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Isolation and spread of enteropathogenic *Yersinia* spp. throughout the pork production chain

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List of abbreviations

ail	attachment invasion locus
BPW	buffered peptone water
CFU	colony forming units
CIN	cefsulodin-irgasan-novobiocin agar
CR-MOX	congo red-magnesium oxalate
CV	crystal violet
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
FT-IR	Fourier transform infrared spectroscopy
HPI	high-pathogenicity island
inv	invasin
ISO	International Organization for Standardization
ITC	irgasan-ticarcillin-potassium chlorate
KIA	kligler iron agar
KOH	potassium hydroxide
Lcr	low-calcium response
LPS	lipopolysaccharide
MAC	MacConkey
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAP	modified atmosphere packaging
MLVA	multilocus variable number of tandem repeat analysis
MRB	modified rappaport broth
Myf	mucoid *Yersinia* factor
OD	optical density
OR	odds ratio
PCA	plate count agar
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMB	peptone-mannitol-bile salts
PSB	peptone-sorbitol-bile salts
PW	peptone water
pYV	plasmid for *Yersinia* virulence
QMRA	quantitative microbiological risk assessment
SSDC	*Salmonella-Shigella* deoxycholate agar
T3SS	type III secretion system
TSB	tryptic soy broth
U	units
virF	virulence factor
yadA	*Yersinia* adhesin A
YeCM	*Y. enterocolitica* chromogenic medium
Yop	*Yersinia* outer protein
YPM	*Y. pseudotuberculosis* derived mitogen
Ysc	Yop secretion

General introduction

1. Taxonomy and characteristics of *Yersinia* spp.

1.1. The genus Yersinia

The genus *Yersinia* is a member of the family *Enterobacteriaceae* in the class *Gammaproteobacteria* of the phylum Proteobacteria. The genus currently comprises 17 species, of which only three are well-characterized human pathogens: *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. *Yersinia pestis* evolved from *Y. pseudotuberculosis* 1,500 to 20,000 years ago and both species share over 90% DNA homology (Achtman *et al.*, 1999). Despite the closer genetic relation with *Y. pestis*, *Y. pseudotuberculosis* behaves clinically similar to *Y. enterocolitica*. *Yersinia ruckeri* is a fish pathogen causing red mouth disease in rainbow trouts and other freshwater fish (Ewing *et al.*, 1978; Oraic *et al.*, 2002). As *Y. ruckeri* is genetically the most distant species within the genus, its classification in the genus *Yersinia* is controversial (Chen *et al.*, 2010; Kotetishvili *et al.*, 2005). The remaining 13 *Yersinia* species (also called ‘‘environmental” *Yersinia* or *Y. enterocolitica*-like species) are either regarded as avirulent or their presumptive pathogenicity has not been studied or confirmed (Sulakvelidze, 2000; Hurst *et al.*, 2011; Merhej *et al.*, 2008; Murros-Kontiainen *et al.*, 2011b; Murros-Kontiainen *et al.*, 2011a; Sprague and Neubauer, 2005; Sprague *et al.*, 2008).

All members of the genus *Yersinia* are non-spore forming, Gram-negative, facultative anaerobic coccobacilli that are catalase-positive and oxidase negative. They are 0.5-0.8 µm in width and 1-3 µm in length and most species are not motile at 37°C, but become motile at 22-30°C due to peritrichous flagella. They grow between 0°C and 45°C, with an optimal growth temperature of 25-30°C.

1.2. Yersinia enterocolitica

The heterogeneous species *Y. enterocolitica* is differentiated in six biotypes and several serotypes, based on biochemical characteristics and lipopolysaccharide (LPS) O-antigens, respectively (see further). Based on their pathogenic potential, these biotypes can also be subdivided in three pathotypes (Table 1). Strains of biotype 1A are also called ‘environmental’ or ‘non-pathogenic’ *Y. enterocolitica* because they are frequently isolated from the environment and lack most virulence markers (Batzilla *et al.*, 2011b; Stephan *et al.*, 2013). Strains of biotype 1B are highly pathogenic, and strains of biotypes 2 to 5 are low to moderately pathogenic. Based on 16S rRNA sequence analysis, Neubauer *et al*. (2000a) subdivided *Y. enterocolitica* in two subspecies: *Y. enterocolitica* subsp. *enterocolitica*, which comprises the highly pathogenic strains of biotype 1B, and *Y. enterocolitica* subsp. *palearctica*, with the low to moderate and non-pathogenic strains. Nevertheless, biotyping provides additional information and is currently still more established than subspecies typing. Historically, biotype 1B strains were predominant in the USA, and were also called ‘American’ strains, whereas the low pathogenic strains were predominant in Europe and were called ‘European’ strains. Nowadays, the low pathogenic strains are also frequently isolated in North America (Lee *et al.*, 1991) and strains of bioserotype 1B/O:8 have recently also been isolated in Europe (Gierczynski *et al.*, 2009; Schubert *et al.*, 2003). Based

on whole-genome comparisons, Howard *et al*. (2006) suggested the existence of three subspecies as they found three distinct clades: a high pathogenicity (biotype 1B), a low pathogenicity (biotypes 2-4), and a more heterogeneous non-pathogenic clade, mainly comprising biotype 1A strains.

Table 1. Relation between biotypes, serotypes, pathogenicity and virulence determinants of *Y. enterocolitica* (adapted from EFSA (2007)).

Biotype	Serotype(s)	Pathogenicity	pYV[b]	HPI[c]
1A	O:4; O:5; O:6,30; O:6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT[a]	None to low	-	-
1B	O:4,32; O:8; O:13a,13b; O:16; O:18; O:20; O:21; O:25; O:41,42; NT	High	+	+
2	O:5,27; O:9; O:27	Low to moderate	+	-
3	O:1,2,3; O:3; O:5,27	Low to moderate	+	-
4	O:3	Low to moderate	+	-
5	O:2,3	Low to moderate	+	-

[a] NT: not typable; [b] pYV: plasmid for *Yersinia* virulence; [c] HPI: high-pathogenicity island.

1.3. Yersinia pseudotuberculosis

Y. pseudotuberculosis is classified into 15 serotypes and several subtypes based on LPS O-antigens (Bogdanovich *et al.*, 2003). *Y. pseudotuberculosis* isolates can be subdivided in four biotypes using melibiose, citrate, and raffinose (Tsubokura and Aleksic, 1995), but unlike *Y. enterocolitica*, these biotypes are not clearly associated with differences in pathogenicity. All *Y. pseudotuberculosis* isolates are regarded as potentially pathogenic for humans (EFSA, 2007), though pathogenicity may differ among strains due to the variable presence and instability of different virulence genes (Ch'Ng *et al.*, 2011) (see further).

2. Pathogenesis and virulence genes of enteropathogenic *Yersinia* spp.

Enteropathogenic *Yersinia* are transmitted via the faecal-oral route and usually infect humans through ingestion of contaminated food or water. After passage through the stomach, the bacteria colonize the intestinal tract, particularly the terminal ileum and proximal colon, where the majority of their pathological effects and hence clinical manifestations occur (Bottone, 1997). Enteropathogenic *Yersinia* penetrate the intestinal mucosa via M-cells overlaying Peyer's patches (Autenrieth and Firsching, 1996; Isberg and Barnes, 2001). After invasion into Peyer's patches, yersiniae are able to resist phagocytosis, which allows them to replicate extracellularly and form microcolonies (Heesemann *et al.*, 2006; Oellerich *et al.*, 2007). Subsequently, *Yersinia* may disseminate via the lymphatics and blood to the mesenteric lymph nodes or other extra-intestinal tissues such as liver and spleen (Autenrieth and Firsching, 1996; Oellerich *et al.*, 2007).

In response to the changing environment in the host, enteropathogenic *Yersinia* can rapidly change gene expression and elaborate virulence factors required to survive the host (Dube, 2009). The pathogenicity of enteropathogenic *Yersinia* is multifactorial and requires several virulence genes, located on the chromosome and on a 70-kb virulence plasmid.

2.1. Chromosomal encoded virulence factors

Initial invasion in the intestinal epithelium requires at least two chromosomal genes, *inv* (invasin) and *ail* (attachment invasion locus). The *inv* gene encodes an outer membrane protein called invasin, which binds to β_1 integrins and mediates efficient translocation through the M-cells of the Peyer's patches (Heesemann *et al.*, 2006; Isberg and Barnes, 2001; Pepe and Miller, 1990). Invasin may also promote dissemination in host tissues through mediation of proinflammatory epithelial cell reactions (Grassl *et al.*, 2003). The *ail* gene encodes Ail, a surface protein with several functions, including serum resistance and adhesion to cells and extracellular matrix (Mikula *et al.*, 2012).

Y. enterocolitica produce a heat-stable enterotoxin, called Yst (*Yersinia* stable toxin), which belongs to the family of STa heat-stable enterotoxins and resembles both structurally and functionally the enterotoxin of enterotoxigenic *Escherichia coli* (Delor *et al.*, 1990; Robins-Browne *et al.*, 1979). STa toxins bind and activate the intestinal receptor guanylyl cyclase C, which leads to cGMP accumulation and extensive water and electrolyte secretion (Lin *et al.*, 2010), and may thus play a role in diarrhoea during infection with *Y. enterocolitica* (Revell and Miller, 2001; Delor and Cornelis, 1992). Within *Y. enterocolitica*, three subtypes of Yst are identified (YstA, YstB and YstC). The *yst*A gene is found in pathogenic biotypes of *Y. enterocolitica*, whereas the *yst*B gene is predominant in *Y. enterocolitica* biotype 1A (Thoerner *et al.*, 2003; Bonardi *et al.*, 2013; Singh and Virdi, 2004; Ramamurthy *et al.*, 1997). *Yst*C is the most toxic enterotoxin, but is uncommon in *Y. enterocolitica* isolates (Yoshino *et al.*, 1995b; Zheng *et al.*, 2008b; Singh and Virdi, 2004).

Lipopolysaccharides (LPS) are the major component of the outer membrane of Gram-negative bacteria and consist of three regions: a lipid A (the endotoxically active part of the molecule), an oligosaccharide core, and an O-polysaccharide (responsible for the serological properties and often referred to as the O-antigen) (Erridge *et al.*, 2002). The O-antigen is necessary for full virulence in both *Y. enterocolitica* and *Y. pseudotuberculosis* and is required for effective colonization of Peyer's patches. Nevertheless, the specific role of O-antigens in *Yersinia* virulence is still unknown (Skurnik and Bengoechea, 2003).

The *myf* and *psa* gene cluster encode proteins for expression and assembly of fibrillar structures called Myf (Mucoid *Yersinia* factor) and pH6 (psa) antigen in *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively (Yang *et al.*, 1996; Iriarte and Cornelis, 1995). The pH6 antigen mediates *in vitro* cell adhesion and hemagglutination of *Y. pseudotuberculosis* (Yang *et al.*, 1996). MyfA is the main subunit of the Myf antigen and is highly conserved in pathogenic biotypes of *Y. enterocolitica* (Stephan *et al.*, 2013), though homologues sequences can be found in strains of biotype 1A (Batzilla *et al.*, 2011b) and its precise role *in vivo* is not clear.

The high-pathogenicity island (HPI) is a chromosomal region that carries genes for Yersiniabactin biosynthesis, transport and regulation (Carniel, 2001). Yersiniabactin is a siderophore responsible for iron uptake, which is a key nutrient for bacterial growth. The HPI correlates with a high pathogenic phenotype and is only present in certain strains of *Y. pseudotuberculosis* and *Y. enterocolitica* biotype 1B (Carniel, 2001; Fukushima *et al.*, 2001), but is absent in *Y. enterocolitica* bioserotype 4/O:3 (Batzilla *et al.*, 2011a).

Some strains of *Y. pseudotuberculosis* produce superantigenic toxins called YPM (*Y. pseudotuberculosis* derived mitogen), which mediate activation of the immune system by excessive stimulation of human T lymphocytes (Müller-Alouf *et al.*, 2001). Currently, three alleles of the superantigen (*ypmA*, *ypmB*, and *ypmC*) have been described, which are located in an unstable locus of the genome (Fukushima *et al.*, 2001; Carnoy *et al.*, 2002). The distribution of *ypm* genes in *Y. pseudotuberculosis* isolates differs geographically as they are more frequently found in clinical strains from the Far East than in European strains (Fukushima *et al.*, 2001; Yoshino *et al.*, 1995a).

2.2. The virulence plasmid

All pathogenic *Yersinia* strains harbour an approximately 70-kb plasmid called pYV (plasmid for *Yersinia* virulence), which is essential for full virulence. The virulence plasmid allows *Yersinia* to survive and replicate extracellular within lymphoid tissues of the host (Heesemann *et al.*, 2006).

The plasmid contains genes encoding a type three secretion system (Ysc T3SS) and a variety of secreted proteins, called *Yersinia* outer proteins or Yops (Bleves and Cornelis, 2000; Dube, 2009; Heesemann *et al.*, 2006; Viboud and Bliska, 2005). Upon host cell contact, Yop effector proteins are injected into the cytoplasm through the Ysc T3SS. Yops are exotoxins that modulate the cytoskeleton and immune signalling pathways, which leads to inhibition of phagocytosis, proinflammatory cytokine production and induction of apoptosis in macrophages. The pYV also encodes an outer membrane protein YadA (*Yersinia* adhesion, encoded by the *yad*A gene), which has many biological functions, including adherence to cells and extracellular matrix components, serum and phagocytosis resistance, and the ability to autoagglutinate (El Tahir and Skurnik, 2001). Despite the high homology, functional differences between *Yad*A of *Y. enterocolitica* and *Y. pseudotuberculosis* exist (Heise and Dersch, 2006).

The transcription of *Yad*A and most of the genes involved in Yop synthesis and secretion is controlled by the transcriptional activator *vir*F (or *lcr*F in *Y. pseudotuberculosis*), which is also located on the virulence plasmid. Yop secretion can be triggered *in vitro* when *Yersinia* is grown at 37°C in a medium depleted of Ca^{2+}, which leads to growth inhibition and is known as ‘low calcium response’ (Cornelis *et al.*, 1998).

3. Yersiniosis in humans

3.1. Clinical manifestations

Enteropathogenic *Yersinia* cause a spectrum of manifestations, ranging from mild self-limiting enteric infections to life-threatening septicaemia. Infections typically manifest as gastro-enteritis, but may also lead to severe extra-intestinal manifestations and a variety of secondary complications. The clinical outcome is determined by host factors, such as the age and the presence of underlying medical conditions, and bacterial specific characteristics, such as species and serotype.

In the gastrointestinal tract, *Y. enterocolitica* can cause acute enteritis, enterocolitis, terminal ileitis, and mesenterial lymphadenitis. The most common symptoms in *Y. enterocolitica* infections are diarrhoea, abdominal pain, and fever (Boqvist *et al.*, 2009; Rosner *et al.*, 2010; Huovinen *et al.*, 2010; Hoogkamp-Korstanje and de Koning, 1991). The diarrhoea, which is sometimes bloody, usually lasts 2 to 4 weeks but has in some patients the tendency to be chronic and recidives (Hoogkamp-Korstanje and de Koning, 1991; Saebo *et al.*, 2005).

The highest incidence of yersiniosis is found in young children under five years of age (Figure 1), where infection mainly manifests as acute gastroenteritis (Verhaegen *et al.*, 1998; Zheng *et al.*, 2008a). Terminal ileitis and mesenterial lymphadenitis are mostly seen in older children and adolescents, in which symptoms may mimic those of acute appendicitis (pseudo-appendicular syndrome) (Shorter *et al.*, 1998; Perdikogianni *et al.*, 2006; Antonopoulos *et al.*, 2008) and may lead to unnecessary appendectomy (Rimhanen-Finne *et al.*, 2009; Rosner *et al.*, 2013; Zheng *et al.*, 2008a; Sakellaris *et al.*, 2004). In rare occasions, enteropathogenic *Yersinia* are implicated in true appendicitis, which is typically granulomatous (Lamps, 2010; Kojima *et al.*, 2007). When symptoms present for weeks to months, this can lead to the misdiagnosis of inflammatory bowel disease as *Yersinia* may mimic Crohn's disease both histologically and clinically (Lamps, 2010).

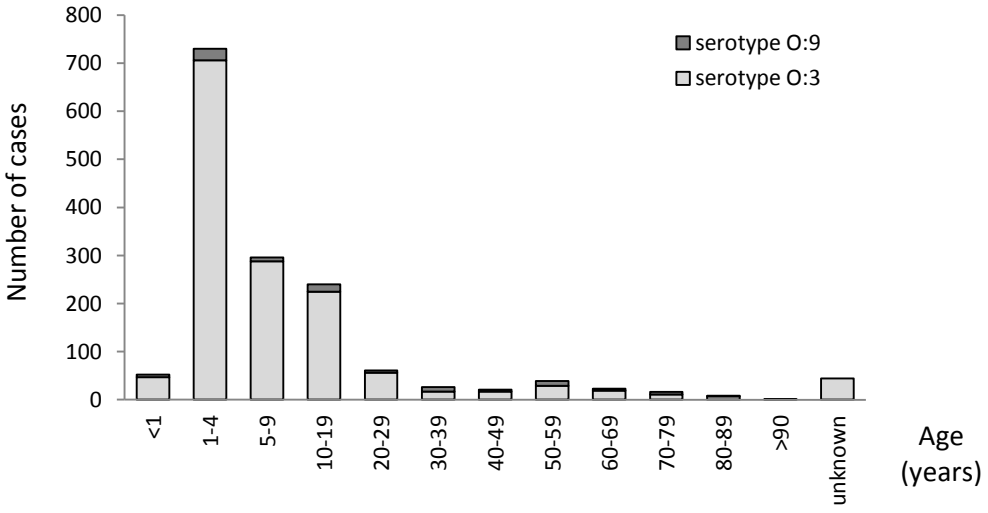

Figure 1. Age distribution of *Y. enterocolitica* serotype O:3 and O:9 infections.
Data from 2006-2011 in Belgium, based on WIV (2012).

Reactive arthritis and erythema nodosum are the most common postinfectious complications of yersiniosis, and can be seen in up to 50% of the reported cases (Hannu *et al.*, 2003; Huovinen *et al.*, 2010; Jalava *et al.*, 2006). Reactive arthritis occurs mainly in adults (Hannu *et al.*, 2003; Rosner *et al.*, 2013), and the incidence is higher among human leucocyte antigen (HLA)-B27-positive patients. However, HLA-B27 does not appear to predispose to infection itself, but increases the risk of developing an arthritis that is more likely to be severe and prolonged (Gaston and Lillicrap, 2003). The clinical presentation of reactive arthritis is usually severe and polyarticular, and predominantly affects the small joints of the hands and feet, knees, ankles and shoulders (Hannu *et al.*, 2003; Zheng *et al.*, 2008a). Reactive arthritis develops about 1-2 weeks after the first symptoms of infection (Hannu *et al.*, 2003; Zheng *et al.*, 2008a; Rosner *et al.*, 2013; Yotsu *et al.*, 2010), and frequently lasts for several months or years (Hannu *et al.*, 2003; Hoogkamp-Korstanje *et al.*, 2000; Reiss-Zimmermann *et al.*, 2007).

Enteropathogenic *Yersinia* may cause infections at extra-intestinal sites, such as abscess formation in liver and spleen (Grigull *et al.*, 2005; Bergmann *et al.*, 2001; Rathmell *et al.*, 1999; Stölzel *et al.*, 2009), pharyngitis (Ito *et al.*, 2012), pneumonia (Wong *et al.*, 2013), septic arthritis (Pouplin *et al.*, 2002), osteomyelitis (Ellenrieder *et al.*, 2010; Katamura *et al.*, 2001; von Eckardstein *et al.*, 2004), encephalitis (Pulvirenti *et al.*, 2007), and endocarditis (Karachalios *et al.*, 2002; Krajinovic *et al.*, 2007). Septicaemia and invasive infections due to *Y. enterocolitica* and *Y. pseudotuberculosis* are not common, but are severe and can have high mortality rates (Bergmann *et al.*, 2001; Mischnik *et al.*, 2012). They are most often described in immunocompromised patients and patients with underlying medical conditions associated with iron overload, such as thalassaemia and haemochromatosis (Zheng *et al.*, 2008a; Vincent *et al.*, 2008; Crowe *et al.*, 1996; Bergmann *et al.*, 2001; van Zonneveld *et al.*, 2002), but may also occur in patients without predisposing factors (Crowe *et al.*, 1996; Kaasch *et al.*, 2012).

Clinical manifestations of *Y. pseudotuberculosis* infections differ geographically and are usually more diverse and severe in the Far East than in Europe, which might be related to the presence of the superantigen YPM in Far Eastern isolates (Fukushima *et al.*, 2001; Yoshino *et al.*, 1995a; Kaito *et al.*, 2012; Abe *et al.*, 1997). *Y. pseudotuberculosis* has also been associated with Kawakasi disease, which is a systemic vasculitis in young children, and the development of serious complications such as coronary artery aneurysms (Vincent *et al.*, 2007; Konishi *et al.*, 1997; Tahara *et al.*, 2006).

Uncomplicated yersiniosis cases are usually self-limiting and do not merit antimicrobial therapy. However, antibiotics may be necessary in immune-compromised patients and patients with more severe invasive infections, such as septicaemia and extra-intestinal manifestations. Enteropathogenic *Yersinia* are generally susceptible to many broad-spectrum antibiotics, although *Y. enterocolitica* is resistant to ampicillin and first-generation cephalosporins due to β-lactamase production (Baumgartner *et al.*, 2007; Bucher *et al.*, 2008; Fredriksson-Ahomaa *et al.*, 2012a; Meyer *et al.*, 2011; Pham *et al.*, 2000). Nevertheless, multiresistant *Y. enterocolitica* have been described, which may be associated with a conjugative plasmid (Sihvonen *et al.*, 2011b). Antimicrobial therapy for yersiniosis infections includes tetracyclines, chloramphenicol,

gentamycin, third generation cephalosporins and fluoroquinolones (Crowe *et al.*, 1996; Gayraud *et al.*, 1993).

3.2. Incidence and species/bioserotype distribution

In 2011, the total number of confirmed yersiniosis cases was 7,017 in the EU, corresponding to a notification rate of 1.63 per 100,000 population (EFSA and CDC, 2013). The number of yersiniosis cases in Belgium varied between 211 to 303 in the period 2005-2010, which corresponds to an incidence of 2 to 3 cases per 100,000 inhabitants (WIV, 2012). Nevertheless, the reported data are believed to underestimate the true incidence of yersiniosis due to difficulties associated with the isolation and confirmation techniques. Moreover, for a yersiniosis case to be reported, an infected person must seek medical care, submit a stool sample, and the causative agent must be identified (Rimhanen-Finne *et al.*, 2009; Scallan *et al.*, 2013). As such, it is estimated that for every laboratory confirmed case of *Y. enterocolitica* in the USA, there are 123 yersiniosis cases that are not confirmed (Scallan *et al.*, 2011).

The vast majority of yersiniosis cases in Belgium and most other European countries are caused by *Y. enterocolitica*, particularly bioserotype 4/O:3 and to a lesser extent also 2/O:9 and 2/O:5,27 (EFSA and CDC, 2013; Rosner *et al.*, 2010; Fredriksson-Ahomaa *et al.*, 2012a; Sihvonen *et al.*, 2009). The relative proportion of serotypes varies according to the age group as the most common serotype O:3 is more prevalent in younger patients, whereas the more rare serotypes are relatively more prevalent in older patients (Rosner *et al.*, 2010; Verhaegen *et al.*, 1998) (Figure 1). Moreover, serotype O:9 is usually more isolated from blood and extra-intestinal samples than serotype O:3 (Verhaegen *et al.*, 1998).

Y. pseudotuberculosis is isolated less frequently than *Y. enterocolitica*, but generally causes more severe and invasive infections (Long *et al.*, 2010; Vincent *et al.*, 2008). Most infections in Europe are caused by serotype O:1 (Vincent *et al.*, 2008; EFSA, 2007; Verhaegen *et al.*, 1998).

Most yersiniosis infections are sporadic (Rosner *et al.*, 2010; Vincent *et al.*, 2008), though some outbreaks with *Y. enterocolitica* and *Y. pseudotuberculosis* have been described (Table 2).

Table 2. Reported outbreaks of *Y. enterocolitica* and *Y. pseudotuberculosis*.

Country	Period	Number of patients	Number of hospitalized patients	Median age of patients in years (range)	Source of infection	Location	Serotype	Reference
Y. enterocolitica								
Croatia	January 2002	22 (17[a])	0	NS (30 – 60)	Unknown	Oil tanker	O:3	Babic-Erceg *et al*. (2003)
Japan	August 2004	42 (16[a])	NS	NS	Salad	School	O:8	Sakai *et al*. (2005)
	July 2006	3[a]	0	5 (0 – 68)	Unknown + person-to-person	Household	O:9	Moriki *et al*., (2010)
Norway	December 2005	11[a]	4 (2†)	44 (10 – 88)	Brawn (ready-to-eat pork)	Households	O:9	Grahek-Ogden *et al*., (2007)
	January 2011	21[a]	NS	30-39 (10 – 63)	Bagged salad mix[d]	Nationwide	O:9	(MacDonald *et al*., 2011)(2011)
USA	October 1995	11[a]	4	7.5 (0 – 41)	Milk[d]	Households	O:8	Shorter *et al*., (1998) and Ackers *et al*., (2000)
	November 2001	12[a,b]	4	0 (0.1 – 0.7)	Chitterlings[d]	Households	O:3	Jones *et al*. (2003)
	November 2002	9[a]	6	0 (0.1 – 1.1)	Chitterlings	Households	O:3	CDC (2003)
	March 2011	16[a]	7	26 (1 – 75)	Pasteurized milk, ice cream	NS	NS	CDC (2011)
Y. pseudotuberculosis								
Canada	November 1998	74[a]	NS	NS	Milk[d]	Nationwide	O:1b	Nowgesic *et al*. (1999)
Finland	October 1998	47[a]	16 (1†)	19 (2 – 77)	Iceberg lettuce[d]	Nationwide	O:3	Nuorti *et al*., (2004)
	May 2003	111 (58[a])	9	10 (4 – 52)	Carrots[a]	School, day care	O:1	Jalava *et al*., (2006)
	March 2004	53 (5[a])	NS	NS	Carrots[a]	School	O:1b	Kangas *et al*., (2008)
	August 2006	427 (119[a])	29	15 (12 – 60)[c]	Carrots[a]	School, day-care	O:1b	Rimhanen-Finne *et al*. (2009)

[a] culture confirmed; [b] different pulsotypes; [c] based on a subpopulation; [d] based on case-control study; NS, not specified; † number of patients that died.

3.3. Transmission routes

Y. enterocolitica is primarily transmitted via the faecal-oral route, most often by the consumption of contaminated food or water. Different case-control studies have been performed, often identifying the consumption of (raw) pork as a significant risk factor for human yersiniosis (Table 3). As such, pork consumption is estimated to be responsible for 71.6 to 77.3% of *Yersinia* infections (Fosse *et al.*, 2008; Batz *et al.*, 2012) and raw pork explains about 30% of all sporadic *Y. enterocolitica* infections in Germany (Rosner *et al.*, 2012).

Table 3. Case-control studies to identify risk factors for human yersiniosis.

Country	Species	Cases	Controls	Risk factor	Reference
Belgium	YE	40	40	Consumption of raw pork	Tauxe *et al*., (1987)
Canada	YE	19	19	Salami consumption	Harb *et al*. (2000)
Finland	YE	54	133	Eating or tasting raw or medium done pork Eating outside home Travelling abroad	Huovinen *et al*., (2010)
Finland	YP	25[a]	71	Eating iceberg lettuce Eating outside home	Jalava *et al*., (2004)
Germany	YE	352	1495	Consumption of raw minced pork Recent preparation of minced pork in the household Playing in a sandbox Contact with birds	Rosner *et al*., (2012)
Israel	YE	16[b]	128	Malnutrition Non-chlorinated water supply	El Quoga *et al*. (2011)
New Zealand	YE	176	379	More than 2 people living in the home No town supply water No reticulated sewerage Not looking after a young child Consumption of pork Eating food from a sandwich bar Eating less fruit and vegetables	Satterthwaite *et al*. (1999)
Norway	YE	67	132	Consumption of pork and sausage Preference of eating undercooked meats Drinking untreated water	Ostroff *et al*. (1994)
Sweden	YE	117[c]	339	Eating food prepared from raw pork or sausages Use of a dummy Contact with domestic animals	Boqvist *et al*. (2009)
USA	YE	10[d]	51	Household preparation of chitterlings	Jones *et al*. (2003)

[a] ≥ 18 years old, including outbreaks; [b] children < 12 years old; [c] children < 6 years old; [d] children < 1 year old.

Besides the foodborne transmission, *Y. enterocolitica* can be transmitted via direct contact with animals. Increased *Y. enterocolitica* serotype O:3 antibodies have been reported in occupations with pig contact, such as pig farmers and abattoir workers (especially those handling throats and intestines) (Seuri and Granfors, 1992; Merilahti-Palo *et al.*, 1991). As pathogenic *Y. enterocolitica* are also found in pet animals (Fredriksson-Ahomaa *et al.*, 2001c; Stamm *et al.*, 2013), close contact between humans and companion animals may facilitate the transmission of the pathogens to humans.

Occasionally, infections may occur through direct inoculation from an environmental source via a wound (*e.g.* finger), which may lead to spread to regional lymph nodes and development of suppurative adenitis (*e.g.* axillary abscess) (Gumaste *et al.*, 2012; Kelesidis *et al.*, 2008; Menzies, 2010). In rare occasions, *Y. enterocolitica* can be transmitted via transfusion of contaminated blood products (Benavides *et al.*, 2003; Guinet *et al.*, 2011). At time of blood sample obtainment, donors may have a transient bacteraemia, after which *Yersinia* may survive and replicate during storage of the blood at 1-4°C. Transfusion of blood products containing large amounts of microorganisms and endotoxin often leads to a rapid and severe sepsis or septic shock, resulting in mortality rates of over 50% (Guinet *et al.*, 2011; Leclercq *et al.*, 2005; Burger *et al.*, 2000).

4. Enteropathogenic *Yersinia* spp. in animals and foods

4.1. Enteropathogenic Yersinia *spp. in animals*

Y. pseudotuberculosis and *Y. enterocolitica* may cause clinical infections in animals (with occasional significant lethality), including ruminants (Movassaghi and Rad, 2002; Seimiya *et al.*, 2005; Wessels *et al.*, 2009; Juste *et al.*, 2009; Zhang *et al.*, 2008), birds (Cork *et al.*, 1999; Csaba *et al.*, 2000), hares (Bartling *et al.*, 2004; Frölich *et al.*, 2003), and a variety of captive animals in zoological gardens (Bielli *et al.*, 1999; Childs-Sanford *et al.*, 2009; Fredriksson-Ahomaa *et al.*, 2007b; Gombac *et al.*, 2008; Owston *et al.*, 2006). Nevertheless, the strains causing these infections generally differ from those found in the majority of human infections, suggesting a limited transmission of these types from animals to humans.

Enteropathogenic *Yersinia* spp. are also detected in animals without showing clinical symptoms of infection. *Y. pseudotuberculosis* is found in a variety of wild animals (Fukushima and Gomyoda, 1991; Lee *et al.*, 2011; Nikolova *et al.*, 2001; Wobeser *et al.*, 2009), including birds (Niskanen *et al.*, 2003), wild boars (Fredriksson-Ahomaa *et al.*, 2009b; Hayashidani *et al.*, 2002; Fredriksson-Ahomaa *et al.*, 2011), and rodents (Backhans *et al.*, 2011), which are the probable reservoir of *Y. pseudotuberculosis* (EFSA, 2007; Kangas *et al.*, 2008). *Y. enterocolitica* has been detected in cattle (Bonardi *et al.*, 2007), sheep (Chenais *et al.*, 2012; Söderqvist *et al.*, 2012), goats (Arnold *et al.*, 2006; Lanada *et al.*, 2005), poultry (Bancerz-Kisiel *et al.*, 2012; Kechagia *et al.*, 2007), dogs (Fredriksson-Ahomaa *et al.*, 2001c), and wild boars (Bancerz-Kisiel *et al.*, 2009; Fredriksson-Ahomaa *et al.*, 2009b). Nevertheless, pigs are the only food-producing animals where pathogenic biotypes of *Y. enterocolitica* are frequently isolated from (Bucher *et al.*, 2008), and, as most of the pig isolates are similar to strains causing human disease (Fredriksson-Ahomaa *et al.*, 2006;

Virtanen *et al.*, 2013), they are regarded as the primary reservoir of human pathogenic *Y. enterocolitica*. Since epidemiologic studies implicate pork as a major risk factor for human yersiniosis, this section will focus on the presence of enteropathogenic *Yersinia* throughout the pork production chain.

4.1.1. Enteropathogenic *Yersinia* spp. in pigs

On pig farms, human pathogenic *Y. enterocolitica* are frequently recovered from growing and finishing pigs, whereas they are rarely isolated from suckling piglets and sows (Bowman *et al.*, 2007; Gürtler *et al.*, 2005; Wehebrink *et al.*, 2008). During the fattening period, the number of pigs shedding pathogenic *Y. enterocolitica* generally increases with age and decreases again by the end of the fattening period (Nesbakken *et al.*, 2006; Virtanen *et al.*, 2012). Although faecal excretion may stop, tonsils remain positive for a longer time period after initial infection (Nielsen *et al.*, 1996). As scraping the tonsils in live animals is not recommended due to animal welfare reasons, studying *Y. enterocolitica* infections at farm level is complicated (Nesbakken *et al.*, 2006). Since the sampling of tonsils is more straightforward during slaughter, most studies isolating pathogenic *Y. enterocolitica* from fattening pigs have been conducted at abattoir level.

Table 4 shows that pathogenic *Y. enterocolitica* are widespread in fattening pigs at slaughter in different countries, though the rate of isolation varies widely between different studies. The proportion of positive tonsils (ranging from 8 to 93%) is generally higher than the frequency found in intestinal content (varying between 0.5 and 30%). Besides tonsils and intestines, pathogenic *Y. enterocolitica* can also be isolated from mesenteric (De Giusti *et al.*, 1995; Gürtler *et al.*, 2005; Nowak *et al.*, 2006) and submaxillary lymph nodes (Nesbakken *et al.*, 2003), although the isolation rate in these lymph nodes is much lower than in tonsils. In most countries, *Y. enterocolitica* bioserotype 4/O:3 is the predominant bioserotype found in fattening pigs (Table 4). However, in Great Britain, a much higher variety of bioserotypes is found in slaughter pigs (McNally *et al.*, 2004; Milnes *et al.*, 2008; Ortiz Martínez *et al.*, 2010). Human pathogenic *Y. enterocolitica* have also been isolated from the tonsils of sows at slaughter, though the isolation rate is significantly lower compared to the prevalence in fattening pigs (Korte *et al.*, 2004).

The proportion of *Y. enterocolitica* positive farms is generally high (Table 4). Specific-pathogen free breeding farms have been negative for pathogenic *Y. enterocolitica* for several years in Norway due to strict hygienic measures that were applied (Nesbakken *et al.*, 2007). Nonetheless, *Yersinia* negative fattening farms may occur, as 16.3% of fattening herds in Germany have no serological positive animals just before or during slaughter (von Altrock *et al.*, 2011). Nevertheless, the within-batch prevalence of *Y. enterocolitica* may vary greatly between farms and even between different batches of one farm (von Altrock *et al.*, 2011; Gürtler *et al.*, 2005). The prevalence in organic pig production is lower than in conventional pig production (Nowak *et al.*, 2006; Virtanen *et al.*, 2011) and within conventional pig production, the prevalence of *Y. enterocolitica* has been shown higher in fattening herds than in farrow-to-finish herds (Nesbakken *et al.*, 2003; Skjerve *et al.*, 1998).

Table 4. Reported isolations of enteropathogenic *Yersinia* spp. from tonsils and intestinal content of fattening pigs at slaughter.

Country	Tonsils		Intestines		Batches		Bioserotypes (number)	Reference
	N	P	N	P	N	P		
Y. enterocolitica								
Estonia	151	0.894	-	-	15	1.000	4/O:3	Ortiz Martínez *et al*. (2009)
Finland	185	0.330	-	-	NS	NS	4/O:3	Fredriksson-Ahomaa *et al*. (2000a)
	210	0.519	-	-	NS	NS	4/O:3	Korte *et al*. (2004)
	350[a]	0.354[b]	358[a]	0.075[b]	15	0.800	4/O:3	Laukkanen *et al*. (2009)
	301	0.588	301	0.299	55	NS	4/O:3	Laukkanen *et al*. (2010b)
	637	0.471	637	0.154	120	0.942	NS	Virtanen *et al*. (2011)
France	900[c]	0.198	-	-	45	0.800	4/NS (146); 3/NS (38)	Fondrevez *et al*. (2010)
	133[d]	0.180	68[d,e]	0.029	21	0.524	4/O:3	Fosse *et al*. (2010)
Germany	164	0.616	164	0.104	NS	NS	4/O:3	Bucher *et al*. (2008)
	50	0.600	50	0.100	19	0.842	4/O:3	Fredriksson-Ahomaa *et al*. (2001a)
	50	0.620	50	0.160	15	0.867	4/O:3	Fredriksson-Ahomaa *et al*. (2009a)
	372	0.384	379	0.005	4	1.000	4/O:3 (144); O:9 (1)	Gürtler *et al*. (2005)
	410[a]	0.166	410[a]	0.078	9	1.000	NS	Nowak *et al*. (2006)
Great Britain	-	-	2509	0.122	NS	NS	3/O:5,27 (142); 3/O:9 (71); 4/O:3 (33); other (46)	McNally *et al*. (2004)
	-	-	2107	0.051	NS	NS	3/O:5,27 (52); 4/O:3 (19); 3/O:9 (13); other (23)	Milnes *et al*. (2008)
	630	0.441	-	-	45	0.689	2/O:9 (124); 2/O:5 (97); 2/O:3 (24); 2/NS (85); 4/O:3 (39); 3/O:3 (1); other (2)	Ortiz Martínez *et al*. (2010)
Greece	455[c]	0.143	-	-	NS	NS	4/O:3 (58); NS (7)	Kechagia *et al*. (2007)
Italy	150	0.147	150	0.040	27	NS	4/O:3 (18); NS/O:3 (5); NS/O:9 (1); other (4)	Bonardi *et al*. (2003)
	-	-	98	0.041	25	NS	3/O:9	Bonardi *et al*. (2007)
	250	0.080	451	0.011	NS	NS	4/O:3 (23); 2/O:9 (2)	Bonardi *et al*. (2013)
	428	0.320	-	-	22	1.000	4/O:3 (136); 2/O:5 (1)	Ortiz Martínez *et al*. (2011)

Table 4 (continued).

Country	Tonsils		Intestines		Batches		Bioserotypes (number)	Reference
	N	P	N	P	N	P		
Latvia	109	0.642	-	-	5	0.600	4/O:3	Ortiz Martínez *et al*. (2009)
	404	0.354	-	-	47	0.754	4/O:3	Terentjeva and Berzins (2010)
Lithuania	-	-	110	0.182	11	0.545	4/O:3	Novoslavskij *et al*. (2013)
The Netherlands	140	0.093	150	0.033	40	NS	4/O:3 (13); 4/O:5 (5)	de Boer *et al*. (2008)
Russia	197	0.335	-	-	10	1.000	4/O:3	Ortiz Martínez *et al*. (2009)
Spain	200	0.925	-	-	14	1.000	4/O:3	Ortiz Martínez *et al*. (2011)
Switzerland	212	0.340	-	-	16	NS	4/O:3 (69); 2/O:5,27 (6); 2/O:9 (1)	Fredriksson-Ahomaa *et al*. (2007c)
Y. pseudotuberculosis								
Great Britain	630	0.181	-	-	45	0.778	2/O:3 (41) ; 1/O:1 (32) ; 1/O:4 (29); 1/O:2 (8); 1/O:3 (6); 2/O:1 (3); 2/O:5 (1); 3/O:3 (1)	Ortiz Martínez *et al*. (2010)
Greece	455[c]	0.007	-	-	NS	NS	NS	Kechagia *et al*. (2007)
Estonia	151	0.013	-	-	15	0.133	2/O:3	Ortiz Martínez *et al*. (2009)
Finland	350[a]	0.097	358[a]	0.067	15	0.400	O:3	Laukkanen *et al*. (2008)
	301	0.027	301	0.043	55	NS	O:3	Laukkanen *et al*. (2010b)
	210	0.038	-	-	NS	NS	2/O:3	Niskanen *et al*. (2002)
Italy	-	-	98	0.010	25	0.040	NS	Bonardi *et al*. (2007)
	428	0.012	-	-	22	0.136	1/O:1 (3) ; 2/O:3 (1); 2/NS (1)	Ortiz Martínez *et al*. (2011)
Latvia	109	0.046	-	-	5	0.600	2/O:3	Ortiz Martínez *et al*. (2009)
	404	0.030	-	-	47	0.128	NS	Terentjeva and Berzins (2010)
Lithuania	-	-	110	0.100	11	0.354	2/O:3	Novoslavskij *et al*. (2013)
Russia	197	0.066	-	-	10	0.600	2/O:3	Ortiz Martínez *et al*. (2009)
Spain	200	0.000	-	-	14	0.000	-	Ortiz Martínez *et al*. (2011)

N, number of samples tested; P, proportion of positives; NS: not specified.

[a] pigs sampled from preselected farms; [b] only pYV-positive samples included; [c] swab samples ; [d] pooled samples (per five); [e] samples taken on-farm.

Table 5. Risk factors associated with the prevalence of enteropathogenic *Yersinia* spp. in pig herds.

Country (reference)	Species	Number of farms	Number of pigs (average; range)	Test (farm/abattoir)	Risk factor (RF)/protective factor (PF)
Finland (Laukkanen *et al.*, 2008)	*Y. pseudotuberculosis*	15[a]	350 (23.3; 21-26)	Bacteriology of rectal swabs (farm) and tonsils (abattoir)	Birds have access to piggery (RF) Outdoor access (RF)
Finland (Laukkanen *et al.*, 2009)	*Y. enterocolitica*	15[a]	350 (23.3; 21-26)	Bacteriology of rectal swabs (farm) and tonsils (abattoir)	Pest animal have access to pig house (PF) Slaughter pigs have coarse feed or bedding (PF) Slaughter pigs drink from nipple (RF)
Finland (Virtanen *et al.*, 2011)	*Y. enterocolitica*	85	519 (6.1; 1-36)	Bacteriology of tonsils and intestinal content (abattoir)	Use of municipal water (PF) Organic production type (PF) Feed from company A (PF) Artificial light (h/day) (RF) Daily/weekly use of antibiotics (RF) Industrial by-products in feed (RF)
Finland (Virtanen *et al.*, 2011)	*Y. enterocolitica*	80	548 (6.8; 1-36)	Bacteriology of intestinal content (abattoir)	Tonsillar carriage (RF) Use of municipal water (PF) Feed from company B (RF) Fasting pigs before slaughter (RF) Health classification (RF) Use of amoxicillin (PF) Snout contacts (RF) Use of tetracycline (RF)
Germany (von Altrock *et al.*, 2011)	Enteropathogenic *Yersinia* spp.[b]	80[c]	2400 (30)	Serology of blood (farm/abattoir)	Fully slatted floor (PF) Use of municipal water (PF) More recurring health problems (RF) Lower daily weight gain (RF)
Lithuania (Novoslavskij *et al.*, 2013)	*Y. enterocolitica*	9	90 (10)	Bacteriology of pooled faeces (farm)	Low biosecurity level (RF)
Norway (Skjerve *et al.*, 1998)	*Y. enterocolitica*[d]	287[e]	1435 (5)	Serology of blood (abattoir)	Farrow-to-finish production (PF) Under-pressure ventilation (PF) Manual feeding of slaughter pigs (PF) Own vehicle for transport to abattoirs (RF) Separation between clean-unclean (RF) Daily observations of a cat with kittens (RF) Straw bedding for slaughter pigs (RF)

[a] five organic and ten conventional farms; [b] antibodies against Yops; [c] only fattening farms; [d] antibodies against *Y. enterocolitica* serotype O:3; [e] only conventional farms.

