Prevalence and Risk Factors of Enteropathogenic *Yersinia* spp. in Pigs at Slaughter Age

Gerty Vanantwerpen

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ail</td>
<td>attachment-invasion locus</td>
</tr>
<tr>
<td>CDE</td>
<td>cleaning-disinfection-stand empty</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CIN</td>
<td>cefsulodin-irgasan-novobiocin agar</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DWG</td>
<td>daily weight gain</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>HPI</td>
<td>high-pathogenicity island</td>
</tr>
<tr>
<td>Inv</td>
<td>invasion</td>
</tr>
<tr>
<td>KIA</td>
<td>kligler iron agar</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCA</td>
<td>plate count agar</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pYV</td>
<td>plasmid for <em>Yersinia</em> virulence</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Syc</td>
<td>specific yop chaperones</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>Ur</td>
<td>Urease Broth</td>
</tr>
<tr>
<td>VirF</td>
<td>virulence factor</td>
</tr>
<tr>
<td>VTEC</td>
<td>Verotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>yadA</td>
<td>Yersinia adhesin A</td>
</tr>
<tr>
<td>Yop</td>
<td><em>Yersinia</em> outer membrane proteins</td>
</tr>
<tr>
<td>YPM</td>
<td><em>Yersinia pseudotuberculosis</em> derived mitogen</td>
</tr>
<tr>
<td>Yst</td>
<td><em>Yersinia</em> stable toxin</td>
</tr>
<tr>
<td>Ysc</td>
<td>Yop secretion</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION
1. Taxonomy and history of the genus *Yersinia*

The genus *Yersinia* is a member of the family of the *Enterobacteriaceae*, the order of the *Enterobacterales*, the class of the γ-proteobacteria and the phylum of the *Proteobacteria*.

In 1934, only two *Yersinia* species (at that time *Pasteurella*) were known, namely *Pasteurella pseudotuberculosis* and *P. pestis*. The first time *Y. enterocolitica* was recognized as a new species, was in 1934 by McIver and Pike who called it *Flavobacterium pseudomallei*. They found this bacterium in two facial abscesses of a farmer. Five years later, in 1939, Schleifstein and Coleman proposed the name *Bacterium enterocoliticum*, due to new resembling isolates originating from enteric content. The name *Yersinia* was first introduced by van Loghem in 1944, who hereby honored the Swiss-born French bacteriologist Alexandre Yersin, who was the first to describe the plague bacteria, *Y. pestis*, in 1894 at the Pasteur Institute in Hong Kong, using the name *Pasteurella pestis* (Solomon, 1995; Bottone *et al.*, 1997; Euzéby, 1997; Prentice and Rahalison, 2007). The name *Y. enterocolitica* was used the first time by Frederiksen in 1964 and he introduced the species to the family of the *Enterobacteriaceae* (Bottone *et al.*, 1997; Bialas *et al.*, 2012).

Brenner *et al.* (1976) distinguished the true *Y. enterocolitica* from the *Y. enterocolitica*-like isolates based on DNA relatedness. Thanks to these findings, Bercovier *et al.* (1980 and 1984), Brenner *et al.* (1980) and Ursing *et al.* (1980) identified four *Y. enterocolitica*-like species, *Y. aldovae*, *Y. intermedia*, *Y. kristensenii* and *Y. frederiksenii*. This differentiation was based on different biochemical reactions (fermentation) of melibiose, rhamnose, raffinose and sucrose. New species were added overtime. Currently, the genus *Yersinia* consists of 18 bacterial species, among which only 3 are pathogenic in humans, i.e., *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. The remaining 15 species: *Y. aldovae*, *Y. intermedia*, *Y. kristensenii*, *Y. frederiksenii*, *Y. rohdei*, *Y. pekkanenii*, *Y. nurmii*, *Y. aleksicai*, *Y. similis*, *Y. bercovieri*, *Y. mollaretii*, *Y. philomiragia*, *Y. ruckeri*, *Y. entomophaga*, *Y. massiliensis* are either regarded as avirulent (‘environmental-type’ bacteria) or their plausible pathogenicity has not yet been studied (Euzéby, 1997; Sprague...
2. Characteristics of *Yersinia* spp.

2.1. In general

*Yersinia* spp. are facultative anaerobic Gram-negative rods. They are 0.5-0.8 µm in diameter and 1-3 µm in length. They are nonmotile at 37°C, but motile by peritrichous flagella when grown below 30°C, except for some *Y. ruckeri* strains and *Y. pestis*, which is always nonmotile. The optimal growth temperature is 28-35°C and can grow on commonly used media for Enterobacteriaceae (e.g. blood agar, MacConkey agar, Salmonella-Shigella Deoxycholate Agar). The colonies are lactose-negative. They are psychrotrophic and able to grow at 4°C, however, this property seems to be more beneficial for environmental species than for pathogenic ones (Carniel and Mollaret, 1990; Holt et al., 1994).

All three human pathogenic *Yersinia* spp. target the lymph tissues during infection and the expression of virulence depends upon the presence of a 70 kb virulence plasmid (pYV), which is essential for infection of these tissues. Loss of the plasmid leads to avirulence of the bacteria. This plasmid and its products, the Yops (for *Yersinia* outer membrane proteins), are specific for the genus (Carniel and Mollaret, 1990; Wren, 2003, Heesemann *et al.*, 2006). *Yersinia pestis* is not a food-born pathogen in comparison with the other two human pathogenic species that are typical enteropathogens (Biohaz, 2007).

*Yersinia pestis* is a clone of *Y. pseudotuberculosis* that diverged 1,500 to 20,000 years ago by picking up two *Y. pestis*-specific plasmids, pFra and pPla, which was the key step for increasing virulence (Achtman *et al.*, 1999; Skurnik *et al.*, 2000; Hinnebusch *et al.*, 2002; Duan *et al.*, 2014). *Yersinia pestis* lost non-essential housekeeping genes and has inactivated certain virulence genes (*inv* and *YadA*) encoding for proteins needed for intestinal pathogenesis (Achtman *et al.*, 1999; Hinnebusch *et al.*, 2002). Rosqvist *et al.* (1988) reported a reduced virulence of *Y. pestis* when activating these inactivated
virulence genes. The plague microorganism is a non-motile, non-acid, non-sporeforming coccobacillus. When stained with aniline dyes the ends of the bacillus will colour more intensely, which is known as ‘bipolar staining’. They have a low resistance to environmental factors: sunlight, high temperatures and desiccation have a destructive effect, and common disinfectants (e.g. lysol) and preparations containing chlorine kill *Y. pestis* within 1 to 10 minutes. The genome consists of a 4.65-Mb chromosome and three plasmids: pMT1 or pFra (96.2 kb), pYV or pCD (70.3 kb), and the species specific pPla or pPCP1 (9.6 kb) (Wren, 2003).

The pathogenicity of *Y. pseudotuberculosis* is associated with several virulence factors that are encoded on a 70 kb virulence plasmid (pYV) (Cornelis *et al.*, 1998). Additionally, a chromosomal high-pathogenicity island (HPI) encodes an iron-uptake system characterized by the siderophore yersiniabactin, the superantigenic toxin *Y. pseudotuberculosis*-derived mitogen (YPM) and invasin, which allows an efficient entry into mammalian cells and plays an important role in systemic infection (Abe *et al.*, 1997; Fukushima *et al.*, 2001; Grassl *et al.*, 2003; Schubert *et al.*, 2004).

Identification of *Y. pseudotuberculosis* is challenging because of its indistinguishable phenotype from the closely related *Yersinia similis* and *Yersinia pekkanenii* (Sprague *et al.*, 2008; Niskanen *et al.*, 2009; Murros-Kontiainen *et al.*, 2011). *Yersinia pseudotuberculosis* can be distinguished from *Y. similis* by 16S rRNA sequencing or by multilocus sequence typing (MLST) based on housekeeping genes (*glnA, thrA, tmk, trpE, adk, argA, aroA*) and from *Y. pekkanenii* by MLST based on other housekeeping genes (*glnA, gyrB, recA* and HSP60) or by DNA-DNA hybridization (Sprague *et al.*, 2008; Laukkanen-Ninios *et al.*, 2011; Murros-Kontiainen *et al.*, 2011).

*Yersinia enterocolitica* is pleomorphic ranging from small coccobacilli with rounded ends and bipolar staining to more elongated bacilli. An intriguing feature of *Y. enterocolitica* is that the motility is temperature regulated: at 25 °C *Y. enterocolitica* is peritrichously flagellated, but at 37 °C they are unflagellated and so nonmotile (Bottone, 1997; Bottone 1999). Based on differences in 16S rRNA and DNA-DNA reassociation values *Y.*
enterocolitica has been subdivided into two subspecies: ssp. enterocolitica and ssp. palearctica (Neubauer et al., 2000a).

Yersinia enterocolitica grows best at a pH between 7.6 and 7.9 and they are highly acid resistant which is mediated by the ability to produce urease (de Koning-Ward and Robins-Browne, 1995; Tennant et al., 2008). At low temperature (4°C), the growth is already inhibited at a pH level that is 0.3-0.5 units higher than the inhibiting pH (on average 4.6) at 25°C. The optimum growth temperature is set from 32 to 35°C, but even at optimal conditions for growth, the generation time is quite long (33-39 min at 32°C) compared to other Enterobacteriaceae (Schiemann, 1980; Adams et al., 1991; Little et al., 1992). They produce pinpoint colonies after 24 h of incubation, which have a typical bull’s eye morphology when grown on selective Cefsulodin-Irgasan-Novobiocin (CIN) agar plates (Schiemann, 1979).

Regarding Y. enterocolitica, there is a high tolerance noticed for surface-active agents like bile salts and sodium desoxycholate. A high tolerance was also observed for magnesium. Inhibitors are cetrimide and potassium tellurite. Irgasan was tolerated at concentrations inhibiting or lethal for other Enterobacteriaceae (Schiemann, 1979; Schiemann, 1980; Brackett, 1986; Bottone, 1999).

The remaining 15 Yersinia spp. have no reported public health significance. Yersinia ruckeri is an important fish pathogen causing enteric red mouth disease. It is important in the aquaculture of rainbow trout in Europe. Symptoms are haemorrhages in various tissues and organs, particularly around the mouth, in the gills, muscles, peritoneum and the lower intestine (Carniel and Mollaret, 1990; Huang et al., 2013).

2.2. Biotyping and serotyping

The species Y. pseudotuberculosis is subdivided in 4 biotypes, based on their different biochemical reaction to raffinose, citrate and melibiose (Fukushima, 2003). Furthermore, there is a very limited genetic variability between the 21 serotypes, in which Y.
*pseudotuberculosis* is currently divided (Palonen *et al.*, 2013). No correlation is found between a given bioserotype and the severity of the disease which is also depending on the susceptibility of the host species. Biotyping is infrequently used due to lack of clear clinical significance (Carniel and Mollaret, 1990). Serotypes O:1a and O:1b are the most common in Europe, Australia, New Zealand, and North America, and O:4b and O:5b in the East Asia. Serotype O:6 is only found in Japan. Serotype O:15 strains are prevalent in human patients in South Korea (Fukushima *et al.*, 2001; Laukkanen-Ninios *et al.*, 2011). Serotypes O:6 to O:14 have been isolated mainly from animals and environmental sources (Laukkanen-Ninios *et al.*, 2011).

*Yersinia enterocolitica* comprises a biochemically heterogeneous collection of organisms. The species has been divided into six biotypes (1A, 1B, 2-5) based on metabolic differences, which can be differentiated by biochemical tests (Table I) (Bottone, 1997; Bottone, 1999). Biotype 1B forms a geographically distinct group of strains that are frequently isolated in North America (the so-called ‘New-World’ strains) and biotypes 2 to 5 are predominantly isolated in Europe and Japan (‘Old- World’ strains). Biotype 1A is commonly found in the environment (Tennant *et al.*, 2003). The biotypes can be placed into three groups: a non-pathogenic group (biotype 1A), which lacks the pYV and seems to be distantly related to the other biotypes; a weakly pathogenic group that is unable to kill mice (biotypes 2 to 5); and a group that has significantly higher virulence for mice (high-pathogenicity group biotype 1B) (Wren, 2003; Schubert et al, 2004; Bhagat and Virdi, 2007). *Yersinia enterocolitica* ssp. *enterocolitica* comprises of biotype 1B and *Y. enterocolitica* ssp. *paleartic* the biotypes 1A and 2-5 (Howard *et al.*, 2006). In whole genome analysis of 100 *Y. enterocolitica* strains belonging to different biotypes it was shown that the biotypes 1A and 1B are more closely related to each other than to other biotypes, and biotypes 2-5 are very closely related (Reuter *et al.*, 2012).
Table I. *Y. enterocolitica* biogrouping scheme (modified from Wauters et al., 1987).

<table>
<thead>
<tr>
<th>Test</th>
<th>Biotype</th>
<th>1A</th>
<th>1B</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase activity</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salicin (acid 24 h)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Esculin hydrolysis (24 h)</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose (acid production)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose (acid production)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/(+)</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/(+)</td>
</tr>
<tr>
<td>Pyrazinamidase activity</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* a: + = positive; - = negative; (+) = delayed positive; v = variable.

The species can also be characterized by serotyping (Bottone, 1999). They are distinguished serologically based on antigenic variation in O-polysaccharides (O-PS; O-antigen), capsules (K-antigens) and flagellae (H-antigens) (Table II). The O-antigens are the most important factors responsible for the serological responses. More than 50 serotypes have by now been distinguished among *Y. enterocolitica*, of which only 11 serotypes have been frequently associated with human infection (Bottone, 1999; Hudson et al., 2008; Virdi and Sachdeva, 2005).

It is well known that certain biotype/serotype combinations are closely correlated with (1) the geographic origin of the isolates, (2) the ecological niches from which they are isolated and (3) their pathogenic significance (Bottone, 1999).
Table II. Pathogenic potential of biotypes and serotypes of *Y. enterocolitica* (modified from Bottone, 1999; Biohaz, 2007; Rastawicki *et al.*, 2013).

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Serotypes</th>
<th>Virulence for humans</th>
<th>pYV</th>
<th>HPI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ecologic/geographic distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>O:8; O:5; O:6,30; O:7; O:13; O:18;...</td>
<td>NP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>Environment, pig, food, water, animal and human faeces / global</td>
</tr>
<tr>
<td>1B</td>
<td>O:7; O:8; O:13; O:18; O:21;...</td>
<td>HP</td>
<td>+</td>
<td>+</td>
<td>Environment, pig (O:8) / United States, Japan, Europe, The Netherlands (O:8-like), Poland (O:8),</td>
</tr>
<tr>
<td>2</td>
<td>O:9; O:5,27</td>
<td>P</td>
<td>+</td>
<td>-</td>
<td>pig / Europe (O:9), United States (O:5,27), Japan (O:5,27)</td>
</tr>
<tr>
<td>3</td>
<td>O:3; O:5,27</td>
<td>P</td>
<td>+</td>
<td>-</td>
<td>Chinchilla (O:1,2,3), pig (O:5,27) / global</td>
</tr>
<tr>
<td>4</td>
<td>O:3; O:2,3; O:1,2,3</td>
<td>P</td>
<td>+</td>
<td>-</td>
<td>pig / Europe, United States, Japan, South Africa, Scandinavia, Canada</td>
</tr>
<tr>
<td>5</td>
<td>O:3; O:2,3; O:1,2,3</td>
<td>P</td>
<td>+</td>
<td>-</td>
<td>Hare / Europe</td>
</tr>
</tbody>
</table>

<sup>a</sup>: NP: Non-Pathogenic; HP: Highly Pathogenic; P: Pathogenic

<sup>b</sup>: HPI: High Pathogenicity Island

2.3. **Virulence genes of human pathogenic *Y. enterocolitica***

*Yersinia enterocolitica* possesses both chromosomal and plasmid-associated virulence determinants. When placed in a medium at 37°C with a low Ca<sup>2+</sup> concentration, human pathogenic *Y. enterocolitica* is producing and secreting certain proteins (Cornelis, 1998). To express the full potential virulence, human pathogenic *Y. enterocolitica* needs a plasmid for *Yersinia* virulence (pYV) encoding approximately 50 proteins (Zink *et al.*, 1980; Portnoy *et al.*, 1981).
A first group of virulence determinants are the adhesins. The chromosomally encoded Invasin (Inv) is a polypeptide of which synthesis in *Y. enterocolitica* is growth phase-dependent. It is maximally expressed when bacteria transit from logarithmic to stationary phase of growth at both 28 and 37°C (Bottone, 1999; Pepe *et al.*, 1994). Inv is directly involved in the first phase of infection and initiates the internalization of the bacteria in small intestine epithelial cells, especially to M cells by binding to β1 integrines (Isberg, 1990; Jepson and Clark, 1998). It stimulates the remodeling of actin filaments in the M-cell cytoskeleton, assists the induction of autophagocytosis in macrophages and initiates the cytokine production (Grassl *et al.*, 2003; Deuretzbacher *et al.* 2009). The chromosomal attachment-invasion locus (Ail), is exclusively produced at 37°C (Miller and Falkow, 1988; Pederson and Pierson, 1995). It supports the adhesion to epithelial cells and it binds to laminin and fibronectin (Miller *et al.*, 2001; Mikula *et al.*, 2013). The third outer membrane adhesin encoded by the chromosome, pH6, supports the resistance to phagocytosis, assists the haemagglutination and tissue adhesion (Chen *et al.*, 2006). The *Yersinia* adhesin (*yadA*) gene is located on the pYV and its expression is mainly temperature-regulated. The proteins are only produced at 37°C. YadA assists the adhesion to epithelial cells, neutrophils, and macrophages, the binding to collagen, fibronectin, and laminin, the invasion of epithelial cells and the Yop (*Yersinia* outer membrane proteins) delivery (Heesemann *et al.*, 1987; Visser *et al.*, 1995; Mikula *et al.*, 2013). It also protects the bacteria against phagocytosis of polymorphonuclear leukocytes and monocytes and initiates the down regulation of *inv* (Mikula *et al.*, 2013). The two regulators of the *yadA* gene transcription are *VirF* (plasmid-borne transcriptional activator of *yadA*) and *YmoA* (chromosome encoded transcriptional repressor of *virF*). The YmoA protein also inhibits the expression of the *inv* gene and the enterotoxin YstA gene (*Yersinia* stable toxins), participates in the temperature-dependent synthesis and secretion of the Yops, and the production of YadA and VirF (Lambert de Rouvroit *et al.*, 1992; Platt-Samoraj *et al.*, 2006; Bancerz-Kisiel *et al.*, 2012). YstB is correlated with *Y. enterocolitica* biotype 1A (Ramamurthy *et al.*, 1997; Bancerz-Kisiel *et al.*, 2012). Lack of pathogenicity in 1A biotype strains is currently under debate, due to the appearance of production of this toxin (Bancerz-Kisiel *et al.*, 2012).
The second virulence determinants, the lipopolysaccharide (LPS) is found on the outer membrane of Gram-negative bacteria. One LPS molecule exists of three parts: 1) lipid A that is anchored in the outer membrane and is an endotoxin, 2) the core oligosaccharide with its internal and external parts and 3) the O-specific polysaccharide that has antigenic properties and is exposed to cell surrounding. The LPS core functions as a bacteriophage receptor, activates the host complement system and interacts with serum proteins other than antibodies (Skurnik et al., 1999; Najdenski et al., 2003; Biedzka-Sarek et al., 2008; Pinta et al., 2009).

The last group of virulence determinants is a set of at least 12 proteins called Yops. Most of the yop genes have been identified and sequenced, and they appeared to be almost identical in the three human pathogenic Yersinia spp. Although initially described as outer membrane proteins, the Yops were also recovered from the culture supernatant, and it was later found that they were actually secreted proteins (Michiels et al., 1990). The Yop virulon consists of two groups of Yops: some are intracellular effectors (effector Yops) delivered inside eukaryotic cells, while others form an extracellular delivery apparatus which is necessary for injecting the effectors across the plasma membrane of eukaryotic cells (translocator Yops) (Cornelis et al., 1998; Cornelis, 2002). The secretion of Yops requires a specific protein pump (Ysc: ‘Yop secretion’), which is part of a type III secretion system (T3SS) and is also encoded by the pYV (Michiels et al., 1991). The secretion requires a complex machinery made of at least 28 proteins. To protect the Yops against degradation before attachment to a cell surface or secretion, chaperones (Syc: ‘specific yop chaperones’) are produced (Wattiau and Cornelis, 1993; Wattiau and Cornelis, 1994; Wattiau et al., 1994; Cornelis et al., 1998; Cornelis, 2002).

Until now, 6 effector proteins (YopE, YopH, YopO, YopM, YopP, and YopT) are known to be translocated through the eukaryotic membrane. The injected Yops support the survival of the invading bacteria, by disturbing the dynamics of the cytoskeleton, disrupting phagocytosis, and blocking the production of pro-inflammatory cytokines (Rosqvist et al., 1994; Persson et al., 1995; Boland et al., 1998; Cornelis et al., 1998; Iriarte and Cornelis, 1998; Cornelis, 2002). YopD assists with the delivery of YopE, while YopB helps deliver YopE and YopH (YopD and YopB are translocator Yops). About 50 genes are involved in
the T3SS, occupying 75% of the pYV (Cornelis, 1998). The production of Yops can be initiated \textit{in vitro} by a low Ca$^{2+}$ concentration (Gemski \textit{et al.}, 1980).

At last, \textit{Y. enterocolitica} 1B have a chromosomally encoded 35 – 45 kb high pathogenicity island (HPI) encoding genes involved in yersiniabactin-mediated iron uptake (Pelludat \textit{et al.}, 1998). This biotype also has \textit{yts1} type II and \textit{ysa} type III secretion systems which enhance virulence (Haller \textit{et al.}, 2000; Iwobi \textit{et al.}, 2003).

3. Epidemiology: distribution and transmission route

3.1. Distribution

\textit{Yersinia pestis}

\textit{Yersinia pestis} caused three pandemics, each started by a different biovar: Antiqua (Mediterranean Sea – 600 AC), Medievalis (Europe – 14$^{\text{th}}$ century) and Orientalis (China – middle 19$^{\text{th}}$ century). Biovar Medievalis, also called the Black Death, decimated the population in Europe (Stenseth \textit{et al.}, 2008). Biovar Orientalis caused outbreaks of plague in Asia until the beginning of the 20$^{\text{th}}$ century. The three pandemics killed more than 200 million people. Today it is believed to exist no longer in Europe or in Australia (Schubert \textit{et al.}, 2004; Stenseth \textit{et al.}, 2008; EFSA and ECDC, 2013).

The pathogen circulates in animal reservoirs, particularly in rodents. They are the main source of \textit{Y. pestis} but they are also sensitive to it. The natural foci are situated in a broad belt in the tropical and sub–tropical latitudes. However, within this belt, many areas are free of the plague (e.g. the desert and large areas of continuous forest) (Schubert \textit{et al.}, 2004).

\textit{Yersinia pseudotuberculosis}

\textit{Yersinia pseudotuberculosis} is an important causal agent of zoonosis with global distribution (Fukushima \textit{et al.}, 2001). \textit{Y. pseudotuberculosis} is able to survive for long
periods in soil and water (river and fresh water), and the contamination of food and water can be a potential source of infection (Fukushima, 1992; Fukushima et al., 1995; Han et al., 2003). The most common reservoirs for *Y. pseudotuberculosis* have been reported to be carrots and lettuce (Jalava et al., 2006). The pathogen has been recovered from diverse animal sources ranging from farm animals, pets and wild animals. It was found in wild mammals like bats, raccoon dogs, deer, hares, rabbits, mouflons, buffaloes, boars and foxes, in birds like ducks and in small rodents (Jerrett et al., 1990; Riet-Correa et al., 1990; Fukushima and Gomyoda, 1991; Nikolova et al., 2001; Backhans et al., 2011; Fredriksson-Ahomaa et al., 2011; Nakamura et al., 2013). Farm animals (pigs, cattle, sheep and goats) are also possible carriers (Philbey et al., 1991; Lanada et al., 2005; Hodges and Carman, 2011; Martinez et al., 2011; Novoslavskij et al., 2013). More information about the presence in pigs and pig batches is given in ‘5. Enteropathogenic *Yersinia* spp. in pigs’.

**Human pathogenic *Y. enterocolitica***

The consumption of pork is the main source for human infection and healthy pigs are known to be the primary reservoir of the human pathogenic types of *Y. enterocolitica*, mainly biotype 4 (serotype O:3) (Tauxe et al., 1987; Ostroff et al., 1994; Bottone, 1999; Fredriksson-Ahomaa et al., 2006; Fosse et al., 2009; Huovinen et al., 2010; EFSA and ECDC, 2013; Rosner et al., 2012). For an exhaustive overview about the presence in pigs and on pig farms, see ‘5. Enteropathogenic *Yersinia* spp. in pigs’. Other food producing animals seldom carry bioserotype 4/O:3. Milnes et al. (2008) found 3.0% of sheep positive for human pathogenic *Y. enterocolitica* biotype 3 (O:4,32, O:5 and O:5,27). McNally et al. (2004) also found bioserotype 3/O:5.27 in 35% of the sheep and 4% of the cattle. Goats can harbor biotypes 2, 3 and 5 (Philbey et al., 1991; Nikolova et al., 2001; Lanada et al., 2005; Milnes et al., 2008; EFSA and ECDC, 2013). High prevalence of anti-YOP antibodies in goats (66%) and sheep (56%) in Northern Germany has been reported (Nikolaou et al., 2005). Chickens can harbor serotypes non O:3 and non O:9, which is not confirmed to be pathogenic (Kechagia et al., 2007).

