Epidemiology of *Yersinia enterocolitica* on pig farms

Sonja Virtanen

ACADEMIC DISSERTATION

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Abstract

*Yersinia enterocolitica* is a foodborne zoonotic pathogen. Among domestic animals, pigs are considered the major reservoir of *Y. enterocolitica* bioivar 4/O:3. The pathogen is found in pig carcasses and pluck sets at slaughterhouses. The contamination of carcasses can be reduced with hygiene measures, such as removing the head with tonsils and bagging the rectum, but these measures cannot completely prevent contamination. Carcass contamination at the slaughterhouse originates from pigs that are already infected on farms. The contamination of carcasses could be reduced by preventing the occurrence of this pathogen already in primary production. Considerable variation exists in the prevalence of *Y. enterocolitica* between different pig farms. The variation partly arises from different conditions and practices in farm management. The aim of this study was to determine the factors in farm management that can be used to prevent the presence and spread of this pathogen within and between pig farms.

Genotyping of strains is needed in outbreak investigations and in epidemiological studies to trace the spread of pathogens. Pulsed-field gel electrophoresis (PFGE) has long been considered a ‘gold standard’ method for genotyping *Y. enterocolitica*. The method has, however, limited discriminatory capacity, is time-consuming, and the results are difficult to compare between laboratories. Thus, more discriminating and sophisticated methods are needed. Multiple-locus variable-number tandem repeat analysis (MLVA) has been developed and used for *Y. enterocolitica* strains of human origin and has proved to have high discriminatory power. This genotyping method was used here to investigate its discriminatory ability, advantages and limitations, and use in genotyping *Y. enterocolitica* strains isolated from pigs.

Among *Y. enterocolitica* 4/O:3 strains that originated from humans, pigs, and pork products from four European countries, the use of MLVA with six VNTR loci (V2A, V4, V5, V6, V7, and V9) was found to have high discriminatory power. Similar MLVA types were detected among humans and pigs, human clinical isolates from limited geographical locations indicating the presence of past unidentified epidemics and also from pigs that originated from the same farms. MLVA proved to be able to detect farm-specific genotypes, but variation in loci V2A, V5, V6, and V7 was common in strains originating from the same farms.

Sampling of the farms revealed the spread of similar MLVA types among farms that had previously transported pigs between each other. Pigs were found to be a major source of transmission of this pathogen between all production types, including farrowing, farrow-to-finish, and fattening farms. Two units of a fattening farm were additionally sampled at two-week intervals to monitor the spread of *Y. enterocolitica* within the fattening units. Piglets from certain breeding farms served as a major source of infection for fattening pigs. These piglets carried farrowing farm-specific MLVA types of *Y. enterocolitica* to the fattening farm, and the infection spread throughout the fattening unit. Finally, by the time of slaughter, 28% of pigs were shedding the pathogen in feces, and antibodies against *Yersinia* were found in 88% of pigs.

Farm management practices and their association with tonsillar carriage and fecal shedding of *Y. enterocolitica* in pigs were studied by a purpose-designed questionnaire for farms whose pigs were previously sampled at slaughterhouses for the presence of this pathogen in tonsil and fecal samples. The use of municipal water, organic production, and purchase of feed...
from a certain feed company were found to be protective factors against the carriage of \textit{Y. enterocolitica}. In contrast, snout-to-snout contacts between pens and buying feed from another company were discovered as risk factors for fecal shedding of the pathogen. In total, 30 farms were further visited and sampled for enteropathogenic \textit{Yersinia}, and the management practices and conditions were recorded during each sampling visit. The use of municipal water, the use of an all-in all-out system in the units of weaned piglets and fattening pigs, buying piglets from no more than one supplier at a time, and generous use of bedding were associated with lower prevalence of \textit{Y. enterocolitica} on farms.
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7 Conclusions
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List of original publications

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


III Virtanen S., Nikunen S., and Korkeala H. The introduction of infected animals to herds is an important route for the spread of *Yersinia enterocolitica* infection on pig farms. *Journal of Food Protection* 2014, 77:116-121.


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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AIAO</td>
<td>All-in all-out</td>
</tr>
<tr>
<td>ail</td>
<td>Gene coding for attachment invasin locus</td>
</tr>
<tr>
<td>CAY</td>
<td>CHROMagar Yersinia</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIN</td>
<td>Cefsulodin-irgasan-novobiosin agar</td>
</tr>
<tr>
<td>DFEH</td>
<td>Department of Food and Environmental Hygiene</td>
</tr>
<tr>
<td>DI</td>
<td>Discriminatory index</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HPI</td>
<td>High pathogenicity island</td>
</tr>
<tr>
<td>inv</td>
<td>Gene coding for invasion</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>ITC</td>
<td>Irgasan-ticarcillin-potassium chlorate</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LRT</td>
<td>Likelihood ratio test</td>
</tr>
<tr>
<td>MAC</td>
<td>MacConkey agar</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multiple-locus variable-number tandem repeat analysis</td>
</tr>
<tr>
<td>MRB</td>
<td>Modified Rappaport broth</td>
</tr>
<tr>
<td>NCFA</td>
<td>Nordic Committee on Food Analysis</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PMB</td>
<td>Peptone-mannitol-bile salts broth</td>
</tr>
<tr>
<td>PSB</td>
<td>Peptone-sorbitol-bile salts broth</td>
</tr>
<tr>
<td>pYV</td>
<td>Plasmid for <em>Yersinia</em> virulence</td>
</tr>
<tr>
<td>REAP</td>
<td>Restriction endonuclease analysis of plasmid</td>
</tr>
<tr>
<td>rho</td>
<td>Within-farm correlation</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SSDC</td>
<td>Salmonella-Shigella deoxycholate calcium agar</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
</tr>
<tr>
<td>VYE</td>
<td>Virulent <em>Yersinia enterocolitica</em> agar</td>
</tr>
<tr>
<td>YeCM</td>
<td><em>Yersinia enterocolitica</em> chromogenic medium</td>
</tr>
<tr>
<td>YER/BOS</td>
<td>Yeast extract rose Bengal-bile oxalate sorbose</td>
</tr>
<tr>
<td>Yop</td>
<td>Yersinia outer protein</td>
</tr>
<tr>
<td>Ysa</td>
<td>Yersinia secretion apparatus</td>
</tr>
<tr>
<td>Ysp</td>
<td>Yersinia secreted protein</td>
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</table>
1 Introduction

*Yersinia enterocolitica* is a foodborne pathogen that causes yersiniosis, which is the third most commonly reported bacterial zoonosis in the European Union (39). Clinical symptoms include gastroenteritis with diarrhea and abdominal pain (51). Most cases caused by *Y. enterocolitica* are considered sporadic. The consumption of undercooked pork has been linked to many yersiniosis cases according to epidemiological investigations (17, 145, 186). Similar *Y. enterocolitica* genotypes obtained from samples of human and pig origin further indicate that pigs and pork are important sources of infection for humans (53, 56, 105).

Pig carcasses are contaminated with *Y. enterocolitica* at slaughterhouses by contaminated tonsils and intestines (103). The origin of *Y. enterocolitica* infection of pigs has been traced back to their farms of origin. However, considerable variation exists in the prevalence of *Y. enterocolitica* on different pig farms (46, 103, 143, 144). Thus far, the origin of *Y. enterocolitica* infection on pig farms has remained unidentified. Contaminated pen floors have been suggested to be the main source of spread of infection (61). Several possible environmental sources have been investigated, but no obvious source has emerged (151).

A few previous studies have clarified the association of farm management practices with prevalence of *Y. enterocolitica* on farms. Close pig-to-pig contacts, absence of coarse feed and bedding, use of straw bedding, drinking from a nipple, high production capacity, wet feeding, access of pest animals to the piggery, and presence of a cat with kittens on the farm have been associated with a high prevalence of pathogenic *Y. enterocolitica* (103, 175). In contrast, organic production, low production capacity, farrow-to-finish production, and manual feeding of slaughter pigs have been associated with a low prevalence of *Y. enterocolitica*.

This study was conducted to identify the farm management factors related to the prevalence of *Y. enterocolitica* on pig farms and to explore the origin and spread of infection within farms in order to find ways to prevent *Y. enterocolitica*. The reduction of the presence of *Y. enterocolitica* in pigs will further reduce the level of contamination at slaughterhouses and help to achieve lower levels of *Y. enterocolitica* in pork products.
2 Review of the literature

2.1 Characteristics of Yersinia enterocolitica

2.1.1 Taxonomy
In the class Gammaproteobacteria of the phylum Proteobacteria, the genus Yersinia belongs to the family Enterobacteriaceae and consists of several species (20). Three of them, Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis are known human pathogens and Y. ruckeri causes disease in fish. In humans, Y. enterocolitica and Y. pseudotuberculosis cause gastroenteritis, whereas Y. pestis is a causative agent of plague (20). Other species belonging to the genus Yersinia include Y. aldovae, Y. aleksiciae, Y. bercovieri, Y. entomophaga, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretii, Y. nurnii, Y. pekkanenii, Y. rohdei, and Y. similis (20, 80, 116, 117, 179, 180).

In 1894, French bacteriologist Alexandre Yersin described the type species of the genus Pasteurella, now known as Yersinia pestis (73). Y. enterocolitica was first documented as Flavobacterium pseudomallei in 1934 in USA by McIver and Pike (18). Five years later, Schleifstein and Coleman isolated an unidentified microorganism from enteric contents and proposed the name Bacterium enterocolicum. Genus Yersinia was proposed in 1944 by van Loghem (199). B. enterocoliticum was renamed Pasteurella x in 1963 and placed in the genus Yersinia in 1964 (38). Y. pseudotuberculosis was described in animals in 1883, known by various names, and placed in the genus Yersinia in 1965 (177).

Based on 16S rRNA analysis, Y. enterocolitica is divided into two subspecies, Y. enterocolitica subsp. enterocolitica and Y. enterocolitica subsp. palearctica (133), the former comprising highly pathogenic biotype 1B strains termed North American and the latter European strains with low pathogenic biotypes 2-5 and nonpathogenic biotype 1A. By using whole-genome comparison with DNA microarrays, clusters composed of nonpathogenic, low pathogenic, and highly pathogenic clades have also been observed (76).

2.1.2 Virulence factors
Y. enterocolitica infection is usually acquired through oral ingestion of contaminated food or drink. The pathogen survives in gastric acid by producing urease, which increases the pH. After entering the small intestine, Y. enterocolitica penetrates M cells, which overlie Peyer’s patches. The bacteria first bind to the mucus layer covering the epithelial cells (19). Plasmid-encoded outer membrane protein YadA enhances the attachment of bacterial cells to the intestinal brush border. YadA is optimally expressed at 37°C, and thus, if Y. enterocolitica cells are ingested in cold food or drink, they must first adapt to the body temperature of the host. Following attachment to M cells, Y. enterocolitica penetrates the tissue in which it multiplies. YadA is a major determinant of complement killing, promoting adherence and invasion (26, 41, 156). It is the most important serum resistance factor and plays an important role in the binding of Y. enterocolitica to the submucosal intestinal tissue (14, 176). YadA is a trimeric protein that has an oval head domain in the N-terminal, a putative coiled-coil rod, and a membrane anchor domain in the C-terminal (75). The head domain is involved in autoagglutination and binding to host cells. The outer membrane C-terminal translocator domain is capable of transporting specific proteins from the periplasm to the bacterial surface (32). Similar amounts of YadA are produced by serotypes O:3 and O:8 (194). Serotypes O:3, O:8, and O:9 bind C4b-binding protein (C4bp), which is an inhibitor of both the classical and lectin pathways of complement (93). YadA also recruits C3b and iC3b directly, without an active complement cascade or additional serum factors (168). As a result, the formation of the
terminal complement complex is limited and bacterial survival is enhanced. Adherence to host cells is promoted by YadA binding to extracellular matrix components (74) such as collagen and laminin (176). YadA is needed to maximize adhesion (194).

Other important plasmid-coded virulence factors include proteins called Yops (Yersinia outer proteins), which are key determinants of pathogenicity. Two types of Yops have been described; translocator and effector Yops (31). These two types work together to help *Y. enterocolitica* persist in host cells, using type III secretion mechanisms to manipulate host cell function. Effector Yops allow bacteria at the cell surface to deliver effector proteins across the cell membrane into the cytosol.

Invasin, a 92-kDa outer membrane protein, is one of the surface structures of *Y. enterocolitica* cells required for virulence. Invasin is encoded by chromosomal virulence gene *inv* and produced at lower temperatures. Therefore, this virulence factor may be present in high concentrations if *Y. enterocolitica* is derived from cold reservoirs of nature or cold-stored contaminated foods. Invasin promotes the penetration of *Y. enterocolitica* into M cells by attaching to B1 integrins, which are located on eukaryotic cell surfaces. *Inv* gene is present in both invasive and noninvasive strains; in noninvasive strains, it is nonfunctional. Alone, without other virulence factors, invasin is not linked to invasiveness (150). Deletion of the *invA* gene has no effect on host cell binding, but prevents *Y. enterocolitica* O:3 strains from invading epithelial cells independently from growth temperature (194). Co-expression of both adhesins YadA and InvA is needed to permit efficient cell binding and invasion of serotype O:3 strains into host cells. Invasin is necessary to initiate the internalization process. Invasin expression at 37°C is a special feature of serotype O:3 strains, as it is not produced in other previously characterized *Yersinia* strains.

Epithelial cell penetration is also enhanced, although more restrictively, by a 17-kDa surface structure Ail, which is encoded by attachment invasion locus *ail* (18, 38). In addition to YadA, *Y. enterocolitica* uses Ail protein to bind C4bp. The presence of Ail alone is insufficient to protect against complement-mediated killing (14). Ail outer membrane protein facilitates the spread of *Y. enterocolitica* to regional lymph nodes, spleen, and liver. *Ail* gene shows greater host specificity with regard to in vitro cell invasion.

Biotype 1B strains have a chromosomal high pathogenicity island (HPI), which encodes siderophore (yersiniabactin) synthesis and a siderophore reuptake mechanism allowing the pathogen to acquire iron from normally inaccessible sources in the host (147). Yersiniabactin also reduces production of reactive oxygen species (ROS) by innate immune effector cells, resulting in reduced bacterial killing. Interestingly, this characteristic is only present in biotype 1B, even though it is encoded by an integrative and conjugative element that can be horizontally spread among bacteria belonging to the family *Enterobacteriaceae* (24). Biotype 1B strains also contain a chromosomal pathogenicity island called *Yersinia* secretion apparatus (Ysa), which contains a second type III secretion system encoding at least 11 different *Yersinia* secreted proteins (Ysps). Although their role in pathogenesis is incompletely understood, they are thought to be important virulence factors (84, 202).

### 2.1.3 Growth conditions

*Y. enterocolitica* is a psychrotrophic pathogen, being able to grow in a temperature range from 0°C to 44°C (51). The optimal growth temperature is 28-30°C (167). At temperatures below 5°C, the growth is quite slow (64). *Y. enterocolitica* typically tolerates freezing for
long periods and can even withstand repeated freezing and thawing (51). *Yersinia* can be destroyed by pasteurization at 72°C for 15-20 s.