Several studies have investigated risk factors that are associated with the presence of human pathogenic *Y. enterocolitica* in (slaughter) pigs (Table 5), though the true infection source for *Yersinia* infections in pig farms remains obscure. Hygienic barriers do not seem to contribute to a lower prevalence of *Y. enterocolitica* in a farm (Skjerve *et al.*, 1998; Virtanen *et al.*, 2011). Water, contact with wild animals, and feed have been indicated as risk factors for *Y. enterocolitica* infections on pig farms (Virtanen and Korkeala, 2012). Nevertheless, environmental sources for the introduction of *Y. enterocolitica* into pig farms remain largely unknown as the pathogens are rarely isolated from the farm environment and feed (Gürtler *et al.*, 2005; Virtanen *et al.*, 2012; von Altrock *et al.*, 2006; Wehebrink *et al.*, 2008). Although pathogenic *Y. enterocolitica* are rarely isolated from sows on-farm, they might be a source of infection for piglets (Bowman *et al.*, 2007; Korte *et al.*, 2004).

Incoming piglets that are infected on breeding farms may be the source of infection in fattening farms and the infection is considered to spread mainly via pig-to-pig transmission (Virtanen *et al.*, 2012). *Y. enterocolitica* seems to spread thoroughly within a farm and generally infects the majority of pigs within a positive herd. Virtanen *et al*. (2012) followed 76 pigs throughout their fattening period in a conventional fattening farm and found that 100% had excreted pathogenic *Y. enterocolitica* at least once during the follow-up. This widespread occurrence is also demonstrated by the high within-herd seroprevalence that is found in fattening pig herds by the time of slaughter. More specifically, von Altrock *et al*. (2011) found that more than half of the fattening herds in Germany had a seroprevalence above 90%.

The number of reports focusing on *Y. pseudotuberculosis* is lower than those studying *Y. enterocolitica*. At farm level, *Y. pseudotuberculosis* is also more frequently found in fattening pigs and only rarely in sows, boars, and piglets (Niskanen *et al.*, 2002; Niskanen *et al.*, 2008). Similar to *Y. enterocolitica*, most studies are conducted at abattoir level, but usually resulting in isolation rates below 10% (Table 4). An exception to this number is Great Britain, where 18% of pig tonsils were found positive by Ortiz Martínez *et al*. (2010). In the study of Laukkanen *et al*. (2009), the prevalence of *Y. pseudotuberculosis* was higher in organic farms than in conventional farms, which is in contrast to *Y. enterocolitica*.

4.1.2. Carcass contamination during pig slaughter

Compared to the large number of reports on the presence of enteropathogenic *Yersinia* in tonsils and faeces, data regarding carcass contamination with enteropathogenic *Yersinia* spp. are scarce. Culture-based reports on carcass contamination with pathogenic *Y. enterocolitica* in different countries vary from 0 to 25.5% (Table 6). A higher contamination is found on edible offal, with isolation rates ranging from 5 to 100%. Parallel to its lower occurrence in pig tissues, carcass contamination with *Y. pseudotuberculosis* is lower than *Y. enterocolitica*, generally being below 4% (Laukkanen *et al.*, 2008; Laukkanen *et al.*, 2010b; Nesbakken *et al.*, 1994; Novoslavskij *et al.*, 2013).

Table 6. Isolations of human pathogenic *Y. enterocolitica* from pig carcasses and edible offal during slaughter.

Country	Number of abattoirs	Number of carcasses	Proportion of positive carcasses	Bioserotype(s)	Sampled sites	Reference
Carcass surface						
Finland	1	80	0.063	4/O:3	Split surface and shoulder	Fredriksson-Ahomaa *et al*. (2000b)
	NS[a]	359	0.064[b]	4/O:3	Thoracic and pelvic cavity	Laukkanen *et al*. (2009)
	1	301	0.216[c]	4/O:3	Pelvis and abdomen, chest and head, skin	Laukkanen *et al*. (2010b)
Germany	NS	383	0.003[b]	4/O:3	Carcass surface	Gürtler *et al*. (2005)
	1	122	0.008	O:3	Belly	Wehebrink *et al*. (2008)
Italy	2	150	0.000	-	Sternal region and throat region	Bonardi *et al*. (2003)
	1	98	0.000	-	Ham	Bonardi *et al*. (2007)
	3	451	0.007	4/O:3; 2/O:9	Hind leg near the tail, back, belly, jowl	Bonardi *et al*. (2013)
Lithuania	2	55	0.255	4/O:3	Thoracic and pelvic cavity	Novoslavskij *et al*. (2013)
Norway	1	24	0.125[b]	O:3	Ham, pelvic duct, kidney region, medial neck	Nesbakken *et al*. (2003)
	1	60	0.150	4/O:3; 2/O:9	Ham	Nesbakken *et al*. (2008)
Sweden	10	541	0.000	-	Ham, back, belly and neck	Lindblad *et al*. (2007)
Edible offal						
Germany	NS	100	0.510	4/O:3	Tongues, lungs, livers, hearts, kidneys	Bucher *et al*. (2008)
	1	20	1.000	4/O:3	Tonsils, tongues, lungs, hearts, diaphragms, livers	Fredriksson-Ahomaa *et al*. (2001a)
	1	20	0.150	4/O:3	Kidneys	Fredriksson-Ahomaa *et al*. (2001a)
	1	1500	0.047	O:3	Livers	von Altrock *et al*. (2010)
Finland	1	51	0.412	4/O:3	Livers, hearts, kidneys, ears	Fredriksson-Ahomaa *et al*. (2000b)
	NS	354	0.093[b]	4/O:3	Livers, lungs, hearts, kidneys	Laukkanen *et al*. (2009)

[a] NS, not specified; [b] pigs from preselected farms; [c] study comparing different slaughter techniques.

Enteropathogenic *Yersinia* are not frequently detected in the abattoir environment (Wehebrink *et al.*, 2008; Sammarco *et al.*, 1997), though *Y. enterocolitica* bioserotype 4/O:3 has already been isolated from the environment and slaughtering equipment, such as the brisket saw, splitting saw, hooks, knives, and air samples (Fredriksson-Ahomaa *et al.*, 2000b; Laukkanen *et al.*, 2010b). Moreover, similar genotypes are found on carcasses and pluck sets at the abattoir and in pigs on-farm (Laukkanen *et al.*, 2008; Laukkanen *et al.*, 2009). Based on the former facts, carcass contamination with enteropathogenic *Yersinia* is believed to originate mainly from incoming pigs.

Enteropathogenic *Yersinia* may disseminate from the tonsils, intestinal content, and other infected tissues to the carcass surface during slaughter and dressing operations (Laukkanen *et al.*, 2008; Laukkanen *et al.*, 2009). Pigs eviscerated by the use of a bung cutter are less frequently contaminated with pathogenic *Y. enterocolitica* than when the rectum is removed manually (Andersen, 1988). Moreover, sealing off the rectum with a plastic bag after loosening further reduces carcass contamination (Laukkanen *et al.*, 2010b; Nesbakken *et al.*, 1994). However, when bagging the rectum, carcass contamination is not completely eliminated, as in Finland still 17% of carcasses was positive after applying this technique (Laukkanen *et al.*, 2010b).

Blast chilling does not reduce the number of *Y. enterocolitica*-contaminated pig carcasses (Nesbakken *et al.*, 2008) and during chilling, cutting, and deboning, a further spread of pathogenic bacteria can occur (Borch *et al.*, 1996).

4.2. Enteropathogenic Yersinia spp. in food

Yersinia is frequently detected in a variety of foods, such as milk and milk products (Najdenski *et al.*, 2012; Ruusunen *et al.*, 2013), vegetables (Cocolin and Comi, 2005; Xanthopoulos *et al.*, 2010), and meats, including beef, poultry, and pork (Bonardi *et al.*, 2010; Sharifi *et al.*, 2011; Lucero Estrada *et al.*, 2012), though the majority of *Yersinia* isolates are non-pathogenic *Yersinia* species or *Y. enterocolitica* biotype 1A (Bonardi *et al.*, 2010; Logue *et al.*, 1996; Wauters *et al.*, 1988).

Compared to the high frequency of human pathogenic *Y. enterocolitica* that is found in pigs and carcasses, the occurrence of these pathogens in retail pork meat products appears to be low. The isolation rates vary greatly depending on the product and applied isolation method. Apart from pig tongues and edible offal, the isolation rates from pork are generally below 10% (Bonardi *et al.*, 2010; Bucher *et al.*, 2008; Lucero Estrada *et al.*, 2012; Fredriksson-Ahomaa *et al.*, 1999b; Fredriksson-Ahomaa *et al.*, 2004; Messelhausser *et al.*, 2011). However, due to a lack of sensitive culture methods, the true contamination level of retail meat is most likely underestimated as the detection limit of culture methods may be 10^3 to 10^6 CFU or more organisms per gram in pork samples (Fredriksson-Ahomaa and Korkeala, 2003).

Enteropathogenic yersiniae are able to survive and grow in foods at refrigeration temperatures, even under modified atmosphere packaging (MAP) (Bergann *et al.*, 1995; Bhaduri and Phillips, 2011; Fredriksson-Ahomaa *et al.*, 2012b; Hayashidani *et al.*, 2008). Whether the number of pathogenic *Y. enterocolitica* will increase or decrease in meat is influenced by a combination of temperature, pH, competitive microflora and the presence of a modified atmosphere (Harrison *et*

al., 2000; Kleinlein and Untermann, 1990; Strotmann *et al.*, 2008). *Y. enterocolitica* also withstands freezing and can survive for extended periods in frozen food (King *et al.*, 2012; Bhaduri, 2006). The heat resistance of enteropathogenic yersiniae is similar to that of other *Enterobacteriaceae* and adequate heating of meat is sufficient to kill *Y. enterocolitica* and *Y. pseudotuberculosis*. The pathogens are inactivated by pasteurization, with *Y. enterocolitica* having a D_{72} value of 0.1 to 3.9 s and D_{65} value of 0.7 to 40 s (Sörqvist, 2003).

5. Detection of enteropathogenic *Yersinia* spp.

5.1. Isolation of enteropathogenic Yersinia spp.

Isolation of pathogenic *Y. enterocolitica* from environmental, animal, and food samples usually involves initial enrichment of the sample followed by plating onto selective agar media (Figure 2). For the isolation of human pathogenic *Y. enterocolitica* from food samples, several standard methods are described, while there are no standardized isolation procedures available for *Y. pseudotuberculosis* (Fredriksson-Ahomaa, 2012). Nevertheless, some isolation methods that were originally developed for *Y. enterocolitica* are also used for *Y. pseudotuberculosis*.

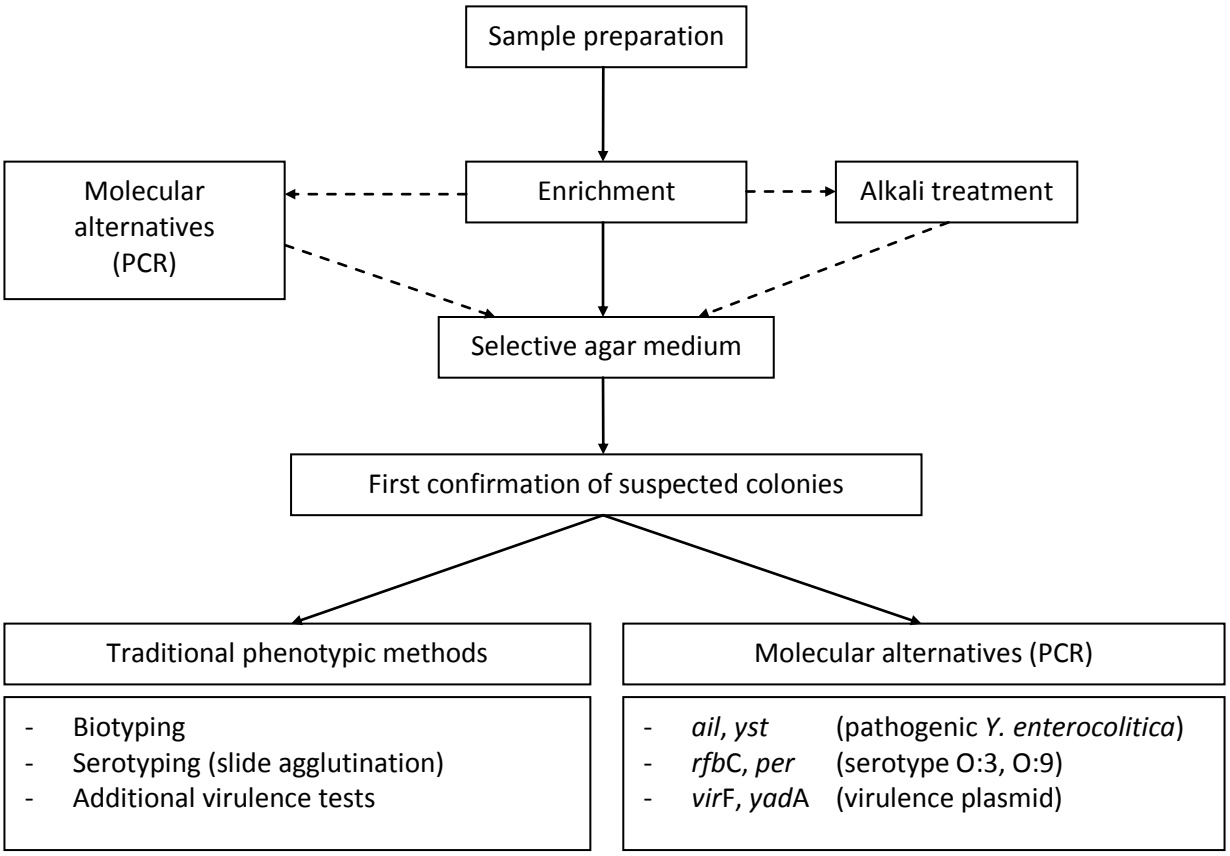

Figure 2. General protocol for the detection, isolation and identification of human pathogenic *Y. enterocolitica*.

As the majority of *Yersinia* isolates from food and environmental sources are non-pathogenic types, it is crucial to discriminate human pathogenic *Y. enterocolitica* from non-pathogenic *Yersinia* strains. Therefore, isolated strains should always be tested for virulence properties after identification at species level.

5.1.1. Enrichment

As the number of pathogens in naturally contaminated samples is generally low, direct isolation without prior enrichment is seldom successful (Wauters *et al.*, 1988; Fukushima, 1985). Numerous enrichment schemes have been described to improve the recovery of *Y. enterocolitica*, and include cold and selective enrichment procedures, or a combination of both (Fredriksson-Ahomaa and Korkeala, 2003). As there is no "universal" enrichment scheme capable to isolate all pathogenic bioserotypes of *Y. enterocolitica*, the use of multiple enrichment broths and plating media are recommended (de Boer, 1992).

Being psychrotrophic, *Yersinia* is able to grow at low temperatures, which is rare among enteric bacteria. This characteristic has provided a successful basis for cold enrichment, by which low to non-selective broths are incubated at 4°C for longer time periods (up to one month) (Fukushima *et al.*, 2011). A drawback of this method is that environmental *Yersinia* and other psychrotrophic bacteria are also able to grow (Fredriksson-Ahomaa and Korkeala, 2003).

Many selective enrichment media have been developed by addition of antimicrobial agents. Selective broths are incubated at higher temperatures, so that *Y. enterocolitica* may be isolated within shorter time periods (Fredriksson-Ahomaa and Korkeala, 2003). Modified Rappaport broth (MRB) contains magnesium chloride, malachite green, and carbenicillin, and is incubated at 22-29°C for 2-4 days (Wauters, 1973). Irgasan-ticarcillin-potassium chlorate (ITC) broth is derived from MRB and is incubated at 24°C for 2 or 3 days (Wauters *et al.*, 1988). These enrichment media have been shown to be effective for the isolation of *Y. enterocolitica* bioserotype 4/O:3, but are less suitable for isolation of *Y. enterocolitica* bioserotype 2/O:9 (Wauters *et al.*, 1988; De Zutter *et al.*, 1994) and *Y. pseudotuberculosis* (Niskanen *et al.*, 2002; Laukkanen *et al.*, 2008).

A disadvantage of enrichment is the additional growth of competing flora, which can complicate selection of *Y. enterocolitica* due to overgrowth on selective agar media. Aulisio *et al*. (1980) observed that both *Y. enterocolitica* and *Y. pseudotuberculosis* are more tolerant to weak alkali solutions than most other Gram-negative bacteria. By mixing the enriched culture with a KOH-solution for a few seconds before plating on an agar medium, back ground flora such *Pseudomonas*, *Proteus* and *Serratia* are suppressed, which facilitates selection of pathogenic *Y. enterocolitica* (Schiemann, 1983).

5.1.2. Selective agar media

Besides the 'traditional' enteric media that can be used for the isolation of *Y. enterocolitica*, (such as MacConkey (MAC) ager and *Salmonella-Shigella* ager) (de Boer, 1992; Schiemann, 1989), many different selective agar media have been developed for the specific isolation of *Y. enterocolitica* (Fredriksson-Ahomaa and Korkeala, 2003).

Salmonella-Shigella deoxycholate calcium chloride (SSDC) agar was developed by adding sodium deoxycholate and $CaCl_2$ to *Salmonella-Shigella* agar (Wauters *et al.*, 1988; Wauters, 1973). *Y. enterocolitica* appears on SSDC agar plates as small, colourless colonies after 24 h of incubation at 30°C. When observing plates through a stereomicroscope with oblique illumination, colonies are non-iridescent, finely granular, and have indistinct edges (Figure 3).

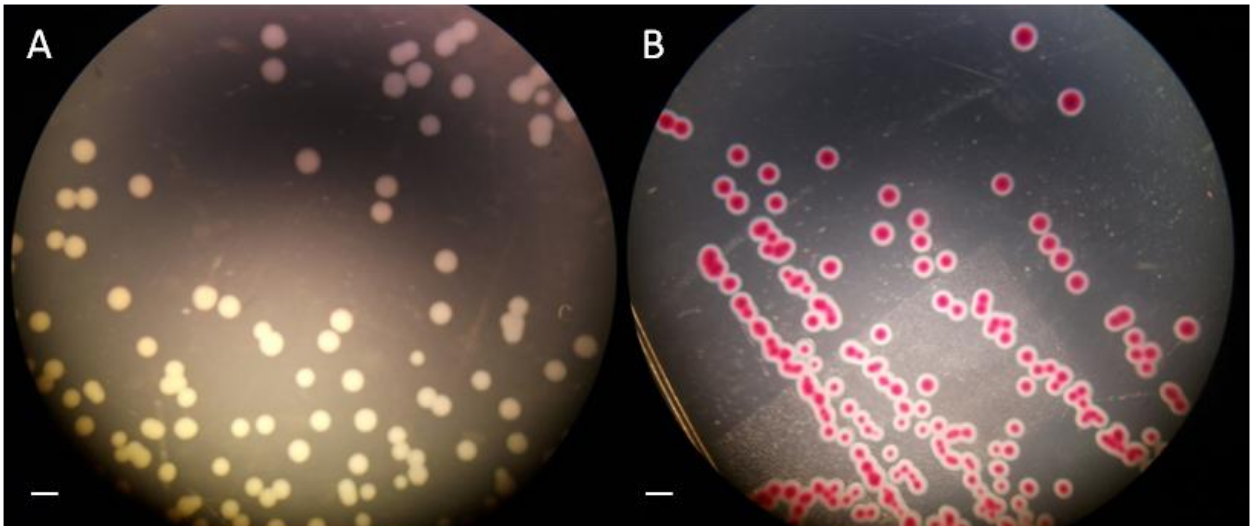

Figure 3. Colony morphology of *Y. enterocolitica* bioserotype 4/O:3 on SSDC (A) and CIN (B) agar plates after 24 h of growth at 30°C (stereomicroscopic view). The white bar represents 1 mm.

Cefsulodin-irgasan-novobiocin (CIN) agar was developed in 1979 (Schiemann, 1979), and is still the most widely used selective agar medium for the isolation of pathogenic *Y. enterocolitica* from human stool samples (Savin *et al.*, 2012) and foods (Fredriksson-Ahomaa, 2012). Nevertheless, the growth of certain *Y. enterocolitica* bioserotype 3/O:3 and *Y. pseudotuberculosis* strains may be inhibited on CIN (Fukushima and Gomyoda, 1986; Savin *et al.*, 2012). Pathogenic *Y. enterocolitica* appear as small colonies with a transparent margin and red centre ('bull's eye' appearance) after incubation at 30°C for 24 h (Figure 3). Non-pathogenic *Yersinia* spp. and *Y. enterocolitica* biotype 1A resemble pathogenic *Y. enterocolitica*, and are difficult to distinguish (Savin *et al.*, 2012). Using a stereo microscope, morphological differentiation is possible as *Y. enterocolitica* serotype O:3 are typically smaller (approximately <1 mm in diameter after 24 h incubation at 30°C), have a deeper red centre, and a sharper border (Hallanvuo *et al.*, 2006; Sihvonen *et al.*, 2009).

Species from several genera can have similar colony morphology on SSDC and CIN agar, and may be difficult to differentiate from pathogenic *Y. enterocolitica*. As such, isolation of pathogenic *Y. enterocolitica* from food and animal samples may be complicated by species belonging to the genera like *Aeromonas*, *Citrobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Providencia* (Arnold *et al.*, 2004; Hilbert *et al.*, 2003; Laukkanen *et al.*, 2010a).

To facilitate the selection of pathogenic *Y. enterocolitica*, several agar media have been developed to differentiate pathogenic from non-pathogenic *Yersinia*. Nevertheless, certain strains may still have a similar appearance as pathogenic *Y. enterocolitica* on these chromogenic

agar media (Table 7). Moreover, Fondrevez *et al*. (2010) used *Y. enterocolitica* chromogenic medium (YeCM) to confirm suspect colonies isolated from CIN agar, though found some strains of the genera *Rahnella*, *Pantoea*, *Citrobacter*, *Vibrio* and *Morganella* to have a similar colony morphology as pathogenic *Y. enterocolitica*.

Table 7. Agar media to differentiate pathogenic *Y. enterocolitica* from non-pathogenic *Yersinia*.

Name	Colony morphology			Reference
	Pathogenic *Y. enterocolitica*	Non-pathogenic *Yersinia*	*Y. pseudotuberculosis*	
Y. enterocolitica chromogenic medium (YeCM)	Red	Purple, blue, green, or red	Blue, green	Weagant (2008)
Y. enterocolitica agar (YECA, AES Chemunex)	Red-fuchsia	Violet, green, pink, yellow or no growth	NS	Denis *et al*. (2011)
CHROMagar *Yersinia* (CAY)	Mauve	Metallic blue, mauve or no growth	No growth	Renaud *et al*. (2013)
Modified virulent *Yersinia enterocolitica* agar (mVYE)	Red	Dark-red with dark peripheral zone or red	Dark pin colonies	Fukushima (1987)

NS, not specified

5.1.3. ISO 10273:2003

The International Standard Organization method (ISO 10273, 2003) for the isolation of presumptive human pathogenic *Y. enterocolitica* from food samples combines two parallel isolation methods: (1) enrichment in ITC with plating on SSDC agar; and (2) enrichment in peptone-sorbitol-bile (PSB) broth with plating on CIN agar (directly and after alkali treatment) (Figure 4). ITC is enriched at 25°C for 48 h and PSB should be enriched at 25°C either for two to three days under continuous agitation, or for five days without agitation. The selective agar plates should be incubated at 30°C for 24 h to 48 h.

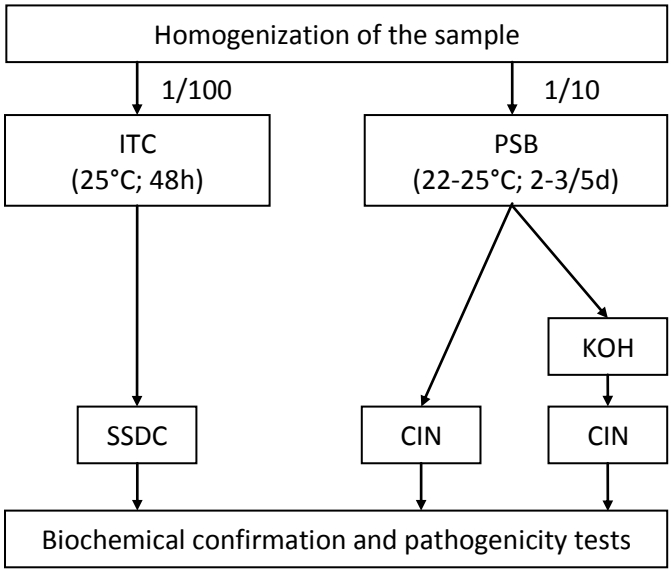

Figure 4. Schematic overview of the ISO 10273:2003 protocol.

5.2. Identification and characterization methods

The identification of *Yersinia* is traditionally based on phenotypic tests, but has been shown difficult due to atypical biochemical reactions (Fredriksson-Ahomaa *et al.*, 2002; Sihvonen *et al.*, 2009) and temperature-dependent biochemical tests. As such, the Voges-Proskauer test in *Y. enterocolitica* is negative when grown at 37°C, but positive at 25-30°C.

5.2.1. Phenotypical identification and virulence tests

Yersinia can be differentiated from other bacteria with similar colony morphology on CIN agar using Kligler iron agar and Christensen's urea agar (Devenish and Schiemann, 1981). Several commercial identification kits, such as API 20E, are suitable for the preliminary identification of *Y. enterocolitica* and *Y. pseudotuberculosis* when incubated at 28°C instead of 37°C (Arnold *et al.*, 2004; Sharma *et al.*, 1990; Neubauer *et al.*, 1998).

Further typing of *Y. enterocolitica*, aiming at the recognition of pathogenic strains, is traditionally based on biotyping according to the revised scheme of Wauters *et al*. (1987) (Table 8). Esculin hydrolysis and pyrazinamidase tests are chromosomal phenotypical criteria to distinguish pathogenic *Y. enterocolitica* biotypes from *Y. enterocolitica* biotype 1A. Biotyping is often accompanied by serotyping, though the serotype alone is not a reliable marker for pathogenicity as several serotypes are common in both pathogenic and non-pathogenic isolates (EFSA, 2007).

Table 8. Biotyping scheme of *Y. enterocolitica* adapted from Wauters *et al*. (1987).

	Biotype					
Test	1A	1B	2	3	4	5
Esculin hydrolysis	(+)	-	-	-	-	-
Pyrazinamidase	+	-	-	-	-	-
Lipase (Tween hydrolysis)	+	+	-	-	-	-
Indole production	+	+	(+)	-	-	-
D-Xylose fermentation	+	+	+	+	-	v
Trehalose fermentation	+	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	-
Voges-Proskauer reaction	+	+	+	+	+	(+)

+, positive; (+), delayed positive; -, negative; v, variable reactions

Alternative phenotypical tests to differentiate between pathogenic and non-pathogenic *Yersinia* are based on the presence of the virulence plasmid pYV, which correlates with several *in vitro* characteristics that are optimally expressed at 37°C (Bhaduri and Smith, 2011). Plasmid-carrying strains show growth restriction at 37°C in media containing a low concentration of Ca^{2+} (the so-called low-calcium response, Lcr), which is associated with the massive production of yops (Bhaduri, 1997; Cornelis *et al.*, 1998; Logue *et al.*, 2006). This low-calcium response is

phenotypically expressed by the formation of pinpoint colonies, whereas plasmidless derivates form much larger colonies. Other pYV-associated characteristics to identify pathogenic isolates include congo red uptake, crystal violet binding, and autoagglutination (Bhaduri and Smith, 2011). The most widely used medium is congo-red magnesium oxalate (CR-MOX) agar, by which both Lcr and congo-red uptake is determined (Sihvonen *et al.*, 2009; Riley and Toma, 1989). Nevertheless, these tests have a limited predictive value for the pathogenicity of *Yersinia* isolates as they are ambiguous to read and may result in false negative findings due to the loss of the virulence plasmid during laboratory cultivation (Thisted Lambertz and Danielsson-Tham, 2005).

5.2.2. Molecular identification and characterization

Neubauer *et al*. (2000b) described a PCR assay targeting the 16S rRNA gene to identify isolates of the species *Y. enterocolitica*. Nevertheless, this PCR assay can only be used for isolates that have already been characterized as member of the genus *Yersinia* as false positive results may occur for *Serratia* and *Citrobacter* species (Arnold *et al.*, 2004).

Several polymerase chain reaction (PCR) assays have been developed targeting both chromosomally and plasmid encoded virulence genes, and are often used for the rapid and accurate identification of enteropathogenic *Yersinia* strains. PCR assays for the specific detection of pathogenic types of *Y. enterocolitica* target chromosomal genes such as *ail* and *yst* (Ibrahim *et al.*, 1997; Nakajima *et al.*, 1992). Identification of *Y. pseudotuberculosis* may be done using primers targeting specific regions of the *inv* gene (Nakajima *et al.*, 1992). The pYV-plasmid in pathogenic *Yersinia* may be identified using PCR assays targeting *yad*A and *vir*F (Wren and Tabaqchali, 1990; Thoerner *et al.*, 2003; Thisted Lambertz *et al.*, 2000). Molecular alternatives for serotyping are available for *Y. enterocolitica* serotypes O:3 (Weynants *et al.*, 1996) and O:9 (Jacobsen *et al.*, 2005) and for all known *Y. pseudotuberculosis* serotypes and subtypes (Bogdanovich *et al.*, 2003).

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Stephan *et al.*, 2011) and Fourier transform infrared spectroscopy (FT-IR) (Kuhm *et al.*, 2009) have been shown to reliably and rapidly identify *Y. enterocolitica* strains to species and even biotype level. Other molecular methods to characterize *Y. enterocolitica* and *Y. pseudotuberculosis* include pulsed-field gel electrophoresis (PFGE) (Asplund *et al.*, 1998; Fredriksson-Ahomaa *et al.*, 1999a; Fredriksson-Ahomaa *et al.*, 2011), multiple-locus variable-number tandem repeats analysis (MLVA) (Gierczynski *et al.*, 2007; Sihvonen *et al.*, 2011b), ribotyping (Lucero Estrada *et al.*, 2011; Martins *et al.*, 2007), and multilocus sequence typing (MLST) (Souza *et al.*, 2010; Kotetishvili *et al.*, 2005).

5.3. Alternative methods for detection

Several PCR assays have been developed as an alternative to traditional culture methods for the detection of pathogenic *Y. enterocolitica* from different sample types. Most PCR assays specifically detect human pathogenic types of *Y. enterocolitica*, using either chromosomal or plasmid encoded virulence genes (Fredriksson-Ahomaa and Korkeala, 2003). Table 9 shows that

PCR assays result in higher detection rates than conventional culture, which is mainly attributed to the low sensitivity of the applied isolation methods. Nevertheless, a disadvantage of PCR is that false-positive results can occur due to detection of DNA from dead bacteria or the presence of homologues target sequences in other bacterial species. As such, *ail* homologues sequences have already been described in *Y. enterocolitica* biotype 1A (Fredriksson-Ahomaa *et al.*, 2012a; Zheng *et al.*, 2008b) and the gene appears to be highly similar to the *ail* gene in pathogenic strains of *Y. enterocolitica* (Sihvonen *et al.*, 2011a; Kraushaar *et al.*, 2011). Moreover, PCR can lead to false-negative results due to the presence of inhibitory substances in clinical, food and environmental samples. To overcome these problems, PCR assays are often applied on enriched samples (Boyapalle *et al.*, 2001; Messelhausser *et al.*, 2011; Thisted Lambertz and Danielsson-Tham, 2005; Vishnubhatla *et al.*, 2001).

Table 9. Comparison of culture and PCR methods for the detection of pathogenic *Y. enterocolitica* from pigs and pork.

Sample type	N	Culture positive	PCR positive	Target gene (PCR type[a])	Reference
Pig samples					
Tonsils	252	0	90	*ail* (q)	Boyapalle *et al*. (2001)
Tonsils	212	72	186	*ail* (q)	Fredriksson-Ahomaa *et al*. (2007c)
Tonsils	1218	68	122	*ail* (q)	Wesley *et al*. (2008)
Lymph nodes	778	0	188	*ail* (q)	Boyapalle *et al*. (2001)
Intestinal content	512	0	138	*ail* (q)	Boyapalle *et al*. (2001)
Faeces	2847	129	372	*ail* (q)	Wesley *et al*. (2008)
Pork samples					
Carcasses	518	0	81	*ail* (c,n)	Lindblad *et al*. (2007)
Carcasses and offal	131	24	42	*yad*A (n)	Fredriksson-Ahomaa *et al*. (2000b)
Pig tongues	51	40	47	*yad*A (n)	Fredriksson-Ahomaa *et al*. (1999b)
Minced meat	350	0	133	*ail* (q)	Boyapalle *et al*. (2001)
Minced meat	225	4	63	*yad*A (n)	Fredriksson-Ahomaa *et al*. (1999b)
Minced meat, sausages	197	5	46	*ail* (c,n)	Thisted Lambertz *et al*. (2007)
Minced meat, sausages	182	5	76	*yad*A (n)	Lucero Estrada *et al*. (2012)
Minced pork, tongues	115	37	57	*yst* (q)	Vishnubhatla *et al*. (2001)
Raw pork	100	0	7	*ail* (q)	Fredriksson-Ahomaa *et al*. (2007a)
Raw pork products	300	6	50	*yad*A (n)	Johannessen *et al*. (2000)
Pork and pork products	446	46	81	*ail* (q)	Messelhausser *et al*. (2011)
Pork meat	118	6	9	*ail* (c)	Thisted Lambertz and Danielsson-Tham (2005)

[a] q: real-time PCR; n: nested PCR; c: conventional PCR.

Aims

Yersiniosis is the third most frequently reported zoonosis in Belgium, and the majority of cases are caused by *Y. enterocolitica* bioserotype 4/O:3. Pigs are considered to be the principal reservoir as they frequently harbour these pathogens in their tonsils and intestinal tract at time of slaughter. Consequently, the pathogens may contaminate the carcass surface during slaughter. Despite the well-known relation between yersiniosis and pork, the current situation of enteropathogenic yersiniae in pigs and pork in Belgium remains largely unknown. Nevertheless, interventions leading to a reduction of pork contamination necessitate a better understanding of the epidemiology of the pathogen. Therefore, the **general aim** of this thesis was to gain more insight in **the occurrence of enteropathogenic *Yersinia* spp. along the pork production chain**.

Adequate **sampling and culture** methods are essential for a reliable isolation of human pathogenic *Yersinia enterocolitica*. During this thesis, different methodologies for the isolation of human pathogenic *Y. enterocolitica* from pig tonsils (Chapters 1 and 2), pig carcasses and minced meat (Chapter 3) were evaluated. These studies particularly focused on the use of the ISO 10273:2003 method, which is recommended for the isolation of human pathogenic *Y. enterocolitica* from food and pig tonsils.

During this thesis, **prevalence estimates** and the **serotype distribution** of enteropathogenic *Yersinia* spp. were obtained throughout the pork production chain in Belgium, including pig tonsils (Chapter 1, 2, and 4), faeces (Chapter 4), carcasses (Chapter 3 and 4), and minced meat (Chapter 3). Additionally, **quantitative** contamination levels were obtained for pig tonsils (Chapter 1 and 2) and different areas of freshly eviscerated pig carcasses (Chapter 4). Moreover, **risk factors** were identified for carcass contamination with human pathogenic *Y. enterocolitica* during the evisceration process (Chapter 4). Finally, **serology** of meat juice was evaluated as an alternative to bacteriological testing of tonsils and faeces for the detection of enteropathogenic *Yersinia* spp. in pigs at slaughter (Chapter 5).

Chapter 1
Recovery of human pathogenic *Yersinia enterocolitica* from pig tonsils - part 1 - enumeration and detection by enrichment versus direct plating

Adapted from: Van Damme I., Habib I., De Zutter L. (2010). *Yersinia enterocolitica* in slaughter pig tonsils: Enumeration and detection by enrichment versus direct plating culture. Food Microbiology 27, 158-161.

Abstract

Tonsil samples from 139 slaughter pigs were examined for the presence of pathogenic *Yersinia enterocolitica* by enrichment procedures based on the standard method ISO 10273:2003. In addition, samples were tested by direct plating method to evaluate its efficiency compared to the enrichment culture methods and to quantify the level of contamination in porcine tonsils.

In total, 52 samples (37.4%) were positive for pathogenic *Y. enterocolitica*, all belonging to bioserotype 4/O:3. Fifty out of the 52 positive samples (96.2%) were detected by direct plating. Enumeration showed an average concentration of 4.5 $\log_{10}$ CFU/g and 4.4 $\log_{10}$ CFU/g tonsil on Salmonella-Shigella-deoxycholate-calcium chloride (SSDC) and cefsulodin-irgasan-novobiocin (CIN) agar plates, respectively. The enrichment procedures recommended by the ISO 10273:2003 method were not optimal for the isolation of pathogenic *Y. enterocolitica* from pig tonsils as two days enrichment in irgasan-ticarcillin-potassium chlorate (ITC) broth resulted in an isolation rate of 84.6%, and 5 days enrichment in peptone-sorbitol-bile (PSB) broth recovered only 59.6% of positive samples. Reducing the enrichment time in PSB from 5 to 2 days resulted in a significantly higher recovery rate (94.2%) and might serve as an appropriate enrichment protocol for the isolation of pathogenic *Y. enterocolitica* from pig tonsils.

Compared to enrichment culture methods, results based on direct plating can be obtained in a shorter time course and provide quantitative data that might be needed for further risk assessment studies.

Keywords: human pathogenic *Yersinia enterocolitica*; isolation; quantification; pig; tonsils.

1. Introduction

Yersinia enterocolitica is a foodborne pathogen causing a variety of symptoms in humans, ranging from mild diarrhoea to immunological complications and potentially lethal septicaemia (Bottone, 1997). The most common clinical manifestation is acute gastroenteritis, which particularly affects young children (Tauxe *et al.*, 1987; Verhaegen *et al.*, 1998). *Y. enterocolitica* is traditionally subdivided into bioserotypes based on the combination of biochemical and serological tests (Wauters *et al.*, 1987). Human pathogenic *Y. enterocolitica* belong to biotypes 1B, 2, 3, 4 and 5, while strains of biotype 1A are generally regarded as non-pathogenic and widely found in the environment. The bioserotypes associated with human disease in Belgium and most other European countries are mainly bioserotype 4/O:3 and to a lesser extent bioserotype 2/O:9 (Verhaegen *et al.*, 1998; EFSA, 2007).

Slaughter pigs are considered to be a major reservoir for the foodborne transmission of *Y. enterocolitica* (Andersen *et al.*, 1991; Fredriksson-Ahomaa *et al.*, 2001b; Fredriksson-Ahomaa *et al.*, 2006) as they are the only food animals that regularly harbour these pathogenic bioserotypes (Bucher *et al.*, 2008). Numerous studies showed that tonsillar tissue is the most reliable sample to detect *Y. enterocolitica* in slaughter pigs (Thibodeau *et al.*, 1999; Nesbakken *et al.*, 2003; Gürtler *et al.*, 2005). Moreover, tonsils are considered as an important source for the contamination of pig offal and carcasses (Fredriksson-Ahomaa *et al.*, 2001a; Fredriksson-Ahomaa *et al.*, 2001b).

Many studies have been conducted on the occurrence of pathogenic *Y. enterocolitica* in pigs at slaughter, but data regarding quantitative contamination are scarce. The contamination level of pathogenic *Y. enterocolitica* in pig organs at slaughter may influence the chance of carcass contamination and subsequently influence the risk of the foodborne transmission to humans. Additionally, with the emergence of quantitative microbiological risk assessment, there is a growing need for quantitative data on contamination levels of *Y. enterocolitica* in slaughter pigs.

For the isolation of pathogenic *Y. enterocolitica*, an International Standard Organization method is described for food samples (ISO 10273, 2003), which is also recommended for pig tonsils (EFSA, 2007). Isolation of pathogenic *Y. enterocolitica* from naturally contaminated samples is generally difficult (Fredriksson-Ahomaa and Korkeala, 2003), as the standard culture methods for the recovery of *Y. enterocolitica* usually involve time-consuming enrichment steps followed by plating on selective media (de Boer, 1992). However, for tonsil samples, direct plating without prior enrichment has been successfully applied (Fredriksson-Ahomaa *et al.*, 2007c; Fredriksson-Ahomaa *et al.*, 2001a) and needs to be evaluated further as it might be a good alternative for the lengthy enrichment procedures.

The aim of the present study was to generate a pilot set of data on quantitative and qualitative levels of pathogenic *Y. enterocolitica* in tonsils of slaughter pigs from Belgium. Additionally, the performance of different isolation methods, including ISO 10273:2003 and direct plating, for the isolation of pathogenic *Y. enterocolitica* from porcine tonsils was evaluated.

2. Materials and methods

2.1. Sampling

Tonsils (*tonsilla veli palatini*) from 139 pigs, slaughtered in two abattoirs in Belgium, were collected during 11 sampling visits. The animals originated from 48 different slaughter-batches, and 1 to 4 animals were randomly sampled per batch. Tonsils were removed aseptically immediately after evisceration, placed into sterile plastic bags and transported to the laboratory under chilled conditions where they were tested within five hours after collection.

2.2. Enumeration and isolation

Tonsil samples were analysed for the presence of pathogenic *Y. enterocolitica* by direct plating and different enrichment protocols based on the ISO method (ISO 10273, 2003) (Figure 5). In addition, using the direct plating method, enumeration of *Y. enterocolitica* in pig tonsils was performed.