Companion animals, like dogs and cats, can carry bioserotype 4/O:3 (Fredriksson-Ahomaa et al., 2001c; Bucher et al., 2008). They possibly receive their *Y. enterocolitica* 4/O:3 from contaminated pork (Fredriksson-Ahomaa et al., 2001c). Wild animals, like rabbits, boars,

3.2. Transmission route

*In general*

*Yersinia pestis* is transmitted by fleas, while the other two species of human pathogenic *Yersinia* are typically transmitted orally. They both can be transmitted by the consumption of contaminated food (most important, see section ‘3.2.2. Presence of enteropathogenic *Yersinia* spp in food’), untreated, contaminated water, direct contact with infected animals or even person-to-person transmission involving faecal-oral contamination (Gutman *et al.*, 1973; Toivanen *et al.*, 1973; Tauxe *et al.*, 1987; Fukushima *et al.*, 1988; Carniel and Mollaret, 1990; Han *et al.*, 2003; Kangas *et al.*, 2008; Stenseth *et al.*, 2008; Rimhanen-Finne *et al.*, 2009; EFSA and ECDC, 2013). Fredriksson-Ahomaa *et al.* (2006) found indistinguishable genotypes between strains from humans and strains from dogs, cats, sheep and wild rodents, indicating that these animals are a possible source for human infections. It is reported that children can get infected by cat-contaminated environmental substances (Fukushima *et al.*, 1989a). At last, blood transfusion is also a possible infection route. Blood transfusion with *Y. enterocolitica* infected blood resulted in 70% of the cases in the death of the receiver. The few bacteria present in the stored blood multiply at 4°C and produce a septic shock shortly after transfusion. The first case of infected blood transfusion ever reported was in 1975. A 57-year-old woman from the Netherlands got a septic shock one hour post transfusion but survived (Jacobs *et al.*, 1989; Mollaret *et al.*, 1989; Guinet *et al.*, 2011).
Presence of enteropathogenic Yersinia spp. in food

A great variety of food can be contaminated with enteropathogenic Yersinia spp. Raw pork and products derived from pork are the most important source of infection: strains isolated from humans have genotypes indistinguishable from the genotypes found in pigs and pork and in case control studies, human infection has been associated with the consumption of pork (products) (Fredriksson-Ahomaa et al., 2001b; Jones et al., 2003; Fearnley et al., 2005; Fredriksson-Ahomaa et al., 2006; Grahek-Ogden et al., 2007; Boqvist et al., 2009; Huovinen et al., 2010). Pig tonsils and intestines are often infected, which can lead to contamination of the carcass during slaughter (Nesbakken, 1985; Nesbakken, 2000; Fredriksson-Ahomaa et al., 2001a; Simonova et al., 2008; Laukkanen et al., 2009; Van Damme et al., 2013). The prevalence on pork carcasses varies between 0 and 63% (Nesbakken, 1988; Fredriksson-Ahomaa et al., 2000b; Boyapalle et al., 2001; Nesbakken et al., 2003; Gürtler et al., 2005; Lindblad et al., 2007; Nesbakken et al., 2008; Wehebrink et al., 2008; Bonardi et al., 2013; Van Damme et al., 2013). Furthermore, human pathogenic Y. enterocolitica have frequently been isolated from pork products and edible offals (Fredriksson-Ahomaa et al., 2007b; Bucher et al., 2008; Hudson et al., 2008; Bonardi et al., 2010; Messelhäusser et al., 2011, Tan et al., 2014). The most contaminated pork products are pig tongues, with an occurrence of 11 to 98%. In minced pork, 0-32% of the samples are contaminated with human pathogenic Y. enterocolitica (Wauters et al., 1988; Kwaga et al., 1990; Fredriksson-Ahomaa et al., 1999; Boyapalle et al., 2001; Vishnubhatla et al., 2001; Bucher et al., 2008; Messelhäusser et al., 2011; Tan et al., 2014). Pathogenic Y. enterocolitica has been isolated from the worktable and the metal glove in a butcher shop, enabling cross-contamination at retail level (Fredriksson-Ahomaa et al., 2004).

Human pathogenic Y. enterocolitica were also detected in meat products, originating from non-porcine animals. In 2011, there was one sample reported of bovine origin containing Y. enterocolitica (EFSA and ECDC, 2013). Tan et al. (2014) detected by PCR 4/6 raw beef samples as positive. Bucher et al. (2008) did not find contaminated raw beef. Yersinia enterocolitica was also demonstrated in meat from goats, sheep, horses, donkeys, bison and water buffalos, as well as in fish (EFSA and ECDC, 2013). Messelhäusser et al. (2011) found three PCR-positive samples out of 51 game meat samples. Also raw poultry samples
can be contaminated, even after they have been frozen (Norberg, 1981; Bonardi et al., 2010; Tan et al., 2014). Even on raw seafood, *Y. enterocolitica* can be found (Tan et al., 2014). At last, contamination of eggs is also possible (Favier et al., 2005).

Milk and milk products (e.g. chocolate milk) can also be contaminated with *Y. enterocolitica*, however in the EU there were no contaminated milk samples reported in 2011 (Black et al., 1978; Schiemann and Toma, 1978; Jayarao and Henning, 2001; EFSA and ECDC, 2013). By using qPCR, it was possible to detect and quantify *ail* positive raw cow milk samples in Belgium (Najdenski et al., 2012) in contrast to Messelhäusser et al. (2011) in Germany. On the other hand, the bacteria were already isolated from pasteurized milk. Since *Y. enterocolitica* does not survive the pasteurization processes of dairy products, the presence of these pathogens in pasteurized milk is probably the result from process failure or recontamination after pasteurization (Lovett et al., 1982; Walker and Gilmour, 1986).

Sometimes, enteropathogenic *Y. enterocolitica* can be found on vegetables (Lee et al., 2004). Tan et al. (2014) did not found contaminated samples. In 2011, there were no positive samples found in the European Union (EU) (EFSA and ECDC, 2013).

4. Human yersiniosis

4.1. Pathogenesis and symptoms of human infection

*Yersinia pestis*

The life cycle of *Y. pestis* is completely distinct from that of enteropathogenic *Yersiniae*. The plague primarily affects wild rodents. It spreads between rodents and to other animals via fleas (most common), cannibalism or (possibly) contaminated soil. The disease spreads among the human population via bites of contaminated fleas and causes bubonic plague, or via aerosols produced by coughing of people with the pneumonic plague. Humans bitten by an infected flea usually develop a bubonic form of plague, which is
characterized by a swelling of the lymph node draining the flea bite site. Initial symptoms of bubonic plague appear 7–10 days after infection. If diagnosed early, bubonic plague can be successfully treated with antibiotics. Most of the time, the bacteria invade the bloodstream and spread to the whole body where they localize mainly in the spleen, liver and lungs. The septicemia is responsible for the fatal outcome of bubonic plague. The mortality rate depends on how soon treatment is started, but is always very high (40%–70% mortality). If the bacteria reach the lungs, the patient develops pneumonia (pneumonic plague). Pneumonic plague is one of the most deadly infectious diseases: patients can die 24 h after infection. During this period, the infected patient is highly infectious. *Y. pestis* is considered one of the most pathogenic bacteria for humans (Carniel and Mollaret, 1990; Stenseth *et al.*, 2008).

*Yersinia pseudotuberculosis* and *Y. enterocolitica*

After oral uptake of the enteropathogenic *Yersinia* spp., they attach to the intestinal brush border of the terminal ileum and proximal colon. This is also the location of the Peyer’s Patches (PP), the preferred place to invade the body and which are covered by M-cells (specialized cells of antigen uptake). *Yersinia enterocolitica* and *Y. pseudotuberculosis* penetration of M-cells is mediated by three invasion genes (*inv*, *ail*, and *yadA*). Bacterial surface structures produced at lower temperatures are already covering the bacterial cell surface before uptake (Pepe and Miller, 1993). YadA mediates mucus and epithelial cell attachment, Inv directly promotes early epithelial cell penetration by attaching to β1 integrins on eukaryotic cell surfaces (Isberg, 1990). Ail, only produced at body temperature, also enhances epithelial cell penetration (Miller and Falkow, 1988; Isberg, 1990). The remodeling of actin filaments in the M-cell induced by Inv is the start of the invagination of the bacteria into the cells, ending within endosomal vesicles. These vesicles transport the bacteria through the M-cell and release them into the lamina propria where they stay from now on mostly extracellular (Autenrieth and Firsching, 1996; Grassl *et al.*, 2003). After this translocation, Inv binds to the β1 integrins, which induces the chemokine production (e.g. IL-8). In the PP, after host cell adhesion, the Ysc is established and initiates the cellular killing. YopH dephosphorylates eucaryotic proteins, especially in phagocytic cells, which interferes with the signal transduction pathways of the target cell and thereby impedes phagocytosis. YopB suppresses the production of the
tumor necrosis factor alpha (TNF-α). Further, to enhance a systemic spread, resistance to complement-mediated has been correlated with the presence of two outer membrane proteins, YadA and Ail (Martinez, 1989). The Yersinias replicate and express YadA, which, as described above, protects against the phagocytosis of recruited polymorphonuclear leukocytes and monocytes (Mikula et al., 2013). Three days post infection, the whole PP is colonized and its normal architecture is destroyed. Moreover, the bacteria create gaps into the basal lamina of the M-cells through which they could freely pass into the lamina propria (Bottone 1997).

There are no striking differences between the enteric symptoms caused by Y. enterocolitica or Y. pseudotuberculosis. Yersinia pseudotuberculosis infections are more common in adults than those caused by Y. enterocolitica which occur mostly in young children. The symptoms for a Y. pseudotuberculosis infection are more severe than the symptoms caused by Y. enterocolitica. Both can present right-sided abdominal pain simulating appendicitis and fever, which is more likely in older children and adults for Y. enterocolitica. Yersinia pseudotuberculosis is clinically manifested as enteritis, mesenteric lymphadenitis and occasionally septicemia. Symptoms of infections with Y. enterocolitica are nausea, diarrhea, sometimes bloody, and in elderly persons and in patients with underlying conditions (iron overload, cirrhosis, diabetes, cancer,...), systemic forms of the disease are often observed. Symptoms typically develop four to seven days after exposure and last on average one to three weeks. The disease is self-limited and rarely lethal for humans (Cover and Aber, 1989; Bottone, 1997; Sakai et al., 2005; EFSA and ECDC, 2013). Due to YadA, its ability to adhere to collagen and the resulting immunologic reaction, Y. enterocolitica can cause reactive arthritis (a sterile, immunomediated inflammation of the joints) and erythema nodosum, which are the two most common sequelae of this infection (Cover and Aber, 1989; Rosner et al., 2013). Reactive arthritis is mostly seen in adults, with a higher incidence in patients who are human leucocyte antigen (HLA)-B27-positive. It develops 1-2 weeks post infection, and lasts for several months or years (Hannu et al., 2003). Other symptoms such as pneumonia, pharyngitis, encephalitis, septicemia and formation of abscesses in liver and spleen may occur (Pulvirenti et al., 2007; Stolzel et al., 2009; Ito et al., 2012; Wong et al., 2013).
4.2. Incidence

The last known figures are those from 2011, since the last joint report of EFSA and ECDC does not mention the incidence of Yersinia spp. (EFSA and ECDC, 2013; 2014). In 2011, there were 7,017 confirmed cases of yersiniosis reported in the EU. The number of cases increased by 3.5% compared to 2010 (n = 6,780), which was the first time a slight increase was observed since 2006. Yersiniosis was the fourth most frequently reported zoonosis in the EU, after Campylobacter, Salmonella and Verotoxigenic Escherichia coli (VTEC). The notification rate in Europe was 1.63 cases per 100,000 inhabitants in 2011, which was very similar in the United States, where it was set at 1.6 (EFSA and ECDC, 2013; Scallan et al., 2013). In Belgium, there were 206 cases registered by the Sentinel Laboratory Network in 2011 (FAVV-AFSCA, 2012). Nevertheless, many infections are not confirmed, reported or even not detected. It is estimated that for each infection with Y. enterocolitica, there are 48 infections not detected (Scallan et al., 2013). Up to 40% of the German population has antibodies against Yersinia (Neubauer et al., 2001).

In Europe, there is a predominance of human pathogenic Y. enterocolitica biotype 4 (serotype O:3) and, less commonly, biotype 2 (serotype O:9, O:5,27). Species information was available for 6,830 of 7,017 confirmed cases: 98.4% were Y. enterocolitica, followed by Y. pseudotuberculosis (0.9%) and other species (0.6%) (EFSA and ECDC, 2013).

The highest country-specific notification rates were observed in Lithuania and Finland, respectively 11.40 and 10.31 cases per 100,000 inhabitants. In 2011, from 773 confirmed yersiniosis cases, 427 (55%) were hospitalized. Most of them (258 cases, 60%) were observed in Lithuania. The highest proportion of hospitalized cases by country was reported in Romania (80.9%) (EFSA and ECDC, 2013). In Ireland, there are very few human cases reported per year, however, based on serology testing, in 25% of the sampled people presence of YOP-antibodies was demonstrated (Ringwood et al., 2012). In the United States, it is also a relatively uncommon cause of sporadic disease, accounting for <0.3% of all foodborne illness (Mead et al., 1999).
The reported outbreaks worldwide of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* occurring this century are listed in Table III. Infection mostly occurs in young children (Jones et al., 2003; Sakai et al., 2005; Moriki et al., 2010). While reports of food borne outbreaks caused by *Y. pseudotuberculosis* are rare worldwide, several outbreaks have been detected in Finland and were caused by the consumption of fresh vegetables (Jalava et al., 2006; Kangas et al., 2008; Rimhamen-Finne et al., 2009).
Table III: Literature reported outbreaks of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* since 2000.

<table>
<thead>
<tr>
<th>Bio/serotype</th>
<th>Period</th>
<th>Patients</th>
<th>Source of infection</th>
<th>Symptoms (% of patients showing symptoms)</th>
<th>Place</th>
<th>Location</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human pathogenic <em>Y. enterocolitica</em> NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>November 2001</td>
<td>12</td>
<td>0.1-0.7 (0)</td>
<td>chitterlings</td>
<td></td>
<td>households</td>
<td>U. S.</td>
<td>Jones et al., 2003</td>
</tr>
<tr>
<td>O:3</td>
<td>January 2002</td>
<td>22</td>
<td>30-60 (NS)</td>
<td>unknown</td>
<td></td>
<td>oil tanker</td>
<td>Croatia</td>
<td>Babic-Erceg et al., 2003</td>
</tr>
<tr>
<td>O:8</td>
<td>August 2004</td>
<td>42</td>
<td>&lt;6 (NS)</td>
<td>salad</td>
<td></td>
<td>school</td>
<td>Japan</td>
<td>Sakai et al., 2005</td>
</tr>
<tr>
<td>O:3</td>
<td>2005</td>
<td>6</td>
<td>NS</td>
<td>raw milk</td>
<td></td>
<td>NS</td>
<td>Austria</td>
<td>Much et al., 2007</td>
</tr>
<tr>
<td>O:9</td>
<td>December 2005</td>
<td>11</td>
<td>10-88 (44)</td>
<td>ready-to-eat pork product</td>
<td></td>
<td>households</td>
<td>Norway</td>
<td>Grahek-Ogden et al., 2007</td>
</tr>
<tr>
<td>2/O:9</td>
<td>July 2006</td>
<td>3</td>
<td>1-68 (5)</td>
<td>pork</td>
<td></td>
<td>one household</td>
<td>Japan</td>
<td>Moriki et al., 2010</td>
</tr>
<tr>
<td>Bio/serotype</td>
<td>Period</td>
<td>Patients</td>
<td>Source of infection</td>
<td>Symptoms (% of patients showing symptoms)</td>
<td>Place</td>
<td>Location</td>
<td>Country</td>
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<tr>
<td>O:9</td>
<td>January 2011</td>
<td>21</td>
<td>10-63 (30-39) ready-to-eat salad mix</td>
<td>gastroenteritis</td>
<td>nationally</td>
<td>Norway</td>
<td>MacDonald et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td>O:1</td>
<td>May 2003</td>
<td>111 4-52 (10) carrots</td>
<td>abdominal illness (53) - erythema nodosum (48) - reactive arthritis (1.5)</td>
<td>school, day-care</td>
<td>Finland</td>
<td>Jalava et al., 2006</td>
<td></td>
</tr>
<tr>
<td>O:1</td>
<td>March 2004</td>
<td>53</td>
<td>7-18 (NS) carrots</td>
<td>gastroenteritis</td>
<td>school</td>
<td>Finland</td>
<td>Kangas et al., 2008</td>
<td></td>
</tr>
<tr>
<td>O:1</td>
<td>August 2006</td>
<td>427</td>
<td>12-60 (15) carrots</td>
<td>fever (95) - acute abdominal pain (97) - back pain (40) - joint pain (38) - diarrhoea (20) - erythema nodosum (15) - vomiting (14)</td>
<td>school, day-care</td>
<td>Finland</td>
<td>Rimhamen-Finne et al., 2009</td>
<td></td>
</tr>
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</table>

*: NS: Not specified
5. Enteropathogenic *Yersinia* spp. in pigs

Pigs are asymptomatic carriers of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in the tonsils and the intestines (Nesbakken, 1985; Bottone, 1999; Nesbakken, 2000; Fredriksson-Ahomaa *et al.*, 2001a; Simonova *et al.*, 2008). They do not develop serious illness because piglets are capable of restricting colonization by *Y. enterocolitica* to the throat and the intestines (Schiemann, 1988).

5.1. Presence in sows, boars and piglets

Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* are detected at a significantly lower rate in sows than in fattening pigs. Previous studies show maximum 14% sows infected with *Y. enterocolitica*, while no sows were found positive for *Y. pseudotuberculosis* (Niskanen *et al.*, 2002; Korte *et al.*, 2004; Gürtler *et al.*, 2005; Bowman *et al.*, 2007; Wehebrink *et al.*, 2008; Farzan *et al.*, 2010). Bowman *et al.* (2007) found that 2.4% of the gestating sows were positive for ail-positive *Y. enterocolitica*, while the pathogen was never detected in sows in the farrowing unit. Some studies suggest that older sows may develop a natural resistance to enteropathogenic *Yersinia* spp. (Fukushima *et al.*, 1984a; Niskanen *et al.*, 2002; Korte *et al.*, 2004; Nesbakken *et al.*, 2006). Niskanen *et al.* (2008) has performed the only reported study on the prevalence in boars. Six boars were studied, none of them were positive.

The prevalence in piglets rose to 3% (Gürtler *et al.*, 2005; Bowman *et al.*, 2007; Wehebrink *et al.*, 2008; Farzan *et al.*, 2009). Bowman *et al.* (2007) suggest that there is a trend of increasing prevalence as piglets get older. They found 0.5% of the suckling piglets and 0.6% of the nursery pigs positive for ail-positive *Y. enterocolitica*. Nevertheless, Gürtler *et al.* (2005) observed no positive suckling piglets at an age of 3 days or 3 weeks, or at an age of 10 weeks (nursery unit).
5.2. **Presence in fattening pigs**

The occurrence of these bacteria in fattening pigs is depending on the age. Bowman *et al.* (2007) found more *Y. enterocolitica* positive pigs in the late fattening stage compared to the early fattening stage. Gürtler *et al.* (2005) obtained similar results, with 2.8% of 14-week old pigs positive and 19.6% of the 20-week old fattening pigs harbored human pathogenic *Y. enterocolitica*.

Two methods can be used to define the prevalence in pigs: a microbiological and a serological way. Serological analysis is based on the antigenic properties of the discussed virulence factors LPS and Yops which are both important for the production of antibodies. These antibodies can be detected by using an enzyme-linked immunosorbent assay (ELISA). An LPS-ELISA only detects antibodies against certain serotypes of which the antigen is included in the ELISA (Thibodeau *et al.*, 2001). An ELISA based on Yops detects all *Yersinia* spp. containing the pYV (*Y. pestis, Y. pseudotuberculosis* and human pathogenic *Y. enterocolitica*) (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany).

There are time-dependent differences in analyzing tonsils, faeces or meat juice/blood (Fig. I).

![Fig. I. The occurrence of *Y. enterocolitica* O:3 in tonsils and faeces and of antibodies against *Y. enterocolitica* O:3 in blood of 60 animals in relation to age (modified from Nesbakken *et al.*, 2006).](image-url)
Some experimental studies about the evolution of antibodies and bacteriology of infected pigs were performed. Nielsen et al. (1996) inoculated 25 pigs with *Y. enterocolitica* O:3. These pigs had culture-positive faeces from day 5 to 21 post infection (p.i.), where after shedding of *Yersinia* declined to <10% of the pigs at day 49 p.i. and to 0% at day 68 p.i. Using an indirect pig LPS-ELISA, sera from all pigs showed an increase of antibody titer. All inoculated pigs had seroconverted at day 19 p.i. and remained seropositive until day 70 p.i. with a maximum level at day 33 p.i. Nesbakken et al. (2006) studied the natural dynamic of infection: between 100 and 180 days of age, serology (LPS-ELISA) could be used to differentiate between infected and non-infected pigs. Bacteriological examination of faeces could be used for the same purpose between 85 and 135 days of age, while bacteriological examination of tonsils could be applied from 85 to 180 days. Vilar et al. (2013) showed a peak in *Yersinia*-excretion in pigs of 2-3 months old, but the antibody-titer was rising till 5 months. Other studies performed on the natural dynamics of infection also show more excreting pigs of 12-21 weeks old, followed by a decrease (Fukushima et al., 1983; Gurtler et al., 2005; Virtanen et al., 2012). Infection can be detected earlier by using the microbiological method instead of serology (Fukushima et al., 1983; Nesbakken et al., 2006; Nielsen et al., 1996). The dilemma of analyzing tonsils or faeces is depending on the time of infection. The carriage of enteropathogenic *Y. enterocolitica* lasts several months in the tonsils, whereas faecal excretion decreases within a few weeks p.i. (Fukushima et al., 1983; Fukushima et al., 1984a; Fukushima et al., 1984b; Nesbakken et al., 2006; Nielsen et al., 1996; Virtanen et al., 2012). Besides, intestinal colonization continues for a long time and does not occur by re-infection (Fukushima et al., 1983). The difference between tonsillar and faecal sampling is not striking for *Y. pseudotuberculosis* (Laukkanen et al., 2008).

There are already many studies performed about the presence of these bacteria in fattening pigs at slaughter in different countries and ranges, based on isolation of tonsils, from 2 to 93%, and based on isolation of faeces, from 4 to 30% (Tables IV and V). Mostly, *Y. enterocolitica* bioserotype 4/O:3 was isolated. The number of studies based on serology is nevertheless very limited. Nesbakken et al. (2006) sampled two farms overtime, 20-100% of the fattening pigs showed antibodies against *Y. enterocolitica* O:3 by LPS-ELISA.
Thibodeau et al. (2001) found an overall prevalence of 66% in 291 tested animals, using an LPS-ELISA. In the study of von Altrock et al. (2011) 80 batches (30 pigs/batch) were tested, with an overall prevalence of 64.1% using an ELISA based on Yops.
Table IV. The reported presence of *Y. pseudotuberculosis* in fattening pigs at slaughter.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of farms</th>
<th>Number of pigs</th>
<th>Positive batches (%)</th>
<th>Number of positive pigs</th>
<th>Infected tonsils (%)</th>
<th>Infected faeces (%)</th>
<th>Bioserotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>10</td>
<td>201</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 (2)</td>
<td>-</td>
<td>1/O:1 (1), 1/O:2 (1), 2/O:3 (3)</td>
<td></td>
<td>Martinez et al., 2011</td>
</tr>
<tr>
<td>China</td>
<td>NS</td>
<td>4495</td>
<td>NS</td>
<td>4 (0.1)</td>
<td>-</td>
<td>NS</td>
<td></td>
<td>Liang et al., 2012</td>
</tr>
<tr>
<td>Estonia</td>
<td>15</td>
<td>151</td>
<td>2 (13)</td>
<td>2 (1)</td>
<td>0</td>
<td>2/O:3</td>
<td></td>
<td>Martinez et al., 2009</td>
</tr>
<tr>
<td>Finland</td>
<td>55</td>
<td>301</td>
<td>6 (40)</td>
<td>34 (10)</td>
<td>-</td>
<td>O:3</td>
<td></td>
<td>Laukkanen et al., 2010b</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>350</td>
<td>4 (27)</td>
<td>-</td>
<td>24 (7)</td>
<td>O:3</td>
<td></td>
<td>Laukkanen et al., 2008</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>358</td>
<td>NS</td>
<td>8 (4)</td>
<td>-</td>
<td>2/O:3</td>
<td></td>
<td>Niskanen et al., 2002</td>
</tr>
<tr>
<td>Great Britain</td>
<td>45</td>
<td>630</td>
<td>35 (78)</td>
<td>114 (18)</td>
<td>-</td>
<td></td>
<td></td>
<td>Martinez et al., 2010</td>
</tr>
<tr>
<td>Greece</td>
<td>NS</td>
<td>455</td>
<td>NS</td>
<td>3 (0.7)</td>
<td>-</td>
<td>NS</td>
<td></td>
<td>Kechagia et al., 2007</td>
</tr>
<tr>
<td>Italy</td>
<td>22</td>
<td>428</td>
<td>3 (14)</td>
<td>5 (1)</td>
<td>-</td>
<td>1/O:1 (3), 2/O:3 (1), 2/NT&lt;sup&gt;b&lt;/sup&gt; (1)</td>
<td></td>
<td>Martinez et al., 2011</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>98</td>
<td>1 (4)</td>
<td>-</td>
<td>1 (1)</td>
<td>NS</td>
<td></td>
<td>Bonardi et al., 2007</td>
</tr>
<tr>
<td>Latvia</td>
<td>47</td>
<td>404</td>
<td>6 (13)</td>
<td>12 (3)</td>
<td>-</td>
<td>NS</td>
<td></td>
<td>Terentjeva and Berzins, 2010</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>109</td>
<td>3 (60)</td>
<td>5 (5)</td>
<td>-</td>
<td>2/O:3</td>
<td></td>
<td>Martinez et al., 2009</td>
</tr>
<tr>
<td>Lithuania</td>
<td>11</td>
<td>110</td>
<td>6 (55)</td>
<td>11 (10)</td>
<td>-</td>
<td>2/O:3</td>
<td></td>
<td>Novoslavskij et al., 2013</td>
</tr>
<tr>
<td>Russia&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
<td>197</td>
<td>6 (60)</td>
<td>13 (7)</td>
<td>-</td>
<td>2/O:3</td>
<td></td>
<td>Martinez et al., 2009</td>
</tr>
</tbody>
</table>

<sup>a</sup>NS: not specified

<sup>b</sup>NT: not typable

<sup>c</sup>Russia: Leningrad Region
Table V. The reported presence of human pathogenic *Y. enterocolitica* in fattening pigs at slaughter by isolation.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of farms</th>
<th>Number of pigs</th>
<th>Positive batches (%)</th>
<th>Positive tonsils (%)</th>
<th>Positive faeces (%)</th>
<th>Bioserotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>10</td>
<td>201</td>
<td>8 (80)</td>
<td>89 (44)</td>
<td>-</td>
<td>3/O:9 (8), 4/O:3 (81)</td>
<td>Martinez et al., 2011</td>
</tr>
<tr>
<td>Canada</td>
<td>264</td>
<td>395</td>
<td>NS*</td>
<td>7 (2)</td>
<td>-</td>
<td>2/O:5,27 (2), 4/O:3 (5)</td>
<td>O’Sullivan et al., 2011</td>
</tr>
<tr>
<td>China</td>
<td>-</td>
<td>4495</td>
<td>-</td>
<td>694 (15)</td>
<td>-</td>
<td>NS</td>
<td>Liang et al., 2012</td>
</tr>
<tr>
<td>Denmark</td>
<td>-</td>
<td>195</td>
<td>-</td>
<td>164 (84)</td>
<td>-</td>
<td>4/O:3</td>
<td>Rasmussen et al., 1995</td>
</tr>
<tr>
<td>Finland</td>
<td>15</td>
<td>151</td>
<td>15 (100)</td>
<td>135 (89)</td>
<td>-</td>
<td>4/O:3</td>
<td>Martinez et al., 2009</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>350</td>
<td>12 (80)</td>
<td>124 (35)</td>
<td>-</td>
<td>4/O:3</td>
<td>Laukkanen et al., 2010b</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>204</td>
<td>-</td>
<td>68 (33)</td>
<td>-</td>
<td>3/O:3 (1); 4/O:3 (67)</td>
<td>Laukkanen et al., 2010a</td>
</tr>
<tr>
<td>Germany</td>
<td>-</td>
<td>164</td>
<td>-</td>
<td>101 (62)</td>
<td>17 (10)</td>
<td>4/O:3</td>
<td>Fredriksson-Ahomaa et al., 2000a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>372</td>
<td>4 (100)</td>
<td>143 (38)</td>
<td>-</td>
<td>4/O:3 (142), O:9 (1)</td>
<td>Gürtler et al., 2005</td>
</tr>
<tr>
<td>Great Britain</td>
<td>45</td>
<td>630</td>
<td>31 (69)</td>
<td>278 (44)</td>
<td>-</td>
<td>2/O:5 (97), 2/O:9 (124), 4/O:3 (39)</td>
<td>Martinez et al., 2010</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2509</td>
<td>-</td>
<td>246 (10)</td>
<td>3/O:5,27 (142), 3/O:9 (71), 4/O:3 (33)</td>
<td>McNally et al., 2004</td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td>-</td>
<td>455</td>
<td>-</td>
<td>58 (13)</td>
<td>-</td>
<td>4/O:3</td>
<td>Kechagia et al., 2007</td>
</tr>
<tr>
<td>Italy</td>
<td>22</td>
<td>428</td>
<td>22 (100)</td>
<td>137 (32)</td>
<td>-</td>
<td>2/O:5 (1), 4/O:3 (136)</td>
<td>Martinez et al., 2011</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>98</td>
<td>NS</td>
<td>-</td>
<td>4 (4)</td>
<td>3/O:9</td>
<td>Bonardi et al., 2007</td>
</tr>
<tr>
<td>Country</td>
<td>Number of farms</td>
<td>Number of pigs</td>
<td>Positive batches (%)</td>
<td>Number of positive pigs</td>
<td>Positive tonsils (%)</td>
<td>Positive faeces (%)</td>
<td>Bioserotypes</td>
</tr>
<tr>
<td>-----------</td>
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<td>-------------------------</td>
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<td>---------------------------------------</td>
</tr>
<tr>
<td>Japan</td>
<td>96</td>
<td>1200</td>
<td>32 (33)</td>
<td>-</td>
<td>89 (7)</td>
<td>2/O:5,27 (1)</td>
<td>4/O:3 (43), 4/O:3 (45)</td>
</tr>
<tr>
<td>Latvia</td>
<td>47</td>
<td>404</td>
<td>35 (74)</td>
<td>143 (35)</td>
<td>-</td>
<td>4/O:3</td>
<td></td>
</tr>
<tr>
<td>Lithuania</td>
<td>11</td>
<td>110</td>
<td>6 (55)</td>
<td>70 (64)</td>
<td>-</td>
<td>4/O:3</td>
<td></td>
</tr>
<tr>
<td>Norway</td>
<td>66</td>
<td>461</td>
<td>NS</td>
<td>67 (15)</td>
<td>-</td>
<td>4/O:3</td>
<td></td>
</tr>
<tr>
<td>Russia(^b)</td>
<td>10</td>
<td>197</td>
<td>10 (100)</td>
<td>66 (34)</td>
<td>-</td>
<td>4/O:3</td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>14</td>
<td>200</td>
<td>14 (100)</td>
<td>185 (93)</td>
<td>-</td>
<td>4/O:3</td>
<td></td>
</tr>
<tr>
<td>Switzerland</td>
<td>16</td>
<td>212</td>
<td>NS</td>
<td>72 (34)</td>
<td>-</td>
<td>2/O:5,27 (6)</td>
<td>2/O:9 (1), 4/O:3 (69)</td>
</tr>
</tbody>
</table>

\(^a\)NS: not specified
\(^b\)Russia: Leningrad Region
5.3. **Presence and control on pig farms**

When the number of farms was taken into account in the study, there happened to be a range from 33 to 100% positive farms (Table V). Sampling the same farms again overtime showed that both enteropathogenic species can persist on farms (Skjerve *et al.*, 1998; Pilon *et al.*, 2000; Niskanen *et al.*, 2008; Poljak *et al.*, 2010). In these positive farms, the number of infected fattening pigs per farm varied (Fukushima *et al.*, 1983; Letellier *et al.*, 1999; Gurtler *et al.*, 2005; Laukkanen *et al.*, 2009; Novoslavkij *et al.*, 2013).

