticarcillin-potassium chlorate (ITC) broth (Wauters *et al.*, 1988a in parallel with peptone, sorbitol and bile salts broth (PSB), and plated on CIN and SSDC agars. The availability of culturing methods for the detection of *Y. pseudotuberculosis* in food samples is more limited. Enrichment in peptone, mannitol and bile salts broth, PMB, (supplemented with 1% mannitol and 0.15% bile salts) for 2 weeks at +4°C and using an alkali treatment before plating on CIN has been successful during outbreak investigations for environmental samples (Jalava *et al.*, 2006; Rimhanen-Finne *et al.*, 2008).

Additionally, a variety of PCR methods have been introduced especially for the detection of pathogenic *Y. enterocolitica* in food and samples of animal and environmental origin (Fredriksson-Ahomaa and Korkeala, 2003a). Recently, real-time PCR methods based on the virulence-associated *ail* gene for detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* allowing also simultaneous detection of both pathogens have been developed (Thisted Lambertz *et al.*, 2008a; Thisted Lambertz *et al.*, 2008b). PCR methods developed for human clinical samples including synovial fluid, blood, stool and tissue have also been described

(Feng *et al.*, 1992; Harnett *et al.*, 1996; Trebesius *et al.*, 1998; Viitanen *et al.*, 1991). Additionally, methods for simultaneous detection of (Fukushima *et al.*, 2003) and differentiation between *Y. enterocolitica* and *Y. pseudotuberculosis* (Trebesius *et al.*, 1998; Weynants *et al.*, 1996) in clinical material have been developed. PCR methods in general have a superior sensitivity compared to the traditional culture methods, but fail to yield bacterial isolates that are essential for further epidemiological studies.

6 Identification and virulence testing

Biochemical identification and virulence testing

Like other members of the family *Enterobacteriaceae*, *Yersinia* are catalase positive and oxidase negative and ferment glucose. *Yersinia* are urease positive, they ferment D-mannitol but most of the strains do not ferment lactose (Table 4). Although commercially available identification systems usually identify the genus *Yersinia* correctly, further identification of the members of this genus is not one of the strengths of these systems. Several major manufacturers cluster *Y. enterocolitica* together with generally non-pathogenic species (for example VITEK with *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia*) and/or do not list all the *Yersinia* species in their database (for example API 20 E lists only six of the currently known 14 species). Nevertheless, widely used API 20 E constitutes a convenient set of useful biochemical tests when interpreted with caution and accompanied by additional tests such as biotyping. Incubation of API 20 E at 28°C instead of 37°C, however, has been shown to yield better identification rates (Archer *et al.*, 1987). *Y. enterocolitica* consists of sucrose and D-sorbitol positive, and L-rhamnose and melibiose negative strains. *Y. pseudotuberculosis*, on the other hand, is easily differentiated from *Y. enterocolitica* by negative reactions for sucrose and sorbitol, and positive reaction for L-rhamnose (Bercovier *et al.* 1980a; Bottone 1997). Fermentation of sucrose has traditionally separated sucrose positive *Y. enterocolitica* and sucrose negative *Y. kristensenii*. However, sucrose negative *Y. enterocolitica* isolates have emerged in pathogenic biotypes, for example, among bioserotype 4/O:3 strains (Fredriksson-Ahomaa *et al.*, 2002). The potential pathogenicity of the sucrose negative *Yersinia* isolates should thus be further evaluated, for example by using the biochemical tests included in the *Y. enterocolitica* biotyping scheme. Fermentation of L-rhamnose and raffinose will separate *Y. frederiksenii* and *Y. intermedia* from *Y. enterocolitica*, and *Y. frederiksenii* and *Y. intermedia* can be further separated by melibiose fermentation. Differentiation by biochemical tests is usually based on a limited set of strains, especially among *Y. enterocolitica* –like species, creating contradictory results as reflected in Table 4.

Phenotypic characteristics such as calcium dependency and autoagglutination at 37°C are associated with the presence of the virulence plasmid (Gemski *et al.*, 1980a; Laird and Cavanaugh, 1980) and have been used in assessing the potential pathogenicity of the *Yersinia* isolate under investigation. Riley and Toma (1989) exploited the assays of calcium dependent growth at 37°C (Gemski *et al.*, 1980a) and Congo red uptake (Prpic *et al.*, 1983), associated with the presence of the virulence plasmid (pYV), and developed Congo-red magnesium oxalate agar (CR-MOX) for detecting pathogenic *Yersiniae. Yersinia* strains harbouring the virulence plasmid grow as pinpoint (calcium dependent growth) red (Congo-red uptake) colonies on this agar. Colourless colonies represent cells that have lost their plasmid or non-pathogenic strains that have never had the plasmid.

Harbouring a virulence plasmid induces metabolic stress for pathogenic *Y. enterocolitica* best evidenced by a decrease in growth rate when growth temperatures increase to 30–35°C (Goverde *et al.*, 1994). This probably explains possible virulence plasmid loss during subculturing of pathogenic *Yersinia* in the laboratory (Berche and Carter, 1982; Li *et al.*, 1998; Prpic *et al.*, 1985). Its presence may vary from approximately 50% to 90% in stock cultures belonging to pathogenic types in *Y. pseudotuberculosis* and even down to 24% in *Y. enterocolitica* as demonstrated in the studies of Fukushima *et al.* (2001) and Farmer *et al.* (1992), respectively. Therefore, it has been advised not to subculture pathogenic *Yersinia* strains at 37°C, but always at 25-28°C (Bottone 2005). Nevertheless, the absence of pYV in the CR-MOX test is not sufficient to indicate that the strain under investigation is non-pathogenic.

Phenotypic testing for virulence plasmid accompanied by tests for pyrazinamidase, salicin fermentation and esculin hydrolysis have been found to be useful in the identification of potential pathogenic types of *Y. enterocolitica* (Chiesa *et al.*, 1993; Farmer *et al.*, 1992). Kandolo and Wauters (1985) found a correlation between the negative reaction in pyrazinamidase (pyrazine-carboxylamidase) testing and bioserotypes of *Y. enterocolitica* that normally harbour the virulence plasmid. Salicin fermentation and esculin hydrolysis have traditionally been part of the biotyping scheme. Farmer *et al.* (1992) used the combination of tests for salicin fermentation and esculin hydrolysis incubated at 25°C for 2 days and correctly identified 97% of the study isolates to pathogenic and non-pathogenic types. The pyrazinamidase test, which does not depend on the pYV, identified strains of pathogenic serotypes with 95% sensitivity (60 of 63 isolates) in that study. In a study by Chiesa *et al.* (1993) only 19 isolates (1%) out of 1,619 tested had discordant results in these reactions. Thus, salicin, esculin and pyrazinamidase tests in combination provide a simple means of distinguishing between potential pathogenic and non-pathogenic strains of *Y. enterocolitica*. Additionally, utilization of sodium acetate has been found promising in the differentiation between pathogenic biotypes and biotype 1A strains of *Y. enterocolitica* (Burnens *et al.*, 1996; Sinha and Virdi, 2000). Since most of the *Y. pseudotuberculosis* clinical isolates belong to pathogenic types (Fukushima *et al.*, 2001), virulence testing of *Y. pseudotuberculosis* is usually unnecessary.

Table 4. Biochemical reactions of *Yersinia* species after incubation at 25–28°C or 35–36°C for 24–48h

Reactions belonging to API 20 E test scheme

	ONPG test	Arginine dihydrolase (ADH)	Lysine decarboxylase (LDC)	Ornithine decarboxylase (ODC)	Citrate (CIT)	Urea hydrolysis (URE)	Phenylalanine/Tryptophane deaminase (TDA)	Indole production (IND)	Voges-Proskauer (+25C) (VP)	D-Mannitol fermentation (MAN)	*myo*-Inositol fermentation (INO)	D-Sorbitol fermentation (SOR)	L-Rhamnose fermentation (RHA)	Sucrose fermentation (SAC)	Melibiose fermentation (MEL)	L-Arabinose fermentation (ARA)
Y.enterocolitica biotype 1A	+	-	-	+	-	+	-	+	[-][b]/+[c]	+	[+][b]/+[c]	+	-	+	-	+
Y.enterocolitica biotype 1B	+	-	-	+	-	+	-	+	[+][b]/+[c]	+	+	+	-	+	-	+
Y.enterocolitica biotype 2	+	-	-	+	-	+	-	w[e,f]/v[c]/+[b]	-[b]/+[c]	+	+	+	-	+	-	+
Y.enterocolitica biotype 3	+	-	-	+	[-]	+	-	-	-[b]/+[c]	+	[+][b]/+[c]	[+]	-	+	-	+
Y.enterocolitica biotype 4	+	-	-	+	-	+	-	-	[-][b]/+[c]	+	[-][b]/+[c]	+	-	+	-	+
Y.enterocolitica biotype 5	[+]	-	-	-[b]/+[c]/(+)[c]	-	+	-	-	-[b]/+[c]/(+)[c]	+	[+][b]/+[c]	[+]	-	[+]	-	[+]
Y. aleksiciae[h]	+	-	+	+	-	+	-	[-]	-	ND	[+]	+	-	-	-	+
Y. frederiksenii	+	-	-	+	v/[+][b]/+[h]/[-][i]	+/[+][i]	-	+	+/[-][b]/-[h]	+	+/[-][i]/-[g]	+	+	+	-	+
Y. intermedia	+	-	[+][b]/-[i]	+	+/-[g,i]	+	-	+	+/[+][b]/-[h]	+	+[b]/[+][g]/[-][i]/-[h]	+	+/[+][b]	+	+	+
Y. kristensenii	+/[+][i]	-	[+][b]/-[i]	+	-	+	-	[+]/v[f]	-	+	[+][b]/[-][i]/-[h]	+	-/[-][b]	-	-/[-][b]	+
Y. rohdei	+/[+][i]	-	-	+/[-][i]/v[f]	+/[+][g]/-[i,f]	+/[+][i]/v[f]	-	-	-	+	-/[-][b]	+	-/[-][b]	+	v[j]	+
Y. aldovae	-	-	-	+/[+][i]	v/[+][b]/+[h,f]/[-][i]	+/[+][i]	-	-	+/[+][b]/-[i,k]	+	+/[+][b,k]/-[i]	+/[+][i]	+/[+][k]/-[g,i]	-/[-][i]/[+][k,b]	-/+[b,h]	+/[+][i]
Y. bercovieri	+/[+][b]	-	-	+	-	+/[+][i]	-	-	-	+	-	+	-	+	-	+
Y. mollaretii	+/[-][i]	-	-	+	-/[-][b]/[+][k]/+[h]	+/[-][i]	-	-	-	+	-/[+][b,k]	+	-	+	-/[-][b]	+
Y. pestis	-/[+][i]	-	-	-	-	-	-	-	-	+	-	-/[+][i]	-	-	v/[-][i]	v[l]
Y. pseudotuberculosis	[+]	-	-	-	v[m]	+	-	-	-/+[h]	+	-	-	+/[+][i]	-	v[m]	+/[+][i]
"Y." ruckeri	+/[+][i]	-	-[h]/[-][k]/[+][i]	+	-/[-][k]/+[f]	-	-	-	-/v[f]	+	-	v[n]	-	-	-	-
Y. massiliensis[o]	+	-	-	+	-[o]	+	-	+	-[o]	+	+	+	-	+	-	+
Y. similis[p]	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	+

Table continues

	Biotyping								Other differential characteristics											
	Esculin hydrolysis	Salicin fermentation	Pyrazinamidase	Tween-Esterase / Lipase (corn oil)[a]	D-Xylose fermentation	Trehalose fermentation	Nitrate > Nitrite	Sorbose fermentation	Raffinose fermentation	Cellobiose fermentation	Lactose fermentation	Maltose fermentation	Malonate fermentation	Mucate fermentation	D-Arabitol fermentation	Fucose fermentation	Glycerol fermentation	alpha-Methyl-D-glucoside fermentation	Motility +37 °C	Motility +25-28 °C
Y.enterocolitica biotype 1A	+	+	+	+	+	+	+	+	-	+	[+]	[+][d]	-	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y.enterocolitica biotype 1B	-	-	-	+	+	+	+	+	-	+	[+]	[+][d]	[+]	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y.enterocolitica biotype 2	-	-	-	-	+	+	+	+	-	+	[+]	[+][d]	[+]	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y.enterocolitica biotype 3	-	[-]	-	-	[+]	+	+	+	-	+	[-]	[+][d]	[+]	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y.enterocolitica biotype 4	-	-	-	-	-	+	+	+	-	[+]	-	[+][d]	-	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y.enterocolitica biotype 5	-	-	-	-	[+][b]/v[g]	-	-	[+][b]/-[g]	-	+	[-]	[+][d]	[+]	-	[+][d]	v[d]	+[d]	-[d]	-	+
Y. aleksiciae[h]	-	-	ND	ND	+	+	ND	+	-	+	+	ND	-	ND	ND	ND	[+]	ND	-	+
Y. frederiksenii	+	+	+	[+]	+	+/v[g]	+	+	-/[+][i]	+	[+]/v[g]/-[h]	+	-	[-][g]/-[i]	+	+	+	-	-	+
Y. intermedia	+	+	+	[-]	+	+	+	+	+/[+][i]/-[h]	+	[+]/v[g]/-[h]	+	-/[-][b]	v[g]/-[i]	[+]	v	+[h]/[+][i]/v[g]	+	-	+
Y. kristensenii	-	-/[-][i]	+	-	+	+/-[g]	+	+/[+][b]	-	+	-/[+][b]/v[h]	+	-	-	[+]	v	[+]/v[g]	-	-	+
Y. rohdei	-/[-][b]/+[h]	-/[-][b]/+[h]	+	-	+/[+][i]	+	+	+/[-][b]	v[j]	+/[-][i]/-[g]	-	-	-	-	-	-	+[h]/[+][i]/v[g]	-	-	+
Y. aldovae	-	-/+[g]	-[g]	-	+/[+][i]	+	+	-/+[e]	-	-/+[b,h]	-/+[b,h]	-	-	v[g]/-[i]	-	v/[+][k]	-	-	-	+
Y. bercovieri	-[g]/[-][b,i]/[+][k]/+[h]	[-]/-[g]/+[h]	v[g]	-	+	+	+	-	-	+	-[k,g]/[-][b,i]/+[h]	+	-	+[g]/-[i]	-	+	-	-	-	+
Y. mollaretii	-/[-][b,k]	-[h,k]/[-][g,i]/[+][b]	-[g]	-	+/[+][b,i]	+	+	+	-	+/[+][b]	-/v[g]/[+][i]	+[k]/[+][i]	-	+[g]/-[i]	-	-	+/[-][i]	-	-	+
Y. pestis	+/[+][i]	-[b,h]/[+][g,i]	-	-	+	+	v[l]	-	-	-	-	+/v[g]	-	-	-	-	v[l]	-	-	-
Y. pseudotuberculosis	+/-[h]	-/[-][i]	-	-	+	+	+	-/+[h]	v[m]	-	-	+	-	-	-	-	+[h]/[+][i]/v[g]	-	-	+
"Y." ruckeri	-	-	+	v[n]	-	+	+/[+][i]	-	-	-	-	+	-	-	-	ND	+[h]/[+][i]/v[g]	-	-	v[n]
Y. massiliensis[o]	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
Y. similis[p]	+	-	-	ND	+	+	ND	-	-	-	[+]	ND	-	ND	ND	ND	ND	ND	ND	+

The (first) result is a combined value from the literature cited (see following page). Contradictory results follow with citation as superscript. +, ≥ 76% of strains positive; -, ≥ 90% of strains negative; [-], 11-25% negative; [+], 26-75% positive; ND, no data; v, variable; w, weakly positive

Table continues

[a] Tween-Esterase (*Y. enterocolitica* and *Y. ruckeri*) / Lipase (corn oil) (other species)

[b] According to Neubauer *et al*. (2000c) (reactions determined after incubation at 28°C for 24h)

[c] According to Bottone (1997); *Y.enterocolitica* biotype reactions according to Wauters *et al.* (1987); (+), delayed positive

[d] Data without consideration of the respective biotypes

[e] According to Aleksic and Bockemühl (1999) (reactions determined after incubation at 25°C for 48h); (+), delayed positive

[f] According to Wanger (2007) (reactions determined after incubation at 35°C, except for VP and CIT at 25°C)

[g] According to Bottone *et al*. (2005)

[h] According to Sprague and Neubauer (2005)

[i] According to Farmer *et al*. (2007) (reactions determined after incubation at 36°C for 48h)

[j] *Y. rohdei* biotype 1: melibiose +, raffinose +; biotype 2: melibiose -, raffinose –; Aleksic and Bockemühl (1999)

[k] According to Stock *et al*. (2002) (reactions determined after incubation at 28°C for 24h)

[l] *Y. pestis* biovar Antiqua: glycerol +, arabinose +, nitrate +; biovar Medievalis: glycerol +, arabinose +, nitrate -; biovar Orientalis: glycerol -, arabinose +, nitrate +; biovar Microtus: glycerol +, arabinose -, nitrate - (Zhou *et al.*, 2004)

[m] *Y. pseudotuberculosis* biotype 1: citrate -, melibiose +, raffinose -; biotype 2: citrate -, melibiose -, raffinose -; biotype 3: citrate +, melibiose -, raffinose -; biotype 4: citrate -, melibiose +, raffinose + (Tsubokura and Aleksic, 1995)

[n] *Y. ruckeri* biotype 1: motility +, tween-esterase +, sorbitol +; Biotype 2: motility -, tween-esterase -, sorbitol – ; according to Davies and Frerichs (1989)

[o] According to Merhej *et al*. (2008). Reactions determined after incubation at 28°C for 24h, 48h and 72h. After 48-72h of incubation, the isolates exhibited citrate utilization and weak acetoin production

[p] According to Sprague *et al*. (2008). Reactions determined after incubation at 28°C for 24h

In addition to biochemical virulence associated tests, genotypic virulence markers based on pYV or chromosomal virulence genes have been exploited as PCR targets in the virulence testing of *Yersinia* isolates. These assays include, separately or in combination, plasmid borne targets like *virF*/*lcrF* and *yadA* genes, and chromosomal targets like *ail*, *inv* and *rfbC* and *yst* genes (Ibrahim *et al.*, 1997a; Nakajima *et al.*, 1992; Thistedt Lambertz and Danielsson-Tham, 2005; Thoerner *et al.*, 2003; Weynants *et al.*, 1996; Wren and Tabaqchali, 1990). For example, Weynants *et al.* (1996) used the combination of *rfbC* (O antigen encoding gene, specific for serotype O:3 representing pathogenic biotypes of *Y. enterocolitica*), *ail*, *inv* and *virF* primers for the detection of and differentiation between *Y. enterocolitica* O:3 (detection of *rfbC* and *ail* genes of pathogenic *Y. enterocolitica*, and the virulence plasmid pYV by *virF*), *Y. pseudotuberculosis* (*virF* and *inv* gene of *Y. pseudotuberculosis*) and pathogenic *Y. enterocolitica* (*virF* and *ail* gene of pathogenic *Y. enterocolitica*).