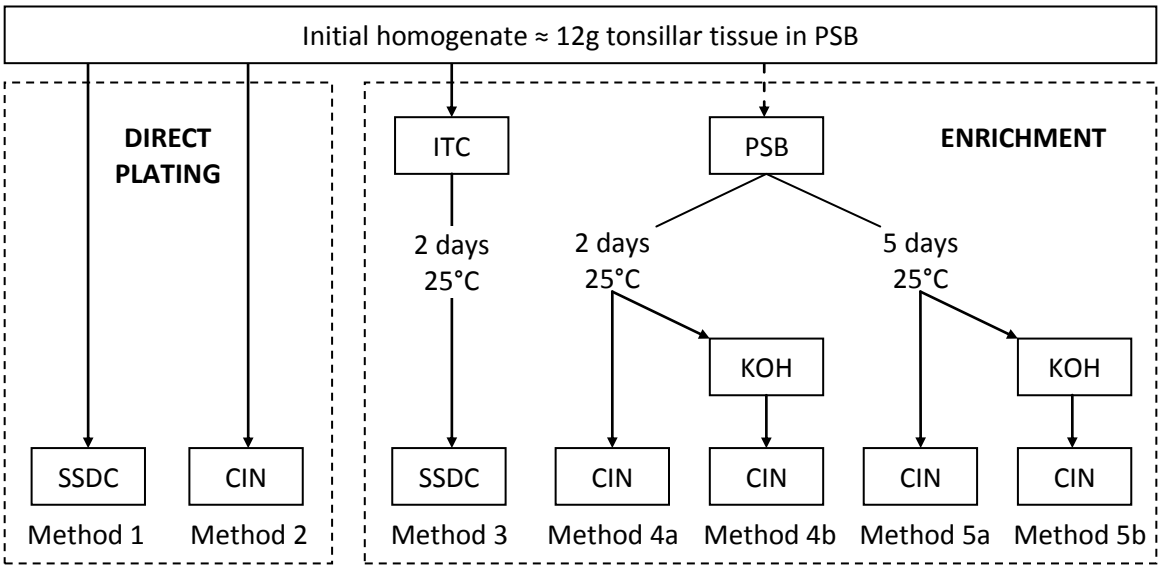

Figure 5. Schematic overview of the isolation protocols for the recovery of pathogenic *Y. enterocolitica* from pig tonsils used in Chapter 1.

Tonsil samples were aseptically cut into small pieces, and 12 g was transferred to a sterile stomacher bag. Samples were homogenized with 108 mL of peptone-sorbitol-bile (PSB) broth (Fluka, Steinheim, Germany) for 4 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). From this initial homogenate, testing was carried out in parallel as follows:

(i) For isolation and enumeration by direct plating (methods 1 and 2), 10 mL was transferred to a sterile tube and 1 mL thereof was spread plated over three (0.3, 0.3 and 0.4 mL) *Salmonella-Shigella*-deoxycholate-calcium chloride (SSDC) (Conda, Madrid, Spain) agar plates. An additional 100 µL of the homogenate was inoculated on one SSDC agar plate by a spiral plate machine

(Eddie Jet, IUL Instruments, Barcelona, Spain) (method 1). The same procedure was repeated using cefsulodin-irgasan-novobiocin (CIN) agar plates (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK) (method 2). All agar plates were incubated at 30°C for 24 h and examined for characteristic *Yersinia* colonies using a stereo microscope with Henry illumination (Olympus). The number of presumptive *Yersinia* colonies was counted and five characteristic colonies were biochemically and serologically confirmed as follows. Prior to biochemical confirmation, colonies were subcultured on Plate Count Agar (PCA, Bio-Rad Laboratories, Marnes-La-Coquette, France) and preliminary identification was carried out using urease broth and Kligler iron agar slants (Oxoid). Colonies that produced typical reaction patterns were further confirmed as *Y. enterocolitica* and biotyped based on the revised scheme of Wauters *et al*. (1987) using the following tests: esculin hydrolysis, indole production, and fermentation of xylose, and trehalose. *Y. enterocolitica* isolates belonging to biotype 4 were serotyped by slide agglutination with a commercial O:3 antiserum (Bio-Rad Laboratories).

(ii) For enrichment procedures (methods 3 to 5), 10 mL of the same initial homogenate was transferred to 90 mL irgasan-ticarcillin-potassium chlorate (ITC) broth [ITC Broth Base (Fluka) supplemented with ticarcillin (Sigma-Aldrich, Steinheim, Germany) and $KClO_3$ (Merck, Darmstadt, Germany)] and incubated at 25°C for two days. After enrichment, ten µL was streaked onto an SSDC agar plate (method 3). The remaining PSB homogenate was incubated at 25°C. After two (method 4a) and five days (method 5a), ten µL was streaked on CIN agar. In parallel to that, the enriched PSB culture was inoculated on CIN agar after alkali treatment (methods 4b and 5b). Therefore, 0.5 mL of the enriched PSB cultures was transferred to 4.5 mL of 0.5% KOH solution and mixed for 20 s. All agar plates were incubated at 30°C for 24 h and *Yersinia* colonies were confirmed as described before.

2.3. Statistical analysis

Quantitative and qualitative testing results were recorded in an Excel spreadsheet and the descriptive data analysis was performed using the software Stata 8.0 (Stata Corporation, College Station, Texas, USA). Correlation between the different enrichment protocols and recovery of *Y. enterocolitica* (presence/absence) was tested using a logistic regression model. Comparison of enumeration results on SSDC and CIN agar plates was performed using Poisson regression.

3. Results

Pathogenic *Y. enterocolitica* were isolated from 52 out of 139 tested tonsil samples (37.4%). All isolates belonged to bioserotype 4/O:3. Fifty samples were positive by direct plating, of which five on SSDC and eight on CIN agar plates could not be enumerated because of background flora. There was no statistical difference between *Yersinia* counts on both media (p = 0.68). The average concentration of *Y. enterocolitica* was 4.5 and 4.4 $\log_{10}$ CFU/g using SSDC and CIN agar plates, respectively, with a standard deviation of 0.9 $\log_{10}$ CFU/g. Eighteen out of 50 tonsils (36.0%) were contaminated within a range of 4-5 $\log_{10}$ CFU/g tonsillar tissue and 16 samples (32.0%) were contaminated with more than 5 $\log_{10}$ CFU/g (Figure 6).

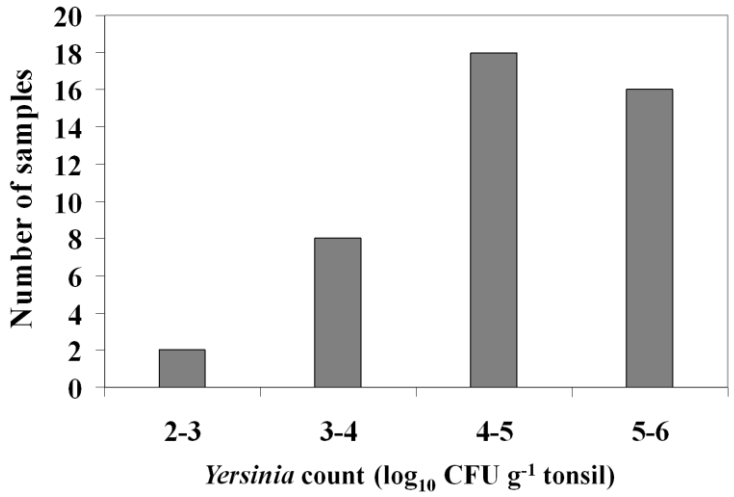

Figure 6. Frequency distribution of *Y. enterocolitica* contamination in tonsil samples recovered by direct plating (n = 50).

Two samples are not included in this figure since enumeration could not be performed on either of the selective plates due to overgrowth of background flora.

The relative sensitivities of the different culture methods for the isolation of pathogenic *Y. enterocolitica* from pig tonsils are summarized in Table 10. None of the isolation procedures detected all positive samples. Out of the 50 tonsils that were positive by direct plating, three were negative using enrichment procedures. On the other hand, two samples were negative by direct plating but positive using enrichment.

Comparing the different enrichment procedures, method 4b (two days enrichment in PSB and KOH-treatment) showed the best performance, recovering 49 positive samples. This was followed by method 3 (two days enrichment in ITC), though the difference between both methods was not statistically significant (p = 0.53). All the samples that were positive using method 3 were also positive using method 4b, whereas five samples that were positive by method 4b tested negative using method 3. With regard to the length of enrichment incubation, five days enrichment in PSB and alkaline treatment (method 5b) was associated with a significant decline (p = 0.02) in *Y. enterocolitica* recovery compared to the two-day enrichment procedure (method 4b).

Results in Table 10 show that alkaline treatment of enriched PSB cultures had a positive impact on *Y. enterocolitica* recovery. The number of pathogenic *Y. enterocolitica* that were detected from pig tonsils after two days enrichment in PSB was significantly higher (p < 0.001) using KOH-treatment than without KOH-treatment.

Table 10. Comparison of different isolation methods for the recovery of pathogenic *Y. enterocolitica* from pig tonsils (n = 139).

Isolation method	Number of positive samples (%)	Relative sensitivity
Method 1 (direct plating on SSDC)	50 (36.0)	96.2
Method 2 (direct plating on CIN)	50 (36.0)	96.2
Method 3 (ITC (2d) + SSDC)[a]	44 (31.7)	84.6
Method 4a (PSB (2d) + CIN)	18 (12.9)	34.6
Method 4b (PSB (2d) + KOH + CIN)	49 (35.3)	94.2
Method 5a (PSB (5d) + CIN)[a]	1 (0.7)	1.9
Method 5b (PSB (5d) + KOH + CIN)[a]	31 (22.3)	59.6
Total[b]	52 (37.4)	100.00

[a] Isolation methods as described by ISO10273:2003; [b] Parallel interpretation, sample is considered positive if at least one test is positive.

4. Discussion

Pathogenic *Y. enterocolitica* belonging to bioserotype 4/O:3 were isolated from 37.4% of the tested samples, indicating that a considerable proportion of Belgian slaughter pigs carries this pathogen in their tonsils. Comparing our findings to previous studies from Belgium, Wauters *et al*. (1976) found pathogenic *Y. enterocolitica* in 47 out of 426 tonsils (11.0%) of Belgian slaughter pigs and in 33 out of 54 tonsils (61.1%) about a decade later (Wauters *et al.*, 1988). Incidence results of pathogenic *Y. enterocolitica* in porcine tonsils vary greatly between different studies, ranging from less than 10% in Poland and the Netherlands (Kot *et al.*, 2007; de Boer *et al.*, 2008) to 89% in Estonia (Ortiz Martínez *et al.*, 2009). However, because there is no uniform method of detection, comparison of reported incidences should be treated with caution.

Quantitative data on the contamination level in tonsils are scarce. To the best of our knowledge, the present study provides the first comprehensive quantitative data set on pathogenic *Y. enterocolitica* in porcine tonsils on an extended number of pigs. Enumeration results revealed that the concentration in positive tonsils of pigs at slaughter is high, showing an average count of more than 10^4 CFU per gram tonsillar tissue. Nesbakken (1988) analysed five tonsil samples using the MPN method, and reported a contamination level up to 5.2×10^4 MPN in whole tonsils with a surface area of 30 cm². Shiozawa *et al*. (1991) reported concentrations between 1.0×10^5 and 8.6×10^6 CFU/g tissue in six naturally infected tonsils. The relatively high proportion of infected pigs and the high amount of bacteria in infected tonsils further support the assumption that tonsils may be an important risk for carcass and offal contamination.

Enumeration was carried out using SSDC and CIN agar plates. Both media can be applied alternatively since there is no statistically significant difference between *Y. enterocolitica* counts on both media. However, in our opinion, enumeration on CIN agar plates is favourable since *Y.*

enterocolitica has a relatively more characteristic colony morphology on this medium (typical "bull's eye" appearance) compared to SSDC.

Considering the growing public health concern for human pathogenic *Y. enterocolitica* in the food chain, more data are needed about the occurrence of this pathogen in its main animal reservoir. In order to monitor pathogenic *Y. enterocolitica* in pigs consistently, suitable and reliable detection methods are essential since the applied detection method may have an impact on the outcome of the results (EFSA, 2007). In view of the fact that tonsils are a valuable diagnostic organ to detect pathogenic *Y. enterocolitica* in pigs at slaughter, the recoverability by different isolation methods based on the ISO 10273:2003 protocol and direct plating methods were evaluated. In 96.2% of culture-positive tonsils, the number of pathogenic *Y. enterocolitica* exceeded detection limit (≥ 10 CFU/g) and the interfering flora on the selective agar plates was minimal, allowing detection by direct plating. Therefore, the direct plating method on SSDC or CIN agar plates seems a good alternative to the lengthy enrichment methods for isolating pathogenic *Y. enterocolitica* from pig tonsils. Using direct plating, presumptive results are available the following day, and pathogenic isolates can be confirmed biochemically within three to four days.

Enrichment in ITC for two days showed satisfactory results, detecting more than 80% of positive samples. However, enrichment in PSB for five days with alkaline treatment resulted in a relative sensitivity of only 59.6%. Without alkaline treatment, this number was even reduced to 1.9%. According to the ISO 10273:2003 method, it is recommended to incubate PSB for two to three days with agitation, or five days without agitation. However, our results indicate that, at least for tonsil samples, shortening the enrichment period without agitation from five to two days results in a higher recovery of human pathogenic *Y. enterocolitica*.

In conclusion, this study shows that a considerable proportion of slaughter pigs in Belgium are carriers of pathogenic *Y. enterocolitica* bioserotype 4/O:3 in their tonsils. In most cases, the pathogen is present in high numbers, thus presenting a potential source of contamination of edible offal and carcasses during slaughter. Direct plating is highly suited for the recovery of pathogenic *Y. enterocolitica* from porcine tonsils since it's less laborious compared to the parallel use of ITC and PSB enrichment, as recommended by ISO 10273:2003, and positive samples are identified within a shorter time period. Moreover, direct plating allows a quantitative estimation, providing additional information about the level of contamination. When using enrichment methods based on ISO 10273:2003 for the isolation of pathogenic *Y. enterocolitica* from porcine tonsils, we recommend reducing the enrichment period in PSB from five to two days, as that will provide a higher recovery rate and a shorter testing period.

Acknowledgements

We thank Sébastien Morio for the technical assistance. The abattoirs are acknowledged for their cooperation in sampling.

Chapter 2
Recovery of human pathogenic *Yersinia enterocolitica* from pig tonsils - part 2 - effect of sampling and short isolation methodologies

Adapted from: Van Damme I., Berkvens D., De Zutter L. (2012). Effect of sampling and short isolation methodologies on the recovery of human pathogenic *Yersinia enterocolitica* from pig tonsils. Foodborne pathogens and disease 9, 600-606.

Abstract

The objective of this study was to determine the effect of sampling (swab samples compared to destructive samples) on isolation rates of human pathogenic *Yersinia enterocolitica* from pig tonsils. Moreover, the relative efficiency of different rapid, routinely applicable isolation methods was evaluated.

Therefore, swab and destructive samples from tonsils of 120 pigs at slaughter were analysed in parallel using direct plating and different enrichment methods. *Salmonella-Shigella*-deoxycholate-calcium chloride (SSDC) agar, cefsulodin-irgasan-novobiocin (CIN) agar, and *Yersinia enterocolitica* chromogenic medium (YeCM) were used as selective agar media. For enrichment, irgasan-ticarcillin-potassium chlorate (ITC) broth and peptone-sorbitol-bile (PSB) broth were incubated at 25°C for 48 h.

Overall, 55 tonsils (45.8%) were positive for *Y. enterocolitica* bioserotype 4/O:3. Recovery was significantly higher using the destructive method compared to the swabbing method. Direct plating resulted in 47 and 28 *Y. enterocolitica*–positive destructive and swab samples, respectively. Alkali treatment of PSB and ITC enrichment broths significantly increased recovery of pathogenic *Y. enterocolitica* from destructive tonsil samples. The performance of YeCM for qualitative and quantitative isolation of pathogenic *Y. enterocolitica* from pig tonsils was equal to SSDC and CIN.

In conclusion, direct plating and ISO 10273:2003 with minor modifications are suitable and rapid methods for isolation of pathogenic *Y. enterocolitica* from destructive tonsil samples.

Keywords: enteropathogenic *Yersinia* spp., pig, tonsils, sampling, isolation.

1. Introduction

Yersinia enterocolitica is a foodborne pathogen that causes enteric infections in humans. In Europe, most infections are attributed to bioserotype 4/O:3. As slaughter pigs are the only food-producing animals that regularly carry this bioserotype, they are regarded as the principal source of pathogenic *Y. enterocolitica* in the human food chain (Bucher *et al.*, 2008; EFSA, 2011; Fredriksson-Ahomaa *et al.*, 2006). Full virulence in pathogenic *Yersinia* requires the presence of a plasmid called pYV (plasmid for *Yersinia* virulence) and several chromosomal genes. Some examples of these chromosomal genes are *ail* (attachment invasion locus), *yst* (*Yersinia* heat-stable enterotoxin), and *inv* (invasin). Based on these genes, polymerase chain reaction (PCR) assays for detection and identification of pathogenic *Yersinia* spp. have been developed, of which several have been shown to be more sensitive than culture methods (Fredriksson-Ahomaa and Korkeala, 2003; Thisted Lambertz *et al.*, 2008a). Nevertheless, culture methods are indispensable when studying the epidemiology of bacterial pathogens to obtain isolates.

Culture methods for recovery of *Y. enterocolitica* rely on initial enrichment followed by plating on selective agar plates. Enrichment at 4°C for several weeks is successful for isolation from different sample types, but for isolation of human pathogenic *Y. enterocolitica*, enrichments in selective media at higher temperatures for shorter time periods have been shown to be as effective as the prolonged cold enrichments (de Boer and Nouws, 1991; De Giusti *et al.*, 1995; Kwaga *et al.*, 1990). Isolation of pathogenic *Y. enterocolitica* from naturally contaminated samples without enrichment is seldom successful (Fredriksson-Ahomaa and Korkeala, 2003). However, for tonsil samples, direct plating is a fast alternative as in most samples background flora on a selective agar medium is minimal and the number of pathogenic *Y. enterocolitica* is sufficiently high (Chapter 1). A disadvantage of current conventional media is the difficulty to distinguish pathogenic *Y. enterocolitica* from non-pathogenic yersiniae and many other Gram-negative bacteria (de Boer, 1992; Fondrevez *et al.*, 2010). To facilitate this differentiation, Weagant (2008) recently designed a chromogenic agar medium, called *Y. enterocolitica* chromogenic medium (YeCM).

Besides sensitive culture methods, adequate sampling procedures are also fundamental for the success of isolation and, therefore, crucial in monitoring programs. When monitoring *Y. enterocolitica* in pigs at slaughter, tonsils are regarded as the sample of choice (EFSA, 2009). Swab samples from tonsils have been used in several studies (Andersen *et al.*, 1991; Fondrevez *et al.*, 2010; Kechagia *et al.*, 2007), since sampling and processing is easier and less time-consuming compared to tissue samples. Therefore, this study evaluates if swab samples have a good predictive value for the presence of pathogenic *Y. enterocolitica* in pig tonsils. Moreover, when screening a large number of animals, short and efficient isolation procedures are indispensable. This study will thus compare different rapid isolation methods to determine the most efficient method for recovery of pathogenic *Y. enterocolitica* isolates from pig tonsils.

2. Materials and methods

2.1. Sample collection and preparation

In a Belgian abattoir, tonsils from 120 fattening pigs were cut out aseptically immediately after removal of the plug set and the surface area of both tonsils was swabbed using a dry sterile cotton swab. The swab sample was placed in 10 mL of peptone-sorbitol-bile broth (PSB), and for the destructive method, 11 g of tonsillar tissue was homogenized in 99 mL PSB. One and 10 mL of the swab and tissue homogenate was transferred into 9 and 90 mL of irgasan-ticarcillin-potassium chlorate (ITC) broth, respectively. Three millilitres of PSB homogenate was used for direct plating, and the remaining homogenate was used for enrichment at 25°C for 48h.

2.2. Isolation and enumeration

All samples were examined using (i) direct plating and (ii) different enrichment methods (Figure 7). Three selective agar media were used: (1) *Salmonella-Shigella*-deoxycholate-calcium chloride agar (SSDC; Conda, Madrid, Spain); (2) cefsulodin-irgasan-novobiocin agar (CIN; Oxoid, Basingstoke, United Kingdom) and (3) *Yersinia enterocolitica* chromogenic medium (YeCM; prepared according to Weagant [2008]).

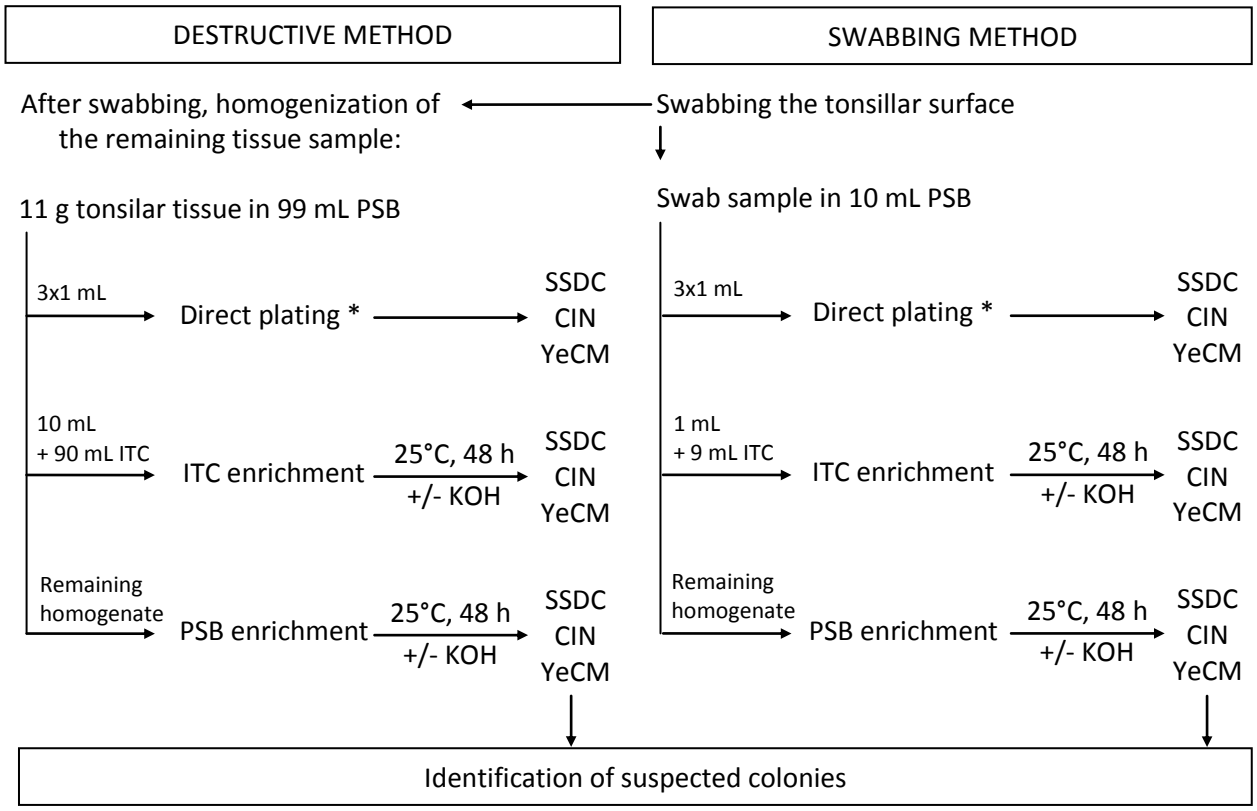

Figure 7. Schematic overview of the isolation protocols for the recovery of pathogenic *Y. enterocolitica* from pig tonsils in Chapter 2.

*Direct plating: 1 mL of PSB homogenate is plated on each of the three selective agar media: 0.9 mL is spread plated over two plates, and 0.1 mL is inoculated on a third plate using the spiral plate machine.

(i) For direct plating, 0.4 and 0.5 mL of the homogenate was spread plated on two plates of each of the three agar media. Moreover, 0.1 mL of the homogenate was inoculated on each of the selective plates using a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). All plates were incubated at 30°C for 24 h and examined for typical *Yersinia* colonies using a stereomicroscope with Henry illumination. Suspected colonies were enumerated and identified as described below.

(ii) After 48 h of enrichment in ITC and PSB at 25°C, a loopful was streaked directly and after alkali treatment onto each of the three different agar plates. All inoculated selective plates were incubated at 30°C for 24 h. Suspected colonies were identified as described below.

2.3. Biochemical identification and biotyping

Suspected *Yersinia* colonies were grown on plate count agar (PCA) and inoculated in urease broth, Kligler Iron agar, and tryptic-casein-soy broth and incubated at 30°C for 24 h. Isolates showing typical reactions were further identified using lysine and ornithine decarboxylase, sucrose fermentation, and Voges-Proskauer reaction at 30°C. Isolates showing atypical reactions were tested using API 20E strips (Bio-Mérieux, Marcy l'Etoile, France), incubated at 25°C for 18–20 h. *Yersinia enterocolitica* isolates were biotyped based on the revised scheme of Wauters *et al*. (1987) using esculin hydrolysis, indole production, and xylose fermentation. *Y. pseudotuberculosis* isolates were biotyped using melibiose, citrate, and raffinose (Tsubokura and Aleksic, 1995) and sent to the Belgian reference laboratory for *Y. pseudotuberculosis* (UCL, St-Luc Hospital, Brussels) for serotyping.

2.4. Molecular identification

The pathogenicity of *Y. enterocolitica* isolates was confirmed using a multiplex PCR with primers targeting the chromosomal virulence genes *ail* and *yst* and the plasmid virulence gene *vir*F according to Harnett *et al*. (1996). A single PCR was performed to identify *Y. enterocolitica* serotype O:3 using primers targeting the *rfb*C gene (Weynants *et al.*, 1996). *Y. pseudotuberculosis* isolates were identified using a PCR assay targeting the *inv*-gene (Nakajima *et al.*, 1992).

DNA was extracted using PrepMan reagent (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions, and 1 µL of the supernatant was used as a template in each PCR assay. DNA amplification was performed in a 50-µL reaction mixture containing 2.5 U GoTaq® Flexi DNA Polymerase, 1 X green reaction buffer, 1.5 mM $MgCl_2$ (Promega, Madison, Wisconsin, USA), 200 µM of each dNTP (Invitrogen, Carlsbad, California, USA), and 0.5 µM of each primer. PCR was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems). Each cycle consisted of pre-denaturation at 94°C for 5 min, 32 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. For *inv*, only the annealing temperature (58°C) was changed. PCR products were electrophoresed in agarose gels, stained with ethidium bromide, and visualized by ultraviolet light.

2.5. Statistical analysis

Statistical analysis was carried out using STATA 11.0 (Stata Corporation, College Station, Texas, USA). Differences between isolation methods were examined using a random-effects logistic regression analysis. The mean load was calculated for each agar medium separately, computing the mean of the log-values, and taking into account the countable plates only. Counts on the three different agar plates were compared using a random-effects negative binomial regression analysis. Samples that were negative for *Y. enterocolitica* by direct plating using the three different agar media were not included in the analysis. Values of plates that were not countable were replaced by the average count on the particular agar medium. For plates that were negative by direct plating, a value of 0 $\log_{10}$ colony-forming units (CFU) per gram or per surface area was used.

3. Results

The effect of sampling on isolation of pathogenic *Y. enterocolitica* from pig tonsils was evaluated by analysing in parallel swab samples and destructive tissue samples from 120 slaughter pig tonsils. In total, *Y. enterocolitica* bioserotype 4/O:3 was isolated from the tonsils of 55 slaughter pigs (45.8%). Recovery using destructive samples was significantly higher compared to swab samples (OR = 14.0 [3.6–54.7]) (Table 11). For 16 tonsils, only the destructive sample was positive, whereas for three tonsils, *Y. enterocolitica* was isolated from the swab sample only.

Table 11. Isolation of *Y. enterocolitica* bioserotype 4/O:3 from porcine tonsils (n=120) using different isolation methods.

	DESTRUCTIVE METHOD			SWABBING METHOD		
	Without alkaline treatment	With alkaline treatment	Total	Without alkaline treatment	With alkaline treatment	Total
Direct plating			47			28
SSDC	45	NT	45	22	NT	22
CIN	46	NT	46	24	NT	24
YeCM	46	NT	46	25	NT	25
ITC enrichment			50			24
SSDC	39	47	49	21	20	23
CIN	38	47	49	17	21	23
YeCM	36	46	46	19	21	23
PSB enrichment			48			37
SSDC	17	42	42	8	31	31
CIN	21	42	44	9	32	33
YeCM	17	43	43	8	30	30
Total[a]			52			39

NT, not tested; [a] Parallel interpretation, sample is considered positive if at least one test is positive.

There was no significant difference in recovery between the three agar plates for any of the isolation procedures ($p > 0.05$). For the majority of enrichment procedures, alkaline treatment resulted in a significant higher recovery ($p < 0.01$) compared to isolation without alkaline treatment. However, alkaline treatment did not enhance recovery after ITC enrichment of swab samples ($p > 0.05$).

For destructive samples, more samples were positive using ITC compared to PSB enrichment. This difference was significant for all plates without alkaline treatment ($p < 0.001$); though, it was not the case when using alkaline treatment ($p > 0.05$). Direct plating on SSDC was more efficient ($p = 0.045$) compared to isolation on SSDC after ITC enrichment without alkaline treatment. Only one sample that was positive after ITC-SSDC was not detected using direct plating on SSDC. Isolation rates after ITC enrichment on CIN and YeCM plates without alkaline treatment were also lower compared to direct plating on CIN ($p = 0.008$) and YeCM ($p = 0.001$), respectively. Nevertheless, for destructive tonsil samples, there was no significant difference between direct plating and any of the enrichment methods when using alkaline treatment ($p > 0.05$).

In contrast to destructive samples, more swab samples were positive after PSB enrichment compared to ITC enrichment ($p < 0.05$). Recovery on SSDC and CIN after PSB enrichment and alkaline treatment was higher than after direct plating on SSDC ($p = 0.003$) and CIN ($p = 0.010$), respectively, though this difference was not significant for YeCM ($p = 0.097$).

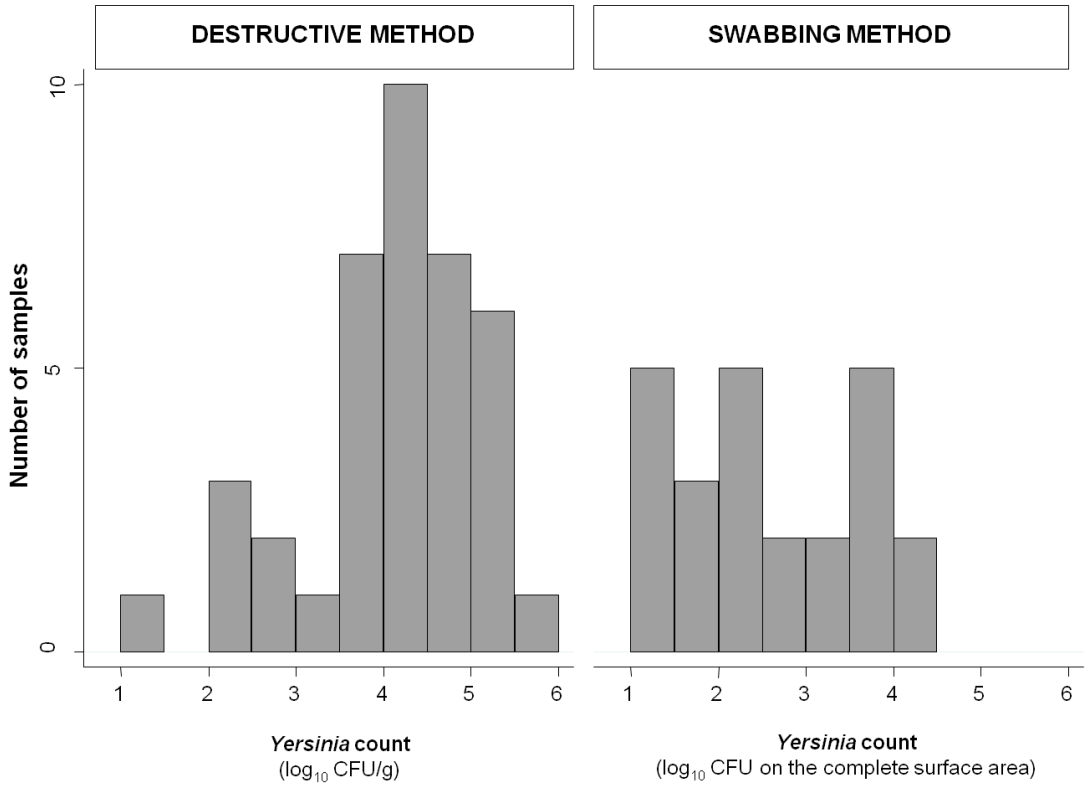

Figure 8. Frequency distribution of *Y. enterocolitica* bioserotype 4/O:3 counts in tonsil samples based on countable CIN agar plates using direct plating (n = 38 for destructive samples, n = 24 for swab samples).

The mean load of *Y. enterocolitica* bioserotype 4/O:3 in destructive samples yielding enumeration data was 4.23 ± 0.99 $\log_{10}$ CFU/g (n = 29) using SSDC, 4.13 ± 0.93 $\log_{10}$ CFU/g (n = 38) using CIN, and 4.25 ± 0.79 $\log_{10}$ CFU/g (n = 37) using YeCM agar plates. For countable swab samples, the number of pathogens on the complete surface area was 2.72 ± 1.09 $\log_{10}$ CFU (n = 16) using SSDC, 2.62 ± 0.99 $\log_{10}$ CFU (n = 24) using CIN, and 2.55 ± 1.10 $\log_{10}$ CFU (n = 22) using YeCM agar plates. No significant differences between counts on the three different agar plates were observed ($p > 0.05$). As such, only the frequency distribution of *Y. enterocolitica* counts using CIN agar plates is shown in Figure 8. Twelve samples that were contaminated with more than 10^2 CFU per gram tonsillar tissue in the destructive sample were negative by the swab sample.

Altogether, 963 *Y. enterocolitica* bioserotype 4/O:3 isolates were recovered, of which 10 isolates (all originating from the same pig) were ornithine negative. All isolates were positive for the *ail* and *yst* gene, and in 780 isolates (81.0%), the *vir*F gene was detected (pYV+). For isolates recovered after ITC and PSB enrichment, 77/393 (19.6%) and 62/337 (18.4%) isolates did not contain the *vir*F gene (pYV-), respectively.

Yersinia pseudotuberculosis bioserotype 1/O:2 was isolated from the destructive tonsil sample of one pig (0.83%) after direct plating on CIN and YeCM agar plates, on which enumeration revealed a concentration of 3.66 and 3.45 $\log_{10}$ CFU/g tonsillar tissue, respectively. The two *Y. pseudotuberculosis* isolates were positive for the *inv* and *vir*F genes, but negative for the *ail*, *yst* and *rfb*C gene.

4. Discussion

Evaluation of sampling methods for pig tonsils showed a higher frequency of *Y. enterocolitica* bioserotype 4/O:3 using destructive samples (43.3%) compared to swab samples (32.5%). Our results are in agreement with the results by Nesbakken (1985), who also found more positive tonsillar tissue samples compared to swab samples, applying different isolation methods. Moreover, several highly contaminated destructive samples were negative when the corresponding swab sample was analysed. As *Y. enterocolitica* is located in the tonsillar crypts and lymph noduli (Shiozawa *et al.*, 1991; Thibodeau *et al.*, 1999), swabbing the tonsillar surface conceivably fails picking up these bacteria. Since swab samples do not represent the internal contamination accurately, they are likely to underestimate the actual carriage of pathogenic *Y. enterocolitica* in pig tonsils.

The International Standard Organization (ISO) method for isolation of presumptive pathogenic *Y. enterocolitica* from food samples (ISO 10273:2003) is also recommended for pig tonsils (EFSA, 2009). This study shows that the ISO method can be used for tonsil samples, though some minor modifications are suggested. First, five-day PSB enrichment was omitted in this study, as five-day enrichment is less efficient than two-day PSB enrichment for the recovery of human pathogenic *Y. enterocolitica* from destructive tonsil samples (Chapter 1). Second, ITC provided better results than PSB for destructive samples, whereas PSB enrichment resulted in more positive swab samples compared to ITC. Remarkably, 15 PSB-positive swab samples that were negative after

ITC enrichment were either negative by direct plating or were contaminated below 20 CFU/surface area (data not shown). Thus, this lower recovery using ITC compared to PSB enrichment for swab samples may rather be due to the smaller sample fraction that is used for ITC than to an inferior efficiency of this medium. Third, ISO 10273:2003 prescribes the use of alkali treatment after PSB enrichment, but not after ITC enrichment. Nevertheless, our data suggest that alkali treatment after ITC enrichment is beneficial for the isolation of pathogenic *Y. enterocolitica* from destructive tonsil samples. Nine destructive samples that were positive by direct plating were not detected by the ITC-SSDC method. However, this number was reduced to three when alkaline treatment was used. As a conclusion, direct plating or additional KOH treatment after ITC enrichment are recommended over the conventional ITC-SSDC method.

Enrichment in ITC is usually followed by plating on SSDC agar plates, as recovery has been shown to be higher compared to the ITC-CIN combination (de Boer and Nouws, 1991; Wauters *et al.*, 1988). In contrast, Fondrevez *et* al. (2010) recovered about three times more positive tonsil swab samples when combining ITC with CIN than with SSDC (without using KOH treatment). However, in this study, similar results were obtained with both agar plates after ITC enrichment. Plating on CIN after ITC enrichment thus seems to be equally good as SSDC for the isolation of *Y. enterocolitica* serotype O:3 from tonsil samples.

Isolation of pathogenic *Y. enterocolitica* from tonsillar tissue using enrichment procedures generally results in more positive samples compared to direct plating (Fredriksson-Ahomaa *et al.*, 2001a; Korte *et al.*, 2004). However, in this study, recovery using direct plating yielded similar results as enrichment procedures, provided quantitative data, and resulted in the most rapid identification of positive samples. This discrepancy might be attributed to different approaches of direct plating. In total, we used one millilitre of the initial homogenate for direct plating, which corresponds to a theoretical detection limit of 10 *Yersinia* per gram. Moreover, spread plating and spiral plating may result in a different outcome compared to traditional streaking using a loop. As the former generally leads to numerous well-isolated colonies due to a homogeneous spread of bacteria, isolation of pathogenic *Yersinia* is less complicated. When applying the direct plating method for isolation of pathogenic *Y. enterocolitica* from tonsils samples, spread plating or spiral plating are thus preferable over traditional streaking.

The performance of YeCM was equal to the standard agar plates SSDC and CIN. When using short isolation procedures for the isolation of pathogenic *Y. enterocolitica* from pig tonsils, as in this study, the benefit of discriminating pathogenic from non-pathogenic *Y. enterocolitica* using YeCM seems to be limited. Nevertheless, YeCM might be advantageous when a large quantity of non-pathogenic *Yersinia* is expected and differentiation of pathogenic from non-pathogenic strains is more problematic. For instance, the applied enrichment procedure can influence the detection of non-pathogenic *Yersinia*. A higher recovery of non-pathogenic *Yersinia* is seen when prolonged cold enrichment is used compared to selective enrichment and direct plating (de Boer and Nouws, 1991; Kwaga *et al.*, 1990; Laukkanen *et al.*, 2009). Additionally, contrary to pig tonsils, in environmental samples and food products, non-pathogenic strains are predominant, whereas the numbers of pathogenic *Y. enterocolitica* are low (Fredriksson-Ahomaa and Korkeala, 2003; Kwaga

et al., 1990). These conditions may complicate the selection of pathogenic strains, in which the use of a selective medium for pathogenic *Y. enterocolitica* such as YeCM can be of interest.

About 80% of *Y. enterocolitica* bioserotype 4/O:3 isolates was PCR-positive for the *vir*F gene. As from many pigs both pYV+ and pYV- isolates were recovered, analysing a limited number of isolates per pig may lead to an underestimation of pYV+ pigs. The plasmid is said to be lost easily under *in vitro* conditions, particularly during repeated culturing in the laboratory at 37°C. However, Li *et al*. (1998) showed that the plasmid is relatively stable at 32°C, and observed only a slight loss of the plasmid after nine days of culture in brain heart infusion broth. In the present study, a considerable proportion of plasmid-negative isolates were detected after direct plating, even though cultures were never incubated at temperatures above 30°C and DNA was extracted no later than 5 days after sampling. Moreover, the proportion of pYV+/pYV- *Y. enterocolitica* isolates after enrichment was similar to the proportion obtained by direct plating. Thus, enrichment in ITC or PSB at 25°C for 48h does not seem to cause an apparent selection towards pYV+ or pYV- strains. Logue *et al*. (2006) observed no significant difference in duration of lag phase or growth rate at 25°C between a plasmid-bearing and plasmid-cured strain, which may suggest that there is no overgrowth by plasmid negative strains during enrichment at 25°C. Hence, it is still unclear in which stage the plasmid is lost.

Sampling methodologies strongly influence the outcome of detection and enumeration of human pathogenic *Y. enterocolitica* from pig tonsils. Although swab samples are easier and faster compared to destructive samples, homogenization of tissue samples is required to obtain reliable results. For direct isolation of pathogenic *Y. enterocolitica* from destructive tonsil samples, we found spread plating to be a suitable method. Not only does it result in a rapid identification of positive samples, it also provides a measure of bacterial counts. Moreover, we found ISO 10273:2003 with minor modifications to be a rapid and routinely applicable isolation method for the recovery of pathogenic *Y. enterocolitica* from destructive tonsil samples.

Acknowledgements

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Chapter 3
Recovery of human pathogenic *Yersinia enterocolitica* from pig carcasses and minced meat

Adapted from: Van Damme I., Berkvens D., Botteldoorn N., Dierick K., Wits J., Pochet B., De Zutter L. (2013). Evaluation of the ISO 10273:2003 method for the isolation of human pathogenic *Yersinia enterocolitica* from pig carcasses and minced meat. Food Microbiology 36, 170-175.

Abstract

Pig carcass swabs (n=254) and minced meat samples (n=82) were examined for pathogenic *Yersinia enterocolitica* using different routinely used enrichment protocols. All samples were obtained in the context of the official *Yersinia* monitoring program in Belgium.

In total, 28 carcasses (11.0%) were contaminated with *Y. enterocolitica* bioserotype 4/O:3 and one (0.4%) with bioserotype 2/O:9. Four minced meat samples (4.9%) tested positive for *Y. enterocolitica* bioserotype 4/O:3. Using the ISO 10273:2003 method, eight out of the 29 *Yersinia*-positive carcasses (27.6%) and none of the contaminated minced meat samples (0.0%) were detected. Reducing the enrichment time in PSB from 5 to 2 days increased the number of positive samples. Overall, enrichment in PSB at 25°C recovered more positive carcasses and minced meat samples than selective enrichment and cold enrichment.

As the exclusive use of the ISO 10273:2003 method results in a strong underestimation of *Y. enterocolitica* positive carcasses and minced meats, efforts are needed to optimize the current version of the ISO method. In addition, isolation of pathogenic *Y. enterocolitica* requires experience and the use of a stereomicroscope to avoid false negative results.

Keywords: pathogenic *Yersinia enterocolitica*; carcass swab; minced meat; isolation; monitoring.