The spread within a pig farm can be caused by environmental factors or by pig-to-pig transmission, but this has not been investigated thoroughly (Fukushima *et al.*, 1983; Pilon *et al.*, 2000; Skjerve *et al.*, 1998). Human pathogenic *Y. enterocolitica* has been isolated from pig house structures such as floors, pen walls, hallways and stairs (Aldova *et al.*, 1980; Pilon *et al.*, 2000; Bolton *et al.*, 2013; Nathues *et al.*, 2013). They were also found on water-dependent materials like nipple drinkers, suckling devices and piping (Pilon *et al.*, 2000; Nathues *et al.*, 2013). At last, they were recovered on boots (Pilon *et al.*, 2000; Vilar *et al.*, 2013). *Yersinia pseudotuberculosis* has only been detected from the pen floor of fattening pigs (Niskanen *et al.*, 2008). Neither of the pathogens was found in the garbage or in the ventilation (Fukushima *et al.*, 1983; Nathues *et al.*, 2013).

The spread between pig farms can originate from incoming, infected piglets that will infect the whole fattening pig unit (Virtanen *et al.*, 2012). Similar genotypes were found between farms that transported piglets between each other (Virtanen *et al.*, 2014). The environment of pig farms was studied for presence of enteropathogenic *Yersinia* spp. They were found more often in the proximity of high infected farms (Fukushima *et al.*, 1983; Pilon *et al.*, 2000). Studies performed on a limited number of pig farms or pigs per farm sampled to search for factors influencing this variation of the prevalence between farms or type of farms, were already conducted (Table VI). Some factors concerning farm management are hard to change and their attributed influence differs between studies. Only Christensen *et al.* (1980), Skjerve *et al.* (1998) and Nesbakken *et al.* (2003) showed a higher prevalence of *Y.*
enterocolitica O:3 in fattening pig farms than in farrow-to-finish farms. Other studies did not find this relation (Andersen et al., 1991; Laukkanen et al., 2010b; Wesley et al., 2008). Also, the influence of the production type (conventional or organic) of pig farms on the prevalence of both enteropathogenic Yersinia spp. has been studied many times, but without a clear outcome. A farm was defined as organic according to the Commission Regulation 889/2008 (EC 889/2008). The difference between conventional and organic pig production concerning the prevalence of enteropathogenic Yersinia spp. has already been studied in countries with a reliable number of organic pig farms. At pig level, Nowak et al. (2006) found Y. enterocolitica in 36 out of 200 examined pigs of organic pig production farms and in 60 out of 210 pigs from conventional housing systems. Virtanen et al. (2011) suggested that the low prevalence of Y. enterocolitica in organic farms is affected by generous use of bedding, limited use of antibiotics and lower animal density. Pigs produced in organic farms have a lower daily weight gain (DWG) and are therefore slaughtered at an older age, which implies less Y. enterocolitica in their tonsils and faeces at time of slaughter, as mentioned above (Nielsen et al., 1996; Nesbakken et al., 2006). Yersinia pseudotuberculosis is more common in organic production systems than in conventional systems (Laukkanen et al., 2008; Ortiz-Martinez et al., 2010). Outdoor access can provide different bioserotypes of enteropathogenic Yersinia spp. to pigs when contact with wild animals occur (Niskanen et al., 2003). Martinez et al. (2010) found a variety of different bioserotypes in English pigs, which could be due to a wider diversity of enteropathogenic Yersinia spp. in English wild animals, subsequently transmitted to pigs. A higher production capacity implies a higher prevalence of both Y. enterocolitica and Y. pseudotuberculosis, due to underlying risk factors, for example larger group sizes and the use of troughs for drinking (Laukkanen et al., 2008; Laukkanen et al., 2009; Laukkanen et al., 2010b).

Measures influencing the prevalence of enteropathogenic Yersinia spp. on pig farms and which are easy to apply do also exist. The factors underlying the measures influencing Y. enterocolitica are first discussed, followed by those influencing Y. pseudotuberculosis, and finally those influencing both species. Regarding Y. enterocolitica, the farm management is an important factor. Purchasing piglets from more than one farm augments the
infection rate on farms. Fattening pig farms should therefore buy their piglets from *Y. enterocolitica*-negative multiplying farms or from just one farm. This declines the risk of introducing *Y. enterocolitica* to the farm (Virtanen *et al*., 2012; Virtanen *et al*., 2014). All-in/all-out management seems to bring down the spread in pigs compared to continuous production (Vilar *et al*., 2013). Secondly, housing also plays a role in the prevalence on pig farms. Snout contact between pigs from adhering pens and the use of bedding material increases the prevalence of *Y. enterocolitica* isolated from faeces or tonsils (Laukkanen *et al*., 2009; Vilar *et al*., 2013; Virtanen *et al*., 2011). A third group of factors are feed related. The use of commercial feed and industrial by-products is related to a higher occurrence of *Y. enterocolitica* in pig herds (Nowak *et al*., 2006; Virtanen *et al*., 2011). In the U.S. the use of meat or bone meal in slaughter pig diet is still allowed and is associated with a higher prevalence (Wesley *et al*., 2008). The administration of a prebiotic element in the diet of piglets helps dropping the occurrence of *Y. enterocolitica* (Virtanen *et al*., 2011). The use of municipal water compared to collected water (rain, ground or well water) is also a protective factor, probably the bacteriological level is controlled more frequently (Virtanen *et al*., 2011; von Altrock *et al*., 2011; Vilar *et al*., 2013). Fourthly, there are factors correlated with the health status of pig farms. Farms with a higher prevalence of *Y. enterocolitica* were using antibiotics frequently due to recurring health problems, had a lower DWG, applied vaccination against *E. coli* and had a higher percentage of deaths due to scours (Wesley *et al*., 2008; Virtanen *et al*., 2011; von Altrock *et al*., 2011). These high-prevalence farms were mostly categorized in the low *Salmonella* risk herd (Nathues *et al*., 2013), while a lower prevalence was found in farms with a positive *Salmonella* status (von Altrock *et al*., 2011). Wesley *et al.* (2008) did not find any relation with the presence of *Salmonella*, roundworms, gastric ulcers, hemolytic bowel syndrome or ileitis. At last, the presence of *Y. enterocolitica* is correlated with the access of pets and pest animals to the stables (Wesley *et al*., 2008; Laukkanen *et al*., 2009; Virtanen *et al*., 2011). These animals possibly spread and maintain infections of *Y. enterocolitica* on the farm. Isolates collected on farm originating from pigs and pest animals possessed the same genotypes (Aldova *et al*., 1980; Backhans *et al*., 2011). Farms dogs did not carry *Y. enterocolitica* (Gürtler *et al*., 2005; Niskanen *et al*., 2008). Only one study examined factors that were only correlated with the presence of *Y. pseudotuberculosis* (Laukkanen...
et al., 2008). The only factor that is easy to apply was the contact with pest animals and the outside environment. Risk factors mentioned in studies concerning both enteropathogenic species are recurring health problems, a lower DWG and a large number of pest animals in the stables (von Altrock et al., 2011; Novoslavkij et al., 2013). These factors have already been mentioned in studies concerning only Y. enterocolitica.

Some factors were not significant according to previous studies, although they should have an influence on the prevalence. An example of such a factor is the cleaning and disinfection of the piggery that does not have an effect on the occurrence of both Y. enterocolitica and Y. pseudotuberculosis on pig farms (Laukkanen et al., 2008; Virtanen et al., 2011).

The presence of Y. enterocolitica and Y. pseudotuberculosis can be influenced actively by applying bacteriocins and bacteriophages, vaccination or competitive exclusion. All of these methods should be studied further. Bacteriocins isolated from Y. kristensenii have already been tested on their influence on pathogenic Y. enterocolitica (Toora et al., 1994). Also the bacteriocin enterocoliticin, isolated from Y. enterocolitica biotype 1A, was tested on its suppression on the growth of pathogenic Y. enterocolitica (Strauch et al., 2001). This bacteriocin has shown effect in vitro, unfortunately, it did not prevent colonization of the intestinal tract by Y. enterocolitica (Damasko et al., 2005). The use of bacteriophages to control infection with Y. enterocolitica in animals and humans has been studied (Skurnik and Strauch, 2006). Yersinia pseudotuberculosis sometimes causes clinical disease in animals. For this reason, vaccines against this pathogen have been developed for administration in zoo and wildlife park animals, maras, deer and horses (Thornton and Smith, 1996; Czernomysy-Furowicz et al., 2010; Quintard et al., 2010). Pseudovac®, a killed whole cell vaccine, is the only registered vaccine in Europe (Y. pseudotuberculosis serotypes O:1-O:6), and its manufacturer, the Department of Veterinary Pathology, Utrecht University, The Netherlands, recommends to perform two subcutaneous injections per year (Quintard et al., 2010). A second vaccine used for deer farms in New Zealand is also a killed vaccine (Yersiniavax®, MSD Animal Health). However, vaccination against the enteropathogenic Yersinia spp. has not yet been developed for pigs. The
studies around competitive exclusion linked with *Yersinia* are very limited. Hussein *et al.* (2003) has found that *Y. enterocolitica* biotype 1A can bring down the adhesion of *Y. enterocolitica* bioserotype 4/O:3 when the latter arrives later at the specific cells. Unluckily, this happens only *in vitro*, not *in vivo*.
Table VI: Risk and protective factors for presence of enteropathogenic *Yersinia* spp. in pig herds.

<table>
<thead>
<tr>
<th>Risk factor (RF) or protective factor (PF)</th>
<th>Farms</th>
<th>Pigs</th>
<th>Type of samples</th>
<th>Country</th>
<th>Reference</th>
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<tr>
<td><em>Y. pseudotuberculosis</em></td>
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<tr>
<td>organic production (RF)</td>
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<tr>
<td>high production capacity (RF)</td>
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<tr>
<td>contact with pest animals and the outside environment (RF)</td>
<td>10-5</td>
<td>3-3</td>
<td>30-60</td>
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<td><em>Y. enterocolitica</em></td>
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<td>own vehicle for transport of slaughter pigs to abattoirs (RF)</td>
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<tr>
<td>separation between clean and unclean section in herds (RF)</td>
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<td>daily observations of a cat with kittens on the farm (RF)</td>
<td>179-86</td>
<td>95-74</td>
<td>53.1-86</td>
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<td>straw bedding for slaughter pigs (RF)</td>
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<tr>
<td>farrow-to-finish production (PF)</td>
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<tr>
<td>under-pressure ventilation (PF)</td>
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<tr>
<td>manual feeding of slaughter pigs (PF)</td>
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<tr>
<td>conventional housing system (RF)</td>
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<td>sourcing pigs from different pig suppliers (RF)</td>
<td>6-3</td>
<td>6-3</td>
<td>100</td>
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<tr>
<td>use of commercial feed (RF)</td>
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<td>location in a central state (RF)</td>
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<td>vaccination for <em>E. coli</em> (RF)</td>
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<tr>
<td>percentage of deaths due to scours (RF)</td>
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<tr>
<td>presence of meat or bone meal in grower-finisher diet (RF)</td>
<td>100</td>
<td>32</td>
<td>32</td>
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References:
- Laukkannen et al., 2008
- Skjerve et al., 1998
- Nowak et al., 2006
- Wesley et al., 2008
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<tr>
<th>Risk factor (RF) or protective factor (PF)</th>
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<th>Country</th>
<th>Reference</th>
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<tr>
<td></td>
<td>$S_f$</td>
<td>$P^b$</td>
<td>$%^c$</td>
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<td>drinking from a nipple (RF)</td>
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<td>10-1</td>
<td>intestinal content</td>
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<td>access of pest animals to pig house (PF)</td>
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<td>8-4</td>
<td>80-80</td>
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<td>coarse feed or bedding for slaughter pigs (PF)</td>
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<td>231-119</td>
<td>106-18</td>
<td>46-15</td>
<td>tonsil</td>
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<tr>
<td>artificial light (h/day) (RF)</td>
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<td></td>
<td>15.4</td>
<td>intestinal content (IC)</td>
<td>Finland$^f$</td>
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<td>daily/weekly use of antibiotics (RF)</td>
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<td>industrial by-products in feed (RF)</td>
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<td>tonsillar carriage (IC) (RF)</td>
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<td>feed from company B (IC) (RF)</td>
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<td>fasting pigs before transport to the slaughterhouse (IC) (RF)</td>
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<td>85</td>
<td>87</td>
<td>94.2</td>
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<td>higher-level farm health classification (IC) (RF)</td>
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<td>318</td>
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<td>snout contact (IC) (RF)</td>
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<td>use of tetracycline (IC) (RF)</td>
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<td>use of municipal water (PF)</td>
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<td>organic production (PF)</td>
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<tr>
<td>buying feed from company A (PF)</td>
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<td>use of amoxicillin (IC) (PF)</td>
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<td>pens without or with sparse amounts of bedding (RF)</td>
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<td>26</td>
<td>994</td>
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<td>faecal samples</td>
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<td>buying piglets from more than one farm (RF)</td>
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<td>using an all-in/all-out (PF)</td>
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<td>use of municipal water (PF)</td>
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<td>Risk factor (RF) or protective factor (PF)</td>
<td>Farms</td>
<td>Pigs</td>
<td>Type of samples</td>
<td>Country</td>
<td>Reference</td>
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<tr>
<td><strong>Y. pseudotuberculosis and Y. enterocolitica</strong></td>
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<tr>
<td>low biosecurity level (RF)</td>
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<td>Lithuania</td>
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<td>a large number of pest animals and pets (RF)</td>
<td></td>
<td></td>
<td></td>
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<td>Novoslavskij et al., 2013</td>
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<td>more recurring health problems (RF)</td>
<td></td>
<td></td>
<td></td>
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<td>Germany</td>
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<tr>
<td>lower daily weight gain (RF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>von Altrock et al., 2011</td>
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<tr>
<td>fully slatted floor (PF)</td>
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<td></td>
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<tr>
<td>use of municipal water (PF)</td>
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<table>
<thead>
<tr>
<th>Sf</th>
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<th>%</th>
<th>Sp</th>
<th>P</th>
<th>%</th>
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<tr>
<td>11</td>
<td>6-4</td>
<td>64-45</td>
<td>110</td>
<td>20-11</td>
<td>18-10</td>
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<td>80</td>
<td>67</td>
<td>83.7</td>
<td>2400</td>
<td>1540</td>
<td>64.1</td>
</tr>
</tbody>
</table>

*Sf*: number of farms sampled  
*P*: number of positive samples  
*%*: percentage of positive samples  
*Sp*: number of pigs sample  
*e*: conventional housing - alternative, organic housing  
*f*: only the number of farms and pigs that were used in the risk factor analysis are given  
*g*: farrow-to-finish herds - slaughter pig production  
*h*: sows and boars were not included; multiplying farms not included  
*i*: Y. enterocolitica 4/O:3 - Y. pseudotuberculosis 2/O:3
AIMS OF THE THESIS
The consumption of raw or undercooked pork is the most important route of human infection with enteropathogenic *Y. enterocolitica*. A possibility of pork getting contaminated on the slaughter line is the (cross)contamination from tonsils and faeces of infected pigs. The risk of (cross)contamination can be decreased by implementing better hygienic measurements in the slaughterhouse and by reducing the number of infected pigs arriving at the slaughterhouse. In Belgium, there is no information available about the prevalence, the number of infected farms or the factors influencing this prevalence.

The general aim of this thesis is to gain insight into the variation of the within-batch prevalence of enteropathogenic *Yersinia* spp. in pigs at slaughter age originating from different farms and the factors influencing this prevalence.

Therefore, specific objectives of this thesis are:

- to investigate the microbiological and serological within-batch prevalence of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* at the time of slaughter and the variation of this prevalence (Chapter 1, 2 and 3)
- to evaluate the microbiological and serological prevalence data, in order to find a comparison between these prevalences and to predict the infection status of pig batches prior to slaughter (Chapter 4)
- to determine risk and protective factors influencing the infection at the moment of slaughter based on microbiological and serological data (Chapter 5 and 6)
EXPERIMENTAL DESIGN
This research was based on two sampling periods.

1. First, a preliminary study to estimate the microbiological within-batch prevalence of enteropathogenic *Yersinia* spp. in tonsils was performed. The number of pigs to be sampled per batch was based on an expected batch prevalence of 50%, a confidence level of 95% and an accepted error of 20% (Chapter 1). The microbiological within-batch prevalence showed a large variation, without clustering around certain percentages.

2. This was the basis of the second and more elaborate sampling period. During these studies, the within-batch prevalence examining both tonsils (Chapter 2) and pieces of diaphragm (Chapter 3) was investigated. The accepted error was adapted to 10%, resulting in more pigs per batch to be sampled. *Yersinia enterocolitica* and *Y. pseudotuberculosis* could be analyzed separately in the study of the microbiological prevalence, while there is no distinction between the two species in the serological study. The microbiological study determines the infection status of the batch at moment of slaughter, while serology at moment of slaughter presents an indication of previous infections. The comparison of both matrixes (tonsil and meat juice) originating from the same pig could be useful to identify microbiologically positive batches based on serology (Chapter 4). Finally, the results of the microbiologically and serologically based within-batch prevalence were used in two separate risk factor analyses. The farms delivering the batches sampled in the slaughterhouse were visited prior to sampling and a questionnaire was filled in. The risk factor analysis based on the microbiological within-batch prevalence of *Y. enterocolitica* (Chapter 5) identifies the factors influencing the presence of this single pathogen in the tonsils at moment of slaughter. The risk factor analysis based on the serological within-batch prevalence of both *Y. enterocolitica* and *Y. pseudotuberculosis* (Chapter 6) points out the factors influencing the risk of infection on-farm.
CHAPTER 1

ESTIMATION OF THE WITHIN-BATCH PREVALENCE AND QUANTIFICATION OF HUMAN PATHOGENIC YERSINIA ENTEROCOLITICA IN PIGS AT SLAUGHTER

1. Abstract

Yersiniosis is the third most common of bacterial zoonosis in the EU. The main source for human infection is pork contaminated with human pathogenic *Yersinia enterocolitica*, for which pigs are the primary reservoir. The aim of this study was to acquire data about the distribution of the prevalence of human pathogenic *Y. enterocolitica* in different batches of slaughter pigs. Between August and October 2011, in five Belgian slaughterhouses tonsils of 1397 fattening pigs, originating from 66 batches, were collected. Samples were plated onto cefsulodin-irgasan-novobiocin agar plates and suspect *Yersinia* colonies were enumerated. *Y. enterocolitica* were found in 375 pig tonsils (26.8%), originating from 46 batches. The within-batch prevalence showed a large variation between the different batches and ranged from 0 to 83.3%. In 20 batches (30.3%), no positive tonsils were detected. The average number of *Y. enterocolitica* was $4.04 \pm 0.97 \log_{10} \text{CFU g}^{-1}$ tonsillar tissue and the mean *Yersinia* count per batch varied between $3.08$ and $5.89 \log_{10} \text{CFU g}^{-1}$. In conclusion, human pathogenic *Y. enterocolitica* is widespread among Belgian pig farms, but there is a large variation in the within-batch prevalence among farms.
2. Introduction

Yersiniosis is the third most common bacterial zoonosis in the EU, after campylobacteriosis and salmonellosis (EFSA and ECDC, 2013). Based on qualitative risk analysis of foodborne hazards in pork, *Yersinia enterocolitica* is considered as a hazard of medium relevance for human health in the EU (Biohaz, 2011). The average EU incidence of yersiniosis is 1.63/100 000 inhabitants. In 2011, *Y. enterocolitica* caused almost 91% of the 7017 reported cases of human yersiniosis in Europe (EFSA and ECDC, 2013). It mainly affects young children of 1-4 years old. Typical symptoms are fever, diarrhea and abdominal pain (Bottone, 1997).

The five human pathogenic *Y. enterocolitica* biotypes (1B, 2-5) possess chromosomal and plasmid-associated virulence factors, including respectively the *ail* and *virF* genes. The species can also be characterized by serotyping, however without virulence determination (Bottone, 1999). The most often detected bioserotype in humans is 4/O:3 (83%), followed by 2/O:9 (15%) (EFSA and ECDC, 2013).

The consumption of pork is the main source for human infection and healthy pigs are known to be the primary reservoir of *Y. enterocolitica* (Tauxe et al., 1987; Bottone, 1999; Fosse et al., 2009; Huovinen et al., 2010; EFSA and ECDC, 2013). Other production animals like ruminants can also be infected, but they seldom carry bioserotype 4/O:3 (Nikolova et al., 2001; Carter and Wise, 2003). Pigs are potential asymptomatic carriers of human pathogenic *Y. enterocolitica* in the tonsils and the intestines, which can lead to contamination of the carcass during slaughter (Nesbakken, 1985; Bottone, 1999; Nesbakken 2000; Fredriksson-Ahoma et al., 2001a; Simonova et al., 2008). Because both ante- and post-mortem meat inspection of pigs currently do not target this contamination, it is suggested that new appropriate procedures should be developed. The occurrence and the level of contamination of *Y. enterocolitica* on pig carcasses are highly variable depending on the origin and the occurrence in pigs prior to slaughter (Biohaz, 2011). A reduction of the prevalence of human pathogenic *Y. enterocolitica* on the pig farms could decrease the (cross-) contamination of the carcasses at the slaughterhouse.
and eventually also the pork meat (Laukkanen et al., 2009). This is also the concept of the future European meat inspection, so the *Y. enterocolitica* status should be known before the pigs arrive at the slaughterhouse (Biohaz, 2011). Serological methods can indicate if pigs have ever been exposed to *Y. enterocolitica* but not a current infection, so the prevalence at slaughter should be established by microbiological examination of tonsils or faeces. The tonsils at time of slaughter are possibly a more significant source of human pathogenic *Y. enterocolitica* than faeces due to a higher number of pigs infected in the tonsils and the higher counts of *Yersinia* spp. in the tonsils than in the faeces (Nesbakken et al., 2003; Nesbakken et al., 2006; Van Damme, 2013).

The overall-prevalence of *Y. enterocolitica* in tonsils has already been determined extensively in different countries (Fredriksson-Ahomaa et al., 2000a; Bonardi et al., 2003; Fredriksson-Ahomaa et al., 2007; de Boer et al., 2008; Simonova et al., 2008; Martinez et al., 2009; Poljak et al., 2010). However, only few studies have reported the within-herd prevalence so far (Skjerve et al., 1998; Nowak et al., 2006; Virtanen et al., 2011). For the risk categorization of slaughter pig batches, the within-herd prevalence should be monitored and new data about the current prevalence at pig farms should be provided (Biohaz, 2011). The aim of this study was to obtain data about the distribution of the prevalence of *Y. enterocolitica* in Belgian slaughter pig batches.

### 3. Materials and methods

#### 3.1. Sampling

Sixty-six batches, each originating from different pig herds, were examined in five Belgian slaughterhouses between August and October 2011. All batches originated from fattening and farrow-to-finish herds. Fattening pigs were slaughtered at an age of 6 to 6.5 months and had an average slaughter weight of 120 kg. The five selected slaughterhouses slaughtered 450 to 600 pigs per hour. The time of transport varied from 30 minutes to 2 hours and all pigs were slaughtered between 30 and 90 minutes after arrival.
Due to the current lack of information about the *Y. enterocolitica* prevalence in pig batches, the sample size per batch was calculated based on an expected batch prevalence of 50%, a confidence level of 95% and an accepted error of 20%.

The tonsils (*tonsilla veli palatini*) were aseptically removed from the head immediately after the removal of the pluck and placed in a sterile stomacher bag. All samples were transported under cooled conditions to the laboratory and examined within 3 h after arrival.