Identification by 16S rRNA gene sequences

The analysis of 16S rDNA sequences is considered a standard in bacterial classification (Stackebrandt *et al.*, 2002) and has become a routine method in bacterial identification at least in reference laboratories. A correlation between DNA-DNA reassociation level and 16rRNA gene sequence similarity exists; a DNA-DNA reassociation level of 70% corresponds to at least 97% 16S rRNA gene sequence similarity. According to the phylogenetic definition, a species (genomospecies) contains strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less difference in the melting temperature of heteroduplexes (ΔTm), which is equivalent to 5% or less sequence divergence (Wayne 1987; Stackebrandt and Goebel 1994). Considering this, a 16S rRNA gene sequence similarity of less than 97% between strains indicates that they represent different species and higher scores generate the need for DNA-DNA hybridization studies for verifying a new species. This guideline established by Stackebrandt and Goebel (1994) was followed for a long time. Because DNA-DNA reassociation analysis is a difficult technique performed in only a few laboratories, 16S rRNA gene sequencing practically took over as a "gold standard" in bacterial identification and produced a vast amount of new information. Subsequently, the criterion of 97% 16S rRNA gene sequence similarity was questioned and new recommendations arose in clinical settings. For example, Drancourt *et al.* (2000) recommended ≥99% sequence similarity of 16S rRNA gene sequences to the sequence deposited in DNA databases for a valid species designation based on a large collection of environmental and clinical unidentifiable bacterial isolates. Finally, Stackebrandt and Ebers (2006) revised the former recommendations and suggested a higher 16S rRNA gene sequence similarity threshold range of 98.7–99% as the point at which DNA-DNA reassociation experiments should be mandatory for testing the genomic uniqueness of novel isolate(s). The applicability of 16S rRNA gene sequence analysis for the identification of a species within the genus *Yersinia* is well established (Ibrahim *et al.*, 1993; Ibrahim *et al.*, 1997b; Neubauer *et al.*, 2000b). In the study by Kotetishvili *et al.* (2005) 16S rRNA gene sequencing data agreed with the biochemical designation of the species in most cases among 58 *Yersinia* strains representing 11 species. However, among the *Y. enterocolitica*–like strains of this study, one strain of *Y. kristensenii* and two strains of *Y. aldovae*, and a second strain of *Y. kristensenii* and a strain of *Y. intermedia* had identical 16S rRNA gene sequences. Thus, 16S rRNA gene sequencing data should be interpreted with care since this analysis does not always unambiguously differentiate the isolates of the closely related species. Another example of this is *Y. pseudotuberculosis* and *Y. pestis*, the two subspecies of the same species, which are identical in DNA-DNA reassociation studies and by 16S rRNA gene sequences (Bercovier *et al.* 1980b; Trebesius *et al.* 1998). On the contrary, the proposed subspecies *Y. enterocolitica* subsp. *palearctica* and *Y. enterocolitica* subsp. *enterocolitica* can be separated by 16S rRNA gene sequencing, if identification to the subspecies level is needed (Neubauer *et al.*, 2000a). Generally, a species may be divided into subspecies based on consistent phenotypic variations or on genetically determined clusters of strains within the species. There are currently no commonly accepted guidelines, however, for the establishment of subspecies. Regarding the ambiguity of the identification by 16S rRNA gene sequences, one of the major drawbacks is the possible bias of sequence comparisons in public databases arising from the quality problems (sequencing errors, incomplete sequences, ambiguities, insufficient strain characterization) of the deposited sequences.

7 Epidemiological typing of foodborne pathogenic *Yersiniae*

7.1 Phenotypic methods

During the history of *Y. enterocolitica*, it was realized very early on that this species was biochemically very heterogeneous compared to *Y. pestis* and *Y. pseudotuberculosis*. This warranted the establishment of several biogroups or biotypes (Niléhn 1969; Wauters 1970; Knapp and Thal 1973) and one of the first suggestions was to divide the strains into five biogroups (Niléhn, 1969). Soon after, Wauters adopted some of the Niléhn's substrates and incorporated lecithinase activity into the new typing scheme (Wauters, 1970). The number of biotypes was subsequently reduced from seven to six (biotypes 1A, 1B and 2-5) when Wauters *et al.* revised the current biotyping scheme and speciated the former biogroups 3A and 3B to species *Y. mollaretii* and *Y. bercovieri*, respectively (Wauters *et al.*, 1988b

Wauters *et al.*, 1987). This typing scheme is currently widely adopted and originally included tween-esterase activity, acid from salicin or esculin hydrolysis, indole production, acid from trehalose and xylose, nitrate reduction, pyrazinamidase activity, ß-D-glucosidase activity, Voges-Proskauer reaction and proline peptidase activity. Isolates comprising biotypes 1B and 2-5 have been associated with disease in humans and animals while biotype 1A is generally regarded as non-pathogenic. Isolates that are most often associated worldwide with *Y. enterocolitica* infections in humans belong to biotype 4.

In *Y. enterocolitica* and related species, at least 76 serotypes based on variability in O-antigen structure have been described (Wauters *et al.*, 1991). In addition, 44 flagellar H–antigens have been described (Aleksic, 1995; Aleksic and Bockemühl, 1987; Aleksic *et al.*, 1986). Capsular K antigen can be associated with different O-serotypes (Aleksic and Bockemuhl, 1984). *Y. enterocolitica* O-antigens have also been detected in other *Yersinia* species, including O:3 in *Y. intermedia*, *Y. kristensenii*, *Y. frederiksenii*, and *Y. mollaretii*, O:9 in *Y. kristensenii*, and *Y. frederiksenii*, and O:8 in *Y. bercovieri* (Aleksic, 1995). By contrast, H-antigens seem to be species and serotype specific, but H-antigen typing is not currently widely adopted. Although the O-antigen structure is not straightforwardly related to pathogenic properties of the strain, there is an association between combined sero- and biotypes and pathogenicity.

The serotyping scheme of *Y. pseudotuberculosis* is based on O-antigenic factors and, more rarely, on H-antigenic factors. Originally this scheme included six serotypes (I to VI) and consisted of 14 O-antigenic factors and 5 H antigenic factors (Thal, 1973; Thal and Knapp, 1971). Thus, among *Y. pseudotuberculosis*, a serotype comprises more than one O-antigenic factor. Later, this scheme was extended by two further serotypes (O:7 and O:8) and 5 O antigen factors (O-16 to O-20) (Tsubokura *et al.*, 1984). Tsubokura *et al.* (1993) subdivided serotype O:1 into O:1a, O:1b and O:1c and described three new serotypes (O:9, O:10, and O:11), as well as four new O antigen factors (O-24 to O-27). Subsequently, a more thorough analysis of O and H antigens by Aleksic *et al.* (1991) extended the antigenic scheme from 13 to 62 serotypes. Finally, Tsubokura and Aleksic (1995) proposed a simplified antigenic scheme for *Y. pseudotuberculosis,* where serotypes O:1 and O:2 are divided into three subgroups a, b and c, and serotypes O:4 and O:5 into subgroups a and b. Thus, the scheme consisted of 20 O-serotypes (O:1a to O:14) and five H-serotypes (a to e) altogether. The O-serotypes in this scheme comprised 30 O-antigen factors. The most recent addition to this scheme is serotype O:15, and the current serotyping scheme of *Y. pseudotuberculosis* thus comprises 21 different O-serotypes (Bogdanovich *et al.*, 2003). Commercially available antisera are available for serotypes O:1 to O:6, excluding subserotypes. However, strains representing rarer O-serotypes, rough strains and a number of cross-reacting strains remain untypeable with these antisera. As a solution to this problem, an O-genotyping method based on multiplex PCR has been developed (Bogdanovich *et al.*, 2003). Although *Y. pseudotuberculosis* is quite homogeneous biochemically, it can be divided into four biotypes based on differences in the fermentation of melibiose, raffinose and salicin (Table 4) (Tsubokura and Aleksic, 1995).

Two bacteriophage typing schemes have been developed in Europe for yersiniae and have been used to relate certain bioserotypes and serotypes of *Y. enterocolitica* to infection sources (Mollaret and Nicolle, 1965; Nicolle, 1973; Nicolle *et al.*, 1968; Nilehn and Ericson, 1969). However, according to Baker and Farmer (1982), the limitations of these schemes have been that one pattern had accounted for 57% (Mollaret and Nicolle, 1965) and 87% (Nilehn and Ericson, 1969) of the strains studied with the two schemes, and they do not lyse serotype O:8 found in the United States. Baker and Farmer (1982) developed a new system for *Y. enterocolitica*, *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia* based on 24 phages. With this scheme, only 22% of strains fell into the most common type and most of the other types contained <5% of strains. In addition, bacteriophage typing has also been used for *Y. pseudotuberculosis* (Nagano *et al.*, 1997a). Bacteriophage typing used to be a common typing method for *Yersinia* species (Toma *et al.* 1979; Shayegani *et al.* 1981; Baker and Farmer 1982). However, maintaining the stock cultures and the control strains adds to the challenges of this method and thus phage typing has had limited availability as a typing method. Today, its importance has diminished due to the arrival of more convenient typing methods, for example, molecular based methods.

7.2 Genotypic methods

While phenotypic methods study the presence or absence of biological and metabolic activities for the characterization of bacteria, genotypic (or DNA-based typing) methods apply more specific characterization and categorization of bacteria at the nucleic acid level. The approach of different genotyping methods in epidemiological studies can be divided into short term (or local) or long term (or global). For example, short-term epidemiology is the confirmation that the two isolates recovered from a localized outbreak of infections represent the same strain. In long-term epidemiology, the relationship of these outbreak isolates to strains of world-wide origin can be studied. In short term approaches in particular, typing methods should be highly discriminatory such that isolates assigned to the same genotype are likely to be descended from a recent common ancestor, and isolates that share a more distant common ancestor are not assigned to the same type (Maiden *et al.*, 1998). In order to have a high-discriminatory typing method, individual loci or uncharacterized regions of the genome that are highly variable within the bacterial population can be identified. For example, in pulsed-field gel electrophoresis (PFGE) and PCR with repetitive element or arbitrary primers (REP and ERIC-PCR, RAPD) the selection of enzymes or primers aims to reveal the maximal variation within the study population. The variation in these applications is usually evolving very rapidly, hindering the applicability of these methods in long-term epidemiology for understanding global population structures of studied organisms. In long-term epidemiology, the aim is to group the strains in order to identify a more distant common ancestor and genomic regions in which the variation is accumulating very slowly (for example housekeeping genes), are usually chosen. By analyzing many loci the discrimination of these methods, like multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST), can be increased.

7.2.1 Pulsed-field gel electrophoresis (PFGE)

The pulsed-field gel electrophoresis method was developed in the mid 1980s (Schwartz and Cantor, 1984) and subsequently applied to molecular epidemiology (Arbeit *et al.*, 1990). In PFGE, the chromosomal DNA of a bacterial cell is released inside agarose plugs and digested with rare-cutting restriction enzymes generating a moderate number of restriction fragments. The restricted DNA inside the agarose plug is then subjected to gel electrophoresis in which the orientation of the electric field alternates in a programmed manner. In conventional electrophoresis, DNA fragments of up to approximately 50 kb readily travel through the gel pore matrix and the movement of fragments larger than this is physically prevented. In PFGE, the changing orientation of the electric field unravels these large ball-like randomly coiled DNA fragments and the time needed for this reorientation is comparable to the size of the DNA fragment. Subsequently, the reorientation in pulsed-fields forces even up to 10 Mb fragments to proceed through the gel pore matrix in a snake-like manner and the fragments still maintain their size dependent electrophoretic mobilities (Herschleb *et al.*, 2007).

The variation in PFGE patterns mainly originates from rearrangements by homologous recombination, insertions and deletions occurring in the chromosomes of the organisms being studied (Barrett *et al.*, 2006). Point mutations were thought to be one of the major contributing factors to PFGE pattern diversity until it was shown in *E. coli* O157:H7 that insertions and deletions have a more important role in creating strain diversity (Kudva *et al.*, 2002). PFGE data is susceptible to errors affecting banding patterns. Among the major sources of errors are the loss of mobile genetic elements (plasmids, pathogenicity islands, etc.) and incomplete restriction digestion resulting in band loss or additional “ghost bands” which naturally are not reproducible in different runs. For example, undigested plasmids that are not in linear conformation may migrate in a gel very unpredictably and create a one-band difference between strains.

Due to its discrimination capacity between related and unrelated isolates and the adequate intra- and interlaboratory reproducibility, PFGE is the most widely used genotyping method of bacterial pathogens. Today, many standardized protocols allow the worldwide comparison of PFGE patterns between different laboratories (Gerner-Smidt and Scheutz, 2006; Martin *et al.*, 2006). The PFGE studies of *Y. enterocolitica* (Asplund *et al.*, 1998; Baumgartner *et al.*, 2007; Buchrieser *et al.*, 1994; Falcao *et al.*, 2006; Fredriksson-Ahomaa *et al.*, 1999; Fredriksson-Ahomaa *et al.*, 2001b; Fredriksson-Ahomaa *et al.*, 2006b; Fredriksson-Ahomaa *et al.*, 2007; Iteman *et al.*, 1991; Iteman *et al.*, 1996; Iwata *et al.*, 2005; Korte *et al.*, 2004; Najdenski *et al.*, 1994; Saken *et al.*, 1994; Thistedt Lambertz and Danielsson-Tham, 2005) have, for example, confirmed the role of pigs as a source of human infection, revealed the prevalence and transmission of different genotypes among humans and animals and observed the overall homogeneity of bioserotype 4/O:3 strains. For example, in the study by Asplund *et al.* (1998), 24 different types were recognized among 106 human and porcine *Y. enterocolitica* bioserotype 4/O:3 isolates when the results with enzymes NotI and XbaI were combined. Most of the isolates belonged to genetically closely related PFGE types among which two PFGE types clearly dominated. Due to the global homogeneity of bioserotype 4/O:3 pulsotypes, however, the typing capacity of PFGE may be limited, especially in outbreak investigations. Fredriksson-Ahomaa *et al.* (1999) were able to increase the discrimination index of PFGE from 0.74 to 0.93 in a set of strains originating from a geographically limited area when isolates harbouring the same NotI pattern were further characterized with ApaI and XhoI.