1. Introduction

Yersiniosis is the third most frequently reported zoonosis in Europe, which is mainly caused by the species *Yersinia enterocolitica* (EFSA and ECDC, 2012). This species is subdivided in six biotypes and several serotypes (Wauters *et al.*, 1987), with bioserotype 4/O:3 being the predominant combination to cause clinical infections in Europe. Infections are usually sporadic and mostly affect young children under five years of age. The most prevalent clinical manifestation is acute enterocolitis, with diarrhoea, abdominal pain, and fever as most common symptoms (Rosner *et al.*, 2010; Verhaegen *et al.*, 1998).

Humans get infected with *Y. enterocolitica* primarily through the consumption of raw or undercooked pork (Ostroff *et al.*, 1994; Tauxe *et al.*, 1987) and slaughter pigs are considered to be the principal animal reservoir for pathogenic strains of *Y. enterocolitica*, mainly bioserotype 4/O:3 (Bucher *et al.*, 2008; Fredriksson-Ahomaa *et al.*, 2006). At time of slaughter, many pigs harbour pathogenic *Y. enterocolitica* in their tonsils, and to a lesser extent also in their intestines and lymph nodes (Gürtler *et al.*, 2005; Nesbakken *et al.*, 2003). Since pigs are asymptomatic carriers, positive animals are not recognised during veterinary inspection. As such, pathogens may spread from infected organs and contaminate the carcass surface during slaughter (Borch *et al.*, 1996; Laukkanen *et al.*, 2009). Moreover, chilling does not reduce the prevalence of pathogenic *Y. enterocolitica* (Nesbakken *et al.*, 2008) and the psychrotrophic character of this organism even allows growth at refrigerated temperatures. Fredriksson-Ahomaa *et al*. (2012b) demonstrated that despite low initial contamination in packaged pork cheeks, numbers of pathogenic *Y. enterocolitica* may exceed 10^4 CFU/g after 12 days of storage at 6°C.

The limited number of pathogens combined with the occurrence of an excessive background flora can complicate the recovery of pathogenic *Y. enterocolitica* from naturally contaminated food matrices (Fredriksson-Ahomaa and Korkeala, 2003) and result in false-negative findings. Therefore, the choice of an adequate testing procedure strongly influences the effectiveness of epidemiological studies. For monitoring and survey purposes of pathogenic *Y. enterocolitica*, the European Food Safety Authority recommends the use of ISO 10273:2003 (EFSA, 2007), which is used in Belgium for the official monitoring of pathogenic *Y. enterocolitica* on pig carcasses and minced meat. Nevertheless, the current ISO method is laborious and has already been shown to be ineffective for isolation of pathogenic *Y. enterocolitica* from pig tonsils (Chapter 1), intestines (Laukkanen *et al.*, 2010a), and various lowly contaminated foods (Fredriksson-Ahomaa *et al.*, 2008; Thisted Lambertz *et al.*, 2008b; Thisted Lambertz *et al.*, 2007). Therefore, this study evaluates the ISO 10273:2003 method and other routinely used isolation methods for the recovery of human pathogenic *Y. enterocolitica* from pig carcasses and minced meat. In addition, the effect of co-incubation of the sponge on the recovery of *Y. enterocolitica* from carcass swabs was assessed after artificial inoculation of pork skin samples.

2. Materials and methods

2.1. Recovery of human pathogenic Y. enterocolitica from naturally contaminated pig carcasses and minced meat samples

2.1.1. Sample collection

In total, 254 pig carcasses and 82 minced meat samples (including pure pork, mixed pork with beef, and pork with veal) were analysed for the presence of pathogenic *Y. enterocolitica*. All samples were taken in the context of the official monitoring program of zoonotic agents in pig carcasses and minced meat in Belgium during 2011. Carcasses were sampled before chilling by meat inspectors from the Federal Agency for the Safety of the Food Chain (FASFC). The following sites of one carcass halve were swabbed: (i) the inner side of the ham (100 cm^2); (ii) the pelvic duct (100 cm^2); (iii) the sternum and neck along the incision line (300 cm^2); and (iv) the foreleg (100 cm^2) (Korsak *et al.*, 2003) using a cellulose sponge (3M, Diegem, Belgium) prehydrated with 10 mL buffered peptone water (BPW). Samples were kept at 4°C until analysis (1 to 6 days, median 2 days).

2.1.2. Isolation of *Y. enterocolitica* from naturally contaminated samples

Samples were enriched in three different broths (Figure 9): irgasan-ticarcillin-potassium chlorate (ITC) broth [ITC Broth Base (Fluka) supplemented with 1 mg/l ticarcillin (Sigma-Aldrich, Steinheim, Germany) and 1 g/l KClO3 (Merck, Darmstadt, Germany)], peptone-sorbitol-bile (PSB; Fluka, Steinheim, Germany) broth, and phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts (PMB) broth. Carcass swabs and 10 g of minced meat were homogenized after the addition of 20 mL and 90 mL of 0.1% peptone water (PW), respectively, using a stomacher apparatus (Colworth Stomacher 400, Seward Ltd, London, UK) for two minutes. For minced meat samples, 10 mL homogenate was transferred into 90 mL of each of the three enrichment broths. For carcass swabs, 10 mL of homogenate was transferred into 90 mL of ITC and PMB, and 90 mL of PSB broth was added to the remaining homogenate (10 mL) with sponge.

ITC and PSB were incubated at 25°C for 2 days, after which a loopful (10 µL) was streaked onto a cefsulodin-irgasan-novobiocin (CIN; Bio-Rad, Marnes-la-Coquette, France) plate. Additionally, 100 µL was streaked onto another CIN agar plate after alkali treatment (0.5 mL of culture mixed with 4.5 mL 0.5% KOH in 0.5% NaCl solution for 20s). After ITC enrichment, an extra *Salmonella-Shigella*-deoxycholate-calcium chloride agar (SSDC; Conda, Madrid, Spain) was inoculated using a loop. For PSB, the same procedure was repeated after 5 days enrichment, with the difference that after alkali treatment a loopful instead of 100 µL was streaked onto a CIN agar plate. PMB broth was incubated at 4°C and a loopful was inoculated on CIN agar plates after 7 and 14 days. Additionally, 0.5 mL of the enriched PMB culture was transferred into 4.5 mL of 0.25% KOH in 0.75% NaCl solution for 20 s, after which 100 µL was streaked onto a CIN agar plate.

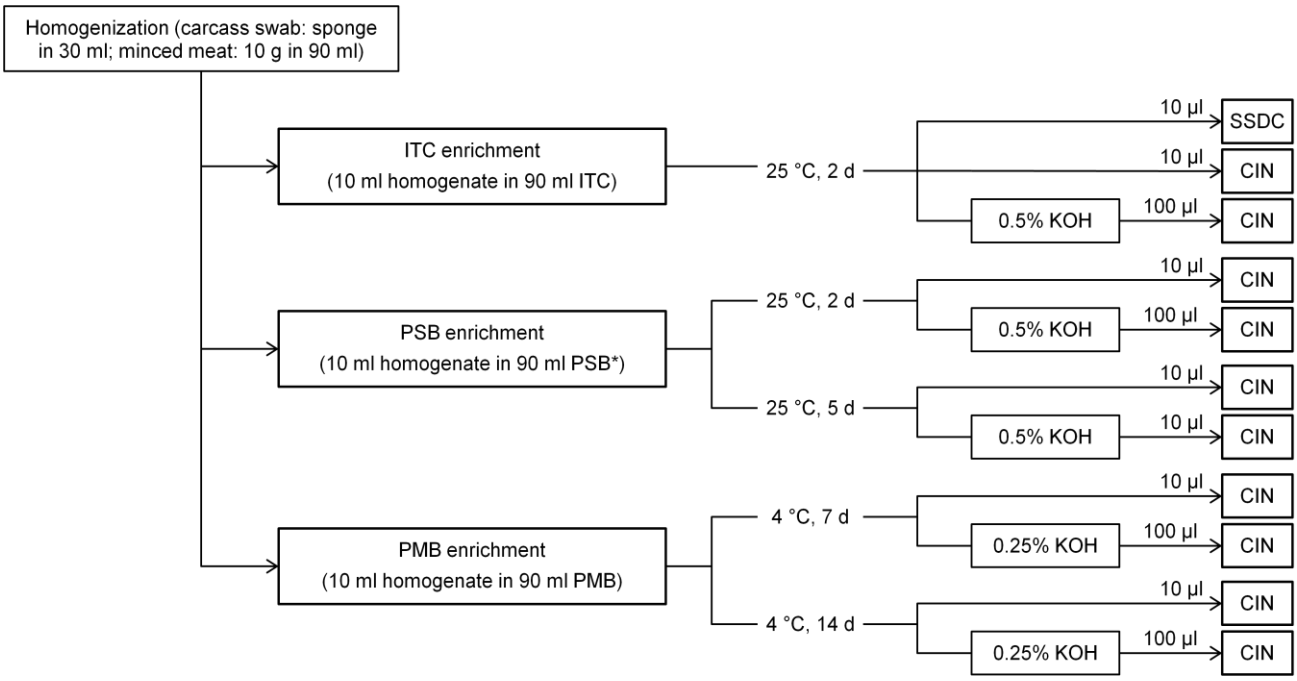

Figure 9. Schematic overview of the isolation protocols for recovery of pathogenic *Y. enterocolitica* from carcass swabs and minced meat samples.

* For carcass swabs: 90 mL PSB was added to the remaining homogenate (10 mL) with sponge.

All agar plates were incubated at 30°C for 24h and examined for *Yersinia* colonies using a stereo microscope with Henry illumination. From each plate, one to five suspected *Yersinia* colonies were streaked on Plate Count Agar (PCA) for pure culture.

2.1.3. Identification of human pathogenic *Y. enterocolitica*

One isolate from each agar plate was transferred to urea broth, Kligler Iron Agar (KIA) and Tryptone Soy Broth (TSB). Isolates showing typical reactions were identified, biotyped and serotyped as described in Chapter 1 and further confirmed using two multiplex PCR assays. Firstly, a virulence PCR was used to detect the *vir*F gene on the virulence plasmid of pathogenic *Yersinia* spp., and the *ail* and *yst* genes on the chromosome of pathogenic biotypes of *Y. enterocolitica* (Harnett *et al.*, 1996). Secondly, a serotype PCR was performed, targeting the *rfb*C gene in *Y. enterocolitica* serotype O:3 (Weynants *et al.*, 1996) and the *per* gene in *Y. enterocolitica* serotype O:9 (Jacobsen *et al.*, 2005). DNA was prepared using 100 µL TSB culture and 50 µL PrepMan^{TM} Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. PCR assays were performed in 25-µL reactions containing one µL of DNA extract, 1.25 U GoTaq® DNA Polymerase, 1X Green GoTaq® Flexi buffer, 2.0 mM $MgCl_2$ (Promega, Madison, USA), 200 µM of each DNTP (Invitrogen, Carlsbad, California, USA), and 0.6 µM or 0.5 µM of primers for *ail* and *vir*F, or *yst*, *rfb*C and *per*, respectively. The PCR reaction consisted of one pre-denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized by UV light. Isolates were regarded as pathogenic *Y.*

enterocolitica when they belong to a pathogenic biotype and the PCR reaction is positive for the *ail* and *yst* gene, regardless of the *vir*F gene.

2.2. Recovery of Y. enterocolitica from artificially contaminated pork skin samples

The effect of co-incubation of the sponge during enrichment in PSB on the recovery of pathogenic *Y. enterocolitica* was evaluated by analysing artificially inoculated pork skin samples (Figure 10). The experimental set-up reproduced the field conditions of the naturally contaminated carcass samples studied above. Three *Y. enterocolitica* bioserotype 4/O:3 strains, recovered from pig carcasses during this study, were grown in TSB at 30°C for 24h, after which cultures were serially diluted for artificial inoculation of 100 cm² pork skin with 5-10 CFU ('low' inoculation dose) and 25-50 CFU ('high' inoculation dose). The concentration was determined by plate counts on PCA incubated at 30°C for 24h. All tests were repeated five times for each experimental setup (i.e. combination of strain and inoculation dose).

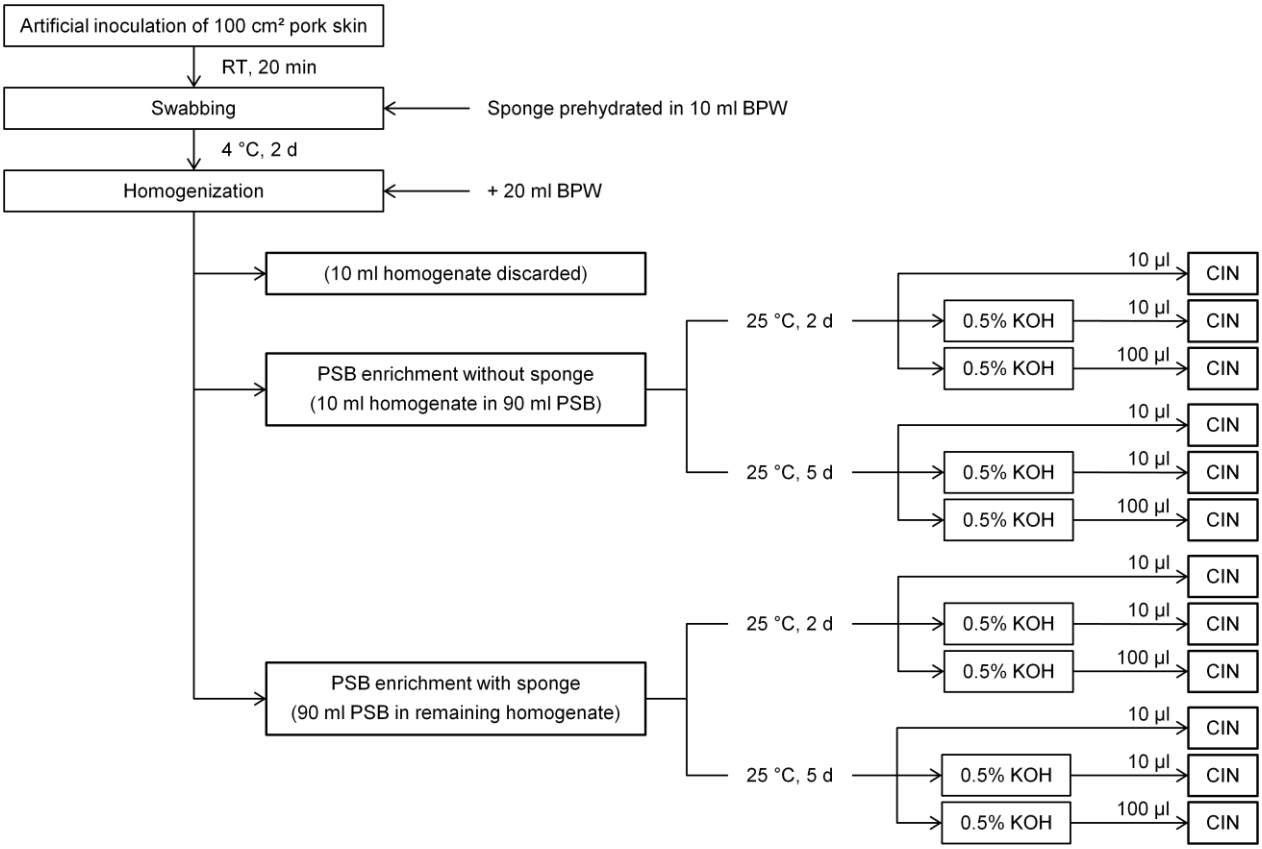

Figure 10. Schematic overview of the isolation protocols for recovery of pathogenic *Y. enterocolitica* from artificially inoculated pork skin samples.

Inoculated pork skin samples were kept at room temperature for 20 minutes (simulating the time between contamination during evisceration and sampling), after which 100 cm² of skin was swabbed using cellulose sponges prehydrated with 10 mL of BPW. Sponges were kept at 4°C for two days (simulating the median time between sampling and start of the analyses), after which 20 mL of PW was added to the sponge. After stomaching for two minutes, 10 mL of homogenate

was discarded, 10 mL was transferred into 90 mL of PSB ('homogenate without sponge'), and 90 mL of PSB was added to the remaining homogenate (10 mL) with sponge ('homogenate with sponge'). After incubation at 25°C for 2 and 5 days, one CIN plate was inoculated directly with a loop (10 µL) and two after alkali treatment (one with 10 µL and one with 100 µL) as described above.

2.3. Statistical analysis

Analyses were carried out using the statistical software package Stata/MP 12.1 (StataCorp, 2011). Results obtained by the different isolation methods were recorded as binary variables (presence or absence of pathogenic *Y. enterocolitica*) and compared using random-effects logistic regression analyses. Bonferroni corrections were applied for multiple testing. For minced meat, isolation methods were not compared statistically due to the limited number of positive samples.

3. Results

3.1. Naturally contaminated pig carcasses and minced meat samples

Pathogenic *Y. enterocolitica* were isolated from 29 carcass swabs (11.4%) and four minced meat samples (4.9%). Twenty-eight carcass swabs were contaminated with *Y. enterocolitica* bioserotype 4/O:3 and one swab was positive for *Y. enterocolitica* bioserotype 2/O:9. All isolates recovered from minced meat belonged to bioserotype 4/O:3. The *vir*F gene was detected in 59 out of 72 *Y. enterocolitica* isolates (81.9%). Additionally, for isolates from one carcass swab and one minced meat sample, a weak amplicon was obtained similar to the one that is expected for the *ail* gene, while they were negative for the *yst* and *vir*F genes. Both isolates were identified as *Yersinia kristensenii* using API 20E (BioMérieux, Marcy l'Etoile, France) at 30°C for 18 to 20h.

Recovery rates of pathogenic *Y. enterocolitica* from naturally contaminated samples by different isolation methods are presented in Table 12. Overall, enrichment in PSB at 25°C yielded more positive swab samples than cold enrichment in PMB ($p = 0.047$) and selective enrichment in ITC ($p = 0.001$). The highest recovery was observed after 2-day PSB enrichment at 25°C with alkali treatment, which yielded 82% of positive samples.

Isolation by ISO 10273:2003 detected 8 out of 29 positive carcasses (27.6%) and none of the positive minced meat samples. Enrichment in PSB for two days with alkaline treatment resulted in a significant higher recovery from carcass swabs than five days enrichment ($p < 0.001$). The ISO-ITC-SSDC method recovered more positive carcasses compared to ITC-CIN, but less when alkaline treatment was used, though the differences were not significant ($p > 0.05$).

Table 12. Number of *Y. enterocolitica*-positive carcass swabs and minced meat samples using different isolation methods.

Isolation method	Carcass swabs	Minced meat
ITC (25°C)[b]	11	0
SSDC (2d)[a]	6	0
CIN (2d)	2	0
KOH + CIN (2d)	10	0
PSB (25°C)[b]	25	3
CIN (2d)	0	0
KOH + CIN (2d)	24	3
CIN (5d)[a]	0	0
KOH + CIN (5d)[a]	2	0
PMB (4°C)[b]	17	1
CIN (7d)	1	0
KOH + CIN (7d)	3	1
CIN (14d)	4	0
KOH + CIN (14d)	16	0
Total[b]	29	4

[a] Isolation methods as described by ISO10273:2003; [b] Parallel interpretation, sample is considered positive if at least one test is positive.

Cold enrichment for 14 days recovered pathogenic *Y. enterocolitica* from significantly more carcass samples than 7 days of cold enrichment when using KOH treatment (p = 0.0092). Overall, cold enrichment in PMB was significantly less effective in isolating pathogenic *Y. enterocolitica* from carcass swabs compared to enrichment in PSB at 25°C (p = 0.047). Thirteen samples were positive by both PSB and PMB enrichment, while twelve and four samples were only positive after PSB and PMB enrichment, respectively (Table 13). Cold enrichment recovered more *Y. enterocolitica* positive carcasses than selective ITC enrichment, but the difference was not significant (p = 0.073).

Table 13. Comparison of *Y. enterocolitica* recovery from carcass swabs by the three enrichment media.

ITC enrichment	PSB enrichment	PMB enrichment	Number of positive samples
+	+	+	8
+	+	-	2
+	-	-	0
+	-	+	1
-	-	+	3
-	+	+	5
-	+	-	10

3.2. Artificially contaminated pork skin samples

The average concentration and standard deviation of the low and high inoculation dose were 8.3 ± 4.3 CFU/100cm² and 40.0 ± 12.6 CFU/100cm², respectively. The results of the different methods of the artificially contaminated pork skin samples are shown in Table 14. In total, 26 out of the 30 inoculated samples (86.7%) tested positive by at least one of the methods. Enrichment of the homogenate with and without sponge resulted in 20 and 24 positive samples, respectively, though the difference was not statistically significant (p = 0.176). After 5 days enrichment in PSB, the inoculation of CIN with the 100-µL volume after alkali treatment recovered significantly more samples than the 10-µL volume (p = 0.042). After two days enrichment, the 100-µL volume was beneficial over the 10-µL volume for lowly contaminated samples, whereas the latter was better for highly contaminated samples, though the differences were not statistically significant (p = 0.232 and p = 0.227, respectively).

Table 14. Recovery of *Y. enterocolitica* from artificially inoculated pork skin samples using PSB enrichment at 25°C.

	Enrichment with sponge			Enrichment without sponge			Total
Method	Low dose	High dose	Total	Low dose	High dose	Total	
PSB (2 days)[b]	7/15[a]	12/15	19/30	11/15	13/15	24/30	26/30
CIN	0/15	0/15	0/30	0/15	3/15	3/30	3/30
KOH (10µL) + CIN	6/15	12/15	18/30	8/15	12/15	20/30	23/30
KOH (100µL) + CIN	7/15	10/15	17/30	11/15	10/15	21/30	25/30
PSB (5 days)[b]	2/15	1/15	3/30	5/15	3/15	8/30	11/30
CIN	0/15	1/15	1/30	0/15	0/15	0/30	1/30
KOH (10µL) + CIN	0/15	0/15	0/30	1/15	1/15	2/30	2/30
KOH (100µL) + CIN	2/15	0/15	2/30	5/15	2/15	7/30	9/30
Total[b]	8/15	12/15	20/30	11/15	13/15	24/30	26/30

[a] Number of positive samples/number of samples that were analysed; [b] Parallel interpretation, sample is considered positive if at least one test is positive.

4. Discussion

Carcass swabs and minced meat were analysed to evaluate the effect of enrichment media for the detection of pathogenic *Y. enterocolitica*. When comparing different isolation methods, several factors should be considered, such as the sample size, dilution factor, and the sampled matrix. Moreover, we believe experience in isolation of pathogenic *Y. enterocolitica* is key in the success of positive sample identification. In several samples, *Y. enterocolitica* appeared on CIN agar plates as small white colonies because of an abundant background flora, and not as the typical “bull’s eyes” colonies. Hence, an adequate training in colony selection and the use of a stereomicroscope with oblique illumination are essential to obtain reliable results.

When selecting an appropriate sample size and sample-to-broth dilution for enrichment broths, a compromise has to be made between a sufficient level of detection and a limitation of overgrowth by competing organisms (de Boer and Seldam, 1987; De Zutter *et al.*, 1995; Lee *et al.*, 1980). The ISO 10273:2003 method prescribes a sample-to-broth ratio of 1:10 for PSB, while for ITC a 1:100 dilution is preferred (ISO 10273, 2003; Wauters *et al.*, 1988). However, no clear indications are given on the dilution of swab samples. Similar to the present study, PSB enrichment was more efficient than ITC enrichment for isolation of *Y. enterocolitica* from porcine tonsil swabs in Chapter 2, albeit different sample sizes and dilutions were used in both media. In contrast, for tonsillar tissue samples, ITC was slightly, yet not significantly better than PSB enrichment. Among other things, this might be explained by different relative concentrations of pathogenic *Y. enterocolitica* and competing bacteria in different sample matrices. In comparison with the high load of pathogens found in pig tonsils (Chapters 1 and 2), the concentration on swabs, carcasses and in food matrices is generally low (Fredriksson-Ahomaa and Korkeala, 2003; Van Damme *et al.*, 2013).

Enrichment in PSB at 25°C yielded more positive samples compared to cold enrichment. Jiang *et al*. (2000) also promoted the use of two-day enrichment at 25°C over two to three weeks of cold enrichment as they found no significant difference in isolation rate of *Y. enterocolitica* from minced meat samples. However, they did not determine the biotype nor the pathogenicity of their *Y. enterocolitica* isolates. Nevertheless, the shortened enrichment at a higher temperature seems to be also preferable over cold enrichment for the isolation of pathogenic isolates, as in the present study it is more efficient and results are obtained in a much shorter time period.

To evaluate the influence of the sponge on the recovery of *Y. enterocolitica* bioserotype 4/O:3 after PSB enrichment, the same sample volume was analysed with and without co-incubation of the sponge. Over 80% of artificially inoculated pork skin samples were recovered using PSB enrichment. The number of positive samples was higher for the homogenate without sponge than when the sponge was co-incubated. However, the samples in the present study were kept at 4°C for two days to simulate the time between sampling and start of the analyses of the official samples. As storage at cold temperatures can be regarded as a pre-enrichment step for *Y. enterocolitica*, the present results may not be extrapolated to freshly analysed samples.

The use of ISO 10273:2003 resulted in a strong underestimation of the number of *Y. enterocolitica*-positive samples. Both for naturally and artificially contaminated samples, a higher recovery was obtained when the enrichment period in PSB was reduced from five to two days. Similar results were also observed for the isolation of pathogenic *Y. enterocolitica* from pig tonsils in Chapter 1. However, in the present study, a 100-µL volume was used after alkali treatment after two days enrichment of the naturally contaminated samples, which is a 10-fold higher volume compared to the loop that was used after five days as described in ISO 10273:2003. During the artificial inoculation of pork skin samples, both volumes gave similar results, though the 100-µL volume was slightly beneficial over a loop when small numbers of *Yersinia* were present after alkali treatment. However, a drawback of this larger volume is the possible overgrowth by background flora, which complicates selection of pathogenic *Yersinia* colonies and

may result in false negative findings. Hence, for lowly contaminated samples with limited competing flora, a 100-µL volume after alkali treatment is advised over a loop.

Pathogenic *Y. enterocolitica* were isolated from 11.4% and 4.9% of pig carcasses and minced meat, respectively, which is within the range of different European studies. Based on culture methods, carcass contamination varies between 0% and 22% (Lindblad *et al.*, 2007; Laukkanen *et al.*, 2010b; Gürtler *et al.*, 2005; Fredriksson-Ahomaa *et al.*, 2000b; Nesbakken *et al.*, 2008; Laukkanen *et al.*, 2009), whereas the occurrence in minced meat samples is generally below 5% (Arnold *et al.*, 2004; Fredriksson-Ahomaa *et al.*, 1999b; Güven *et al.*, 2010; Messelhausser *et al.*, 2011; Thisted Lambertz *et al.*, 2007). As a higher prevalence of pathogenic *Y. enterocolitica* has been obtained using PCR detection (Fredriksson-Ahomaa *et al.*, 1999b; Lindblad *et al.*, 2007; Lucero Estrada *et al.*, 2012; Thisted Lambertz *et al.*, 2007), there is probably an underestimation of the true prevalence on carcasses and meats due to the low sensitivity of the culture methods (Fredriksson-Ahomaa and Korkeala, 2003). Nevertheless, culture methods are less sensitive to false positive results and isolates are useful when studying the epidemiology of a pathogen, so that culture methods remain indispensable.

In conclusion, the exclusive use of ISO 10273:2003 resulted in a prevalence of only 3% and 0% for carcass swabs and minced meat, respectively. This methodological problem contributes to an underestimation of the true prevalence of pathogenic *Y. enterocolitica* in foods and complicates comparison of data that are acquired using different isolation methods. As current methods are not sufficient for the isolation of pathogenic *Y. enterocolitica* from food samples, there is a need for new sensitive culture methods. Moreover, to reduce the number of false-negative results of currently used methods, reading of plates should be done by experienced laboratory staff using a stereomicroscope.

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Chapter 4
Contamination of freshly slaughtered pig carcasses with enteropathogenic *Yersinia* spp. and investigation of associated risk factors during slaughter

Abstract

A cross-sectional survey was undertaken to determine the distribution of enteropathogenic *Yersinia* spp. in pigs at slaughter in Belgium. Moreover, factors associated with *Yersinia* contamination of pig carcasses were studied.

Tonsils, faeces and carcass swabs from 360 carcasses were sampled in nine pig abattoirs. All samples were tested for the presence of enteropathogenic *Yersinia* spp. using direct plating, selective enrichment and cold enrichment. *Yersinia enterocolitica* serotype O:3 was isolated from the tonsils and faeces of 55.3% and 25.6% of pigs, and *Y. pseudotuberculosis* from 1.4% and 0.6%, respectively.

The pathogens were recovered from 39.7% of the carcass surfaces. The highest prevalence was found for the mandibular region (28.9%), followed by the sternal region (16.4%), pelvic duct (7.8%), and split surface near the sacral vertebrae (6.9%). The quantitative load was the highest for the mandibular region, though the vast majority of carcass samples (96.9%) had *Yersinia* concentrations below the detection limit of enumeration.

The presence of *Y. enterocolitica* in the faeces was significantly associated with carcass contamination at all sampled areas. Additionally, the presence of the pathogen in tonsils was associated with *Y. enterocolitica* contamination of the mandibular region and pelvic duct. The contamination with *Y. enterocolitica* at the split surface was also higher when the head was split together with the carcass compared to when the head remained intact. Cleaning and disinfection of the knife before the removal of the plug set was a protective factor for contamination of the sternal region. Moreover, a higher sternal contamination was seen in high-capacity than in low-capacity abattoirs. For the mandibular region, incision of the tonsils during removal of the plug set and splitting of the head were risk factors associated with *Y. enterocolitica* contamination.

Keywords: enteropathogenic *Yersinia* spp., pigs, slaughter, carcass contamination, risk factors.

1. Introduction

Human pathogenic *Y. enterocolitica* are commonly found in tonsils of pigs at slaughter, in which the number can reach up to 10^6 CFU per gram (Chapters 1 and 2). Furthermore, infected pigs may carry pathogenic *Y. enterocolitica* in their intestinal content, mesenterial lymph nodes and other lymph nodes. From these tissues, carcasses may become contaminated with enteropathogenic *Yersinia* spp. during slaughter (Borch *et al.*, 1996; Laukkanen *et al.*, 2008).

The European Food Safety Authority identified *Y. enterocolitica*, after *Salmonella*, as the most relevant bacterial pathogen related to pigs and pork (EFSA, 2011). In order to control the spread of the pathogen to carcasses, a better understanding of the epidemiology of enteropathogenic yersiniae is required. Knowledge on risk factors, which may affect the occurrence of *Yersinia* on carcasses is required to implement effective control measures. To our knowledge, no comprehensive study has yet been conducted to determine risk factors for *Yersinia* carcass contamination during evisceration.

The aim of this study was to determine the occurrence, contamination level and bioserotype distribution of enteropathogenic *Yersinia* spp. on freshly eviscerated pork carcasses in Belgium. Additionally, risk factors associated with the presence of pathogenic *Y. enterocolitica* on pork carcass during slaughter were identified.

2. Material and methods

2.1. Sampling

2.1.1. Study design

The primary selection of the abattoirs was based on the annual numbers of slaughtered pigs (fattening pigs only) in 2009. Only abattoirs that had slaughtered more than 100,000 pigs were included (22 abattoirs in total), out of which 9 abattoirs were randomly selected. The annual number of slaughtered fattening pigs in these selected abattoirs varied from about 135,000 to 1,250,000. Each abattoir was visited 4 times during the period of August 2010 to August 2011. All sampling visits were performed on Mondays and were evenly distributed over the one-year-period to account for seasonal differences in prevalence. On each sampling day, ten pigs in one abattoir were sampled during normal slaughter operations. For each sampled pig, specific slaughter practices were recorded (Table 19).

2.1.2. Collection of samples at the abattoir

Tonsils, faeces and carcass swabs from 360 pigs were sampled during 36 different visits. The first pig to be sampled on each sampling day was randomly selected within 15 minutes after evisceration of the first pig and the following nine pigs were sampled every 15 minutes. The selected carcasses were marked just before the start of evisceration and the corresponding batch number was recorded. The whole intestinal tract was put in a plastic bag immediately after evisceration and the rectum was closed with threads, after which it was excised and put in a

different plastic bag. Tonsils were excised aseptically after the removal of the plug set and put in a sterile plastic bag. The corresponding carcasses were swabbed after splitting of the carcasses, but prior to cooling. Four different areas of approximately 100 cm² were swabbed using cellulose sponges (3M™ Sponge-Stick, 3M, Diegem, Belgium): (1) the pelvic duct, (2) the split surface near the sacral vertebrae, (3) the sternal region (breast cut and surrounding skin), and (4) the mandibular region, but avoiding the region of the tonsils. Each sponge was hydrated by adding 20 mL PMB (phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts) to the sample bag immediately before sampling. Alternating between each pig, all swabs were taken either from the right or left carcass half. All samples were transported to the laboratory under cooled conditions and processed immediately upon arrival.

2.2. Isolation and enumeration of enteropathogenic Yersinia *spp.*

From tonsils and faeces, a 11-g subsample was weighed aseptically and homogenized (Stomacher 400, Seward, Worthing West Sussex, United Kingdom) in 99 mL of PMB for one minute. Swab sponges were stomached for one minute just before starting the analyses.

All samples were analysed for enteropathogenic *Yersinia* spp. using (i) direct plating, (ii) selective enrichment and (iii) cold enrichment. (i) For direct plating, 500 µL of PMB homogenate was spread plated onto a cefsulodin-irgasan-novobiocin (CIN; Bio-Rad, Nazareth, Belgium) agar plate in duplicate. (ii) For selective enrichment, 10 mL of PMB homogenate from tonsils and faeces was transferred into 90 mL of irgasan-ticarcillin-potassium chlorate (ITC) broth. For carcass samples, 5 mL of PMB was transferred into 45 mL ITC broth. After 2 days enrichment at 25°C, a loopful (10 µL) was streaked onto a CIN agar plate. Additionally, after alkali treatment (0.5 mL of ITC culture mixed with 4.5 mL 0.5% KOH in 0.5% NaCl solution for 20 s), 100 µL was streaked onto another CIN agar plate. (iii) For cold enrichment, the remaining PMB homogenate was incubated at 4°C for 7 and 14 days. After 7 days, a loopful (for tonsils and carcass samples) or 100 µL (for faecal samples) of enriched culture was streaked onto a CIN agar plate. After 14 days enrichment, 0.5 mL of the enriched PMB culture was transferred into 4.5 mL of 0.25% KOH in 0.75% NaCl solution for 20 s, after which 100 µL was streaked onto a CIN agar plate.

All CIN agar plates were incubated at 30°C for 24h and examined for *Yersinia* colonies using a stereo microscope with Henry illumination. All plates were additionally incubated at room temperature for 24h and examined again for the presence of *Y. pseudotuberculosis*. From each CIN agar plate, at least one suspect *Yersinia* colony was selected and streaked on plate count agar (PCA) for further identification.

2.3. Identification and virulence properties of enteropathogenic Yersinia *spp.*

Presumptive positive isolates were tested for biochemical characteristics using urea broth and Kligler Iron Agar (KIA) and transferred into Tryptone Soy Broth (TSB) for molecular identification. One-hundred µL of incubated TSB culture was used to extract DNA using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, USA), following the manufacturer's instructions. *Y. enterocolitica* isolates were confirmed by a virulence and serotype PCR assay as

described earlier (Chapter 3). *Y. pseudotuberculosis* isolates were confirmed using a PCR assay targeting the *inv* gene (Nakajima *et al.*, 1992) and sent to the Belgian reference laboratory for *Y. pseudotuberculosis* (UCL, St-Luc Hospital, Brussels) for serotyping.

2.4. Statistical analyses

All statistical analyses were carried out using Stata/MP 12.1 (StataCorp, 2011). Differences between carcass sites were analysed using a logistic regression, including carcass as random-effect.

Univariate logistic regressions were used to examine the association between the presence of *Y. enterocolitica* on each of the four sampled carcass sites as dependent variable and different slaughter practices, chain speed, and the presence in tonsils and rectum as independent variables. A backwards elimination process was used in which all variables, significant at $p < 0.05$ in a univariate analysis, were included in the initial multivariate model (including all possible interactions), excluding at each step the non-significant variable until only significant main effects and interactions remained. Multilevel models including abattoir and farm as random effects were tested for each model and retained where necessary.

Quantitative data obtained by direct plating were converted to $\log_{10}$ CFU/g for tonsils and intestinal content, and $\log_{10}$ CFU/swab for carcass swabs to calculate the mean and standard deviation.

3. Results

3.1. Presence of enteropathogenic Yersinia *spp. in pigs at slaughter*

In total, enteropathogenic *Yersinia* spp. were isolated from the tonsils and/or faeces of 234 slaughter pigs (65.0%) (Table 15). No significant differences between seasons were observed ($p > 0.05$).

Table 15. Number of pig samples positive for enteropathogenic *Yersinia* spp. (n=360).

Sample type	*Y. enterocolitica*[a]	*Y. pseudotuberculosis*[b]	Total (enteropathogenic *Yersinia* spp.)
Tonsils	199	5	203
Faeces	92	2	93
Total[c]	230	7	234

[a] *ail*, *yst*, and *rfb*C-positive isolates; [b] *inv*-positive isolates; [c] parallel interpretation: a pig was considered positive if at least one of the samples was positive.

Pathogenic *Y. enterocolitica* serotype O:3 were isolated from the tonsils and faeces of 199 and 92 slaughtered pigs, respectively. Of these animals, 61 were positive for pathogenic *Y. enterocolitica* in both tonsils and faeces, whereas 138 and 31 were only *Y. enterocolitica*-positive in tonsils and

faeces, respectively. *Y. pseudotuberculosis* was isolated from the tonsils and faeces of five and two pigs, respectively. A concurrent contamination of *Y. pseudotuberculosis* and *Y. enterocolitica* serotype O:3 was found in one tonsil and one faecal sample. In one pig, *Y. enterocolitica* was isolated from the tonsils, while from the faeces *Y. pseudotuberculosis* was the only pathogenic *Yersinia* species recovered.

Table 16 presents, per abattoir, the number of samples positive for pathogenic *Y. enterocolitica* serotype O:3. In all abattoirs, tonsils resulted in the highest prevalence, varying between 47.5 and 65.0%. The prevalence of positive carcasses varied from 22.5 to 57.5% per abattoir. In total, 143 pig carcasses (39.7%) were contaminated with pathogenic *Y. enterocolitica* serotype O:3 at one or more of the 4 sampled sites. Twenty-nine of these positive pig carcasses (20.3%) did not carry pathogenic *Y. enterocolitica* in the tonsils nor the faeces. Moreover, in 116 animals, pathogenic *Y. enterocolitica* were recovered from tonsils and/or faeces whereas no pathogenic *Y. enterocolitica* were recovered from the carcass surface. In total, 101 pigs were negative for enteropathogenic *Yersinia* spp. in the tonsils, faeces or any of the sampled carcass sites.

Table 16. Number of *Y. enterocolitica*[a]-positive samples in 9 different abattoirs.

	Number of samples per abattoir	Abattoir									Total
		A	B	C	D	E	F	G	H	I	
Tonsils	40	26	24	19	24	21	20	25	19	21	199
Faeces	40	5	6	12	15	14	13	12	10	5	92
Carcasses (total)[b]	40	16	17	13	23	15	9	20	9	21	143
Pelvic duct	40	1	4	2	5	4	4	3	4	1	28
Split surface	40	1	5	2	3	2	2	6	1	3	25
Sternal region	40	6	3	8	15	7	3	6	5	6	29
Mandibular region	40	10	14	7	16	8	8	17	4	20	104

[a] *ail*, *yst*, and *rfb*C-positive isolates; [b] parallel interpretation: a carcass was considered positive when at least one of the four sampled sites was positive.

Pathogenic *Yersinia* were mostly recovered from the mandibular region (28.9%), followed by the sternal region (16.4%), pelvic duct (7.8%), and split surface (6.9%) (Table 17). The proportion of carcasses positive at the mandibular region was significantly higher than the other regions ($p < 0.001$). Presence of enteropathogenic yersiniae on the sternal region was significantly higher than on the pelvic duct and split surface ($p < 0.001$), though counts there appeared to be slightly lower (no statistical analysis possible). Based on direct plating, the average load was the highest for the mandibular region, with a maximum of 3.42 $\log_{10}$ CFU/100cm². Nevertheless, the vast majority of carcass samples (1395/1440, 96.9%) had *Yersinia* concentrations below the detection limit of direct plating (20 CFU/100 cm²).

Table 17. Qualitative and quantitative contamination of pig carcasses with enteropathogenic yersiniae.

Carcass area	Number of positive samples	Countable samples[a]	Mean[b]	Standard deviation	Maximum
Pelvic duct	28	10	1.53	0.49	2.66
Split surface	25	3	1.58	0.49	2.15
Sternal region	59	2	1.30	NC[c]	1.30
Mandibular region	104	28	1.90	0.60	3.42

[a] Number of samples that were positive by direct plating and where enumeration was possible; [b] Numbers are in $\log_{10}$ CFU/swab (100 cm²); [c] Not calculated.

The *Y. pseudotuberculosis* positive carcasses originated from 7 different farms, sampled in 5 different abattoirs (Table 18). All isolates tested positive for the *inv*-gene and strains belonged to serotype O:1 and O:2. From one pig, the mandibular region was *Y. pseudotuberculosis*-positive, whereas only *Y. enterocolitica* serotype O:3 was isolated from its tonsils.

Table 18. Distribution of enteropathogenic *Yersinia* spp. in *Y. pseudotuberculosis*-positive pig carcasses.

Carcass number	Abattoir	Farm	Tonsils	Faeces	Pelvic duct	Split surface	Sternal region	Mandibular region
32	D	1	YP^{II}		YE			
114	B	2	YE	YP^{I}				
184	H	3	YE, YP^{II}					
195	I	4	YP^{II}					
197	I	4	YE				YE	YE, YP^{II}
200	I	5	YP^{I}			YE		YE
251	F	6	YP^{II}					
258	F	7		YE, YP^{II}				

YE, *Y. enterocolitica* serotype O:3 (*ail*, *yst*, and *rfb*C-positive); YP^{I}, *Y. pseudotuberculosis* serotype O:1 (*inv*-positive); YP^{II}, *Y. pseudotuberculosis* serotype O:2 (*inv*-positive).

3.2. Risk factors for carcass contamination

For each of the four carcass sites, risk factors for contamination with *Y. enterocolitica* serotype O:3 are shown in Table 19. The presence of *Y. enterocolitica* in the faeces was positively associated with carcass contamination at each of the four tested sites. Moreover, a significant positive association was found between the occurrence of *Y. enterocolitica* on the pelvic duct and its presence in tonsils. However, the odds ratio was lower for tonsils than for faeces.

Table 19. Results of the multivariate analyses for the association between the occurrence of *Y. enterocolitica*[a] on carcasses and its presence in tonsils and rectum, different slaughtering techniques, and chain speed.