*Y. enterocolitica* is able to grow at a pH of 4 to 10, with the optimum pH being 7.6 (51). While *Y. enterocolitica* is able to grow in a salt concentration of 5%, a salt concentration higher than 7% will prevent growth (159). Tolerance to different salt concentrations depends on the surrounding temperature (51). *Y. enterocolitica* is a facultatively anaerobic bacterium, being able to grow in both aerobic and anaerobic conditions and also in modified atmospheres. However, increasing levels of carbon dioxide (CO₂) will lengthen the lag phase and retard the growth (152).

*Y. enterocolitica* grows well in most enteric media (18); however, on Salmonella-Shigella (SSDC) agar, the growth is poor. On MacConkey agar (MAC), *Y. enterocolitica* colonies are colorless due to slow fermentation of lactose. After incubation at 37°C, *Y. enterocolitica* colonies are very small and easily hidden under more dominant enteric bacterial colonies in fecal samples. Therefore, to detect *Y. enterocolitica*, fecal samples should be incubated at 25°C (18).

### 2.2 Yersiniosis

#### 2.2.1 Symptoms and post-infectious sequelae

The most frequently encountered clinical manifestation of yersiniosis is acute uncomplicated gastroenteritis (181). Acute enteritis with fever and occasionally bloody, watery diarrhea are the most common symptoms in young children (18, 91). Other symptoms, most often encountered in young adults, include abdominal pain, acute mesenteric lymphadenitis, and terminal ileitis, which can be confused with appendicitis (154). Adults over 25 years of age are more likely to develop serious enteritis, ileitis, and colitis (181). The symptoms can persist from a few days to a couple of weeks. However, asymptomatic carriage of *Y. enterocolitica* in humans is also considered possible (200).

Rarely, septicemia may occur in immunocompromised hosts such as those who have previously received chemotherapy (107). Septicemia caused by *Y. enterocolitica* has also been associated with iron overload (18). In patients with primary hereditary hemochromatosis, the occurrence of secondary hepatic abscesses caused by *Y. enterocolitica* is rare but possible (11). An underlying disorder with diabetes mellitus has also been associated with the occurrence of hepatic *Y. enterocolitica* abscesses. Transfusion of blood contaminated with *Y. enterocolitica* or containing bacterial endotoxin occasionally causes acute septic shock, with a fatality rate as high as 55% (67).

Post-infectious sequelae, such as reactive arthritis, erythema nodosum, Reiter’s syndrome, glomerulonephritis, myocarditis, or uveitis, may occur. Among patients with post-infectious reactive arthritis, HLA-B27 positivity is especially common (18, 51). The joint pain typically develops from one week to one month after infection, most commonly in knees, ankles, and wrists (66, 71). Erythema nodosum appears two weeks after the initial *Y. enterocolitica* infection (212), but can also be a sequel of several other diseases such as streptococcal infections and sarcoidosis (33). Erythema nodosum occurs more commonly in women that in men.
2.2.2 Sporadic infections and outbreaks

Most yersiniosis cases caused by *Y. enterocolitica* are sporadic, even though small outbreaks have also been reported. Sources of infection in outbreaks have included water and contaminated dairy and pork products (1, 88, 192). In Hungary, an outbreak caused by food prepared by stuffing small pieces of boiled chitterling into a coat made of pig stomach has been described (109). Eight people from five different families got sick. Symptoms of children were enteritis with vomiting and diarrhea, fever, and sore throat. In adults, only sore throat, extremital pain, and fever were documented. *Y. enterocolitica* 4/O:3 was isolated from the suspected food.

Outbreak caused by pasteurized milk has been reported in the USA (184). Fever, abdominal pain, and diarrhea were described in 86% of the 172 patients from whom *Y. enterocolitica* agglutinating most strongly with O:13 and O:18 antisera was isolated. Epidemiological investigation revealed pasteurized milk from a certain company as a source, but no *Y. enterocolitica* isolates were confirmed in the sampling. A random survey for households revealed yersiniosis-like symptoms in 8.3% of people who had consumed the same milk at the same time. Most cases with enteritis symptoms were reported in children aged less than five years. Extraintestinal infections, such as pharyngitis, sepsis, wound infection, and urinary tract infection, were more common in adults. The most common symptoms were fever, abdominal pain, and diarrhea. Vomiting, sore throat, rash, bloody stools, and joint pain were also reported. Marked leukocytosis was observed in many of the patients.

An outbreak of 50 cases in the Washington state, USA, was caused by contaminated soybean curd from a certain plant (183). *Y. enterocolitica* serotype O:8 was isolated not only from the patients and the tofu but also from the untreated spring water of the plant, which seemed to be the main source of contamination. Enteric infections were reported in young children, with a median age of three years. In young adults (median age 28 years), extraintestinal infections were predominant. Some clinical or laboratory evidence of secondary spread among family members who did not eat tofu was also observed. Contaminated well water also caused a small family outbreak of *Y. enterocolitica* bioserotype 4/O:3 in the USA (192). *Y. enterocolitica* was isolated from two patients and the well. Previously, heavy rains may have had contributed to the surface water runoff to the well.

In Japan, in a follow-up in 1973-1985, most yersiniosis cases (98%) were caused by *Y. enterocolitica* serotype O:3 (110). During this time period yersiniosis accounted for only about 0.5% of all reported diarrhea cases in adults and 0.9% of diarrhea cases in children. Thus, the incidence of yersiniosis was almost two times higher in children. Because *Y. enterocolitica* infection does not always cause diarrhea, the true incidence was suspected to be two to three times higher. Most cases were considered sporadic. Outbreaks were mainly observed in primary schools. Besides milk in one outbreak, the original source of infection remained unknown.

Yersiniosis can also be caused by *Y. pseudotuberculosis*. In contrast to yersiniosis caused by *Y. enterocolitica*, these cases are most often reported as outbreaks, especially in Finland and Japan (86, 141, 158, 193). In Finnish outbreaks, the sources have included vegetables linked to institutional kitchens. In Japanese outbreaks, the sources have included water and barbequed food, but in many cases the vehicle of infection was unknown (81, 193).
2.2.3 Yersiniosis in different countries

In the EU, yersiniosis is the third most commonly reported bacterial zoonosis (40) after salmonellosis and campylobacteriosis. In the EU, 1.58 cases per 100 000 population were reported in 2010 (40). Of all reported yersiniosis cases, 91% were caused by *Y. enterocolitica*, 1.7% by *Y. pseudotuberculosis*, and the remaining cases were caused by unspecified species. Of the reported *Y. enterocolitica* cases, 83% were caused by bioserotype 4/O:3 and 15% by bioserotype 2/O:9. Most cases occurred in patients less than 14 years of age. No deaths due to yersiniosis were reported in 2010. The overall yersiniosis incidence rates have been decreasing in the EU since 2006. The highest reported within-country incidences have been observed in Lithuania and in Finland, where the annual incidences were 12.9 and 9.8 per 100 000 population, respectively, followed by Luxembourg (7.0), Estonia and the Czech Republic (4.3), Germany (4.1), and Denmark (3.5).

In Finland, the number of annually reported *Y. enterocolitica* cases has stayed relatively stable, varying from approximately 400 to 600 cases in 2001-2011 (Figure 1), corresponding to an annual incidence of 8-10 cases per 100 000 inhabitants (77, 78, 85, 120-124). More variation is noted in the number of cases caused by *Y. pseudotuberculosis*, due to a varying number of outbreaks in different years. Geographical variation is detected in yersiniosis cases within the country. Recently, *Y. enterocolitica* infection has been reported to occur most often in people aged 45-54 years and over 75 years. However, *Y. enterocolitica* strains isolated from patients older than 75 years have been considered apathogenic, whereas pathogenic strains have been overrepresented in patients under two years of age. Serotype O:9 infections have been on the rise in recent years in both Finland and elsewhere (108, 115, 174).

![Figure 1](image_url)

Figure 1. Annual number of yersiniosis cases caused by *Y. enterocolitica* and *Y. pseudotuberculosis* reported to the National Institute for Health and Welfare in Finland in 2001-2011.

In the USA, 96400 yersiniosis cases were reported in 1996-1997 (112). Foodborne transmission is considered the vehicle in 90% of cases. The hospitalization rate has been 24% and case-fatality rate 0.5%. The total number of cases is estimated to be 38 times higher than the number of reported cases because most yersiniosis cases are self-limiting and people visit doctors only in more severe cases.
2.2.4 Sources of sporadic infections
In many sporadic yersiniosis cases, the source of infection could not be traced back to a particular item (18). In epidemiological case-control studies, consumption of food prepared from undercooked pork products has been a major risk factor for yersiniosis (17, 65, 145, 160, 186). In addition, exposure during the preparation of chitterlings, which are prepared by cleaning and boiling the intestines of pigs, has been a constitutional risk factor for yersiniosis (88, 104). Indistinguishable genotypes of Y. enterocolitica have been found in samples of human and pig origin (47, 56), indicating that pigs are an important source of sporadic yersiniosis cases. Y. enterocolitica 4/O:3 has also been found in foodstuffs such as edible pig offal and raw pork (49).

The high prevalence of Y. enterocolitica in pigs can pose a risk of infection for people in contact with the animals. Transmission from pig to man has been described on a pig farm (154). Elevated antibodies against Yersinia have been reported in pig farmers, butchers, and slaughterhouse employees (113, 154, 169).

2.3 Isolation of Y. enterocolitica
2.3.1 Enrichment and selective media
Isolation of Y. enterocolitica from samples is often based on combining both enrichment and selective plating. Enrichment in liquid media is typically used in isolation of Y. enterocolitica from samples (51). The most commonly used selective enrichment medium is irgasan-ticarcillin-potassium chlorate broth (ITC), which is especially productive for the recovery of bioserotype 4/O:3 strains (51). By reducing the concentration of chlorate, MgCl₂, and malachite green, the growth of bioserotype 2/O:9 can be improved (36). Compared with enrichment in yeast extract rose bengal-bile oxalate sorbose (YER/BOS), ITC has been found to be superior in detecting Y. enterocolitica 4/O:3 and 2/O:9 strains (98). By using YER/BOS, other Y. enterocolitica and Yersinia species were detected. ITC enrichment is typically used for food samples (82).

A phosphate-buffered saline with peptone, sorbitol, and bile salts (PSB) is a less selective enrichment medium than ITC, but is often used for food, water, and environmental samples. Sorbitol can be replaced with 1% mannitol (PMB) (135). These enrichment media are typically used for cold enrichment. Cold enrichment from one to three weeks has proved efficient in isolating pathogenic Y. enterocolitica from pig fecal samples, carcass swabs, oral cavities and tonsils (101, 125, 131, 140, 144, 196) and also from human fecal samples (94).

As Y. enterocolitica tolerates an alkaline environment, treatment with potassium hydroxide (KOH) can be used to reduce other microbial populations after cold enrichment (165). A 20-s treatment in 0.25% KOH has been used for fecal and tonsil samples after 14 days of cold treatment (101, 142, 144). From naturally and artificially contaminated food samples, the treatment with 0.5% KOH was found to increase the yield of Yersinia spp. fourfold and the sensitivity up to 100-fold (8).

Selective culture media for the detection of Y. enterocolitica have been developed. Of these, cefsulodin-irgasan-novobiosin (CIN) agar developed by Schiemann et al. (163) is one of the most commonly used. CIN agar contains cefsulodin, irgasan, and novobiosin as selective antimicrobials and is selective against Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis, but not Serratia. Mannitol fermentation permits the discrimination of Y. enterocolitica from several other Gram-negative bacteria. From fecal
samples using direct plating or cold enrichment, a higher recovery of \textit{Y. enterocolitica} has been obtained on CIN agars than on Salmonella-Shigella (SSDC) agar or MacConkey agar (MAC) because of a reduction of the level of background microbial organisms. Colonies resembling \textit{Y. enterocolitica} but deemed not to be this pathogen have commonly been identified as \textit{Citrobacter} (101, 163). \textit{Y. enterocolitica} grows on CIN as colonies that have a deep red center with a sharp border and are surrounded by an outer translucent zone. Pigmentation as a result of mannitol fermentation is stronger and more complete when incubated at 22°C for 48 h than at 32°C for 24 h. The edge of the colony may be intact or irregular depending on the strain (163).

MacConkey agar (MAC) is one of the most widely used traditional enteric media for isolation of pathogenic \textit{Yersinia} (51). On MAC, \textit{Y. enterocolitica} forms small, flat, and colorless or pale pink lactose-negative colonies that are 1 to 2 mm in diameter. \textit{Y. pseudotuberculosis} has been detected by using modified MacConkey containing 1% sorbitol (172). In this medium, \textit{Y. pseudotuberculosis} colonies are easily detected as they appear colorless, and thus, are easier to distinguish from other \textit{Yersinia} species.

Fukushima et al. (59) developed virulent \textit{Yersinia enterocolitica} (VYE) agar for detection of virulent \textit{Y. enterocolitica} by adding esculin and ferric citrate to CIN. Black esculin hydrolysis produced by non-\textit{Yersinia} colonies was, however, noted to mask potentially virulent esculin-negative \textit{Y. enterocolitica} (209).

Two chromogenic media have been developed for the detection of \textit{Y. enterocolitica}. \textit{Y. enterocolitica} chromogenic medium (YeCM) was published by Weagant (209) for isolation of potentially virulent \textit{Y. enterocolitica}. The agar contained cellobiose as a fermentable sugar, a chromogenic substrate, and selective inhibitors to suppress several competing bacteria. Strains of potentially virulent \textit{Y. enterocolitica} biotypes 1B and 2-5 formed red bulls-eye colonies on YeCM that were similar to those described for CIN agar. However, \textit{Y. enterocolitica} biotype 1A and other apathogenic \textit{Yersinia} species that formed typical red bulls-eye colonies on CIN agar grew as blue or purple colonies on YeCM. Additionally, biotype 1B colonies were easily distinguished from other strains on YeCM, but not on CIN.