The early PFGE studies of *Y. pseudotuberculosis* (Iteman *et al.*, 1991; Iteman *et al.*, 1995) focused mainly on assessing the genomic stability and typeability of this species. In a study by Iteman *et al.* (1995) of 30 strains (10 each of serotypes O:1, O:2 and O:3), almost every strain had a specific pulsotype and the authors suggested that genomic rearrangements occurring frequently in this species could be the reason for the polymorphism. The *in vitro* stability of the pulsotypes of *Y. pseudotuberculosis* was also studied, and the authors observed genomic instability that was strain dependent rather than related to the length of storage of the bacteria. Subsequently, PFGE have been exploited in studies of the distribution of *Y. pseudotuberculosis* in pigs and poultry at farm and slaughterhouse level and in wild animals (Kardos *et al.*, 2007; Laukkanen *et al.*, 2008; Niskanen *et al.*, 2002; Niskanen *et al.*, 2008; Niskanen *et al.*, 2003), and during outbreak investigations (Jalava *et al.*, 2006; Rimhanen-Finne *et al.*, 2008) (this study).

7.2.2 Ribotyping

In ribotyping, the genotype pattern obtained reflects the restriction fragment length polymorphisms (RFLPs) of the neutrally evolving housekeeping genes typically found to flank chromosomal rRNA gene sequences (Bouchet *et al.*, 2008). Ribotyping involves the isolation and restriction of genomic DNA, separation of the

DNA fragments in gel electrophoresis, and transferring the separated fragments to a nylon membrane by Southern blot (Southern, 1975). The transferred membrane containing genomic DNA digests is hybridized with a labelled ribosomal operon probe, visualized according to the label used, and the resulting banding pattern is analyzed manually or with the aid of fingerprint analysis software. More recently, the PCR application of ribotyping termed rRNA spacer length polymorphism, or intergenic transcribed sequence (ITS) profiling, or PCR-ribotyping has been used. Since the initial studies by Andersen and Sauders (1990) and Blumberg *et al.* (1991), ribotyping has constantly been used in the epidemiology of *Y. enterocolitica* (Gulati and Virdi, 2007; Iteman *et al.*, 1996; Iwata *et al.*, 2008) and *Y. pseudotuberculosis* (Martins *et al.*, 2007; Voskressenskaya *et al.*, 2003). In a study applying conventional and PCR-ribotyping (Lobato *et al.*, 1998) 11 conventional riboprofiles for 56 strains of *Y. enterocolitica* 4/O:3 were generated. Although PCR ribotyping increased the total number of ribotypes to 17, the majority of strain clustered within one PCR ribotype. The study by Gulati and Virdi (2007) gave evidence of the presence of two clonal groups among strains of *Y. enterocolitica* biotype 1A. Although only a limited number of ribotypes was identified for the strains representing various geographic origins, PCR ribotyping was able to slightly increase the discrimination of conventional ribotyping in this study. Similarly among 68 *Y. pseudotuberculosis* strains isolated in Brazil, only four ribotypes were identified (Martins *et al.*, 2007). The limited diversity of genotypes is considered a major weakness when using ribotyping for example in outbreak investigations. On the other hand, ribotyping is a suitable tool for long-term epidemiology and may reveal important information on taxonomic relationship and global spread of strains. For example among *Y. pseudotuberculosis*, quite a limited number of ribotypes were identified (Voskressenskaya *et al.*, 2003) but evidence of global dissemination of the organism and circulation in different animal and human hosts was obtained.

7.2.3 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism typing is based on the amplification of subsets of total genomic restriction fragments using PCR (Vos *et al.*, 1995). In AFLP, DNA is cut with restriction enzymes (with a frequent cutter and a rare cutter) and the ends of these DNA fragments are ligated to double-stranded oligonucleotide adapters. In the next step, the sequence of the adapters and the adjacent restriction site serve as a primer binding sites in the amplification of the restriction fragments. Selective nucleotides are included at the 3’ ends of PCR primers to ensure that only restriction fragment in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. This limits the amplification to a certain subset of the restriction fragment. The number of restriction fragments to be amplified is fine-tuned by using rare cutting and frequent cutting restriction enzymes and adapters in combination, since only the rare cutter/frequent cutter fragments will be amplified. After polyacrylamide gel electrophoresis, or more recently, after the detection of fragments in an automatic sequence apparatus, a highly informative pattern of 40 to 200 bands is observed (Savelkoul *et al.*, 1999; Vos *et al.*, 1995). AFLP is considered more reproducible and robust than random amplified polymorphic DNA (RAPD) analysis because of the chance to use stringent PCR annealing temperatures in AFLP compared to RAPD. Like RFLP analysis, RAPD analysis, PFGE and rep-PCR, AFLPs correspond to mutations that are dispersed over the genome, and variation by AFLP analysis is based on the mutations in restriction sites or length variation of restriction fragments. In addition, AFLP analysis also exploits the variation in the nucleotides that match the selective 3′ ends and displays more fragments than other fingerprinting techniques. AFLP is suitable for short-term epidemiological studies; it seems to have the same taxonomic range as other fingerprinting techniques like PFGE (Savelkoul *et al.*, 1999). AFLP has been used to genotype *Y. enterocolitica* strains of human and animal origin at least in the United Kingdom (Fearnley *et al.*, 2005) and Switzerland (Kuehni-Boghenbor *et al.*, 2006). In both studies, AFLP primarily clustered the *Y. enterocolitica* strains according to the biotype and demonstrated, according to the previous observations by PFGE, that pathogenic bioserotypes were more clonal than non-pathogenic bioserotypes. Additionally, the study by Fearnley *et al.* (2005) suggested that some strains causing human disease do not come from veterinary sources identifiable at this time.

7.2.4 Multiple loci-based genomic typing

Multilocus sequence typing (MLST) examines the nucleotide sequences of multiple loci encoding housekeeping genes (or fragments of them) and indexes the variation in these sequences as sequence types (STs) (Maiden *et al.*, 1998). MLST loci, or the housekeeping genes, encode proteins that are under stabilizing selection for conservation of metabolic function and thus makes MLST suitable for both epidemiological and population genetic studies. In practice, the method is better suited to the investigation of bacterial phylogeny and the evolution of population lineages than to typing of strains, for example, in foodborne outbreaks. MLST was derived from **Multilocus enzyme electrophoresis (MLEE)**, a method first applied to prokaryotes by Selander *et al.* (1986), that uses the relative electrophoretic mobilities of intracellular enzymes to characterize and differentiate organisms by generating an electromorph type (ET). As in MLST, the differentiation is based on allelic variation at an individual gene locus, but in MLEE only genetic changes that alter the electrophoretic properties of proteins, only about one twentieth of all possible mutations, are detected (Maiden, 2006). MLEE (which actually is characterized as a phenotypic typing method) has been applied to *Y. enterocolitica* and many *Y.*

enterocolitica-like species (Caugant *et al.*, 1989; Dolina and Peduzzi, 1993; Goullet and Picard, 1984, 1988), including *Y. ruckeri* (Schill *et al.*, 1984). Similarly, MLST was applied recently to study a set of 58 strains representing 11 species of *Yersinia* in a study by Kotetishvili *et al.* (2005) and for *Y. pestis* (Revazishvili *et al.*, 2008). These studies (Dolina and Peduzzi, 1993; Kotetishvili *et al.*, 2005) along with the comparative analysis of 16S RNA and *gyrB* sequences (Demarta *et al.*, 2004) have confirmed the existence of three (or four) genomospecies among *Y. frederiksenii* indistinguishable based on phenotypic characteristics and first revealed by DNA-DNA hybridisation (Ursing and Aleksic, 1995; Ursing *et al.*, 1980). Among *Y. enterocolitica* biotype 4 and *Y. pseudotuberculosis*, human strains were recognized as being closely related to animal strains of the same species by MLEE (Dolina and Peduzzi, 1993).

Repetitive DNA varying in size, location and complexity has been used more extensively in subtyping bacteria recently. For example, PCR typing methods based on families of short intergenic repeated sequences have been developed. **Repetitive Extragenic Palindromic (REP)** elements, also known as palindromic units, were first described as potential regulatory sequences within untranslated regions of operons (Higgins *et al.*, 1982) and a consensus REP sequence was formulated by the multiple alignments of REP-like sequences from *Escherichia coli* and *Salmonella* Typhimurium (Gilson *et al.*, 1984; Stern *et al.*, 1984). **Enterobacterial Repetitive Intergenic Consensus (ERIC)** sequences, also known as intergenic repeat units, are larger 126 bp elements containing a highly conserved central inverted repeat located in extragenic regions and has been defined using genomic sequence information obtained primarily from *E. coli* and *S.* Typhimurium (Hulton *et al.*, 1991; Sharples and Lloyd, 1990). Both types contain highly conserved palindromic inverted repeat sequences that can be used as primer binding sites for PCR fingerprinting of different bacterial genomes. The resulting fingerprint patterns reveal inter-REP or inter-ERIC distances (within the limitations of polymerase extension) and patterns specific to bacterial species and strains (Versalovic *et al.*, 1991). REP and /or ERIC PCR has been applied for both *Y. enterocolitica* (Aarts *et al.*, 2001; Falcao et al., 2006; Sachdeva and Virdi, 2004; Wojciech *et al.*, 2004) and *Y. pseudotuberculosis* (Kim *et al.*, 2003). In a ERIC PCR study of *Y. enterocolitica* strains (Falcao *et al.*, 2006) two strains of biotype 1A isolated from foods and harbouring the *ail* and *ystA* genes usually associated with *Y. enterocolitica* virulence, were grouped closer (almost 70% fingerprint similarity) to the pathogenic strains isolated from humans and animals than to the other food strains (similarity lower than 50%). The authors concluded that ERIC-PCR could possibly be used as a tool to group *Y. enterocolitica* strains by their virulence potential.

Variable number of tandem repeat regions (VNTRs) are short sequence motifs in bacterial genes or intergenic regions which may be variable among strains with respect to the number of repeat units present or their individual primary structure (van Belkum, 2007). **Multiple-locus variable number tandem**

repeat (MLVA) analysis detects this variability for a number of different loci and has proven particularly effective for assessing genetic diversity in bacterial species that are highly monomorphic, such as *Bacillus anthracis* and *Y. pestis* (Lindstedt, 2005). Today, MLVA usually involves multiplex PCR amplification with specific primers and various fluorescent dyes coupled with sizing the amplicons with high-resolution capillary electrophoresis providing the resolution required for sizing fragments that differ by only a few base pairs in size. Even though MLVA has been found useful for differentiating between foodborne bacterial isolates in outbreak settings, it can overestimate the divergence between very similar isolates originating from different ecological niches because the generation of variation in some VNTRs can be under selection. Therefore, some guidelines for MLVA data interpretation have been suggested (Noller *et al.*, 2006) emphasizing that isolates diverging by a single or double tandem repeat in a single VNTR locus might have arisen from a point source and should be investigated accordingly. Consequently, each MLVA scheme addressed for epidemiological investigations should be pre-validated with a known group of epidemiologically linked strains. For typing of *Y. enterocolitica* 4/O:3, de Benito *et al.* (2004) used the polymorphic tandem repeat region (VNTR) that was able to increase the discrimination capacity of PFGE. MLVA genotyping for *Y. enterocolitica* 4/O:3 (Gierczynski *et al.*, 2007) appeared to be a promising tool for outbreak investigations, and it distinguished 45 genotypes among 62 *Y. enterocolitica* 4/O:3 strains of worldwide origin. MLVA seemed to be especially helpful for distinguishing *Y. enterocolitica* subsp. *palearctica* isolates that are difficult to differentiate by PFGE.

7.2.5 Other genotyping methods used for *Yersiniae*

RAPD (random amplified polymorphic DNA) typing or arbitrary primed PCR (AP-PCR) is based on the amplification of genomic DNA with a single short (9-10 bp) primer of arbitrary nucleotide sequence (Welsh and McClelland, 1990; Williams *et al.*, 1990). The PCR is carried out at low annealing temperatures (36°C) under conditions that favour relatively non-specific binding of the primer to multiple sites of the template DNA. The resulting multiple PCR products are then separated in agar gel electrophoresis. RAPD requires no previous sequence information and it studies the whole genome instead of relying on hypervariability within one specific gene. Discrimination between isolates is usually accomplished by combining RAPD results obtained with several primers. However, RAPD has been criticised for the lack of reproducibility, because PCR conditions and the concentration of different PCR reagents, for example, have an impact on the resulting RAPD patterns. Blixt *et al.* (2003) evaluated these factors and developed an interlaboratory RAPD typing method for *Y. enterocolitica*. In that study, serotype O:3 strains clustered according to their geographic origin, so that isolates originating from Finland, Sweden and

Norway fell into one subcluster, and the isolates from Denmark and England fell into the other subcluster together with O:9 and O:5,27 strains. In addition, Rasmussen *et al*. (1994) and Odinot *et al*. (1995) have evaluated the use of RAPD in epidemiological investigations. RAPD has been used for *Y. pseudotuberculosis* during Izumi fever outbreak investigations (Makino *et al.*, 1994) and for studying the source of infection in breeding monkeys (Kageyama *et al.*, 2002).

DNA microarrays allow genome-wide comparison of the presence or absence of similar DNA regions in sufficiently related microorganisms. In this technique, typically a collection of DNA probes manufactured based on a previously sequenced bacterial isolate are fixed on a solid support followed by a genomic hybridization of a study isolate. DNA microarrays are expensive for routine use, and the future use of this technology in epidemiological studies and molecular typing of bacterial pathogens will largely depend on the cost-effectiveness of new protocols. However, Howard *et al*. (2006) constructed a microarray with duplicated reporter elements representing all chromosomal and plasmid-predicted (4,291) coding sequences (CDSs) of the sequenced strain *Y. enterocolitica* 8081 1B. By applying comparative phylogenomics, the authors confirmed the highly heterogeneous nature of this species and observed three distinct statistically supported clusters composed of a non-pathogenic clade, a low-pathogenicity clade and a highly pathogenic clade, supporting the existence of three subspecies among *Y. enterocolitica*.

In addition to the afore-mentioned techniques, various others have been applied for pathogenic *Yersiniae*. For example, analysis of genes encoding **virulence factors pYV, HPI and YPM** among 2,235 *Y. pseudotuberculosis* strains of global origin enabled the establishment of six genetic groups and gave an insight into the evolution and spread of this organism (Fukushima *et al.*, 2001). In addition, typing based on **CRISPRs** (clustered regularly interspaced short palindromic repeats) has been established for *Y. pseudotuberculosis* and *Y. pestis* (Vergnaud *et al.*, 2007) and a **single nucleotide polymorphism (SNP)** detection assay has been developed for population structure studies of highly monomorphic *Y. pestis* North American isolates (Touchman *et al.*, 2007). For *Y. pseudotuberculosis,* typing with an **IS*200*-like element** present in multiple copies in the genome resulted in discriminatory capacity greater than ribotyping and almost equal to PFGE (Odaert *et al.*, 1996). In addition, ***gyrB* sequence** analysis and RFLP genotyping have been used for *Y. frederiksenii* (Demarta *et al.*, 2004) and *Y. enterocolitica* 1A strains (Gulati and Virdi, 2007), respectively.

AIMS OF THE STUDY

The aims of this study were:

- To develop a simplified phenotypic approach for identification of *Y. enterocolitica* and *Y. enterocolitica* –like species that could be used in routine laboratories with basic equipment, in order to produce correct information on the prevalence and clinical significance of *Yersinia* isolates.

- To develop a genotyping method for *Y. enterocolitica* bioserotype 4/O:3 for epidemiological investigations and to evaluate the discriminatory power of the method together with PFGE.

- To develop and optimize a PFGE protocol for the epidemiological typing of *Y. pseudotuberculosis* isolates.

- To evaluate the usefulness of PFGE in epidemiological surveillance and outbreak investigations of *Y. pseudotuberculosis.*

- To gain information on the genotypes of *Y. pseudotuberculosis* outbreak and non-outbreak isolates in Finland where outbreaks of *Y. pseudotuberculosis* have been recurring.

MATERIALS AND METHODS

1 Bacterial strains (I–IV)

The study included 444 bacterial strains originating from human, food, animal and environmental samples and reference culture collections. The strains studied (n = 338, Table 5) represented eight species, and the reference strains (n = 106, Table 6) nine species, of the genus *Yersinia* and belonged to several bioserotypes. The reference strains originated from the American Type Culture Collection (ATCC, USA), National Collection of Type Cultures (NCTC, HPA, London, UK), reference culture collection of Enteric Bacteria Laboratory (EBL) of the National Public Health Institute (KTL, Helsinki, Finland) and the culture collection of Prof. Mikael Skurnik (Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Finland). The strains had been stored at -70°C in sterilized skim milk, except for studies I-II, in which some of the strains had also been stored at room temperature in nutrient deep agar tubes.