### 3.2. Detection and enumeration of human pathogenic *Y. enterocolitica*

The tonsils (1.0-10.0g) were divided into small pieces, diluted in 0.1% peptone water (1/10, w/v) and homogenized in a stomacher blender (Colworth Stomacher 400, Seward, London, U.K.) for 2 min. One hundred microliter from each homogenate was plated onto a cefsulodin-irgasan-novobiocin (CIN) agar plate (*Yersinia Selective Agar Base* and *Yersinia Selective Supplement*, Oxoid, Basingstoke, UK) with a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain), allowing the detection as well as the enumeration of *Y. enterocolitica*. After incubation at 30°C for 24 h, all CIN plates were examined using a stereo microscope with Henry illumination (Olympus, Aartselaar, Belgium) and the number of suspect *Yersinia* colonies (typically bull’s eye colonies with a red centre) was counted. From each plate, one suspect colony was streaked onto a Plate Count Agar (PCA) plate (Bio-Rad, Marnes-La-Coquette, France) and incubated at 30°C for 24 h. Then, cultures were transferred into Urease Broth (Ur), Kligler Iron Agar (KIA) (Oxoid) and Tryptone Soy Broth (TSB) (Bio-Rad) and incubated at 30°C for 24 h. When fermentation of glucose, no fermentation of lactose and no development of gas or H$_2$S on KIA and a degradation of urea was observed, the isolates were considered as presumptive *Y. enterocolitica* and were subsequently confirmed using a Polymerase Chain Reaction (PCR) to detect the *ail* gene (Harnett *et al.*, 1996). One hundred microliter from each TSB culture was centrifuged at 12 000 rpm for 2 min. The supernatant was removed, 50 μl PrepMan® Ultra (Applied Biosystems, Foster City, U.S.) was used to suspend the pellet and the tubes were put in a heat-block (Grant, Cambridge, U.K.) for 10 min at 100°C. After 2 min of
cooling down, the tubes were again centrifuged at 12 000 rpm for 2 min. Thirty microliter of the supernatant was stored at -20°C as DNA template in the PCR assay. One microliter of the template was added to 24 μl of the PCR mix (Promega, Leiden, The Netherlands), which contained the ail primer set (Invitrogen, Paisley, U.K.).

3.3. Statistical analysis

Results were recorded in an Excel spreadsheet and the quantitative data were log transformed. Only the samples with countable numbers were taken into account to calculate the mean count for each batch. To identify the limit of detection of infection in batches without any positive tonsil sample, Win Episcope was used (Thrusfield et al., 2001).

4. Results

Samples were collected from 1397 fattening pigs originating from 66 batches. The size of the batches varied from 34 to 930 animals, with a mean batch size of 228. The number of pigs to be sampled varied from 17 to 24, with an average of 21 pigs per batch.

In total, *ail* gene positive *Y. enterocolitica* was recovered from 375 pig tonsils (26.8%; 95% confidence interval (CI): 24.5-29.1%). The within-batch prevalence showed a large variation between the different batches and ranged from 0 to 83.3% (Fig. II). The data presented a bimodal distribution, with modes at the classes 0% (20/66) and 35-39.9% (9/66). In 69.7% of all slaughter pig batches at least the tonsils of one pig was found positive. In 20 batches (30.3%), no *Y. enterocolitica* was isolated from the tonsils. Based on an average of 21 samples per batch, the upper 95% CI limit for the prevalence of negative batches was 12.7%.
The average number of *Y. enterocolitica* in tonsil tissue was 4.04 log$_{10}$ CFU g$^{-1}$ with a standard deviation of 0.97 log$_{10}$ CFU g$^{-1}$ and a maximum of 5.99 log$_{10}$ CFU g$^{-1}$ (Fig. II). In 14 samples, the number of colonies could not be counted due to overgrowth of accompanying flora. Seventy of the 375 *Yersinia* positive tonsils (18.7%) contained between 5.00 and 5.49 log$_{10}$ CFU g$^{-1}$. Eight tonsils (0.02%) were contaminated over 5.49 log$_{10}$ CFU g$^{-1}$ tonsillar tissue.

Figure II. Frequency distribution of the presence of *Y. enterocolitica* in batches (n=66) of slaughter pigs.
The mean *Yersinia* count per batch varied between 3.08 and 5.89 log\(_{10}\) CFU g\(^{-1}\) tonsillar tissue with a standard deviation of 0.54 log\(_{10}\) CFU g\(^{-1}\) (Fig. IV). Out of the 46 infected batches, most (n=18) had a contamination level between 4.00-4.49 log\(_{10}\) CFU g\(^{-1}\).
5. Discussion

In consequence of the concept of the future European meat inspection, determining the microbiological prevalence in batches could allow division of farms into different risk groups (Biohaz, 2011).

Nonetheless, the information about the distribution of the prevalence of human pathogenic *Y. enterocolitica* within pig batches at slaughter is limited. Therefore the number of samples to be taken per herd was determined at the beginning of this study, based on an expected batch prevalence of 50%. In 20 pig herds (30.3%), no infected pigs were found. Nevertheless, batches with a prevalence below 12.7% might not be detected due to the sample size used. Moreover, only tonsil samples were examined in the present study, as tonsils are the most reliable samples to detect *Y. enterocolitica* in pigs (Nesbakken et al., 2003; Gürtler et al., 2005). Therefore, information about other tissues or organs could have resulted in more positive batches. Hence, we have no information whether the negative batches in the present study are indeed free of *Y. enterocolitica* or have a prevalence below the detection limit.

In the present study, 69.7% of the batches was positive for human pathogenic *Y. enterocolitica*, which is within the range of other studies. The number of positive batches varies between 15 and 94% among different studies (Skjerve et al., 1998; Martinez et al., 2010; Poljak et al., 2010; Terentjeva and Berzins, 2010; Virtanen et al., 2011). Several factors may attribute to this variation, such as the number of samples per herd, the sample matrix and the test method. Skjerve et al. (1998) found pigs with positive serum samples in 63% of the herds in Norway analyzing 5 to 43 pigs per herd. Virtanen et al. (2011) isolated *Y. enterocolitica* from 94% of Finnish farms testing the tonsils and faeces of 2 to 86 pigs per herd.

The prevalence within batches in the current study varied from 0 to 83.0%. Similarly, Gürtler et al. (2005) examined pig tonsils of six batches at slaughter, which resulted in a within-batch prevalence between 8 and 69%. Based on serology, von Altrock et al. (2011)
investigated 80 farms (30 samples per herd) which resulted in a within-batch prevalence of 0 to 100%. In contrast, Nowak et al. (2006), found only a small number of pigs per farm to be infected with *Y. enterocolitica* in the tonsils. Except for the study of Nowak *et al.* (2006), the within-batch prevalence seems to vary greatly among different farms.

Destructive tissue samples were used in this study as swabbing the tonsil has already been shown to be insufficient for the detection of *Y. enterocolitica* in pig tonsils (Nesbakken *et al.*, 1985; Van Damme *et al.*, 2012). Both studies yielded more positive tonsillar tissue samples compared to swab samples, although each study applied different isolation methods. Moreover, direct plating was used as the only isolation method as it has already been shown to be an adequate method for isolation of *Y. enterocolitica* from pig tonsils and additionally quantitative data can be obtained (Van Damme *et al.*, 2010). Moreover, because the main goal of the present study was to gain insight in the distribution of the prevalence in pigs at slaughter, positive samples due to cross-contamination occurring at the slaughterhouse has to be excluded as much as possible.

To our knowledge, for the first time, quantitative data per herd was obtained. Highly contaminated pigs are more likely to cause cross-contamination to the carcass. There is a low within-batch prevalence observed when there is a high mean count. This might be explained by the moment of infection (Nesbakken *et al.*, 2006). A recent infection of pigs might cause colonization of a limited number of pigs within a group, leading to high *Yersinia* count in the tonsils. This hypothesis has to be confirmed by serology, since recent infected animals would lack antibodies.

In conclusion, there is a wide distribution of the prevalence of human pathogenic *Y. enterocolitica* in batches of pigs at slaughter. Therefore it may be interesting to include in risk factor studies beside the *Y. enterocolitica* status of the batches of pigs also the prevalence in the batches. In such studies sufficient samples have to be collected in order to estimate the prevalence more accurately so that potential risk factors can be better linked to the observed prevalences in the studied batches.
WITHIN-BATCH PREVALENCE AND QUANTIFICATION OF HUMAN PATHOGENIC
YERSINIA ENTEROCOLITICA AND Y. PSEUDOTUBERCULOSIS IN TONSILS OF PIGS AT SLAUGHTER

1. Abstract

Yersiniosis is a common bacterial zoonosis in Europe and healthy pigs are known to be the primary reservoir of human pathogenic *Yersinia enterocolitica* and *Y. pseudotuberculosis*. However, little information is available about the prevalence of these pathogens within pig batches at time of slaughter. The tonsils of 7047 fattening pigs, belonging to 100 farms, were aseptically collected immediately after evisceration in two Belgian slaughterhouses. The batch size varied between 70 and 930 pigs. On average, 70 pigs were sampled per batch. The tonsils were examined by direct plating on cefsulodin-irgasan-novobiocin (CIN) agar plates and the number of suspect *Yersinia* colonies was counted. Pathogenic *Y. enterocolitica* serotype O:3 were found in tonsils of 2009 pigs (28.5%), originating from 85 farms. The within-batch prevalence in positive farms ranged from 5.1 to 64.4%. The number of *Y. enterocolitica* in positive pigs varied between 2.01 and 5.98 $\log_{10}$ CFU g$^{-1}$ tonsil, with an average of 4.00 $\log_{10}$ CFU g$^{-1}$ tonsil. *Y. pseudotuberculosis* was found in seven farms, for which the within-batch prevalence varied from 2 to 10%. In five of these farms, both *Y. enterocolitica* and *Y. pseudotuberculosis* were simultaneously present. Human pathogenic *Yersinia* spp. are widespread in slaughter pig batches in Belgium as 87% of the tested batches were infected with these pathogens at time of slaughter. The large variation of the prevalence between batches may lead to different levels of contamination of carcasses and risks for public health.
2. Introduction

Yersiniosis is a significant foodborne disease in Europe, primarily caused by the species *Yersinia enterocolitica* (98%) and to a lesser account by *Y. pseudotuberculosis*. The average EU incidence of yersiniosis is 1.63 per 100,000 inhabitants (EFSA and ECDC, 2013). Clinical manifestation is symptomized by diarrhea, abdominal pain and fever and occurs mostly in young children (Bottone, 1999). In children (> 4 years) and adults, pain in the lower right abdomen and fever may be the predominant symptoms and therefore it is often confused with appendicitis (Bottone, 1999).

The pathogenicity of *Yersinia* spp. is determined by the presence of virulence genes encoded on the chromosome and on the pYV. The sequence of the chromosomally encoded virulence genes *ail*, *yst* and *inv* are species-dependent and sometimes even vary within one species. Different PCR based assays have been developed for the identification of enteropathogenic *Yersinia* spp. with several PCR assays targeting the pYV-encoded genes, such as *yadA* and *virF* (Miller et al., 1989; Cornelis, 1998; Bottone, 1999; Revell and Miller, 2001).

The species *Y. enterocolitica* is divided into six biotypes (1A, 1B, 2-5), of which only biotype 1A does not carry pYV and is therefore considered apathogenic. Further differentiation into serotypes is not biotype-dependent, though some combinations, such as bioserotypes 4/O:3 and 2/O:9, are more frequently distributed in Europe and cause most human infections (EFSA and ECDC, 2013).

Although many animal species may carry pathogenic yersiniae, pigs are known as the main reservoir of human pathogenic *Y. enterocolitica*, especially of bioserotype 4/O:3 (EFSA and ECDC, 2013). Yersiniae are predominantly present in pigs’ tonsils and are shed in the faeces (Fredriksson-Ahomaa et al., 2001a). During slaughter, carcasses can be faecally contaminated or contaminated by infected tissues of its own or its near neighbors. The risk of (cross-)contamination in the slaughterhouse can be reduced by a decrease of the within-batch prevalence (Laukkonen et al., 2009).
In a qualitative risk analysis of foodborne hazards in pork performed by the European Food Safety Authority (EFSA), *Y. enterocolitica* ranked the second most important pork-related bacterial pathogen (Biohaz, 2011). EFSA proposed to categorize slaughter pig batches in risk classes, in order to facilitate appropriate actions taken at slaughterhouse-level. Therefore, information regarding the within-herd prevalence is required (Biohaz, 2011).

Few studies have reported the within-herd prevalence so far. Skjerve et al. (1998) and von Altrock et al. (2011) determined this within-batch prevalence serologically. The other researchers used a microbiological pathway, using different sample matrices (Nowak et al., 2006; Laukkanen et al., 2009, Fondrevez et al., 2010; Virtanen et al., 2011; Novoslavskij et al., 2012; Chapter 1). As tonsils remain infected for a longer period than faeces, the within-batch prevalence of pigs at slaughter is preferably assessed by microbiological examination of tonsils rather than faeces (Nesbakken et al., 2006).

As little information is available about the prevalence of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* within pig batches, the aim of this study was to determine the within-batch prevalence of these pathogens in the tonsils of pigs at slaughter.

### 3. Materials and methods

#### 3.1. Sampling

From January until December 2012, in two Belgian slaughterhouses (A and B), tonsils from 7047 fattening pigs, representing 100 pig batches, were aseptically removed from the head immediately after evisceration. All batches originated from 100 different conventional farrow-to-finish and fattening pig farms. The pigs were slaughtered at an age of 6 to 6.5 months with a live body weight of about 120 kg. The number of pigs to be sampled per batch was calculated based on an expected batch prevalence of 50%, with a
confidence level of 95% and an accepted error of 10%. The tonsils were transported under cooled conditions to the laboratory and analyzed within the same day.

3.2. **Detection and enumeration of human pathogenic Y. enterocolitica and Y. pseudotuberculosis**

The detection and enumeration are described in Chapter 1.

3.3. **Confirmation by PCR**

DNA was extracted as described by Van Damme *et al.* (2013). A first PCR targeting the virulence genes *ail*, *yst* and *virF* was performed (Harnett *et al.*, 1996). A second multiplex PCR was processed to determine the serotype with *rfbC* and *per* primer sets (Weynants *et al.*, 1996; Jacobsen *et al.*, 2005). When no fragments or only the fragment generated for the *virF* was present, a third PCR assay was performed targeting the *inv* gene to identify *Y. pseudotuberculosis* (Nakajima *et al.*, 1992).

3.4. **Statistical analysis**

Results were registered in an Excel spreadsheet and the quantitative data were log transformed. The detection limit of the direct plating method was $2 \log_{10}$ CFU g$^{-1}$ tonsil. To calculate the mean count for each batch, only the samples with countable numbers were taken into account. Win Episcope was used (Thrusfield *et al.*, 2011) to identify the limit of detection of infection in batches without any positive tonsil sample. A batch was considered positive when at least one pig within the batch carried *Y. enterocolitica* or *Y. pseudotuberculosis* in the tonsils. The overall prevalence and the 95% confidence interval (CI) for *Y. enterocolitica* and *Y. pseudotuberculosis* was calculated using Stata/MP 12.1 (StataCorp, 2011), declaring batches as the primary sampling units (clusters).
4. Results

Pigs from 100 farms were sampled in two Belgian slaughterhouses. The batch size ranged from 70 to 930, with a mean of $314 \pm 169$ pigs per batch. The calculated sample size varied from 41 to 88 pigs per batch (mean: $70 \pm 10$). In total, tonsils from 7047 pigs were collected.

In 2009 tonsils (28.5%; 95% CI: 24.9-32.2%), *ail*- and *yst*-positive *Y. enterocolitica* serotype O:3 was present (Table VII). In 85 of the 100 batches, at least one pig carried *Y. enterocolitica* in the tonsils. The mean within-batch prevalence of positive batches was $33.5\% \pm 17.6\%$ and ranged from 5.1 to 64.4% (Fig. V). The data presented a bimodal distribution, with modes at classes 0% (15/100) and 25-35% (22/100). The smallest and largest negative batch (n=15) included 170 and 540 pigs respectively, which represents a variation of the upper 95% CI limit for the prevalence in negative batches between 4.12 and 3.33%.
Table VII. Presence and quantification of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in pigs (n=7047) and batches (n=100).

<table>
<thead>
<tr>
<th></th>
<th>Number of positive pigs (%)</th>
<th>Quantification in positive tonsils (log_{10} CFU g^{-1} tonsillar tissue)</th>
<th>Number of positive batches (%)</th>
<th>Within-batch prevalence (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantification in positive batches (log_{10} CFU g^{-1} tonsillar tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Range</td>
<td></td>
<td>Mean Range</td>
<td>Mean Range</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>2009 (28.5)</td>
<td>4.00 ±0.96 2.01-5.98</td>
<td>85 (85.0)</td>
<td>33.5 ±17.6 5.1-64.4</td>
<td>3.96 ±0.29 2.91-4.67</td>
</tr>
<tr>
<td><em>Y. pseudotuberculosis</em></td>
<td>23 (0.3)</td>
<td>3.60 ±0.94 2.01-5.49</td>
<td>7 (7.0)</td>
<td>5.3 ±25.3 2-10</td>
<td>3.55 ±0.61 2.48-4.34</td>
</tr>
<tr>
<td>Human pathogenic Yersinia spp.</td>
<td>2031 (28.8)</td>
<td>3.97 ±0.96 2.01-5.98</td>
<td>87 (87.0)</td>
<td>33.2 ±17.2 4.1-64.6</td>
<td>3.92 ±0.29 2.91-4.67</td>
</tr>
</tbody>
</table>

<sup>a</sup>: only counting for positive batches

Table VIII. Comparison between the two slaughterhouses.

<table>
<thead>
<tr>
<th>S&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Number of-positive pigs (95% CI)</th>
<th>Proportion of positive pigs (95% CI)</th>
<th>Number of positive batches</th>
<th>Mean within-batch prevalence&lt;sup&gt;d&lt;/sup&gt; (95% CI)</th>
<th>Y. pseudotuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50</td>
<td>3488</td>
<td>290 (251; 328)</td>
<td>29.0 (23.6; 34.5)</td>
<td>41</td>
<td>35.9 (5.4;66.4)</td>
<td>0.4 (0-0.9)</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>3559</td>
<td>337 (283; 392)</td>
<td>28.0 (23.2; 32.8)</td>
<td>44</td>
<td>31.3 (3.1;59.5)</td>
<td>0.1 (0-0.5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: slaughterhouse  <sup>b</sup>: number of batches  <sup>c</sup>: number of pigs  <sup>d</sup>: based on positive batches only
The number of *Y. enterocolitica* in positive tonsils and positive batches is shown in Table VII. In 87 samples, the number of colonies was not countable due to the overgrowth by accompanying flora. The number of *Y. enterocolitica* in positive tonsils varied between 2.01 and 5.98 log$_{10}$ CFU g$^{-1}$ tissue, with a mean of 4.00 ± 0.96 log$_{10}$ CFU g$^{-1}$ tonsil. The frequency distribution of *Y. enterocolitica* counts in the 2009 contaminated tonsils is shown in Figure VI. At batch-level, the mean number ranged from 2.91 to 4.67 with an overall mean of 3.96 ± 0.96 log$_{10}$ CFU g$^{-1}$ tonsil. The frequency distribution of *Y. enterocolitica* mean numbers in positive batches is shown in Figure VII.

Only seven pig batches were positive for *Y. pseudotuberculosis* and showed a within-batch prevalence ranging from 2 to 10% (Table VII). In 23 tonsils (0.33%; 95% CI: 0.05-0.60%), inv-positive *Y. pseudotuberculosis* was present. From five batches, both *Y. enterocolitica* and *Y. pseudotuberculosis* were isolated, though both pathogens were only simultaneously present in one pig. In the 23 tonsils infected with *Y. pseudotuberculosis*, the contamination level varied between 2.01 and 5.49 log$_{10}$ CFU g$^{-1}$ tonsillar tissue with an
average of $3.60 \pm 0.94 \log_{10} \text{CFU g}^{-1} \text{tonsil}$. At batch level, the mean count of the *Y. pseudotuberculosis* positive batches ranged from $2.48$ to $4.34 \log_{10} \text{CFU g}^{-1} \text{tonsillar tissue}$, with an average of $3.55 \pm 0.61 \log_{10} \text{CFU g}^{-1} \text{tonsil}$.

**Figure VI.** Frequency distribution of *Y. enterocolitica* counts in tonsillar tissue.

**Figure VII.** Frequency distribution of the mean *Y. enterocolitica* counts in tonsillar tissue per pig batch.
Comparing the two slaughterhouses, the overall proportion of positive pigs was very similar in both slaughterhouses (Table VIII). The mean batch size and the number of *Y. enterocolitica* positive batches were slightly higher in slaughterhouse B than in slaughterhouse A. The mean within-batch prevalence of *Y. enterocolitica* positive batches was higher in slaughterhouse A, though no significant differences were observed.

In total, 3930 *Y. enterocolitica* O:3 isolates were collected, of which 260 (6.6%) showed no degradation of urea. All of these urea-negative isolates harbored *ail*, *yst*, *rfbC* and most of them (188/260) also *virF*. Only urea-negative isolates were present in three batches, 14 batches yielded both urea-positive and negative isolates whereas all isolates from 68 batches were able to degrade urea. Fifteen pig tonsils harbored urea-negative as well as urea-positive isolates. One out of 46 *Y. pseudotuberculosis* isolates did not show degradation of urea.

The presence of the pYV was verified by PCR targeting the *virF*-gene. Nine hundred and three *Y. enterocolitica* isolates (23%) were *virF*-negative (Table IX). The prevalence of *virF*-negative isolates per batch ranged from 0 to 60%. In seven batches all isolates were *virF*-positive. Both types of isolates could be present in the same pig tonsil. Only 3 pig tonsils harbored *Y. pseudotuberculosis* without *virF*.

Table IX. The presence of *virF* in *Y. enterocolitica* and *Y. pseudotuberculosis* at isolate-, pig- and batch-level.

<table>
<thead>
<tr>
<th></th>
<th><em>Y. enterocolitica</em></th>
<th></th>
<th><em>Y. pseudotuberculosis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolate (n=3930) (%)</td>
<td>Pig (n=7047) (%)</td>
<td>Batch (n=100) (%)</td>
</tr>
<tr>
<td><em>virF</em> -</td>
<td>903 (23.0)</td>
<td>572 (8.1)</td>
<td>78 (78.0)</td>
</tr>
<tr>
<td><em>virF</em> +</td>
<td>3027 (77.0)</td>
<td>1688 (24.0)</td>
<td>85 (85.0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3930 (100)</td>
<td>2009 (28.5)</td>
<td>85 (85.0)</td>
</tr>
</tbody>
</table>
5. Discussion

The present study demonstrates that fattening pig batches were frequently infected with human pathogenic *Y. enterocolitica* O:3. However within a positive batch the number of colonized pigs varied widely. Due to the possible cross-contamination in the slaughterhouse, presence of a high proportion of infected pig batches at slaughter represents a potential risk for public health (Nesbakken *et al.*, 2003). The high prevalence of enteropathogenic *Y. enterocolitica* at farm-level can result in a high contamination rate of the carcass (Laukkanen *et al.*, 2009).

The batch-level prevalence of batches positive for *Y. enterocolitica* ranged in the present study from 5.1 to 64.4%, which is very similar to the result of Gurtler *et al.* (2005) (5.6 to 68.8%). This is in contrast to the analysis of Poljak *et al.* (2010) and Terentjeva and Bezins (2010), where the within-batch prevalence ranged from 7 to 100% and from 0 to 100% respectively. Several factors may attribute to this variation, such as the sample size (410-2400), sample matrix (tonsils, faeces or blood) and the test method (PCR, cold enrichment, ELISA). The variation in the within-batch prevalence in a study can be explained by the variability in the farm management and within-farm factors, like the use of fully slatted floors and municipal water (Nowak *et al.*, 2006, Virtanen *et al.*, 2011, von Altrock *et al.*, 2011).

In the present study, 15% of the batches were *Y. enterocolitica* negative which suggests a widespread infection of pig batches at slaughter with *Y. enterocolitica*. This result is comparable with the study of Martinez *et al.* (2011) which was performed in the same region and where 2 of the 10 farms (20%) were *Yersinia*-free. Other studies reported an amount of negative batches varying from 5.8% to 46.7% (Bhaduri *et al.*, 2005; Martinez *et al.*, 2009; Terentjeva and Bezins, 2010; Virtanen *et al.*, 2011; Novoslavskij *et al.*, 2013).

In the current study the Belgian overall-prevalence of *Y. enterocolitica* was 28.5%. This study was set up to determine the prevalence at batch-level. In Chapter 1, the pig-level prevalence was 26.8%. The result is comparable to other studies reported in the same
country. Van Damme et al., (2010) and Martinez et al. (2011) estimated this prevalence at 37.4% and 44% respectively. This variation could be due to a different study design (determining within-batch prevalence by stratified random sampling versus overall-prevalence by random sampling) and the isolation method (direct plating, selective and cold enrichment: via enrichment procedures it is possible to detect low concentrations) used. Between pig-producing countries a large variation is noticed when considering the results of prevalence studies in Greece (13%), Lithuania (25%), Italy (32%), Switzerland (34%), Latvia (35-64%), Finland (49.9%), Estonia (89%), Spain (93%), Canada (5.1-35.1%) and the U.S. (13.1%) (Gürtler et al., 2005; Fredriksson-Ahomaa et al., 2007; Kechagia et al., 2007; Martinez et al., 2009; Poljak et al., 2010; Terentjeva and Bezins, 2010; Martinez et al., 2011; Virtanen et al., 2011; Novoslavskij et al., 2012).

Little information is available about the within-batch prevalence of *Y. pseudotuberculosis*. In the current study, it ranged from 2 to 10%. This contrasts with the findings of Terentjeva and Bezins (2010), were the prevalence peaked at 40% in one batch. Laukkanen et al. (2008) found a within-farm prevalence up to 75%, and a higher prevalence in pig farms with an organic compared to a conventional management. These different management systems may have a different input of *Yersinia* spp. because organic systems are more influenced by environmental conditions (climate, wild animals) than conventional systems. In the present study, only conventional farms were included as organic farms are very rare in Belgium. Due to this lower within-batch prevalence, a large number of samples need to be taken to detect batches infected with *Y. pseudotuberculosis* than with *Y. enterocolitica*. The *Y. pseudotuberculosis* negative batches were more common in the present study, as only 7 batches were infected, which is a much lower prevalence compared to the study of Martinez et al. (2011). The latter study detected 8 out of 10 farms positive for the presence of *Y. pseudotuberculosis*. Studies carried out in other regions (Estonia, Latvia and Lithuania), showed a higher number of infected batches, varying from 13 to 60% positive batches (Martinez et al., 2009; Terentjeva and Bezins, 2010; Novoslavskij et al., 2012). In the present study, only 23 out of 7047 pigs (0.3%) carried *Y. pseudotuberculosis*. This is a similar result like in most other European countries as Spain (0%), Italy (1%), Estonia (1%), Latvia (3-5%), Finland (4%) and as reported in Russia.
(7%) (Martinez et al., 2009; Terentjeva and Bezins, 2010; Martinez et al., 2011). This variance could be explained by the differences in management system (organic versus conventional) like mentioned earlier.