A chromogenic medium CHROMagar Yersinia (CAY) has recently been developed for the detection of \textit{Y. enterocolitica} in stool samples (157). In total, 1494 samples were tested from hospitalized human patients and CAY was found to be as sensitive as the reference medium CIN. However, CAY was significantly more specific, with a low false-positive rate; the specificity using CAY was 99\%, compared with 90\% using CIN. Pathogenic \textit{Y. enterocolitica} grew as colorless colonies after a 24-h incubation and as purple colonies with a diameter of 1-2 mm after a 48-h incubation. In contrast, nonpathogenic \textit{Y. enterocolitica} grew as metallic blue colonies, 1-3 mm in diameter after 24- and 48-h incubations. The growth of \textit{Y. pseudotuberculosis}, \textit{Y. kristensenii}, \textit{Y. aldovae}, \textit{Y. intermedia}, and \textit{Y. mollaretii} was inhibited on CAY. \textit{Y. frederiksenii} grew as metallic blue colonies resembling apathogenic \textit{Y. enterocolitica}. The only detected confounding pathogen was \textit{Y. bercovieri}, which had a growth similar to \textit{Y. enterocolitica}. \textit{Citrobacter} spp., which commonly give false-positive results in CIN (163), were clearly distinguished from pathogenic \textit{Y. enterocolitica} in CAY. This method has also been found to be suitable for rapid detection of \textit{Y. enterocolitica} in pig tonsils (37).
2.3.2 Isolation from porcine samples
Tonsils are considered one of the most frequent sites for *Y. enterocolitica*. Histologically, in tonsils, *Y. enterocolitica* causes mild inflammation with mononuclear infiltration and epithelial cell destruction around the crypts (172). *Y. enterocolitica* cells are observed at the epithelium of tonsillar crypts, which are often filled with degenerated lymphocytes, cellular debris, and numerous other Gram-negative bacteria.

Because of the low number of pathogenic *Y. enterocolitica* and the high number of other bacteria in food and environmental samples, the detection of *Y. enterocolitica* by direct plating, even on selective media, can be difficult (51). Traditional culture methods lack sensitivity in detecting enteropathogenic *Yersinia*. In the studies of pork products, pig tonsils, and pig feces, the detection rate of *Y. enterocolitica* by real-time PCR is higher than by using conventional culture methods (22, 50, 57). By using the culture method, *Y. enterocolitica* could be isolated from 4% of pig fecal samples, whereas the detection rate using fluorogenic PCR was 12% (13). In samples from pig tonsils and raw pork, 88% and 7%, respectively, were positive using real-time PCR, whereas the corresponding detection rates were only 35% and 0% using culture methods (48).

In Japan, *Y. enterocolitica* has been detected in 24% of tonsil samples from pigs (172). Bacterial counts were highest in tonsils, where $10^5$ to $10^6$ *Y. enterocolitica* cells were found in one gram of sample. Of cecal contents, 24% were positive for *Y. enterocolitica*, with $10^2$ to $10^5$ cells per gram detected. In total, 85% of oral cavity swabs and 36% of masseter muscles were positive for *Y. enterocolitica*, and the bacterial counts were $10^2$ to $10^3$ per gram of sample (172).

Combined samples of tonsil and tongue tissue have yielded higher isolation rates of *Y. enterocolitica* than either alone. More positive isolation findings have been gained from destructive tissue samples than from superficial swabs (125). The highest number of positive samples has been detected with three weeks’ cold enrichment in PSB, followed by plating onto CIN, but three weeks’ enrichment is time-consuming and relatively slow in urgent cases such as epidemics. A combination of direct plating onto CIN or eight days’ cold pre-enrichment in PSB, followed by four days’ enrichment in modified Rappaport broth (MRB) at room temperature and plating onto CIN is preferred to increase the sensitivity of the culture. In comparison of two isolation methods, PBS cold enrichment at 4°C for 21 days, followed by MRB enrichment at room temperature for two days and plating onto MacConkey agars that were incubated at room temperature for two days yielded higher number of positives from processed than from raw pork products (164). More *Y. enterocolitica* was detected from raw pork by enrichment at MRB at room temperature for seven days.

In comparison of four different isolation methods: International Organization for Standardization (ISO) (82), modified ISO, modified method of Nordic Committee on Food Analysis (NCFA) (137), and the isolation method of the Department of Food and Environmental Hygiene (DFEH) for pig fecal samples, the DFEH method proved to have the highest sensitivity, 78%, in detection of pathogenic *Y. enterocolitica* (Figure 2) (101). The sensitivity of the ISO method for fecal samples was only 38%, modified ISO 43%, and modified NCFA 69%. Most of the false-positive colonies were detected as *Citrobacter* spp. and other nonpathogenic *Yersinia*.
Figure 2. Comparison of different isolation protocols for detection of *Y. enterocolitica* from pig fecal samples (101).

In a vast comparison of isolation methods for the detection of *Y. enterocolitica* from swabs and destructive samples from tonsils of 120 pigs, direct plating and enrichment both in ITC and PSB at 25°C for 48 h were used (197). The samples were plated onto SSDC, CIN, and YeCM agars. Overall, 55 (45.8%) of the tonsils were positive for *Y. enterocolitica* bioserotype 4/O:3. Destructive samples yielded significantly higher recovery. Alkali treatment was found to increase the recovery of *Y. enterocolitica* from both ITC and PSB enrichment of destructive samples. No differences were observed between the performances of the agars. In the study of Van Damme et al. (198), direct plating was found to be efficient in isolation of *Y. enterocolitica* from pig tonsils. Reducing the enrichment time in PSB to two days was found to increase the efficiency from enrichment compared with five days. In a recent study, cold enrichment in PMB for 14 days and the use of alkali treatment with 0.25% KOH was more efficient in isolating *Y. enterocolitica* from tonsil, fecal, and carcass swab samples than direct plating and seven days’ cold enrichment in PMB (196).

In the study of Schiemann et al. (164), 7% of processed pork and 49% of raw pork products tested positive for *Y. enterocolitica* by using MRB enrichment at 22°C for seven days and
parallel enrichment in phosphate-buffered saline (PBS) at 4°C for 21 days, followed by 1:10 dilution and enrichment in MRB at 22°C for two days. Both enrichments were plated onto MAC and incubated at 22°C for two days. Mainly serotype O:3 was found from pork tongues and serotype O:5 from different raw pork products. Many of the isolates were nontypable.

2.4 Characterization of Y. enterocolitica

2.4.1 Serotyping

Y. enterocolitica-like species were originally divided into 54 different serotypes according to the O antigens by Wauters et al. (5). Aleksic et al. (4) further divided Y. enterocolitica into 117 different serovars according to flagellar H antigens. Pathogenic strains belong to serotypes O:1, 2, 3; O:2,3; O:3; O:4,32; O:5,27; O:8; O:9; O:13a,13b; O:18; O:20; and O:21 (159, 208). Serotype O:3 is most commonly detected in pigs and is often associated with human disease (19, 51). However, serotype O:9 infections have been on the rise in recent years both in Europe and in Japan (108, 174). Serotype O:8 is considered a North American type and is highly pathogenic (171).

2.4.2 Biotyping

A biotyping scheme has been developed for Y. enterocolitica by Wauters et al. (208) (Table 1). Most environmental Y. enterocolitica strains that are considered apathogenic are esculin- and salicin-positive within one day, but delayed reactions with esculin and salicin are seen in all strains, even pathogenic ones, within 2-10 days. A strong association has been found between apathogenic strains and lack of pyrazinamidase activity (208). This was only seen in environmental strains of biotype 1A and strains of biogroup 3A-3B, nowadays known as Y. mollaretii and Y. bercovieri (207). Biotype 1B strains also have β-D-glucosidase activity and strains of Y. mollaretii and Y. bercovieri have a strong reaction for proline-peptidase enzyme.

Table 1. Revised biotyping scheme of Y. enterocolitica by Wauters et al. (208).

<table>
<thead>
<tr>
<th>Biotypes</th>
<th>1A</th>
<th>1B</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Esculin/salinic 24 h</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-D-glucosidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Proline peptidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Biotype 1B strains mainly belong to serotype O:8. Biotype 2 only includes serotypes O:9 and O:5,27. Bioserotype 4/O:3 is most common in human illness (111, 161), but 1B/O:8, 3/O:3, 2/O:9, and 2/O:5,27 have also been documented (60). Biotype 1A strains have traditionally been considered apathogenic, but signs of possible virulence, such as the presence of virulence gene ail, has been documented in these strains (25, 173, 187, 188). Biotype 1A strains may be considered opportunistic pathogens since these strains are often isolated from patients with gastroenteritis (51, 78).
2.4.3 Genotyping

Subtyping of *Y. enterocolitica* according to biochemical properties has only a low discriminatory power. Therefore, DNA-based molecular methods are needed. Of these, pulsed-field gel electrophoresis (PFGE) has been considered a ‘gold standard’ for genotyping *Y. enterocolitica* (55, 118). The method is based on cutting the genomic DNA by a rare-cutting restriction enzyme and the obtained DNA fragments, representing the whole bacterial DNA, are separated on an agarose gel by pulsed electrophoresis. The result is an image on a gel, which makes comparison of results difficult between different laboratories, despite computerized gel scanning and creation of data banks of PFGE patterns (55). One of the most often used restriction enzymes for *Y. enterocolitica* has been *NotI*, which results in a discriminatory capacity of 74%. By using three enzymes, the discriminatory index can be increased up to 94% (44). Compared with ribotyping and restriction endonuclease analysis of plasmid (REAP), PFGE has been found to be the most suitable for epidemiological tracing of *Y. enterocolitica* (83).

Amplified fragment length polymorphism (AFLP) has been used to discriminate several bacterial species (205). AFLP is also based on cutting the DNA of the studied organism with the use of restriction enzymes. Usually, two restriction enzymes, a rare cutter and a frequent cutter, are used. Specific adapters are designed to attach to the digested DNA fragments, and the fragments with ligated adapters are multiplied in two PCR reactions. The first pre-selective PCR reaction is targeted to exponentially amplify the fragments, and the second selective PCR run selectively amplifies the target fragments. Primer sequences with a fluorescent label and an additional nucleotide added to the end are typically used in selective amplification. The fragments are traditionally separated in denaturing gel electrophoresis, or in a more sophisticated approach, by capillary gel electrophoresis, where the results are gained electronically and can be analyzed with computers and easily compared between different laboratories. An application of AFLP has been developed for *Y. enterocolitica* (42, 97), but the principle of this application differs from the general AFLP protocol presented above. Only a single adapter and one PCR run are used. The method has, however, been able to discriminate the studied strains according to their serotype, and different *Yersinia* species are divided separately.

Multiple-locus variable-number tandem repeat analysis (MLVA) is a modern genotyping method developed for several bacterial species (106, 195). The method is based on finding variable-number tandem repeats (VNTR) in the genome and multiplying these fragments in a PCR reaction. Different genotypes are distinguished by the number of repeats in each locus (195). In 2007, the first application of MLVA was published for *Y. enterocolitica* 4/O:3 (62). The application uses six VNTR regions (V2A, V4, V5, V6, V7, and V9). The method has been found to be applicable in detecting both sporadic and outbreak-related strains (174). The discriminatory ability is superior to that of PFGE, being as high as 0.999. A separate MLVA application has also been developed for *Y. enterocolitica* biotype 1A (68).

2.5 Reservoirs of *Y. enterocolitica*

2.5.1 *Y. enterocolitica* in pigs and on farms

Among domestic animals, pigs are considered the major reservoir of *Y. enterocolitica* 4/O:3 (23, 92). Pigs are thought to be asymptomatic carriers of this pathogen since no signs of illness have been documented. When colonization in piglets was tested with several serotypes, colostrum-deprived piglets born by Cesarean section were more colonized with *Y.
*Enterococlitica* than those born normally and with colostrum; however, none of the piglets developed signs of illness (166).

In Europe, bioserotype 4/O:3 predominates in pigs in many countries such as Belgium (91%), Italy (99%), Spain (100%), Russia (100%), Estonia (100%), and Latvia (100%) (142, 143). Bioserotypes 3/O:9 and 2/O:5 are only rarely found in Belgian (9%) and Italian (1%) pigs (142). Compared with other European countries, a higher variety of *Y. enterocolitica* bioserotypes is detected in pigs in England, where bioserotypes 2/O:9 (33%) and 2/O:5 (26%) are the most commonly found (144). In the USA, among *Y. enterocolitica* strains from conventional and antimicrobial-free production systems, 43% belonged to serotype O:3, 26% to serotype O:5, and 4% to serotype O:9 (185). Only 13% of these strains were *yadA*-positive and 40% were *ail*-positive.

To determine the presence of *Y. enterocolitica* in pigs in China, 8773 samples were evaluated from 11 provinces between 2009 and 2011 (105). In total, 20% of the 4495 oropharyngeal swabs, 8% of the 1239 intestinal contents, and 5% of the 3039 fecal samples from slaughterhouse pens tested positive for *Y. enterocolitica*. Most of the isolates were of bioserotype 3/O:3. In addition, a few bioserotype 4/O:3 and 2/O:9 strains were detected. In most provinces, pathogenic strains were found, but in one province, mainly biotype 1A was detected. PFGE divided these strains into 49 genotypes.

In a study in Sao Paulo, Brazil, *Y. enterocolitica* bioserotype 4/O:3 was found in 30% of pig samples collected at two slaughterhouses, but in none of the pork samples collected from markets (148). Only two of the 120 slaughterhouse environment samples tested positive for *Y. enterocolitica* 4/O:3. Instead, biotype 1A was frequently found in slaughterhouses and markets. The presence of virulence genes in 122 biotype 1A strains isolated from pig slaughterhouses and meat markets were characterized. A total of 94 strains (77%) were positive for at least one of the virulence genes *ail*, *virF*, or *ystA* (149). Twenty-two biotype 1A strains were submitted to PFGE genotyping, resulting in 22 distinct pulsotypes, with genetic similarity ranging from 50% to 84%.

Variation exists in the prevalence of enteropathogenic *Yersinia* in pigs and pig farms in different countries (Table 2). However, since in some countries, the number of sampled animals and farms is low and the sampling frames and isolation methods differ between studies, the figures presented in Table 3 do not represent true national prevalences, but show how widespread *Y. enterocolitica* is in pigs in several countries. The use of various isolation methods in different studies obviously has an impact on the reported prevalences. In addition, the sampling site of the pig has a major effect on the results. Tonsils are the most reliable site of sampling (46, 129), and therefore, only culture results obtained from tonsil samples are included in Table 2.
Table 2. Prevalence of pathogenic *Y. enterocolitica* in tonsil samples of pigs in different countries using culture.