Study I. The 70 *Y. enterocolitica* 4/O:3 strains listed in Table 5 were previously genotyped by PFGE into 23 types (Asplund *et al.*, 1998) and therefore were chosen to test the capability of YeO:3RS genotyping to further discriminate this group of strains. In addition, isolates of pathogenic serotypes O:9, O:5,27 and O:8, non-pathogenic biotype 1A strains, other *Yersinia* strains and three control strains (*Salmonella enterica*) were studied. A set of reference strains for different pathogenic and non-pathogenic *Yersinia* bioserotypes originating from the culture collection of Prof. Mikael Skurnik were studied for the presence of YeO:3 RS sequence (Table 6).

Study II. Unserotypeable *Y. enterocolitica* strains (n = 67) isolated by Finnish hospital laboratories during 1988-2000 were included in this study. The strains represented *Y. enterocolitica* biotype 1A (n = 55), nonbiotypeable *Y. enterocolitica* (n = 1), *Y. bercovieri* (n = 5), *Y. mollaretii* (n = 5) and *Y. rohdei* (n = 1), listed in Table 5 as they were recategorised after this study.

Study III. All *Y. pseudotuberculosis* serotype O:3 isolates (n = 27) from case patients available for testing during an outbreak investigation from October to November 1998 were investigated in this study. For comparison, one unrelated sporadic strain isolated in September 1997 was also included (Table 5).

Study IV. *Y. pseudotuberculosis* isolates (n = 89) submitted to Enteric Bacteria Laboratory of KTL during outbreak investigations from March to August 2001 were studied. The strains represented serotypes O:1 (n = 55) and serotype O:3 (n = 34) (Table 5).

2 Case-control study on *Y. pseudotuberculosis* outbreaks (III, IV)

A case was defined as an illness in which *Y. pseudotuberculosis* serotype O:3 was isolated by culturing of stool or blood specimens from 15 October to 6 November 1998 (Study III) or as a resident of Finland aged 18 years or older with isolation of *Y. pseudotuberculosis* from stool or blood culture from 1 May to 31 July 2001 (Study IV). Control subjects were identified from the general population through the national population register. Two (Study III) or five (Study IV) controls per case were matched for year of birth, gender and postal code of residence.

Informed consent was obtained from the case and control patients. The data was collected using a standard questionnaire during telephone interviews by trained personnel (Study III) or by post (Study IV). The patients were asked about illness, consumption of fresh produce, meat products, and untreated water, shopping locations, meals eaten outside the home (Study III–IV) and specific diets (Study IV). For case patients, the questions referred to the two weeks before the onset of symptoms. Controls were asked about the two weeks before the questionnaire completion or interview.

Table 5. Bacterial strains included in the study. The *Y. bercovieri*, *Y. mollaretii* and *Y. rohdei* strains of Study II are named in the table as they were recategorised after the study

Strain	Serotype	Biotype	Origin of the isolate[1]	No. of isolates (n = 338)	Study
Y. enterocolitica	O:3	4		**73**	
			human	70	I
			human	3	II
Y. enterocolitica	O:9	2		**12**	
			human	9	I
			human, Germany	2	
		3	human	1	II
Y. enterocolitica	O:5,27	2		**5**	
			human, Canada	1	I
			human, USA	2	
			coypu, France	1	
		3	human	1	II
Y. enterocolitica	O:5	1A		**14**	
			human	13	I
			minced meat	1	
Y. enterocolitica	NT[2]	1A		**65**	
			human	6	I
			bacon	1	
			porcine	1	
			gull	1	
			well water	1	
			human	55	II
Y. enterocolitica	NT	NBT[3]	human	1	II
Y. pseudotuberculosis	O:1	ND[4]	human	**57**	
			human	55	IV
			human	2	
Y. pseudotuberculosis	O:3	ND		**93**	
			human	2	I
			human	30	
			human	27	III
			human	32	IV
			human (blood)	2	IV
Y. pseudotuberculosis	O:2	ND	iceberg lettuce	**1**	III
Y. pseudotuberculosis	NT	ND		**3**	
			iceberg lettuce	1	III
			soil	1	
			irrigation water	1	
Y. bercovieri	ND	NBT[4,5]	human	**5**	II
Y. mollaretii	ND	NBT[5]	human	**4**	II
Y. mollaretii	ND	3[5]	human	**1**	II
Y. rohdei	ND	NBT[5]	human	**1**	II
Y. kristensenii	ND	ND	gull	**1**	I
Y. frederiksenii	ND	ND	water	**1**	I
Y. intermedia	ND	ND	well water	**1**	I

[1] All strains were isolated in Finland and all human strains were isolated from stool samples, unless otherwise stat The strains originating outside Finland were received from the culture collection of Prof. Mikael Skurnik.

[2] NT (not typeable); either cross-reacting or not agglutinating with the *Y. enterocolitica* antisera O:3, O:5, O:8, O:9.

[3] NBT (not biotypeable); at least two reactions diverging from *Y. enterocolitica* biotypes (Wauters *et al.*, 1987).

[4] ND (not determined).

[5] Biotyping refers to *Y. enterocolitica* biotyping scheme (see Chapter 6).

Table 6. Reference strains used as controls in the study

Strain	**Category/characteristics of the strains**	**No. of strains (n = 106)**	**Reference/origin**	**Study**
Y. enterocolitica	Reference for colony morphology, sequencing and biochemical testing	1	ATCC^{T} 9610	II
Y. enterocolitica		1	NCTC 11176	
Y. enterocolitica		1	RH 4823[1]	
Y. mollaretii		1	ATCC 43969^{T}	
Y. bercovieri		1	ATCC 43970^{T}	
Y. rohdei		1	ATCC 43380^{T}	
Y. rohdei		1	ATCC 43872	
Y. aldovae		1	ATCC 35236^{T}	
Y. frederiksenii		1	ATCC 33641^{T}	
Y. intermedia		1	ATCC 29909^{T}	
Y. kristensenii		1	ATCC 33638^{T}	
Y. pseudotuberculosis		1	ATCC 29833^{T}	
Y. pseudotuberculosis		1	RH 3526[1]	
Y. ruckeri		1	ATCC 29473^{T}	
Y. enterocolitica	Reference for serotype O:9	1	NCTC 11174	I
Y. enterocolitica	Reference for serotype O:5,27	1	NCTC 10463	I
Y. enterocolitica	Reference for serotype O:8	1	NCTC 10938	I
Y. enterocolitica	European pathogenic *Y. enterocolitica* bioserotypes (3/O:1, nb[2]/O:1,2,3, 5/O:2, 4/O:3, 2/O:9, 2/O:5,27)	13	Culture collection of Prof. Skurnik	I
Y. enterocolitica	Other pathogenic *Y. enterocolitica* bioserotypes (1B/O:4,32, 1B/O:8, 1B/O:13, 1B/O:13a,13b, nb[2]/O:13,18, 1B/O:20, 1B/O:21)	15	Culture collection of Prof. Skurnik	I
Y. enterocolitica	Biotype 1A serotypes (4; 5; 6; 6,30; 6,31; 7,8; 10; 13,7; 25; 25,26; 26,44;28,50; 34; 35,52; 35,36;; 41(27)K1; 41(27),42K1; 41(27), 43; 41,43; 50; NT,K1; NT)	37	Culture collection of Prof. Skurnik	I
Y. bercovieri *Y. kristensenii* *Y. frederiksenii* *Y. intermedia* *Y. mollaretii* *Y. ruckeri* *Y. pseudotuberculosis*	Other *Yersinia* species	21	Culture collection of Prof. Skurnik	I
Salmonella Newport	Negative control strain	2	IH 69594, IH 69604[1]	I
Salmonella Typhimurium	Negative control strain	1	IH 110663	I

[1] Control strain (RH) or culture collection strain (IH) of EBL, KTL

[2] nb = not biotyped, pathogenicity determined previously (Kay *et al.*, 1983; Lee *et al.*, 1981; Schiemann and Devenish, 1982)

3 Probes and primers (I–II)

Three plasmids containing genomic fragments of *Y. enterocolitica* O:3 cloned into pBR322 were used as probes (Table 7) (Study I). The plasmids were isolated using a Qiagen midi plasmid DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition, eight different fragments of the restriction enzyme digested plasmid pAY100 were used as probes (Study I). The fragments were isolated on 1% low-melting-point agarose (SeaPlaque agarose; FMC BioProducts, Rockland, Maine) gel electrophoresis. The isolated fragments were purified from the excised agarose blocks with the QIAquick gel extraction kit (Qiagen). The probes were finalized by labelling the purified plasmid DNAs and the isolated fragments of pY100 with digoxigenin (DIG-High Prime labelling kit, Roche Molecular Biochemicals, Boehringer, Germany; according to the manufacturer's instructions).

The primers used for amplification of the *Y. enterocolitica* O:3 repeated sequence (YeO:3RS) (Study I) were based on the plasmid pAY100 sequence upstream of the O-antigen gene cluster of *Y. enterocolitica* serotype O:3 (GenBank/EMBL/DDBJ accession number Z18920) (Table 8). The primers described by Jalava (2000) and Kotilainen *et al.* (1998) were used for sequencing the 16S rRNA gene of *Y. enterocolitica* and *Y. enterocolitica* –like strains (Table 8) (Study II).

Table 7. Probes used in Study I

Probe	**Description**	**Reference**
pAY100	Gene cluster (12.5 kb) for the biosynthesis of *Y. enterocolitica* O:3 LPS O-antigen cloned in plasmid pBR322	Al-Hendy *et al.*, 1991
pRV7	Gene cluster (12.4 kb) for the biosynthesis of *Y. enterocolitica* O:3 LPS outer core cloned in plasmid pBR322	Skurnik *et al.*, 1995
p19kd-15	Gene cluster (13.4 kb) for the biosynthesis of *Y. enterocolitica* O:3 urease enzyme core cloned in plasmid pBR322	Skurnik *et al.*, 1993
ClaI$_{16700}$-ClaI$_{1348}$[1,2]	1.65 kb restriction digest of pAY100	Study I
ClaI$_{1348}$-ClaI$_{1918}$	0.57 kb restriction digest of pAY100	
ClaI$_{1918}$-EcoRI$_{4598}$	2.68 kb restriction digest of pAY100	
EcoRI$_{4598}$-EcoRI$_{5800}$	1.2 kb restriction digest of pAY100	
EcoRI$_{4598}$-EcoRI$_{16650}$	12.05 kb restriction digest of pAY100	
EcoRI$_{5800}$-ClaI$_{8458}$	2.66 kb restriction digest of pAY100	
SphI$_{7450}$-SphI$_{11214}$	3.76 kb restriction digest of pAY100	
ClaI$_{8458}$-EcoRI$_{16650}$	8.19 kb restriction digest of pAY100	
SphI$_{11214}$-EcoRI$_{12400}$	1.19 kb restriction digest of pAY100	

[1] Restriction sites as subscripts, referring to the restriction map of plasmid pAY100

[2] See Table 9 for details of the restriction enzymes

Table 8. Primers used in the PCR detection and sequencing in this study

Primers (5'→3')	**Location[1]**	**Target**	**Size (bp)**	**Description**	**Study**	**Reference**
Pr-HC4-(1):GTG CCG TAG CTC AGC TCG (forward)	439 – 456 (pAY100)	*orf0.0-orf0.67*	490	Detection of repeated sequence upstream of the O-antigen gene cluster of *Y. enterocolitica* O:3	I	This study
Pr-CC8-(1): AAC GCC GCC GTG GAG GCC (reverse)	929 – 912 (pAY100)					
FD 1 MOD[2]: AGA GTT TGA TC(TC) TGG (TC)T (TC)AG (forward)	8 - 27 (*E. coli*)	16S rRNA	450	Amplicon for the sequencing of 16S rRNA gene (with primer 533r)	II	Jalava 2000 Kotilainen *et al*. 1998 Weisburg *et al*. 1991 Lane *et al*. 1985
533r: GTG CCA GCA GCC GCG GTA A (reverse)	515 - 533 (*E.coli*)					

[1] pAY100, pAY100 sequence upstream of the O-antigen cluster of *Y. enterocolitica* O:3 (GeneBank accession number: Z18920) (Zhang *et al.*, 1993) or *E. coli*, *E. coli* 16S rRNA (Gutell *et al.*, 1985).

[2] The primer is a mixture of oligonucleotides. The positions indicated by parentheses are occupied by either of the nucleotides inside the parentheses.

4 Restriction enzymes and molecular weight standards (I–IV)

Of the six restriction enzymes used in the preliminary YeO:3RS genotyping experiments (Study I), two enzymes were selected for further typing studies (Table 9) (Study I–II). Similarly, of the three restriction enzymes used in the preliminary PFGE typing experiments (unpublished data), two were chosen for subsequent genotyping (Table 9) (Study III–IV).

A digoxigenin (DIG)-labelled DNA molecular weight marker III (Roche) (Size range 0.3-1.5 kb) was used as a size marker for each gel in the YeO:3RS genotyping studies (Study I–II). In PFGE typing, Low Range PFG Marker (New England BioLabs Inc., Beverly, Mass., USA) (Size range 0.1-200 kb) was used as a size marker (Study III-IV).

Table 9. Restriction enzymes used in this study

Method	Enzyme[1]	Status	Recognition site	Study
YeO:3RS genotyping	EcoRI	Probe synthesis	5'...G↓AATTC…3'	I, II
	ClaI	Probe synthesis	5'...AT↓CGAT…3'	I, II
	SphI	Probe synthesis	5'...GCATG↓C…3'	I, II
	AvaI	Pre[2]	5'...C↓PyCGPuG…3'	I, II
	HaeIII	Pre	5'...GG↓CC…3'	I, II
	HindIII	Pre	5'...A↓AGCTT…3'	I, II
	MspI	Pre	5'...C↓CGG…3'	I, II
	BglI	Pre+Genotyping	5'...GCCNNNN↓NGGC…3'	I, II
	NciI	Pre+Genotyping	5'...CC↓SGG…3'	I, II
PFGE	XbaI	Pre	5'...T↓CTAGA…3'	III[3]
	SpeI	Pre+Genotyping	5'...A↓CTAGT…3'	III, IV
	NotI	Pre+Genotyping	5'...GC↓GGCCGC…3'	III, IV

[1] Roche Molecular Biochemicals, Boehringer, Germany
[2] Enzymes were used in preliminary genotyping studies
[3] Unpublished data

5 Identification of *Yersinia* isolates (I–IV)

The species identification of the isolates was carried out by standard protocols (Bottone 1997; Farmer 1999) (see Table 4 in Review of the literature). In Study II, however, strains isolated and identified by Finnish hospital clinical microbiology laboratories were retested at EBL (KTL) with API 20 E (bioMérieux, France) at 30°C. Identification to the species level was further confirmed by combining the results of API 20 E, biotyping and additional sugar fermentation tests. These tests were performed in sugar fermentation broth with Bromothymol blue indicator and supplemented with 0.5% of one of the following sugars: sorbose, raffinose, cellobiose, maltose, D-arabitol, fucose, glycerol and α-methyl-D-glucoside. The sugar broths were incubated at 25°C and read after 24 and 48 hrs.

For examination of the colony morphology through a stereo microscope (Olympus zoom stereo microscope SZH10, Tokyo, Japan), the strains were streaked on cefsulodin-irgasan-novobiocin (CIN) agar (Oxoid) and incubated at 30°C for 22-24 hrs. The morphology of the strains tested was compared to those of the ATCC type strains, NCTC reference strain and one control strain of EBL (Table 6).

Identification of these strains was verified with 16S rRNA sequencing (see Chapter 10).

6 Biotyping of *Y. enterocolitica* (I, II)

Y. enterocolitica strains were biotyped according to Wauters *et al.* (1987) (Table 10). The biotyping consisted of tests for esculin hydrolysis, acid-production from salicin, trehalose and xylose, pyrazinamidase and lipase (tween-esterase) activities, and production of indole and acetoin (Voges-Proskauer reaction). The biotyping tests were incubated at 25°C and read after 24 and 48 hrs.