	Pelvic duct		Split surface		Sternal region[b]		Mandibular region[c]	
Independent variable	$OR_{adjusted}$ (95% CI)	*p*	$OR_{adjusted}$ (95% CI)	*p*	$OR_{adjusted}$ (95% CI)	*p*	$OR_{adjusted}$ (95% CI)	*p*
Presence of *Y. enterocolitica* in tonsils[d]	3.03 (1.27-7.24)	0.013	NS[e]		NS		3.73 (2.03-6.86)	<0.001
Presence of *Y. enterocolitica* in faeces	5.59 (2.45-12.77)	<0.001	2.62 (1.13-6.07)	0.024	2.60 (1.22-5.53)	0.013	2.54 (1.27-5.08)	0.009
Mechanical removal of the rectum	NS		NS		NS		NS	
Incision of the gut	NS		NS		NS		NS	
Cleaning and disinfection of the knife before evisceration	NS		NS		NS		NS	
Cleaning and disinfection of the knife before removal of the plug set	NS		NS		0.29 (0.09-0.99)	0.049	NS	
Incision of the tonsils during removal of the plug set	NS		NS		NS		3.91 (1.39-11.05)	0.010
Splitting of the head	NS		2.90 (1.17-7.17)	0.022	NS		3.19 (1.26-8.04)	0.014
Incision of submandibular lymph nodes during veterinary inspection	NS		NS		NS		NS	
Slaughter rate (<200 pigs/hour)	NS		NS		1.00	-	NS	
Slaughter rate (200-500 pigs/hour)	NS		NS		0.84 (0.29-2.45)	0.742	NS	
Slaughter rate (>500 pigs/hour)	NS		NS		2.83 (1.01-7.94)	0.047	NS	

[a] *ail*, *yst* and *rfbC*-positive isolates; [b] clustering by farm; [c] clustering by abattoir and farm; [d] tonsils that were positive by direct plating; [e] NS, odds ratio in univariate and/or multivariate analysis is not significantly different from 1.00 ($p > 0.05$).

When the head was split together with the carcass, 18 out of 180 carcasses (10.0%) were positive at the split surface, compared to only 7 (3.9%) when the head was left intact during splitting of the carcass (OR_{adj} = 2.90; p = 0.022). Cleaning the knife before removal of the plug set was a protective factor for contamination at the sternal region (OR_{adj} = 0.29, p = 0.049). Moreover, significantly more animals were positive at the sternum in abattoirs with a high slaughter rate (>500/h) (29/120) compared to abattoirs with medium (200-500/h) (14/120) or low (<200/h) (16/120) capacity.

When the head was split together with the carcass, 66/180 (36.7%) carcasses were positive at the mandibular region compared to 38/180 (21.1%) when the head was intact (p = 0.014). When the tonsils were incised during removal of the plug set, the odds of contamination of the mandibular region was 3.91 times that of carcasses where the tonsils were not incised.

4. Discussion

4.1. Presence of enteropathogenic Yersinia spp. in pigs and on pig carcasses at slaughter

This study shows that the occurrence of enteropathogenic yersiniae in pigs at slaughter in Belgium is high as the organisms were isolated from 56% of tonsils and 26% of faecal samples, with 65% of pigs being positive in at least one of both samples. The prevalence of enteropathogenic *Yersinia* spp. varies greatly between different European studies, with numbers ranging from 8 to 93% in tonsils and from 1 to 30% in faeces (Table 4).

The isolation of pathogenic *Y. enterocolitica* from pork carcasses in different European studies varies between 0 and 26% (Novoslavskij *et al.*, 2013; Gürtler *et al.*, 2005), which is lower than the proportion of positive carcasses in this study (40%). Nevertheless, large variations were found between the different regions, ranging from 7% at the split surface to 29% at the mandibular region.

To our knowledge, this is the first report gathering a comprehensive set of quantitative data on carcass contamination during slaughter using direct plating on selective agar media. The numbers of pathogenic *Y. enterocolitica* on carcass surfaces were typically low as most of the positive samples were negative by direct plating, for which the detection limit in the current study was 20 bacteria per 100 cm². Nevertheless, certain regions (particularly the mandibular region) may be highly contaminated, with concentrations above 10^3 CFU/100cm².

The prevalence of *Y. pseudotuberculosis* in pig tonsils and faeces at slaughter in the present study was lower compared to *Y. enterocolitica*, which is in accordance with other studies (Laukkanen *et al.*, 2010b; Novoslavskij *et al.*, 2013; Ortiz Martínez *et al.*, 2009; Ortiz Martínez *et al.*, 2011; Terentjeva and Berzins, 2010). Moreover, carcass contamination was low as only one carcass (0.3%) was found positive for this pathogen, which is similar to other studies where isolation rates below 5% are found (Laukkanen *et al.*, 2010b; Nesbakken *et al.*, 1994; Novoslavskij *et al.*, 2013).

4.2. Risk factors for carcass contamination with Y. enterocolitica *serotype O:3*

Pork carcasses may get contaminated with enteropathogenic yersiniae during slaughter as a result of the spread of the bacteria from tonsils, lymph nodes and intestinal contents. Identification of risk factors for carcass contamination may contribute to a better understanding of the dissemination of pathogenic *Y. enterocolitica* during slaughter.

Contamination of faeces and tonsils were positively associated with carcass contamination after slaughter, which indicates that infected pigs are an important source for carcass contamination. Laukkanen *et al*. (2009) observed that all positive carcasses originated from farms with a higher within-farm prevalence and that genotypes found on carcasses could also be found in pigs infected on the farms. The former findings all support the hypothesis that *Yersinia* contamination of pig carcasses originates mainly from positive pigs delivered to the abattoir. This also suggests that transfer of the pathogens over different batches may be limited.

Despite the association between carcass contamination and *Yersinia* presence in tonsils and faeces, contaminated carcasses also derived from uninfected pigs. Besides contamination from infected tissues, cross-contamination may also originate from previously slaughtered pigs. In fact, the split surface and pelvic duct may rather originate from a previous pig than from the sampled pig. Even though some associations were found between positive tissues, the correlation may have been higher when tonsils of the previous pig had been sampled. For contamination of the split surface, *Yersinia* positive tonsils were not a significant risk factor, whereas splitting of the head was positively correlated with contamination at this site. Nevertheless, the risk factor 'splitting of the head' is presumably related to tonsil contamination of previous pigs.

A positive association was found between the split surface and faeces, though contamination at this site is less likely to have originated from faeces. An association is not necessarily a causal relationship and presence in the faeces is thus probably more a risk indicator than a risk factor.

Abattoir personnel or processing equipment may transfer pathogens to subsequent carcasses. Pathogenic *Y. enterocolitica* have already been recovered from brisket saws, knives, hooks, and splitting saws in pig abattoirs (Fredriksson-Ahomaa *et al.*, 2000b; Laukkanen *et al.*, 2010b). Accordingly, cleaning of knives was a protective factor in the present study, whereas splitting of the head together with the carcass was associated with a higher *Yersinia* contamination. During the splitting of the head, tonsils may contaminate the splitting machine (Fredriksson-Ahomaa *et al.*, 2000b), and contaminate the split surface of the following carcass.

Since the submaxillary lymph nodes may harbour pathogenic *Yersinia* spp., the compulsory incision of the submaxillary lymph nodes during veterinary inspection has been proposed as a risk for *Yersinia* carcass contamination (Nesbakken *et al.*, 2003). In 57.1% of sampled carcasses, the submaxillary lymph nodes were incised during veterinary inspection (data not shown), but we were not able to show a statistical association between carcass contamination and incision of these lymph nodes. Among other things, the relatively low number of *Yersinia* positive lymph nodes and/or a limited transmission by knifes may result in a low association between incision of the lymph nodes and carcass contamination.

Carcasses eviscerated by the use of a mechanical bung cutter were less frequently contaminated with pathogenic *Y. enterocolitica* at the pelvic duct compared to manual evisceration, though the effect was not statistically significant. Manual removal of the bung was performed in only two abattoirs, corresponding to 80 of the sampled carcasses (22%). Similar to our results, Andersen (1988) found lower frequencies of *Yersinia* on the carcass surface when a mechanical bung cutter than when the rectum was removed using a knife. Moreover, an additional reduction was obtained when a plastic bag was used to enclose the rectum immediately after loosening, which has been confirmed by others (Laukkanen *et al.*, 2010b; Nesbakken *et al.*, 1994). Enclosure of the rectum is a common practice in Sweden and Norway (Borch *et al.*, 1996), but it is not a standard slaughter routine in Belgium as none of the sampled abattoirs performed this technique. Despite a significant reduction, carcass contamination is not completely eliminated and still remains relatively high (Laukkanen *et al.*, 2010b).

The sternum was the second most frequently contaminated area in this study. This region may get contaminated with faecal material during removal of the intestines and is extensively contacted by hands and equipment of workers during removal of the plug set and the following dressing operations. Differences between high capacity and low capacity abattoirs in sternal *Yersinia* contamination might be explained by differences in slaughter techniques. As such, slaughterhouses with a higher slaughter rate may require more personnel to eviscerate one carcass, which may promote transfer of the pathogen.

Incision of the intestines was observed during evisceration of 16.6% of the sampled carcasses (data not shown), though no significant differences in contamination were found between carcasses with lacerated or intact gut. This may be related to the low presence or low number of human pathogenic *Y. enterocolitica* in different parts of the intestinal tract (Thibodeau *et al.*, 1999; Nesbakken *et al.*, 2003). The caecum may contain up to 10^5 *Y. enterocolitica* per gram (Shiozawa *et al.*, 1991), though a comprehensive set of qualitative and quantitative data on the presence of the pathogen in different segments of the intestinal tract is lacking.

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Chapter 5
Correspondence between serology of meat juice and bacteriology of tonsils and faeces for the detection of enteropathogenic *Yersinia* spp. in pigs at slaughter

Abstract

The aim of the present study was to determine the association between serology and *Yersinia* detection by bacterial culture in individual pigs at slaughter.

Diaphragm muscles, tonsils, and faecal samples were collected from 370 fattening pigs during slaughter. Detection of anti-*Yersinia* antibodies in meat juice samples was done using an indirect enzyme-linked immunosorbent assay (ELISA) based on Yops (*Yersinia* outer proteins). Tonsils and faeces were tested for the presence of enteropathogenic *Yersinia* spp. by direct plating on cefsulodin-irgasan-novobiocin (CIN) agar plates.

Out of the 370 meat juice samples, 222 (60.0%) gave a positive serological reaction using a cut-off value of 30%. Enteropathogenic *Yersinia* spp. were found in the tonsils and faeces of 161 and 30 pigs, respectively. Recovery of enteropathogenic yersiniae from the tonsils was highly correlated with positive serotiters, whereas no correlation was found with faecal excretion.

Results demonstrated that serology has an acceptable sensitivity, but a relatively low specificity for the rapid detection of enteropathogenic *Yersinia* spp. in tonsils of pigs at slaughter.

Keywords: enteropathogenic *Yersinia* spp., serology, meat juice, Yops.

1. Introduction

Enteropathogenic *Yersinia* spp. (pathogenic types of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) are foodborne pathogens that primarily affect the gastrointestinal tract, but have a strong tendency for extraintestinal spread under certain host conditions. All human pathogenic *Yersinia* harbour a virulence plasmid (pYV) that encodes a "*yop* virulon" which consists of a type III secretion system and *Yersinia* outer proteins (Yops).

Yersinia enterocolitica is mainly transmitted to humans through the consumption of contaminated pork. A high proportion of pigs at slaughter are infected with enteropathogenic *Yersinia* spp. in their tonsils and intestines, and pathogens may spread to the carcass during slaughter and dressing operations. A major difficulty is that pigs carry *Yersinia* spp. without showing any symptoms. This results in the fact that infected pigs can not be identified by current meat inspection methods (EFSA, 2011).

To ensure an effective control of the main biological hazards in pig carcasses, preventive measures and controls should be applied both on-farm and at abattoir level (EFSA, 2011). As carcass contamination with pathogenic *Y. enterocolitica* depends on the contamination status of pigs delivered to the abattoirs (Chapter 4), incoming pig batches should be differentiated with respect to the risk they pose for public health (EFSA, 2011). Although bacteriological monitoring of tonsils provides the best indication for the identification of *Yersinia* infections in slaughter pigs, conventional culture methods are time-consuming and labour-intensive (Fredriksson-Ahomaa and Korkeala, 2003). In contrast, serological methods for the screening of antibodies are more convenient and faster alternatives compared to bacteriological examination on the condition that the correlation between antibody titres and culture is high. Hence, the current study was conducted to assess the agreement between serological and bacteriological tests at animal level under field conditions. Accordingly, *Yersinia* antibodies in meat juice and the presence of enteropathogenic *Yersinia* spp. in tonsils and faeces of pigs at slaughter were determined to estimate the relative sensitivity and specificity of the serology test for predicting the presence of *Yersinia* in slaughter pigs.

2. Materials and Methods

2.1. Serological and bacteriological analyses

Tonsils, faeces, and ten gram of the diaphragm muscle of 370 pig carcasses, originating from 194 farms (1 - 14 pigs per farm), were sampled in ten pig abattoirs in Belgium and transported to the laboratory under refrigerated temperatures. Diaphragm muscles were frozen (-20°C) immediately upon arrival for 24h and then thawed at 4°C for 24h, after which the meat juice was collected and stored at -20°C until analysis. Samples were examined for antibodies against pathogenic *Yersinia* using YOPSCREEN Pig ELISA (Labor Diagnostik, Leipzig, Germany) according to the manufacturer's instructions. The presence of antibodies against Yops (*Yersinia* Outer Proteins) was determined by measuring the optical density (OD) at 450 nm. Activity values (OD%) were calculated based on

OD values, relative to the mean OD values of positive and negative controls. An OD% ≥ 30% was used as cut-off value for a positive result. Tonsils and faeces were examined for enteropathogenic *Yersinia* spp. by direct plating on cefsulodin-irgasan-novobiocin (CIN) agar plates and isolates were confirmed using PCR assays as described earlier. In short, *Y. enterocolitica* isolates were confirmed by a virulence and serotype PCR assay as described in Chapter 3. *Y. pseudotuberculosis* isolates were confirmed using a PCR assay targeting the *inv* gene as described in Chapter 4.

2.2. Statistical analyses

Statistical analyses were performed using Stata/MP 12.1 (StataCorp, 2011). The association between serology and culture results was determined using a logistic regression, including farm as random effect. Based on bacteriological results of tonsils and faeces, animals were classified in four different groups (group 1: tonsils and faeces negative, group 2: tonsils negative and faeces positive, group 3: tonsils and faeces positive, and group 4: tonsils positive and faeces negative). The seroprevalences within these groups was compared using a logistic regression. The diagnositic sensitivity and specificity of the ELISA test were estimated relative to the culture of pathogenic *Yersinia* spp. from the tonsils and faeces as gold standard.

3. Results

A histogram of the activity values (OD%) showed a bimodal distribution of the serological data (Figure 11). In total, 222 pigs (60.0%) were seropositive based on a cut-off value of 30%. Thirty-three samples (8.92%) had an activity value between 10% and 20%, and 19 samples (5.1%) between 20 and 30%.

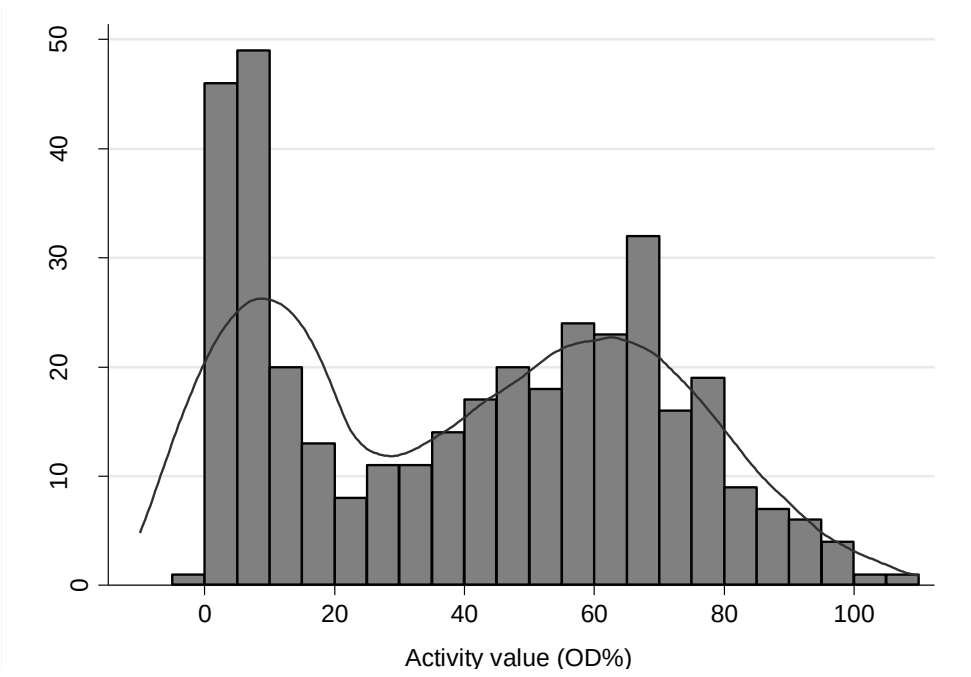

Figure 11. Distribution of activity values (OD%) of meat-juice samples in 370 pigs at slaughter.

In total, enteropathogenic *Yersinia* spp. were recovered from 161 tonsils (43.5%) and 30 faecal samples (8.1%). Overall, 171 pigs (46.2%) carried enteropathogenic *Yersinia* spp. in the tonsils and/or excreted them in the faeces. The number of pigs showing serological evidence of infection was significantly higher ($p < 0.001$), with 222 meat juice samples (60.0%) giving a positive reaction (Figure 11).

Table 20. Bacteriological and serological results of 370 pigs at slaughter.

	Bacteriology[a]			Serology (cut-off 30%)		
Group	Tonsils	Faeces	*n*[b]	Positive	Negative	%[c]
1	Negative	Negative	199	74	125	37.19
2	Negative	Positive	10	5	5	50.00
3	Positive	Positive	20	14	6	70.00
4	Positive	Negative	141	129	12	91.49
	Total		370	222	148	60.00

[a] presence of enteropathogenic *Yersinia* spp. by direct plating ; [b] number of samples ; [c] seroprevalence within each group.

From the 161 pigs that carried *Yersinia* in the tonsils, 143 (88.8%) tested serologically positive and 18 pigs (11.2%) tested negative (Table 20). Out of the 30 pigs that excreted *Yersinia* in the faeces, 19 (63.3%) were seropositive and 11 (36.7%) were seronegative. Seventy-four of the tested pigs (20.0%) were serologically positive but showed no evidence of a current infection based on bacteriological results. A strong positive association was found between serology and the presence of *Yersinia* in tonsils ($OR_{adjusted}$ = 39.76; 95%CI: 13.73-115.13; $p < 0.001$) whereas no relation was found for rectal samples ($OR_{adjusted}$ = 0.40; 95%CI: 0.11-1.47, $p = 0.169$). The specificities and sensitivities for serology relative to bacteriological examination of tonsils and faeces are shown in Table 21.

Table 21. Sensitivity and specificity of serology based on bacteriological results of tonsils, faeces, or both.

Matrix	Sensitivity	Specificity	Correctly classified
Tonsils	88.8	62.2	73.8
Faeces	63.3	40.3	42.2
Both[a]	86.6	62.8	73.8

[a] both: tonsils, faeces, or at least one test is positive.

4. Discussion

Several factors may influence **serological results**, such as the applied antigen, cut-off value and sample type.

The ELISA that is used here contains **Yop antigens**, so that both human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* antibodies are detected. Contrarily, ELISA assays using specific antigens against *Y. enterocolitica* serotype O:3 (Nielsen *et al.*, 1996) may detect antibodies against the most common pathogenic serotype in pigs, but not the more rare serotypes and *Y. pseudotuberculosis*.

The distribution of the OD% values showed a bimodal distribution with modes at about 10% and 60%, which may represent the seronegative and seropositive population, respectively. If so, this distribution suggests that the **cut-off value** for the ELISA test should be between 20 and 30%, and indicates that the proposed cut-off value of 30% corresponds with the field data.

Muscle fluid is a faster and easier alternative to **serum** for the detection of antibodies. Nielsen *et al*., (1998) found that meat juice is a good alternative to serum for the detection of *Salmonella* antibodies in finishing pigs during slaughter whereas Vico and Mainar-Jaime (2011) described a low correlation between both sample matrices. Moreover, the release of muscle fluid may be influenced by many factors, including the level of preslaughter stress, the residual content of blood in the tissues, and the presence of fascias in the tissues (Nielsen *et al.*, 1996).

Besides test-related factors, **biological aspects** such as age of the pigs and prevalence differences (*e.g.* between regions and farms) may also influence the serological outcome. Nevertheless, despite many influencing factors, the proportion of slaughter pigs with *Yersinia* antibodies are usually very similar among different studies as the seroprevalence found here (65.1%) agrees with the 66% in Canada (Thibodeau *et al.*, 2001) and 64% found in fattening pigs just before or during slaughter in Germany (von Altrock *et al.*, 2011).

In contrast to **bacteriological examination**, a serological reaction is a historical measure of infection. Pathogenic *Y. enterocolitica* can be recovered from tonsils and intestines within hours after oral inoculation of pigs (Thibodeau *et al.*, 1999), whereas seroconversion is observed after 12 to 19 days of experimental infection (Nielsen *et al.*, 1996). Similarly, Nesbakken *et al*. (2006) followed pigs on two *Yersinia* positive farms and observed that contamination of tonsils and faeces started around the same time whereas seroconversion occurred about two weeks later. In the present study, 6.22% (23/370) of the tested pigs were serologically negative while they carried and/or excreted pathogenic *Yersinia* in the tonsils and faeces, respectively, which may indicate a recent infection. Initial infection may have taken place on-farm during the final stage of the fattening period, but also during transport or lairage at the abattoir. The average lairage time for all pigs sampled was approximately 3 hours (data not shown), which is sufficiently long for *Yersinia* to colonize the tonsils (Thibodeau *et al.*, 1999). Nevertheless, our data indicate that presumably only a minority (6.2%) of the pigs are infected within the two weeks before slaughter.

The biological difference between a 'historical' serological reaction and the actual presence of pathogens automatically results in dissimilarities between both tests. Nevertheless, when serology and bacteriology were compared for detection of enteropathogenic yersiniae in fattening pigs at the time of slaughter, a high association was found between seropositivity and isolation of *Yersinia* from the tonsils. Nevertheless, despite the high correlation that was found between positive tonsil culture and serological findings, several animals would be misclassified during slaughter using serology testing. This misclassification is mainly the result of false positive results due to the low diagnostic specificity of the test. Although some animals may test negative for enteropathogenic yersiniae in the tonsils after a certain period, the serological reaction remains positive for at least 70 days after infection (Nielsen *et al.*, 1996).

The results of the present study indicate that there is no correlation between serology and faecal *Yersinia* excretion at time of slaughter. Similarly, von Altrock *et al*. (2006) analysed blood and faeces from 900 pigs in 30 fattening pig herds shortly before slaughter and no correlation was found between serological and bacteriological results. Pigs can carry the pathogen in tonsils for several months whereas faecal excretion declines more rapidly (Nielsen *et al.*, 1996; Virtanen *et al.*, 2012). Nevertheless, the time period after experimental infection in which *Yersinia* are detectable in the faeces is influenced by the isolation method used, and possibly results from a decline in numbers over time (Nielsen *et al*., 1996). As only direct plating was used in the present study, only animals excreting higher numbers of *Yersinia* in the faeces were detected, which may indicate that they were relatively more recently infected. Recently infected animals are less likely to have seroconverted, which might partly explain the low diagnostic sensitivity of the serological test to detect excretion of enteropathogenic *Yersinia* spp. in the faeces. This is further supported by the changing seroprevalence over the different groups. The increase in seroprevalence over the different groups suggests that, in general, pigs under field conditions initially excrete *Yersinia* in the faeces. Then, *Yersinia* is present in the faeces and tonsils. Finally, the tonsils remain positive for a certain period of time.

In conclusion, serology of meat juice of pigs at slaughter correlates well with the carriage of pathogenic *Yersinia* in tonsils, whereas serological findings show no relation with *Yersinia* excretion in the faeces. The meat juice ELISA can thus be used as an indicator for *Yersinia* carriage at individual level, though the low specificity may result in many false positive reactions.

General discussion

Slaughter pigs are the major reservoir for human pathogenic *Y. enterocolitica* and pork is responsible for about 70% of human yersiniosis cases. With the purpose of reducing the number of human infections, establishment of effective measures to control *Y. enterocolitica* in the pork production chain requires a better understanding of the epidemiology of the pathogen. In order to obtain reliable results on the occurrence of pathogenic *Y. enterocolitica* throughout the production chain, adequate testing methods are necessary.

At the start of this thesis, information about the current situation of enteropathogenic *Yersinia* spp. in pigs and pork in Belgium was limited. During this dissertation, both qualitative and quantitative data were gathered about the presence of these pathogens in pork from slaughter to retail, *i.e.* pigs at slaughter (tonsils (Chapters 1, 2 and 4) and faeces (Chapter 4)), carcasses (Chapter 3 and 4), and minced meat (Chapter 3). Special attention was paid to different sampling and isolation methods for the recovery of human pathogenic *Y. enterocolitica* from pig and pork matrices, and revealed several limitations of standard isolation procedures (Chapters 1-3). Moreover, data were gathered about the spread of enteropathogenic yersiniae during evisceration of pig carcasses through the identification of risk factors for carcass contamination (Chapter 4). Finally, serology of meat juice collected during slaughter was evaluated as an alternative to bacteriological testing (Chapter 5).

Sampling, isolation and identification of enteropathogenic *Yersinia* spp.

Obtaining reliable results is fundamental to gain a better insight in the epidemiology of enteropathogenic *Yersinia* spp. in pigs and pork. Both sampling and isolation methods were demonstrated to influence the final outcome of contamination of different porcine matrices.

Sampling

In Chapter 2, **destructive samples** were significantly better than swab samples to recover human pathogenic *Y. enterocolitica* from **pig tonsils**. Although swab samples are easier and faster than destructive samples, homogenization of tissue samples is required to obtain reliable results, most likely because the pathogens are not at the surface of the tonsil but inside the crypts. On pig carcasses, human pathogenic *Y. enterocolitica* may also be located in deeper tissue structures of the muscles and skin (Morild *et al.*, 2011), though swabbing the surface is the standard procedure here as damaging the carcass is detrimental.

For carcasses, the contamination rate varied widely between **different areas** of the **carcass** (Chapter 4). The high overall proportion of positive carcasses in Chapter 4 (40%) was primarily attributed to the high contamination rate of the mandibular region (29%), whereas the prevalence of the pelvic duct, split surface, and sternum was significantly lower (varying between 7% and 16%). Even though the number of isolation methods was higher (see further) and the carcass area that was sampled was larger, the prevalence estimate in Chapter 3 was lower than in Chapter 4 (Table 22), which could be (partly) explained by the absence of the highly contaminated mandibular region in the former study. Similarly, Laukkanen *et al*. (2010b) and Nesbakken (1988) also found the highest number of carcasses contaminated at the chest and

head region. Therefore, inclusion of the head area when analysing carcasses for the presence of *Y. enterocolitica* might result in a considerable increase of the overall carcass prevalence. Nevertheless, two areas that were sampled (the pelvic duct and sternum) were similar in Chapter 3 and 4. Combining the results from both regions from Chapter 4 would result in 20% positive carcasses, which is higher than the proportion obtained in Chapter 3. Therefore, other factors such as the study design and person-to-person variation in swabbing may also influence results. Furthermore, the use of pooled samples in Chapter 3 compared to individual samples in Chapter 4 may have decreased the number of positive carcasses due to a larger background flora that may be present in pooled samples.

Table 22. Carcass contamination with pathogenic *Y. enterocolitica* in Belgium.

Study	N	P	Isolation methods	Sampled sites
Chapter 3[a]	254	0.114	PSB (25°C, 2,5d) +/- KOH + CIN ITC (25°C, 2d) + SSDC/CIN PMB (4°C, 7,14d) +/- KOH + CIN	Inner side of the ham; pelvic duct; sternum and neck along the incision line; foreleg (600 cm²)
Chapter 4[b]	360	0.397	Direct CIN ITC (25°C, 2d) +/- KOH + CIN PMB (4°C, 7,14d) (+KOH) + CIN	Pelvic duct; split surface; sternum; mandibular region (400 cm²)

N, number of samples; P, proportion of positive samples.
[a] samples taken in the context of the official monitoring program; [b] samples taken to determine risk factors for carcass contamination during slaughter.

Isolation of human pathogenic Y. enterocolitica

As **pig tonsils** are often highly contaminated, isolation of *Y. enterocolitica* from these tissues is generally straightforward. In fact, **direct plating** is a fast and easily applicable method and shows a good efficiency for the recovery of *Y. enterocolitica* from pig tonsils (Chapters 1 and 2). As direct plating resulted in a similar percentage of positive pigs tonsils than the standard ISO 10273 method (Chapter 1), EFSA also advises direct plating for monitoring purposes of *Y. enterocolitica* in pig tonsils (EFSA, 2009).

Contrary to pig tonsils, testing of **pig carcasses and pork** requires more sensitive (enrichment) methods as the quantitative contamination level is usually low (Chapter 4). To detect pathogenic *Y. enterocolitica* in food samples, the **ISO 10273:2003 method** is the reference method worldwide. Nevertheless, this method has been shown **ineffective** to detect human pathogenic *Y. enterocolitica* from artificially and naturally contaminated samples (Chapters 1 and 3) (Laukkanen *et al.*, 2010a; Thisted Lambertz *et al.*, 2007; Fredriksson-Ahomaa *et al.*, 2008). The ISO method prescribes incubation of PSB for five days, whereas under continuous agitation, PSB should be incubated for two days. Nevertheless, for the isolation of pathogenic *Y. enterocolitica* from pig tonsils, carcass swabs, and minced meat, shortening the incubation period of PSB from five to two days (without agitation) resulted in considerably higher recovery rates (Chapters 1 and 3). Based on results from Chapter 3, 3% of pig carcasses and 0% of minced meat samples was positive using the ISO 10273:2003 method, whereas the shortened method resulted in an

increase of the prevalence estimate to 10% and 4%, respectively. The ISO method is used in the frame of the official monitoring program in Belgium and thus results in an underestimation of the true number of positive samples. The ISO 10273:2003 method is currently under revision, and a revised version can be expected in the near future.

Isolation of Y. pseudotuberculosis

No standard isolation methods are currently described for the isolation of *Y. pseudotuberculosis*. The isolation methods used for *Y. enterocolitica* are also used for *Y. pseudotuberculosis*, but are generally insufficient for the latter species as selective enrichment is inefficient for the recovery of this species (Laukkanen *et al.*, 2008; Niskanen *et al.*, 2002), and CIN agar has an inhibitory effect on the growth of some *Y. pseudotuberculosis* strains (Savin *et al.*, 2012). Several samples that were positive by direct plating (indicating a high contamination level) were negative after selective enrichment (Chapter 2 and unpublished results), which confirms the low efficiency of selective enrichment. The recovery of *Y. pseudotuberculosis* from pig samples requires the use of cold enrichment at 4°C for several weeks (Niskanen *et al.*, 2002), which is also the method by which we found most positive *Y. pseudotuberculosis* samples (unpublished results). *Y. pseudotuberculosis* was not recovered from pig tonsils using direct plating by Niskanen *et al*. (2002), though we found four out of the six positive tonsils using this method, with quantitative levels similar to those obtained for pathogenic *Y. enterocolitica* (Chapters 2 and unpublished results). All of these findings suggest that the prevalence of *Y. pseudotuberculosis* in animal and food samples is largely underestimated when the traditional methods for human pathogenic *Y. enterocolitica* are used.

Selection of the appropriate colonies

A major difficulty encountered during conventional culture of pathogenic *Yersinia* is the difficulty of selecting suspected colonies, particularly in the presence of a large amount of background flora. Pathogenic ***Y. enterocolitica*** have typically a bull's eye appearance on CIN agar, though they are small (< 1mm after 24h of incubation at 30°C) and many other bacteria may have a rather similar morphology, so that differentiation requires experience. Moreover, in combination with abundant accompanying flora, the pathogens may not present as the typical colonies, but as small colourless colonies (Figure 12). The use of a stereomicroscope with oblique illumination facilitates and is often required for the detection of suspected colonies, and should be combined with a thorough training in colony selection to obtain reliable results. Nevertheless, although a stereomicroscope with oblique illumination is indispensable, it is not used in most routine laboratories.

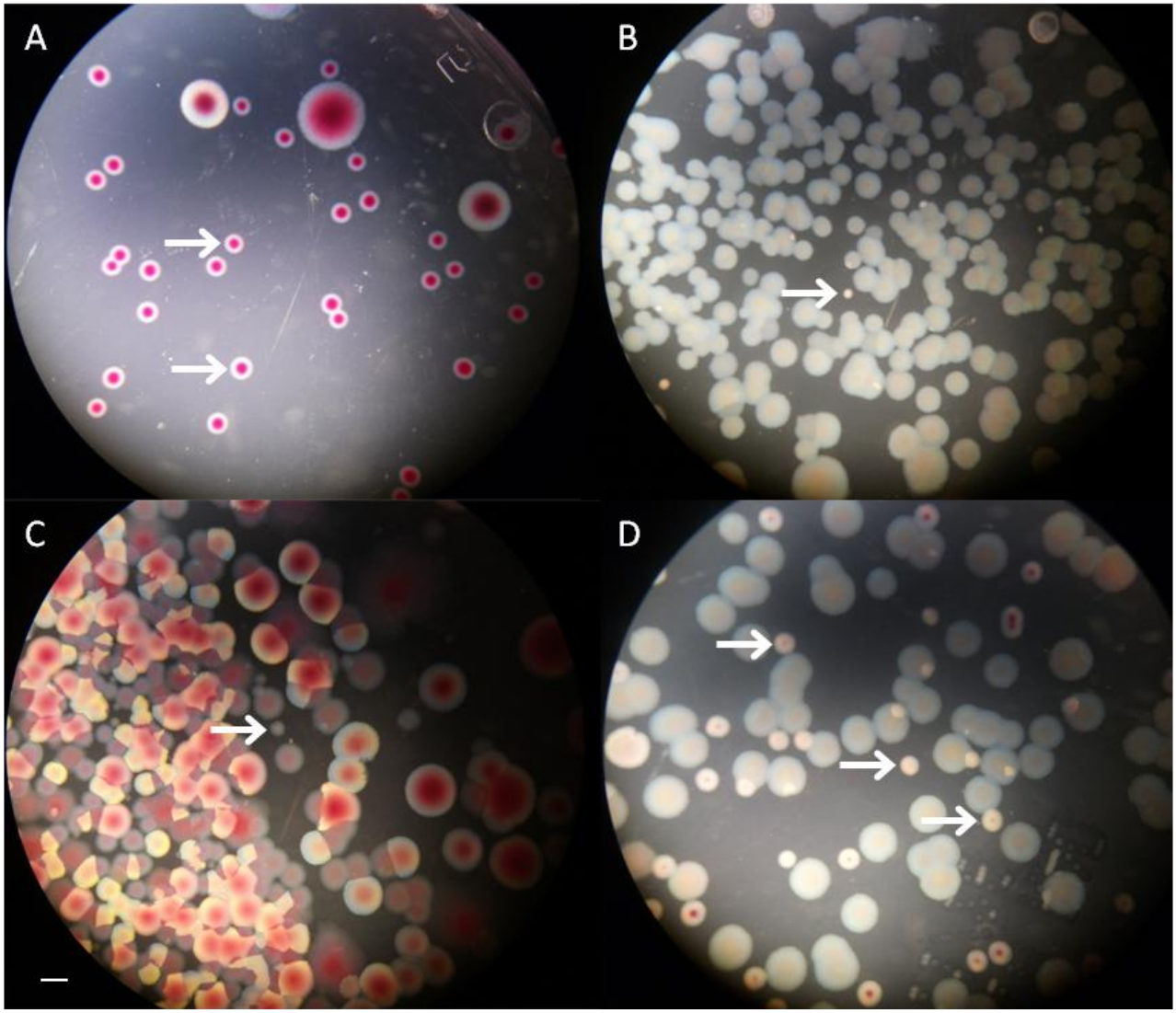

Figure 12. Colony morphology of *Y. enterocolitica* serotype O:3 (white arrows) in the presence of background flora after 24 h incubation at 30°C on CIN agar plates (stereomicroscopic view).
A: typical bull's eye appearance with limited background flora; B, C, and D: non-typical appearance due to the presence of abundant background flora. The white bar represents 1 mm.

Similar to *Y. enterocolitica*, the 'recognition' of suspected ***Y. pseudotuberculosis*** isolates is difficult. After 24h incubation at 30°C, *Y. pseudotuberculosis* colonies appear as white pinpoint colonies on CIN agar (Figure 13), and are easily overlooked. Therefore, an additional incubation for 24h at room temperature is proposed for the detection of *Y. pseudotuberculosis*. Nonetheless, the majority of *Y. pseudotuberculosis* positive samples in this thesis were obtained after 24h of incubation of the CIN agar plates (data not shown). After 48h, *Y. pseudotuberculosis* colonies are larger, though a concurrent growth of accompanying flora is inevitable, which may complicate the isolation.

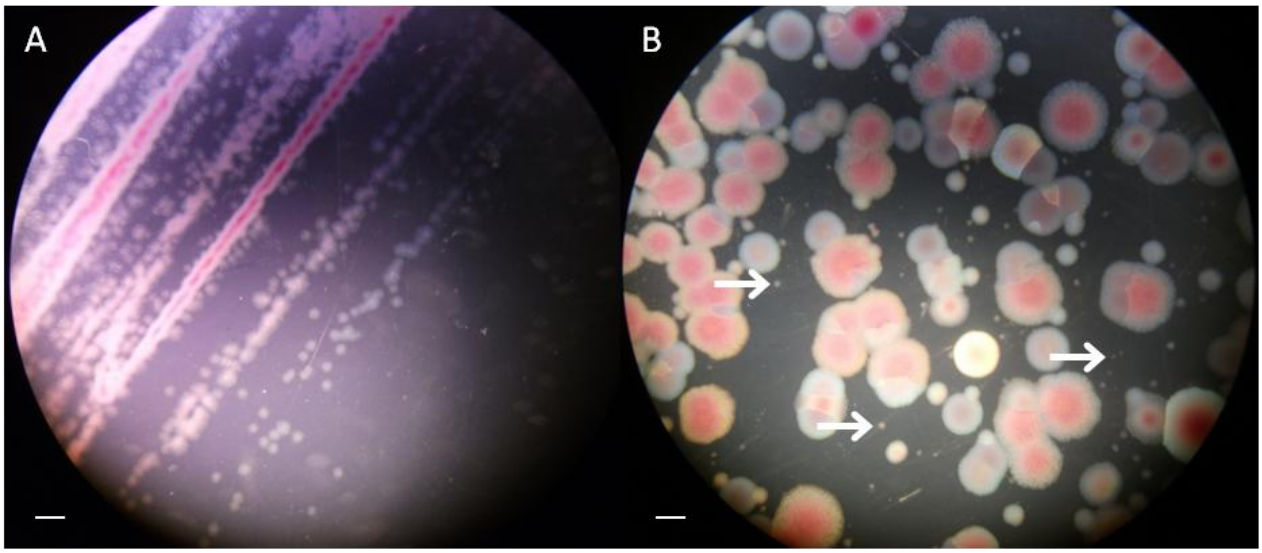

Figure 13. Colony morphology of *Y. pseudotuberculosis* after 24 h incubation at 30°C on CIN agar plates in pure culture (A) and in the presence of background flora (B) (stereomicroscopic view).
Y. pseudotuberculosis forms colourless pinpoint colonies and are indicated with white arrows. The white bar represents 1 mm.

Y. pseudotuberculosis is not frequently isolated from humans, animals, food and the environment. Besides the limited occurrence, the low reported isolation rates might also be related to the difficulties associated with isolation of this pathogen. Moreover, this underestimation might be more pronounced in samples where the numbers of pathogens are low and the presence of high background flora, such as carcasses and minced pork.

Identification

Y. enterocolitica isolates are traditionally subdivided in six biotypes. Biotyping of *Y. enterocolitica* isolates is associated with a specific difficulty, being the differentiation between biotype 2 and biotype 3, which is based on the difference in indole production (Table 8), that is respectively weak and absent. When using standard indole tests in biotyping, stains of biotype 2 are frequently regarded as indole negative and misidentified as biotype 3. The positive identification of weak indole producers requires a more sensitive method to differentiate weak producers from true negative strains by using a heavy suspension and the addition of tryptophan to the test medium (Wauters and Vaneechoutte, 2011).

The pYV plasmid is found in pathogenic biotypes of *Y. enterocolitica* and *Y. pseudotuberculosis*, and is sometimes used as the single criterion to determine the pathogenicity of a strain. However, as the plasmid can be lost during repeated laboratory cultivation, this may lead to false-negative results. Loss of the virulence plasmid during cultivation is more common at 37°C, whereas the plasmid is usually retained at temperatures below 32°C (Goverde *et al.*, 1994; Li *et al.*, 1998). Nevertheless, despite incubation temperatures below 30°C, plasmid negative isolates are rather frequently recovered from pig tissues as the proportion of plasmidless *Y. enterocolitica* isolates in tonsils of pigs at slaughter is usually about 20% (Ortiz Martínez *et al.*, 2011; Van Damme *et al.*, 2013; Ortiz Martínez *et al.*, 2009). Moreover, the present study showed that these

plasmid-negative isolates are not randomly distributed and are more common in certain samples than in others (unpublished results; Van Damme *et al*. (2013)). This observation indicates that plasmidless findings may not be explained by a random loss during laboratory cultivations only and suggests that the plasmid may be absent *in vivo* under certain conditions or that other unknown factors trigger plasmid loss. Therefore, further research is needed to clarify the presence of plasmidless isolates in pig tissues and to elucidate factors influencing their recovery.

At 37°C, pYV-carrying strains grow slower than their plasmid-cured derivates, whereas the growth characteristics are generally very similar at temperatures of 20-25°C (Goverde *et al.*, 1994; Logue *et al.*, 2006; Sheridan *et al.*, 1998). Throughout this thesis, no clear differences were observed in plasmid-carriage between the applied isolation methods. In contrast, Laukkanen *et al*. (2010a) found most plasmid-negative isolates in pig intestines after cold enrichment. Variations in the recovery of plasmid-carrying isolates between studies may be explained by differences in the subjective selection of 'typical' colonies. Occasionally, larger colonies with a somewhat translucent border were seen after cold and selective enrichment (Figure 14). When such colonies were observed during this thesis, both typical and aberrant colonies were tested, the latter being generally more likely to be plasmid-negative. Therefore, selection of colonies of different morphology (including smaller colonies) may reduce the number of plasmid-negative samples (Van Damme *et al.*, 2013). Nevertheless, as in many cases both plasmid-carrying and plasmid negative isolates can be recovered from the same sample (Chapter 2; Fredriksson-Ahomaa *et al*. (2000b); Laukkanen *et al*. (2010a); Van Damme *et al*. (2013)), a sufficient number of isolates should be tested for the presence of the virulence plasmid when only plasmid-carrying isolates are considered pathogenic.