<table>
<thead>
<tr>
<th>Country</th>
<th>Pigs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Farms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>37% (516/1737)</td>
<td>75% (54/76)</td>
<td>(142, 198, 201)</td>
</tr>
<tr>
<td>Brazil</td>
<td>30% (144/480)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(148)</td>
</tr>
<tr>
<td>Canada</td>
<td>24% (162/888)</td>
<td>ND</td>
<td>(72, 146, 190)</td>
</tr>
<tr>
<td>China</td>
<td>13% (1132/8773)</td>
<td>ND</td>
<td>(105)</td>
</tr>
<tr>
<td>Denmark</td>
<td>25% (554/2218)</td>
<td>82% (81/99)</td>
<td>(6)</td>
</tr>
<tr>
<td>England</td>
<td>44% (278/630)</td>
<td>69% (31/45)</td>
<td>(144)</td>
</tr>
<tr>
<td>Estonia</td>
<td>89% (135/151)</td>
<td>100% (15/15)</td>
<td>(143)</td>
</tr>
<tr>
<td>Finland</td>
<td>37% (68/185)</td>
<td>71% (34/48)</td>
<td>(7, 45)</td>
</tr>
<tr>
<td>France</td>
<td>20% (178/900)</td>
<td>ND</td>
<td>(43)</td>
</tr>
<tr>
<td>Germany</td>
<td>62% (101/164)</td>
<td>84% (16/19)</td>
<td>(23, 46)</td>
</tr>
<tr>
<td>Greece</td>
<td>13% (58/455)</td>
<td>ND</td>
<td>(92)</td>
</tr>
<tr>
<td>Italy</td>
<td>32% (209/684)</td>
<td>72% (34/49)</td>
<td>(16, 35, 142)</td>
</tr>
<tr>
<td>Japan</td>
<td>24% (34/140)</td>
<td>ND</td>
<td>(172)</td>
</tr>
<tr>
<td>Latvia</td>
<td>50% (213/513)</td>
<td>67% (38/52)</td>
<td>(143, 189)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>43% (37/86)</td>
<td>ND</td>
<td>(34)</td>
</tr>
<tr>
<td>Norway</td>
<td>75% (18/24)</td>
<td>100% (3/3)</td>
<td>(128)</td>
</tr>
<tr>
<td>Poland</td>
<td>4% (3/80)</td>
<td>67% (2/3)</td>
<td>(96)</td>
</tr>
<tr>
<td>Russia</td>
<td>34% (66/197)</td>
<td>100% (10/10)</td>
<td>(143)</td>
</tr>
<tr>
<td>Spain</td>
<td>93% (185/200)</td>
<td>100% (14/14)</td>
<td>(142)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>25% (52/212)</td>
<td>ND</td>
<td>(57)</td>
</tr>
<tr>
<td>USA</td>
<td>10% (122/1218)</td>
<td>20% (20/102)</td>
<td>(211)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Prevalence %, (No. of positives/No. of sampled). In case of several studies per country, the median prevalence is reported. <sup>b</sup>ND, no data

*Y. enterocolitica* is present on several pig farms in Europe, as shown in Table 3. Prevalences from 4% to 93% have been found in individual pigs. In addition, regional variation in the prevalence is detected within countries (12, 45, 95, 211). The regional differences could indicate possibilities for control of *Yersinia* in pigs. *Y. enterocolitica* is isolated more frequently during the winter months (213).

*Y. enterocolitica* is seldom found in newborn piglets (21, 210). Piglets shed the organism in feces beginning from 14 weeks of age (69, 129). In an experimental study, antibodies were shown to take at least 12 days to develop after an artificial infection (134). Antibodies have been detected in blood samples of 15-weeks-old piglets (129), indicating that the infection of piglets commences around the age of 13 weeks. This is the typical age that piglets are removed from farrowing farms to fattening units. By the time of slaughter, 66-80% of exposed fattening pigs have antibodies against *Yersinia* (129, 191). In contrast to low fecal prevalence, the occurrence of *Y. enterocolitica* is high in tonsils of finishing pigs at slaughter (58, 191).

The prevalence of *Y. enterocolitica* in tonsils of sows is significantly lower than in fattening pigs at the slaughterhouse (95), possibly due to naturally developed immunity. Sows may still be a source of infection for piglets. In a cross-sectional study in farrow-to-finish farms in the USA, *Y. enterocolitica* was occasionally found in fecal and oral-pharyngeal swabs of pregnant sows, but not in fecal samples of farrowing sows (21). Due to the lack of oral-pharyngeal samples from farrowing sows, the true prevalence of *Y. enterocolitica* may have
been underestimated. However, the low prevalence of *Y. enterocolitica* in farrowing sows and piglets suggests that the former may not be the main source of infection for piglets.

Several studies have investigated factors in pig production and management and their association with within-farm prevalence of pathogenic *Y. enterocolitica* (103, 140, 175, 204). In some of these studies, the within-farm prevalence has been defined by serology (175, 204), whereas in other studies bacteriological cultures have been taken (103, 140). This may be reflected in the results obtained. Serology is a more sensitive method in detection of herds with a history of exposure to *Yersinia*. In a study conducted at a slaughterhouse, *Y. enterocolitica* was isolated from tonsils or intestinal samples of 27% of pigs, whereas antibodies against *Yersinia* were found in 66% of the same pigs (191). However, depending on the method used, antibodies against *Y. enterocolitica* and *Y. pseudotuberculosis* may both give a positive result in serology, not allowing these two pathogens to be distinguished from each other (204).

Several factors in farm management and conditions have been associated with the presence of *Y. enterocolitica* on farms (Tables 3 and 4). Organic production, high production capacity, and contacts with pest animals and the outside environment have been associated with increased prevalence of *Y. pseudotuberculosis* in pigs (102). Interestingly, organic production is considered a protective factor against *Y. enterocolitica* (140). Transportation to slaughter and high production capacity are risk factors for *Y. enterocolitica* (103, 140). Low biosecurity level has been associated with high prevalence of *Y. enterocolitica* on pig farms in Lithuania (139).

Table 3. Factors associated with low prevalence of *Y. enterocolitica* on pig farms.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Farm management factor</th>
<th>Sample material</th>
<th>Sampling place</th>
<th>No. of farms studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>(103)</td>
<td>Organic production, use of coarse feed or bedding</td>
<td>Fecal, tonsil, intestinal samples, pluck set and carcass swabs</td>
<td>Farms, slaughterhouses</td>
<td>15</td>
</tr>
<tr>
<td>(204)</td>
<td>Use of municipal water, fully slatted floor</td>
<td>Blood, antibodies</td>
<td>Farms, slaughterhouses</td>
<td>80</td>
</tr>
<tr>
<td>(175)</td>
<td>Farrow-to-finish production, manual feeding of slaughter pigs, under-pressure ventilation</td>
<td>Blood, antibodies</td>
<td>Slaughterhouse</td>
<td>387</td>
</tr>
<tr>
<td>(140)</td>
<td>Alternative (organic) housing, transportation to slaughterhouse by farmer</td>
<td>Tonsils, cecal contents, lymph nodes</td>
<td>Slaughterhouse</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 4. Factors associated with high prevalence of *Y. enterocolitica* on pig farms.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Farm management factor</th>
<th>Sample material</th>
<th>Sampling place</th>
<th>No. of farms studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>(103)</td>
<td>High production capacity, drinking from a nipple</td>
<td>Fecal, tonsil, intestinal samples, pluck set and carcass swabs</td>
<td>Farms, slaughter-houses</td>
<td>15</td>
</tr>
<tr>
<td>(139)</td>
<td>Low biosecurity level</td>
<td>Feces and carcass swabs</td>
<td>Farms, slaughter-houses</td>
<td>11</td>
</tr>
<tr>
<td>(204)</td>
<td>Low daily weight gain, recurring health problems</td>
<td>Blood, antibodies</td>
<td>Farms, slaughter-houses</td>
<td>80</td>
</tr>
<tr>
<td>(175)</td>
<td>Slaughter pig production, transportation to slaughterhouse by farmer, separation of clean and unclean sections, presence of cat with kittens on a farm, use of straw bedding</td>
<td>Blood, antibodies</td>
<td>Slaughterhouse</td>
<td>387</td>
</tr>
<tr>
<td>(140)</td>
<td>Several pig suppliers Use of commercial feed</td>
<td>Tonsils, cecal contents, lymph nodes</td>
<td>Slaughterhouse</td>
<td>9</td>
</tr>
</tbody>
</table>

Inconsistent findings have emerged in the use and amount of different bedding materials and their association with the presence of *Y. enterocolitica*. In one study, the absence of coarse feed and bedding was related to a higher prevalence of *Y. enterocolitica* (103), whereas the housing of pigs on a fully slatted floor was associated with low within-farm prevalence of *Yersinia* (204). The use of straw as bedding has been found to increase the risk of presence of *Y. enterocolitica* antibodies on farms (175).

Water is considered a possible source of transmission of *Y. enterocolitica* on pig farms (151). The use of municipal water has been found to be a protective factor against *Y. enterocolitica* (204). However, none or only 1% of water samples collected from pig farms have tested positive for *Y. enterocolitica* (29, 151), indicating that water is unlikely to contribute markedly to the transmission of the infection.

The effect of feed on the presence of *Y. enterocolitica* on pig farms has been investigated in several studies. The use of commercial feed has been identified as a risk factor for the presence of *Y. enterocolitica* (140), whereas manual feeding of slaughter pigs has been considered a protective factor (175). The factor ‘manual feeding’ has in fact been related to smaller herd size and farrow-to-finish production, which actually reflect a lower number of contacts between animals from different farms. Open management type and various origins of piglets have been associated with the presence of *Y. enterocolitica* on a farm (29). Farrow-to-finish combined production type is considered a protective factor for the presence of *Y. enterocolitica* within a farm (175).
Contaminated pen floors are suggested a source of infection for piglets (61). However, while several samples from farm environments have been studied, no obvious environmental source of *Y. enterocolitica* has been found (29, 151, 203). Thus, *Y. enterocolitica* may survive poorly in farm environments. The occurrence of *Y. enterocolitica* in the farm environment is mostly considered a consequence of fecal contamination with the pathogen that is carried in the intestines of pigs (151). In Norway, specific pathogen-free herds have been established and successfully maintained free from *Y. enterocolitica* for years by strict biosecurity measures (130).

### 2.5.2 Other sources of *Y. enterocolitica*

Besides pigs, human pathogenic *Y. enterocolitica* can be isolated from cattle, sheep, and goats (63, 111). Serotypes have usually included O:5,27 and O:9. In New Zealand, 60% of goat farms have been discovered to be positive for *Yersinia*, and the majority of isolates have belonged to bioserotype 5/O:2,3 (99). Similarly to pigs, the prevalence of *Y. enterocolitica* in goats declines as their age increases (99). Pathogenic *Y. enterocolitica* was mainly found from young goats below one year of age. Recently, pathogenic *Y. enterocolitica* serotype O:3 has been detected in hunted wild alpine ibex in Switzerland (89). Serotypes O:3 and O:5,27 have been isolated from dogs and cats (54).

Environmental isolates of *Y. enterocolitica* are usually considered nonpathogenic. In Sweden, nonpathogenic *Y. enterocolitica* biotype 1A has been recovered from sheep (178). In rats and mouse-like rodents, *Y. enterocolitica* serotypes other than O:3 have been found (3). In one study, the isolation rate was even higher in rats (35.2%) than in pigs (8.3%), but *Y. enterocolitica* serotypes present in rats included O:6 and O:7, and many isolates were untypable (213). In chicken, *Y. enterocolitica* serotypes other than O:3 and O:9 have been isolated in Greece (92).

*Y. enterocolitica* has been isolated from 8% of bulk tank milk samples from dairy farms in Finland. However, none of the isolates was pathogenic because virulence genes were not found (162). In the USA, likely pathogenic *Y. enterocolitica* was detected in 6% of bulk tank milk samples (87). Furthermore, 30%, 6%, and 4% of raw milk, fermented milk, and raw milk cheese samples, respectively, were contaminated with *Y. enterocolitica*, but most of the isolates belonged to biotype 1A (70). However, two biotype 2 and one biotype 3 isolates were also found in raw milk samples.

### 2.6 Transmission of *Y. enterocolitica* in the food chain

#### 2.6.1 Transmission from farms to slaughterhouses

Contamination of pig carcasses with *Y. enterocolitica* has been shown to originate from pigs infected already on farms (103). Similar genotypes of the pathogen are found in batches of pigs on farms and in the carcasses and pluck sets of the corresponding pigs at slaughterhouses. When fecal samples of the same fattening pigs were collected at farms prior to slaughter, indistinguishable farm-specific genotypes of *Y. enterocolitica* were found using PFGE in the carcasses of the corresponding pigs later at the slaughterhouse (103).

To avoid the transmission of *Y. enterocolitica* infection from one pig to another, only herds that are free of infection should be in contact with each other during transport and they should be slaughtered at the same time (128). Herds that carry *Y. enterocolitica* should be transported to the slaughterhouses and slaughtered separately. Holding the animals overnight...
at the slaughterhouse before slaughtering has been found to be a risk factor for carriage of *Y. enterocolitica* (114).

### 2.6.2 Contamination and reduction of *Y. enterocolitica* at slaughterhouses

Pig carcasses are widely contaminated with *Y. enterocolitica* 4/O:3 at the slaughterhouses. Enteropathogenic strains can be isolated from carcasses, ears, livers, kidneys, and hearts of the pigs (50). Tonsils are considered a major source of carcass contamination. Common genotypes of *Y. enterocolitica* found in tonsils are also found in pluck sets and carcasses of the pigs. During the slaughtering process tonsils can be removed along with the pluck sets, which are hung on a hook after removal. In this process, the spread of *Y. enterocolitica* from contaminated tonsils to pluck sets is inevitable (50). *Y. enterocolitica* can also be found in the lymph nodes of slaughtered pigs (140), and thus, the incision of lymph nodes during meat inspection may contribute to cross-contamination. In addition, the slaughterhouse environment and slaughtering equipment are frequently contaminated with *Y. enterocolitica* (50).

The same genotypes of pathogenic *Y. enterocolitica* 4/O:3 have been isolated from the pluck sets and the rectal swabs of pigs originating from the same farms in the majority of the cases (103), indicating that the pig itself is the most frequent source of carcass contamination. In addition to direct contamination, different genotypes of *Y. enterocolitica* that were not found on the sampled farms were isolated in pluck sets of the pigs (103). Pluck set contamination seems therefore to originate both directly from intestinal contents and tonsils of the pigs and from the contaminated equipment of the slaughterhouse.

Carcass contamination can be reduced at slaughterhouses by using such hygiene measures as removing the head and bagging the rectum early in the slaughterhouse processing line (46, 100, 132, 155). In evisceration where the head is not split, the tongue is left in the head and the head is removed with the tongue and tonsils, 75% reduction of the contamination of front carcasses and 50% reduction of the contamination of liver and diaphragm with *Y. enterocolitica* can be achieved (28). In a study of 30 pig carcasses, the head and tongue were considered the major sources of carcass contamination since contamination with *Y. enterocolitica* 4/O:3 seemed to decrease along with increasing distance from the head (126). *Y. enterocolitica* was found in 83% of oral cavities, 47% of cranial incision sites in the carcasses, 43% of abdominal incision sites, and 27% of circumanal incision sites.

By closing the rectum with a plastic bag immediately after circumanal incision, the prevalence of *Y. enterocolitica* in pig carcasses has been reduced from 10% to 0.8% (132). In Norway, the prevalence of *Y. enterocolitica* in carcasses has been 12% without closing of rectum with a plastic bag, whereas none of the carcasses were found contaminated when the plastic bag was used (127). In Sweden, the introduction of plastic bags reduced the prevalence of carcass contamination from 8% to 1.7% (127). In the study of Laukkonen et al. (100), closing of the rectum reduced the number of carcasses contaminated with *Y. enterocolitica* from 26% to 17%.