Table 10. Biochemical tests used for biotyping *Y. enterocolitica*[1]

	Biotype Reaction[2]					
Test	1A	1B	2	3	4	5
Esculin hydrolysis (24 h)	+/-	-	-	-	-	-
Salicin (acid production 24h)	+	-	-	-	-	-
Pyrazinamidase[3]	+	-	-	-	-	-
Lipase (Tween-esterase)	+	+	-	-	-	-
Xylose (acid production)	+	+	+	+	-	v
Trehalose (acid production)	+	+	+	+	+	-
Indole production	+	+	(+)	-	-	-
Voges-Proskauer test	+	+	+	+	+	+/(+)

[1] Modified from Wauters *et al.* (1987)

[2] Symbols: +, positive; -, negative; (+), delayed positive; v, variable

[3] According to Kandolo and Wauters (1985)

7 Serotyping (I–IV)

Y. enterocolitica strains were serotyped by slide agglutination with antisera against *Y. enterocolitica* O:3, O:5, O:8 and O:9 (Denka Seiken, Tokyo, Japan). Similarly, *Y. pseudotuberculosis* strains were serotyped by slide agglutination with antisera against *Y. pseudotuberculosis* serotypes 1-6 (Denka Seiken). The strains were streaked on nutrient agar or Drigalski-Conradi agar plates and incubated overnight at 30°C or 48±4 h at 25°C before serotyping.

8 Detection of the virulence plasmid-associated phenotype

For the presence of the virulence plasmid (pYV), the strains of Study II were further studied on Congo-Red Magnesium Oxalate agar (CR-MOX) as described by Riley and Toma (1989). The plates were incubated at 37°C and read after 24 and 48 hrs.

9 Chromosomal DNA extraction (I, II)

For Study I, chromosomal DNA isolation was carried out essentially as described previously (Rappuoli *et al.*, 1988). For this purpose, bacterial strains were grown overnight (18-22 h) in BHI broth at +30°C with shaking. Contaminating DNase activity found in the DNA of some of the strains was inactivated by an additional phenol extraction procedure with phenol-chloroform-water (25:24:1) and chloroform-isoamylalcohol (24:1) according to standard protocols. For sequencing in Study II, DNA was extracted from overnight cultures grown at 28°C on sheep blood agar. Cells were harvested in a TE buffer and boiled for 15 min. Cell debris was centrifuged (13000 rpm, 2 min) and supernatant was used as a template in the PCR reactions (1 and 5 µl in 50 µl reaction).

10 16S rRNA sequencing (II)

Primers FD1 MOD (forward) and 533r (reverse) (Table 8) were used to amplify the beginning of the 16S rDNA sequence (Jalava, 2000; Kotilainen *et al.*, 1998). The reaction mixture contained 5 µl of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2 \times 6 H_2O$, 0.01 % gelatin), 100 µM of each dNTP, 250 nM of each primer and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Warrington, UK). Amplification was performed in the MasterCycler thermocycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation for 10 min at 94°C, followed by 34 cycles of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C and final extension 10 min at 72°C. PCR reaction products were purified with Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Sequences of one strand (with 533r primer) were determined by the ABI Prism 310 Genetic Analyzer using the BigDye fluorescent terminator chemistry (Applied Biosystems, Warrington, UK). The 16S rRNA gene sequences obtained (450 bp) in this study were aligned against sequences of *Yersinia* type strains using Vector NTI Suite v.6 software (InforMax Inc., USA). Sequences obtained were also compared to those available in the GeneBank database of the National Center for Biotechnology Information (NCBI) by using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/).

11 YeO:3RS genotyping (I)

11.1 Digestion of chromosomal DNA and gel electrophoresis

Restriction enzymes (Table 9) were used to digest 2 µg of DNA according to the recommendations of the manufacturer (Roche). The restriction fragments were separated by 1% agarose (SeaKem ME agarose, FMC BioProducts) gel electrophoresis at (22V) 1.6 V/cm for 18–19 h in 0.5 × TBE (0.045 M Tris-borate, 0.001 M EDTA) buffer.

11.2 Southern blotting and hybridization

DNA from electrophoresis gels was blotted onto hybridization membranes (Hybond-N+, 0.45 µm, Amersham, UK) by the Southern (1975) method using a vacuum transfer system. Blotted membranes were fixed with 0.4 M NaOH and rinsed briefly in 5 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate). Prehybridization (2 h) and hybridization (18 h) were carried out at 60°C in standard hybridization buffer (5 × SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent (Roche)). After hybridization, the membranes were washed twice at 60°C for 15 minutes in 2 × SSC, 0.1% SDS. Colorimetric detection of the digoxigenin-labelled probes was done according to the manufacturer's instructions (Roche).

12 YeO:3RS PCR (I)

PCR amplifications were performed in 0.2 ml polypropylene tubes with a MJ Research Minicycler equipped with a hot bonnet lid (Waltham, MA). A DNA template from bacteria for PCR was prepared from bacteria grown overnight in 0.3 ml of Luria broth. The bacteria were pelleted and resuspended into 40 µl of water and 14 µl of phenol:chloroform (1:1) was added, the tube was vortexed for 20 s, centrifuged for 3 min at 14000 rpm and 20 µl of the upper phase was recovered for use as a template in PCR. The PCR conditions were as follows: the 50 µl reaction mixture contained a template (1-2 µl) and primers (10 pmol per reaction), 1 unit of DynaZyme II DNA polymerase (FinnZymes, Espoo, Finland), 200 µM of each dNTP in a reaction buffer provided with the DNA polymerase. The PCR cycle, 94°C 15 sec, 55°C 15 sec and 72°C 30 sec, was repeated 25 times. The primers Pr-HC4-(1) and Pr-CC8-(1) (Table 8) used for amplification were based on the O-antigen gene cluster sequence of *Y. enterocolitica* serotype O:3 (GenBank/EMBL/

DDBJ accession number Z18920). After PCR, 5 µl of the reactions were analysed in 1% agarose gels to detect the amplified products.

13 PFGE (III, 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 essentially as described by Lukinmaa *et al.* (1999). An 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. Chromosomal DNA was digested overnight at 37°C with 10 U of restriction enzymes NotI and SpeI (Roche). Pulsed-field gel 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, Calif.) in running conditions of 7 to 15 s, 30 h, 6 V/cm, 120°, 14 °C.

14 Assurance of the repeatability of the typing result and subtype naming (I–IV)

In order to evaluate the stability of the banding patterns and repeatability of the YeO:3RS and PFGE typing methods, the typing procedures described above (Chapters 11 and 13) were repeated at least three times for the probe pAY100 (Study I, II) and twice for PFGE (Study III, IV), starting from DNA isolation or restriction digestion, for every typeable strain studied.

An isolate differing from the others by at least one band was assigned to a subtype of its own (Study 1–IV). Previously proposed criteria (Tenover *et al.*, 1995) were also considered in designating the PFGE subtypes of *Y. pseudotuberculosis* (Study III, IV). Serotype O:1 PFGE types were named, for example, S11 (S, digested with SpeI; 1, serotype O:1; 1, type detected first), and serotype O:3 PFGE types were named, for example, N32 (N, digested with NotI; 3, serotype O:3; 2, second detected type).

For designation of the YeO:3RS subtypes (Study I, II), NciI types were numbered and *Bgl*I types lettered according to the order of detection in the laboratory. The serotype of a strain was indicated by a prefix (for example serotype O:3, types 3.1 and 3.a; serotype O:5,27, types 5.1 and 5.a; and serotype O:9, types 9.1 and 9.a).

15 Calculation of the discrimination index (I)

The discrimination index (DI), that is, the probability that two unrelated strains sampled from the test population will be placed into different typing groups, was calculated by an application of Simpson's index of diversity (Hunter and Gaston, 1988) for the estimation of the discriminatory power of YeO:3RS typing method or the combined power of YeO:3RS and PFGE typing methods. The index was calculated by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of genotypes described, and n_j is the number of strains belonging to the jth type.

RESULTS

1 Applicability of colony morphology in the preliminary species identification of *Yersiniae*

Of the 67 strains that were identified as *Y. enterocolitica* by Finnish clinical microbiology laboratories and were unserotypeable by the antisera used, 55 belonged to biotype (BT) 1A, one belonged to BT 3, and the remaining 11 strains were not biotypeable (at least two reactions diverged from the established biotypes). The identification of unserotypeable BT 3 strain and 11 unbiotypeable strains was thus considered doubtful. When comparing the colony morphology through a strereomicroscope of these 12 strains to reference strains and previously bioserotyped control strains, 8 strains could be tentatively identified as *Y. bercovieri* or *Y. mollaretii* (Study II, Table 1). Only one of the 12 strains had colony morphology suggestive of *Y. enterocolitica*.

API 20E identified 9 of these 12 strains as *Y. enterocolitica* with >90% certainty (Study II, Table 1), whereas 16S rRNA gene sequencing confirmed only one *Y. enterocolitica* strain. Additionally, five strains were considered as *Y. mollaretii,* five strains as *Y. bercovieri*, and one strain as *Y. rohdei* among the 12 doubtfully identified strains based on sequencing. Consequently, all of the *Y. mollaretii* strains and three of the five *Y. bercovieri* strains were correctly identified when the colony morphology was examined prior to any other testing. It was not possible to identify the remaining three strains by colony morphology and they included *Y. rohdei*, *Y. bercovieri*, and one biochemically atypical strain of *Y. bercovieri*. The latter strain differed morphologically, biochemically, and by sequence (3 nucleotide difference to the type strain) from other *Y. bercovieri* strains (Study II, Tables 1 and 2, Fig. 1). The growth of *Y. rohdei* was poor on CIN agar hampering the morphological examination (the appearance of every strains studied was unique). All 11 non-*Y. enterocolitica* strains had colony morphology clearly different from *Y. enterocolitica*, thus it was possible to avoid misidentification for all 11 strains by colony morphology, but only for 3 strains with API 20 E.

Y. bercovieri and *Y. mollaretii* colonies (approximately 1.5 mm in diameter) had typical morphology in the microscopic examination with eroded edges and ground-glass appearance of the translucent zone surrounding the red centre of the colonies (best seen in slightly oblique illumination). These features distinguished them from the colonies of *Y. enterocolitica* BT 1A (approximately 2 mm in diameter, larger centre of the colony, and the surrounding zone devoid of ground-glass appearance), bioserotype 4/O:3 (approximately <1 mm in diameter, smaller, deeper red centre of the colony with sharper border) (Study II, Fig. 1) and bioserotype 3/O:9 (data not shown).

In biotyping according to the *Y. enterocolitica* scheme, the strains that later were considered *Y. bercovieri* and *Y. mollaretii* were always esculin, salicin and lipase negative but had a positive pyrazinamidase reaction (Study II, Table 1). An exception was strain IH 111767 which differed from the other *Y. bercovieri* and *Y. mollaretii* strains by a negative pyrazinamidase reaction placing it in *Y. enterocolitica* BT 3 (Study II, Table 1), but considered *Y. mollaretii* after sequencing. In VP test, two and three of the five *Y. bercovieri* and *Y. mollaretii* strains studied, respectively, gave positive results. Type strains ATCC 43969 (*Y. mollaretii*) and ATCC 43970 (*Y. bercovieri)* gave a negative reaction in this test, as expected (data not shown). In the test for fucose, four of the five *Y. bercovieri* strains gave a positive reaction and all five *Y. mollaretii* strains gave a negative reaction (Study II, Table 1). Similarly in the test for sorbose, four of the five *Y. bercovieri* strains gave a negative reaction and all five *Y. mollaretii* strains gave a positive reaction. However, both of the type strains ATCC 43969 (*Y. mollaretii*) and ATCC 43970 (*Y. bercovieri)* gave a positive reaction in the test for fucose. All of the *Y. rohdei* strains studied (including ATCC type strains) were maltose positive and sorbose negative. The test for glycerol also gave a high proportion of positive reactions for *Y. bercovieri* and *Y. mollaretii* (Table 11).

2 Distribution of YeO:3RS genotypes among *Y. enterocolitica* strains

The YeO:3RS probe (plasmid pAY100) comprises a 12.5 kb genomic fragment of *Y. enterocolitica* O:3 lipopolysaccharide O-antigen gene cluster cloned into plasmid pBR322. In Study I, the genotyping potential of YeO:3RS probe was evaluated in hybridization and PCR experiments with 203 bacterial strains altogether. YeO:3RS genotyping identified 15 different typing patterns with the restriction enzymes NciI and BglI among 70 *Y. enterocolitica* 4/O:3 human isolates. Altogether 27 different subtypes were identified among these isolates when typing results with PFGE (Asplund *et al.*, 1998) and YeO:3RS genotyping were combined. YeO:3RS genotyping was able to divide both largest PFGE groups A1 and B1 into six different subtypes (Study I, Table 1). PFGE type B2 was divided into three and F1 into two different YeO:3RS types. Thus, the discrimination of both YeO:3RS (DI 0.73) and PFGE (DI 0.69) was increased to DI 0.85. By YeO:3RS typing, genotypes 3.1b and 3.2a were most prevalent, including 28 (40 %) and 23 (33 %) isolates, respectively. Genotype 3.5f included five isolates (7 %), genotypes 3.7b and 3.3a each included two isolates (3 %), and all remaining 10 genotypes contained one strain each (1 %).

Table 11. Biochemical features of Study II *Yersinia* strains (excluding biochemical tests of Table 1 of Study II)

Species[1]	**Sorbose**	**Raffinose**	**Cellobiose**	**Maltose**	**D-Arabitol**	**Fucose**	**Glycerol**	**α-Methyl-D-Glucoside**
Strains isolated from patients								
Y. mollaretii (n = 5)	100[2]	0	100	100	0	0	100	0
Y. bercovieri (n = 5)	0 (20)[3]	0	100	100	0 (20)[3]	83	100	0
Y. rohdei (IH 41571)	-	+	+	+	-	-	+	-
Y. enterocolitica (IH 111298)	+	+	+	+	+	+	+	+
Y. enterocolitica (IH 111778)	+	-	+	+	+	+	+	-
Control strains								
Y. mollaretii (ATCC 43969)	+	-	+	+	-	+	+	-
Y. bercovieri (ATCC 43970)	-	-	+	+	-	+	+	-
Y. rohdei (ATCC 43380)	-	+	+	+	-	-	-	-
Y. rohdei (ATCC 43872)	-	-	+	+	-	-	-	-
Y. enterocolitica (ATCC 9610)	+	-	+	+	-	-	+	-
Y. enterocolitica (NCTC 11176)	+	-	+	+	-	-	+	-
Y. enterocolitica (RH 4823)	+	-	+	+	+	+	+	-
Y. aldovae (ATCC 35236)	-	-	-	-	-	+	-	-
Y. frederiksenii (ATCC 33641)	+	-	+	+	+	+	+	-
Y. intermedia (ATCC 29909)	+	+	+	+	-	+	+	+
Y. kristensenii (ATCC 33638)	+	-	+	+	+	+	+	-
Y. pseudotuberculosis (ATCC 29833)	-	-	-	+	-	-	-	-
Y. pseudotuberculosis (RH 3526)	-	-	-	+	-	-	-	-
Y. ruckeri (ATCC 29473)	-	-	-	+	-	-	-	-

[1] Containing strains of Study II, Table 1 and the reference strain of Table 6 (materials and methods). Identity verified by sequencing the beginning of the 16S rRNA gene (Jalava 2000). RH, control strain or IH, culture collection strain of EBL, KTL

[2] Values are percentages of strains tested positive

[3] One (atypical) patient strain out of 5 strains (20%) tested positive for both sorbose and D-arabitol. This strain differed also morphologically and by sequence (3 nucleotide difference to the type strain) from other *Y. bercovieri* strains.

The genotyping potential of probe YeO:3RS was also tested on isolates of the other prevalent European pathogenic bioserotypes, that is, 2/O:9 and 2/O:5,27. The profiles generated were clearly distinguishable from 4/O:3 profiles. Twelve tested *Y. enterocolitica* 2/O:9 isolates (Study I, Table 2) gave five different YeO:3RS genotypes when the results of the banding patterns of BglI and NciI were combined. Five tested 2/O:5,27 isolates yielded five different YeO:3RS genotypes (Study I, Table 2, Fig. 3). The 2/O:5,27 profiles were more closely related to each other than to either the 4/O:3 or 2/O:9 profiles. Among other strains tested, YeO:3 genotyping resulted in either incomplete typing patterns or weak hybridization with both enzymes used. These strains included one bioserotype 1B/O:8 strain, 14 bioserotype 1A/O:5 strains, 10 non serotypeable biotype 1A strains, five strains of other *Yersinia* species and three *Salmonella* strains (Study I, Table 2, Fig. 4). When studying the distribution of YeO:3RS region by PCR among 136 different *Yersinia* strains (Study I, Tables 2 and 3), strains of European pathogenic bioserotypes 4/O:3, 2/O:9 and 2/O:5,27 were always positive in accordance with the hybridization results. In addition, this genomic region was present in the European pathogenic *Y. enterocolitica* bioserotypes 3/O:1 (n = 2) and 5/O:2 (n = 2), but only in 3 out of 104 other *Yersinia* strains tested. The American pathogenic serotypes belonging to biotype 1B were PCR negative (Study I, Tables 2 and 3). The stability of the banding patterns and repeatability of the YeO:3RS typing was confirmed in repeated (at least three times for probe pAY100, Study I, II) experiments for every typeable strain studied.