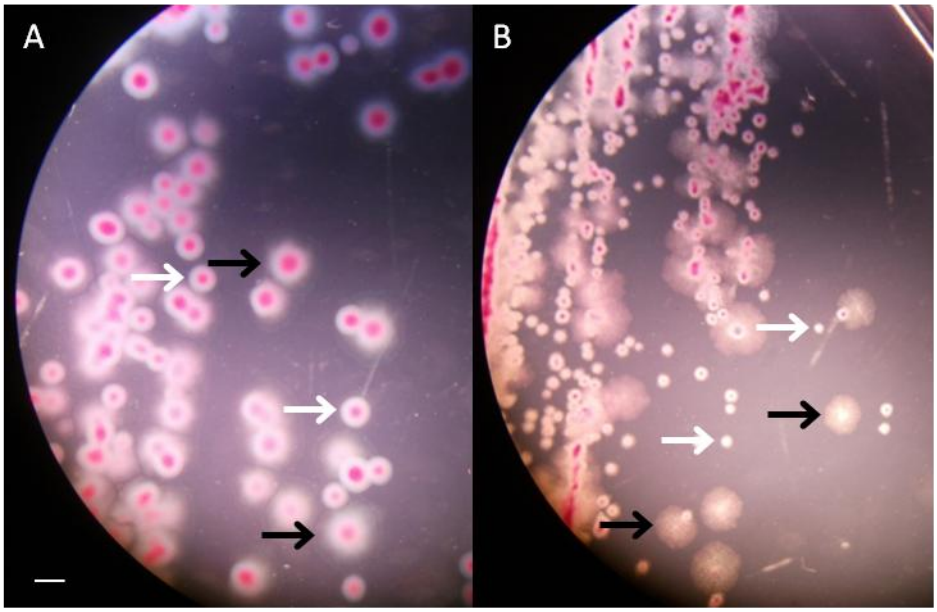

Figure 14. Stereomicroscopic view of typical (white arrows) and 'large' (black arrows) *Y. enterocolitica* (A) and *Y. pseudotuberculosis* (B) colonies on CIN after 24h at 30°C.

Occurrence of enteropathogenic *Yersinia* spp. along the Belgian pork production chain

Table 23 gives an overview of the isolations of enteropathogenic *Yersinia* throughout this thesis. As discussed above, the final outcome of the studies is influenced by different factors, including differences in sampling, isolation methodologies, and study design. An overall decreasing trend was seen in the contamination along the pork production chain, which can be partly explained by the difficulties that are associated with isolation from the latter, more 'complex' samples (Fredriksson-Ahomaa *et al.*, 2004).

Table 23. Isolation of enteropathogenic yersiniae from pigs and pork throughout this thesis.

	Pigs[a]			Pig carcasses			Minced meat		
	n	Positives	Proportion	n	Positives	Proportion	n	Positives	Proportion
Chapter 1[b]	139	52	0.374			-			-
Chapter 2[b]	120	56	0.467			-			-
Chapter 3[b]			-	254	29	0.114	82	4	0.049
Chapter 4[c]	360	234	0.650	360	143	0.397			-
Total	619	342	0.553	614	172	0.280	82	4	0.049

[a] Chapter 1 and 2: tonsils; Chapter 4: tonsils and faeces; [b] samples taken to compare different isolation methods; [c] samples taken to determine risk factors for carcass contamination.

Since tonsils are not for human consumption, the risk they pose for public health only relies in the potential source for contamination of pig carcasses and edible offal with foodborne pathogens. As shown in Chapters 1 and 2, *Y. enterocolitica* positive tonsils are in the majority of cases highly contaminated and these highly contaminated tonsils may thus be more important for contamination of carcasses than lowly contaminated samples. Therefore, tonsils positive by direct plating are suitable for epidemiological studies of human pathogenic *Y. enterocolitica* in pig tonsils at abattoir level. The prevalence of pathogenic *Y. enterocolitica* in pig tonsils ranged between 37 and 55% over the different studies (Table 23). However, when only the data obtained by direct plating of the destructive method are taken into account, the prevalence over the three studies varied less (from 36 to 43%).

Quantitative data on the contamination of pig carcasses with human pathogenic *Y. enterocolitica* are limited, but results from Chapter 4 showed that the contamination level of pig carcasses is generally low. The infectious dose in humans is not clearly demonstrated, but is expected to be high. Nevertheless, even small numbers of *Yersinia* on carcass surfaces may pose a risk for public health because of the potential growth to high numbers, even when packaged under modified atmosphere during refrigerated storage (Fredriksson-Ahomaa *et al.*, 2012b).

Table 24. Distribution of enteropathogenic yersiniae in humans, pigs and pork in Belgium during 2009-2011.

	Pigs (tonsils)[a]		Pig carcasses[b]		Minced pork[c]		Human cases[d]	
Species/bioserotype	n	%	n	%	n	%	n	%
Y. enterocolitica bioserotype 4/O:3	306	98.08	171	98.84	4	100.00	654	90.08
Y. enterocolitica bioserotype 2/O:9	0	0.00	1	0.58	0	0.00	50	6.89
Y. enterocolitica (other)	0	0.00	0	0.00	0	0.00	6	0.83
Y. pseudotuberculosis	6	1.98	1	0.58	0	0.00	16	2.20
Total (enteropathogenic yersiniae)	312	100.0	173	100.00	4	100.00	726	100.00

[a] number of positive samples based on results of Chapter 1, 2, and 4; [b] based on results from Chapter 3 and 4; [c] based on results from Chapter 3; [d] number of reported human yersiniosis cases during 2009-2011 based on WIV (2012).

Y. enterocolitica serotype O:3, which is the most frequent serotype associated with human yersiniosis cases, was also the dominant human pathogenic *Yersinia* type recovered from pigs and pork during this thesis (Table 24). Nevertheless, the relative proportion of 'other' pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* (slightly) differed between pigs, pork, and humans.

As indicated above, methodological problems may contribute to the observed differences in bioserotype and species distribution between matrices. Moreover, enrichment methods and selective agar plates have been shown to favour particular strains and inhibit others (Savin *et al.*, 2012; De Zutter *et al.*, 1994). In humans with acute clinical symptoms, pathogenic *Yersinia* may be the predominant bacteria, whereas in carcass swabs and meat samples the concentration of pathogenic *Yersinia* is generally low (Chapter 4), requiring enrichment and selective agar media.

Y. enterocolitica bioserotype 2/O:9 was isolated from one pig carcass in Chapter 3, whereas from pig tissues, no *Y. enterocolitica* other than bioserotype 4/O:3 were found. Contrarily, Ortiz Martínez *et al*. (2011) found that 9% of all *Y. enterocolitica* isolates obtained from slaughter pig tonsils collected in Belgium belonged to bioserotype 3/O:9. However, all bioserotype 3/O:9-positive pigs originated from one out of the ten farms that were tested (Ortiz-Martinez, personal communication). This indicates that 'other' serotypes (particularly serotype O:9) are overall rare in Belgian slaughter pigs, but they may be prevalent on certain pig farms. Although *Y. enterocolitica* bioserotype 4/O:3 is generally the utter most frequently type in pigs at slaughter, in some countries, other bioserotypes predominate (McNally *et al.*, 2004; Ortiz Martínez *et al.*, 2010). Therefore, import of contaminated pig carcasses and pork (products) from certain countries may result in infections with 'other' bioserotypes. Finally, although pigs are assumed to be the main reservoir for human pathogenic *Y. enterocolitica*, other sources might also account for a number of human infections. Especially cattle and sheep are occasionally associated with *Y. enterocolitica* serotype O:9 as it may be recovered after false-positive serological reactions for brucellosis due to cross-reacting LPS (Chenais *et al.*, 2012; Gourdon *et al.*, 1999). Nevertheless, data regarding the presence of human pathogenic *Y. enterocolitica* in the environment, animals

or foodstuff other than pork in Belgium are scarce (Najdenski *et al.*, 2012), which hampers the study of source attributions in Belgium.

Domestic pigs are not assumed to be the main reservoir for ***Y. pseudotuberculosis***, as this species is also found in many environments and animals (Fredriksson-Ahomaa *et al.*, 2011; Han *et al.*, 2003). Nevertheless, the ratio of *Y. pseudotuberculosis* relative to *Y. enterocolitica* in pigs was similar to the proportion in human infections (Table 24). Overall, *Y. pseudotuberculosis* serotypes O:1 and O:2 were found in 1.29% of pigs throughout this thesis. Ortiz Martínez *et al*. (2011) found *Y. pseudotuberculosis* serotypes O:1, O:2, and O:3 in the tonsils of 5 pigs at slaughter (5/201, 2%) in Belgium, which originated from 8 (*sic*) out of 10 farms tested (80%). The prevalence of *Y. pseudotuberculosis* has been shown to be higher in organic than in conventional pig farms (Laukkanen *et al.*, 2009). In England, a *Y. pseudotuberculosis* prevalence as high as 18% has been found in slaughter pig tonsils, which might be related to differences in pig farming as British pigs are often raised outdoors (Ortiz Martínez *et al.*, 2010). Since the number of organic pig farms and outdoor raised pigs in Belgium are limited, this might explain the lower prevalence of *Y. pseudotuberculosis* in Belgian pigs. Nevertheless, a shift towards more organic production may result in an emergence of different *Yersinia* species and subtypes.

Strategies to reduce *Yersinia* contamination and future perspectives

Meat borne pathogens should to be controlled through a complete, continuous **farm-to-fork** system (Norrung and Buncic, 2008). As pork is responsible for the major part of *Y. enterocolitica* infections in humans, preventive measures throughout the pork industry to reduce contamination of the end product, combined with an increased consumer awareness of enteropathogenic *Yersinia* spp. and pork may be effective means to reduce the number of human yersiniosis cases.

Primary production

As shown in Chapter 4, the main risks for carcass contamination with human pathogenic *Y. enterocolitica* seem to be directly related to the carrier status of **incoming pigs**. Logistic slaughter may be useful to reduce slaughterline contamination though may currently have a limited benefit due to the widespread occurrence of *Yersinia* spp. in Belgian pig farms. Thus, **on-farm interventions** decreasing the number of *Yersinia* positive pigs may be of great value to reduce the percentage of *Yersinia*-positive carcasses which might be challenging due to the prevalent distribution in slaughter pigs. Moreover, the introduction and transmission routes of enteropathogenic yersiniae on pig farms remain largely unknown and further research on its dynamics in pig herds is required before proper control actions may be realized.

As current meat inspection methods fail to identify *Yersinia* infected pigs, **risk categorisation of pig farms** might be helpful to control *Yersinia* (EFSA, 2011). To overcome the problems associated with bacterial culture, **serology of meat juice** samples was tested as an alternative to bacterial culture for the identification of *Yersinia* positive pigs (Chapter 5). Serology may be used for the rapid screening for positive animals as it had a satisfactory sensitivity to detect bacteriological

positive pigs, though the diagnostic specificity was low. Nonetheless, serology might be a promising tool to determine the *Yersinia* infection status of a herd as the determination of antibodies in meat juice has the advantage of being more practical and time-saving than bacteriological analyses. Whether this is a good method for detection at herd-level and whether there is a stable result over different herds from one farm needs to be investigated.

Salmonella and *Y. enterocolitica* are indicated by EFSA (2011) as the main bacterial pathogens related to pork. Several measures have been undertaken to reduce *Salmonella* in pigs, though the effects on the occurrence of enteropathogenic *Yersinia* spp. in Belgian pigs are not known. As von Altrock *et al*. (2011) found a negative correlation between the *Yersinia* seroprevalence and the herd's serological *Salmonella* status in German fattening farms, both pathogens may require different control measures at farm level. Therefore, the introduction of a combined control programme for both pathogens demands further research regarding the association between *Salmonella* and *Y. enterocolitica* in pigs. As a start, the implementation of new strategies to reduce *Salmonella* in multiplying herds may also be evaluated for enteropathogenic yersiniae.

Slaughter and dressing

To control meatborne pathogens throughout the production process, efficient **HACCP**-based process hygiene management systems should be implemented to **limit contamination of carcasses** (Norrung and Buncic, 2008).

The use of a plastic bag after removal of the rectum reduces carcass contamination with human pathogenic *Y. enterocolitica*, and is commonly applied in Norway and Sweden (Borch *et al.*, 1996). Moreover, leaving the tongue (including the tonsils) inside the head during evisceration may also decrease carcass contamination, and is frequently used in France (Denis *et al.*, 2012). Nevertheless, both methods are currently not routinely used in Belgium. Considerable changes in the production process and employment of additional personnel to provide effective reductions of *Y. enterocolitica* are difficult to implement due to the high economical burden. Contrary, the risk factors in Chapter 4 were determined during routine slaughter practices, so that all investigated factors were existing variations of normal slaughter activities in Belgian slaughterhouses. This may be an important step toward potential interventions that are easily applicable at slaughterhouse level.

The identification of risk factors showed that **tonsils** were related to carcass contamination, either directly or indirectly (*e.g. via* incision of the tonsils during plug set removal, cleaning of knives before plug set removal, or splitting of the head) (Chapter 4). In addition to the well-known intestinal role in carcass contamination, slaughterhouse personnel should thus be educated in the importance of tonsils in relation to foodborne pathogens. This also applies further down the production chain, since the presence of (residuals of) tonsils in pig carcasses might also contribute to the high number of human pathogenic *Y. enterocolitica* that are found in meat and the environment in butcher shops (Fredriksson-Ahomaa *et al.*, 2004). Finally, hygienic rules should be respected to avoid contamination as human pathogenic *Y. enterocolitica* have already been detected on slaughtering equipment, such as knives, hooks, air samples, but also on

aprons, gloves, computers and coffeemakers used by abattoir workers and butchers (Fredriksson-Ahomaa *et al.*, 2000b; Fredriksson-Ahomaa *et al.*, 2004; Laukkanen *et al.*, 2010b).

Alternative strategies and enhancements of current **pig slaughter and processing practices** should still be evaluated for their effectiveness in reducing carcass and pork contamination. Different slaughter practices, particularly related to tonsil and head management on carcass contamination should be assessed for enteropathogenic *Yersinia* spp. as well as other pathogens that can be present in pig tonsils (Fredriksson-Ahomaa *et al.*, 2009a; O'Sullivan *et al.*, 2011).

The carcass swabs in Chapter 4 were mostly taken from inner sides of the carcass and not from the skin, so that the contamination that was detected most likely occurred in the clean zone (evisceration, removal of the pluck set, and splitting of the carcass). Nevertheless, information on the occurrence of enteropathogenic *Yersinia* spp. in production steps before evisceration of pig carcasses (**dirty zone**) is lacking. Carcasses may already be contaminated during stunning and sticking, or become contaminated during scalding (Bonardi *et al.*, 2013), dehairing and polishing. Nevertheless, with the exception of polishing, these factors are probably negligible (Bolton *et al.*, 2013).

The designated variations in *Yersinia* contamination of the different carcass areas in Chapter 4 also implies that different **meat cuts** may display different contamination levels and a resulting risk for humans. At the mandibular region, both qualitative and quantitative *Y. enterocolitica* contamination were the highest. As human pathogenic *Y. enterocolitica* on cheek muscles can grow to high numbers during storage (Fredriksson-Ahomaa *et al.*, 2012b), they may thus pose a greater *Yersinia* risk for public health than meat cuts from other areas. Similar to a risk categorisation at slaughterhouse level, in meat cutting plants, risk categorisation of different areas or meat cuts may be used. Meats intended to be consumed raw should preferably be prepared from low risk areas, whereas portions of high risk areas should be heat-treated or undergo a decontamination treatment. Accordingly, head meat should not be used for products that are potentially eaten raw, such as minced meat.

Finally, this thesis provided valuable quantitative and qualitative data to study *Yersinia* in the pork production chain using **quantitative microbiological risk assessment** (QMRA). QMRAs require detailed production step-specific data concerning the same individual animals (Ranta *et al.*, 2010). Based on QMRA, risk managers may decide the **most efficient steps** during pork production to implement control measures.

Consumer level: towards an increased public awareness?

Finally, increased consumer awareness may be effective to decrease the number of yersiniosis cases in humans. Heating results in an adequate reduction of *Yersinia*, so that sufficient cooking of pork and pork products is a suitable preventive measure at consumer level. Nevertheless, raw pork is still frequently consumed, even by young children. As such, Rosner *et al*. (2012) found that 4% of German children younger than 1 year consumed raw minced pork. However, in children with yersiniosis, the percentage of children eating raw minced pork in the 7 days preceding onset of illness was 28% and 35% in the age group of 0-2 years and 2–4 years, respectively.

Additionally, the use of a dummy has been shown a risk factor for yersiniosis (Boqvist *et al.*, 2009), so young parents should be informed regarding the risk of cross contamination during the preparation of raw pork (products). Thus, public awareness about *Y. enterocolitica* and education of consumers about the risks associated with consumption of raw pork products may be effective to reduce yersiniosis in humans. Nevertheless, transfer of scientific knowledge to the public to change consumer behaviour has often limited success.

References

Abe,J., Onimaru,M., Matsumoto,S., Noma,S., Baba,K., Ito,Y., Kohsaka,T., Takeda,T. (1997) Clinical role for a superantigen in *Yersinia pseudotuberculosis* infection. *Journal of Clinical Investigation* **99:** 1823-1830.

Achtman,M., Zurth,K., Morelli,C., Torrea,G., Guiyoule,A., Carniel,E. (1999) *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 14043-14048.

Ackers,M.L., Schoenfeld,S., Markman,J., Smith,M.G., Nicholson,M.A., DeWitt,W., Cameron,D.N., Griffin,P.M., Slutsker,L. (2000) An outbreak of *Yersinia enterocolitica* O:8 infections associated with pasteurized milk. *Journal of Infectious Diseases* **181:** 1834-1837.

Andersen,J.K. (1988) Contamination of freshly slaughtered pig carcasses with human pathogenic *Yersinia enterocolitica*. *International Journal of Food Microbiology* **7:** 193-202.

Andersen,J.K., Sorensen,R., Glensbjerg,M. (1991) Aspects of the epidemiology of *Yersinia enterocolitica*: a review. *International Journal of Food Microbiology* **13:** 231-237.

Antonopoulos,P., Constantinidis,F., Charalampopoulos,G., Dalamarinis,K., Karanicas,I., Kokkini,G. (2008) An emergency diagnostic dillemma: a case of *Yersinia enterocolitica* colitis mimicking acute appendicitis in a beta-thalassemia major patient: the role of CT and literature review. *Emerg Radiol* **15:** 126.

Arnold,T., Neubauer,H., Ganter,M., Nikolaou,K., Roesler,U., Truyen,U., Hensel,A. (2006) Prevalence of *Yersinia enterocolitica* in goat herds from northern Germany. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **53:** 382-386.

Arnold,T., Neubauer,H., Nikolaou,K., Roesler,U., Hensel,A. (2004) Identification of *Yersinia enterocolitica* in minced meat: A comparative analysis of API 20E, *Yersinia* identification kit and a 16S rRNA-based PCR method. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **51:** 23-27.

Asplund,K., Johansson,T., Siitonen,A. (1998) Evaluation of pulsed-field gel electrophoresis of genomic restriction fragments in the discrimination of *Yersinia enterocolitica* O:3. *Epidemiology and Infection* **121:** 579-586.

Aulisio,C.C.G., Mehlman,I.J., Sanders,A.C. (1980) Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from foods. *Applied and Environmental Microbiology* **39:** 135-140.

Autenrieth,I.B., Firsching,R. (1996) Penetration of M cells and destruction of Peyer's patches by *Yersinia enterocolitica*: an ultrastructural and histological study. *Journal of Medical Microbiology* **44:** 285-294.

Babic-Erceg,A., Klismanic,Z., Erceg,M., Tandara,D., Smoljanovic,M. (2003) An outbreak of *Yersinia enterocolitica* O:3 infections on an oil tanker. *European Journal of Epidemiology* **18:** 1159-1161.

Backhans,A., Fellstrom,C., Lambertz,S.T. (2011) Occurrence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in small wild rodents. *Epidemiology and Infection* **139:** 1230-1238.

Bancerz-Kisiel,A., Szczerba-Turek,A., Lipczynska,K., Stenzel,T., Szweda,W. (2012) Bioserotypes and virulence markers of *Yersinia enterocolitica* strains isolated from mallards (*Anas platyrhynchos*) and pheasants (*Phasianus colchicus*). *Journal of Food Protection* **75:** 2219-2222.

Bancerz-Kisiel,A., Szczerba-Turek,A., Platt-Samoraj,A., Socha,P., Szweda,W. (2009) Application of multiplex PCR for the evaluation of the occurrence of ail, ystA, and ystB genes in *Yersinia enterocolitica* strains isolated from wild boars (*Sus scrofa*). *Bulletin of the Veterinary Institute in Pulawy* **53:** 351-355.

Bartling,C., Wolfel,R., Nikolaou,K., Petry,T., Thiede,S., Hildebrandt,T., Fassbender,M., Goritz,F., Blottner,S., Spittler,H., Neubauer,H. (2004) Prevalence of anti-*Yersinia* antibodies in European brown hares in North-Rhine Westphalia, Germany. *Deutsche Tierarztliche Wochenschrift* **111:** 259-264.

Batz,M.B., Hoffmann,S., Morris,J.G. (2012) Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protection* **75:** 1278-1291.

Batzilla,J., Antonenka,U., Hoper,D., Heesemann,J., Rakin,A. (2011a) *Yersinia enterocolitica palearctica* serobiotype O:3/4 - a successful group of emerging zoonotic pathogens. *Bmc Genomics* **12:** 348.

Batzilla,J., Heesemann,J., Rakin,A. (2011b) The pathogenic potential of *Yersinia enterocolitica* 1A. *International Journal of Medical Microbiology* **301:** 556-561.

Baumgartner,A., Kuffer,M., Suter,D., Jemmi,T., Rohner,P. (2007) Antimicrobial resistance of *Yersinia enterocolitica* strains from human patients, pigs and retail pork in Switzerland. *International Journal of Food Microbiology* **115:** 110-114.

Benavides,S., Nicol,K., Koranyi,K., Nahata,M.C. (2003) *Yersinia* septic shock following an autologous transfusion in a pediatric patient. *Transfusion and Apheresis Science* **28:** 19-23.

Bergann,T., Kleemann,J., Sohr,D. (1995) Model investigations into the psychrotrophic growth of *Yersinia enterocolitica*. *Journal of Veterinary Medicine Series B-Zentralblatt fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health* **42:** 523-531.

Bergmann,T.K., Vinding,K., Hey,H. (2001) Multiple hepatic abscesses due to *Yersinia enterocolitica* infection secondary to primary haemochromatosis. *Scandinavian Journal of Gastroenterology* **36:** 891-895.

Bhaduri,S. (2006) Enrichment, isolation, and virulence of freeze-stressed plasmid-bearing virulent strains of *Yersinia enterocolitica* on pork. *Journal of Food Protection* **69:** 1983-1985.

Bhaduri,S. (1997) Presence of virulence plasmid in *Yersinia enterocolitica* after its expression at 37C. *Journal of Rapid Methods and Automation in Microbiology* **5:** 29-36.

Bhaduri,S., Phillips,J.G. (2011) Growth model of a plasmid-bearing virulent strain of *Yersinia pseudotuberculosis* in raw ground beef. *Zoonoses and Public Health* **58:** 77-84.

Bhaduri,S., Smith,J.L. (2011) Virulence plasmid (pYV)-associated expression of phenotypic virulent determinants in pathogenic *Yersinia* species: a convenient method for monitoring the presence of pYV under culture conditions and its application for isolation/detection of *Yersinia pestis* in food. *Journal of Pathogens* **2011:** 727313.

Bielli,M., Lauzi,S., Pratelli,A., Martini,M., Dall'Ara,P., Bonizzi,L. (1999) Pseudotuberculosis in marmosets, tamarins, and Goeldi's monkeys (*Callithrichidae*/*Callimiconidae*) housed at a European Zoo. *Journal of Zoo and Wildlife Medicine* **30:** 532-536.

Bleves,S., Cornelis,G.R. (2000) How to survive in the host: the *Yersinia* lesson. *Microbes and Infection* **2:** 1451-1460.

Bogdanovich,T., Carniel,E., Fukushima,H., Skurnik,M. (2003) Use of O-antigen gene cluster-specific PCRs for the identification and O-genotyping of *Yersinia pseudotuberculosis* and *Yersinia pestis*. *Journal of Clinical Microbiology* **41:** 5103-5112.

Bolton,D.J., Ivory,C., McDowell,D. (2013) Thermal inactivation of *Yersinia enterocolitica* in pork slaughter plant scald tank water. *Meat Science* **95:** 668-671.

Bonardi,S., Bassi,L., Brindani,F., D'Incau,M., Barco,L., Carra,E., Pongolini,S. (2013) Prevalence, characterization and antimicrobial susceptibility of *Salmonella enterica* and *Yersinia enterocolitica* in pigs at slaughter in Italy. *International Journal of Food Microbiology* **163:** 248-257.

Bonardi,S., Brindani,F., Pizzin,G., Lucidi,L., D'Incau,M., Liebana,E., Morabito,S. (2003) Detection of *Salmonella* spp., *Yersinia enterocolitica* and verocytotoxin-producing *Escherichia coli* O157 in pigs at slaughter in Italy. *International Journal of Food Microbiology* **85:** 101-110.

Bonardi,S., Paris,A., Bacci,C., D'Incau,M., Ferroni,L., Brindani,F. (2007) Detection and characterization of *Yersinia enterocolitica* from pigs and cattle. *Veterinary Research Communications* **31:** 347-350.

Bonardi,S., Paris,A., Bassi,L., Salmi,F., Bacci,C., Riboldi,E., Boni,E., D'Incau,M., Tagliabue,S., Brindani,F. (2010) Detection, semiquantitative enumeration, and antimicrobial susceptibility of *Yersinia enterocolitica* in pork and chicken meats in Italy. *Journal of Food Protection* **73:** 1785-1792.

Boqvist,S., Pettersson,H., Svensson,A., Andersson,Y. (2009) Sources of sporadic *Yersinia enterocolitica* infection in children in Sweden, 2004: a case-control study. *Epidemiology and Infection* **137:** 897-905.

Borch,E., Nesbakken,T., Christensen,H. (1996) Hazard identification in swine slaughter with respect to foodborne bacteria. *International Journal of Food Microbiology* **30:** 9-25.

Bottone,E.J. (1997) *Yersinia enterocolitica*: the charisma continues. *Clinical Microbiology Reviews* **10:** 257-276.

Bowman,A.S., Glendening,C., Wittum,T.E., LeJeune,J.T., Stich,R.W., Funk,J.A. (2007) Prevalence of *Yersinia enterocolitica* in different phases of production on swine farms. *Journal of Food Protection* **70:** 11-16.

Boyapalle,S., Wesley,I.V., Hurd,H.S., Reddy,P.G. (2001) Comparison of culture, multiplex, and 5 ' nuclease polymerase chain reaction assays for the rapid detection of *Yersinia enterocolitica* in swine and pork products. *Journal of Food Protection* **64:** 1352-1361.

Bucher,M., Meyer,C., Grotzbach,B., Wacheck,S., Stolle,A., Fredriksson-Ahomaa,M. (2008) Epidemiological data on pathogenic *Yersinia enterocolitica* in Southern Germany during 2000-2006. *Foodborne Pathogens and Disease* **5:** 273-280.

Burger,R., Gerlich,W., Gurtler,L., Heiden,M., Jansen,B., Kretschmer,V., Lefevre,H., Lower,J., Montag-Lessing,T., Neumann,R., Pauli,G., Seitz,R., Schlenkrich,U., Werner,E., Willkommen,H. (2000) *Yersinia enterocolitica*. *Infusion Therapy and Transfusion Medicine-Infusionstherapie und Transfusionsmedizin* **27:** 58-66.

Carniel,E. (2001) The *Yersinia* high-pathogenicity island: an iron-uptake island. *Microbes and Infection* **3:** 561-569.

Carnoy,C., Floquet,S., Marceau,M., Sebbane,F., Haentjens-Herwegh,S., Devalckenaere,A., Simonet,M. (2002) The superantigen gene *ypm* is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis*. *Journal of Bacteriology* **184:** 4489-4499.

CDC (Centers for Disease Control and Prevention) (2003) *Yersinia enterocolitica* gastroenteritis among infants exposed to chitterlings - Chicago, Illinois, 2002. *MMWR (Morbidity and Mortality Weekly Report)* **52:** 956.

CDC (Centers for Disease Control and Prevention) (2011) *Yersinia enterocolitica* infections associated with pasteurized milk - southwestern Pennsylvania, March-August, 2011. *MMWR (Morbidity and Mortality Weekly Report)* **60:** 1428.

Ch'Ng,S.L., Octavia,S., Xia,Q.Y., An,D., Tanaka,M.M., Fukushima,H., Lan,R.T. (2011) Population structure and evolution of pathogenicity of *Yersinia pseudotuberculosis*. *Applied and Environmental Microbiology* **77:** 768-775.

Chen,P.E., Cook,C., Stewart,A.C., Nagarajan,N., Sommer,D.D., Pop,M., Thomason,B., Thomason,M.P.K., Lentz,S., Nolan,N., Sozhamannan,S., Sulakvelidze,A., Mateczun,A., Du,L., Zwick,M.E., Read,T.D. (2010) Genomic characterization of the *Yersinia* genus. *Genome Biology* **11**.

Chenais,E., Bagge,E., Thisted-Lambertz,S., Artursson,K. (2012) *Yersinia enterocolitica* serotype O:9 cultured from Swedish sheep showing serologically false-positive reactions for *Brucella melitensis*. *Infection Ecology and Epidemiology* **2:** 19027.

Childs-Sanford,S.E., Kollias,G.V., bou-Madi,N., McDonough,P.L., Garner,M.M., Mohammed,H.O. (2009) *Yersinia pseudotuberculosis* in a closed colony of Egyptian fruit bats (*Rousettus aegyptiacus*). *Journal of Zoo and Wildlife Medicine* **40:** 8-14.

Cocolin,L., Comi,G. (2005) Use of a culture-independent molecular method to study the ecology of *Yersinia* spp. in food. *International Journal of Food Microbiology* **105:** 71-82.

Cork,S.C., Collins-Emerson,J.M., Alley,M.R., Fenwick,S.G. (1999) Visceral lesions caused by *Yersinia pseudotuberculosis*, serotype II, in different species of bird. *Avian Pathology* **28:** 393-399.

Cornelis,G.R., Boland,A., Boyd,A.P., Geuijen,C., Iriarte,M., Neyt,C., Sory,M.P., Stainier,I. (1998) The virulence plasmid of *Yersinia*, an antihost genome. *Microbiology and Molecular Biology Reviews* **62:** 1315-1352.

Crowe,M., Ashford,K., Ispahani,P. (1996) Clinical features and antibiotic treatment of septic arthritis and osteomyelitis due to *Yersinia enterocolitica*. *Journal of Medical Microbiology* **45:** 302-309.

Csaba,N., Tibor,B., Robert,G., Attila,C., Sandor,V. (2000) Disease in large number in a broiler turkey flock caused by *Yersinia pseudotuberculosis*. *Magyar Allatorvosok Lapja* **122:** 579-585.

de Boer,E. (1992) Isolation of *Yersinia enterocolitica* from foods. *International Journal of Food Microbiology* **17:** 75-84.

de Boer,E., Nouws,J.F.M. (1991) Slaughter pigs and pork as a source of human pathogenic *Yersinia enterocolitica*. *International Journal of Food Microbiology* **12:** 375-378.

de Boer,E., Seldam,W.M. (1987) Comparison of methods for the isolation of Yersinia enterocolitica from porcine tonsils and pork. *International Journal of Food Microbiology* **5:** 95-101.

de Boer,E., Zwartkruis-Nahuis,J.T.M., Lesuis,R. (2008) Prevalence of human pathogenic *Yersinia enterocolitica* in pigs. *Tijdschrift Voor Diergeneeskunde* **133:** 938-941.

De Giusti,M., De Vito,E., Serra,A., Quattrucci,B., Boccia,A., Pacifico,L., Ranucci,A., Ravagnan,G., Chiesa,C. (1995) Occurrence of pathogenic *Yersinia enterocolitica* in slaughtered pigs and pork products. *Contributions to Microbiology and Immunology* **13:** 126-129.

De Zutter,L., Janssens,M., Wauters,G. (1995) Detection of *Yersinia enterocolitica* serogroup O:3 using different inoculation methods of the enrichment medium iragasan ticarcillin chlorate. *Contributions to Microbiology and Immunology* **13:** 123-125.

De Zutter,L., Le,M.L., Janssens,M., Wauters,G. (1994) Short-comings of irgasan ticarcillin chlorate broth for the enrichment of *Yersinia enterocolitica* biotype 2, serotype 9 from meat. *International Journal of Food Microbiology* **23:** 231-237.

Delor,I., Cornelis,G.R. (1992) Role of *Yersinia enterocolitica* Yst toxin in experimental infection of young rabbits. *Infection and Immunity* **60:** 4269-4277.

Delor,I., Kaeckenbeeck,A., Wauters,G., Cornelis,G.R. (1990) Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and nonpathogenic Yersiniae. *Infection and Immunity* **58:** 2983-2988.

Denis,M., Houard,E., Labbe,A., Fondrevez,M., Salvat,G. (2011) A selective chromogenic plate, YECA, for the detection of pathogenic *Yersinia enterocolitica*: specificity, sensitivity, and capacity to detect pathogenic *Y. enterocolitica* from pig tonsils. *Journal of Pathogens* **2011:** 296275.

Denis,M., Minvielle,B., Feurer,C., Desmonts,M.H., Carniel,E. (2012) Epidémiologie de *Yersinia enterocolitica* chez le porc, réservoir animal de souches pathogènes pour l'Homme. *Bulletin épidemiologique, santé animale et alimentation* **50**.

Devenish,J.A., Schiemann,D.A. (1981) An abbreviated sceme for identification of *Yersinia enterocolitica* isolated from food enrichments on CIN (cefsulodin-irgasan-novobiocin) agar. *Canadian Journal of Microbiology* **27:** 937-941.

Dube,P. (2009) Interaction of *Yersinia* with the gut: mechanisms of pathogenesis and immune evasion. In: *Molecular mechanisms of bacterial infection via the gut.* Sasakawa C (ed), pp. 61-91. Springer-Verlag Berlin Heidelberg, Berlin.

EFSA (2007) Scientific opinion of the panel on biological hazard (BIOHAZ) on monitoring and identification of human enteropathogenic *Yersinia* spp. *The EFSA Journal* **595:** 1-30.

EFSA (2009) Technical specifications for harmonised national surveys of *Yersinia enterocolitica* in slaughter pigs on request of EFSA. *The EFSA Journal* **7(11):** 1374 [23pp].

EFSA (2011) Scientific opinion on the public health hazards to be covered by inspection of meat (swine). *The EFSA Journal* **9:** 2351 [198 pp].

EFSA, CDC (2013) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal* **11(4):** 3129 [250 pp.].

EFSA, ECDC (2012) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal* **10(3):** 2597 [442 pp.].

El Qouqa,I.A., El Jarou,M.A., bu Samaha,A.S., Al Afifi,A.S., Al Jarousha,A.M.K. (2011) *Yersinia enterocolitica* infection among children aged less than 12 years: a case-control study. *International Journal of Infectious Diseases* **15:** E48-E53.

El Tahir,Y., Skurnik,M. (2001) YadA, the multifaceted *Yersinia* adhesin. *International Journal of Medical Microbiology* **291:** 209-218.

Ellenrieder,M., Zautner,A.E., Podbielski,A., Bader,R., Mittelmeier,W. (2010) Spondylodiscitis of the lumbar spine in a non-immunocompromised host caused by *Yersinia enterocolitica* O:9. *Archives of Orthopaedic and Trauma Surgery* **130:** 469-471.

Erridge,C., nett-Guerrero,E., Poxton,I.R. (2002) Structure and function of lipopolysaccharides. *Microbes and Infection* **4:** 837-851.

Ewing,W.H., Ross,A.J., Brenner,D.J., Fanning,G.R. (1978) *Yersinia ruckeri* sp. nov., Redmouth (Rm) bacterium. *International Journal of Systematic Bacteriology* **28:** 37-44.

Fondrevez,M., Labbe,A., Houard,E., Fravalo,P., Madec,F., Denis,M. (2010) A simplified method for detecting pathogenic *Yersinia enterocolitica* in slaughtered pig tonsils. *Journal of Microbiological Methods* **83:** 244-249.

Fosse,J., Laroche,M., Oudot,N., Rossero,A., Seegers,H., Magras,C. (2010) Farm to slaughter study of pigs batches infection by *Staphylococcus aureus* and *Yersinia enterocolitica*: an exploratory study. *Revue de Medecine Veterinaire* **161:** 20-29.

Fosse,J., Seegers,H., Magras,C. (2008) Foodborne zoonoses due to meat: a quantitative approach for a comparative risk assessment applied to pig slaughtering in Europe. *Veterinary Research* **39**.

Fredriksson-Ahomaa,M. (2012) Isolation of enteropathogenic *Yersinia* from non-human sources. *Adv.Exp.Med.Biol.* **954:** 97-105.

Fredriksson-Ahomaa,M., Autio,T., Korkeala,H. (1999a) Efficient subtyping of *Yersinia enterocolitica* bioserotype 4/O:3 with pulsed-field gel electrophoresis. *Letters in Applied Microbiology* **29:** 308-312.

Fredriksson-Ahomaa,M., Bjorkroth,J., Hielm,S., Korkeala,H. (2000a) Prevalence and characterization of pathogenic *Yersinia enterocolitica* in pig tonsils from different slaughterhouses. *Food Microbiology* **17:** 93-101.

Fredriksson-Ahomaa,M., Bucher,M., Hank,C., Stolle,A., Korkeala,H. (2001a) High prevalence of *Yersinia enterocolitica* 4:O3 on pig offal in Southern Germany: a slaughtering technique problem. *Systematic and Applied Microbiology* **24:** 457-463.

Fredriksson-Ahomaa,M., Cernela,N., Hachler,H., Stephan,R. (2012a) *Yersinia enterocolitica* strains associated with human infections in Switzerland 2001-2010. *European Journal of Clinical Microbiology & Infectious Diseases* **31:** 1543-1550.

Fredriksson-Ahomaa,M., Gerhardt,M., Stolle,A. (2009a) High bacterial contamination of pig tonsils at slaughter. *Meat Science* **83:** 334-336.

Fredriksson-Ahomaa,M., Hallanvuo,S., Korte,T., Siitonen,A., Korkeala,H. (2001b) Correspondence of genotypes of sporadic *Yersinia enterocolitica* bioserotype 4/O : 3 strains from human and porcine sources. *Epidemiology and Infection* **127:** 37-47.

Fredriksson-Ahomaa,M., Hartmann,B., Scheu,P., Stolle,A. (2007a) Detection of pathogenic *Yersinia enterocolitica* in meat using real-time PCR. *Journal fur Verbraucherschutz und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety* **2:** 202-208.

Fredriksson-Ahomaa,M., Hartmann,B., Wacheck,S., Stolle,A. (2008) Evaluation of the ISO 10273 method for isolation of *Yersinia enterocolitica* 4/O : 3 in food samples. *Archiv fur Lebensmittelhygiene* **59:** 99-102.

Fredriksson-Ahomaa,M., Hielm,S., Korkeala,H. (1999b) High prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tongues and minced meat at the retail level in Finland. *Journal of Food Protection* **62:** 123-127.

Fredriksson-Ahomaa,M., Koch,U., Klemm,C., Bucher,M., Stolle,A. (2004) Different genotypes of *Yersinia enterocolitica* 4/O:3 strains widely distributed in butcher shops in the Munich area. *International Journal of Food Microbiology* **95:** 89-94.

Fredriksson-Ahomaa,M., Korkeala,H. (2003) Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clinical Microbiology Reviews* **16:** 220-229.

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

Fredriksson-Ahomaa,M., Korte,T., Korkeala,H. (2001c) Transmission of *Yersinia enterocolitica* 4/O:3 to pets via contaminated pork. *Letters in Applied Microbiology* **32:** 375-378.

Fredriksson-Ahomaa,M., Murros-Kontiainen,A., Sade,E., Puolanne,E., Bjorkroth,J. (2012b) High number of *Yersinia enterocolitica* 4/O:3 in cold-stored modified atmosphere-packed pig cheek meat. *International Journal of Food Microbiology* **155:** 69-72.

Fredriksson-Ahomaa,M., Naglic,T., Turk,N., Seol,B., Grabarevic,Z., Bata,I., Perkovic,D., Stolle,A. (2007b) Yersiniosis in zoo marmosets (*Callitrix jacchuss*) caused by *Yersinia enterocolitica* 4/O : 3. *Veterinary Microbiology* **121:** 363-367.

Fredriksson-Ahomaa,M., Niskanen,T., Neubauer,H., Laukkanen,R., Korkeala,H. (2002) Characterisation of sucrose-negative *Yersinia enterocolitica* 4/O : 3 isolates recovered from pig tonsils. *International Journal of Food Microbiology* **75:** 19-25.

Fredriksson-Ahomaa,M., Stolle,A., Siitonen,A., Korkeala,H. (2006) Sporadic human *Yersinia enterocolitica* infections caused by bioserotype 4/O:3 originate mainly from pigs. *Journal of Medical Microbiology* **55:** 747-749.

Fredriksson-Ahomaa,M., Stolle,A., Stephan,R. (2007c) Prevalence of pathogenic *Yersinia enterocolitica* in pigs slaughtered at a Swiss abattoir. *International Journal of Food Microbiology* **119:** 207-212.

Fredriksson-Ahomaa,M., Wacheck,S., Bonke,R., Stephan,R. (2011) Different enteropathogenic *Yersinia* strains found in wild boars and domestic pigs. *Foodborne Pathogens and Disease* **8:** 733-737.

Fredriksson-Ahomaa,M., Wacheck,S., Koenig,M., Stolle,A., Stephan,R. (2009b) Prevalence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in Switzerland. *International Journal of Food Microbiology* **135:** 199-202.

Frölich,K., Wisser,J., Schmüser,H., Fehlberg,U., Neubauer,H., Grunow,R., Nikolaou,K., Priemer,J., Thiede,S., Streich,W.J., Speck,S. (2003) Epizootiologic and ecologic investigations of European brown hares (Lepus europaeus) in selected populations from Schleswig-Holstein, Germany. *Journal of Wildlife Diseases* **39:** 751-761.

Fukushima,H. (1985) Direct isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from meat. *Applied and Environmental Microbiology* **50:** 710-712.

Fukushima,H. (1987) New selective agar medium for isolation of virulent *Yersinia enterocolitica*. *Journal of Clinical Microbiology* **25:** 1068-1073.

Fukushima,H., Gomyoda,M. (1986) Growth of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* biotype 3B serotype O:3 inhibited on cefsulodin-irgasan-novobiocin agar. *Journal of Clinical Microbiology* **24:** 116-120.

Fukushima,H., Gomyoda,M. (1991) Intestinal carriage of *Yersinia pseudotuberculosis* by wild birds and mammals in Japan. *Applied and Environmental Microbiology* **57:** 1152-1155.