The two slaughterhouses showed no significant variation in their results, so the different input of pigs has no influence (Table VIII). Fredriksson-Ahomaa et al. (2000), who used different isolation and PCR methods, found a significant difference between slaughterhouses, which may be due to differences in slaughtering procedures, hygiene measurements and origin of incoming pigs.

Only few data are available about the number of *Y. enterocolitica* and *Y. pseudotuberculosis* in slaughter pig tonsils at pig- and batch-level (Van Damme et al., 2010; Van Damme et al., 2012; Chapter 1). Tonsils of 328 pigs contained more than 5.00 log$_{10}$ *Y. enterocolitica* g$^{-1}$ tissue. Highly contaminated pigs may be more likely to cause cross-contamination during slaughter. To our knowledge, the present study was the first comprehensive study enumerating *Y. pseudotuberculosis* in pig tonsils. In the case tonsils were positive for *Y. pseudotuberculosis* comparable numbers were present as for *Y. enterocolitica*.

In the present study, only *Y. enterocolitica* serotype O:3 was detected. The dominance in Belgium of bioserotype 4/O:3 has also been described in Van Damme (2013) and in Chapter 1. However, Martinez et al. (2011) found bioserotype 3/O:9 in 8 (9%) of the 89 positive samples collected in Belgium. Martinez et al. (2011) did not mention if all bioserotype 3/O:9 were isolated from pigs originating from the same batch or the same farm. The latter data indicates that other bioserotypes than 4/O:3 are presented in the Belgian pig population.

To our knowledge, it is the first time that the presence of the enzyme urease was considered in isolates derived from pigs on a large scale. This has an implication for the first screening of potential colonies (Devenish and Schiemann, 1981): to avoid false negatives, also urease negative colonies should be retained for further testing.
Almost 23% of the isolates were virF-negative, which is similar compared to the study of Martinez et al. (2009, 2011) and Van Damme et al. (2013) where 19%, 23% and 18.5% of the isolates from tonsil samples did not carry the pYV respectively. The wide range of presence of virF-negative samples within a batch raises the question whether loosing the pYV during isolation is the only cause of pYV-negative human pathogenic Y. enterocolitica (Van Damme et al., 2013). It can be argued that the inability to produce urease and the absence of pYV are related, but this is not correlated (data not shown). There is even no batch included in this study that was completely virF- and urease-negative. Similar to the results of Martinez et al., (2009), the amount of Y. enterocolitica virF-negative samples is larger than the Y. pseudotuberculosis virF-negative samples.

In conclusion, there can be a large variation in the within-batch prevalence of human pathogenic Y. enterocolitica and Y. pseudotuberculosis in pig batches at time of slaughter. The proposal of the biohazard panel of EFSA is to collect data about the infection rate of pig batches prior to slaughter (Biohaz, 2011). Due to the large amount of positive batches, a risk based classification of pig batches before slaughter, as also proposed by EFSA, will be challenging. Nevertheless, this variation may be used to determine factors which are influencing this prevalence.
SEROPREVALENCE OF ENTEROPATHOGENIC YERSINIA SPP. IN PIG BATCHES AT SLAUGHTER

1. Abstract

Enteropathogenic *Yersinia* spp. are one of the main causes of foodborne bacterial infections in Europe. Slaughter pigs are the main reservoir and carcasses are contaminated during a sub-optimal hygienically slaughtering-process. Serology is potentially an easy option to test for the *Yersinia*-status of the pig (batches) before slaughter. A study of the variation in activity values (OD%) of *Yersinia* spp. in pigs and pig batches when applying a serological test was therefore conducted. In this study, pieces of the diaphragm of 7047 pigs, originating from 100 farms, were collected and meat juice was gathered, where after an enzyme-linked immunosorbent assay (ELISA) Pigtype Yopscreen (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany) was performed. The results were defined positive if the activity values exceeded the proposed cut-off value of 30 OD%. Results at pig level displayed a bimodal-shaped distribution with modes at 0 to 10% (n=879) and 50 to 60% (n=667). The average OD% was 51% and 66% of the animals tested positive. The within-batch seroprevalence ranged from 0 to 100% and also showed a bimodal distribution with modes at 0% (n=7) and 85-90% (n=16). On 7 farms, no single seropositive animal was present and in 22 farms, the mean OD% was below 30%. Based on the results obtained at slaughter, 66% of the pigs had contact with enteropathogenic *Yersinia* spp. at farm level. The latter occurred in at least 93% of the farms. Many farms are harboring enteropathogenic *Yersinia* spp.
2. Introduction

Enteropathogenic Yersinia spp. are an important cause of foodborne bacterial infections in Europe. Two species, human pathogenic Y. enterocolitica and Y. pseudotuberculosis, caused 7017 infections or 1.63 infections/100000 inhabitants in 2011 in Europe, with human pathogenic Y. enterocolitica responsible for more than 98% of these infections (EFSA and ECDC, 2013). Symptoms range from mild, self-limiting diarrhea to mesenteric lymphadenitis. However, other chronic disorders like reactive arthritis or erythema nodosum can also emerge (Bottone, 1997; EFSA and ECDC, 2013). Many animal species may be carrier of these pathogens, but pigs are regarded as the main reservoir. Handling and consumption of raw or undercooked pork are the primary risk factors for human infection (Tauxe et al., 1987; Nikolova et al., 2001; Boqvist et al., 2009; EFSA and ECDC, 2013).

Yersinia strains harboring the virulence plasmid (pYV) are pathogenic for humans. Strains lacking the pYV like Y. enterocolitica biotype 1A are considered apathogenic (Revell and Miller, 2001). Pathogenic strains trigger an antibody response against for example an integrated antihost system encoded on the pYV, the Yop (Yersinia outer membrane proteins) virulon, which consists of (1) a delivery apparatus and (2) a specialized type III Yop secretion system (Ysc) allowing the delivery of (3) effector Yops into eukaryotic cells, the latter controlled by YopN. The effector Yops have different functions: they can disrupt the cytoskeleton (YopE), round up cells (YopE, YopO) and induce apoptosis of macrophages (YopP, YopJ) (Rosqvist et al., 1991; Cornelis and Wolf-Watz, 1997; Mills et al., 1997; Cornelis et al., 1998; Heesemann et al., 2006).

On-farm infections are still active when pigs are delivered to the slaughterhouse. Due to slaughter procedures, bacteria present in faeces and in the throat of infected pigs can contaminate other carcasses during processing (Laukkanen et al., 2009). In a recent qualitative risk analysis of foodborne hazards in pork, human pathogenic Y. enterocolitica is indicated as a risk of medium relevance for human health in the EU, based on the probability of occurrence, the severity of consequences and the proportion of cases
caused by pig meat (Biohaz, 2011). One of the recommendations was to modernize current meat inspection towards a more risk-based approach. For this reason, it would be useful to have information about the infection status of slaughter pig batches before their arrival at the slaughterhouse. After comparing bacteriology and serology, serological data should allow the identification of high and low risk farms, which creates the opportunity to implement a risk based porcine meat inspection. Currently, it remains difficult to determine the infection status of fattening pigs on farms because microbiological isolation before slaughter requires sampling of the tonsils. Sampling of the faeces is not informative at moment of slaughter, the pigs may still carry *Y. enterocolitica* in the tonsils, but not shed them in the faeces (Nesbakken, 2006). Moreover, since microbiological isolation is time consuming, serology of meat juice samples from pigs during slaughter may be used to determine the infection rate of farms. By using serology, it should be possible on a later stage to link these results with the on-farm infections. Therefore, the aim of this study was to evaluate if there is variation in the serological prevalence of human pathogenic *Y. enterocolitica* in pigs at slaughter.

### 3. Material and methods

The pigs sampled for the study of Chapter 2 are the same pigs sampled for this study. A piece of the diaphragm pillar ($\pm$ 10g) was collected immediately after splitting the carcass. Upon arrival at the laboratory, all samples were stored at -20°C. After two to three weeks, the samples were thawed during 48 h at 4°C and 2 ml of meat juice was collected and stored at -20°C until analysis. The enzyme-linked immunosorbent assay (ELISA) Pigtype Yopscreen (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany) was performed according to the manufacturer’s instructions. The amount of antibodies against Yops, expressed by the optical density (OD), was determined with a spectrophotometer (Tecan SpectraFluor, MTX Lab Systems, Virginia, U.S.) at 450 nm. Activity values (OD%) were calculated based on the measured OD values, and the mean OD values of positive and negative controls using the following formula:
Activity value (OD%) = \frac{OD_{sample} - OD_{neg}}{OD_{pos} - OD_{neg}}

The results were considered positive if the activity value exceeded the cut-off value of 30 OD% as proposed by the manufacturer. The within-batch seroprevalence is the comparison of the number of positive pigs to the total number of sampled pigs (1/0 cut-off 30 OD%). A batch was classified as positive when at least one pig within the batch had an activity value above the cut-off value.

4. Results

Pigs from 100 batches were examined in two Belgian slaughterhouses. The batch size varied from 70 to 930 pigs, with a mean batch size of 314 ± 169 pigs. According to previous calculations, the sample size varied from 41 to 88 pigs per batch (mean: 70 ± 10). In total, pieces of the diaphragm of 7047 pigs were collected.

The OD% of the individual pigs showed a large variation ranging from -4.9 to 181.6 OD% (Fig. VIII). The results displayed a bimodal-shaped distribution with modes at 0-10% (n=879) and 50-60% (n=667). The average OD% was 51%. Sixty-six percent of the animals were classified as positive according to the used cut-off value. The mean OD% per batch was also very variable (from -1 to 95). In 22 batches the mean OD% was less than 30% (Fig. IX).
The within-batch seroprevalence (pigs with OD% > 30% / total pigs sampled per batch) ranged from 0 to 100% and displayed a bimodal distribution with modes at 0% (n=7) and 85-90% (n=16) (Fig. X). On only seven farms, no seropositive animal was present. The mean within-batch seroprevalence was 66.4%.

Figure VIII. Frequency distribution of activity values (OD%) in meat juice originating from pigs at slaughter (dashed line: cut-off value of 30 OD%).

Figure IX. Frequency distribution of activity values (OD%) in meat juice of batches (n = 100) of slaughter pigs (dashed line: cut-off 30 OD%).
Chapter 3

5. Discussion

In the present study, the seroprevalence of 66% of pigs at slaughter pointed towards a frequent contact with enteropathogenic *Yersinia* spp. during the rearing period. Few studies reported on the overall prevalence. In the study of Thibodeau et al. (2001) performed in Canada, an overall prevalence of 66% in 291 tested animals was presented, using a lipopolysaccharide (LPS)-ELISA. In the study of von Altrock et al. (2011) 80 batches (30 pigs/batch) were tested, with an overall prevalence of 64.1%. This study was performed in Germany (Lower Saxony) with the same ELISA kit as the present study. These results are similar with the one found in the present study.

Few studies are currently available about the within-batch seroprevalence. A research of Skjerve et al. (1998), conducted in Norway using a LPS-ELISA, sampled 287 batches (5 to...
43 pigs/batch), from which 182 (63.5%) were found positive, though the within-batch prevalence ranged from 0 to 100%. Meemken and Blaha (2011) studied the *Yersinia* within-herd seroprevalence in 6 herds, which also ranged from 0 to 100% (10 to 108 pigs/herd), using the same ELISA kit as the present study. In the research of von Altrock *et al.* (2011) the average within-herd seroprevalence was 65.7% which is similar to the present findings. They found 16% of the batches seronegative in contrast to 7% in the present study. This lower number of seronegative batches could be due to the higher number of pigs that were sampled per batch.

As shown in Figure IX, many batches show a high mean OD%, which is correlated with a high level of antibodies in the meat juice. This was also the case with the results found by von Altrock *et al.* (2011). However, the number of batches with a seroprevalence (1/0 cut-off 30 OD%) above the 90% was much higher (52.2%) in the study of von Altrock *et al.* (2011) in contrast to the present study (28%). Nevertheless, 44% of the farms in the present study have a seroprevalence above the 85%, which indicates that positive farms often deliver a high number of seropositive slaughter pigs.

In line with the EFSA proposal to implement a more risk based porcine meat inspection (Biohaz, 2011), a categorization of the infection status of batches arriving at the slaughterhouse should be made. This classification can be either based on microbiological examination or on serological data. The prevalence based on microbiological analysis of the tonsils can only be determined pain and stress free and is easy to perform when samples are taken at slaughterhouse level. Therefore, the prevalence based on serological analysis of meat juice should be preferred: it could be determined prior to slaughter, obtaining blood samples from living pigs is less stressful for these pigs compared to collecting tonsil samples and it can be combined with serological tests for other pathogens. Nesbakken *et al.* (2006) stated that serological testing of pigs from 100 days of age until slaughter age can be applied as a basis for classification between herds free from *Yersinia* spp. and infected herds. In the present study, meat juice was selected instead of blood serum samples as Meemken and Blaha (2011) showed an excellent agreement between blood serum collected at slaughter age and meat juice.
A disadvantage of using serology is the possibility that pigs can already be infected but do not have antibodies yet. It takes 12 to 19 days for seroconversion by experimental infection -which could be longer in practice-, but only a few hours for contamination of the tonsils in five-week old piglets by oral inoculation (Nielsen et al., 1996; Thibodeau et al., 1999). Moreover, one has to take into account that when the infection is finished and the pigs do not harbor *Yersinia* spp. any longer, antibodies are still present. The association between the within-batch microbiological (based on tonsils) and serological (based on meat juice) prevalence is still not clear. Research about this relationship at time of slaughter should be considered.

The seroprevalence of pigs at slaughter varies widely between pig batches of different farms. Due to this large variety, the seroprevalence offers the opportunity to determine factors that influence this prevalence. Further research about risk factors that determine the within-batch prevalence should be performed.
CHAPTER 4

PREDICTION OF THE INFECTION STATUS OF PIGS AND PIG BATCHES AT SLAUGHTER WITH HUMAN PATHOGENIC *YERSINIA* SPP. BASED ON SEROLOGICAL DATA
1. Abstract

Pigs are the main reservoir of *Y. enterocolitica*, and the microbiological and serological prevalence of this pathogen differs between farms. The infection status of pig batches arriving at the slaughterhouse is largely unknown. Moreover, a link between the presence of human pathogenic *Yersinia* spp. and the presence of antibodies is missing. A relation between the microbiological and serological prevalence could help to predict the infection of the pigs prior to slaughter. Pigs from 100 different batches were sampled. Tonsils and pieces of diaphragm were collected from 7047 pigs (on average 70 pigs per batch). The tonsils were analyzed using a direct plating method and confirmed with a multiplex Polymerase Chain Reaction (*ail*, *yst*, *virF*). The meat juice of the diaphragm pillars was analyzed by Enzyme Linked ImmunoSorbent Assay Pigtype Yopscreen (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany). The bacteriological and serological results were compared using a mixed-effects logistic regression at the pig and the batch level. *Yersinia* spp. were found in 2009 pigs, of which 1872 also had antibodies against *Yersinia* spp. According to the logistic regression, the microbiological contamination could not be predicted by the presence of antibodies at pig level. At the batch level, a relation was observed. A mean activity value of 37 Optical Density (OD)% indicates a microbiological positive farm. The equation could predict whether a pig batch will include infected pigs before it arrives at the slaughterhouse.
2. Introduction

Pigs are identified as the main reservoir of human pathogenic *Yersinia enterocolitica* (Tauxe *et al.*, 1987). These *Yersinia* spp. represent 98.4% of the 7000 confirmed human yersiniosis cases in the European Union each year, and most of the remaining cases (1.6%) is caused by *Y. pseudotuberculosis* (EFSA and ECDC, 2013). Human pathogenic *Y. enterocolitica* is also responsible for more than 14000 cases in young children in the United States each year (Scallan *et al.*, 2013).

Pigs infected at farm level are the main source for the (cross-)contamination of pig carcasses at the slaughterhouse (Laukkanen *et al.*, 2009). The knowledge of the infection status of pig batches slaughter may allow a distinction of non-infected and infected pig batches. This way, the status of pigs and pig batches could be taken into account for the slaughter ranking in order to decrease the number of contaminated carcasses. A relation between the presence of *Yersinia* spp. in pigs at the moment of slaughter and the presence of antibodies, which can be obtained prior to slaughter, is therefore needed. There are two common methods to assess the prevalence of human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in pigs and pig batches at slaughter: microbiological analysis of tonsillar tissue or faeces, or serological analysis of meat juice or blood. Pigs at slaughter age are not shedding *Y. enterocolitica* frequently, while their tonsils are still positive and contain a higher number of *Yersinia* spp. (Nesbakken *et al.*, 2006; Van Damme, 2013). Meat juice and blood have a proven excellent agreement (Meemken and Blaha, 2011). The prevalence obtained by both methods in pigs at slaughter display a great variation between pig farms (Fukushima *et al.*, 1983; Letellier *et al.*, 1999; Gurtler *et al.*, 2005; Laukkanen *et al.*, 2009; Novoslavkij *et al.*, 2011). The microbiological and the serological method also show some discrepancies. Depending on the age of the pigs to be sampled and the time of primary infection, a choice of method has to be made. Primarily, the presence of human pathogenic *Yersinia* spp. in pigs and the production of antibodies does not follow the same timeframe. A study performed by Nielsen *et al.*, (1996) followed-up the evolution of antibodies and bacteriology in experimentally infected pigs. Culture-positive faeces were obtained from day 5 to 21 post
infection (p.i.), where after no pigs were shedding *Yersinia* at day 68 p.i. All inoculated pigs had seroconverted at day 19 p.i. and remained seropositive until day 70 p.i. Nesbakken *et al.* (2006) and Vilar *et al.* (2013) studied the natural dynamic of infection. Both studies found a rising antibody-titer starting at an age of 100 days till 5 months. Nesbakken *et al.* (2006) indicated the bacteriological examination of faeces useful between 85 and 135 days of age, while, according to Vilar *et al.* (2013), the *Yersinia*-excretion was peaking in pigs of 2-3 months old. Other studies performed on the natural dynamics of infection also show a peak in excretion, however at an older age (12-21 weeks) (Fukushima *et al.*, 1983; Gurtler *et al.*, 2005; Virtanen *et al.*, 2012). These studies indicate that shedding *Y. enterocolitica* in the faeces happens before antibodies are produced, resulting in an earlier detection of infection by using the microbiological method than serology (Fukushima *et al.*, 1983; Nesbakken *et al.*, 2006; Nielsen *et al.*, 1996). The evolution of antibodies and bacteriology in pigs concerning *Y. pseudotuberculosis* was never studied. Secondly, the dilemma of analyzing tonsils or faeces is also depended on the time of infection. The carriage of enteropathogenic *Y. enterocolitica* last several months in the tonsils, whereas faecal excretion decrease within a few weeks p.i. (Fukushima *et al.*, 1983; Fukushima *et al.*, 1984a; Fukushima *et al.*, 1984b; Nielsen *et al.*, 1996; Nesbakken *et al.*, 2006; Virtanen *et al.*, 2012). The difference between tonsillar and faecal sampling is not obvious for *Y. pseudotuberculosis* (Laukkanen *et al.*, 2008).

The aim of this study is to provide a predictive value based on serology for the prognosis of the microbiological status of pigs and pig batches at slaughter age.

**3. Material and methods**

**3.1. Sample collection, microbiological analysis and serological analysis**

Sample collection and the microbiological and serological analyses have been already described in Chapters 2 and 3.
3.2. **Statistical analysis**

Three different cut-off values were considered in the interpretation of the serological results: 25 OD%, 30 OD% and 35 OD%. The cut-off of 30 OD% is the one proposed by the manufacturer. The value of 25 OD% was previously recommended by the same manufacturer and because this was 5 OD% lower, also a 5 OD% higher value was taken into account. Values lower than the cut-off value were considered as negative, values equal to or higher than the cut-off value were considered as positive. A batch was considered as positive when at least one sample -tonsil or meat juice- was positive. The results of both prevalence-determining methods were compared using a mixed-effects logistic regression at pig and batch level by using Stata/MP 12.1 (StataCorp, 2011). The freedom of disease as well as the sensitivity and the specificity of the test with the microbiology as golden standard was calculated by Win Episcope for both batch and individual level (Thrusfield et al., 2011). The positive and negative predictive values depend on the prevalence. They were also calculated using Win Episcope. The normality tests (Kolmogorov-Smirnov and Shapiro-Wilk test) were calculated by SPSS version 21 (IBM, Armonk, New York, U.S.).

4. **Results**

*Yersinia enterocolitica* was present in 2009 pigs and 23 animals harbored *Y. pseudotuberculosis*. In one pig, both *Y. enterocolitica* and *Y. pseudotuberculosis* were recovered, which makes the total *Yersinia* spp. infected pigs to 2031 (Chapter 2). Of these pigs, 1872 also had a level of antibodies against *Yersinia* spp. above the proposed cut-off of 30 OD% (Table X). In total, there were 4851 pigs positive in at least one sample matrix.
Table X. The categorization of slaughter pigs (n=7047) depending on their microbiological and serological status.

<table>
<thead>
<tr>
<th>Antibodies in meat juice</th>
<th>Human pathogenic Yersinia spp. in tonsils</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent*</td>
<td>2196</td>
<td>159</td>
</tr>
<tr>
<td>Present*</td>
<td>2820</td>
<td>1872</td>
</tr>
<tr>
<td>Total</td>
<td>5016</td>
<td>2031</td>
</tr>
</tbody>
</table>

*based on the activity value using a cut-off of 30 OD%

Out of the 5016 pigs without *Yersinia* spp. in their tonsils, 2196 had no antibodies against these bacteria and 2820 pigs possessed the antibodies. The bacteriological negative pigs displayed a large variation in activity value (-4.90 – 169.08 OD%). However, the majority of the bacteriological negative pigs (n=5016) yielded a low activity value (Fig. XI). The number of pigs with an activity value between -5 and 5 OD% is 1105. Most pigs classified as bacteriological positive (n=2031) also have positive antibody titers (Fig. XII). The results of the microbiological positive pigs are normally distributed (mean=72.14; standard deviation=31.80): the result of the Kolmogorov-Smirnov test was 0.033 (P<0.005) and the result of the Shapiro-Wilk test was 0.994 (P<0.005). Applying the logistic regression, the microbiological contamination could not be predicted by the presence of antibodies at the pig level (Fig. XIII). At the pig level, the sensitivity of the test is 92.2% and the specificity 43.8% when the cut-off value is 30 OD% (Table XI). When this cut-off is changed to 25 OD%, the sensitivity slightly increased to 94.2% and the specificity decreased to 40.0%. If the cut-off is changed to 35 OD%, the sensitivity drops to 88.2%, the specificity increases to 47.6%.
Figure XI. Distribution of the individual pig activity values of microbiological negative pigs (n=5016).

Figure XII. Distribution of the individual pig activity values of microbiological positive pigs (n=2031).
Table XI. The sensitivity, specificity, positive and negative predictive value at different cut-off values (25, 30 and 35 OD%) at pig and batch level with microbiology in tonsils as golden standard.

<table>
<thead>
<tr>
<th></th>
<th>25 OD%</th>
<th>30 OD%</th>
<th>35 OD%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pig level (%)</td>
<td>Batch level (%)</td>
<td>Pig level (%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>94.2</td>
<td>93.1</td>
<td>92.2</td>
</tr>
<tr>
<td>Specificity</td>
<td>40.0</td>
<td>100</td>
<td>43.8</td>
</tr>
<tr>
<td>Positive</td>
<td>39.5</td>
<td>100</td>
<td>39.9</td>
</tr>
<tr>
<td>predictive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>94.3</td>
<td>68.4</td>
<td>93.2</td>
</tr>
<tr>
<td>predictive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>value</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure XIII. Scatter plot of the concentration of *Yersinia* spp. in the tonsils and the activity value of the antibodies against *Yersinia* spp. in meat juice collected from diaphragm at pig level.
At batch level, 87 batches were infected with *Yersinia* spp. from which 78 batches had a mean within-batch OD% above 30 OD% (Table XII). There were 87 batches that had at least one pig positive in one sample matrix. None of the negative batches for *Yersinia* spp. in the tonsils had activity values of more than 30 OD% in the meat juice. At batch level, the sensitivity of the serology test (30 OD%) was 89.7%, while the specificity was calculated at 100%. These values remain the same when the cut-off value is changed to 35 OD%. However, the sensitivity increases when the cut-off value is altered to 25 OD%.

The mixed-effects logistic regression resulted in the following formula:

\[
\text{within-batch microbiological prevalence} = \frac{0.444}{1 - e^{-0.063 \times (\text{mean OD\%} - 37.069)}}
\]

for which the cut-off value for a positive farm is 37 OD% (Fig. XIV).

Table XII. The categorization of batches of slaughter pigs (n=100) depending on their microbiological and serological status.

<table>
<thead>
<tr>
<th>Antibodies in meat juice</th>
<th>Human pathogenic <em>Yersinia</em> spp. in tonsils</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Absent*</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Present*</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>87</td>
</tr>
</tbody>
</table>

* based on the activity value using a cut-off of 30OD%
5. Discussion

Since pork is a main source for human yersiniosis, it is important to reduce the prevalence of *Yersinia* spp. in pork as much as possible. A main strategy of the reduction of the risk of pig carcass contamination is the decrease of the number of infected slaughter pigs (Laukkanen et al., 2009). In Europe, there is no control program available or in use to decrease this on-farm prevalence yet. Another possibility is to reduce the contamination during slaughter by bagging the rectum (Nesbakken et al., 1984; Laukkanen et al., 2010b). The presence of *Yersinia* spp. in a batch is important regarding contamination of carcasses and is the basis when logistic slaughtering is applied. Therefore, knowledge of the infection status is needed before pigs are slaughtered. The comparison between microbiology and serology for *Yersinia* spp. at pig and batch level only results in a relation.

![Scatter plot of the mean within-batch activity value of the antibodies against *Yersinia* spp. in meat juice collected from diaphragm and the within-batch bacteriological prevalence of *Yersinia* spp. in the tonsils at batch level.](image)
at batch level. Overall, a weak agreement was found between bacteriology and serology for *Yersinia* spp. diagnosis. This is in line with Nollet *et al.* (2005) who compared the presence of *Salmonella* spp. and of antibodies against *Salmonella* spp. in slaughter pigs. There was also a weak agreement between these two diagnostic procedures.

Pigs without human pathogenic *Yersinia* spp. in their tonsils, most (56.2%) are serologically classified as positive due to an activity value above the cut-off value of 30 OD%. So the proposed cut-off of 30 OD% is not reliable to detect microbiological negative pigs. Decreasing the cut-off activity value (e.g. 25 OD%) leads to a higher detection of infected pigs (sensitivity is 94.2%), which is the most important. However, this also leads to indicating more non-infected pigs as infected. Microbiological negative pigs can present a wide range of activity values. An already cleared infection could be an explanation for the presence of these antibodies. Nielsen *et al.* (1996) showed that antibodies are still present 70 days p.i. (end of the experiment) while excretion was finished at 68 days p.i., which is comparable with the study of Vilar *et al.* (2013), where antibodies were still present 5 months p.i., while excretion was finished after 2-3 months p.i. To our knowledge, a(n) (experimental) study of the evolution of *Yersinia* spp. in the tonsils instead of faecal excretion and serology do not exist.