To detect which part of the YeO:3RS probe (plasmid pAY100) was responsible for the genotyping potential, fragments of plasmid pAY100 were used separately as probes and hybridized to the DNA of four serotype O:3 strains representing four different *Bgl*I patterns. Of the plasmid pAY100 fragments, probe ClaI_{16700} - ClaI_{1348} hybridised to all the essential bands of the complete pAY100 banding pattern, and in particular, to the differentiating bands, so that the four different patterns were regenerated (Study I, Fig. 1a). In conclusion, these analyses mapped the genotyping potential of pAY100 to the 1.65 kb region upstream of the O-antigen biosynthetic genes, that is, to *orf0.0-orf0.67* sequences that apparently form the core of the repeated sequence region. The *orf0.0-orf0.67* sequences were present in many copies in the genome of the strains studied representing *Y. enterocolitica* subsp. *palearctica* (European pathogenic serotypes). The typing potential of two additional probes was also tested in preliminary studies. Probes pRV7 and p19kd-15, carrying the gene clusters for the biosynthesis of the LPS outer core and the urease enzyme, respectively, gave banding patterns which did not differentiate between strains (data not shown) and were therefore no longer used.

3 Molecular epidemiological characteristics of *Y. pseudotuberculosis* outbreaks

During outbreak investigations in Studies III and IV, the PFGE patterns of selected sporadic strains and strains related to previous outbreaks were also investigated (Table 12, Figure 3, Study IV, Figure 1). Outbreak A occurred in August, 1997, and affected 35 schoolchildren in Pirkkala, Tampere region. Four *Y. pseudotuberculosis* O:3 isolates available for typing from this study had indistinguishable genotyping patterns S31N31 in PFGE (Figure 3). Outbreak B occurred in August-September 1998, and affected 53 schoolchildren in Mänttä, also in Tampere region. As with outbreak A, the four *Y. pseudotuberculosis* O:3 isolates available for typing had indistinguishable genotyping patterns S31N31 in PFGE. The sources of outbreaks A and B could not be resolved, but epidemiological investigation suggested vegetables eaten in a school canteen as possibly related to these outbreaks.

Outbreak C started only a month after outbreak B in October 1998 and lasted for three weeks. This outbreak (Study III) comprised four geographically separate clusters of cases in Southern Finland among which 47 culture confirmed *Y. pseudotuberculosis* O:3 infections were identified. All 27 isolates from case patients available for PFGE had the indistinguishable genotyping patterns S32N32. A population-based case control study identified iceberg lettuce as a potential source of the infections. During the trace-back investigations, 4 farms in the southwest archipelago were identified as possible sources of iceberg lettuce sold to the cafeterias associated with clusters of case patients. The implicated lot of iceberg lettuce was no longer available for culture. *Y. pseudotuberculosis* was isolated from one soil and one irrigation water sample taken from one of the suspected farms in November 1999. In subsequent sampling in October 2000, *Y. pseudotuberculosis* was additionally isolated from two iceberg lettuce samples. One strain isolated from iceberg lettuce was serotype O:2, but the other strains did not agglutinate with O:1-O:6 antisera. The PFGE patterns of environmental strains differed from the outbreak strain patterns.

Outbreak D in October, 1999, comprised three geographically separate clusters of 31 cases also in southern Finland. The 17 isolates available for typing from this outbreak had indistinguishable genotyping patterns S32N32. Although the source of this outbreak could not be identified, iceberg lettuce was again suspected. The outbreak A and B genotype S31N31 had 3 band difference with the corresponding outbreak C and D genotype S32N32.

During 2001, 89 culture-confirmed cases of *Y. pseudotuberculosis* were reported in Finland; 55 (62 %) were of serotype O:1, and 34 (38 %) were of serotype O:3. After notification of a suspected food-borne outbreak among schoolchildren from one municipality on 30 May, a case control study and a PFGE study, including *Y. pseudotuberculosis* isolations from 1 May to 31 July 2001 were conducted.

Table 12. Distribution of PFGE types among *Y. pseudotuberculosis* outbreak strains (Studies III and IV) and selected non-outbreak strains (Hallanvuo *et al*., 2002)

Origin of the strain	Time of isolation	Serotype	No of strains	PFGE type	Study
Non-outbreak strains	February 1994	O:3	1	S32 N32	Hallanvuo *et al*., 2002
	March 1995	O:3	1	S34 N32	
	April 1997	O:3	1	S32 N32	
Outbreak A	August 1997	O:3	4	S31 N31	Hallanvuo *et al*., 2002
Outbreak B	September 1998	O:3	4	S31 N31	Hallanvuo *et al*., 2002
Outbreak C from 4 regions	November 1998	O:3	27	S32 N32	Study III
Non-outbreak strain	July 1999	O:3	1	S33 N33	Hallanvuo *et al*., 2002
Outbreak D from 3 regions	October 1999	O:3	17	S32 N32	Hallanvuo *et al*., 2002
Non-outbreak strains	June 2000	O:3	1	S32 N32	Hallanvuo *et al*., 2002
	May 2000	O:1	1	S11 N11	
	July 2000	O:1	1	S12 N12	
Outbreak E from several regions	Summer 2001	O:3	13	S32 N32[1]	Study IV
			5	S33 N32[2]	
			10	S33 N33[3]	
			3	S32 N34	
			1	S34 N31	
			1	S34 N32	
			1	S33 N34	
		O:1	16	S11 N11	
			32	S12 N12	
			2	S13 N13	
			1	S12 N15	
			1	S15 N16	
			1	S17 N17	
			1	S121 N12	
			1	S122 N122	

[1] Genotype of strains originating from the Western Finland infection clusters (see Figure 3).

[2] Genotype of strains originating from the city of Mikkeli infection cluster.

[3] Genotype of strains originating from the Kainuu (region in Northern Finland) infection cluster.

The sources of the multiple outbreaks during summer 2001 were not identified. Iceberg lettuce was again suspected, but eating outside the home was most strongly associated with the infections.

During the summer 2001 outbreak E, the four most common *Y. pseudotuberculosis* serotype O:1 and O:3 profiles (S11N11, S12N12, S32N32, and S33N33) accounted for 80% (16 + 32 +13 + 10/89) of the isolates. Conversely, 20% of the strains were divided mostly into single strain genotypes (Table 12). Clustering of serotype O:3 cases were noticed in Western Finland, Mikkeli region and Kainuu region (Figure 3). The serotype O:3 PFGE type S32N32 that was involved in the previous outbreaks, C and D, appeared in strains representing infections scattered around Western Finland. These infections occurred within two weeks (data not shown). Genotype S33N33 that differed by two bands (SpeI enzyme) and by four bands (NotI enzyme) from the Western Finland genotype appeared in a regional cluster of infections in Kainuu (region in Northern Finland). Almost simultaneously, genotype S33N32, that differed by two bands (SpeI) from the Western Finland genotype, appeared in a cluster of infections around the city of Mikkeli in mid Eastern Finland. All the serotype O:3 major cluster genotypes differed from each other by 2-4 bands when the results from both enzymes were considered separately (Figure 3). The dominating type S32N32 was also found among sporadic strains isolated before the outbreaks (in 1994 and 1997).

Strains from serotype O:1 infections diverged into two main PFGE types (S11N11 and S12N12) which differed from each other by only one band (Figure 3; Study IV, Figure 1). These two types had been detected previously among sporadic strains in summer 2000. The infections were concentrated mainly in Southern Finland, but were more separate than O:3 infections in time and geography. An exception to this was a cluster of five infections in the Tampere region. Among the rest of serotype O:1 strains in summer 2001, the diversity was higher. For example, even up to 6 and 10 band differences were seen between SpeI profile S13 and major types S11 and S12, respectively (Table 12, data not shown).

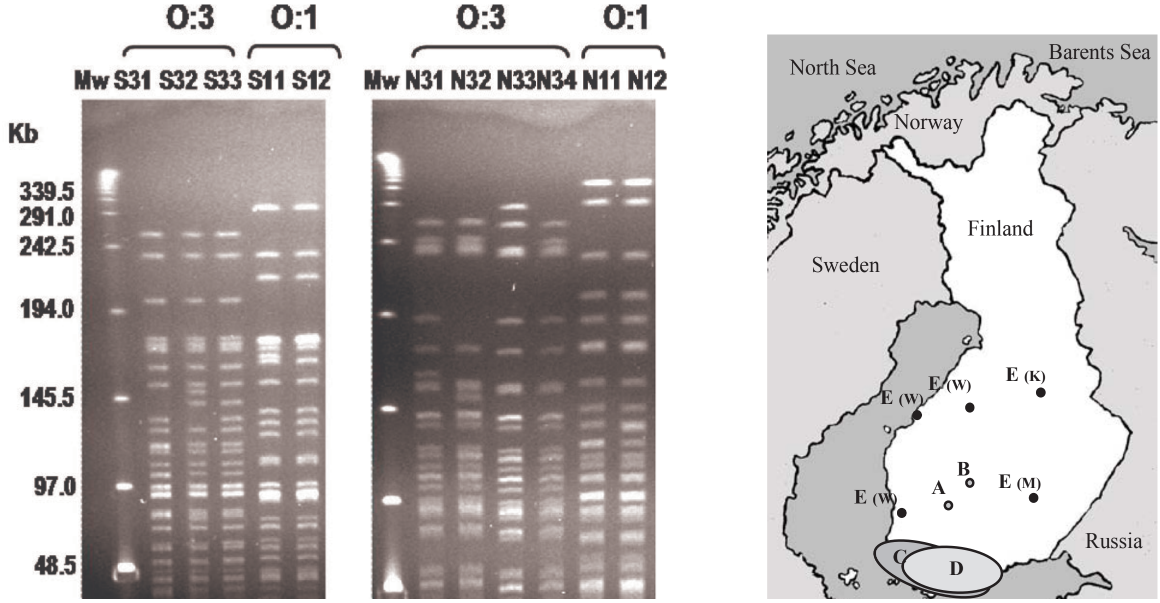

Figure 3. Left panel: PFGE banding patterns of outbreak and non-outbreak strains of *Y. pseudotuberculosis* (Table 12). Mw, Low Range PFG Marker (NEB); S, pattern with enzyme SpeI; N, pattern with enzyme NotI. Right panel: Different clusters of cases in outbreaks of Y. pseudotuberculosis in Finland. A, outbreak A in Tampere region (August 1997); B, outbreak B in Tampere region (September 1998); C, outbreak C in four different regions in southern Finland (November 1998, Study III); D, outbreak D in three different regions in southern Finland (October 1999); E, outbreak E (summer 2001, Study IV), serotype O:3 infection clusters of Western Finland (W), Mikkeli region (M) and Kainuu region (K) are shown (See also Study IV, Figure 1).

DISCUSSION

1 Aspects of valid species identification and clinical significance of *Y. enterocolitica* and *Y. enterocolitica*–like isolates

Y. enterocolitica is the third most commonly reported zoonosis in Europe with 8,979 reported cases of yersiniosis in 2006 (EFSA, 2007a). The incidence figures of *Y. enterocolitica* should include only the strains potentially pathogenic to humans but suffer worldwide from inclusion of also clinically non-significant strains. Relatively simple ways, like biotyping, for assessing the potential pathogenicity of *Yersinia* strains, have long existed. However, the implementation of biotyping as a guideline to reporting the strains has only recently been suggested (EFSA, 2007b). Adding to the complexity of assessing the clinical significance of this species, biotype 1A traditionally regarded as non-pathogenic, has recently been suggested to harbour a "clinical" subgroup potentially pathogenic to humans and indistinguishable from the non-pathogenic subgroup of this biotype by currently available identification and typing methods (Tennant *et al.*, 2003). For example, in the study by Noble *et al*. (1987), strains of *Y. enterocolitica* and *Y. enterocolitica*-like species lacking many virulence factors were significantly associated with the occurrence of diarrhoea in patients in whom no other infectious or non-infectious causes of enteric disease could be identified. According to Bottone (1997), the recovery of the *Y. enterocolitica* strain or other *Yersinia* species from the stool of a symptomatic patient by direct cultivation or after minimal (24 to 48 h) cold enrichment and the absence of another potential etiologic agent may imply significance regardless of the virulence attributes. However, according to Tennant *et al*. (2003), many of the studies suggesting pathogenicity of *Y. enterocolitica* biotype 1A have been uncontrolled clinical observations. Therefore, more data is needed to prove the causative association between *Y. enterocolitica* strains of biotype 1A and gastrointestinal complaints.

In Finland, *Yersinia* infections are notifiable and the number of *Y. enterocolitica* cases has gradually decreased since 1995 (873 cases). After the year 2006 (533 cases), the number of cases fell by 22% in 2007 (414 cases), bringing the incidence down below 8/100,000 (Anonymous, 2008b). Some of the hospital laboratories have voluntarily submitted *Yersinia* isolates to EBL of KTL for further identification. Of the approximately 300 *Y. enterocolitica* strains that arrived in 2000, biotyping revealed that 40 % were non-pathogenic BT 1A strains, 10 % did not belong to any of the established biotypes, and only half of the strains belonged to pathogenic bio-or serotypes (Hallanvuo and Siitonen, 2002). However, voluntary submission of the strains may cause overrepresentation of atypical strains among those submitted to EBL if a laboratory chooses to send only the problematic strains. Most of the hospital laboratories in Finland use direct plating on CIN agar but some of them also use cold enrichment in the isolation of *Yersinia.* Prolonged cold enrichment is thought to favour fast-growing non-pathogenic *Yersinia* species at the expense of pathogenic bioserotypes in the isolation process.

Y. enterocolitica–like species have not yet been demonstrated to cause human disease. However, according to Sulakvelidze (2000) some of these organisms may be potential emerging pathogens harbouring putative virulence factors that are different from those of the classical "pathogenic" *Yersinia* strains and may be overlooked by the traditional virulence assays. For example, *Y. bercovieri* and *Y. mollaretii* produce novel heat stable enterotoxins that are putative virulence markers of these species (Sulakvelidze, 2000; Sulakvelidze *et al.*, 1999). Evaluation of the clinical significance of the *Y. enterocolitica*–like organisms is critically disabled by the fact that these species are misidentified as *Y. enterocolitica* by most of the commercially available identification systems. This also adds a source of error to the annual incidence figures of *Y. enterocolitica*. Additionally, the clinical "non-existence" of *Y. enterocolitica*–like species leads to obscure estimations of the prevalence and significance of these species until they are properly identified in routine clinical laboratories.

In this study, a significant proportion of the "*Y. enterocolitica*" strains that were not typeable by the antisera available belonged to *Y. enterocolitica*–like species when they were identified by sequencing of the beginning of the 16S rRNA gene. Thus, identification based on a diagnostic kit like API 20 E and commercial serotyping antisera is inadequate in order to avoid misidentifications. Furthermore in serotyping, the occurrence of O-antigens typical of pathogenic species (for example O:3, O:9, and O:8) has been demonstrated in *Y. enterocolitica*–like strains and *Y. enterocolitica* biotype 1A (Aleksic, 1995; Wauters *et al.*, 1988b. Because these strains can account for a significant proportion of the incoming *Yersinia* isolates in the clinical laboratory (McNally *et al.*, 2004; Sihvonen *et al.*, 2007), the applicability of serotyping in primary diagnostics can be further questioned. The results of this study point out the effective role of the examination of colony morphology and using the biochemical reactions included in *Y. enterocolitica* biotyping scheme in aiding the correct identification of *Y. enterocolitica*–like strains. This study initiated further studies of profiling *Y. enterocolitica* and *Y. enterocolitica*–like organisms (Sihvonen *et al.*, 2009) and eventually led to changing identification practices in Finnish clinical microbiology laboratories.