Pig carcasses are routinely scalded at the slaughterhouses, and scald tank water has been proposed as a possible source of cross-contamination. Time-temperature combinations to ensure the thermal inactivation of *Y. enterocolitica* have been established (15). A time-temperature combination of 2.7 min at 60°C is required to achieve a one log reduction in *Y. enterocolitica* in pork scald tank water. The predicted equivalent at 65°C was 0.6 min. The
study provided data and a model to enable pork processors to apply parameters to limit the risk of carcass cross-contamination with *Y. enterocolitica* in scald tanks.

### 2.6.3 *Y. enterocolitica* in pork products

*Y. enterocolitica* is a frequent finding on pig tongues (49). The contamination of pig tongues occurs easily if the pathogen is carried in the tonsils of the pig (46). On the surface of edible pig offal, which includes tongues, hearts, liver, and kidneys, an isolation rate of *Y. enterocolitica* 4/O:3 as high as 51% has been described in Southern Germany (23). Correspondingly, the pathogen has been isolated in 14-18 % of raw pork samples in Germany and overall in the European Union (23, 138). In minced meat containing pork, 25% of the samples have been positive in the PCR method and 2% in culture method (49).

Packaging pork under modified atmosphere is used as a preventive measure to inhibit the growth of spoilage-causing microbes. However, the use of 30% carbon dioxide (CO2) and 70% oxygen (O2) could not significantly inhibit the growth of *Y. enterocolitica* 4/O:3 when the pork products were stored at 2°C for 12 days (182). Furthermore, high numbers of *Y. enterocolitica* have been isolated from pig cheek meat stored under 30% CO2 and 70% O2 (52). Interestingly, no *Y. enterocolitica* was isolated from pork strips from the hind leg in the same study. Therefore, the location of meat in the pig carcass appears to be significantly associated with the level of contamination with *Y. enterocolitica*. Cheek meat should only be used for heated products.

The transmission of *Y. enterocolitica* 4/O:3 in the pork production chain is illustrated in Figure 3.

![Figure 3. Transmission route of *Y. enterocolitica* from farm to fork.](image-url)
3 Aims of the study

The objective of this study was to identify the factors on farms that contribute to the prevalence of pathogenic *Y. enterocolitica* within farms and those that can be used to prevent the occurrence of the pathogen at farm level. Additionally, the aim was to establish the most discriminating method for genotyping *Y. enterocolitica* that could be used to study the epidemiology of this pathogen on farms. Specific aims were as follows:

1. To evaluate the use of multiple-locus variable-number tandem repeat analysis (MLVA) in genotyping *Y. enterocolitica* strains from different sources and to apply the method to investigate the epidemiology of *Y. enterocolitica* on pig farms (I, II, III)
2. To follow the spread of *Y. enterocolitica* within a pig farm and between different farms (II, III)
3. To find factors in farm management practices and conditions that can be used in preventing *Y. enterocolitica* at the farm level (IV, V)
4 Materials and methods

4.1 Sampling
In Study I, to investigate the use of MLVA as a genotyping tool for *Y. enterocolitica*, 379 *Y. enterocolitica* strains originating from Finland (n=288), Germany (n=46), England (n=34) and Russia (n=11) were genotyped using six different VNTR loci (V2A, V4, V5, V6, V7, and V9) (62). The strains were isolated from pig fecal samples (n=183), human patients (n=150), and pork samples collected from meat stores or slaughterhouses (n=46) between 1995 and 2007. Both sporadic (n=278) and epidemiologically linked strains of pigs that originated from the same farms (n=101) were included. The strains belonged to serotypes O:3 (n=363), O:9 (n=9), and O:5,27 (n=7).

In Study II, two units on a fattening-farm were separately monitored in a follow-up study. Seventy-six piglets were sampled via feces at two-week intervals throughout the fattening period. Blood samples were collected at the beginning and the end of the study. In one unit, the first samples were collected two weeks after the piglets arrived at the fattening farm and in the other unit, the first samples were collected the day after arrival. The last samples were collected at the slaughterhouse.

To investigate the prevalence and spread of *Y. enterocolitica* on pig farms in practice, in Studies III and V, a total of 32 farms were visited, and fecal (n=1545) and blood samples (n=334) were collected from 1564 pigs. At the visited farms, a proportion of pens from different units were sampled throughout the study. Age groups of 1) less than two months, 2) two to three months, 3) three to five months, 4) fattening pigs older than five months, and 5) sows were sampled. Additionally, environmental samples (n=120) and water samples (n=16) were collected. Management practices and conditions were recorded during each sampling visit (Table 5).

<p>| Table 5. Management practices and conditions recorded during the sampling visits to farms. |</p>
<table>
<thead>
<tr>
<th>Category</th>
<th>Subcategory</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>General management</td>
<td>Production type</td>
<td>Farrow-to-finish, fattening, farrowing</td>
</tr>
<tr>
<td></td>
<td>Animal flow</td>
<td>All-in all-out</td>
</tr>
<tr>
<td></td>
<td>Animal density and contacts</td>
<td>Origin and purchase of pigs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contacts with other farms of the study</td>
</tr>
<tr>
<td>Production capacity</td>
<td>Herd size</td>
<td>Number of sows, piglets, and fattening pigs</td>
</tr>
<tr>
<td></td>
<td>Animal density and contacts</td>
<td>Number of animals in sampled pens</td>
</tr>
<tr>
<td>Bedding and water</td>
<td>Source of bedding</td>
<td>Snout contacts and moving of fecal material between pens</td>
</tr>
<tr>
<td></td>
<td>Source of water</td>
<td>Straw, shavings, sawdust, peat, newspaper</td>
</tr>
<tr>
<td>Other animals</td>
<td></td>
<td>Little, sparse, plenty</td>
</tr>
<tr>
<td>General health</td>
<td></td>
<td>Municipal or own; type of well</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence of birds, rodents, cats, and dogs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use of antimicrobials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presence of weak piglets</td>
</tr>
</tbody>
</table>
In Study IV, previous culture results from intestinal contents and tonsils of 788 healthy fattening pigs from 120 different farms that were sampled at six different slaughterhouses in Finland were checked for the presence of *Y. enterocolitica*. On average, 13.1 samples were collected per farm (range 2 to 86, standard deviation 15.68). Farms from which sampled animals originated were contacted by telephone and mailed a questionnaire that contained questions about production. In total, data on 166 variables were collected. The questionnaire contained questions about general management (production type, animal flow strategies, use of employees and substitute workers, amount of artificial light, type of ventilation), production capacity (herd size, group size, unit size, animal density), hygiene in the piggery (washing and disinfection, manure removal, contacts between pigs), hygiene in the pens (cleanliness, manure moving between pens, amount and source of bedding), feed and drink (origin of feed, feeding type, water source), pest and pet animals (access of birds, rodents, flies, cats, and dogs to the piggery, feed storage, bedding storage) and general health (diseases and medications, health classification). To clarify whether the origin of piglets affects the carriage or shedding of *Y. enterocolitica*, the origins of examined fattening pigs were traced. These data were available for four of six slaughterhouses, comprising 73 of the 120 farms (61%).

4.2 Isolation and identification of *Y. enterocolitica*

The same isolation method was used throughout the study. Ten grams of feces were mixed with 90 ml of peptone-mannitol-bile salts broth (PMB), and three isolation methods were used. A volume of 100 µl was i) immediately plated onto cefsulodin-irgasan-novobiosin (CIN) agar (Oxoid, Cambridge, UK) (101) and additional cold enrichment at 4°C for ii) 7 days and iii) 14 days were used and followed by plating on CIN agars. With 14 days’ enrichment, alkali treatment with 0.25% KOH solution was used. The CIN agars were incubated at 28°C for 24 h and further for 24-48 h at room temperature. Three typical ‘bull’s eye’ colonies grown on each of CIN agars were further cultured on tryptic soy agars (TSA) (Difco, Lawrence, KS, USA) and incubated at 28°C for 24 h to obtain pure cultures. Isolates were tested for urea hydrolysis by culturing on agars containing urea and indicator.

4.3 Biotyping, serotyping, and detection of virulence genes

The pathogenicity of the urea-hydrolyzing isolates was confirmed using PCR to detect the chromosomal virulence gene *ail* (119) and the *virF* (90) gene located in virulence plasmid pYV. Only the isolates that contained both *ail* and *virF* genes were considered pathogenic *Y. enterocolitica* in the results. Strains were further tested using API 20E (BioMeriéux, Marcy l’Etoile, France) incubated at 28°C. The isolates were biotyped according to a modified biotyping scheme published by Wauters et al. (208) and serotyped by using commercial antisera (Denka Seiken, Tokyo, Japan).

4.4 MLVA genotyping

The strains were genotyped by using multiple-locus variable-number tandem repeat analysis (MLVA). The MLVA analysis was carried out based on the method described by Gierczynski et al. (62). VNTR loci V2A, V4, V5, V6, V7, and V9 were used. The forward primers were labeled with fluorescent ABI PRISM® dyes, PET, NED, 6-FAM, or VIC (Applied Biosystems Foster City, CA, USA).

4.4.1 Extraction of DNA

The DNA of examined strains was extracted by using either guanidium thiocyanate (153) or a colony of pure culture was transferred with a peak of a pipette tip to a mixture of 100 µl of 1
x Buffer for DyNAzyme (Thermo Fisher Scientific Inc., Vantaa, Finland) containing 0.6 U of Proteinase K (Thermo Fisher Scientific). The mixture was incubated at 37°C for 60 min and further heated at 95°C for 10 min.

4.4.2 PCR of VNTR regions
Primers were divided into two separate multiplex PCRs with V2A (6-FAM), V4 (NED), and V6 (PET), as well as V5 (NED), V7 (VIC), and V9 (6-FAM). PCR mastermix contained 5 µl of template, 0.5 µl of 10 mM dNTP mix (Thermo Fisher Scientific), 1 U of DyNAzyme II (Thermo Fisher Scientific), and 2.5 µl of 10 x Buffer for DyNAzyme (Thermo Fisher Scientific), all diluted into 16 µl of Sigma H2O (Sigma-Aldrich Co., Ayrshire, UK), resulting in a total PCR volume of 25 µl. A primer concentration of 5 pmol was used for primers V2A, V4, V5, and V6, 7 pmol for primer V7, and 3 pmol for primer V9.

PCR conditions were as follows: initial denaturation at 94°C for 1 min, followed by 9 cycles of denaturation at 94°C for 30 s, annealing at 63–55°C (decreasing 1°C with each cycle) for 30 s, extension at 72°C for 30 s, and 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min (174). A volume of 1.5 µl of 10-fold diluted PCR end-products was denatured with 10 µl of HiDi formamide (Applied Biosystems) mixed with 0.3 µl of the internal GeneScan-500 LIZ standard (Applied Biosystems), boiled for 3 min, and placed on ice.

4.4.3 Capillary electrophoresis
Capillary electrophoresis was run with ABI Prism® 310 Genetic Analyzer (Applied Biosystems), with POP-4™ Performance Optimized Polymer (Applied Biosystems) and 1 x genetic analyzer running buffer with EDTA (Applied Biosystems). The run time was 28 min, the voltage was 15 kV, and the run temperature was set at 60°C.

The data were collected with GeneScan software (Applied Biosystems). VNTR data were input into Bionumerics 5.2 (Applied Maths, Kortrijk, Belgium) and analyzed. Clustering of the MLVA results was calculated using Euclidian distance.

4.5 Collection of blood samples and determination of Yersinia antibodies
Blood samples were collected from pigs into evacuated blood collection tubes that contained clotting activator. In piglets and fattening pigs, blood was drawn from the right jugular vein, and in sows, the saphenous vein was used. Samples were transported to the laboratory under refrigeration, and the tubes were centrifuged at 3600 rpm for 10 min. Serum was transferred to 1.5 ml Eppendorf® tubes and stored at -20°C.

The presence of Yersinia antibodies was determined by a commercially available ELISA kit (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany) according to the manufacturer’s instructions. The ELISA test detects antibodies against Yersinia Outer Proteins (YOPs), which are only expressed by pathogenic strains. The optical density (OD) was measured in a spectrophotometer (Multiskan Ascent, Thermo Fisher Scientific Inc., Waltham, MA, USA) and an OD value of 0.2 was used as the cut-off.

4.6 Statistical analysis
To analyze the discriminatory ability of MLVA, Simpson’s discriminatory indices (DIs) were calculated for each loci according to Hunter and Gaston (79). In Study IV, a univariate statistical analysis with the carriage and shedding of Y. enterocolitica of individual pigs as outcomes was conducted with random effects logistic regression with the farm as a clustering
factor. Variables with a p-value < 0.15 were included. A pig being a carrier was determined by the pig having any positive sample. Both models were built by a forward stepwise approach, and a likelihood ratio test (LRT) \( p \leq 0.05 \) was used as an inclusion criterion. The reliability of the model estimates was assessed by comparing relative differences in the parameter estimates obtained by using different quadrature points, and by graphical exploration of deviance residuals and fitted probabilities. STATA 9 software (StataCorp LP, College Station, TX, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analyses.

In Study V, data were analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and the statistical software package SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The pen was considered the experimental unit (27). A pen was regarded as positive when any fecal or blood sample tested positive. Seroprevalences were calculated assuming sensitivity and specificity of 100%, as indicated in the manufacturer’s validation report. The exact binomial 95% confidence intervals (CIs) formula described by Agresti and Coull (2) was used. Epidemiological data were gathered by using an on-farm interview and by observation at the time of sampling. The Spearman test was performed to identify correlations between the independent variables \( (r > 0.8, p < 0.01) \). Pearson’s chi-square \( (\chi^2) \) test was used, and variables with a \( p \)-value < 0.2 were included in the multivariate analysis. A log-linear analysis model selection and a general log-linear analysis were used to examine the relationship between variables and to control the response rate. In both steps, a main-effects Poisson mode was applied.

The Kruskal-Wallis test denoted by the test statistic \( H(x) \), where \( x \) is the degrees of freedom and the Jonckheere-Terpstra tests were used to investigate the effect of animal age. To determine the exact position of the differences, post hoc tests for Kruskal-Wallis were used. The Wilcoxon signed-rank test was used to evaluate the relationship between the spread of \( Y.\ enterocolitica \) in fecal samples and the level of antibodies.
5 Results

5.1 MLVA method in genotyping porcine isolates

In Study I, 379 *Y. enterocolitica* strains were characterized with MLVA by using six loci (V2A, V4, V5, V6, V7, and V9). Of these strains originating from four countries and from both humans and pigs, 317 different MLVA types were detected. Similar MLVA types were observed between 1) strains isolated from human fecal samples and pigs, 2) clinical human samples from different years, and 3) pigs originating from the same farms. After strains with known epidemiological links were excluded, 262 different MLVA types of 278 strains remained. For sporadic strains, MLVA was found to have a discriminatory index of 0.999. All MLVA types were discovered to be country-specific, as none of the types was detected in several countries. Compared with the results previously generated by PFGE, MLVA was superior in its discriminatory ability. In total, 105 different genotypes among 206 strains were discovered by using PFGE with three enzymes *NotI*, *ApaI*, and *XhoI*, while 193 genotypes were found using MLVA.