A very low amount of the microbiological positive pigs (7.8%) presented an activity value below the proposed cut-off of 30 OD%. An explanation for the discrepancy is the biological difference between the presence of the pathogen and the serological reaction in the animal. Presence of the *Yersinia* spp. in the tonsils is due to a recent infection, whereby no antibodies will be detected in the serum of these infected animals. According to Thibodeau *et al.* (1999), tonsils can already be colonized a few hours post infection, while infected pigs are all seroconverted around 19 days p.i. (Nielsen *et al.*, 1996).

Based on serology results it is possible to detect infected batches prior to slaughter by using the presently proposed equation: when the mean activity value is more than 37 OD%, the batch is indicated positive. Knowing the microbiological prevalence prior to slaughter, infected pig batches can be delivered to the abattoir and slaughtered after non-
infected pig batches to avoid cross-contamination in the slaughterhouse. To prove freedom of disease, from a batch of 50 pigs, 12 pigs should be sampled (based on a mean within-batch bacteriologic prevalence of 30%). When the batch counts more pigs, 13 pigs should be sampled. It is more important to detect microbiological positive batches than pigs, since separating infected and non-infected pigs at the slaughterhouse is impossible. If pig batches are categorized in the slaughterhouse based on serological testing, it should be taken into account that slaughter pigs can still harbor *Yersinia* spp. in the tonsils, without seroconversion (9 of the 87 batches). The sensitivity of the serological test at batch level was 89.7% using a cut-off of 30 OD%. The use of the lowest cut-off value (25 OD%) is recommended in order to increase the probability of classifying positive herds correctly based on serological testing. However, the lower the cut-off value, the higher the number of seropositive batches detected although the animals are not harboring the bacteria (the highest mean activity value of a microbiological negative batch was 20.6 OD%). Though *Yersinia* spp. are no longer present in those kind of batches, a positive serological result at the batch level means that *Yersinia* is or has been present in the batch and the farmer needs to lower this prevalence. As shown in Fig. XIV, batches with a low mean activity value, were also infected at a low level. This is fundamental when dividing batches as low or high infected. To the contrary, low infected batches could have a high mean activity value.

To decrease the infection in a batch, the number of piglet suppliers can be decreased, the presence of other pig farms in the area should be as low as possible, semi slatted floors in the fattening pig unit should be available (Chapter 5). Logistic slaughtering has been also proven to be useful in blocking the spread and (cross)-contamination of *Salmonella* spp. on pig carcasses during slaughter (Swanenburg *et al.*, 2001; Arguello *et al.*, 2014). Both studies indicate that, next to an accurate batch separation according to their seroprevalence levels, strict measures for cleaning and disinfection in the lairage and the slaughterhouse facilities are needed when logistic slaughter is performed. Due to a decreasing prevalence of *Salmonella* at herd level, infections at transport and lairage became more and more important. A separation based on the herd status and executed
while transporting the pigs, significantly decreased the percentage of infected pigs that became infected at lairage (Hotes et al., 2011).

In conclusion, only a weak agreement was found between the results of both methods at individual level. Serological screening methods could be useful at batch level to distinguish infected from non-infected batches. In order to correctly classify batches, 13 samples per batch (batches larger than 100 pigs) should be sampled. Serological testing of pigs prior to slaughter followed by classifying the batches (infected and non-infected) and adapting the slaughter order could be useful to decrease the risk of contamination during slaughtering procedures.
ASSESSMENT OF RISK FACTORS FOR A HIGH WITHIN-BATCH PREVALENCE OF YERSINIA ENTEROCOLITICA IN PIGS BASED ON MICROBIOLOGICAL ANALYSIS AT SLAUGHTER
1. Abstract

The purpose of the current study was to find factors at farm-level influencing the bacteriological prevalence of *Y. enterocolitica* in pigs at time of slaughter. On 100 farms, data concerning housing, ventilation, biosecurity, management, feeding and disease control were collected using a face-to-face questionnaire. At the slaughterhouse, tonsils of on average 70 slaughter pigs per batch were sampled to determine the infection status of pigs. After univariate mixed effect logistic regressions, variables which were related to the *Yersinia* prevalence (P < 0.05) were included in a multivariate model. In this model, the factors remaining positively associated with a higher *Y. enterocolitica* carriage in the tonsils (P<0.1) were an increasing number of piglet suppliers, a high density of pig farms in the area and the use of semi slatted floors in the fattening pig unit. The proper use of a disinfection bath before entering the stables and a poor biosecurity level were protective factors, although a higher prevalence was associated with a significant positive interaction between the presence of pets in the stables and a poor biosecurity level. Reducing the number of piglet suppliers and using a disinfection bath properly could be easily implemented by pig farmers to lower the prevalence of *Y. enterocolitica* in pigs at slaughter.
2. Introduction

Yersiniosis is an important food-borne disease responsible for about 7000 confirmed human cases per year in the European Union and around 14600 infections in young children in the U.S. each year (EFSA and ECDC, 2013, Scallan et al., 2013). Infections are mainly caused by the human pathogenic biotypes of \textit{Yersinia enterocolitica} (98.4%) and to a lesser extent, \textit{Y. pseudotuberculosis} (0.9%). Pork and products thereof are considered as the most important infection source. Contamination of the meat occurs mainly by (cross-)contamination during slaughter by pigs harboring Yersiniae in their tonsils or by faecal shedding (Laukkanen et al., 2009). Since pigs have been identified as the main reservoir of these pathogens, the European Food Safety Authority (EFSA) has published in a recent scientific opinion (Biohaz, 2011) to assist the reduction of the contamination risk in the pig slaughterhouses by suggesting to decrease the number of infected animals at farm-level. Decreasing the occurrence of \textit{Y. enterocolitica} in pigs and ultimately on pork is considered as the most important control step towards reducing the number of human infections, therefore the infection rate of delivered batches prior to slaughter should be known so that appropriate actions in the slaughterhouse can be taken.

Studies of risk factors for the occurrence of \textit{Y. enterocolitica} at pig farm level have been performed in several countries, but, except for the study of Wesley et al. (2008) in the U.S., these were always based on a limited number of farms or pigs per farm sampled (Nowak et al., 2006; Laukkanen et al., 2008; Laukkanen et al., 2009; Virtanen et al., 2012; Novoslavskij et al., 2013). So-far, common findings are that the use of bedding material, the purchase of piglets originating from more than one farm, daily observation of the presence of a cat in the stables, drinking from nipples and snout contact between pigs from adhering pens in the fattening pig unit are risk factors for infection. The use of municipal water, a farrow-to-finish approach and organic farming acted as protective factors (Laukkanen et al., 2009; Virtanen et al., 2012; Novoslavskij et al., 2013; Vilar et al., 2013). Certain intervention strategies based on these factors, could reduce the within-batch prevalence prior to slaughter, but have not been implemented so far.
As transmission already starts at farm-level, several studies have assessed the prevalence prior to slaughter (Nowak et al., 2006; Laukkanen et al., 2009; Fondrevez et al., 2010; Virtanen et al., 2011; Novoslavskij et al., 2012). They revealed a large variation in prevalence between pig batches originating from different farms allowing determination of factors influencing the infection status.

The aim of the present study is to assess different farm factors influencing the microbiological prevalence of human pathogenic *Y. enterocolitica* in pigs at slaughter to obtain interventions to reduce this prevalence and that finally decrease the level of *Y. enterocolitica* (cross-)contamination in the slaughterhouse.

### 3. Material and methods

#### 3.1. Study design and sample collection

The collection of samples has already been discussed in Chapter 2. The farms were scattered all over Belgium, with most batches located in West-Flanders related to the high density of pig farms in this province (more than 50% of all Belgian pig farms).

#### 3.2. Collection of questionnaire data

In the present study, shortly before the pigs were slaughtered, each farm was visited and a face-to-face questionnaire was filled in combined with a guided tour on the farm. The *Yersinia* status of the farm was still unknown and the questioning was always performed by the same investigator. In total, 59 fattening pig herds and 41 farrow-to-finish herds were included. The number of sows in the farrow-to-finish farms varied between 80 and 750, and the size of the fattening pig farms ranged from 297 to 10,500 slaughter pigs. The items in the questionnaire were related to the following aspects: production parameters, type of farm management and housing system, biosecurity and hygiene measurements, animal management, origin of the drinking water, type of feed and veterinary support (Table XIII). In total, sixty-eight questions were asked to the farmer or evaluated during
the visit. Most of the data were binomial (questions answered by yes or no), some categorical (e.g. feed: (1) grain, (2) meal, (3) porridge) or continuous (e.g. daily weight gain). A Specific Pathogen Free (SPF) farm is free from Mycoplasma hyopneumoniae (enzootic pneumonia), certain serotypes of Actinobacillus pleuropneumoniae, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Sarcoptes scabiei (mange) and lice. The yearly average Salmonella result, expressed by the ratio of sample to positive (S/P), was calculated based on farm specific official reports.

3.3. **Statistical method**

Excel software and Stata/MP 12.1 (StataCorp, 2011) were used for all analyses. The dependent variable was defined as the infection status of the animals (presence/absence of *Y. enterocolitica*). Independent variables included categorical, continuous and binomial farm-level variables. When the correlation coefficient between farm variables was high (\(r \geq |0.7|\)), only one of the variables was further included in the model. The decision regarding which variable to include depended on the biological plausibility. In this study, the number of slaughter pigs present was chosen instead of the number of sows (Table XIII).

The association between each independent variable and the outcome was first screened using an univariate mixed effect logistic regression. Variables with \(P < 0.05\) in this univariate analysis were retained for the multivariate model with farm as random effect. In this model, only items which remained significantly associated with *Y. enterocolitica* carriage in the tonsils \(P < 0.1\) were determined either as risk or as protective factors. All possible interactions were evaluated and included when significant \((P < 0.1)\). The mean within-batch prevalence was calculated based on the number of piglet suppliers and differences were evaluated using one sample t-tests \((P < 0.1)\).
Table XIII. List of variables used in the questionnaire and their reason of exclusion from the analysis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Modality</th>
<th>Reason of exclusion(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General information</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Batch size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Number of slaughter pigs present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Number of sows present</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>• Specific Pathogen Free farm</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Production parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Daily weight gain</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>• Mortality ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Feed conversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Management and housing system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Type of farm</td>
<td>Farrow-to-finish – fattening pig farm</td>
<td></td>
</tr>
<tr>
<td>• Floor type in slaughter pig stables</td>
<td>Fully slatted floor – semi-slatted floor</td>
<td></td>
</tr>
<tr>
<td>• Snout contact possible between pigs from adhering pens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Use of straw bedding in the nursery or fattening pig unit</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• Appropriate temperature change in fattening pig unit</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• Appropriate humidity in the stables</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Biosecurity and hygiene measurements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• all-in/all out system in fattening pig unit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• rodents visible in the stable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• rodent control program</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• large amount of flies in the stables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• outside the stable: hard and clean ground</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• pets allowed in the stable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• birds present in the fattening unit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• grid present in the air openings</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• wild boars present in the neighborhood</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Characteristic</td>
<td>Modality</td>
<td>Reason of exclusion^a</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>• cleaning after each rearing round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• disinfection after each rearing round</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>• stable stays empty during ≥ 3 days after each rearing round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• control cleaning and disinfection with hygienogram</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• presence of other pig farms in the area (closer than 500m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• bath placed inside the stable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• bath content renewed at least every 2 weeks</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>• farm clothes available for visitors</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• other clothes for each compartment</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• presence of a hygiene lock before entering the stables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• need to shower before entering the stables</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• use of separate clean and dirty road</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• use of other cleaning material for each compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• disinfection material to compel pigs</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• disinfection loading place</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• farm specific material to fix pigs</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• different shoes for entering the pen with ill pigs</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>• farm specific manure tubes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Animal management

• number of piglet suppliers
• age arriving piglets
• farm of origin ≥ health statute
• smaller pigs are relocated in new pens
• maximum capacity load fattening pig unit reached

Drinking water

• origin of water supply
  - Rain – municipal water
• use of acidified water in the nursery
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Modality</th>
<th>Reason of exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>use of acidified water in the sow unit</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>use of acidified water in the fattening pig unit</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>yearly cleaning and disinfection of the pipes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yearly bacteriologic control of the water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>daily control of functioning nipples</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Feed**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Modality</th>
</tr>
</thead>
<tbody>
<tr>
<td>type of feed: fattening pigs</td>
<td>Grain – meal - porridge</td>
</tr>
<tr>
<td>type of feed: sows</td>
<td>Grain – meal - porridge</td>
</tr>
<tr>
<td>type of feed: piglets</td>
<td>Grain – meal - porridge</td>
</tr>
<tr>
<td>Acidified feed: fattening pigs</td>
<td></td>
</tr>
<tr>
<td>Acidified feed: sows</td>
<td></td>
</tr>
<tr>
<td>Acidified feed: piglets</td>
<td></td>
</tr>
<tr>
<td>Origin feed</td>
<td>Commercial – tailor-made</td>
</tr>
<tr>
<td>Type of feed supply</td>
<td>Automatic - manual</td>
</tr>
</tbody>
</table>

**Veterinary support**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Reason of exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>presence of <em>Brachyspira</em> spp.</td>
<td>1</td>
</tr>
<tr>
<td>presence of <em>Lawsonia</em> intracellularis</td>
<td>1</td>
</tr>
<tr>
<td>mean S/P <em>Salmonella</em> ratio last year</td>
<td></td>
</tr>
<tr>
<td>past standardized antibiotic treatments</td>
<td></td>
</tr>
<tr>
<td>use of anthelmintica in fattening pig unit</td>
<td></td>
</tr>
</tbody>
</table>

**End of rearing period**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Reason of exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>feed stop 12-16 h before loading</td>
<td>1</td>
</tr>
<tr>
<td>transport vehicle cleaned and disinfected</td>
<td>3</td>
</tr>
</tbody>
</table>

*a1: farms applying the factor: five or less, or 95 or more
2: pooled factors
3: missing values
4: correlation coefficient ≥ |0.7|
4. Results

When visiting the pig farms, it was noticed that pigs belonging to the same slaughtering batches could be derived from different stables. The answers to the questions were sometimes different between these stables (e.g. floor type, type of feed). In this case, the stable where the majority of the pigs were staying, was chosen to fill in the questionnaire. After a first evaluation of the 100 questionnaires, some of the collected items turned out to be unusable due to the low variance between farms, as for instance the use of straw bedding in the nursery or fattening pig unit (only applied by one of the 100 farms), the obligated use of a shower for visitors before entering the stables (3%), the use of different shoes when entering the pen with the ill pigs (1%) and the use of rainwater tanks or wells as drinking water supply (100%). Therefore, in the complete study, items with five or less farms applying this item were ruled out, resulting in the exclusion of 19 out of 68 items.

Some close-related items were pooled in the univariate analysis, e.g. the item ‘cleaning-disinfection-stand empty (‘CDE’) consists of (1) the cleaning and (2) the disinfection of the stable after each rearing round and (3) the time period in which the stable stood empty after the pigs were slaughtered (cut-off: ≥ 3 days). A ‘proper use of a disinfection bath’ consists of (1) whether the bath is placed inside the stable, so it is protected from weather conditions and one is obliged to step in it and (2) the bath is cleaned at least every two weeks. This limit of two weeks was based on the efficacy data provided by the most frequently used products (e.g. MS Kiemkill tabs, Schippers, Arendonk, Belgium). Items with more than 15 missing responses, due to a clueless farmer or person responsible for the stable, were omitted. As a result, only 36 explanatory items were taken further into account (Table XIV).
Table XIV. Description of the remaining binomial and categorical pig farm characteristics in the 100 visited farms.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Modality</th>
<th>Number of farms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Management and housing system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• farrow-to-finish production</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>• fully slatted floor in fattening pig unit</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>• snout contact possible between pigs from adhering pens</td>
<td></td>
<td>67</td>
</tr>
<tr>
<td><strong>Biosecurity and hygiene measurements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• all-in/all out system in fattening pig unit</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>• rodents visible in the stable</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>• proper rodent control</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>• pets allowed in the stable</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>• birds present in the fattening unit</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>• poor CDE*</td>
<td>• no cleaning</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>• no disinfection</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>• stable empty during &lt; 3days</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>• total poor CDE</td>
<td>45</td>
</tr>
<tr>
<td>• presence of other pig farms in the area (closer than 500m)</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>• correct use of disinfection bath</td>
<td>• bath inside the stable</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>• bath cleaned at least every 2 weeks</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>• total</td>
<td>31</td>
</tr>
<tr>
<td>• farm clothes available for visitors</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>• presence of a hygiene lock before entering the stables</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td><strong>Animal management</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• number of piglet suppliers</td>
<td>• 0</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>• 1</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>• 2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>• 3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>• 4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>• &gt;5</td>
<td>6</td>
</tr>
<tr>
<td><strong>Drinking water</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• use of municipal water</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>• use of acidified water in the nursery</td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>
After the univariate analysis, only seven factors were significant (P < 0.05), which were all included in the multivariate analysis. During this analysis, the element ‘snout contact possible between pigs from adhering pens’ (P-value\textsubscript{univariate} = 0.045) was excluded due to a P-value\textsubscript{multivariate} > 0.1. In the final logistic regression model, three risk factors, two protective factors and one significant interaction were identified (Table XV). Significant risk factors were ‘the use of a semi-slatted floor in the fattening pig unit’, ‘presence of other pig farms in the area (closer than 500m)’ and ‘the number of piglet suppliers’ (P < 0.1). Protective factors were ‘the proper use of a disinfection bath’ and a ‘poor CDE’. A significant interaction (P < 0.1) was observed between a ‘poor CDE’ and ‘the presence of pets in the stable’, which led to an increasing prevalence.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Modality</th>
<th>Number of farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• type of feed fattening pigs</td>
<td>• grain</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>• meal</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>• porridge</td>
<td>2</td>
</tr>
<tr>
<td>• commercial feed</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>• rodent-free feed storage</td>
<td></td>
<td>93</td>
</tr>
<tr>
<td>Veterinary support</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• past standardized antibiotic treatments</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>• use of anthelmintica</td>
<td></td>
<td>94</td>
</tr>
</tbody>
</table>

*CDE: cleaning-disinfection-stand empty
Table XV. Logistic regression model with a random effect for farm, of variables significantly ($P \leq 0.1$) associated with the presence of human pathogenic \emph{Y. enterocolitica} in Belgian pig batches at slaughter ($n = 100$ farms).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi slatted floor in fattening unit</td>
<td>1.65</td>
<td>0.085</td>
<td>[1.03; 2.64]</td>
</tr>
<tr>
<td>Presence of other pig farms in the area (closer than 500m)</td>
<td>1.63</td>
<td>0.036</td>
<td>[1.03; 2.59]</td>
</tr>
<tr>
<td>Number of piglet suppliers</td>
<td>1.15</td>
<td>0.019</td>
<td>[1.02; 1.28]</td>
</tr>
<tr>
<td>Proper use of disinfection bath</td>
<td>0.58</td>
<td>0.032</td>
<td>[0.36; 0.95]</td>
</tr>
<tr>
<td>Poor Cleaning-Disinfection-Stand empty (CDE)</td>
<td>0.50</td>
<td>0.022</td>
<td>[0.28; 0.90]</td>
</tr>
<tr>
<td>Interaction: poor CDE and pets</td>
<td>2.39</td>
<td>0.065</td>
<td>[1.05; 5.53]</td>
</tr>
</tbody>
</table>

The mean within-batch prevalence increased with an increasing number of piglet suppliers (Fig. XV). Nevertheless, there was no significant difference between the mean within-batch prevalence of farrow-to-finish farms (25.9%; $n=41$ farms) and fattening pig farms with only one supplier (25.2%; $n=34$ farms) ($P > 0.1$). When more suppliers are involved, the mean within-batch prevalence increased for two suppliers to 29.7% (eight farms), for three suppliers to 36.9% (five farms) and for four or more suppliers to 42.5% (12 farms) ($P < 0.1$).
The mean within-batch prevalence of both binary outcomes of the risk and protective factors was determined (Fig. XVI). The difference between the use of a semi (n=19) and fully (n=81) slatted floor was stated by a prevalence of 36.0% and 26.7% respectively (P < 0.1). With other pig farms in the area, the prevalence increased from 24.5% (n=37) towards 30.9% (n=63) (P > 0.1). A third difference between the mean within-batch prevalence in farms using a disinfection bath properly (n=31) was 20.4% compared to 32.2% in farms without the proper use of such a bath (n=69) (P < 0.1). The impact of the second protective factor, a poor CDE, was demonstrated by a prevalence of 24.2% for farms with a poor CDE (n=45) compared to 32.0% in farms which applied good CDE (n=55) (P < 0.1). When pets were allowed in the stables (n=43), the prevalence was 32.1% compared to 25.8% when no pets were found in the stables (n=57) (P > 0.1). Comparing the two latter factors, 31 farms had a poor CDE and no pets in their stables, what resulted in a mean within-batch prevalence of 20.3% (Fig. XVII). Fourteen farms also had a poor CDE, but did allow pets in the stables. These farms obtained a prevalence (32.9%) similar to those farms with good CDE and pets present in the stables (n=29; 31.8%) and the farms with good CDE and no pets (n=26; 32.2%). When there was a possible snout contact in the fattening pig unit, the prevalence was higher (33.1%; n=67) than when there was no possibility to have snout contact (26.2%; n=33).
Figure XVI. Comparison of the mean within-batch prevalence between the two possible outcomes of seven variables.

Figure XVII. Comparison of the mean within-batch prevalence depending on the presence of poor Cleaning-Disinfection-Stand empty (CDE-) and pets in the stables.
5. Discussion

The present study assessed different risk factors based on the presence of human pathogenic *Y. enterocolitica* in pig tonsils at time of slaughter originating from 100 farms, taking into account clustering per batch. As the prevalence based on bacteriological data at time of slaughter leads to the final carcass contamination (Laukkanen *et al.*, 2009), risk factors related to this prevalence, are also affecting this contamination.

Based on a much larger number of farms, as well as pigs examined in the present study, some previous factors could be confirmed, other factors were new or even contradicted previous studies.

In the present study, factors increasing the within-batch prevalence at time of slaughter are identified as the presence of semi-slatted floor in the fattening pig unit and the presence of other pig farms in the neighborhood (<500m). The occurrence of *Y. enterocolitica* in a batch is also augmented with the number of piglet suppliers. The proper use of a disinfection bath and a poor CDE, without pets in the stable, are other factors that have a decreasing influence on the prevalence.

The first two risk factors, ‘the use of a semi-slatted floor in the fattening pig unit’ and ‘presence of other pig farms in the area (<500m)’ can probably not be remediated immediately. The use of a semi slatted floor increases the prevalence based on bacteriological results compared to a fully slatted floor, represented by an 10% increase in mean within-batch prevalence of 36% and 26.6% respectively. The influence of the type of floor can be explained by the contact time with pig faeces, in which *Y. enterocolitica* is present. Semi-slatted floors create a more constant contact with the pig faeces (Fukushima *et al.*, 1983; Nielsen *et al.*, 1996; Nesbakken, 2006). The second risk factor ‘presence of other pig farms in the area (<500m)’ was never studied before and indicates a possible transmission route between nearby farms. The origin of this spread is however still unknown.
The third risk factor, ‘the number of piglet suppliers’, differed between farms with none or one supplier, and farms with more suppliers. The maximum number of piglet suppliers in this study was 11 per farm. It is known that incoming piglets, potentially infected on multiplying farms are a possible source for spreading and infection on fattening pig farms (Virtanen et al., 2012). When piglets arrive from different multiplying farms, there is more chance to purchase infected, *Yersinia*-excreting piglets. Purchasing piglets from more than one farm was also identified as a risk factor in the studies of Nowak et al., (2006) and Vilar et al., (2013). The present study indicates an equal risk between fattening farms when purchasing piglets from one supplier and farrow-to-finish farms, which is similar to the study of Vilar et al. (2013).

In addition, two new protective factors were identified and both are related to hygienic measurements. The first one, ‘proper use of a disinfection bath’, decreases the spread between different stables on the same farm, so infections stay more localized. The difference with the ‘farm clothes available for visitors’, which is not a significant factor, is that the disinfection bath is also used by the farmer, and not only by the farm visitors. In this way, infections introduced from outside the farm, and even within the farm but from different stables, may be eliminated by this disinfection bath. The second protective factor, a ‘poor CDE’, is more difficult to interpret. Similar results are available for the presence of *Salmonella* on pig farms. Van der Wolf et al. (2001) reported that the omission of disinfection of pig stables was associated with a lower *Salmonella* seroprevalence compared to herds that sometimes or always applied disinfection. Moreover, Poljak et al. (2008) showed that increased frequency of cleaning with cold water and disinfection was positively correlated with *Salmonella* shedding. Moreover, when pets are allowed into the stable, ‘poor CDE’ is a risk factor for a higher prevalence. When pets are allowed in and move between stables, they keep the infection going as they act as carriers and transmitters of *Y. enterocolitica* (Yanagawa et al., 1977; Fredriksson-Ahomaa, 2001; Murphy et al., 2010). The use of an all-in/all-out management was also identified as protective factor in a more limited study of Vilar et al. (2013). Novoslavskij et al. (2013) reported a low biosecurity level as a risk factor. However, in the latter study, the biosecurity factor included other factors than these in the present study.
Some factors were not included in the analysis or were not significant in the present study, but turned out to be risk or protective factors in other studies. The use of a straw bedding was initially taken into account in the questionnaire, but as applied in only one farm, was not further included. The use of a bedding was in other studies identified as a risk factor (Laukkanen et al., 2009; Vilar et al., 2013). Moreover, the use of municipal water is considered as a protective factor (Virtanen et al., 2012; Vilar et al., 2013), though this factor was excluded, as all farms used rainwater tanks or wells as drinking water supply. It is an interesting subject to take into account in future studies. A higher production capacity has been associated with a higher prevalence of *Y. enterocolitica*, due to underlying risk factors inherent in a higher production (Laukkanen et al., 2009; Laukkanen et al., 2010b). Nevertheless, no differentiation was found in the present study. Feeding factors have also been related with the prevalence of *Y. enterocolitica*. Manual feeding of slaughter pigs has been determined as a protective factor. Use of commercial feed, presence of meat or bone meal in grower-finisher diet and industrial by-products in feed have been associated with a higher *Y. enterocolitica* infection rate. Feed producing companies can have a positive or negative influence on the prevalence (Nowak et al., 2006; Wesley et al., 2008; Virtanen et al., 2011). In the present study, no feed related significant differences were found.

To our knowledge, there are two studies available that performed a risk factors analysis based on serology (Skjerve et al., 1998; von Altrock et al., 2011). von Altrock et al. (2011) mentioned the use of a fully slatted floor and the use of municipal water as protective factors in German pig farms. Skjerve et al. (1998) identified the daily presence of a cat with kittens in the stable and the use of a bedding as risk factors, while a farrow-to-finish farm was seen as a protective factor.