Fukushima,H., Matsuda,Y., Seki,R., Tsubokura,M., Takeda,N., Shubin,F.N., Paik,I.K., Zheng,X.B. (2001) Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. *Journal of Clinical Microbiology* **39:** 3541-3547.

Fukushima,H., Shimizu,S., Inatsu,Y. (2011) *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* detection in foods. *Journal of Pathogens* **2011:** 735308.

Gaston,J.S.H., Lillicrap,M.S. (2003) Arthritis associated with enteric infection. *Best Practice & Research in Clinical Rheumatology* **17:** 219-239.

Gayraud,M., Scavizzi,M.R., Mollaret,H.H., Guillevin,L., Hornstein,M.J. (1993) Antibiotic treatment of *Yersinia enterocolitica* septicemia - a retrospective review of 43 cases. *Clinical Infectious Diseases* **17:** 405-410.

Gierczynski,R., Golubov,A., Neubauer,H., Pham,J.N., Rakin,A. (2007) Development of multiple-locus variable-number tandem-repeat analysis for Y*ersinia enterocolitica* subsp *palearctica* and its application to bioserogroup 4/O3 subtyping. *Journal of Clinical Microbiology* **45:** 2508-2515.

Gierczynski,R., Szych,J., Rastawicki,W., Wardak,S., Jagielski,M. (2009) Molecular characterization of human clinical isolates of *Yersinia enterocolitica* bioserotype 1B/O8 in Poland: emergence and dissemination of three highly related clones. *Journal of Clinical Microbiology* **47:** 1225-1228.

Gombac,M., Svara,T., Zdovc,I., Juntes,P., Pogacnik,M. (2008) Yersiniosis in captive Capybaras (Hydrochaeris Hydrochaeris). *Slovenian Veterinary Research* **45:** 135-140.

Gourdon,F., Beytout,J., Reynaud,A., Romaszko,J.P., Perre,D., Theodore,P., Soubelet,H., Sirot,J. (1999) Human and animal epidemic of *Yersinia enterocolitica* O:9, 1989-1997, Auvergne, France. *Emerging Infectious Diseases* **5:** 719-721.

Goverde,R.L.J., Kusters,J.G., Huis in't Veld,J.H. (1994) Growth rate and physiology of *Yersinia enterocolitica*; influence of temperature and presence of the virulence plasmid. *Journal of Applied Bacteriology* **77:** 96-104.

Grahek-Ogden,D., Schimmer,B., Cudjoe,K.S., Nygard,K., Kapperud,G. (2007) Outbreak of *Yersinia enterocolitica* serogroup O : 9 infection and processed pork, Norway. *Emerging Infectious Diseases* **13:** 754-756.

Grassl,G.A., Bohn,E., Muller,Y., Buhler,O.T., Autenrieth,I.B. (2003) Interaction of *Yersinia enterocolitica* with epithelial cells: invasin beyond invasion. *International Journal of Medical Microbiology* **293:** 41-54.

Grigull,L., Linderkamp,C., Sander,A., Schmid,H., Mutschler,U., Welte,K., Beilken,A. (2005) Multiple spleen and liver abscesses due to *Yersinia enterocolitica* septicemia in a child with congenital sideroblastic anemia. *Journal of Pediatric Hematology Oncology* **27:** 624-626.

Guinet,F., Carniel,E., Leclercq,A. (2011) Transfusion-transmitted *Yersinia enterocolitica* sepsis. *Clinical Infectious Diseases* **53:** 583-591.

Gumaste,P., Boppana,V.S., Garcha,A.S., Blair,D. (2012) Thigh abscess caused by *Yersinia enterocolitica* in an immunocompetent host. *Case Reports in Medicine* **2012:** article ID 259475.

Gürtler,M., Alter,T., Kasimir,S., Linnebur,M., Fehlhaber,K. (2005) Prevalence of *Yersinia enterocolitica* in fattening pigs. *Journal of Food Protection* **68:** 850-854.

Güven,A., Sezer,C., Duman Aydin,B., Oral,N.B., Vatansever,L. (2010) Incidence and pathogenicity of *Yersinia enterocolitica* isolates from foods in Turkey. *Kafkas Universitesi Veteriner Fakultesi Dergisi* **16:** S107-S112.

Hallanvuo,S., Peltola,J., Heiskanen,T., Siitonen,A. (2006) Simplified phenotypic scheme evaluated by 16S rRNA sequencing for differentiation between *Yersinia enterocolitica* and *Yersinia enterocolitica*-like species. *Journal of Clinical Microbiology* **44:** 1077-1080.

Han,T.H., Paik,I.K., Kim,S.J. (2003) Molecular relatedness between isolates *Yersinia pseudotuberculosis* from a patient and an isolate from mountain spring water. *Journal of Korean Medical Science* **18:** 425-428.

Hannu,T., Mattila,L., Nuorti,J.P., Ruutu,P., Mikkola,J., Siitonen,A., Leirisalo-Repo,M. (2003) Reactive arthritis after an outbreak of *Yersinia pseudotuberculosis* serotype O : 3 infection. *Annals of the Rheumatic Diseases* **62:** 866-869.

Harb,J., Fyfe,M., Patrick,D., Trinidad,A., Hockin,J., Masoud,N., Louie,K., Kurzac,C. (2000) Case-control study assessing the association between yersiniosis and exposure to salami. *Canada Communicable Disease Report* **26:** 161-164.

Harnett,N., Lin,Y.P., Krishnan,C. (1996) Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction. *Epidemiology and Infection* **117:** 59-67.

Harrison,W.A., Peters,A.C., Fielding,L.M. (2000) Growth of *Listeria monocytogenes* and *Yersinia enterocolitica* colonies under modified atmospheres at 4 and 8°C using a model food system. *Journal of Applied Microbiology* **88:** 38-43.

Hayashidani,H., Iwata,T., Yamaguchi,S., Hara-Kudo,Y., Okatani,T.A., Watanabe,M., Lee,K.I., Kumagai,S. (2008) Survival of pathogenic *Yersinia enterocolitica* in vacuum-packed or non-vacuum-packed pork at low temperature. *Biocontrol Science* **13:** 139-144.

Hayashidani,H., Kanzaki,N., Kaneko,Y., Okatani,A.T., Taniguchi,T., Kaneko,K., Ogawa,M. (2002) Occurrence of Yersiniosis and Listeriosis in wild boars in Japan. *Journal of Wildlife Diseases* **38:** 202-205.

Heesemann,J., Sing,A., Trülzsch,K. (2006) *Yersinia*'s stratagem: targeting innate and adaptive immune defense. *Current Opinion in Microbiology* **9:** 55-61.

Heise,T., Dersch,P. (2006) Identification of a domain in *Yersinia* virulence factor YadA that is crucial for extracellular matrix-specific cell adhesion and uptake. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 3375-3380.

Hilbert,F., Mayrhofer,S., Smulders,F.J.M. (2003) Rapid urease screening of *Yersinia* on CIN agar plates. *International Journal of Food Microbiology* **84:** 111-115.

Hoogkamp-Korstanje,J.A., de Koning,J. (1991) *Yersinia enterocolitica* infections; clinical and histological characteristics, diagnosis and therapy. *NederlandsTijdschrift voor Geneeskunde* **135:** 2218-2223.

Hoogkamp-Korstanje,J.A.A., Moesker,H., Bruyn,G.A.W. (2000) Ciprofloxacin upsilon placebo for treatment of *Yersinia enterocolitica* triggered reactive arthritis. *Annals of the Rheumatic Diseases* **59:** 914-917.

Howard,S.L., Gaunt,M.W., Hinds,J., Witney,A.A., Stabler,R., Wren,B.W. (2006) Application of comparative phylogenomics to study the evolution of *Yersinia enterocolitica* and to identify genetic differences relating to pathogenicity. *Journal of Bacteriology* **188:** 3645-3653.

Huovinen,E., Sihvonen,L.M., Virtanen,M.J., Haukka,K., Siitonen,A., Kuusi,M. (2010) Symptoms and sources of *Yersinia enterocolitica*-infection: a case-control study. *Bmc Infectious Diseases* **10:** 122.

Hurst,M.R.H., Becher,S.A., Young,S.D., Nelson,T.L., Glare,T.R. (2011) *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub Costelytra zealandica. *International Journal of Systematic and Evolutionary Microbiology* **61:** 844-849.

Ibrahim,A., Liesack,W., Griffiths,M.W., Robinsbrowne,R.M. (1997) Development of a highly specific assay for rapid identification of pathogenic strains of *Yersinia enterocolitica* based on PCR amplification of the *Yersinia* heat-stable enterotoxin gene (*yst*). *Journal of Clinical Microbiology* **35:** 1636-1638.

Iriarte,M., Cornelis,G.R. (1995) MyfF, an element of the network regulating the synthesis of fibrillae in *Yersinia enterocolitica*. *Journal of Bacteriology* **177:** 738-744.

Isberg,R.R., Barnes,P. (2001) Subversion of integrins by enteropathogenic *Yersinia*. *Journal of Cell Science* **114:** 21-28.

ISO 10273. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of presumptive pathogenic *Yersinia enterocolitica* (ISO 10273:2003). 2003. Geneva, Switzerland, International Organization for Standardization.

Ito,T., Suzuki,T., Kawase,J., Fukushima,H., Nanao,K. (2012) *Yersinia enterocolitica* bacteremia and enterocolitis in a previously healthy 20-month-old girl. *Journal of Infection and Chemotherapy* **18:** 756-759.

Jacobsen,N.R., Bogdanovich,T., Skurnik,M., Lübeck,P.S., Ahrens,P., Hoorfar,J. (2005) A real-time PCR assay for the specific identification of serotype O : 9 of *Yersinia enterocolitica*. *Journal of Microbiological Methods* **63:** 151-156.

Jalava,K., Hakkinen,M., Valkonen,M., Nakari,U.M., Palo,T., Hallanvuo,S., Ollgren,J., Siitonen,A., Nuorti,J.P. (2006) An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis*. *Journal of Infectious Diseases* **194:** 1209-1216.

Jalava,K., Hallanvuo,S., Nakari,U.M., Ruutu,P., Kela,E., Heinasmaki,T., Siitonen,A., Nuorti,J.P. (2004) Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. *Journal of Clinical Microbiology* **42:** 2789-2791.

Jiang,G.C., Kang,D.H., Fung,D.Y.C. (2000) Enrichment procedures and plating media for isolation of *Yersinia enterocolitica*. *Journal of Food Protection* **63:** 1483-1486.

Johannessen,G.S., Kapperud,G., Kruse,H. (2000) Occurrence of pathogenic *Yersinia enterocolitica* in Norwegian pork products determined by a PCR method and a traditional culturing method. *International Journal of Food Microbiology* **54:** 75-80.

Jones,T.F., Buckingham,S.C., Bopp,C.A., Ribot,E., Schaffner,W. (2003) From pig to pacifier: chitterling-associated yersiniosis outbreak among black infants. *Emerging Infectious Diseases* **9:** 1007-1009.

Juste,R.A., Minguijon,E., Arranz,J., Fuertes,M., de Heredia,I.B. (2009) Lamb mortality in an outbreak of *Yersinia pseudotuberculosis* mastitis, as a collateral effect of colostrum feeding for Lentivirus-control. *Small Ruminant Research* **86:** 46-51.

Kaasch,A.J., Dinter,J., Goeser,T., Plum,G., Seifert,H. (2012) *Yersinia pseudotuberculosis* bloodstream infection and septic arthritis: case report and review of the literature. *Infection* **40:** 185-190.

Kaito,H., Kamei,K., Ogura,M., Kikuchi,E., Hoshino,H., Nakagawa,S., Matsuoka,K., Abe,J., Ito,S. (2012) Acute encephalopathy and tubulointerstitial nephritis associated with *Yersinia pseudotuberculosis*. *Pediatrics International* **54:** 926-928.

Kangas,S., Takkinen,J., Hakkinen,M., Nakari,U.M., Johansson,T., Henttonen,H., Virtaluoto,L., Siitonen,A., Ollgren,J., Kuusi,M. (2008) *Yersinia pseudotuberculosis* O:1 traced to raw carrots, Finland. *Emerging Infectious Diseases* **14:** 1959-1961.

Karachalios,G., Bablekos,G., Karachaliou,G., Charalabopoulos,A.K., Charalabopoulos,K. (2002) Infectious endocarditis due to *Yersinia enterocolitica*. *Chemotherapy* **48:** 158-159.

Katamura,K., Okazaki,S., Usami,I., Tsuboyama,T., Takeda,N. (2001) Multifocal osteomyelitis and erythematous plaque associated with *Yersinia pseudotuberculosis* infection. *Pediatrics International* **43:** 711-713.

Kechagia,N., Nicolaou,C., Ioannidou,V., Kourti,E., Ioannidis,A., Legakis,N.J., Chatzipanagiotou,S. (2007) Detection of chromosomal and plasmid - encoded virulence determinants in *Yersinia enterocolitica* and other *Yersinia* spp. isolated from food animals in Greece. *International Journal of Food Microbiology* **118:** 326-331.

Kelesidis,T., Balba,G., Worthington,M. (2008) Axillary abscess in a patient with *Yersinia enterocolitica* infection as a result of exposure to pork. *The American Journal of Medicine* **121:** e1.

King,A.M., Miller,R.K., Castillo,A., Griffin,D.B., Hardin,M.D. (2012) Effects of lactic acid and commercial chilling processes on survival of *Salmonella*, *Yersinia enterocolitica*, and *Campylobacter coli* in pork variety meats. *Journal of Food Protection* **75:** 1589-1594.

Kleinlein,N., Untermann,F. (1990) Growth of pathogenic *Yersinia enterocolitica* strains in minced meat with and without protective gas with consideration of the competitive background flora. *International Journal of Food Microbiology* **10:** 65-71.

Kojima,M., Morita,Y., Shimizu,K., Yoshida,T., Yamada,I., Togo,T., Johshita,T. (2007) Immunohistological findings of suppurative granulomas of *Yersinia enterocolitica* appendicitis: a report of two cases. *Pathology Research and Practice* **203:** 115-119.

Konishi,N., Baba,K., Abe,J., Maruko,T., Waki,K., Takeda,N., Tanaka,M. (1997) A case of Kawasaki disease with coronary artery aneurysms documenting *Yersinia pseudotuberculosis* infection. *Acta Paediatrica* **86:** 661-664.

Korsak,N., Jacob,B., Groven,B., Etienne,G., China,B., Ghafir,Y., Daube,G. (2003) *Salmonella* contamination of pigs and pork in an integrated pig production system. *Journal of Food Protection* **66:** 1126-1133.

Korte,T., Fredriksson-Ahomaa,M., Niskanen,T., Korkeala,H. (2004) Low prevalence of *yad*A-positive *Yersinia enterocolitica* in sows. *Foodborne Pathogens and Disease* **1:** 45-52.

Kot,B., Trafny,E.A., Jakubczak,A. (2007) Application of multiplex PCR for monitoring colonization of pig tonsils by *Yersinia enterocolitica*, including biotype 1A, and *Yersinia pseudotuberculosis*. *Journal of Food Protection* **70:** 1110-1115.

Kotetishvili,M., Kreger,A., Wauters,G., Morris,J.G., Sulakvelidze,A., Stine,O.C. (2005) Multilocus sequence typing for studying genetic relationships among *Yersinia* species. *Journal of Clinical Microbiology* **43:** 2674-2684.

Krajinovic,V., Andrasevic,A.T., Barsic,B. (2007) Tricuspidal valve endocarditis due to Yersinia enterocolitica. *Infection* **35:** 203-205.

Kraushaar,B., Dieckmann,R., Wittwer,M., Knabner,D., Konietzny,A., Made,D., Strauch,E. (2011) Characterization of a *Yersinia enterocolitica* biotype 1A strain harbouring an *ail* gene. *Journal of Applied Microbiology* **111:** 997-1005.

Kuhm,A.E., Suter,D., Felleisen,R., Rau,J. (2009) Identification of *Yersinia enterocolitica* at the species and subspecies levels by Fourier transform infrared spectroscopy. *Applied and Environmental Microbiology* **75:** 5809-5813.

Kwaga,J., Iversen,J.O., Saunders,J.R. (1990) Comparison of two enrichment protocols for the detection of *Yersinia* in slaughtered pigs and pork products. *Journal of Food Protection* **53:** 1047-1049.

Lamps,L.W. (2010) Infectious causes of appendicitis. *Infectious Disease Clinics of North America* **24:** 995-1018.

Lanada,E.B., Morris,R.S., Jackson,R., Fenwick,S.G. (2005) Prevalence of *Yersinia* species in goat flocks. *Australian Veterinary Journal* **83:** 563-566.

Laukkanen,R., Hakkinen,M., Lundén,J., Fredriksson-Ahomaa,M., Johansson,T., Korkeala,H. (2010a) Evaluation of isolation methods for pathogenic *Yersinia enterocolitica* from pig intestinal content. *Journal of Applied Microbiology* **108:** 956-964.

Laukkanen,R., Martinez,P.O., Siekkinen,K.M., Ranta,J., Maijala,R., Korkeala,H. (2008) Transmission of *Yersinia pseudotuberculosis* in the pork production chain from farm to slaughterhouse. *Applied and Environmental Microbiology* **74:** 5444-5450.

Laukkanen,R., Martinez,P.O., Siekkinen,K.M., Ranta,J., Maijala,R., Korkeala,H. (2009) Contamination of carcasses with human pathogenic *Yersinia enterocolitica* 4/O:3 originates from pigs infected on farms. *Foodborne Pathogens and Disease* **6:** 681-688.

Laukkanen,R., Ranta,J., Dong,X.J., Hakkinen,M., Martínez,P.O., Lundén,J., Johansson,T., Korkeala,H. (2010b) Reduction of enteropathogenic *Yersinia* in the pig slaughterhouse by using bagging of the rectum. *Journal of Food Protection* **73:** 2161-2168.

Leclercq,A., Martin,L., Vergnes,M.L., Ounnoughene,N., Laran,J.F., Giraud,P., Carniel,E. (2005) Fatal *Yersinia enterocolitica* biotype 4 serovar O:3 sepsis after red blood cell transfusion. *Transfusion* **45:** 814-818.

Lee,K., Iwata,T., Nakadai,A., Kato,T., Hayama,S., Taniguchi,T., Hayashidani,H. (2011) Prevalence of *Salmonella*, *Yersinia* and *Campylobacter* spp. in feral raccoons (*Procyon lotor*) and masked palm civets (*Paguma larvata*) in Japan. *Zoonoses and Public Health* **58:** 424-431.

Lee,L.A., Taylor,J., Carter,G.P., Quinn,B., Farmer,J.J., Tauxe,R.V. (1991) *Yersinia enterocolitica* O:3 - An Emerging Cause of Pediatric Gastroenteritis in the United-States. *Journal of Infectious Diseases* **163:** 660-663.

Lee,W.H., Harris,M.E., McClain,D., Smith,R.E., Johnston R.W. (1980) Two modified selenite media for the recovery of *Yersinia enterocolitica* from meats. *Applied and Environmental Microbiology* **39:** 205-209.

Li,H.Y., Bhaduri,S., Magee,W.E. (1998) Maximizing plasmid stability and production of released proteins in *Yersinia enterocolitica*. *Applied and Environmental Microbiology* **64:** 1812-1815.

Lin,J.E., Valentino,M., Marszalowicz,G., Magee,M.S., Li,P., Snook,A.E., Stoecker,B.A., Chang,C., Waldman,S.A. (2010) Bacterial heat-stable enterotoxins: translation of pathogenic peptides into novel targeted diagnostics and therapeutics. *Toxins* **2:** 2028-2054.

Lindblad,M., Lindmark,H., Lanibertz,S.T., Lindqvist,R. (2007) Microbiological baseline study of swine carcasses at Swedish slaughterhouses. *Journal of Food Protection* **70:** 1790-1797.

Logue,C.M., Sheridan,J.J., Wauters,G., McDowell,D.A., Blair,I.S. (1996) *Yersinia* spp. and numbers, with particular reference to *Y. enterocolitica* bio/serotypes, occurring on Irish meat and meat products, and the influence of alkali treatment on their isolation. *International Journal of Food Microbiology* **33:** 257-274.

Logue,C.M., Sherwood,J.S., Doetkott,C. (2006) Growth studies of plasmid bearing and plasmid cured *Yersinia enterocolitica* GER O:3 in the presence of cefsulodin, irgasan and novobiocin at 25 and 37°C. *Journal of Applied Microbiology* **100:** 1299-1306.

Long,C., Jones,T.F., Vugia,D.J., Scheftel,J., Strockbine,N., Ryan,P., Shiferaw,B., Tauxe,R.V., Gould,L.H. (2010) *Yersinia pseudotuberculosis* and *Y. enterocolitica* infections, FoodNet, 1996-2007. *Emerging Infectious Diseases* **16:** 566-567.

Lucero Estrada,C.S., Velazquez,L.C., Favier,G.I., Di Genaro,M.S., Escudero,M.E. (2012) Detection of *Yersinia* spp. in meat products by enrichment culture, immunomagnetic separation and nested PCR. *Food Microbiology* **30:** 157-163.

Lucero Estrada,C.S.M., Velázquez,L.D., Escudero,M.E., Favier,G.I., Lazarte,V., De Guzman,A.M.S. (2011) Pulsed field, PCR ribotyping and multiplex PCR analysis of *Yersinia enterocolitica* strains isolated from meat food in San Luis Argentina. *Food Microbiology* **28:** 21-28.

MacDonald,E., Heier,B.T., Stalheim,T., Cudjoe,K.S., Skjerdal,T., Wester,A., Lindstedt,B.A., Vold,L. (2011) *Yersinia enterocolitica* O:9 infections associated with bagged salad mix in Norway, February to April 2011. *Eurosurveillance* **16:** article 19866.

Martins,C.H.G., Bauab,T.M., Leite,C.Q.F., Falcao,D.P. (2007) Ribotyping and virulence markers of *Yersinia pseudotuberculosis* strains isolated from animals in Brazil. *Memorias do Instituto Oswaldo Cruz* **102:** 587-592.

McNally,A., Cheasty,T., Fearnley,C., Dalziel,R.W., Paiba,G.A., Manning,G., Newell,D.G. (2004) Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999-2000. *Letters in Applied Microbiology* **39:** 103-108.

Menzies,B.E. (2010) Axillary abscess due to *Yersinia enterocolitica*. *Journal of Clinical Microbiology* **48:** 3438-3439.

Merhej,V., Adekambi,T., Pagnier,I., Raoult,D., Drancourt,M. (2008) *Yersinia massiliensis* sp nov., isolated from fresh water. *International Journal of Systematic and Evolutionary Microbiology* **58:** 779-784.

Merilahti-Palo,R., Lahesmaa,R., Granfors,K., Gripenberglerche,C., Toivanen,P. (1991) Risk of Yrsinia infection among butchers. *Scandinavian Journal of Infectious Diseases* **23:** 55-61.

Messelhausser,U., Kampf,P., Colditz,J., Bauer,H., Schreiner,H., Holler,C., Busch,U. (2011) Qualitative and quantitative detection of human pathogenic *Yersinia enterocolitica* in different food matrices at retail level in Bavaria. *Foodborne Pathogens and Disease* **8:** 39-44.

Meyer,C., Stolle,A., Fredriksson-Ahomaa,M. (2011) Comparison of broth microdilution and disk diffusion test for antimicrobial resistance testing in *Yersinia enterocolitica* 4/O:3 strains. *Microbial Drug Resistance* **17:** 479-484.

Mikula,K.M., Lolodziejczyk,R., Goldman,A. (2012) *Yersinia* infection tools-characterization of structure and function of adhesins. *Frontiers in Cellular and Infection Microbiology* **2:** article 169.

Milnes,A.S., Stewart,I., Clifton-Hadley,F.A., Davies,R.H., Newell,D.G., Sayers,A.R., Cheasty,T., Cassar,C., Ridley,A., Cook,A.J.C., Evans,S.J., Teale,C.J., Smith,R.P., McNnally,A., Toszeghy,M., Futter,R., Kay,A., Paiba,G.A. (2008) Intestinal carriage of verocytotoxigenic *Escherichia coli* O157, *Salmonella*, thermophilic *Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and pigs at slaughter in Great Britain during 2003. *Epidemiology and Infection* **136:** 739-751.

Mischnik,A., Dahme,T., Bekeredjian,R., Zimmermann,S. (2012) Haemophilia-associated *Yersinia pseudotuberculosis* serotype O:1 septicaemia: the role of iron. *Journal of Medical Microbiology* **61:** 157-159.

Moriki,S., Nobata,A., Shibata,H., Nagai,A., Minami,N., Taketani,T., Fukushima,H. (2010) Familial outbreak of *Yersinia enterocolitica* serotype O9 biotype 2. *Journal of Infection and Chemotherapy* **16:** 56-58.

Morild,R.K., Olsen,J.E., Aabo,S. (2011) Change in attachment of *Salmonella Typhimurium*, *Yersinia enterocolitica*, and *Listeria monocytogenes* to pork skin and muscle after hot water and lactic acid decontamination. *International Journal of Food Microbiology* **145:** 353-358.

Movassaghi,A.R., Rad,M. (2002) An outbreak of *Yersinia pseudotuberculosis* infection in lambs. *Journal of Applied Animal Research* **22:** 125-128.

Müller-Alouf,H., Carnoy,C., Simonet,M., Alouf,J.E. (2001) Superantigen bacterial toxins: state of the art. *Toxicon* **39:** 1691-1701.

Murros-Kontiainen,A., Fredriksson-Ahomaa,M., Korkeala,H., Johansson,P., Rahkila,R., Bjorkroth,J. (2011a) *Yersinia nurmii* sp nov. *International Journal of Systematic and Evolutionary Microbiology* **61:** 2368-2372.

Murros-Kontiainen,A., Johansson,P., Niskanen,T., Fredriksson-Ahomaa,M., Korkeala,H., Bjorkroth,J. (2011b) *Yersinia pekkanenii* sp nov. *International Journal of Systematic and Evolutionary Microbiology* **61:** 2363-2367.

Najdenski,H., Heyndrickx,M., Herman,L., Werbrouck,H., Van Coillie,E. (2012) Quantification of *Yersinia enterocolitica* in raw milk using qPCR. *Veterinary Microbiology* **160:** 428-434.

Nakajima,H., Inoue,M., Mori,T., Itoh,K., Arakawa,E., Watanabe,H. (1992) Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *Journal of Clinical Microbiology* **30:** 2484-2486.

Nesbakken,T. (1988) Enumeration of *Yersinia enterocolitica* O : 3 from the porcine oral cavity, and its occurrence on cut surfaces of pig carcasses and the environment in a slaughterhouse. *International Journal of Food Microbiology* **6:** 287-293.

Nesbakken,T. (1985) Comparison of sampling and isolation procedures for recovery of *Yersinia enterocolitica* serotype O:3 from the oral cavity of slaughter pigs. *Acta Veterinaria Scandinavica* **26:** 127-135.

Nesbakken,T., Eckner,K., Hoidal,H.K., Rotterud,O.J. (2003) Occurrence of *Yersinia enterocolitica* and *Campylobacter* spp. in slaughter pigs and consequences for meat inspection, slaughtering, and dressing procedures. *International Journal of Food Microbiology* **80:** 231-240.

Nesbakken,T., Eckner,K., Rotterud,O.J. (2008) The effect of blast chilling on occurrence of human pathogenic *Yersinia enterocolitica* compared to *Campylobacter* spp. and numbers of hygienic indicators on pig carcasses. *International Journal of Food Microbiology* **123:** 130-133.

Nesbakken,T., Iversen,T., Eckner,K., Lium,B. (2006) Testing of pathogenic *Yersinia enterocolitica* in pig herds based on the natural dynamic of infection. *International Journal of Food Microbiology* **111:** 99-104.

Nesbakken,T., Iversen,T., Lium,B. (2007) Pig herds free from human pathogenic *Yersinia enterocolitica*. *Emerging Infectious Diseases* **13:** 1860-1864.

Nesbakken,T., Nerbrink,E., Rotterud,O.J., Borch,E. (1994) Reduction of *Yersinia enterocolitica* and *Listeria* spp. on pig carcasses by enclosure of the rectum during slaughter. *International Journal of Food Microbiology* **23:** 197-208.

Neubauer,H., Aleksic,S., Hensel,A., Finke,E.J., Meyer,H. (2000a) *Yersinia enterocolitica* 16S rRNA gene types belong to the same genospecies but form three homology groups. *International Journal of Medical Microbiology* **290:** 61-64.

Neubauer,H., Hensel,A., Aleksic,S., Meyer,H. (2000b) Identification of *Yersinia enterocolitica* within the genus *Yersinia*. *Systematic and Applied Microbiology* **23:** 58-62.

Neubauer,H., Sauer,T., Becker,H., Aleksic,S., Meyer,H. (1998) Comparison of systems for identification and differentiation of species within the genus *Yersinia*. *Journal of Clinical Microbiology* **36:** 3366-3368.

Nielsen,B., Ekeroth,L., Bager,F., Lind,P. (1998) Use of muscle fluid as a source of antibodies for serologic detection of *Salmonella* infection in slaughter pig herds. *Journal of Veterinary Diagnostic Investigation* **10:** 158-163.

Nielsen,B., Heisel,C., Wingstrand,A. (1996) Time course of the serological response to *Yersinia enterocolitica* O:3 in experimentally infected pigs. *Veterinary Microbiology* **48:** 293-303.

Nikolova,S., Tzvetkov,Y., Najdenski,H., Vesselinova,A. (2001) Isolation of pathogenic Yersiniae from wild animals in Bulgaria. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **48:** 203-209.

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

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

Niskanen,T., Waldenstrom,J., Fredriksson-Ahomaa,M., Olsen,B., Korkeala,H. (2003) *virF*-positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* found in migratory birds in Sweden. *Applied and Environmental Microbiology* **69:** 4670-4675.

Norrung,B., Buncic,S. (2008) Microbial safety of meat in the European Union. *Meat Science* **78:** 14-24.

Novoslavskij,A., Serniene,L., Malakauskas,A., Laukkanen-Ninios,R., Korkeala,H., Malakauskas,M. (2013) Prevalence and genetic diversity of enteropathogenic *Yersinia* spp. in pigs at farms and slaughter in Lithuania. *Research in Veterinary Science* **94:** 209-213.

Nowak,B., von Mueffling,T., Caspari,K., Hartung,J. (2006) Validation of a method for the detection of virulent *Yersinia enterocolitica* and their distribution in slaughter pigs from conventional and alternative housing systems. *Veterinary Microbiology* **117:** 219-228.

Nowgesic,E., Fyfe,M., Hockin,J., King,A., Ng,H., Paccagnella,A., Trinidad,A., Wilcott,L., Smith,R., Denney,A., Struck,L., Embree,G., Higo,K., Chan,J.L., Markey,P., Bush,M.S.D. (1999) Outbreak of *Yersinia pseudotuberculosis* in British Columbia - November 1998. *Canada Communicable Disease Report* **25:** 97-100.

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

O'Sullivan,T., Friendship,R., Blackwell,T., Pearl,D., McEwen,B., Carman,S., Slavic,D., Dewey,C. (2011) Microbiological identification and analysis of swine tonsils collected from carcasses at slaughter. *Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire* **75:** 106-111.

Oellerich,M.F., Jacobi,C.A., Freund,S., Niedung,K., Bach,A., Heesemann,J., Trülzsch,K. (2007) *Yersinia enterocolitica* infection of mice reveals clonal invasion and abscess formation. *Infection and Immunity* **75:** 3802-3811.

Oraic,D., Zrncic,S., Sostaric,B., Bazulic,D., Lipej,Z. (2002) Occurrence of enteric redmouth disease in rainbow trout (*Oncorhynchus mykiss*) on farms in Croatia. *Acta Veterinaria Hungarica* **50:** 283-291.

Ortiz Martínez,P., Fredriksson-Ahomaa,M., Pallotti,A., Rosmini,R., Houf,K., Korkeala,H. (2011) Variation in the prevalence of enteropathogenic *Yersinia* in slaughter pigs from Belgium, Italy, and Spain. *Foodborne Pathogens and Disease* **8:** 445-450.

Ortiz Martínez,P., Fredriksson-Ahomaa,M., Sokolova,Y., Roasto,M., Berzins,A., Korkeala,H. (2009) Prevalence of enteropathogenic *Yersinia* in Estonian, Latvian, and Russian (Leningrad region) pigs. *Foodborne Pathogens and Disease* **6:** 719-724.

Ortiz Martínez,P., Mylona,S., Drake,I., Fredriksson-Ahomaa,M., Korkeala,H., Corry,J.E.L. (2010) Wide variety of bioserotypes of enteropathogenic *Yersinia* in tonsils of English pigs at slaughter. *International Journal of Food Microbiology* **139:** 64-69.

Ostroff,S.M., Kapperud,G., Hutwagner,L.C., Nesbakken,T., Bean,N.H., Lassen,J., Tauxe,R.V. (1994) Sources of sporadic *Yersinia enterocolitica* infections in Norway: a prospective case-control study. *Epidemiology and Infection* **112:** 133-141.

Owston,M.A., Wu,C.C., Ramos-Vara,J.A. (2006) Hepatic yersiniosis in a cougar (Felis concolor). *Journal of Veterinary Diagnostic Investigation* **18:** 511-513.

Pepe,J.C., Miller,V.L. (1990) The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. *Journal of Bacteriology* **172:** 3780-3789.

Perdikogianni,C., Galanakis,E., Michalakis,M., Giannoussi,E., Maraki,S., Tselentis,Y., Charissis,G. (2006) *Yersinia enterocolitica* infection mimicking surgical conditions. *Pediatric Surgery International* **22:** 589-592.

Pham,J.N., Bell,S.M., Martin,L., Carniel,E. (2000) The beta-lactamases and beta-lactam antibiotic susceptibility of *Yersinia enterocolitica*. *Journal of Antimicrobial Chemotherapy* **46:** 951-957.

Pouplin,S., Daragon,A., Cambon-Michot,C., Dujardin,F., Biga,N., Pons,J.L., Le Loet,X. (2002) Septic arthritis of the hip caused by *Yersinia enterocolitica*: a case report. *Joint Bone Spine* **69:** 604-606.

Pulvirenti,D., Aikaterini,T., Neri,S. (2007) Septicemia, hepatic abscess, and encephalitis due to *Yersinia enterocolitica*. *Journal of Clinical Gastroenterology* **41:** 333-334.

Ramamurthy,T., Yoshino,K., Huang,X.Z., Nair,G.B., Carniel,E., Maruyama,T., Fukushima,H., Takeda,T. (1997) The novel heat-stable enterotoxin subtype gene (*ystB*) of *Yersinia enterocolitica*: nucleotide sequence and distribution of the *yst* genes. *Microbial Pathogenesis* **23:** 189-200.

Ranta,J., Siekkinen,K.M., Nuotio,L., Laukkanen,R., Hellstrom,S., Korkeala,H., Maijala,R. (2010) Causal hidden variable model of pathogenic contamination from pig to pork. *Statistical Modelling* **10:** 69-87.

Rathmell,W.K., Arguin,P., Chan,S., Yu,A. (1999) *Yersinia pseudotuberculosis* bacteremia and splenic abscess in a patient with non-insulin-dependent diabetes mellitus. *Western Journal of Medicine* **170:** 110-112.

Reiss-Zimmermann,M., Sorge,I., Schille,R., Beer,J., Hirsch,W. (2007) *Yersinia enterocolitica*-associated generalized microinfarctions of bone and spleen in a child. *Pediatric Radiology* **37:** 1294-1297.

Renaud,N., Lecci,L., Courcol,R.J., Simonet,M., Gaillot,O. (2013) CHROMagar Yersinia, a new chromogenic agar for screening of potentially pathogenic *Yersinia enterocolitica* isolates in stools. *Journal of Clinical Microbiology* **51:** 1184-1187.

Revell,P.A., Miller,V.L. (2001) *Yersinia* virulence: more than a plasmid. *Fems Microbiology Letters* **205:** 159-164.

Riley,G., Toma,S. (1989) Detection of pathogenic Yersinia enterocolitica by using congo red-magnesium oxalate agar medium. *Journal of Clinical Microbiology* **27:** 213-214.

Rimhanen-Finne,R., Niskanen,T., Hallanvuo,S., Makary,P., Haukka,K., Pajunen,S., Siitonen,A., Ristolainen,R., Poyry,H., Ollgren,J., Kuusi,M. (2009) *Yersinia pseudotuberculosis* causing a large outbreak associated with carrots in Finland, 2006. *Epidemiology and Infection* **137:** 342-347.

Robins-Browne,R.M., Still,C.S., Miliotis,M.D., Koornhof,H.J. (1979) Mechanism of action of *Yersinia enterocolitica* enterotoxin. *Infection and Immunity* **25:** 680-684.

Rosner,B.M., Stark,K., Höhle,M., Werber,D. (2012) Risk factors for sporadic *Yersinia enterocolitica* infections, Germany 2009-2010. *Epidemiology and Infection* **140:** 1738-1747.

Rosner,B.M., Stark,K., Werber,D. (2010) Epidemiology of reported *Yersinia enterocolitica* infections in Germany, 2001-2008. *Bmc Public Health* **10:** 337.

Rosner,B.M., Werber,D., Höhle,M., Stark,K. (2013) Clinical aspects and self-reported symptoms of sequelae of *Yersinia enterocolitica* infections in a population-based study, Germany 2009-2010. *Bmc Infectious Diseases* **13:** 236.

Ruusunen,M., Salonen,M., Pulkkinen,H., Huuskonen,M., Hellstrom,S., Revez,J., Hanninen,M.L., Fredriksson-Ahomaa,M., Lindstrom,M. (2013) Pathogenic bacteria in Finnish bulk tank milk. *Foodborne Pathogens and Disease* **10:** 99-106.

Saebo,A., Vik,E., Lange,O.V., Matuszkiewicz,L. (2005) Inflammatory bowel disease associated with *Yersinia enterocolitica* O:3 infection. *European Journal of Internal Medicine* **16:** 176-182.

Sakai,T., Nakayama,A., Hashida,M., Yamamoto,Y., Takebe,H., Imai,S. (2005) Outbreak of food poisoning by *Yersinia enterocolitica* serotype O8 in nara prefecture: The first case report in Japan. *Japanese Journal of Infectious Diseases* **58:** 257-258.

Sakellaris,G., Kakavelakis,K., Stathopoulos,E., Michailidou,H., Charissis,G. (2004) A palpable right lower abdominal mass due to *Yersinia* mesenteric lymphadenitis. *Pediatric Surgery International* **20:** 155-157.

Sammarco,M.L., Ripabelli,G., Ruberto,A., Iannitto,G., Grasso,G.M. (1997) Prevalence of Salmonellae, Listeriae, and Yersiniae in the slaughterhouse environment and on work surfaces, equipment, and workers. *Journal of Food Protection* **60:** 367-371.

Satterthwaite,P., Pritchard,K., Floyd,D., Law,B. (1999) A case-control study of *Yersinia enterocolitica* infections in Auckland. *Australian and New Zealand Journal of Public Health* **23:** 482-485.

Savin,C., Leclercq,A., Carniel,E. (2012) Evaluation of a single procedure allowing the isolation of enteropathogenic *Yersinia* along with other bacterial enteropathogens from human stools. *Plos One* **7:** e41176.

Scallan,E., Hoekstra,R.M., Angulo,F.J., Tauxe,R.V., Widdowson,M.A., Roy,S.L., Jones,J.L., Griffin,P.M. (2011) Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases* **17:** 7-15.

Scallan,E., Mahon,B.E., Hoekstra,R.M., Griffin,P.M. (2013) Estimates of illnesses, hospitalizations and deaths caused by major bacterial enteric pathogens in young children in the United States. *Pediatric Infectious Disease Journal* **32:** 217-221.

Schiemann,D.A. (1989) *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. In: *Foodborne bacterial pathogens.* Doyle MP (ed), pp. 601-672. Marcel Dekker, Inc., New York.

Schiemann,D.A. (1979) Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Canadian Journal of Microbiology* **25:** 1298-1304.

Schiemann,D.A. (1983) Alkalotolerance of *Yersinia enterocolitica* as a basis for selective isolation from food enrichments. *Applied and Environmental Microbiology* **46:** 22-27.

Schubert,S., Bockemühl,J., Brendler,U., Heesemann,J. (2003) First isolation of virulent *Yersinia enterocolitica* O8, biotype 1B in Germany. *European Journal of Clinical Microbiology & Infectious Diseases* **22:** 66-68.

Seimiya,Y.M., Sasaki,K., Satoh,C., Takahashi,M., Yaegashi,G., Iwane,H. (2005) Caprine enteritis associated with *Yersinia pseudotuberculosis* infection. *Journal of Veterinary Medical Science* **67:** 887-890.

Seuri,M., Granfors,K. (1992) Antibodies against *Yersinia* among farmers and slaughterhouse workers. *Scandinavian Journal of Work Environment & Health* **18:** 128-132.

Sharifi,Y.M.K., Soltan-Dallal,M.M., Zali,M.R., Avadisians,S., Bakhtiari,R. (2011) Incidence and antibiotic susceptibilities of *Yersinia enterocolitica* and other *Yersinia* species recovered from meat and chicken in Tehran, Iran. *African Journal of Microbiology Research* **5:** 2649-2653.

Sharma,N.K., Doyle,P.W., Gerbasi,S.A., Jessop,J.H. (1990) Identification of *Yersinia* species by the API 20E. *Journal of Clinical Microbiology* **28:** 1443-1444.

Sheridan,J.J., Logue,C.M., McDowell,D.A., Blair,I.S., Hegarty,T. (1998) A study of the growth kinetics of *Yersinia enterocolitica* serotype O:3 in pure and meat culture systems. *Journal of Applied Microbiology* **85:** 293-301.

Shiozawa,K., Nishina,T., Miwa,Y., Mori,T., Akahane,S., Ito,K. (1991) Colonization in the tonsils of swine by *Yersinia enterocolitica*. *Contributions to Microbiology and Immunology* **12:** 63-67.

Shorter,N.A., Thompson,M.D., Mooney,D.P., Modlin,J.F. (1998) Surgical aspects of an outbreak of *Yersinia* enterocolitis. *Pediatric Surgery International* **13:** 2-5.

Sihvonen,L.M., Hallanvuo,S., Haukka,K., Skurnik,M., Siitonen,A. (2011a) The *ail* gene is present in some *Yersinia enterocolitica* biotype 1A strains. *Foodborne Pathogens and Disease* **8:** 455-457.

Sihvonen,L.M., Haukka,K., Kuusi,M., Virtanen,M.J., Siitonen,A. (2009) *Yersinia enterocolitica* and *Y. enterocolitica*-like species in clinical stool specimens of humans: identification and prevalence of bio/serotypes in Finland. *European Journal of Clinical Microbiology & Infectious Diseases* **28:** 757-765.

Sihvonen,L.M., Toivonen,S., Haukka,K., Kuusi,M., Skurnik,M., Siitonen,A. (2011b) Multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. *Bmc Microbiology* **11:** 42.