Farm-specific MLVA groups of *Y. enterocolitica* strains were also observed. However, among strains originating from one farm, variation was commonly found in tandem repeat numbers of loci V2A, V5, V6, and V7, but not loci V4 and V9. Within strains from 15 farms, we observed variation in one to three of these loci (Table 6). Due to using the MLVA method for genotyping in Study II, monitoring of the spread of farm-specific MLVA types within a fattening farm was possible. In Study II, all analyzed strains had the same repeat numbers in VNTR loci V4 and V9.

In Study III, when analyzing *Y. enterocolitica* strains isolated from 22 Finnish pig farms positive for *Y. enterocolitica*, on average 4.3 MLVA types were found on each farm (range 1 to 14, standard deviation 3.67) when two strains differing by only one number in one of the VNTR loci were considered different MLVA types. Only one MLVA type was detected on five farms (23%). However, farm-specific MLVA types that contained mild variation in MLVA profiles were common on many farms, and variation in the VNTR loci was again more commonly detected in VNTR loci V2A, V5, V6, and V7 than in loci V4 and V9.
Table 6. Variation detected in MLVA profiles of *Y. enterocolitica* originating from the same pig farms.

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. of VNTRs in each locus</th>
<th>No. of strains</th>
<th>Bioserotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V2A</td>
<td>V4</td>
<td>V5</td>
</tr>
<tr>
<td>FI1</td>
<td></td>
<td>17-18</td>
<td>2</td>
</tr>
<tr>
<td>FI3</td>
<td></td>
<td>14-15</td>
<td>2</td>
</tr>
<tr>
<td>FI5</td>
<td></td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>FI6</td>
<td></td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>FI10</td>
<td></td>
<td>12-13</td>
<td>9</td>
</tr>
<tr>
<td>FI12</td>
<td></td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>FI14</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>FI17</td>
<td></td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>FI19</td>
<td></td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>FI20</td>
<td></td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>FI24</td>
<td></td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>GB1</td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>GB3</td>
<td></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>GB4</td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>RU1</td>
<td></td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

Among 100 strains in Study I, the obtained V2A VNTR fragments were markedly shorter than originally expected, as their length was less than 263 bp. However, the lengths of these exceptionally short fragments varied in six basepair intervals, as the results were 232, 238, 244, 250, 256, and 262 bp. The short V2A fragments of each length were sequenced in order to clarify the results. The actual repeat numbers of these fragments confirmed by sequencing were 2, 3, 4, 5, 6, and 7 when the corresponding VNTR fragment lengths were 232, 238, 244, 250, 256, and 262 bp (Table 7). According to the sequencing results, new guidelines for the interpretation of MLVA VNTR V2A results was devised and used in this study.
Table 7. Discrimination and distribution of tandem repeat numbers in each VNTR locus.

<table>
<thead>
<tr>
<th>Locus</th>
<th>V2A</th>
<th>V4</th>
<th>V5</th>
<th>V6</th>
<th>V7</th>
<th>V9</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIa</td>
<td>b</td>
<td>c</td>
<td>b</td>
<td>c</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>Repeat number bp bp bp bp bp bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>232b</td>
<td>5</td>
<td>113</td>
<td>187</td>
<td>176</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>238b</td>
<td>4</td>
<td>120</td>
<td>7</td>
<td>188</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>244b</td>
<td>19</td>
<td>127</td>
<td>30</td>
<td>194</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>250b</td>
<td>12</td>
<td>134</td>
<td>19</td>
<td>200</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>256b</td>
<td>19</td>
<td>141</td>
<td>60</td>
<td>206</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>262b</td>
<td>41</td>
<td>148</td>
<td>44</td>
<td>212</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>268</td>
<td>26</td>
<td>155</td>
<td>0</td>
<td>218</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>274</td>
<td>27</td>
<td>162</td>
<td>15</td>
<td>224</td>
<td>58</td>
</tr>
<tr>
<td>10</td>
<td>280</td>
<td>36</td>
<td>169</td>
<td>12</td>
<td>230</td>
<td>43</td>
</tr>
<tr>
<td>11</td>
<td>286</td>
<td>36</td>
<td>176</td>
<td>2</td>
<td>236</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>292</td>
<td>47</td>
<td>183</td>
<td>2</td>
<td>242</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>298</td>
<td>22</td>
<td>190</td>
<td>2</td>
<td>248</td>
<td>37</td>
</tr>
<tr>
<td>14</td>
<td>304</td>
<td>22</td>
<td>197</td>
<td>2</td>
<td>254</td>
<td>18</td>
</tr>
<tr>
<td>15</td>
<td>310</td>
<td>20</td>
<td>204</td>
<td>0</td>
<td>260</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>316</td>
<td>12</td>
<td>211</td>
<td>0</td>
<td>266</td>
<td>7</td>
</tr>
<tr>
<td>17</td>
<td>322</td>
<td>13</td>
<td>218</td>
<td>0</td>
<td>272</td>
<td>10</td>
</tr>
<tr>
<td>18</td>
<td>328</td>
<td>10</td>
<td>225</td>
<td>1</td>
<td>278</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>334</td>
<td>5</td>
<td></td>
<td></td>
<td>284</td>
<td>5</td>
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<tr>
<td>20</td>
<td>340</td>
<td>4</td>
<td></td>
<td></td>
<td>290</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>346</td>
<td>1</td>
<td></td>
<td></td>
<td>296</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>352</td>
<td>1</td>
<td></td>
<td></td>
<td>302</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>358</td>
<td>1</td>
<td></td>
<td></td>
<td>308</td>
<td>5</td>
</tr>
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<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>326</td>
<td>1</td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>332</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>338</td>
<td>1</td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a DI, Simpson’s Discriminatory Index
b Confirmed by sequencing
c n, No. of strains having the particular repeat number

The discriminatory ability of each VNTR locus is presented in Table 7. Locus V2A was found to have the highest DI. Approximately the same level of discrimination was detected in loci V5, V6, and V7, whereas loci V4 and V9 were clearly the least discriminating.

5.2 Presence of *Y. enterocolitica* on pig farms

In all experiments in this study, Finnish pig farms were found to be highly contaminated with *ail*- and *virF*–positive *Y. enterocolitica*. *Y. enterocolitica* was isolated from 69% (22/32) of sampled farms and from 24% (369/1546) of individual pigs. The within-farm fecal prevalence varied from 0% to 97% between farms. The vast majority of farms was contaminated with *Y. enterocolitica* bioserotype 4/O:3. Of all 30 farms sampled, only one farm had bioserotypes 2/O:9 and 2/O:5,27.
Yersinia antibodies were found in the majority (88%) of the studied farms. The average within-farm seroprevalence was 60%, varying between 0% and 100%. However, the presence of Yersinia antibodies was significantly associated with the age of the sampled pigs (Figure 4, Study V). Significant differences in the occurrence of antibodies and fecal samples testing positive for Y. enterocolitica were observed in different age groups.

The prevalence of pathogenic Y. enterocolitica in the feces of fattening pigs varied from 3% to 89% for different sampling times and was the highest at about 4 weeks after arrival to the fattening farm (Table 8). This corresponds to the pigs having an approximate age of 16 weeks. Intermittent fecal shedding of Y. enterocolitica was observed in some of the pigs.

Table 8. Fecal excretion of pathogenic Y. enterocolitica by pigs during the follow-up.

<table>
<thead>
<tr>
<th>Unit</th>
<th>No. of pigs</th>
<th>No. of fattening pigs excreting Y. enterocolitica (fecal prevalence, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wk 2</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>26 (65%)</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>21 (58%)</td>
</tr>
<tr>
<td>1-2</td>
<td>76</td>
<td>47 (62%)</td>
</tr>
</tbody>
</table>

a NS, not studied.

5.3. Origin of infection on fattening farms

In Study II, where two units of the same fattening farms were monitored at two-week intervals during the whole fattening period of one batch of pigs in each unit, piglets were found to be infected in their original farms and carried farm-specific genotypes of Y. enterocolitica to the fattening farm. Eight piglets that had originated from three farms had antibodies against Yersinia when they arrived at one unit in the fattening farm (Table 9). The day after their arrival, 17 piglets in the same unit excreted ail- and virF-positive Y. enterocolitica in their feces. Eventually, the pathogen was isolated in the fecal samples of every pig in both units during the fattening period. Only bioserotype 4/O:3 pathogenic strains were detected. By the time of slaughter, 82% of the sampled pigs had raised antibodies against Yersinia.
Table 9. *Yersinia* antibodies in serum samples and MLVA types of *Yersinia enterocolitica* isolates from fecal samples of piglets taken the day after arrival at unit 1 of the fattening farm.

<table>
<thead>
<tr>
<th>Farm of origin of piglets</th>
<th>No. of piglets</th>
<th>Seropositive piglets (positive pigs/all pigs studied)</th>
<th>MLVA types(^a) of the isolates (No. of <em>Y. enterocolitica</em>-positive piglets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>38% (3/8)</td>
<td>A (5), B (1)</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0% (0/3)</td>
<td>A (1)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>67% (2/3)</td>
<td>B (2)</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0% (0/3)</td>
<td>No <em>Y. enterocolitica</em> detected</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0% (0/3)</td>
<td>C (1)</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0% (0/4)</td>
<td>B (1)</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>50% (3/6)</td>
<td>C (4), A (1)</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0% (0/3)</td>
<td>No <em>Y. enterocolitica</em> detected</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0% (0/2)</td>
<td>No <em>Y. enterocolitica</em> detected</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0% (0/2)</td>
<td>A (1)</td>
</tr>
</tbody>
</table>

\(^a\) MLVA types, A (12-2-12-13-12-3), B (12-2-12-29-7-3), C (9-2-20-27-8-3).

Three different MLVA types A (12-2-12-13-12-3), B (12-2-12-29-7-3), and C (9-2-20-27-8-3), were isolated from the fecal samples of pigs in unit 1. Piglets from farms 1, 3, and 7 carried the genotypes A, B, and C, respectively. These genotypes had subsequently rapidly spread among the other pigs in the fattening unit since they were isolated from fecal samples of pigs already the day after arrival into the farm. Different genotypes were isolated from the same pigs at different sampling times, but dominant strains were detected within each pen. These strains differed between the two units. In unit 2, four different MLVA genotypes, A (12-2-12-13-12-3), D (11-2-15-10-6-3), E (13-2-10-15-4-3), and F (15-2-9-7-6-3), were found. Type A was found in both units. This genotype originated from a certain farm which had delivered piglets to both units. The same MLVA types were present in each unit from the beginning until the end of the study and no new genotypes emerged during the follow-up.

### 5.4 Spread of infection between farms of different production types

In Study V, among 1118 fecal samples of pigs from 22 farms, 369 samples tested positive for *Y. enterocolitica* (Table 10). In total, 86 different MLVA types were found. One to fourteen different MLVA types were found on each farm. On farms positive for *Y. enterocolitica*, the within-farm prevalence varied from 2% to 97%.
The same genotypes of *Y. enterocolitica* were incidentally found on some of the farms. A link was established in the animal transportation between the farms sharing similar MLVA types (Figure 5). Two fattening farms, F1 and F20, bought piglets from the same origin and MLVA type 12-2-12-13-12-3 was found on both of these farms. Farrowing farm F7 appeared to distribute MLVA types 10-4-10-11-7-5, 10-5-10-11-7-5, and 11-5-10-11-7-5 to fattening farms F2 and F12. Farrow-to-finish farm F8 and fattening farm F9, which shared MLVA type 18-6-15-11-9-7, were located close to each other and had the same owner, who continuously transported piglets from farm F8 to farm F9. Farrow-to-finish farm F6 had sold sows to farm F4 seven years before the sampling of this study. MLVA types 7-2-6-9-6-3 and 7-2-6-10-6-3 were found on these farms. Once farm F4 was additionally sampled one year later the same

<table>
<thead>
<tr>
<th>Farm</th>
<th>Total no. of samples</th>
<th>No. of <em>Y. enterocolitica</em>-positive samples</th>
<th>No. of MLVA types</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>29</td>
<td>9 (31%)</td>
<td>2</td>
</tr>
<tr>
<td>F2</td>
<td>55</td>
<td>36 (65%)</td>
<td>12</td>
</tr>
<tr>
<td>F3</td>
<td>50</td>
<td>18 (36%)</td>
<td>1</td>
</tr>
<tr>
<td>F4</td>
<td>58</td>
<td>26 (45%)</td>
<td>4</td>
</tr>
<tr>
<td>F5</td>
<td>53</td>
<td>22 (42%)</td>
<td>1</td>
</tr>
<tr>
<td>F6</td>
<td>50</td>
<td>14 (28%)</td>
<td>2</td>
</tr>
<tr>
<td>F7</td>
<td>69</td>
<td>33 (48%)</td>
<td>8</td>
</tr>
<tr>
<td>F8</td>
<td>43</td>
<td>1 (2%)</td>
<td>1</td>
</tr>
<tr>
<td>F9</td>
<td>27</td>
<td>7 (26%)</td>
<td>5</td>
</tr>
<tr>
<td>F10</td>
<td>45</td>
<td>14 (31%)</td>
<td>6</td>
</tr>
<tr>
<td>F11</td>
<td>32</td>
<td>2 (6%)</td>
<td>2</td>
</tr>
<tr>
<td>F12</td>
<td>34</td>
<td>33 (97%)</td>
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</tr>
<tr>
<td>F13</td>
<td>62</td>
<td>37 (60%)</td>
<td>14</td>
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<tr>
<td>F14</td>
<td>56</td>
<td>21 (38%)</td>
<td>5</td>
</tr>
<tr>
<td>F15</td>
<td>23</td>
<td>11 (48%)</td>
<td>2</td>
</tr>
<tr>
<td>F16</td>
<td>52</td>
<td>10 (19%)</td>
<td>2</td>
</tr>
<tr>
<td>F17</td>
<td>140</td>
<td>6 (4%)</td>
<td>4</td>
</tr>
<tr>
<td>F18</td>
<td>62</td>
<td>26 (42%)</td>
<td>1</td>
</tr>
<tr>
<td>F19</td>
<td>70</td>
<td>4 (6%)</td>
<td>1</td>
</tr>
<tr>
<td>F20</td>
<td>40</td>
<td>17 (43%)</td>
<td>9</td>
</tr>
<tr>
<td>F21</td>
<td>34</td>
<td>7 (21%)</td>
<td>2</td>
</tr>
<tr>
<td>F22</td>
<td>34</td>
<td>15 (44%)</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>1118</td>
<td>369</td>
<td>86</td>
</tr>
</tbody>
</table>
MLVA types were still found. Certain MLVA types of *Y. enterocolitica* seemed to persist on farms and pigs were considered the main source of these strains because no additional genotypes emerged from possible environmental sources.