In the general picture of pig producing management, another food borne pathogen, *Salmonella*, is also very important. The risk and protective factors for a higher prevalence of *Y. enterocolitica* on pig farms could be conflicting with factors influencing the prevalence of *Salmonella* on pig farms. In a study of Vico et al. (2011) the mesenteric lymph nodes of pigs were collected in the slaughterhouse and analyzed for the presence
of \textit{Salmonella}. Risk factors in this study were lack of rodent control programs, fattening pig herds, herds managed by more than one full-time worker, municipal water as drinking water supply and relatively long fattening times. Only one factor is similar between the study about \textit{Salmonella} and the present study: pig herds with one or more piglet suppliers (fattening pig herds) are a risk for a higher prevalence of \textit{Y. enterocolitica} and \textit{Salmonella}. Cardinale \textit{et al.} (2010) performed a risk factor study based on the bacteriological \textit{Salmonella} status of 60 farms by analyzing faecal samples and socks. The prevalence increased when there was no disinfection at the farrowing stage, when large numbers of cockroaches were present and when birds were seen in the stable. A lower level of \textit{Salmonella} was reached when the technical personnel visited the stable less than once a month, when castration of piglets was done after 1 week of age and when the all-in all-out system was respected. Garcia-Feliz \textit{et al.} (2009) analyzed faecal samples for the presence of \textit{Salmonella}. The only two risk factors given in this study were the feeding of pelleted feed and a high production rate. No factors of the two latter studies were similar compared to the present study.

In conclusion, reducing the number of piglet suppliers, using a disinfection bath properly and prohibiting the entrance of pets into the stable are factors that are easily implemented to lower the prevalence of \textit{Y. enterocolitica} in pigs at slaughter.
CHAPTER 6

FACTORS INFLUENCING THE SEROPREVALENCE OF HUMAN PATHOGENIC YERSINIA SPP. IN FATTENING PIGS AT SLAUGHTER
1. Abstract

The main objective of this study was to analyze potential herd-level factors associated with the detection of *Yersinia* antibodies in fattening pigs at time of slaughter which gives the opportunity to create interventions to decrease the presence of these antibodies. The seroprevalence of *Yersinia* spp. varies greatly between pig farms. Due to this variation, risk factors can be determined which may be applied in the pig farm management so the seroprevalence prior to slaughter will decrease. One hundred farms were visited and during a face-to-face questionnaire data concerning housing, ventilation, biosecurity, management, feeding and disease control were collected. At the slaughterhouse, pieces of diaphragm were collected, where after the meat juice was gathered and an ELISA was performed to determine the seroprevalence. After using univariate mixed effect logistic regressions, variables which were related to the *Yersinia* prevalence (*P* < 0.05) were included in a multivariate model. In this model, four risk factors and one protective factor remained significantly associated with antibodies against *Yersinia* species in meat juice (*P*<0.1). Many piglet suppliers, a high density of pig farms in the area and the use of semi-slatted floors in the fattening pig unit were risk factors. The possibility of snout contact in the fattening pig unit was a protective factor, although a significant positive interaction between the presence of pets in the stables and snout contact was observed.
2. Introduction

Human pathogenic *Y. enterocolitica* (98.4%) and *Y. pseudotuberculosis* (0.9%) are causing the third most important bacterial food-borne disease which leads to about 7000 confirmed human cases in the European Union each year (EFSA and ECDC, 2013). Pigs are the main reservoir of these pathogens (Thibodeau *et al.*, 1999; Fredriksson-Ahoma, 2001). The infection is mainly caused by consumption of pork and products thereof. Contamination of pork occurs by (cross-)contamination during slaughter or following steps, due to infected pigs (Laukkonen *et al.*, 2009). A reduction of the number of infected pigs at farm-level would reduce the amount of contaminated carcasses. To know the number of infected pigs prior to slaughter, there are three possible samples: tonsils, faeces or blood. Microbiological isolation before slaughter requires sampling of the tonsils as pigs are intermittent shedders and most pigs no longer shed enteropathogenic *Yersinia* spp. in the faeces at slaughter age, thus resulting in an underestimation of the prevalence when analyzing faecal samples. Nevertheless, sampling of tonsils in living pigs is not animal-friendly (Fukushima *et al.*, 1983; Thibodeau *et al.*, 1999; Nesbakken, 2006), so, if sampling happens before slaughter, serological analysis is the most appropriate method.

The number of studies based on the serological prevalence of *Yersinia* spp. are limited (Skjerve *et al.*, 1998; von Altrock *et al.*, 2011). The study of Skjerve *et al.* (1998) was based only on the antibodies against *Y. enterocolitica* serotype O:3 and the risk factor analysis was based on 265 slaughter pig producing farms (conventional and farrow-to-finish production) and sampled 5 pigs per herd, while the study of von Altrock *et al.* (2011) assessed the level of antibodies against both enteropathogenic *Yersinia* spp., analyzing in 80 herds 30 blood samples per herd. Snout contact, use of tetracycline, fasting pigs before slaughter, the use of bedding material, daily observation of a cat in the stables and drinking from nipples were identified as risk factors. Common protective factors were the use of municipal water, a farrow-to-finish farm and manual feeding of slaughter pigs.

It is important to determine farm factors influencing this seroprevalence, so farmers could adapt their farm management by introducing these factors to decrease the within-batch
prevalence. The aim of this study is to gain information about these farm factors to influence the within-batch seroprevalence of pigs at time of slaughter so that measurements can be taken to reduce this within-batch seroprevalence.

3. Material and methods

3.1. Study design and sample collection

The study design and the sample collection have been described in Chapter 3.

3.2. Collection of questionnaire data and statistical method

The same questionnaire data and statistical method as described in Chapter 5 are used.

4. Results

An overview of the collected farm data showed that some factors were not useful for the analysis due to different reasons. When there were 5 farms or less applying a certain factor, it was excluded. Examples for exclusion were: being a Specific Pathogen Free farm (only one SPF-farm), the use of a new set of clothes when changing house (only 3 farms) and the use of acidified feed in the nursery (only 3 farms). This resulted in exclusion of 19 factors. Moreover, variables with a high number of missing values (more than 15 missing) were omitted. Most of these missing values were due to the interviewed person, who was in these cases often the keeper and not the owner of the stables or the keeper/owner did not record certain information (e.g. daily weight gain).

Some variables were pooled, e.g. the factor ‘cleaning-disinfection-empty’ ('CDE') consists of (1) the cleaning and (2) the disinfection of the stable after each rearing round and (3) the time period in which the stable remained empty after the pigs were slaughtered (at
least 3 days). A ‘proper use of a disinfection bath’ exists of (1) where the bath was placed and (2) over what time period the bath was renewed.

According to the reducing measurements mentioned above, only 36 explanatory factors were retained for the analyses (Table XIV).

Only 6 factors remained significant after the univariate analyses (P < 0.05). During the multivariate analysis, the factors ‘pets allowed in the stable’ (univariate analysis: P-value = 0.019) and the ‘proper us of a disinfection bath’ (univariate analysis: P-value = 0.031) were eliminated. The final logistic regression model yielded three risk factors, one protective factor and one significant interaction (Table XVI).

The risk factors were ‘the use of a semi-slatted floor in the fattening pig unit’, ‘presence of other pig farms in the area (closer than 500m)’ and ‘the number of piglet suppliers’. As protective factor there was ‘snout contact possible between pigs from adhering pens’ that, in relation with the presence of pets in the stable, turned into a risk factor.

Table XVI. Final logistic regression model with a random effect for farm, of variables significantly (P ≤ 0.1) associated with the presence of antibodies against human pathogenic *Yersinia* spp. in Belgian pig batches at slaughter (n = 100 farms).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi slatted floor in fattening pig unit</td>
<td>3.78</td>
<td>0.022</td>
<td>[1.21; 11.82]</td>
</tr>
<tr>
<td>Presence of other pig farms in the area (closer than 500m)</td>
<td>2.32</td>
<td>0.076</td>
<td>[1.01; 5.31]</td>
</tr>
<tr>
<td>Number of piglet suppliers</td>
<td>1.43</td>
<td>0.003</td>
<td>[1.13; 1.82]</td>
</tr>
<tr>
<td>Snout contact possible between pigs from adhering pens</td>
<td>0.10</td>
<td>0.001</td>
<td>[0.03; 0.37]</td>
</tr>
<tr>
<td>Interaction: snout contact and pets</td>
<td>17.64</td>
<td>0.004</td>
<td>[2.56; 121.51]</td>
</tr>
</tbody>
</table>

There were 41 farrow-to-finish farms included in this study, that had a mean within-batch seroprevalence of 60% (Fig. XVII). The fattening pig farms with just one piglet supplier (n=34) had a slightly higher prevalence of 64% (P > 0.1). The prevalence increased for
farms with 2 (n=8), 3 (n=5) or more (n=12) piglet suppliers to 68, 78% and 89% respectively (P < 0.1).

The mean within-batch seroprevalence of the assessed binary risk and protective factors are shown in Fig. XVIII. Nineteen farms had a semi slatted floor in the fattening stables, which resulted in a mean within-batch seroprevalence of 74%, whereas the other 81 farms with a fully slatted floor had a mean seroprevalence of 65% (P < 0.1). When there were other pig farms in the surroundings (n=63), these farms had a higher seroprevalence compared to farms without other pig farms in the area (n=37) (75 and 60% respectively) (P < 0.1). Farms with the possibility of snout contact between pigs in adherent pens (n=67) obtained a lower seroprevalence (61%) compared to stables with a closed pen separation (n=33; 78%) (P < 0.1). Looking closer to the interaction of snout contact and pets in the stable, it is noticed that out of the 67 farms with possible snout contact, 27 did not allow pets in the stables (Fig. XIX). These farms had a mean within-batch seroprevalence of 51% (P < 0.1). The other 40 farms had a prevalence of 75% which is similar to the farms with no possibility of snout contact in the fattening pig stables. Sixteen of them had pets in the

Figure XVII. Distribution of the mean within-batch seroprevalence (%) over the different number of piglet suppliers per farm
stable and had a mean seroprevalence of 74%, while the 17 remaining farms with no snout contact and no pets had a mean within-batch seroprevalence of 81%.

Figure XVIII. Comparison of the mean within-batch seroprevalence between the binary outcome of the assessed risk and protective factors.

Figure XIX. Comparison of the mean within-batch seroprevalence between the four categories of possible snout contact and pets present in the stable.
5. Discussion

The aim of this study was to investigate multiple risk factors for the presence of antibodies against YOP’s of *Yersinia* spp. in meat juice of pigs at slaughter, while controlling for clustering by farm. This analysis was based on the infection status of the pigs, specifying a positive animal having an activity value of 30 OD%. The number of pigs sampled per batch was established very precise and accurate (number of pigs to be sampled per batch was calculated based on an expected batch prevalence of 50%, a confidence level of 95% and an accepted error of 10%). The risk factors based on the serological results show the possible risks for infection during the whole rearing period, even when the infection is already finished, which is in contrast to risk factors based on the microbiological prevalence at time of slaughter.

The first two risk factors, ‘the use of a semi-slatted floor in the fattening pig unit’ and ‘presence of other pig farms in the area (closer than 500m)’ could be explained easily. Using a semi-slatted floor allows accumulation of faeces on the pen floor. Since pig faeces can contain human pathogenic *Yersinia* spp., the contact between these faeces and pigs may lead to infection (Fukushima *et al.*, 1983; Nielsen *et al.*, 1996; Nesbakken, 2006). The use of a fully slatted floor was mentioned as a protective factor by von Altrock *et al.* (2011), which is similar to the present study. The presence of other pig farms closer than 500m to the investigated farm is also a risk factor for an increasing prevalence. This could be due to close contact between farmers, the nearby passage of farm vehicles or even the spread by rodents (Backhans *et al.*, 2011). These two factors are difficult to adjust by the farmer.

The third risk factor in the current study, ‘the number of piglet suppliers’, was also noted by Vilar *et al.* (2013) and may also represent a difference between the farrow-to-finish farms (zero suppliers) and the fattening pig farms (1-11 suppliers in the current study). Figure XVII shows the more piglet suppliers there are at one farm, the higher the seroprevalence is. Skjerve *et al.* (1998) indicated being a farrow-to-finish farm is a protective factor, which also aligns to the present study. Virtanen *et al.* (2012) showed
that incoming piglets, possibly infected on their multiplying farm of origin, are a feasible source of infection for other piglets originating from other farms. The more piglets are purchased by different suppliers, the greater the risk of buying infected pigs and of spreading *Yersinia* spp. in the pen.

The only protective factor that was present in the current study was ‘snout contact possible between pigs from adhering pens’. This contact could lead to a higher chance of spread between pens, leading to a higher prevalence. Nevertheless, if this snout contact could lead to infections at very young age, resulting in a decrease of antibody response below the cut-off value by the time the pigs are slaughtered. A study of Nesbakken *et al.* (2006) mentioned an increasing level of titers from an age of 80 days to 162 days in two multiplying herds, and a study of Fukushima *et al.* (1983) presented an increasing level of seropositive pigs at day 77. But when pigs are slaughtered at an age of 6-6.5 months (183-195 days), these levels may be already decreasing. Nielsen *et al.* (1996) showed a decreasing antibody titer over time. In the study of Virtanen *et al.* (2012), based on the microbiological prevalence, snout contact was mentioned a risk factor. It is possible that the tonsils remain infected, while the antibody titer is decreasing. If there is a pet allowed in the stable, the possible snout contact becomes a risk factor. Pets can enter different stables and may carry pathogens originating from these stables, possibly resulting in a continuous re-infection (Yanagawa *et al.*, 1977; Fredriksson-Ahomaa, 2001; Murphy *et al.*, 2010). Nevertheless, Fukushima *et al.* (1983) did not find an increasing antibody titer after re-infection. More research is needed about the fluctuation of antibody titer throughout the rearing period and after re-infection. Moreover, a comparison between microbiological and serological results at pig- and batch-level should be made.

A limited number of studies about risk factors at farm-level were based on a serological prevalence (Skjerve *et al.*, 1998; von Altrock *et al.*, 2011). In these studies, other risk factors were presented. Straw bedding was categorized as a risk factor in these studies, but such a bedding is not common in Belgian pig farms (only applied by one farm in this research). The use of municipal water was regarded as a protective factor in these studies, but was not significant in the present study.
At pig farm level, there is a second foodborne zoonosis to be taken into account. The risk factors of a high seroprevalence of *Salmonella* have been assessed many times. The identified risk factors are: the moving of individual animals during the fattening period, not having a separate transporter for different age groups, pigs having contact to other animals, application of antibiotics, being situated less than 2 km away from another pig farm, an increased number of pig farms within a 10 km radius and the use of granulated feed instead of flour. The following factors were identified as being protective: not cleaning the transporter, not having clean boots available, fully slatted floor, use of protective clothing, cleaning the feed tube, administer liquid feeding instead of dry feeding, feeding homemix and barley and being a conventional pig farm (Hotes *et al.*, 2010; Smith *et al.*, 2010; Gotter *et al.*, 2012). Some of these factors (the proximity of other pig farms and the fully slatted floor) affect the antibody level of both *Salmonella* as *Yersinia* spp. von Altrock *et al.* (2011) insinuated that the *Yersinia* seroprevalence is inversely associated with the serological *Salmonella* status. This has to be taken into account when setting up a combined zoonotic control program for *Salmonella* and *Yersinia*.

To conclude, in the present study, farm factors influencing the seroprevalence of human pathogenic *Yersinia* spp. in pig batches at slaughter, were analyzed. Determined risk factors are the use of a semi slatted floor in the fattening pig unit, the presence of other pig farms in the area, an increasing number of piglet suppliers and the possible snout contact in the presence of pets (interaction). The only protective factor is the possibility of snout contact in the fattening pig unit. When using this knowledge to change some factors in pig farms, the prevalence at farm level should decrease.
GENERAL DISCUSSION
Yersiniosis is in Europe and the U.S. still one of the most common bacterial zoonosis due to consumption of contaminated food (Scallan et al., 2013; EFSA and ECDC, 2013), with pork identified as the most important source for human infection (Tauxe et al., 1987; Ostroff et al., 1994; Fredriksson-Ahomaa et al., 2006; Boqvist et al., 2009; Huovinen et al., 2010). Therefore, the general aim of this thesis was to clarify the infection status of pigs with enteropathogenic Yersinia spp. at slaughter age and to assess the factors influencing the prevalence.

The (cross-)contamination of pork mostly occurs during transformation of the meat as the bacteria can still be present in the throat and less frequent in the faeces of infected pigs presented for slaughter (Laukkanen et al., 2009). A reduction of the number of human cases could be accomplished when less pig carcasses would be contaminated. It has been demonstrated that hygienic measurements in the slaughterhouse alone will fail to significantly reduce the carcass contamination (Nesbakken et al., 1994; Laukkanen et al., 2010b; Ranta et al., 2010). Therefore, lowering the number of infected pigs prior to slaughter is a major intervention strategy. This requires however as a start basic information about the prevalence at slaughter age as well as a predictive tool for this prevalence. This tool can help to identify the infection status of pig batches at slaughter prior to slaughter, which can influence the order of slaughtering.

1. A predictive tool for the infection status at slaughter age

To determine the prevalence of enteropathogenic Yersinia spp. in pig batches, two methods are currently applicable. Microbiological analysis indicates a momentary infection, while serology symbolizes previous infection(s). Microbiological analysis is based on the detection of Yersinia in two different matrices: faeces or tonsils. Analyzing faeces at slaughter age is possible prior to slaughter in contrast to analyzing tonsils. Collecting a piece of the tonsils in the live animal may negatively affect animal welfare. Faecal analysis is however less relevant than studying tonsils because the faecal shedding has ended while the infected tonsils are still detectable (Nielsen et al., 1996; Nesbakken
et al., 2006; Virtanen et al., 2012). Therefore, studies in the thesis focused on the tonsils as matrix to detect the presence of *Yersinia* spp.

Based on the microbiological analysis of tonsils, a large variation seems to be present in the within-batch prevalence of pig batches at slaughter (Chapters 1 and 2). Reasons for this variation are, however, still unknown. The moment of initial infection is likely to be of major significance. In batches with low microbiologically based prevalence, it is not certain if this is due to a recent infection with a low number of animals infected, or if the infection started long time ago with still some infected animals present. The porcine tonsils could also become contaminated during transport or in the lairage when pigs are picking up contaminated faeces. When batches are delivered to the slaughterhouse, some infected pigs will still shed the bacteria in the faeces. In this way, they can infect other pigs or contaminate the pens of the lairage area, and infect pigs of subsequent batches that enter these pens. Nevertheless, since the faeces contain a low number of *Yersinia* bacteria at moment of slaughter (Nesbakken et al., 2003; Nesbakken et al., 2006; Van Damme, 2013), the number of *Yersinia* found in the tonsils at the slaughterline after possible contamination during transport or in the lairage will likely also be low. When observing the *Yersinia* count of microbiologically positive and serologically negative pigs (Fig. XX), 121 out of the 159 were harboring a high *Yersinia* count (> 3 log\(_{10}\) CFU/g tonsil). The mean *Yersinia* count in pig faeces is 3 log\(_{10}\) CFU/g faeces (Van Damme et al., 2013). The time of transportation and the time spent in the lairage are too short for the *Yersinia* to reach large numbers (growth rate: 33-39 min at 32°C) (Schiemann, 1980). This suggests that these 121 microbiologically positive and serologically negative pigs were likely infected (serologically negative), shortly before transport to slaughter (count was too high).
Fig. XX. The *Yersinia* count of microbiologically positive and serologically negative (activity value < 30 OD%) pigs.

Reducing the risk of contamination in the lairage, cleaning and disinfecting (a part of) the lairage are interesting options. However, from a practical viewpoint, this is impossible to accomplish during the slaughter day. Consequently, the (small) risk to infect new pigs in the lairage will remain. Nevertheless, when negative batches would be slaughtered first (preferably at the beginning of the week), and there would be a thorough cleaning and disinfection at the end of every day or at least every week, the probability that pigs would get infected in the lairage should already decrease.

Also a wide range (0 to 64.4%) of the microbiological prevalence was observed without clustering around certain percentages, which implies that there is no accurate distinction possible between low and highly infected batches. Another possibility to make a distinction based on infection rate is the use of serology. Therefore, two matrices are available. Blood can be obtained prior to and during slaughter, in contrast to meat juice that can only be collected after slaughter. According to Meemken and Blaha (2011), these two matrices show an excellent agreement, hence meat juice can be used instead of blood. In Chapter 3, the observed within-batch seroprevalence showed an even larger variation (0 to 100%) than the microbiological prevalence. The comparison of the microbiological and serological within-batch prevalence resulted in similarities as well as
discrepancies (Chapter 4): there were batches microbiologically negative and serologically
negative (13%), which implies that these farms are enteropathogenic *Yersinia* spp. free. A
low seroprevalence always indicates a low microbiological prevalence though not
necessarily *Yersinia* free. Using serology to distinguish low and highly infected batches, is
possible. However, a low microbiological prevalence can imply a low or a high
seroprevalence. Batches with a low microbiological prevalence and low seroprevalence
point towards a recent infection, it is even possible this happened in the lairage (Nielssen
*et al.*, 1996; Nesbakken *et al.*, 2006), while a low microbiological prevalence combined
with a high seroprevalence suggests an infection that took place earlier. The analytical
results at slaughter age depend on the moment of the initial infection and on its course.
It is not well known yet at which specific age pigs become infected. If piglets introduce
*Yersinia* spp. in pig farms (Virtanen *et al.*, 2014), the infection is expected to take place at
a young age. Moreover, according to Fukushima *et al.* (1983), who experimentally re-
infected pigs, *Yersinia* spp. did not reappear in the faeces, concluding that re-infection
does not occur. The influence of a second infection on the infection status of the tonsils
is still unknown. Consequently, without re-infection and after clearing the infection, many
pigs should become *Yersinia* free prior to slaughter. In the present studies however, many
pigs are still infected in their tonsils prior to slaughter. This could be due to re-infection or
to the carrier status of the pigs. In contrast to the hypothesis of Fukushima *et al.* (1983),
re-infection probably does occur. The study of Fukushima *et al.* (1983) was performed a
long time ago. It would be interesting to verify whether the results are still applicable and
to further elucidate the possibility of re-infection. Secondly, if re-infection exists, its
frequency will have an influence on the bacteriological and serological prevalence at the
moment of slaughter.

The implementation of the prediction of the infection rate of pigs prior to slaughter by
serology is matrix depending. The advantages of using serology are the low cost, and the
applicability prior to slaughter while microbiological analysis of the tonsils is more
expensive, time consuming and can only be carried out after slaughter. When the
information about the prevalence is available after slaughter, it is already too late to
differentiate between low and highly infected batches prior to slaughter. Also, it is too
late to avoid (cross-)contamination at the slaughterline and to have an impact on the meat contamination status. A low seroprevalence was always related to a low microbiological prevalence (Chapter 4). In contrast, a high seroprevalence stands for either a low or a high microbiological prevalence. Before the collection of data overtime (historical data) can be introduced, the fluctuation of the prevalence of subsequent batches from the same farms should be studied. For instance, it could be that the batches coming from the same farm will always show the similar prevalence. However, it is also possible that there is a big variation between these batches. Using historical data based on microbiology of tonsils to categorize the following batches is only useful when the within-batch microbiological prevalence is stable over time. This should be studied. The result should be included in the food chain information document. The farmer can assess the infection rate and the effect of preventive measurements by checking the results of the within-batch prevalence provided by the slaughterhouse.

Since batches with a low seroprevalence also have a low microbiological prevalence, serological analysis is a tool to assure the microbiological prevalence at slaughter. Serological based categorization of batches with a low or high infection rate, creates the opportunity of logistic slaughtering, which is a new possibility to lower the risk of (cross-) contamination in the slaughterhouse. The formulated equation in Chapter 4 is the basis to this categorization.

\[
\text{mean within – batch microbiological prevalence} = \frac{0.444}{1 - e^{-0.063 \times (\text{OD}\% - 37.069)}}
\]

This equation seems difficult, but it is usable. When a farm for example has a mean activity value of 20 OD%, the mean within-batch microbiological prevalence is -0.23, which means there are no infected pigs in this batch. When, on the other hand, the mean activity value is 50 OD%, the mean within-batch microbiological prevalence is 0.80. Many pigs in this batch are infected at slaughter age.
2. The possibilities of the farmer to influence the prevalence

At farm level, potential opportunities are present to decrease the number of infected pigs delivered to the slaughterhouse. Risk and protective factors were studied in Chapters 5 and 6. Three risk factors were the same in both studies: the use of a semi slatted floor, the presence of pig farms in the area and the number of piglet suppliers. Two factors, the proper use of a disinfection bath and the possible snout contact between pigs from adherent pens, were significant protective factors in the multivariate analysis based on the microbiological and serological prevalence, respectively, while they were both significant protective factors in the univariate analysis of the other study. The CDE is only a significant protective factor in the study based on the microbiological prevalence. CDE and the possible snout contact both interact with the presence of pets, resulting in the change from protective to risk factor. Differences between the results of the microbiological and serological risk factor study have two explanations. Firstly, the species on which the study was based on. The microbiological factors are based only on the prevalence of *Y. enterocolitica*, while the serological factors are based on human pathogenic *Yersinia* spp. Secondly, the microbiologically based risk factors indicate the possibilities why pigs could be infected at moment of slaughter, while the factors based on serology show the factors why pigs could get infected during rearing. Pig farmers should take into account the risk and protective factors, which are indicated in this thesis, to reduce the number of infected pigs. Some factors cannot be implemented straight away. The proximity of pig farms in the area (closer than 500m) is not usable because the location of a farm cannot be changed. This factor is a borderline risk factor (0.05 < P-value < 0.1) in the study based on the serological prevalence, but is significantly associated (Odds Ratio = 1.63; P-value = 0.036) in the risk factor study based on the microbiological prevalence. Nevertheless, this factor was never identified as a risk factor in other studies, maybe because some regions or countries have a very low density of pig farms (e.g. Finland). There is still no clarification why this factor was significant in the present study. The transmission of enteropathogenic *Yersinia* spp. between pig farms (e.g. emission of air, pest animals, persons,...) has not been studied so far, except for transporting piglets between farms (Virtanen *et al.*, 2014). *Yersinia* spp. are maybe transferred aerogenic. In
the present study, only the proximity of the neighbours (500 m) was included. As supposed by the fact that nearby farms are a risk factor for infection, there are possible transmission routes. The direction of wind, the number of neighbours or their biosecurity were not taken into account in this study. Theses three items are however important when try to elucidate the influence of aerogenic spread. *Yersinia* spp. could also be transferred by pest animals (insects or rodents), be due to contact between farms or a minor biosecurity (e.g. same boots used in different compartments or contaminated boots worn by visiting farmers). The possible transmission routes should be examined more in detail. Pest animals could move between farms and transfer pathogens. Contact between farms, especially between low and high infected farms, should be minimized. In the studies described in Chapters 5 and 6, many biosecurity measurements showed to be not significant. Even when biosecurity was at a high level, farms could still be *Yersinia* positive. When a compartment contains only pigs from the same origin, and the internal biosecurity is maintained, there should be no transmission possible between compartments. Even these farms harbored pigs microbiologically positive in the tonsils. Maybe these pigs were already infected when they arrived at the farm or were infected by their mother. Another possibility is that *Yersinia* spp. could be transferred by air, by flies or is part of the farm microbiota. The possibility of *Yersinia* staying on-farm for years has never been investigated. The infection route at farms with a high biosecurity level is still unknown. When the farmer does not use an all-in/all-out system, newly introduced pigs can easily be infected by the elderly animals. However, this factor showed to be not significant and many (80%) farmers did apply an all-in/all-out system in the fattening pig unit. Pigs introduced in an empty compartment are still at risk for infection. Genotyping *Yersinia* strains of subsequent batches of a fattening pig farm could indicate the possibility of persisting strains. Also genotyping strains of nearby farms could demonstrate possible transmissions between these farms.