Singh,I., Virdi,J.S. (2004) Production of *Yersinia* stable toxin (YST) and distribution of *yst* genes in biotype 1A strains of *Yersinia enterocolitica*. *Journal of Medical Microbiology* **53:** 1065-1068.

Skjerve,E., Lium,B., Nielsen,B., Nesbakken,T. (1998) Control of *Yersinia enterocolitica* in pigs at herd level. *International Journal of Food Microbiology* **45:** 195-203.

Skurnik,M., Bengoechea,J.A. (2003) Biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic Yersiniae. *Carbohydrate Research* **338:** 2521-2529.

Söderqvist,K., Boqvist,S., Wauters,G., Vagsholm,I., Thisted-Lambertz,S. (2012) *Yersinia enterocolitica* in sheep - a high frequency of biotype 1A. *Acta Veterinaria Scandinavica* **54:** 39.

Sörqvist,S. (2003) Heat resistance in liquids of *Enterococcus* spp., *Listeria* spp., *Escherichia coli*, *Yersinia enterocolitica*, *Salmonella* spp. and *Campylobacter* spp. *Acta Veterinaria Scandinavica* **44:** 1-19.

Souza,R.A., Pitondo-Silva,A., Falcão,D.P., Falcão,J.P. (2010) Evaluation of four molecular typing methodologies as tools for determining taxonomy relations and for identifying species among *Yersinia* isolates. *Journal of Microbiological Methods* **82:** 141-150.

Sprague,L.D., Neubauer,H. (2005) *Yersinia aleksiciae* sp nov. *International Journal of Systematic and Evolutionary Microbiology* **55:** 831-835.

Sprague,L.D., Scholz,H.C., Amann,S., Busse,H.J., Neubauer,H. (2008) *Yersinia similis* sp nov. *International Journal of Systematic and Evolutionary Microbiology* **58:** 952-958.

Stamm,I., Hailer,M., Depner,B., Kopp,P.A., Rau,J. (2013) Yersinia enterocolitica in diagnostic fecal samples from European dogs and cats: identification by Fourier transform infrared spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology* **51:** 887-893.

StataCorp. Stata: Release 12. Statistical Software. [Release 12]. 2011. College Station, TX: StataCorp LP.

Stephan,R., Cernela,N., Ziegler,D., Pfluger,V., Tonolla,M., Ravasi,D., Fredriksson-Ahomaa,M., Hachler,H. (2011) Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF Mass spectrometry. *Journal of Microbiological Methods* **87:** 150-153.

Stephan,R., Joutsen,S., Hofer,E., Säde,E., Björkroth,J., Ziegler,D., Fredriksson-Ahomaa,M. (2013) Characteristics of *Yersinia enterocolitica* biotype 1A strains isolated from patients and asymptomatic carriers. *European Journal of Clinical Microbiology & Infectious Diseases* **32:** 869-875.

Stölzel,F., Pursche,S., Bruckner,S., Bornhauser,M., Ehninger,G., Schaich,M. (2009) *Yersinia pseudotuberculosis* causing abscesses in a 31-year-old patient in the post-immunosuppression period after allogeneic HSCT. *Bone Marrow Transplantation* **43:** 515-516.

Strotmann,C., von Mueffling,T., Klein,G., Nowak,B. (2008) Effect of different concentrations of carbon dioxide and oxygen on the growth of pathogenic *Yersinia enterocolitica* 4/O : 3 in ground pork packaged under modified atmospheres. *Journal of Food Protection* **71:** 845-849.

Sulakvelidze,A. (2000) Yersiniae other than *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*: the ignored species. *Microbes and Infection* **2:** 497-513.

Tahara,M., Baba,K., Waki,K., Arakaki,Y. (2006) Analysis of Kawasaki disease showing elevated antibody titres of *Yersinia pseudotuberculosis*. *Acta Paediatrica* **95:** 1661-1664.

Tauxe,R.V., Wauters,G., Goossens,V., Van Noyen,R., Vandepitte,J., Martin,S.M., De Mol,P., Thiers,G. (1987) *Yersinia enterocolitica* infections and pork: the missing link. *Lancet* **329:** 1129-1132.

Terentjeva,M., Berzins,A. (2010) Prevalence and antimicrobial tesistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in slaughter pigs in Latvia. *Journal of Food Protection* **73:** 1335-1338.

Thibodeau,V., Frost,E.H., Chenier,S., Quessy,S. (1999) Presence of *Yersinia enterocolitica* in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter. *Canadian Journal of Veterinary Research* **63:** 96-100.

Thibodeau,V., Frost,E.H., Quessy,S. (2001) Development of an ELISA procedure to detect swine carriers of pathogenic *Yersinia enterocolitica*. *Veterinary Microbiology* **82:** 249-259.

Thisted Lambertz,S., Danielsson-Tham,M.L. (2005) Identification and characterization of pathogenic *Yersinia enterocolitica* isolates by PCR and pulsed-field gel electrophoresis. *Applied and Environmental Microbiology* **71:** 3674-3681.

Thisted Lambertz,S., Granath,K., Fredriksson-Ahomaa,M., Johansson,K.E., Danielsson-Tham,M.L. (2007) Evaluation of a combined culture and PCR method (NMKL-163A) for detection of presumptive pathogenic *Yersinia enterocolitica* in pork products. *Journal of Food Protection* **70:** 335-340.

Thisted Lambertz,S., Lindqvist,R., Ballagi-Pordany,A., Danielsson-Tham,M.L. (2000) A combined culture and PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *International Journal of Food Microbiology* **57:** 63-73.

Thisted Lambertz,S., Nilsson,C., Hallanvuo,S. (2008a) TaqMan-based real-time PCR method for detection of *Yersinia pseudotuberculosis* in food. *Applied and Environmental Microbiology* **74:** 6465-6469.

Thisted Lambertz,S., Nilsson,C., Hallanvuo,S., Lindblad,M. (2008b) Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Applied and Environmental Microbiology* **74:** 6060-6067.

Thoerner,P., Bin Kingombe,C.I., Bogli-Stuber,K., Bissig-Choisat,B., Wassenaar,T.M., Frey,J., Jemmi,T. (2003) PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Applied and Environmental Microbiology* **69:** 1810-1816.

Tsubokura,M., Aleksic,S. (1995) A simplified antigenic scheme for serotyping of *Yersinia pseudotuberculosis*: phenotypic characterization of reference strains and preparation of O and H factor sera. *Contributions to Microbiology and Immunology* **13:** 99-105.

Van Damme,I., Berkvens,D., Baré,J., De Zutter,L. (2013) Influence of isolation methods on the occurrence of plasmid-carrying *Yersinia enterocolitica* serotype O:3 in slaughter pig tonsils, faeces and carcass surface swabs. *International Journal of Food Microbiology* **164:** 32-35.

van Zonneveld,M., Droogh,J.M., Fieren,M.W.J.A., Gyssens,I.C., van Gelder,T., Weimar,W. (2002) *Yersinia pseudotuberculosis* bacteraemia in a kidney transplant patient. *Nephrology Dialysis Transplantation* **17:** 2252-2254.

Verhaegen,J., Charlier,J., Lemmens,P., Delmée,M., Van Noyen,R., Verbist,L., Wauters,G. (1998) Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1967-1996. *Clinical Infectious Diseases* **27:** 59-64.

Viboud,G.I., Bliska,J.B. (2005) *Yersinia* outer proteins: Role in modulation of host cell signaling responses and pathogenesis. *Annual Review of Microbiology* **59:** 69-89.

Vico,J.P., Mainar-Jaime,R.C. (2011) The use of meat juice or blood serum for the diagnosis of *Salmonella* infection in pigs and its possible implications on *Salmonella* control programs. *Journal of Veterinary Diagnostic Investigation* **23:** 528-531.

Vincent,P., Leclercq,A., Martin,L., Duez,J.M., Simonet,M., Carniel,E. (2008) Sudden onset of pseudotuberculosis in humans, France, 2004-05. *Emerging Infectious Diseases* **14:** 1119-1122.

Vincent,P., Salo,E., Skurnik,M., Fukushima,H., Simonet,M. (2007) Similarities of Kawasaki disease and *Yersinia pseudotuberculosis* infection epidemiology. *Pediatric Infectious Disease Journal* **26:** 629-631.

Virtanen,S., Korkeala,H. (2012) Enteropathogenic *Yersinia* in pig production. *Wiener Tierarztliche Monatsschrift* **99:** 298-305.

Virtanen,S., Laukkanen-Ninios,R., Ortiz Martínez,P., Siitonen,A., Fredriksson-Ahomaa,M., Korkeala,H. (2013) Multiple-locus variable-number tandem repeats analysis in genotyping *Yersinia enterocolitica* strains from human and porcine origin. *Journal of Clinical Microbiology* **51:** 2154-2159.

Virtanen,S., Salonen,L., Laukkanen-Ninios,R., Fredriksson-Ahomaa,M., Korkeala,H. (2012) Piglets are a source of pathogenic *Yersinia enterocolitica* on fattening-pig farms. *Applied and Environmental Microbiology* **78:** 3000-3003.

Virtanen,S.E., Salonen,L.K., Laukkanen,R., Hakkinen,M., Korkeala,H. (2011) Factors related to the prevalence of pathogenic *Yersinia enterocolitica* on pig farms. *Epidemiology and Infection* **139:** 1919-1927.

Vishnubhatla,A., Oberst,R.D., Fung,D.Y.C., Wonglumsom,W., Hays,M.P., Nagaraja,T.G. (2001) Evaluation of a 5 '-nuclease (TaqMan) assay for the detection of virulent strains of *Yersinia enterocolitica* in raw meat and tofu samples. *Journal of Food Protection* **64:** 355-360.

von Altrock,A., Louis,A.L., Rosler,U., Beyerbach,M., Kreienbrock,L., Waldmann,K.H. (2006) Study on the bacteriological and serological prevalence of *Campylobacter* spp. and *Yersinia enterocolitica* in fattening pig herds in Lower Saxony. *Berliner und Munchener Tierarztliche Wochenschrift* **119:** 391-399.

von Altrock,A., Roesler,U., Merle,R., Waldmann,K.H. (2010) Prevalence of pathogenic *Yersinia enterocolitica* strains on liver surfaces of pigs and their antimicrobial susceptibility. *Journal of Food Protection* **73:** 1680-1683.

von Altrock,A., Roesler,U., Waldmann,K.H. (2011) Herd factors associated with the serological *Yersinia* prevalence in fattening pig herds. *Foodborne Pathogens and Disease* **8:** 1249-1255.

von Eckardstein,K., Spuler,A., Brauer,C., Mehl,M., Kiwit,J. (2004) Spontaneous cervical osteomyelitis due to *Yersinia enterocolitica* in a non-immunocompromised host. *European Journal of Clinical Microbiology & Infectious Diseases* **23:** 66-68.

Wauters,G. (1973) Diagnostic biologique des infections à *Yersinia enterocolitica*. *Medecine et Maladies Infectieuses* **3:** 437-441.

Wauters,G., Goossens,V., Janssens,M., Vandepitte,J. (1988) New enrichment method for isolation of pathogenic *Yersinia enterocolitica* serogroup O:3 from pork. *Applied and Environmental Microbiology* **54:** 851-854.

Wauters,G., Kandolo,K., Janssens,M. (1987) Revised biogrouping scheme of *Yersinia enterocolitica*. *Contributions to Microbiology and Immunology* **9:** 14-21.

Wauters,G., Pohl,P., Stevens,J. (1976) Portage de *Yersinia enterocolitica* par le porc de boucherie. I. Recherche de *Yersinia enterocolitica* dans les matières fécales et les amygdales de porc. *Medecine et Maladies Infectieuses* **6:** 484-485.

Wauters,G., Vaneechoutte,M. (2011) Approaches to the identification of aerobic gram-negative bacteria. In: *Manual of Clinical Microbiology, 10th Edition.* Versalovic J (ed), ASM Press.

Weagant,S.D. (2008) A new chromogenic agar medium for detection of potentially virulent *Yersinia enterocolitica*. *Journal of Microbiological Methods* **72:** 185-190.

Wehebrink,T., Kemper,N., Beilage,E.G., Krieter,J. (2008) Prevalence of *Campylobacter* spp. and *Yersinia* spp. in the pig production. *Berliner und Munchener Tierarztliche Wochenschrift* **121:** 27-32.

Wesley,I.V., Bhaduri,S., Bush,E. (2008) Prevalence of *Yersinia enterocolitica* in market weight hogs in the United States. *Journal of Food Protection* **71:** 1162-1168.

Wessels,M.E., Payne,J.H., Bannerman,R.P. (2009) Oculoglandular syndrome caused by *Yersinia pseudotuberculosis* in a dairy goat. *Journal of Comparative Pathology* **141:** 190-194.

Weynants,V., Jadot,V., Denoel,P.A., Tibor,A., Letesson,J.J. (1996) Detection of *Yersinia enterocolitica* serogroup O:3 by a PCR method. *Journal of Clinical Microbiology* **34:** 1224-1227.

WIV. Surveillance van infectieuze aandoeningen door een netwerk van laboratoria voor microbiologie 2011 - Epidemiologische trends 1983-2010. Ducoffre, G. D/2012/2502/34. 2012. Wetenschappelijk Instituut Volksgezondheid (WIV),OD Volksgezondheid en Surveillance.

Wobeser,G., Campbell,G.D., Dallaire,A., McBurney,S. (2009) Tularemia, plague, yersiniosis, and Tyzzer's disease in wild rodents and lagomorphs in Canada: a review. *Canadian Veterinary Journal-Revue Veterinaire Canadienne* **50:** 1251-1256.

Wong,K.K., Fistek,M., Watkins,R.R. (2013) Community-acquired pneumonia caused by *Yersinia enterocolitica* in an immunocompetent patient. *Journal of Medical Microbiology* **62:** 650-651.

Wren,B.W., Tabaqchali,S. (1990) Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. *Lancet* **336:** 693.

Xanthopoulos,V., Tzanetakis,N., Litopoulou-Tzanetaki,E. (2010) Occurrence and characterization of *Aeromonas hydrophila* and *Yersinia enterocolitica* in minimally processed fresh vegetable salads. *Food Control* **21:** 393-398.

Yang,Y.X., Merriam,J.J., Mueller,J.P., Isberg,R.R. (1996) The *psa* locus is responsible for thermoinducible binding of *Yersinia pseudotuberculosis* to cultured cells. *Infection and Immunity* **64:** 2483-2489.

Yoshino,K.-I., Ramamurthy,T., Nair,G.B., Fukushima,H., Ohtomo,Y., Takeda,N., Kaneko,S., Takeda,T. (1995a) Geographical heterogeneity between Far East and Europe in prevalence of *ypm* gene encoding the novel superantigen among *Yersinia pseudotuberculosisi* strains. *Journal of Clinical Microbiology* **33:** 3356-3358.

Yoshino,K.-I., Takao,T., Huang,X., Murata,H., Nakao,H., Takeda,T., Shimonishi,Y. (1995b) Characterization of a highly toxic, large molecular size heat-stable enterotoxin produced by a clinical isolate of *Yersinia enterocolitica*. *Febs Letters* **362:** 319-322.

Yotsu,R., Mii,S., Hayashi,R., Harada,H., Furukawa,K., Eto,H. (2010) Erythema nodosum associated with *Yersinia enterocolitica* infection. *Journal of Dermatology* **37:** 819-822.

Zhang,S.P., Zhang,Z.Y., Liu,S.L., Bingham,W., Wilson,F. (2008) Fatal yersiniosis in farmed deer caused by *Yersinia pseudotuberculosis* serotype O:3 encoding a mannosyltransferase-like protein WbyK. *Journal of Veterinary Diagnostic Investigation* **20:** 356-359.

Zheng,H., Sun,Y., Lin,S., Mao,Z., Jiang,B. (2008a) *Yersinia enterocolitica* infection in diarrheal patients. *European Journal of Clinical Microbiology & Infectious Diseases* **27:** 741-752.

Zheng,H.X., Sun,Y., Mao,Z.G., Jiang,B. (2008b) Investigation of virulence genes in clinical isolates of *Yersinia enterocolitica*. *Fems Immunology and Medical Microbiology* **53:** 368-374.

Summary

Yersiniosis, the third most frequently reported zoonosis in Belgium, is caused by human pathogenic biotypes of *Yersinia enterocolitica* and to a lesser extent also by *Yersinia pseudotuberculosis*. These so-called enteropathogenic *Yersinia* spp. harbour several virulence genes, located on the chromosome and on a 70-kb virulence plasmid, which are reviewed in the **general introduction**. Enteropathogenic *Yersinia* may cause a wide spectrum of manifestations, but mainly manifest in young children under five years of age as gastro-enteritis, with fever, diarrhoea and abdominal pain as most common symptoms. Most yersiniosis cases in Belgium are caused by *Y. enterocolitica* bioserotype 4/O:3, which is primarily transmitted to humans *via* consumption of contaminated water or food, particularly pork. Enteropathogenic Yersinia are found in a variety of animals, though pigs are regarded as the principal reservoir of these pathogens. Hence, this doctoral thesis focuses on the occurrence of enteropathogenic *Yersinia* throughout the pork production chain. The detection of enteropathogenic *Yersinia* in living animals is difficult, so most studies are conducted at abattoir level. At time of slaughter, many pigs harbour these pathogens in their tonsils and intestinal tract, from which the pathogens may be transferred to the carcass during slaughter. Despite the well-known relation between yersiniosis and pork, the current situation of enteropathogenic *Yersinia* in pigs and pork in Belgium remains largely unknown. Nevertheless, implementation of interventions leading to a reduction of pork contamination necessitates a better understanding of the epidemiology of the pathogen. Therefore, the **aim** of this thesis was to gain more insight in the occurrence of enteropathogenic *Yersinia* along the pork production chain.

As adequate sampling and culture methods are essential to obtain reliable results, different methodologies were evaluated during this thesis for the isolation of human pathogenic *Y. enterocolitica* from pig matrices. The ISO 10273:2003 method was used as a reference method to compare different isolation methods. The ISO method combines two parallel methods: enrichment in irgasan-ticarcillin-potassium chlorate (ITC) broth and peptone-sorbitol-bile (PSB) broth and plating on respectively *Salmonella-Shigella* deoxycholate calcium chloride (SSDC) agar and cefsulodin-irgasan-novobiocin (CIN) agar.

In **Chapter 1**, tonsil samples from pigs at slaughter were examined by direct plating and enrichment procedures based on the ISO 10273:2003 method. In total, 37.4% of the tonsil samples were positive for human pathogenic *Y. enterocolitica*, all belonging to bioserotype 4/O:3. Fifty out of the 52 positive samples (96.2%) were detected by direct plating, for which enumeration showed an average concentration of 4.5 $\log_{10}$ colony forming units (CFU) per gram. The enrichment procedures recommended by the ISO 10273:2003 method were not optimal for the isolation of pathogenic *Y. enterocolitica* from pig tonsils, though reducing the enrichment time in PSB from 5 to 2 days resulted in a significantly higher recovery rate. Direct plating was shown to be an effective and rapid culture method for the isolation of human pathogenic *Y. enterocolitica* from pig tonsils, and provided quantitative data.

The objective of **Chapter 2** was to determine the effect of sampling on the recovery of human pathogenic *Y. enterocolitica* from pig tonsils. Therefore, swab and destructive samples were analysed in parallel using direct plating and different short enrichment methods. Although swab

samples are easier and faster compared to destructive samples, homogenization of tissue samples was required to obtain reliable results. The performance of *Y. enterocolitica* chromogenic medium (YeCM) for qualitative and quantitative isolation of pathogenic *Y. enterocolitica* from pig tonsils was equal to that of the standard media SSDC and CIN.

In **Chapter 3**, pig carcass swabs and minced meat samples were examined for pathogenic *Y.* enterocolitica using different enrichment protocols. In total, 28 carcasses (11.0%) were contaminated with *Y. enterocolitica* bioserotype 4/O:3 and one (0.4%) with bioserotype 2/O:9. Four minced meat samples (4.9%) tested positive for *Y. enterocolitica* bioserotype 4/O:3. Using the ISO 10273:2003 method, eight out of the 29 *Yersinia*-positive carcasses and none of the contaminated minced meat samples were detected. Reducing the enrichment time in PSB from 5 to 2 days also increased the number of positive carcass and minced meat samples. Overall, enrichment in PSB at 25°C recovered more positive samples than selective enrichment in ITC and cold enrichment.

A cross-sectional survey was undertaken in **Chapter 4** to determine the distribution of enteropathogenic *Yersinia* spp. in pigs at slaughter in Belgium and to study factors associated with pig carcass contamination. *Y. enterocolitica* serotype O:3 was isolated from the tonsils and faeces of 55.3% and 25.6% of the tested pigs, and *Y. pseudotuberculosis* from 1.4% and 0.6%, respectively. The pathogens were also recovered from 39.7% of the carcass surfaces. The highest occurrence was found for the mandibular region, followed by the sternal region, pelvic duct, and split surface near the sacral vertebrae. The quantitative load was the highest for the mandibular region, though the vast majority of carcass samples had *Yersinia* concentrations below the detection limit of enumeration (<5 CFU/100cm^2). The presence of human pathogenic *Y. enterocolitica* in the faeces was significantly associated with carcass contamination at all sampled areas. Other risk factors for carcass contamination included the presence of the pathogen in tonsils, incision of the tonsils during removal of the plug set, and splitting of the head together with the carcass, whereas cleaning and disinfection of knifes before removal of the plug set was a protective factor. Moreover, differences between high-capacity and low-capacity abattoirs were found for contamination of the sternum.

The aim of **Chapter 5** was to determine the association between serology and bacteriology for the detection of enteropathogenic *Yersinia* in individual pigs at slaughter. Diaphragm muscles, tonsils, and faecal samples were collected from fattening pigs during slaughter. Anti-*Yersinia* antibodies in meat juice samples were detected using an indirect enzyme-linked immunosorbent assay (ELISA) based on Yops (*Yersinia* outer proteins). Positive serotiters were highly correlated with the recovery of enteropathogenic *Yersinia* from the tonsils, whereas no correlation was found with faecal excretion. Results demonstrated that serology has an acceptable sensitivity, but a relatively low specificity for the rapid detection of enteropathogenic *Yersinia* in tonsils of pigs at slaughter.

As described in the **general discussion**, this thesis revealed several limitations of standard isolation procedures and demonstrated that both sampling and isolation methods have a

significant influence on the final outcome of a study. The contamination rate varied widely between different areas of the carcass, and inclusion of the head region resulted in a higher number of *Yersinia* positive carcasses. Using the standard culture methods available for the recovery of pathogenic *Y. enterocolitica*, the use of a stereomicroscope and experience in the selection of suspected colonies are essential to obtain reliable results. Throughout this thesis, it was demonstrated that enteropathogenic *Yersinia*, particularly *Y. enterocolitica* bioserotype 4/O:3, are common in Belgian pigs at slaughter, and that tonsils are often contaminated in high numbers. Reducing the number of pork-related yersiniosis cases requires an integrated control from farm to fork. The excretion or carriage of *Y. enterocolitica* in pig faeces and tonsils are important factors for carcass contamination and spread of the pathogens may be reduced by proper hygienic practices and enhanced slaughter techniques. Further research should focus on the spread of enteropathogenic *Yersinia*, both on-farm and during slaughter.

Samenvatting

Yersiniose is de derde meest gerapporteerde zoönose in België en wordt veroorzaakt door humaan pathogene biotypes van *Yersinia enterocolitica* en in mindere mate ook door *Yersinia pseudotuberculosis*. Deze enteropathogene *Yersinia* species bezitten verschillende virulentiegenen, die zowel chromosomaal als op een 70-kb virulentieplasmide gelegen zijn en worden besproken in de **algemene inleiding**. Infecties met enteropathogene *Yersinia* kunnen zich uiten in verschillende vormen en komen voornamelijk voor bij jonge kinderen (onder de vijf jaar). Hierbij manifesteert de ziekte zich voornamelijk als een gastro-enteritis met als belangrijkste symptomen koorts, diarree en buikpijn. De meeste *Yersinia* infecties in België worden veroorzaakt door *Y. enterocolitica* bioserotype 4/O:3. De mens wordt voornamelijk besmet door consumptie van gecontamineerd water en voedsel, varkensvlees in het bijzonder. Enteropathogene *Yersinia* worden teruggevonden bij verschillende diersoorten, maar varkens worden aanzien als het belangrijkste reservoir. Om deze reden is deze doctoraatsthesis gericht op het voorkomen van enteropathogene *Yersinia* doorheen de productieketen van varkensvlees. Het aantonen van enteropathogene *Yersinia* bij levende dieren is moeilijk, waardoor de meeste studies worden uitgevoerd in het slachthuis. Op het moment van slachten zijn varkens frequent drager in de amandelen en darmen, van waaruit deze pathogenen tijdens het slachten kunnen worden overgedragen op het karkas. Ondanks de duidelijke associatie tussen yersiniose en varkensvlees, is er in België weinig gekend over het voorkomen van enteropathogene *Yersinia* in varkens en varkensvlees. Een betere kennis over de epidemiologie van deze pathogenen is echter onontbeerlijk om tot een reductie van de contaminatie van varkensvlees te kunnen komen. Daarom was het **doel** van deze thesis een beter inzicht te verkrijgen in het voorkomen van enteropathogene *Yersinia* doorheen de productieketen van varkensvlees.

Aangezien geschikte staalname- en cultuurmethoden essentieel zijn om betrouwbare resultaten te bekomen, werden tijdens deze thesis verschillende methoden geëvalueerd om humaan pathogene *Y. enterocolitica* uit varkensmatrices te isoleren. De ISO 10273:2003 werd als referentiemethode gebruikt om verschillende isolatiemethoden te vergelijken. De ISO methode omvat twee parallelle methoden: aanrijking in irgasan-ticarcillin-potassium chlorate (ITC) bouillon en peptone-sorbitol-bile (PSB) bouillon met uitplating op respectievelijk *Salmonella-Shigella* deoxycholate calcium chloride (SSDC) agar en cefsulodin-irgasan-novobiocin (CIN) agar.

In **hoofdstuk 1** werden varkensamandelen bemonsterd in het slachthuis en onderzocht met behulp van directe uitplating en aanrijkingsmethoden gebaseerd op de ISO 10273:2003 methode. In totaal werden 37.4% van de amandelen positief bevonden op de aanwezigheid van humaan pathogene *Y. enterocolitica*, allen behorend tot bioserotype 4/O:3. Vijftig van de 52 positieve stalen (96.2%) werden gedetecteerd met directe uitplating, waarbij de gemiddelde besmetting 4.5 $\log_{10}$ kolonievormende eenheden (KVE) per gram was. De voorgeschreven ISO 10273:2003 methode bleek niet optimaal te zijn voor de isolatie van pathogene *Y. enterocolitica* uit varkensamandelen. Een verkorting van de aanrijkingstijd in PSB van 5 naar 2 dagen leidde tot een significante verhoging van het aantal positieve stalen. Tijdens deze studie werd ook aangetoond dat directe uitplating een efficiënte en snelle cultuurmethode is voor de isolatie van humaan pathogene *Y. enterocolitica* uit varkensamandelen, waarbij bovendien de kiemen gekwantificeerd kunnen worden.

Het doel van **hoofdstuk 2** was om het effect van bemonsteringsmethoden op de isolatie van humaan pathogene *Y. enterocolitica* uit varkensamandelen te bepalen. Hiervoor werden swab en destructieve stalen onderzocht d.m.v. directe uitplating en enkele korte aanrijkingsmethoden. Alhoewel swabs gemakkelijker en sneller zijn in vergelijking met destructieve stalen, bleek deze laatste methode echter noodzakelijk om betrouwbare resultaten te bekomen. Het chromogeen medium voor *Y. enterocolitica* (YeCM) bleek even goed te zijn als de standaard media SSDC en CIN voor zowel de kwalitatieve als kwantitatieve isolatie van pathogene *Y. enterocolitica* uit varkensamandelen.

In **hoofdstuk 3** werden karkasswabs en gehaktstalen onderzocht op de aanwezigheid van pathogene *Y. enterocolitica* door middel van verschillende aanrijkingsprotocols. In totaal waren 28 karkassen (11.0%) gecontamineerd met *Y. enterocolitica* bioserotype 4/O:3 en één (0.4%) met bioserotype 2/O:9. Vier gehakstalen (4.9%) testten positief op *Y. enterocolitica* bioserotype 4/O:3. Met de ISO 10273:2003 methode werden slechts acht van de 29 *Yersinia* positieve karkassen en geen enkele van de besmette gehaktstalen gedetecteerd. Wanneer de aanrijkingstijd in PSB ingekort werd van 5 naar 2 dagen, verhoogde het aantal positieve karkassen en gehaktstalen.

Er werd een dwarsdoorsnede-studie uitgevoerd in **hoofdstuk 4** om de verspreiding van enteropathogene *Yersinia* species bij slachtvarkens in België te bepalen en factoren te bestuderen die gerelateerd zijn met de contaminatie van varkenskarkassen. *Y. enterocolitica* serotype O:3 werd geïsoleerd uit de amandelen en mest van respectievelijk 55.3% en 25.6% van de onderzochte varkens en *Y. pseudotuberculosis* uit respectievelijk 1.4% en 0.6% van de stalen. De kiemen werden tevens geïsoleerd van 39.7% van de karkassen. De kaakregio was het meest besmet, gevolgd door de borst, het bekkenkanaal en het kliefvlak ter hoogte van het sacrum. De kwantitatieve contaminatie was het hoogst ter hoogte van de kaakregio, maar de overgrote meerderheid van de karkasstalen bevatte aantallen onder de telbare detectielimiet (<5 KVE/100cm²). De aanwezigheid van humaan pathogene *Y. enterocolitica* in de mest was een significante risicofactor voor karkasbesmetting voor alle bemonsterde zones. Overige risicofactoren voor karkascontaminatie waren de aanwezigheid van de kiem in de amandelen, het insnijden van de amandelen tijdens het verwijderen van de hartslag en het klieven van de kop. Het reinigen en desinfecteren van messen voor de uitsnijden van de hartslag was daarentegen een beschermende factor. Bovendien werden voor karkascontaminatie ter hoogte van de borst verschillen tussen grote en kleine slachthuizen gevonden.

Het doel van **hoofdstuk 5** was om het verband te bepalen tussen serologie en bacteriologie voor de detectie van enteropathogene *Yersinia* bij individuele varkens. Tijdens het slachten werden een deel van het middenrif, de amandelen en de mest verzameld en werden anti-*Yersinia* antistoffen in vleessap bepaald d.m.v. een indirecte enzyme-linked immunosorbent assay (ELISA) die gebaseerd was op Yops (*Yersinia* outer proteins). Positieve serumtiters waren sterk gecorreleerd met de isolatie van enteropathogene *Yersinia* uit de amandelen, terwijl er geen correlatie gevonden werd met uitscheiding in de mest. De resultaten toonden aan dat serologie

een aanvaardbare gevoeligheid heeft, maar een relatief lage specificiteit voor de snelle detectie van enteropathogene *Yersinia* in varkensamandelen.

Zoals besproken wordt in de **algemene discussie**, toonde deze thesis verschillende beperkingen van standaard gebruikte isolatiemethoden aan en bewees dat zowel de bemonsterings- als de isolatiemethoden een belangrijke invloed hebben op de bekomen resultaten. De contaminatie varieerde sterk tussen verschillende zones van het karkas, en het bemonsteren van de kopregio resulteerde in een hoger aantal *Yersinia* positieve karkassen. Wanneer de standaard isolatiemethoden gebruikt worden om *Y. enterocolitica* aan te tonen, is het noodzakelijk om een stereomicroscoop te gebruiken en is voldoende kennis nodig om verdachte kolonies te herkennen om tot betrouwbare resultaten te komen. Tijdens deze studie werd aangetoond dat enteropathogene *Yersinia*, en meer bepaald *Y. enterocolitica* bioserotype 4/O:3, veel voorkomt bij Belgische slachtvarkens, en dat amandelen vaak met hoge aantallen besmet zijn. Om tot een reductie te komen van het aantal yersiniose gevallen, zijn maatregelen van riek tot vork noodzakelijk. Vernieuwde slachttechnieken, vooral gericht op het beperken van contaminatie via mest en amandelen zijn mogelijke interventies op slachthuisniveau. Verder onderzoek naar de verspreiding van enteropathogene *Yersinia*, zowel in de primaire fase als op slachthuis-niveau, is echter aangewezen.

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Inge

Curriculum vitae

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Inge Van Damme werd geboren op 25 juni 1984 te Sint-Niklaas. Na het beëindigen van haar secundaire studies Wetenschappen-Wiskunde aan de Broederschool Humaniora te Sint-Niklaas, startte ze in 2002 met de studies Diergeneeskunde aan de Universiteit Gent. Zij behaalde in 2008 het diploma van Dierenarts (optie Onderzoek) met onderscheiding. In februari 2009 startte zij als bursaal van het Bijzonder Onderzoeksfonds van de UGent haar doctoraatsonderzoek bij de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid van de faculteit Diergeneeskunde. Dit onderzoek handelde over het voorkomen van enteropathogene *Yersinia* species (*Y. enterocolitica* en *Y. pseudotuberculosis*) tijdens het slachten van varkens. Het onderzoek werd begeleid door Prof. Dr. L. De Zutter en Prof. Dr. D. Berkvens. In 2011 won zij de IMS prijs voor Meat Science and Technology en tevens vervolledigde zij in 2013 het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de UGent. Inge Van Damme is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam actief deel aan meerdere nationale en internationale congressen.

Scientific publications

Van Damme I., Habib I., De Zutter L. (2010). *Yersinia enterocolitica* in slaughter pig tonsils: Enumeration and detection by enrichment versus direct plating culture. Food Microbiology 27, 158-161.

Van Damme I., Berkvens D., De Zutter L. (2012). Effect of sampling and short isolation methodologies on the recovery of human pathogenic *Yersinia enterocolitica* from pig tonsils. Foodborne pathogens and disease 9, 600-606.

Van Damme I., Berkvens D., Baré J., De Zutter L. (2013). Influence of isolation methods on the occurrence of plasmid-carrying *Yersinia enterocolitica* serotype O:3 in slaughter pig tonsils, faeces and carcass surface swabs. International Journal of Food Microbiology 164, 32-35.

Vanantwerpen G., Houf K., **Van Damme I.**, Berkvens D., De Zutter L. (2013). Estimation of the within-batch prevalence and quantification of human pathogenic *Yersinia enterocolitica* in pigs at slaughter. Food Control 34, 9-12.

Van Damme I., Berkvens D., Botteldoorn N., Dierick K., Wits J., Pochet B., De Zutter L. (2013). Evaluation of the ISO 10273:2003 method for the isolation of human pathogenic *Yersinia enterocolitica* from pig carcasses and minced meat. Food Microbiology 36, 170-175.

Lambrecht E., Baré J., **Van Damme I.**, Bert W., Sabbe K., Houf K. (2013). *Yersinia enterocolitica* behavior in the presence of the bacterivorous *Acanthamoeba castellanii*. Applied and Environmental Microbiology, in press, doi:10.1128/AEM.01915-13.

Van Damme I., Berkvens D., Vanantwerpen G., Wauters G., De Zutter L. Risk factors associated with pig carcass contamination by enteropathogenic *Yersinia* spp. in Belgium. In preparation.

Van Damme I., Vanantwerpen G., Berkvens D., De Zutter L. Correspondence between serology and bacteriology of enteropathogenic *Yersinia* spp. in pigs at slaughter. In preparation.

Abstracts and proceedings

Van Damme I., Habib I., De Zutter L. (2008) Quantitative and qualitative estimation of the occurrence of pathogenic *Yersinia enterocolitica* in tonsils from Belgian slaughter pigs. 13th Conference on Food Microbiology, 11-12 September 2008, Ghent, Belgium.

Van Damme I., De Zutter L. (2010). Detection and enumeration of human pathogenic *Yersinia enterocolitica*: comparison of direct plating and enrichment, VIIème Congrès de la SFM, 2-4 June 2010, Marseille, France.

Van Damme I., De Zutter L. (2010). Comparison of swab and destructive samples for recovery of pathogenic *Yersinia enterocolitica* from pig tonsils, 22nd International ICFMH Symposium (FoodMicro 2010), 30 August – 3 September 2010, Copenhagen, Denmark.

Van Damme I., De Zutter L. (2010). Isolation of pathogenic *Yersinia enterocolitica* from porcine tonsils: comparison of swabbing and destructive samples, 15th Conference on Food Microbiology, 16-17 September 2010, Ghent, Belgium.

Van Damme I., De Zutter L. (2010). Comparison of swabbing and destructive samples for recovery of pathogenic *Yersinia enterocolitica* from pig tonsils, 10th International Symposium on *Yersinia*, 23-27 October 2010, Recife, Brazil.

Van Damme I., De Zutter L. (2011). Occurrence of human enteropathogenic *Yersinia* spp. in Belgian pigs and contamination of pork carcasses during slaughter, 9th International Conference on the Epidemiology and Control of biological, chemical and physical hazards in pigs and pork (SafePork 2011), 19-22 June 2011, Maastricht, The Netherlands, Proceedings book p 126-129.

Van Damme I., De Zutter L. (2011). Occurrence of human enteropathogenic *Yersinia* spp. in pigs and contamination of pork carcasses during slaughter, 4th Congress of European Microbiologists (FEMS 2011), 26-30 June 2011, Geneva, Switzerland.

Van Damme I., De Zutter L. (2011). Occurrence of human enteropathogenic *Yersinia* spp. in pigs and contamination of pork carcasses during slaughter, SfAM Summer Conference 2011, 4 July 2011, Dublin, Ireland.

Van Damme I., De Zutter L. (2011). Occurrence of human enteropathogenic *Yersinia* spp. in pigs and contamination of pork carcasses during slaughter, 57th International Congress of Meat Science and Technology, 7-12 August 2011, Ghent, Belgium.

Van Damme I., De Zutter L. (2011). Human pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in Belgium: prevalence in pigs and contamination of pork carcasses during slaughter, 16th Conference on Food Microbiology, 22-23 September 2011, Brussels, Belgium.

Van Damme I., Berkvens D., Vanantwerpen G., Baré J., De Zutter L. (2012). Risk factors for carcass contamination with pathogenic *Yersinia enterocolitica* in Belgian pig slaughterhouses, 17th Conference on Food Microbiology, 20-21 September 2012, Brussels, Belgium.

Van Damme I., Berkvens D., Botteldoorn N., Dierick K., Pochet B., De Zutter L. (2012). Isolation of pathogenic *Yersinia enterocolitica* from pork carcasses and minced meat samples in the context of official monitoring in Belgium, 17th Conference on Food Microbiology, 20-21 September 2012, Brussels, Belgium.

Lambrecht E., Baré J., **Van Damme I.**, Sabbe K., Houf K. (2013). Intra- and extracellular survival of human pathogenic *Yersinia enterocolitica* in coculture with the bacterivorous protozoon *Acanthamoeba castellanii*, IAFP European Symposium, 15-17 May 2013, Marseille, France.

Seliwiorstow T., Baré J., **Van Damme I.**, Uyttendaele M., De Zutter L. (2013). *Campylobacter* transmission between batches in a Belgian broiler slaughterhouse. IAFP European Symposium, 15-17 May 2013, Marseille, France.

Vanantwerpen G., Houf K., **Van Damme I.**, De Zutter L. (2013). Within-batch prevalence and quantification of human enteropathogenic *Yersinia* spp. in pigs at slaughter, IAFP European Symposium, 15-17 May 2013, Marseille, France.

Vanantwerpen G., Houf K., **Van Damme I.**, De Zutter L. (2013). Seroprevalence of enteropathogenic *Yersinia* spp. in batches of pigs at slaughter, IAFP European Symposium, 15-17 May 2013, Marseille, France.

Van Damme I., Berkvens D., Baré J., Botteldoorn N., Dierick K., Pochet B., De Zutter L. (2013). Influence of isolation procedures on official monitoring data of pathogenic *Yersinia enterocolitica*, IAFP European Symposium, 15-17 May 2013, Marseille, France.

Van Damme I., Berkvens D., Vanantwerpen G., Baré J., De Zutter L. (2013). Effect of slaughter practices on contamination of pig carcasses with pathogenic *Yersinia enterocolitica*, IAFP European Symposium, 15-17 May 2013, Marseille, France.

Lambrecht E., Baré J., **Van Damme I.**, Sabbe K., Houf K. (2013). Influence of environmental factors and bacterial strain identity on the association of *Yersinia enterocolitica* with the bacterivorous protozoon *Acanthamoeba castellanii*, 11th International Symposium on *Yersinia*, 24-28 June 2013, Suzhou, China.

Vanantwerpen G., Houf K., **Van Damme I.**, De Zutter L. (2013). Risk factors for a high batch-level bacteriologic prevalence of *Y. enterocolitica* in Belgian pigs at slaughter, 11th International Symposium on *Yersinia*, 24-28 June 2013, Suzhou, China.

Vanantwerpen G., Houf K., **Van Damme I.**, De Zutter L (2013). Within-batch prevalence and quantification of human enteropathogenic *Yersinia* spp. in pigs at slaughter, 11th International Symposium on *Yersinia*, 24-28 June 2013, Suzhou, China.

Vanantwerpen G., Houf K., **Van Damme I.**, De Zutter L. (2013). Seroprevalence of enteropathogenic *Yersinia* spp. in batches of pigs at slaughter, 11th International Symposium on *Yersinia*, 24-28 June 2013, Suzhou, China.

Van Damme I., Berkvens D., Vanantwerpen G., Baré J., De Zutter L. (2013). Risk factors for carcass contamination with *Yersinia enterocolitica* serotype O:3 in Belgian pig slaughterhouses, 11th International Symposium on *Yersinia*, 24-28 June 2013, Suzhou, China.

Oral presentations

Van Damme I., De Zutter L. Detection and enumeration of human pathogenic *Yersinia enterocolitica* : comparison of direct plating and enrichment, VIIème Congrès de la SFM, 2-4 June 2010, Marseille, France.

Van Damme I., De Zutter L. Occurrence of human enteropathogenic *Yersinia* spp. in Belgian pigs and contamination of pork carcasses during slaughter, 9th International Conference on the Epidemiology and Control of biological, chemical and physical hazards in pigs and pork (SafePork2011), 19-22 June 2011, Maastricht, The Netherlands.

Van Damme I., De Zutter L. Occurrence of human enteropathogenic *Yersinia* spp. in pigs and contamination of carcasses during slaughter, 57th International Congress of Meat Science and Technology, 7-12 August 2011, Ghent, Belgium (Winner of the 2011 IMS prize for Science and Technology).

Van Damme I., De Zutter L. Human pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in Belgium: prevalence in pigs and contamination of pork carcasses during slaughter, 16th Conference on Food Microbiology, 22-23 September 2011, Brussels, Belgium.

Van Damme I., De Zutter L. Enteropathogenic *Yersinia* spp. in pigs and pork, World Meat Congress, 5-6 June 2012, Paris, France.

Van Damme I., Berkvens D., Baré J., Botteldoorn N., Dierick K., Pochet B., De Zutter L. (2013). Influence of isolation procedures on official monitoring data of pathogenic *Yersinia enterocolitica*, IAFP European Symposium, 15-17 May 2013, Marseille, France.