The number of MLVA types within a farm was found to be significantly associated with regular purchase of new animals (p<0.05). On average, 2.3 MLVA types of *Y. enterocolitica* were evident on farms that were not purchasing new animals (standard deviation 1.5), compared with 6.7 MLVA types (standard deviation 4.1) on farms that regularly purchased and transported new pigs to the farm. Furthermore, on 75% of the farms that used municipal water, only one MLVA type of *Y. enterocolitica* was obtained, whereas several MLVA types were found on 88% of farms that had a private well as the source of water. The difference between the number of MLVA types on farms having different water sources was significant (p<0.05). The number of MLVA types was also significantly associated with the number of fattening pigs (p<0.05), but not with the number of sows on farms (p>0.05).

### 5.5 Farms factors associated with the prevalence of *Y. enterocolitica* on farms

The association of farm factors with the presence of *Y. enterocolitica* was investigated in two separate studies. In Study IV, based on the results from on-farm questionnaires, the farms that
used municipal water and organic production and bought feed from a certain feed company (company “A”) were less likely to have pigs as carriers of *Y. enterocolitica*. An increasing amount of artificial light in hours per day as a measure of pig activity, daily or weekly use of antimicrobials, and use of industrial by-products in feed were found to be risk factors for the carriage of the pathogen (Table 11). Tonsillar carriage of *Y. enterocolitica*, using commercial feed from another feed company (company “B”), fasting pigs before slaughter, and higher level of health classification were risk factors for fecal shedding, whereas the use of municipal water was a protective factor for fecal shedding, as was the use of amoxicillin medication (Table 12). A higher number of snout contacts between pigs and use of tetracycline was associated with increased fecal shedding.

Table 11. Results from the random effects logistic regression model for a pig being a carrier (positive in any sample) of *ail-* and *virF*-positive *Yersinia enterocolitica*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cluster-specific Odds Ratio</th>
<th>95% Confidence interval</th>
<th>Standard error</th>
<th>P-value from Wald test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of municipal water</td>
<td>0.10</td>
<td>0.03–0.31</td>
<td>0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Organic production type</td>
<td>0.02</td>
<td>0.002–0.24</td>
<td>0.03</td>
<td>0.002</td>
</tr>
<tr>
<td>Feed from company “A”</td>
<td>0.27</td>
<td>0.09–0.82</td>
<td>0.15</td>
<td>0.021</td>
</tr>
<tr>
<td>Artificial light (h/day)</td>
<td>1.14</td>
<td>1.02–1.28</td>
<td>0.07</td>
<td>0.023</td>
</tr>
<tr>
<td>Daily/weekly use of antimicrobials</td>
<td>3.56</td>
<td>1.12–11.37</td>
<td>2.11</td>
<td>0.032</td>
</tr>
<tr>
<td>Industrial by-products in feed</td>
<td>4.44</td>
<td>1.07–18.46</td>
<td>3.23</td>
<td>0.040</td>
</tr>
<tr>
<td>rho*</td>
<td>0.46</td>
<td>0.30–0.64</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

*rho = within-farm correlation*

Table 12. Results from the random effects logistic regression model for a pig shedding *ail-* and *virF*-positive *Yersinia enterocolitica* in feces.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cluster-specific Odds Ratio</th>
<th>95% Confidence interval</th>
<th>Standard error</th>
<th>P-value from Wald test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsillar carriage</td>
<td>3.09</td>
<td>1.33–7.18</td>
<td>1.33</td>
<td>0.009</td>
</tr>
<tr>
<td>Municipal water</td>
<td>0.25</td>
<td>0.08–0.71</td>
<td>0.13</td>
<td>0.009</td>
</tr>
<tr>
<td>Feed from company “B”</td>
<td>16.79</td>
<td>1.87–151.03</td>
<td>18.82</td>
<td>0.012</td>
</tr>
<tr>
<td>Fasting pigs before slaughter</td>
<td>3.95</td>
<td>1.29–12.06</td>
<td>2.25</td>
<td>0.016</td>
</tr>
<tr>
<td>Health classification</td>
<td>2.83</td>
<td>1.06–7.54</td>
<td>1.41</td>
<td>0.038</td>
</tr>
<tr>
<td>Use of amoxicillin</td>
<td>0.13</td>
<td>0.02–1.08</td>
<td>0.14</td>
<td>0.059</td>
</tr>
<tr>
<td>Snout contacts between pens</td>
<td>2.48</td>
<td>0.94–6.52</td>
<td>1.22</td>
<td>0.067</td>
</tr>
<tr>
<td>Use of tetracycline</td>
<td>2.91</td>
<td>0.82–10.30</td>
<td>1.88</td>
<td>0.098</td>
</tr>
<tr>
<td>rho*</td>
<td>0.35</td>
<td>0.17–0.59</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

*rho = within-farm correlation*

The use of municipal water was associated with lower levels of *Yenterocolitica* in pigs also in Study V, which was carried out by sampling at farms. Water samples were simultaneously collected at farms, but no pathogenic *Yersinia* were found in water samples even by detection with real-time PCR. On-farm sampling also revealed the use of an all-in all-out management system, generous use of bedding, purchase of pigs from no more than one supplier at a time, and having an adjacent pen negative for *Y. enterocolitica* to be associated with the detection of lower levels of *Yenterocolitica* (Table 13).
Table 13. Farm management practices associated with the presence of *Y. enterocolitica* in fecal samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of pens positive / no. of pens tested (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All in/All out system</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Use</td>
<td>31/188 (16.5)</td>
<td></td>
</tr>
<tr>
<td>Non-use</td>
<td>94/328 (28.7)</td>
<td></td>
</tr>
<tr>
<td>Source of water</td>
<td></td>
<td>0.048</td>
</tr>
<tr>
<td>Municipal</td>
<td>33/252 (13.1)</td>
<td></td>
</tr>
<tr>
<td>Own</td>
<td>74/390 (19)</td>
<td></td>
</tr>
<tr>
<td>Drill well</td>
<td>23/202 (11.4)</td>
<td></td>
</tr>
<tr>
<td>Ring well</td>
<td>9/77 (11.7)</td>
<td></td>
</tr>
<tr>
<td>Spring well</td>
<td>11/18 (61.1)</td>
<td></td>
</tr>
<tr>
<td>Unknown type</td>
<td>31/93 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Amount of bedding</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Absent</td>
<td>65/400 (16.3)</td>
<td></td>
</tr>
<tr>
<td>Sparse</td>
<td>69/215 (32.1)</td>
<td></td>
</tr>
<tr>
<td>Plentiful</td>
<td>4/48 (8.3)</td>
<td></td>
</tr>
<tr>
<td>No. of pigs’ suppliers</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>No purchase</td>
<td>22/203 (10.8)</td>
<td></td>
</tr>
<tr>
<td>1 supplier</td>
<td>99/406 (24.4)</td>
<td></td>
</tr>
<tr>
<td>≥ 1 supplier</td>
<td>21/44 (47.7)</td>
<td></td>
</tr>
<tr>
<td>Adjacent pen</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Positive</td>
<td>51/69 (73.9)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8/138 (5.8)</td>
<td></td>
</tr>
</tbody>
</table>
6 Discussion

6.1 MLVA method in genotyping porcine isolates
In Study I, a large number of *Y. enterocolitica* strains of various origins were typed using MLVA with six VNTR loci. The method showed high discriminatory capacity since 312 different MLVA types were found among 379 *Y. enterocolitica* strains. In the study of Gierczynski et al. (62), MLVA detected 45 genotypes among 62 *Y. enterocolitica* 4/O:3 strains, while Sihvonen et al. found 77 types among 88 *Y. enterocolitica* strains (174). As several previous studies have noted, MLVA evidently has a higher discriminatory power than PFGE (136, 174, 206). A discriminatory index of 0.999 was seen in this study; Sihvonen et al. previously reported a similar discriminatory index (174). Interestingly, we observed in the MLVA results a significant (p<0.05) association between PFGE type NA/AA/HA and repeat numbers two in locus V4 and three in locus V9. By using NotI restriction enzyme, the studied strains were divided into three major groups using letters NA/NB/NC and further to subtypes by numbers. Similarly, by using enzymes ApaI and XhoI, the studied strains were divided into two major groups using letters AA/AB and HA/HB (44).

Performing MLVA was faster than PFGE. With the protocols used, PFGE analysis took five days, whereas MLVA results were ready within 24 h. PFGE is based on an image on a gel and relies on the correct interpretation of the image (106). Interpreting PFGE results is difficult to automate, especially if several enzymes are used. MLVA results can be analyzed with automated software, which reduces the possibility for human error. Between laboratories, MLVA results can be compared more easily than PFGE gel images, but this requires universal guidelines for interpreting MLVA results.

Similar MLVA types of strains were isolated from humans and pigs in a corresponding time period and within the same geographical region. Similar PFGE types have previously been shown in strains isolated from human and porcine sources (47, 56). Since a markedly higher discriminatory capacity is observed in MLVA than in PFGE, our results further show that edible pig offal is an important source in the transmission of *Y. enterocolitica* between pigs and humans.

Among strains originating from pigs, similar MLVA types were mainly detected from pigs that originated from the same farms. However, some MLVA types could be detected from pigs from several farms. In Studies II and III, *Y. enterocolitica* strains were found to be transmitted by infected pigs from one farm to another. After pigs from different origins were mixed on a farm, different MLVA types were incorporated and rapidly spread in the pig population within each unit. Previous contacts and animal sales between the farms may have enabled the spread of similar MLVA types on these farms. Interestingly, similar MLVA types found on several farms were country-specific; they predominated in each country. In the USA, by using AFLP method for genotyping, 172 *Y. enterocolitica* strains were grouped into nine clades (185). Isolates from the same production system showed clonal relatedness, and more than one AFLP genotype of *Y. enterocolitica* was found to circulate within a farm.

Among the six VNTR loci used in this study, the discriminatory power of VNTR locus V2A was the highest, whereas VNTR loci V4 and V9 showed the lowest discrimination. This finding is in agreement with that of a previous study (174). Regional differences seem to exist in the discriminatory ability of different loci, as among Chinese strains, locus V5 exhibited the highest discriminatory power and the least variation occurred in loci V4 and V2A (206).
Among MLVA results from the same pig farms, variation was commonly detected in four more discriminating VNTR loci (V2A, V5, V6, and V7). Recently, allelic variation of a single VNTR locus has been reported in *Mycoplasma pneumoniae* (10). Such variation was detected in our study in several VNTR loci in *Y. enterocolitica*. Both deletions and insertions of tandem repeats were observed. Variation was mainly evident in strains originating from pigs from the same farms. Since the same MLVA types seem to persist on farms for years, the occurrence of mutations in these strains over time is unsurprising and may also lead to variation in VNTRs. Variation in one or more VNTRs can cause the MLVA results of identical clones to appear different (106). However, we found no variation in loci V4 and V9, which had lower discriminatory power. These loci also have VNTR unit lengths of 7 and 12 bp, whereas the VNTR unit length of the rest of the loci is 6 bp. These less discriminating loci are thus longer than the other loci. The more discriminating loci appear occasionally to be even hypervariable and overly discriminating for long-term use. This should be considered when interpreting results, especially from pig farms. Within strains of a single epidemiological origin, differences in VNTR numbers of one or several loci can occur in the long run.

The minimum length of a V2A fragment has originally been reported to be 263 bp (62). Compared with the tandem repeat numbers and their corresponding amplicon lengths reported in previous studies (62, 174), V2A primers produced PCR products that were unexpectedly short, as their lengths were only 232-262 bp. However, the sequencing results of our study showed that these 232- to 262-bp-sized fragments contained two to seven VNTR units. When investigating the previously published *Y. enterocolitica* subsp. *palearctica* 4/O:3 Y11 genome (9), we also noticed that the size of the V2A amplicon of 274 bp equaled the number of nine repeats.

The results of Study II were interpreted before the sequencing results of unexpectedly short V2A VNTR fragments in Study I were obtained. Therefore, in the original results, the repeat numbers of locus V2A are six figures too small, and thus, number six should be added to each result from locus V2A. In addition, one should be added to locus V4 results to obtain the correct number of VNTR units.

Especially in short-term epidemiological studies, MLVA appeared to be a practical tool in genotyping *Y. enterocolitica*. Among strains that originated from pigs, MLVA distinguished the strains according to their farm of origin. However, variation was commonly created in more discriminating VNTR loci in long-term studies. Several environmental stresses may cause variation within a genotype (30). Due to its sensitivity and high discriminatory power, MLVA may be able to detect these effects caused by different stresses in the environment. Reliable comparison of the results between laboratories will require uniform interpretation of the results.

Purchase of new animals was significantly associated with the farms having a higher number of *Y. enterocolitica* MLVA types. Such an association was also found with the number of fattening pigs on the farm. Introducing new animals into the pig herd seems to increase the spread of different *Y. enterocolitica* strains and further increase the variation in MLVA results. However, variation in more discriminating VNTR loci was also observed on farms where only one *Y. enterocolitica* MLVA type was found. Different MLVA types of *Y. enterocolitica* may have previously been carried by newly purchased animals and mixed at farms at the time of transferring new animals to the herds. At the time of sampling, farm-
specific, although variable, MLVA results were detected on some of these farms.

6.2 Presence of *Y. enterocolitica* on pig farms

*Y. enterocolitica* was isolated from 69% (22/32) of sampled farms (Studies II and III) and from 24% (369/1546) of fecal samples of individual pigs. This study was not designed to be a prevalence study, but rather a study of the spread of *Y. enterocolitica* between farms and the association of farm management practices with the occurrence and spread of *Y. enterocolitica* within farms. Earlier prevalence studies of *Y. enterocolitica* in Finland have described a farm prevalence of 71% and a prevalence of 37% in individual pigs when tonsil samples were collected at slaughterhouses (7, 45). The previous farm prevalence is very close to the prevalence obtained here. Differences in individual prevalence may partially be explained by different sample material used since the previous prevalence was based on tonsil samples whereas fecal samples were collected in our study.

The within-farm fecal prevalence varied from 0% to 97% between farms and was likely affected by the age of the sampled pigs since pigs from all age groups were sampled during the farm visits. Recently, variation from 0% to 83% in within-batch prevalence of *Y. enterocolitica* has been reported in Belgium (201). In the present study, bioserotype 4/O:3 was the most commonly detected, as has been the case in most European countries (142, 143). Bioserotypes 2/O:9 and 2/O:5,27 were isolated from only one farm. Among European pigs, these bioserotypes are particularly common in England (144).

*Yersinia* antibodies were found in 88% of the studied farms (Study V). Similarly in Germany, *Yersinia* antibodies were detected on 84% of farms (204). In Norway, 63% of pig herds were seropositive (175). In the present study, the within-farm seroprevalence varied between 0% and 100%, the average being 60%. Previously, an average within-herd seroprevalence of 67% was detected in Germany (204). A significant association was noted between the presence of *Yersinia* antibodies and the age of the sampled pigs (Study V). Significant differences were also observed in the occurrence of antibodies, and fecal samples testing positive for *Y. enterocolitica* were observed in different age groups, although care should be taken in the interpretation of this result because serological tests are not completely equivalent to the isolation of *Y. enterocolitica*. Delay exists in the time of infection. The manufacture validation report of the ELISA indicates a period of 14 days post-infection, which is the time required for the detectable levels of antibodies to develop (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany).