Together with colony morphology, the tests for esculin, salicin and pyrazinamidase, in particular, revealed the strains misidentified as *Y. enterocolitica* by API 20E. Furthermore, the tests for fucose and sorbose were the most useful biochemical tests in differentiating *Y. bercovieri* and *Y. mollaretii* isolates from each other, although the test reaction for fucose did not differ between the type strains (ATCC 43969 and 43970) of these species. The results of clinical isolates for fucose and sorbose were in agreement with previous results (Aleksic and Bockemühl, 1999; Stock *et al.*, 2002; Wanger, 2007). Stock *et al.* (2002) concluded that, in particular, the tests for urease and the fermentation of cellobiose, fucose, maltose, sorbitol, sorbose, sucrose and D-xylose were key reactions in the identification of *Y. bercovieri*, *Y. mollaretii*, *Y. aldovae*, and *Y. ruckeri* to species level. Furthermore in our study, the positive reaction for raffinose, in addition to melibiose, seemed to be useful for differentiating *Y. intermedia* from *Y. frederiksenii* (and other *Yersinia* species). The unexpected positive results for maltose in our study among *Y. rohdei* strains and the high proportion of positive reactions in glycerol, especially among *Y. bercovieri* and *Y. mollaretii* strains, are in contrast to the data of Farmer *et al.* (at 36°C) (Farmer *et al.*, 2007) (see Table 4 in the review of the literature) but can be explained by the lower incubation temperature (25°C) used in our study. Namely, many metabolic reactions of *Yersiniae* are increased at lower temperatures and the increase in the proportion of positive reactions of maltose at lower temperatures has also been previously demonstrated (Stock *et al.*, 2002). However, the proportion of positive VP results for *Y. bercovieri* and *Y. mollaretii* strains was higher than in the data of Aleksic and Bockemühl (1999), although the incubation temperature was the same. With respect to new *Yersinia* species (described after Study II) *Y. aleksiciae* (Sprague and Neubauer, 2005), *Y. massiliensis* (Merhej *et al.*, 2008), and *Y. similis* (Sprague *et al.*, 2008), most of the strains in this study had a clearly different API 20 E profile (1114703 for *Y. aleksiciae*; 1154723 for *Y. massiliensis*, and 0014112 for *Y. similis*) and the possibility of these species could be excluded without sequencing.

Identification by 16SrRNA sequencing has been used widely in species identification and the criteria of ≥99% sequence similarity to the sequence deposited in DNA databases for valid species designation has been established (Clarridge, 2004; Drancourt *et al.*, 2000). However, in some genera the 1% difference for defining species is invalid and 0.5% difference is used instead (Clarridge, 2004). It has been proposed that a difference of at least 5 to 15 bp in the whole 16S rRNA gene sequence would be needed for defining species (Fox *et al.*, 1992). Recently, Stackebrandt and Ebers (2006) revised former recommendations and suggested a 16S rRNA sequence similarity threshold range of 98.7–99 % as the point at which DNA-DNA reassociation experiments should be mandatory for testing the genomic uniqueness of a novel isolate(s). However, no criterion whether to use just the forward or reverse sequences has been set. Many laboratories use just the forward sequence and, for example, in one study of 50 strains it was shown that either the forward or reverse sequence could be used to assign a correct species identification, with less than 1% difference between sequences (Clarridge, 2004). In Study II, we used the 450 bp initial reverse sequence of 16SrRNA gene, an approach that has been found to provide adequate differentiation for identification for most clinical bacterial isolates (Clarridge, 2004). Similarly, we expected a 16S rRNA gene sequence similarity of ≥99% for species designation (Clarridge, 2004; Drancourt *et al.*, 2000). The drawbacks of the 16S rRNA gene sequencing have been identified and discussed (Boudewijns *et al.*, 2006; Clarridge, 2004; Drancourt *et al.*, 2000; Fox *et al.*, 1992; Patel, 2001). One of the major drawbacks is the quality problems in the sequences deposited in the public databanks like GenBank, especially related to deposits older than 10–15 years (Clayton *et al.*, 1995). To minimize erroneous interpretations and to validate the system, we also sequenced and compared the reference strains and used them for pairwise comparisons as recommended (Boudewijns *et al.*, 2006). We found at most three nucleotide difference of Study II clinical strains to the type strains used, which corresponds to <1% difference and thus validates the species definition.

A simplified phenotypic scheme based on the Study II results and hands-on experience in EBL was introduced for differentiation between *Y. enterocolitica* and *Y. enterocolitica*–like species. In EBL, it has been found useful to start the identification of *Yersinia* strains (verified in routine hospital laboratories by API 20 E to belong to the *Yersinia* species) by examining the microscopic colony morphology on CIN agar and the presence of the virulence plasmid on CR-MOX agar. The strains are then forwarded to serotyping (that is, those with the appearance of pathogenic bioserotypes 4/O:3 and 2 or 3/O:9), biotyping (appearance differing from bioserotypes 4/O:3 and 2 or 3/O:9) and additional biochemical testing (*Y. enterocolitica*–like appearance and presence of non-biotypeable strains) if necessary. For example, *Y. bercovieri* and *Y. mollaretii* strains misidentified as *Y. enterocolitica* are easily revealed by colony morphology and by negative reactions for esculin, salicin and lipase and positive reaction for pyrazinamidase (included in *Y. enterocolitica* biotyping scheme). The *Y. enterocolitica* bioserotype 3/O:5,27, which is generally less frequently isolated, has a colony morphology sometimes similar to that of *Y. bercovieri* and *Y. mollaretii*, but it can be distinguished by a negative pyrazinamidase reaction in the *Y. enterocolitica* biotyping scheme. Distinguishing between *Y. bercovieri* and *Y. mollaretii* can be made by tests for fucose and sorbose. Sequencing is necessary for only a few strains that remain unidentified after these steps; including rare cases of *Y. rohdei*, pyrazinamidase negative *Y. bercovieri* or *Y. mollaretii* with a similar colony morphology to *Y. enterocolitica* O:5,27 and *Y. enterocolitica* with coinciding atypical morphology and biotyping reactions not common to any of the established biotypes.

2 Molecular epidemiology of *Y. enterocolitica* 4/O:3 infections

Among *Y. enterocolitica*, genomic polymorphisms appear to be higher for non-pathogenic strains of biotype 1A found in the environment than for pathogenic strains of bioserotypes 4/O:3 and 2/O:9 (Fredriksson-Ahomaa *et al.*, 2006a). Due to the low degree of polymorphism, the search for an epidemiological typing method discriminatory enough for *Y. enterocolitica* 4/O:3 can be challenging. A novel epidemiological typing method based on the use of a repeated genomic region (YeO:3RS) as a probe was developed in Study II for the detection and differentation between strains of European pathogenic *Y. enterocolitica* bioserotypes 4/O:3, 2/O:5,27 and 2/O:9. Of the three *Y. enterocolitica* O:3 genomic clusters evaluated as genotyping probes, probe pAY100 carrying a copy of YeO:3RS proved to be useful. In a previous PFGE study of Finnish bioserotype 4/O:3 strains, it was noticed that two main PFGE groups dominated, comprising 72% of the 106 strains studied (Asplund *et al.*, 1998). NciI-BglI genotyping of the representative PFGE types of these 106 strains with pAY100 divided each of the two major XbaI-NotI PFGE types into six different subtypes, thus increasing the discrimination of PFGE. Irrespective of the subtyping capacity, it was noticed that two genomic groups (3.1b and 3.2a) also dominated in pAY100 genotyping. Interestingly, the main groups of these different methods were related to each other, suggesting the existence of two major genomic lineages among *Y. enterocolitica* 4/O:3. Similarly in studies by PFGE, restriction enzyme analysis of the virulence plasmid (REAP) and ribotyping, Iteman *et al.* (1996) noticed the divergence of *Y. enterocolitica* 4/O:3 genome into two groups represented by strains of phage type IXb and strains of other phage types.

In addition to bioserotype 4/O:3, probe pAY100 was efficient in genotyping strains of bioserotypes 2/O:9 and 2/O:5,27. Furthermore, PCR experiments showed that the *orf0.0-orf0.67* genes upstream of the O-antigen gene cluster of serotype O:3 (YeO:3RS region) were also present in the European pathogenic bioserotypes 3/O:1 and 5/O:2. The stability and repeatability of YeO:3RS typing patterns were confirmed. However, a major factor affecting the typeability of a strain observed during typing procedures in both YeO:3RS (Study I) and PFGE (Studies III and IV) typing was the degradation of extracted chromosomal DNA of some strains after cell lysis. This was probably due to DNAses produced by the strains (Nakajima *et al.*, 1994) and was avoided by additional phenol extraction procedures and proteinase K treatment in YeO:3RS and PFGE typing procedures, respectively.

The lack of polymorphism in the hybridisation patterns with probes pRV7 and p19kd-15, and also when the parts of pAY100 containing O:3 O-antigen biosynthetic genes were used as probes, suggests that the gene clusters for LPS O-antigen, outer core and urease are conserved in *Y. enterocolitica* 4/O:3 strains.

On the contrary, the polymorphisms in pAY100 patterns potentially arise from repetitions of the 1.65 kb *orf0.0-orf0.67* sequences (YeO:3RS region) and their different locations in the genome. The functions of *orf0.0-orf0.67* genes upstream of the O-antigen gene cluster of serotype O:3 are not yet known (Zhang *et al.*, 1993). Transposon mutagenesis has shown that they are not directly involved in O-antigen biosynthesis (al-Hendy *et al.*, 1991). The partial similarity of the deduced amino acid sequence of *orf0.67* to hypothetical proteins in the *Y. pestis* genome and *Shigella* bacteriophage suggests that YeO:3RS may be part of a bacteriophage sequence present in several copies in the genome.

Bioserotype 1B/O:8 hybridised only weakly with the 1.65 kb *orf0.0-orf0.67* fragment and gave negative PCR results. Nevertheless, partially similar YeO:3RS sequences were present in at least two copies in bioserotype 1B/O:8 when compared to the sequenced genome of the 1B/O:8 strain 8081 (the nucleotide sequence identity of approximately 65-70% explained the weak hybridisation results). Strains of bioserotype 1B/O:8, as well as of bioserotype 1B/O:13a,13b, are rare in Europe, but they have caused foodborne outbreaks in the USA (Bissett *et al.*, 1990; Lee *et al.*, 1990). Our hybridisation and PCR results agree with the division of the pathogenic *Y. enterocolitica* serotypes into two evolutionary lineages, that is, the American and the European lineages (Caugant *et al.*, 1989; Ibrahim *et al.*, 1992; Miller *et al.*, 1989; Skurnik and Toivanen, 1991) on the basis of which the division of *Y. enterocolitica* into subspecies *enterocolitica* and *palearctica* has been established (Neubauer *et al.*, 2000a). Prevalence of the YeO:3RS sequence follows the same division; it is present in weakly pathogenic bioserotypes (subspecies *palearctica*) and absent in biotype 1B strains (subspecies *enterocolitica*). Therefore, it could be also useful in diagnostic differentiation between these two subspecies. After Study II, a method based on a similar hybridisation approach and the presence of a repetitive sequence, an insertion sequence ISYen2, was described by Golubov *et al.* (2005). As with YeO:3RS, ISYen2 was present only in weakly pathogenic types of *Y. enterocolitica.* A large set of *Y. enterocolitica* strains and some other strains of *Yersinia* species originating from different countries was analysed and the authors suggested ISYen2 be used as a signature marker of weakly pathogenic bioserotypes of *Y. enterocolitica*. However, compared to YeO:3RS typing, the number of hybridization bands present in ISYen2 typing patterns was reduced, resulting probably in limited epidemiological typing capacity of ISYen2 typing.

Reproducibility and discriminatory power are key features in an evaluation of an epidemiological typing system for bacteria (Tenover *et al.*, 1995; Tyler *et al.*, 1997) Probe YeO:3RS fulfilled these criteria in genotyping *Y. enterocolitica* bioserotype 4/O:3 isolates in the present study. Combining YeO:3RS typing with PFGE shows an example how two different typing methods can efficiently complement each other in molecular epidemiology. Polymorphism that was masked in the total genomic digestions of PFGE typing was successfully revealed by focusing on the polymorphism introduced by the YeO:3RS fragment upstream of the O:3

O-antigen gene cluster. Similarly, for increasing the discrimination capacity of PFGE, de Benito *et al.* (2004) used polymorphic tandem repeat region *orf528* for typing *Y. enterocolitica* 4/O:3. In that study, eight bioserotype 4/O:3 strains with the same PFGE pattern were distributed into seven different *orf528* types thus greatly improving the discriminatory capacity of PFGE in a set of strains originating from a geographically limited area. In our study, the discrimination of PFGE (DI 0.69) was increased to DI 0.85 by YeO:3RS genotyping in a set of Finnish clinical *Y. enterocolitica* 4/O:3 isolates, and the method was also applicable to other European pathogenic bioserotypes of *Y. enterocolitica*.

3 Molecular epidemiology of *Y. pseudotuberculosis* outbreaks

According to the widely accepted Tenover criteria for bacterial strain typing (Tenover *et al.*, 1995), an isolate is closely related to an outbreak strain if the profiles differ from each other in the position of up to three bands, possibly related if the difference is from four to six bands, and unrelated if the difference is seven bands or more. The variation in PFGE patterns originates mainly from rearrangements by homologous recombination, insertions and deletions occurring in the chromosomes of the organisms in the study (Barrett *et al.*, 2006). Considering the Tenover criteria, the strains in serotype O:3 infection clusters in all outbreaks (A to E), with one exception, were either identical or closely related. The only exception was one serotype O:3 cluster in outbreak E (2001), the strains of which could be considered possibly related (four band difference) to strains of outbreaks C, D, and E (Western Finland and Mikkeli clusters) and closely related (three band difference) to strains of outbreaks A and B. Furthermore, during 1997-2001 sporadic strains of serotype O:3 harbouring indistinguishable or closely related genotypes to outbreak genotypes were seen. Among serotype O:1, the strains in two major clusters in summer 2001 were also closely related. Additionally, strains that belonged to single genotypes were also observed during that summer. These strains were clearly non-related (7 to 10 band differences) to the strains of two major O:1 clusters, and were considered not part of the outbreak.

Recently, the Tenover criteria received some criticism based on 10 years experience in the PulseNet network (Barrett *et al.*, 2006). The authors pointed out the importance of considering the reproducibility of the PFGE method for different organisms, the quality of PFGE gels, the variability of the organism being subtyped and the prevalence of the pattern in question. During our investigations, the PFGE patterns were reproducible in different runs, and the possible quality problems of PFGE gels were overcome by slightly changing the running parameters for verifying band differences. As stated, the variability of the organism studied should be considered when interpreting the PFGE patterns. This variability may be low among *Y. enterocolitica* (Najdenski *et al.*, 1995), but occur more frequently among *Y. pseudotuberculosis* genomic rearrangements. Iteman *et al.* (1995) observed strain dependent genomic instability introducing polymorphism into the PFGE patterns of *Y. pseudotuberculosis* strains. The frequent genomic rearrangements might explain the 1-4 band difference in PFGE patterns of clusters of strains with an epidemiological connection during outbreak investigations in summer 2001. For example, the only common factor between children in two infection clusters (S33N33 and S33N32) was the school. Thus, the four-band difference with the NotI enzyme in patterns of these clusters does not necessarily exclude a common source contamination. One possibility might have been contaminated food served at school; however, the food items served were no longer available for culture. Considering the frequent genomic rearrangements in *Y. pseudotuberculosis*, one serotype O:3 clone and one serotype O:1 clone could have been responsible for all of the outbreaks described.

The prevailing *Y. pseudotuberculosis* serotype during 1990s was O:3. Serotype O:1 was hardly detected among those strains arriving in EBL from clinical laboratories in 1990s. In 1980s, however, both serotypes (O:3 and O:1) were involved in *Y. pseudotuberculosis* outbreaks (Tertti *et al.*, 1984; Tertti *et al.*, 1989). However, serotype O:1 emerged in 1999 with genotypes differing from the outbreak types S11N11 and S12N12 (data not shown). During summer 2000, the serotype O:1 outbreak types (S11N11 and S12N12) were first recognized in strains originating from a few clustered infections around the same time that were not studied further. The following summer, both serotypes prevailed in geographically separate clusters of infections. Looking back, this situation of many simultaneously circulating genotypes causing clusters of infection was exceptional compared to outbreaks that have occurred recently. After 2001, serotype O:3 outbreaks disappeared and the outbreak diversity seems to have been lost. Recent outbreaks (2003, 2004, 2006 and 2008) have involved only strains of serotype O:1 forming genetically tight clusters of a single, recurring genotype S12N12 (Jalava *et al.*, 2006; Rimhanen-Finne *et al.*, 2006; Rimhanen-Finne *et al.*, 2008). However, the considerable diversity of the genotypes among serotype O:1 strains in summer 2001 should have guaranteed a chance of more variation also among strains associated with coming outbreaks. Interestingly in one recent outbreak caused by serotype O:1 strains (S12) (Rimhanen-Finne *et al.*, 2008), a serotype O:3 strain of the previous outbreak genotype (S32) was co-isolated with O:1 strains from carrot storage facilities during environmental investigations (unpublished data). This raises a question as to whether the recurrent appearance of S12 genotype as an outbreak-associated type is more of a coincidence or organized process based on, for example, advantageous surviving properties of a strain in carrot related outbreak settings. Whether these strains have some selective advantage over strains representing other types in these settings or not, could be a subject of further studies. Considering sporadic infections, the collective solid data of the prevalence of different serotypes in Finland is currently unavailable. However, it is clear that outbreak types were present among sporadic strains before the outbreaks occurred and in the time between outbreaks. The point when a "sporadic" strain turned into an outbreak associated strain could have even been related to cold autumn wheather conditions prevailing before harvesting the contaminated lettuce in this study. The temperature drop below 0°C for a couple of nights before harvesting the lettuce could have provided a selective advantage for cold-adapted *Y. pseudotuberculosis* to multiply at the expense of the other microbial population to the levels needed for the outbreak to occur.