Changing a semi into a fully slatted floor type and creating the possibility of snout contact between pens in the fattening pig unit can only be implemented when the farmer is willing to cooperate or when building or renewing a stable. This could however be introduced in the legislation when building new stables. The use of a fully slatted floor was also
indicated as a risk factor for a higher prevalence of *Salmonella* by Hotes *et al.* (2010). The use of a semi slatted floor was a borderline risk factor (0.05 < P-value < 0.1) in the study based on the microbiological prevalence of tonsils. But this same factor was strongly associated (Odds Ratio = 3.78) as risk factor to the serological prevalence. This suggests that this factor is meaningful in both studies. Laukkanen *et al.* (2009) indicated the use of bedding material as a protective factor. Nevertheless, the use of bedding material is not compatible with a fully slatted floor. The possible snout contact in the fattening pig unit is strongly associated with a lower seroprevalence (Odds Ratio = 0.1), while this factor was only significant in the univariate analysis based on microbiological prevalence (Odds Ratio = 0.6). The low seroprevalence of pigs raised on farms allowing snout contact, points to an infection at a young age (antibodies are decreasing) or very recently (still low antibody levels). A very recent infection due to snout contact is unlikely since the pigs are having this contact since they entered the fattening pig unit. In the univariate analysis based on microbiological prevalence it seems that snout contact is also a protective factor. In the multivariate analysis, the factor was not significant anymore. Distinguishing low from highly infected farms at moment of slaughter based on serology is difficult in farms allowing snout contact. They have more chance of having a low seroprevalence, but there is no relation with the microbiological prevalence.

An applicable option at every pig farm to reduce the number of on-farm infections with enteropathogenic *Yersinia* spp., is placing a disinfection bath inside, at the entrance of the stable and replace the fluid at least every two weeks, based on the time of activity of the products used (e.g. MS Kiemkill tabs, Schippers, Arendonk, Belgium). The use of a disinfection bath was a significant protective factor (Odds Ratio = 0.58) in the risk factor analysis based on the microbiological prevalence and also a significant protective factor in the univariate analysis based on the seroprevalence (Odds Ratio = 0.31) (Chapters 5 and 6). Both studies indicate that the proper use of a disinfection bath will decrease the prevalence. This is a low-cost effective measurement that also helps to avoid or diminish the spread of other pathogens.
An increasing number of piglet suppliers was indicated in both studies as a risk factor (Odds Ratio = 1.15 in Chapter 5; Odds Ratio = 1.43 in Chapter 6). Decreasing the number of piglet suppliers is an easily applicable measure to reduce the microbiologically and serologically based prevalence and was also mentioned by Vilar et al. (2013). The difference of the within-batch prevalence of farms with one versus two or three suppliers indicates that though even with a minor effort, it is effective in reducing this prevalence. This is probably due to the smaller chance of buying piglets from an infected farm. When reducing the number of suppliers, it is also important that farmers select the *Yersinia* negative suppliers. If piglets from different suppliers should be placed in different compartments, with a satisfying internal biosecurity, the risk of spread will decrease.

In the thesis, there were some contradictions with the literature, as some factors studied were not significant to have an influence on the microbiologically or serologically based within-batch prevalence, whereas they were significant in other studies. The presence of pest animals in the stables, for example, was not significant in the present study. However, Laukkanen et al. (2009) found that their presence was a protective factor, while Novoslavskij et al. (2013) indicated it as a risk factor. Another factor, like the feed producer, turned out to be too diverse to take into account. Finally, Virtanen et al. (2011) pointed fasting pigs before transport to the slaughterhouse as a risk factor for faecal shedding at time of slaughter. Maybe, it is due to the stress induced by fasting, that starts 24 h before the transport to the slaughterhouse, that increases the faecal shedding of *Y. enterocolitica*. However, farmers are obliged to fasten all pigs at slaughter before transport.

*Yersinia enterocolitica* free pig herds can however be raised. Such herds have already been established and maintained for many years in Norway, where Specific Pathogen Free (SPF) herds do not contain *Y. enterocolitica* (Nesbakken et al., 2007). When the top of a breeding pyramid is free from human pathogenic *Y. enterocolitica*, the prevalence of *Y. enterocolitica* will decrease in the general pig population. The proportion of infected sows variates between 0-14% (Niskanen et al., 2002; Korte et al., 2004; Gürtler et al., 2005; Bowman et al., 2007; Wehebrink et al., 2008; Farzan et al., 2010). Keeping batches or
herds non- or low infected, is also depending on the risk of transmitting \textit{Y. enterocolitica} to this farm. Pilon \textit{et al}. (2000) has found a limited number of positive environmental samples, indicating that infections are potentially coming from outside (pigs, persons or pets). Nesbakken \textit{et al}. (2007) suggested that these human pathogenic \textit{Y. enterocolitica}-free segments of the pig population could be the beginning of providing human pathogen–free (HPF) pork on the market. At this time, the Belgian Federal Agency for the Safety of the Food Chain (FAVV) is trying to produce \textit{Salmonella}-free piglets by clearing the top of the breeding pyramid from \textit{Salmonella} (Sci Com 2013/06). As stated by Nesbakken \textit{et al}. (2007), pig herds can also become \textit{Y. enterocolitica}-free. Maybe the methods used to reduce the prevalence of \textit{Salmonella}, may be similar to those decreasing the prevalence of \textit{Yersinia} spp.. Producing slaughter pigs free from both important pigborne pathogens should be possible. It is still uncertain whether the goal of producing \textit{Salmonella}- and \textit{Y. enterocolitica}-free slaughter pigs could be achieved by only reducing the prevalence of both pathogens in the breeding herds and reduce or eliminate the transfer of these pathogens in the feed. Other transmission routes could also be important.

3. Final impact on carcass contamination

The slaughterhouse can adopt its slaughter activities on the within-batch prevalence of arriving pigs at slaughter and apply appropriate slaughter techniques and logistic slaughtering. 

As mentioned by EFSA, and if pig batches are categorized according to the risk for human health they imply, there are two options. First, the low infected pig batches could be slaughtered, followed by the high infected batches. Then, it is of great importance to obtain a strict separation between these two types of batches and the lairage area should be cleaned thoroughly every day. Another possibility is to slaughter them on different days. Logistic slaughtering has already been introduced for high \textit{Salmonella} prevalence in pig herds. This logistic slaughtering is only based on the seroprevalence of \textit{Salmonella}, without a relation with the microbiological prevalence. This resulted in the observation of no clear effects on carcass contamination (Arguello \textit{et al}., 2013). The seroprevalence does
not indicate the infection rate of a pig batch at slaughter. Slaughtering batches having a low seroprevalence still ends by slaughtering low and highly infected batches, which results in an equal risk to contaminate carcasses during slaughter procedures as when all batches are slaughtered independent of their serological status.

Adapting slaughtering techniques can be advantageous to avoid (cross-)contamination to pig carcasses. Nesbakken et al. (1984) and Laukkanen et al. (2010) showed that bagging of the rectum assists in the reduction of the faecal contamination of the carcasses. This method is common in the Nordic countries. This practice is also useful to protect the carcass from contamination with other bacteria. The tonsils are also an important source of carcass contamination and the splitting machine is a transporter of bacteria from one pig to another when the head is also splitted (Van Damme, 2013). In order to avoid carcass contamination by the tonsils, EFSA suggests to open the head away from the slaughter line. This practice however, has never been investigated thoroughly. Nesbakken et al. (2003) showed that the incision of the submaxillary lymph nodes and touching carcasses by the meat inspection personnel also allowed (cross-)contamination. At last, the previous meat inspection was adapted to avoid transferring as less as possible bacteria between pig carcasses by preventing contact of the inspector with the carcass. According to EFSA (Biohaz, 2011), palpating and making incisions in the carcass is a risk factor of (cross-)contamination. At this moment, only a visual meat inspection is allowed (EC 219/2014). Carcasses are only palpated and incised when abnormalities have been discovered and when the pig is removed from the slaughter line.
4. Future perspectives

There are still some uncertainties remaining on the infection of pigs with enteropathogenic *Yersinia* spp. which can be studied in the future:

- When does the pig get infected for the first time? Where does this initial infection come from? Is the moment farm-dependent? Is there a difference between farrow-to-finish and fattening pig farms?
- Do pigs get re-infected? If it exists, where does this infection comes from? How does the pig react on this second infection?
- Concerning both mentioned future studies, the possible transmission routes of infection are still unknown.
- What is the antimicrobial resistance of *Y. enterocolitica*? Is there a difference between farms? Is this linked to the antimicrobial use on a farm?
- Is the introduction of *Y. enterocolitica* on a farm accompanied by *Salmonella*?
- Nearby farms are indicated as a risk factor. It is possible that those farms harbor the same genotypes of *Yersinia* spp. This can also indicate a possible transmission route.
- Does the prevalence fluctuate between subsequent batches? Maybe historical data can be used to categorize batches.
SUMMARY
There are three human pathogenic species in the genus *Yersinia*: *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. While *Y. pestis* is no longer present in Europe, human pathogenic *Y. enterocolitica* is the most important of the two enteropathogenic *Yersinia* spp. due to its high number of human cases. The animal host species of *Y. pseudotuberculosis* are birds and pest animals, whereas *Y. enterocolitica* is mostly found in pigs. This latter species is also responsible for most of the foodborne outbreaks caused by *Yersinia* spp., originating from pork products whereas the outbreaks caused by *Y. pseudotuberculosis* are mostly derived from vegetables. The prevalence of human pathogenic *Y. enterocolitica* in pigs varies greatly among countries and pig farms (0-100%), but statistically based studies about the within-batch prevalence were lacking. The way of farming could have an influence on the presence of *Y. enterocolitica*.

A preliminary study of the within-batch prevalence of *Y. enterocolitica* in Belgian pig batches at slaughter was performed to show the variation of this prevalence and is discussed in Chapter 1. Tonsils of 1397 pigs from 66 batches were collected. On average 21 tonsils per batch were sampled. These tonsils were analyzed using the direct plating method on cefsulodin-irgasan-novobiocin (CIN) agar plates, and these results were verified performing a Polymerase Chain Reaction (PCR) (detecting the *ail* gene). Pathogenic *Y. enterocolitica* were found in 375 pig tonsils (26.8%). The within-batch prevalence ranged from 0 (20 batches) to 83.3%. The number of colonies varied between $2.01$ and $6.00 \log_{10} \text{CFU} \text{ g}^{-1} \text{ tonsil}$. This preliminary study revealed a great variety in presence of *Y. enterocolitica* among pig batches without clustering around a certain prevalence.

These results were taken into account in the following study, where more samples per batch were taken to detect low infected farms. The number of pigs per batch sampled was calculated based on an expected batch prevalence of 50%, a confidence level of 95% and an accepted error of 10%. Tonsils (Chapter 2) and pieces of diaphragm (Chapter 3) were collected from each of the 7047 fattening pigs at slaughter, originating from 100 farms. On average, 70 pigs were sampled per batch. Again, direct plating on CIN agar plates was performed on the homogenate of the tonsils, followed by a PCR(species and
serotype). The pieces of the diaphragm (± 10g) were first stored at -20°C, where after they were thawed and 2 ml of meat juice was collected, which was used in the ELISA Pigtype Yopscreen (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany). This ELISA targets antibodies against all human pathogenic Yersinia spp. The optical density (OD) was determined at 450 nm. The results were positive if the absorbance exceeded the proposed cut-off value of 30%. Pathogenic Y. enterocolitica serotype O:3 were found in tonsils of 2009 pigs (28.5%), originating from 85 different farms, with a count between 2.01 and 5.98 log_{10} CFU g^{-1} tonsil (on average 4.00 log_{10} CFU g^{-1} tonsil). The within-batch prevalence in positive farms ranged from 5.1 to 64.4%. Yersinia pseudotuberculosis was found in seven farms, for which the within-batch prevalence varied from 2 to 10%. The serological tests resulted in a binomial-shaped distribution with modes at 0-8.3 OD% and 58.3-66.6 OD% for the individual pigs with an average of 51 OD%. Sixty-six percent of the animals tested positive according to the used criterion. At batch level, there was also a binomial distribution with modes at 0-5% (n=12) and 85-90% (n=16). The within-batch seroprevalence ranged from 0 (n=7) to 100% (n=1). The results of these studies revealed that many batches are microbiologically as well as serologically positive for Y. enterocolitica and Y. pseudotuberculosis and that there is a difference between farms. This variation could be due to certain farm factors.

The two methods, microbiology and serology, of obtaining the within-batch prevalence were compared (Chapter 4). If there is a relation, the microbiological prevalence at time of slaughter and important for food safety, can be predicted by the serological prevalence obtained before slaughter. The results of these prevalences were compared using a mixed-effect logistic regression at pig and batch level. Of the 2009 pigs positive for Y. enterocolitica, 1872 also had antibodies against Yersinia spp. Unfortunately, at pig level, the microbiological contamination could not be predicted by the presence of antibodies. Nevertheless, at batch level, a relation was observed (prevalence =0.444/(1-e^{-0.063*(Optical Density-37.069)}), cut-off value for a positive farm is 37 OD%). This formula could predict whether a pig batch will contain infected pigs before they arrive at the slaughterhouse. This way eventually, infected batches could be slaughtered last so the risk of cross-contamination in the slaughterhouse could be avoided or diminished.
The results of the study on the within-batch prevalence of *Y. enterocolitica* (and *Y. pseudotuberculosis*) in pigs at slaughter were the basis of the risk factor analysis at farm level. In Chapter 5, the analysis was performed on the bacteriological results discussed in Chapter 2, but only based on *Y. enterocolitica*. Chapter 6 is looking at the risk factors for a high seroprevalence, based on both enteropathogenic *Yersinia* spp. Each farm was visited and data concerning housing, ventilation, biosecurity, management, feeding and disease control were collected using a face-to-face questionnaire. The number of positive animals per batch was the outcome variable. First, variables were submitted to a univariable analysis using a mixed effect logistic regression, with farm as random effect. Variables which were related to the *Yersinia* prevalence (P < 0.05) were included in a multivariable model, excluding at each step the non-significant variable until only significant main effects and interactions remained. In the multivariable model based on bacteriology, three risk factors, two protective factors and one interaction remained significantly associated with *Y. enterocolitica* carriage in the tonsils (P < 0.1). More piglet suppliers, a high density of pig farms in the surroundings and semi slatted floors in the fattening pig stables were positively associated with a higher infection level whereas the use of a disinfection bath before entering the stables and a poor biosecurity level were protective factors. The latter protective factor showed a significant interaction with the factor ‘presence of pets in the stables’, which increases the prevalence. Based on serological results, four risk factors, one protective factor and one interaction remained significantly associated with the presence of antibodies against enteropathogenic *Yersinia* spp. in meat juice (P < 0.1). Many piglet suppliers, a high density of pig farms in the area and the use of semi slatted floors in the fattening pig unit were considered risk factors. The only protective factor was the possibility of snout contact in the fattening pig unit, although a significant positive interaction between the presence of pets in the stables and snout contact was observed. The risk factors are similar between microbiological and serological prevalence results. Nevertheless, a poor biosecurity level has an influence on the bacteriological prevalence, but not on the serological prevalence. Otherwise, snout contact decreases the antibody level in pig batches, but has no influence on the presence of *Y. enterocolitica* in tonsils of pigs at slaughter.
At last, a proposal is formulated in the general discussion to reduce the amount of contaminated carcasses in the slaughterhouse. This proposal is based on a control at farm- and slaughterhouse level. The pig farmer can, after analyzing the risk and protective factors, indicate what has to/should be changed at the farm. At the end of the rearing period, blood samples should be collected to obtain an impression of the infection rate in a batch of slaughter pigs. The results can help the slaughterhouse to indicate non- or low-infected batches and to slaughter them first, so contamination in the slaughterhouse can be reduced. More research should be performed about the dynamics of infection at farm level, what happens with the antibody titer after re-infection and subsequent batches of the same farm should be observed to indicate the variation of the prevalence over time.
SAMENVATTING
Binnen het genus *Yersinia* bestaan er 15 species die geen infectie bij de mens kunnen veroorzaken en drie humaan pathogene species: *Y. pestis*, *Y. pseudotuberculosis* en *Y. enterocolitica*. Alle drie bezitten ze minstens één virulentieplasmide, dat naast de chromosomale virulentiegenen, verantwoordelijk zijn voor het ontstaan van infecties. *Yersinia enterocolitica* is onderverdeeld in zes biotypes, waarvan enkel 1A niet humaan pathogeen is (mist het virulentieplasmide). *Yersinia pestis* is niet meer aanwezig in Europa waardoor deze niet meer verder besproken wordt. De humaan pathogene *Y. enterocolitica* is de belangrijkste van de twee enteropathogene *Yersinia* species door zijn relatief hoog aantal humane casussen. Beiden geven gastro-intestinale symptomen. De dierlijke gastheren van *Y. pseudotuberculosis* zijn vogels en knaagdieren, terwijl *Y. enterocolitica* vooral teruggewonden wordt bij varkens. Dit laatste species is ook verantwoordelijk voor de meeste voedselgebonden uitbraken veroorzaakt door *Yersinia*, uitgaande van gecontamineerd varkensvlees. Uitbraken veroorzaakt door *Y. pseudotuberculosis* zijn meestal het gevolg van de consumptie van besmette groenten.

De prevalentie van humaan pathogene *Y. enterocolitica* in varkens varieert sterk tussen verschillende landen en varkensbedrijven (0-100%). Studies over de binnenlotprevalentie gebaseerd op statistische aantallen waren echter niet. De manier waarop een varkensbedrijf gerund wordt, kan, naast de infrastructuur, een grote invloed hebben op de aanwezigheid van *Y. enterocolitica*.

In een preliminaire studie bij verschillende loten van Belgische varkens werd aangetoond dat er een variatie is in deze prevalentie op het moment van slachten (Hoofdstuk 1). Tonsillen van 1397 varkens, afkomstig van 66 loten, werden verzameld. Gemiddeld werden er 21 tonsillen per lot bemonsterd. Deze tonsillen werden geanalyseerd via de directe uitplatingsmethode op cefsulodin-irgasan-novobiocin (CIN)-agar platen. Humaan pathogene *Y. enterocolitica* werden teruggevonden in 375 varkenstonsillen (26,8%). De binnenlotprevalentie varieerde van 0 (20 loten) tot 83,3%. Het aantal kolonies per gram tonsillair weefsel varieerde van 2,01 tot 6,00 log_{10} CFU. Deze preliminaire studie toonde een grote spreiding aan in de aanwezigheid van *Y. enterocolitica* tussen varkensloten afkomstig van verschillende bedrijven waarbij ook een grote variatie aanwezig was (geen clustering rond bepaalde prevalenties).
Deze resultaten werden meegenomen naar een tweede studie, waar meer stalen per lot werden genomen om laag-geïnfecteerde bedrijven te detecteren. Het te bemonsteren aantal varkens per lot werd berekend op een verwachte binnenlotprevalentie van 50% met een betrouwbaarheidsinterval van 95% en een aanvaarde fout van 10%. Tonsillen (Hoofdstuk 2) en een stuk van het diafragma (Hoofdstuk 3) werden verzameld van elk van de 7047 vleesvarkens op het moment van slachten. Deze varkens waren afkomstig van 100 loten die elk van een ander varkensbedrijf kwamen. Gemiddeld werden 70 varkens per lot bemonsterd. Opnieuw werd het homogenaat van de tonsillen gebruikt bij de directe uitplating op CIN-agar platen, gevolgd door een PCR op species- en serotypeniveau. De stukjes diafragma (± 10g) werden eerst bewaard bij -20°C, waarna ze ontdooid werden. Op het verzamelde vleesvocht (2 ml) werd de ELISA Pigtype Yopscreen (Labor Diagnostik Leipzig, Qiagen, Leipzig, Germany) uitgevoerd. Deze ELISA detecteert antistoffen tegen alle humaanpathogene Yersinia soorten. Wanneer de absorptie de voorgesteld cut-off van 30% overschreed, waren de resultaten positief. Pathogene Y. enterocolitica serotype O:3 werden teruggevonden in de tonsillen van 2009 varkens (28.5%), komende van 85 verschillende bedrijven, met een telling tussen 2,01 en 5,98 log₁₀ CFU g⁻¹ tonsil (gemiddeld 4,00 log₁₀ CFU g⁻¹ tonsil). De binnenlotprevalentie in positieve bedrijven varieerde van 5,1 tot 64,4%. Yersinia pseudotuberculosis werd gevonden in zeven bedrijven. Daarbij varieerde de binnenlotprevalentie van 2 tot 10%. De serologische tests resulteerden in een binomiaal gevormde distributie met modi bij 0-8,3 OD% en 58,3-66,6 OD% voor de individuele varkens, met een gemiddelde van 51 OD%. Zesenzestig procent van de onderzochte dieren testte positief volgens het vooropgestelde criterium. Op lotniveau was er eveneens een binomiale distributie met modi bij 0-5% (n=12) en 85-90% (n=16). De binnenlotseroprevalentie varieerde van 0 (n=7) tot 100% (n=1). Het resultaat van deze studies was dat veel bedrijven zowel microbiologisch als serologisch positief zijn voor Y. enterocolitica en Y. pseudotuberculosis en dat er een verschil is tussen bedrijven. De variatie zou het gevolg kunnen zijn van bedrijfsfactoren.

De beide methoden waarmee de prevalentie bepaald werd, werden vergeleken (Hoofdstuk 4). Indien er een relatie is, kan de microbiologische prevalentie op het moment van slachten, die belangrijk is voor de voedselveiligheid, voorspeld worden door
de serologische prevalentie voor het slachten. De resultaten van deze prevalenties werden vergeleken via een mixed-effect logistische regressie op dier- en lotniveau. Van de 2009 varkens die positief waren voor Y. enterocolitica, hadden 1872 ook antistoffen tegen Yersinia spp. Op dierniveau kon de microbiologische contaminatie niet voorspeld worden aan de hand van de aanwezigheid van antistoffen. Daarentegen werd er op lotniveau wel een relatie waargenomen (prevalentie =0.444/(1-e^{-0.063*(Optische Densiteit-37.069)})), met een cut-off waarde voor een positief bedrijf 37 OD%. Deze formule kan voorspellen of een lot geïnfecteerde varkens zal bevatten voordat ze aankomen in het slachthuis. Op deze manier kunnen de geïnfecteerde loten eventueel als laatste geslacht worden zodat kruiscontaminatie in het slachthuis vermeden of verminderd kan worden.

De resultaten van de studie over de microbiologische en serologische binnenlotprevalentie van Y. enterocolitica (en Y. pseudotuberculosis) in varkens op slachtleefijd vormden de basis van de risicofactoren analyse op bedrijfsniveau. In Hoofdstuk 5 werd de analyse uitgevoerd op de bacteriologische resultaten van Y. enterocolitica. Hoofdstuk 6 beschrijft de risicofactoren voor een hoge seroprevalentie, gebaseerd op beide enteropathogene Yersinia spp. Elk varkensbedrijf werd persoonlijk bezocht en info met betrekking tot het huisvesting, de ventilatie, de bioveiligheid, het management, de voeding en de ziektebestrijding werden verzameld via een enquête met de varkenshouder. Het aantal positieve dieren per lot was de uitkomst variabele. Eerst werden de variabelen onderworpen aan een univariabele analyse gebruik makend van een mixed-effect logistische regressie, met het bedrijf als random effect. Variabelen die gerelateerd waren met de Yersinia prevalentie (P < 0.05) werden meegenomen in het multivariabele model. Bij elke stap werden de niet-significante variabelen uitgesloten, totdat enkel significante variabelen en interacties overbleven. In het multivariabele model gebaseerd op bacteriologie, bleven drie risicofactoren, twee beschermende factoren en één interactie over die significant geassocieerd waren met de aanwezigheid van Y. enterocolitica in de tonsillen (P < 0.1). Meerdere biggenleveranciers, veel varkensbedrijven in de omgeving (dichter dan 500m) en halfvolle vloeren in de mestvarkensstallen waren positief geassocieerd met een hoger infectieniveau terwijl het gebruik van een desinfectiebad voor het betreden van de stallen en een slechte
bioveiligheid beschermende factoren waren. Deze laatste vertoonde een positieve, significante interactie met de factor ‘aanwezigheid van huisdieren in de stallen’. Gebaseerd op de serologische resultaten, vier risicofactoren, één beschermende factor en één interactie waren geassocieerd met de aanwezigheid van antistoffen tegen enteropathogene *Yersinia* in vleessap (P < 0.1). Meerdere biggenleveranciers, veel varkensbedrijven in de omgeving (dichter dan 500m) en halfvolle vloeren in de mestvarkensstallen werden geïdentificeerd als risicofactoren. De enige beschermende factor was de mogelijkheid van snuitcontact in de vleesvarkensstallen. Een significant positieve interactie tussen de aanwezigheid van huisdieren in de stallen en snuitcontact werd gezien. Deze risicofactoren zijn gelijklopend tussen de microbiologische en serologische prevalentieresultaten. Hoe dan ook, een slechte bioveiligheid heeft een invloed op de bacteriologische prevalentie, maar niet op de serologische resultaten. Aan de andere kant, vermindert snuitcontact het niveau van aanwezige antistoffen in loten, maar heeft geen invloed op de aanwezigheid van *Y. enterocolitica* in tonsillen van varkens op slachtleeftijd.

Tot slot wordt er in de algemene discussie een voorstel gedaan dat kan leiden tot een reductie van het aantal gecontamineerde karkassen in het slachthuis. Dit voorstel is gebaseerd op een controle op bedrijfs- en slachthuisniveau. De varkenshouder kan, na het analyseren van de risico- en beschermende factoren, bekijken wat er op zijn bedrijf veranderd kan/moet worden. Op het einde van de vetmestingfase kan er per bedrijf bloed verzameld worden om zo een indruk te krijgen van de infectiestatus van een lot varkens. Deze uitslag kan gebruikt worden door het slachthuis om niet- of laag-besmette loten eerder te slachten zodat contaminatie in het slachthuis gereduceerd kan worden. Daarnaast moet er nog verder onderzoek gebeuren naar de dynamiek van de infectie binnen een bedrijf, wat er met de antistoffentiter gebeurt na herinfectie en opeenvolgende loten zouden opgevolgd moeten worden om eventuele variatie op te sporen tussen loten van hetzelfde bedrijf.


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CURRICULUM VITAE


Gerty Vanantwerpen is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Ze nam actief deel aan meerdere internationale en nationale congressen.

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Vanantwerpen, G., De Zutter, L. Estimation of the Within-batch Prevalence of Human Pathogenic Yersinia Enterocolitica in Pigs at Slaughter. Seventeenth Conference on Food


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Buitenlandse dienstreizen

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ECVPH AGM and Conference. 19-20 September 2013, Turin, Italy.
Food Micro 2014. 1-4 September 2014, Nantes, France.
ECVPH AGM and Conference. 6-8 October 2014, Copenhagen, Denmark.
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