Significant differences in prevalence between phases of production were detected, with a peak of excretion of *Y. enterocolitica* occurring in pigs aged 2-3 months. Diverse values of *Y. enterocolitica* prevalence in fecal samples among different age groups have been reported in other studies (61, 69, 210), but most did not report whether these differences were statistically significant. *Y. enterocolitica* was isolated with peak numbers of positive animals occurring between the ages of 91 and 133 days (61). In a study including four farms, a higher prevalence was reported in 20-week-old than in 14-week-old fattening pigs and non-occurrence of *Y. enterocolitica* in sows or piglets (69). The presence of *Y. enterocolitica* was only detected in fecal samples of growing and finishing pigs of fattening herds (210), and in a study of two herds, in pigs older than 80 days (129). For the first time, significant differences in seroprevalence values were observed between age groups in Study V. Except for sows, the number of seropositive animals increased with increasing age. Blood samples from pigs younger than one month were not collected, and thus, their exposure or the presence of maternal antibodies against pathogenic *Yersinia* in piglets could not be evaluated. In total,
67% of sows were seropositive and 4% of sows excreted *Y. enterocolitica* in feces.

6.3 Origin of infection on fattening farms

Piglets from certain farms were found to have been infected by *Y. enterocolitica* before transportation to the fattening farm. Antibodies against pathogenic *Yersinia* were detected in blood samples, and farm-specific *Y. enterocolitica* MLVA types were carried in intestines of these piglets who brought the pathogen to the fattening farm. Subsequently, on the fattening farm, the infection spread extremely effectively. Only a minority of piglets seemed to be carriers of the infection, but these piglets soon transmitted the infection to the rest of the piglets. By the time of arrival to the fattening farm, 12-week-old piglets from three specific farms were found to have raised antibodies against pathogenic *Y. enterocolitica*. In a previous study, pigs were not seropositive until the age of 102-107 days (129), equalling an age of 14-15 weeks. After oral contamination, antibodies have been shown to take as long as 12 days to develop (134).

The same MLVA type A originated from farrowing farm 1 and was present in both units. Farm 1 seems to be a reservoir of this genotype and apparently the piglets from this farm continuously transmit the contamination when delivered to different fattening farms. In fact, pigs originating from farm 1 were incidentally sampled in Study III, and the same MLVA type was found. Farm-specific MLVA genotypes of *Y. enterocolitica* appear to exist and persist in some farrowing farms.

The same MLVA types circulated within each unit and were present in the unit throughout the fattening period. No external source of *Y. enterocolitica* seemed to exist on the fattening farm since after the beginning of the follow-up, no additional genotypes emerged. The infection sources therefore seemed to be on-site since the beginning of the study. During the follow-up the incidence of *Y. enterocolitica* in fecal samples of the pigs was 100%, demonstrating that the pathogen spreads thoroughly within a pig herd.

In Study V, when the farms purchased new piglets from more than one supplier, higher numbers of pens having *Y. enterocolitica*-positive pigs were observed. Piglets from infected breeding farms also demonstrably introduced and spread *Y. enterocolitica* throughout the fattening units of pig farms. In farrowing farms, 60% of pigs belonging to the age group 2-3 months old excreted *Y. enterocolitica* in their feces. This is exactly the age when they were introduced into the fattening farms. These results highlight the importance of limiting the number of suppliers providing new piglets to reduce the risk of introduction of *Y. enterocolitica* to fattening herds.

The incidence of zoonotic human diseases could be regulated by preventing the occurrence and spread of zoonotic pathogens in primary production. Results of Study II show that piglets from certain farms are an important source of *Y. enterocolitica* for fattening farms. To reduce the level of this pathogen in pig production, mixing piglets from *Y. enterocolitica*-positive farms with piglets negative for *Y. enterocolitica* should be avoided, and the prevention methods should be targeted at piglet production units.

6.4 Spread of infection between farms of different production types

Transportation of infected pigs from one farm to another seemed to spread similar *Y. enterocolitica* strains between farms. Transfer of animals thus is an important source of transmission of this pathogen on pig farms. In addition to findings of Study II where piglets were found to carry *Y. enterocolitica* infection from farrowing farms to fattening units, in
Study III, carriage of *Y. enterocolitica* by pigs was shown to occur in all production types. The introduction of infected animals to herds seems to be the main route for the spread of the infection between farms. Apparently, due to using the MLVA method, which has a high discriminatory power (174), generation and comparison of farm-specific results were possible.

In Study III, which detected the spread of different *Y. enterocolitica* strains on farms, MLVA types were considered different when even a one-number difference was noted in the MLVA profiles. So far, no guidelines exist for the correct interpretation of the results. Variation in more discriminating VNTR loci was evident in Studies I and III and was especially common among strains originating from the same pig farms. If mild variation in MLVA results was included in the same genotype in Study III, the total number of MLVA types would have been reduced.

After *Y. enterocolitica* contamination, the farm seems to remain contaminated for years. In Study III, a similar *Y. enterocolitica* MLVA type was found on farms F4 and F6. The only previous contact between these two farms was the purchase of sows from farm F6 to farm F4 seven years before this study. The purchased sows may have brought *Y. enterocolitica* infection to farm F4 at that time, and the same MLVA type was still circulating among the pigs of both farms. This MLVA type also appeared relatively uniformly on these farms for years. Because no additional genotypes emerged from the environment, the pig population seems to be the main source and reservoir of *Y. enterocolitica* on farms. Ideally, farms with no purchase of new animals and free from contamination are likely to maintain their non-infected status (130).

To prevent spread of *Y. enterocolitica* on pig farms, pigs, and further in the food chain, the results of Study III suggested that one of the main goals is to apply a production system that limits the purchase of new animals and minimizes the transport of pigs between farms.

### 6.5 Farms factors associated with the prevalence of *Y. enterocolitica* on farms

Several protective factors against pathogenic *Y. enterocolitica* 4/O:3 were identified by both questionnaires and on-farm observations. Organic production type was one of the most significant protective factors and has previously been associated with low within-farm prevalence of *Y. enterocolitica* (103, 140). Based on the findings of Study I, factors behind organic production and the lower prevalence of *Y. enterocolitica* on organic farms included generous use of bedding, limited use of antimicrobials, and lower animal density. Furthermore, organic pigs had lower daily weight gain than conventional pigs and were therefore obviously slaughtered at an older age. Thus, organic pigs may carry less *Y. enterocolitica* in their tonsils and intestine at the time of slaughter due to development of antibodies and natural resistance of infection (69, 129). However, lower daily weight gain has been associated with a higher prevalence of *Yersinia* antibodies, but this may have been related to recurrent health problems in the herds (204).

The use of municipal water was a significant protective factor for both carriage and shedding of *Y. enterocolitica*. *Y. enterocolitica* is considered a possible waterborne pathogen (170) and has previously been isolated from tap water on a pig farm (151). The results of Studies IV and V were similar to those reported by von Altrock et al. (204) who found that the use of municipal water was associated with a lower level of *Yersinia* antibodies in pigs and lower within-farm prevalence of *Yersinia*. Water is likely to be a source of *Y. enterocolitica*.
infection for pigs. However, all water samples collected at our sampling visits tested negative for pathogenic *Yersinia*. The use of municipal water was also associated with the presence of only one MLVA type in pigs on a farm (p<0.05) (Study III). Water from private wells of the farms is untreated, and the use of chlorination treatment could reduce the number of *Y. enterocolitica*.

Regular purchase of animals was significantly associated with increasing number of MLVA types on farms (Study III). In addition, an association was also noted between the number of MLVA types and the number of fattening pigs on the farm.

Buying commercial feed from company A was found to be a protective factor against *Y. enterocolitica*, whereas buying feed from company B was a risk factor. The use of commercial feed has been associated with the herds being highly contaminated with *Y. enterocolitica* (140). The protective effect of feed from company A was evident when the feed was fed to piglets on farms with farrow-to-finish production. The feed from company A for piglets contained a prebiotic hydrolyzed yeast component designed to prevent colonization of the intestine with other Gram-negative pathogenic bacteria such as *Salmonella* and *Escherichia coli*. Thus, the use of a dietary supplement is likely to reduce the presence of pathogenic *Y. enterocolitica* in pigs. In Study IV, the proportions of own grain and commercial feed in diet had no association with the occurrence of *Y. enterocolitica*, but the use of industrial by-products, such as whey or barley starch, was found to be a risk factor for fecal shedding of *Y. enterocolitica*. Industrial by-products are typically used with wet feeding and their use was also associated with larger herd size and higher daily weight gain of the pigs.

Hygienic barriers built to prevent the spread of infectious diseases appear to fail in preventing *Y. enterocolitica* (175). In fact, a higher animal health classification was a predisposing factor for fecal shedding in Study IV. In contrast, in the study of Novoslavkij et al. (139), a low biosecurity level was associated with an increased risk for the presence of *Y. enterocolitica* on farms. The association was not detected in farmers reporting washing and disinfecting a piggery carrying or shedding *Y. enterocolitica*. However, some farmers only reported disinfecting the pens without first washing. The procedure of disinfecting without washing is probably not useful in destruction of pathogenic bacteria since organic material on pen floors and pen walls is likely to inactivate the disinfectant; this approach should therefore not be used. In contrast to the failure of disinfection in the prevention of the spread of *Y. enterocolitica* on farms in Study IV, in Study V we found a significant association between an all-in all-out (AIAO) system in weaning and fattening units and a lower prevalence of *Y. enterocolitica* within the farm. Pens were less likely to be *Y. enterocolitica*-positive when farms practiced AIAO (p<0.05). AIAO is a factor that directly prevents the dissemination of *Y. enterocolitica* by eliminating contacts between different batches of pigs. Proper cleaning and disinfection of the units are undoubtedly needed between batches.

The prevalence of *Y. enterocolitica* has been significantly lower in production systems with a limited number of piglet suppliers (140, 175). In Study IV, no association was found between the levels of *Y. enterocolitica* on farms and the number of piglet suppliers, but the information was traced long after the fact. In Study V, the purchase of piglets from no more than one supplier at a time was significantly associated with a lower prevalence of *Y. enterocolitica*. In Study IV, the duration of light exposure, used as a measure of pig activity, and the possibility for snout contacts between pens were associated with higher fecal shedding of *Y. enterocolitica*. Activity time and snout contacts between pigs may increase the
risk of direct transmission of the pathogen between pigs. In addition to the snout-to-snout contacts between pigs, open slatted walls between adjacent pens also permit the transfer of fecal material between pens and therefore increase the transmission of *Y. enterocolitica*. Moreover, a protective effect was observed when adjacent pens were negative for *Y. enterocolitica* in Study V.

Daily and weekly use of antimicrobials was related to higher carriage of *Y. enterocolitica*, but was also associated with larger herd size, and thus, higher predisposition to diseases. The use of tetracycline was related to higher carriage and shedding prevalence of *Y. enterocolitica* in Study IV. In contrast, in Study V, the use of antimicrobial treatment had no association with the prevalence of *Y. enterocolitica*.

In sampling results of Study V, the lowest prevalence of *Y. enterocolitica* in fecal samples was associated with the generous use of bedding. The most common bedding materials were straw, shavings, or sawdust. Similarly, in Study IV, low prevalence of *Y. enterocolitica* on organic farms resulted from generous use of bedding. Previously, in the study of Laukkanen et al. (103), an absence of bedding in slaughter pigs was associated with a high prevalence of pathogenic *Y. enterocolitica*, whereas von Altrock et al. (204) observed an association between low serological *Yersinia* prevalence and housing on a fully slatted floor with no bedding. The use of a fully slatted floor is likely to allow continuous removal of fecal material, thus decreasing the likelihood of contamination. However, the use of fully slatted floors also decreases the welfare of the animals. In contrast, generous use of bedding material is beneficial for animal welfare and from this perspective advisable for the prevention of *Y. enterocolitica*. 
7 Conclusions

The MLVA genotyping method was found to have good resolution in the typing of *Y. enterocolitica* strains. By using MLVA for strains originating from pigs, farm-specific genotypes were obtained. Similar MLVA types were found in strains of human and pig origin and from pigs originating from the same but also from different farms. In several cases, the observation of similar MLVA types of *Y. enterocolitica* in several farms was preceded by transportation of animals between these farms. However, variation in some VNTR loci was commonly observed in MLVA results of the same farms. A new observation concerning the interpretation of the results of a certain VNTR locus was made, and this will also have an effect on future studies.

*Y. enterocolitica* strains were transported from one farm to another via pigs. Infected piglets from certain farrowing farms transmitted the infection to fattening farms, where it rapidly spread throughout the unit. Purchase of gilts to farrowing or farrow-to-finish farms from infected breeding farms was also found to be a source of the spread of infection from one farm to another. To prevent the transmission of this pathogen among pig farms, the origin of purchased animals plays an essential role. *Y. enterocolitica*-free piglet production is an important starting point.

In prevention of the spread of *Y. enterocolitica* within a pig farm, transmission between age groups should be restricted by using batch production and all-in all-out systems. Using the all-in all-out management system in both weaning and fattening units when introducing new pigs was identified as an important management practice that can reduce the spread of *Y. enterocolitica* within and between farms. Additionally, the use of municipal water on pig farms was observed to be a protective factor against *Y. enterocolitica*. The use of scarce bedding and the purchase of new animals from more than one supplier at a time were identified as factors contributing to the occurrence of *Y. enterocolitica* on farms. Significant differences in the presence of *Y. enterocolitica* in pigs of different ages were seen, with peak excretion occurring in pigs aged 2-3 months.
8 References


82. International Organization for Standardization. 2003. Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of presumptive pathogenic Yersinia enterocolitica. ISO 10273. ISO 10273:


determinants in *Yersinia enterocolitica* and other *Yersinia* spp. isolated from food animals in Greece. Int. J. Food Microbiol. **118**:326-331.


137. Nordic Committee of Food Analysis. 1996. *Yersinia enterocolitica*. Detection in foods. 117:


Kondadi, Pradeep Kumar. 2013. Genomics and functional genetics of the zoonotic pathogen *Helicobacter bizzozeronii*.


Lindén, Jere. 2013. TCDD-induced changes in the expression of selected hypothalamic feeding-regulatory genes and mRNA quantification using reverse transcription qPCR.


González, Manuel. 2012. Characterization of *Campylobacter jejuni* strains from different hosts and modelling the survival of *C. jejuni* in chicken meat and in water.


Hakkinen, Marjaana. 2010. Finnish cattle as reservoir of *Campylobacter spp*.


Ortiz Martínez, Pilar. 2010. Prevalence of enteropathogenic *Yersinia* in pigs from different European countries and contamination in the pork production chain.


Sjöman, Maria. 2010. The use of serotyping and PFGE-typing of *Listeria monocytogenes* in food processing contamination studies and human foodborne infections.

Niskanen, Taina. 2010. Diagnostics and epidemiology of *Yersinia pseudotuberculosis*.