4 Sources and vehicles of *Y. pseudotuberculosis* outbreaks

Since 1994, the Finnish clinical microbiology laboratories have to report their *Y. pseudotuberculosis* findings to the National Infectious Diseases Register of KTL. Before that time, infections caused by *Y. pseudotuberculosis* were mainly sporadic and only occasional, small outbreaks were reported (Tertti *et al.*, 1984). During 1997–2008, however, *Y. pseudotuberculosis* caused 10 outbreaks of infections in Finland, altogether with approximately 500 microbiologically confirmed cases. Culture confirmed infections probably represent only a proportion of the disease burden, because clinical diagnosis is difficult, and routine stool cultures may not detect the organism (Leino *et al.*, 1987). The patients usually only have fever with abdominal pain in the absence of diarrhoea (Jalava *et al.*, 2006; Smego *et al.*, 1999; Tertti *et al.*, 1989), and stool cultures may not be requested. Most of the clinical laboratories routinely submit their isolates to KTL for serotyping, and when necessary, the isolates can be genotyped by PFGE. This valuable voluntary work of the clinical laboratories facilitates the rapid detection of the increased number of infections caused by a certain serotype and further investigations.

During the past few years, fresh produce has increasingly been identified as a source of outbreaks of different foodborne pathogens. Between 1992 -2000 in England, salad vegetable or fruit products served as a vehicle in 5.5% of the reported 1,518 general outbreaks of infectious diseases (Long *et al.*, 2002). Since 1995 in the U.S., 16 outbreaks of *E. coli* O157:H7 associated with spinach or lettuce have been reported prior to 2006 (USDA, 2006). Furthermore, fresh produce was stated as the most important vehicle of foodborne illnesses in 2005 in the U.S. (Gourabathini *et al.* 2008). In Finland, fresh vegetables and vegetable products (including salads and carrots) were the most common reported food group causing infection outbreaks in 2006 and were associated with 31% of all outbreaks that year. Furthermore, the outbreaks related to fresh vegetables that year were the most extensive ones; norovirus and *Y. pseudotuberculosis* both caused an outbreak with over 400 illnesses (Niskanen *et al.*, 2007). Vegetable products (iceberg lettuce and carrots) have either been suspected or epidemiologically demonstrated as the source of the recurring *Y. pseudotuberculosis* outbreaks in Finland. Iceberg lettuce was revealed as a source of the geographically dispersed outbreak in Study III in 1998. By extensive trace-back investigations, the source of the contaminated iceberg lettuce could be narrowed down to 4 farms in the southwest archipelago. Although no implicated iceberg lettuce was available for culture, *Y. pseudotuberculosis* of different serotypes to the outbreak type was isolated from one soil, one irrigation water sample and two iceberg lettuce samples taken from one of the suspected farms in November 1999 and October 2000. Considering the different serotypes of the isolates and the time between outbreak and sampling, the PFGE patterns of environmental strains logically differed from the outbreak strain patterns.

The mechanism for contamination of the iceberg lettuce (Study III) remained open but the use of irrigation water contaminated by animal faeces was strongly suspected. Wild roe deer found excessively in the area have access to the lettuce fields and irrigation water sources, and large quantities of deer faeces were found in lettuce fields and around all the irrigation water sources. Deer are known reservoirs of *Y. pseudotuberculosis* and outbreaks of infections, subclinical infections or asymptomatic carriage is common among deer (Jerrett *et al.*, 1990; Sanford, 1995). The carrier animals may start to excrete the bacterium if exposed to stress related to, for example, cold weather, weaning, or starvation. Interestingly, the lettuce implicated in Study III was harvested during inclement weather after a sudden cold snap following relatively mild autumn weather; a contributing factor identified previously in the outbreaks of *Y. pseudotuberculosis* in deer (Sanford, 1995). Furthermore, wild animals (especially feral cats and rodents) have been suggested to serve as a source of environmental contamination associated with *Y. pseudotuberculosis* infection in deer (Mackintosh and Henderson, 1984). During a *Y. pseudotuberculosis* infection peak in 2004-2005 in France, a sudden increase in the rodent reservoir, mainly in rural areas, was suggested as a probable cause for the increase in the number of human infections (Vincent *et al.*, 2008). The authors speculated that changing agricultural practices (that drive away the natural predators of rodents) and reduction of pesticide use may favour the expansion of rodent populations. In the case of pig farms, pest animals seem to have a substantial role in spreading and maintaining the *Y. pseudotuberculosis* contamination on the farm (Laukkanen *et al.*, 2008). During the 2004 outbreak investigations in Finland, a *Y. pseudotuberculosis* genotype identical to patient strains was found among the strains isolated from the fluid of spoiled carrots on the infected farm and the shrews caught in the carrot field (Anonymous, 2005a; Kangas *et al.*, 2008). This emphasizes the possible role of rodents in the initial contamination of carrot storage facilities and carrots in recent carrot related outbreaks in Finland. Thus, the control of the population of small mammals in storage and production facilities, in addition to other hygienic measures during carrot processing, might help to limit the incidence of *Y. pseudotuberculosis* infections in humans.

Recently, the Finnish Food Safety Authority Evira conducted a study to reveal the contamination rate of foodborne pathogenic *Yersinia* in domestic carrots. The results indicated that the carrots are not regularly contaminated with *Y. pseudotuberculosis*. Namely, foodborne pathogenic *Yersinia* species could not be detected during the six month survey period (Niskanen, 2007). To prevent the outbreaks in the future, the Finnish Food Safety Authority Evira has informed the farmers, vegetable-processing plants and institutional kitchens of the risk of *Y. pseudotuberculosis* infection arising from domestic carrots stored over winter. Additionally, instructions to improve hygiene practices during carrot processing and handling has been given nationally. For example, farmers and distributors have been advised to remove poor quality carrots during storage and before processing, and voluntary microbial testing of carrots that have been stored until late spring has been recommended. Institutional kitchens have been advised to wash the carrots; even those they receive peeled and washed, before use.

However, many different factors, in addition to contaminating animals, contribute before infection in human occurs due to the consumption of contaminated vegetables. Due to the increasing importance of vegetables as vehicle for outbreaks globally, research has also focused recently on plant associated factors and the harvesting process as contributors to multiplication of pathogenic bacteria in vegetables. Plant tissue damage of various types during harvesting and processing has been shown to promote significant multiplication of *E. coli* O157:H7 over a short time in lettuce (Brandl, 2008), and a similar process may have contributed to the number of *Y. pseudotuberculosis* bacteria in the iceberg lettuce in our study. More specifically, it has been shown that leaf age and nitrogen content contribute to shaping the bacterial communities of preharvest and postharvest lettuce and with *E. coli* O157:H7, young lettuce leaves may be associated with a greater risk of contamination (Brandl and Amundson, 2008). Additionally, it has been shown that even protozoa present on wet surfaces of fresh produce can interact with enteric pathogens; recent study showed that *E. coli* O157:H7 can multiply in, and exit from, the protozoan vesicles and most probably be protected this way from harsh environmental conditions resulting from, for example, the use of sanitizers in fresh produce processing (Gourabathini *et al.*, 2008).

In general, *Y. pseudotuberculosis* circulates in the environment and infects wild animals that may then contaminate the lettuce or carrots in the field or storage. *Y. pseudotuberculosis* is tolerant to environmental conditions: it can survive for a long period of times in environmental waters, well water, and soil (Inoue *et al.*, 1988a; Jalava *et al.*, 2006). This way *Y. pseudotuberculosis* is not dependent on restricted reservoir species, and can circulate between many animals and environment. An investigation of *Y. pseudotuberculosis* in water and soil samples in Poland by the PCR targeting *ypm* gene yielded 4% and 3% of the samples positive, respectively

(Czyzewska and Furowicz, 2003). Similarly in Finland, *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* were detected by the real-time PCR targeting *ail* gene (Thisted Lambertz *et al.*, 2008a; Thisted Lambertz *et al.*, 2008b) in 6% and 12% of 17 well water samples, respectively, in a project studying the quality of well water intended for children's consumption involving camp centres, private day care, and schools with private wells (S. Hallanvuo, unpublished results). In Japan, the isolation of *Y. pseudotuberculosis* from river water samples has been successful only during colder months from November (51.7% of the rivers) to May (17.5%) (Vincent *et al.*, 2007). Because of the cold adaptation, prolonged carrot storage for up to 10–12 months, as observed in some of the recent outbreaks in Finland, probably allows *Y. pseudotuberculosis* to "cold-enrich" to a hazardous level. Additionally, the persistence of *Y. pseudotuberculosis* in carrot processing facilities has been detected during recent Finnish outbreak investigations (Jalava *et al.* 2006, S. Hallanvuo; unpublished observations). This leads to speculation that *Y. pseudotuberculosis* could have a substantial role as an endogenous process contaminant of such product facilities. Further investigations to reveal this role and to learn more about the persistence of *Y. pseudotuberculosis* in such production facilities could be one way to help to prevent outbreaks in the future.

Although *Y. pseudotuberculosis* has been isolated from environmental samples during several recent outbreak investigations in Finland, detection and isolation by culture methods from grated carrots samples representing the epidemiologically implicated lot served to the patients has never succeeded. After implementation of the real-time PCR method targeting part of the *ail* gene specific for *Y. pseudotuberculosis* (Thisted Lambertz *et al.*, 2008a), *Y. pseudotuberculosis* has been detected in samples of grated carrots representing the epidemiologically implicated lot in the two most recent outbreaks. In both cases, real-time PCR analysis suggested small bacterial numbers beyond the limit of detection of the culture method in these samples (approximately 1 to 10^1 cells/25 g of sample) (S. Hallanvuo, unpublished results). The small number of *Y. pseudotuberculosis* bacteria present in the epidemiologically implicated samples of Finnish outbreaks suggests either an uneven distribution of the bacteria in the lots of grated carrots serving as a vehicle for infection or a very small infectious dose for this bacterium.

During 2001, the outbreak-associated serotype in Finland changed from O:3 to O:1. Similarly, the vehicle of infections changed from iceberg lettuce to carrots. Since 2003, five outbreaks of *Y. pseudotuberculosis* O:1 infections associated with grated carrots have been described (Anonymous, 2005a, 2008a; Jalava *et al.*, 2006; Kangas *et al.*, 2008; Rimhanen-Finne *et al.*, 2006; Rimhanen-Finne *et al.*, 2008). The mechanism for the emergenge of serotype O:1 strains harbouring the outbreak genotype in the end of 1990s is unknown. Among *Y. pseudotuberculosis*, the strains capable of causing Far East scarlet-like fever (FESLF) syndrome, first detected in Japan and later on in Far East Russia, have been suggested to slowly migrate further to west probably among wildlife (EFSA, 2007b; Eppinger *et al.*, 2007). A similar migration of outbreak strains from east to west is conceivable in Finland, for example, among rodents or other animal reservoir, and is supported by the high incidence of *Y. pseudotuberculosis* human infections in Russia (Anonymous, 2005c, 2006) compared to the low incidence in Sweden and other Nordic countries.

CONCLUSIONS

The strains of *Y. enterocolitica* that are unserotypeable by commercially available antisera are a very common faecal finding in many clinical laboratories. A significant proportion of unserotypeable and unbiotypeable "*Y. enterocolitica*" strains belonged to *Y. enterocolitica*–like species when the identity was confirmed by sequencing the beginning of the 16S rRNA gene (and ≥ 99% similarity threshold was used for species designation). Thus, identification based on a diagnostic kit like API 20 E and commercial serotyping antisera is inadequate. The group of untypeable *Y. enterocolitica*, identified by a commercial diagnostic kit, hid *Y. bercovieri*, *Y. mollaretii*, and *Y. rohdei* strains. In Finland, most of the clinical isolates of *Y. enterocolitica* belong to biotype 1A, among which strains harbouring O-antigens typical of pathogenic species (for example O:3, O:9, and O:8) are relatively common. This further compromises the diagnosis based on serotyping. Biotyping is a relative simple way of assessing the potential pathogenicity of *Yersinia* strains and should be implemented as a principal typing method over serotyping in routine diagnostic laboratories.

Y. enterocolitica biotype 1A, along with *Y. enterocolitica*–like species, has not yet been clearly demonstrated to cause human disease, but there are suggestions that some of these organisms may cause disease with different mechanisms other than *Y. enterocolitica* strains representing pathogenic biotypes. The prevalence of strains of *Y. enterocolitica*–like species cannot be validly evaluated and more thorough studies about their clinical significance will not be motivated, until *Yersiniae* are reliably identified in routine clinical microbiology laboratories. In the meantime, these organisms, along with non-pathogenic *Y. enterocolitica*, add a source of error to the annual incidence figures of *Y. enterocolitica*.

Comparing the colony morphology through a strereomicroscope turned out to be a better tool for avoiding misidentification than the commercially available biochemical test kit. It was possible to avoid misidentification for all 11 non-*Y. enterocolitica* strains by colony morphology, but only for three strains with API 20 E. Consequently, a simplified phenotypic scheme for differentiation between *Y. enterocolitica* and *Y. enterocolitica*–like species was developed. At its simplest, this differentiation could be achieved by examining the colony morphology in tandem with the tests for esculin, salicin and pyrazinamidase. For further differentiating between *Y. bercovieri* and *Y. mollaretii* isolates, the tests for fucose and sorbose were the most useful biochemical tests. For laboratories that have limited capacity for biotyping, the simplest way to avoid misidentifications would be to compare the colony morphology of a preliminary API 20 E-identified *Y. enterocolitica* strain with the *Y. enterocolitica* reference strains representing at least the most commonly encountered bioserotypes 4/O:3, 2/O:9, and BT1A. This study initiated further studies that have led to a change in identification protocols in Finnish clinical microbiology laboratories.

A novel epidemiological typing method based on the use of a repeated genomic region (YeO:3RS) as a probe was developed for the detection and differentiation between strains of European pathogenic *Y. enterocolitica* bioserotypes 4/O:3, 2/O:5,27, and 2/O:9. The genotyping potential of the YeO:3RS typing method was based on the repetitions of the *orf0.0–orf0.67*genes upstream of the O-antigen gene cluster present in pathogenic *Y. enterocolitica* subspecies *palearctica* strains. YeO:3RS genotyping was able to increase the discrimination in a set of 106 previously PFGE-typed Finnish *Y. enterocolitica* bioserotype 4/O:3 strains among which two main PFGE genotypes had prevailed. Both methods also gave evidence of the existence of two major genomic lineages among *Y. enterocolitica* 4/O:3 strains.

Early recognition of apparently sporadic and geographically dispersed outbreaks of *Y. pseudotuberculosis* infections was dependent on notifications from clinical laboratories and active laboratory-based surveillance using serotyping and PFGE subtype analysis. It was shown that the ongoing laboratory-based surveillance played a key role in linking the geographically dispersed and apparently unrelated cases as parts of the same outbreak. Also, to our knowledge, this was the first study to epidemiologically link an outbreak of human illnesses to a specific food item serving as a vehicle for *Y. pseudotuberculosis* infection in humans.

Globally, the importance of fresh produce as a vehicle of foodborne illness has greatly increased during the past decade. During 1997– 2008, *Y. pseudotuberculosis* has caused 10 outbreaks of human infections in Finland, with approximately 500 microbiologically confirmed cases representing only a proportion of the disease burden. During the study period, the serotype responsible for the outbreaks changed from O:3 to O:1. Before the year 2001, the strains of serotype O:3 were responsible for the outbreaks and iceberg lettuce was identified as a vehicle. During 2001, the strains of serotypes O:3 and O:1 prevailed as outbreak causing types. Since 2001, one genotype of serotype O:1 has been solely responsible for outbreaks and carrots have been repeatedly identified as the source.

A genotypic study by PFGE revealed that outbreaks of *Y. pseudotuberculosis* infections of a certain serotype were caused by closely related strains. In fact, one serotype O:3 clone and one serotype O:1 clone could have been responsible for all of the outbreaks described. On the other hand, evidence of a higher diversity of genotypes among strains outside the outbreak clusters was obtained. Outbreak genotypes were present among sporadic strains before the outbreaks occurred. Transformation from a "sporadic" strain to a strain associated with outbreaks may have occurred randomly during suitable cold autumn wheather prevailing before harvesting the contaminated lettuce in this study. The cold wheather may have provided a selective advantage for cold-adapted *Y. pseudotuberculosis* to multiply to the levels needed for the outbreak to occur.

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