Development of a PCR-based method for detection of pathogenic *Yersinia enterocolitica* in pork

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Abstract

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During the last decade, *Yersinia enterocolitica* has been reported to cause between 500 and 800 cases of human gastroenteritis per year in Sweden. As pigs are the only animals in human food production that regularly harbour the pathogen, pork is probably an important source of infection. Earlier it has only rarely been possible to recover the bacterium from pork, but in the last few years this was made possible by DNA-based technology. In this project, a PCR-based method for the detection of pathogenic *Y. enterocolitica* in pork was developed.

The chromosome-located gene attachment invasion locus (*ail*) was chosen as the PCR-target. The *ail* PCR assay was evaluated according to criteria for a standardised PCR-based method set by the European research project FOOD-PCR. In a trial involving 14 European laboratories, the *ail* PCR assay showed high repeatability and robustness.

The complete PCR-based method comprises a sample treatment step prior to the *ail* PCR assay. The assay consists of either one (single) or two (nested) PCR analyses and an internal amplification control for monitoring false-negative results. The detection limit of the complete (single) PCR method, using inoculated enriched homogenates, was established to 10 cfu or less per gram. An increased sensitivity in the form of a nested PCR was required to enable detection of the bacterium in naturally contaminated pork. This is in practice very important.

Finally, for characterisation of isolated strains, a multiplex PCR assay was developed, directed towards four different virulence-associated genes (*yst*, *rfb*C, *ail* and *vir*F). As presence or absence of the four PCR targets was established, the following groups were identified: pathogenic *Y. enterocolitica* 4/O:3 strains, pathogenic *Y. enterocolitica* serotypes other than 4/O:3, *Y. pseudotuberculosis* strains and nonpathogenic strains.

The method does not allow for confirmation of the viability of the pathogen, the reason being that the bacterium cannot be isolated by traditional culture. The method can therefore preferably be used where information about viability is not important, for example in studies to identify the critical points during slaughter, important to limit contamination by the bacterium.

Keywords: PCR; *Yersinia enterocolitica* 4/O:3; foodborne pathogen; Sample treatment; buoyant density centrifugation; internal amplification control, pork.

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To my dear family

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Appendix

Paper I-V

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Thisted Lambertz, S., Ballagi-Pordány, A., Nilsson, A., Norberg, P. & Danielsson-Tham, M-L. 1996. A comparison between a PCR method and a conventional culture method for detecting pathogenic *Yersinia enterocolitica* in food. *Journal of Applied Bacteriology 81,* 303-308.

II. Thisted Lambertz, S., Ballagi-Pordány, A. & Lindqvist R. 1998. A mimic as internal standard to monitor PCR analysis of food-borne pathogens. *Letters in Applied Microbiology 26,* 9-11.

III. Thisted Lambertz, S., Lindqvist, R., Ballagi-Pordány, 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.

IV. Thisted Lambertz, S. & Danielsson-Tham, M-L. 2005. Identification and characterization of pathogenic *Yersinia enterocolitica* by PCR and PFGE. *Applied and Environmental Microbioogyl 71,* 3674-3681.

V. Thisted Lambertz, S., Granath, K., Fredriksson-Ahomaa, M., Johansson, K-E. & Danielsson-Tham, M-L. Evaluation of a combined culture and PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *In manuscript.*

Abbreviations

ail	attachment invasion locus
API	analytical profile index
BD	buoyant density
BDC	buoyant density centrifugation
BHI	brain heart infusion
BOS	oxalate sorbose broth
CFU	colony forming units
CIN	cefsaludin irgasan novobiosin
CR-BHO	Congo red brain heart infusion agarose
DNA	deoxyribonucleic acid
Escul	esculin
PFGE	pulsed-field gel electrophoresis
Pyz	pyrazinamidase
ISO	International Organisation of Standardisation
ITC	irgasan ticarcillin chlorate
KOH	potassium hydroxide
Lip	lipase
MRB	modified rappaport broth
NFA	National Food Administration
NMKL	Nordic Committee on Food Analysis
*Not*I	*Nocardia otitidis-caviarum*, restriction endonuclease
PBS	phosphate buffer saline
PCR	polymerase chain reaction
REAP	restriction enzyme analysis of the plasmid
*rfb*C	the O:3-antigen gene
Sal	salicin
SIK	Swedish Institute for Food and Biotechnology
SMI	Swedish Institute for Infectious Disease Control
SSDC	salmonella shigella agar with deoxycholate
Taq	*Thermus aquaticus*
Treh	trehalose
TSB	trypton soya broth
USDA	US Department of Agriculture
*vir*F	virulence-regulatory factor
VP	voges-proskauer
Xyl	xylose
yst	yersinia heat-stable toxin

Introduction

General background

The genus *Yersinia,* named in 1944 after the French bacteriologist Alexandre Yersin, comprises eleven species, of which three are human pathogens: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Table 1). *Y. pestis* causes bubonic and pneumonic plague, is transmitted by flea bites or aerosols, and has a narrow range of animal reservoirs and a restricted flea-rodent-flea life cycle. In contrast, *Y. enterocolitica* and *Y. pseudotuberculosis* behave like true enteropathogens. They cause intestinal symptoms of moderate intensity, they are transmitted by the faecal-oral route and they spend a part of their life cycle in the environment. The reasons for these dramatic differences in epidemiological characteristics and clinical symptoms remain largely unknown (Carniel, 2003).

Classification

According to Bergey´s Manual of Systematic Bacteriology, the genus *Yersinia* belongs to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacteriales*, family *Enterobacteriaceae*. The genus currently consists of the following species: *Y. pseudotuberculosis*, *Y. pestis*, *Y. enterocolitica*, *Y. intermedia*, *Y. kristensenii*, *Y. frederiksenii*, *Y. aldovae*, *Y. rohdei*, *Y. mollaretii*, *Y. bercovieri* and *Y. ruckeri*. The species *Y. ruckeri* is included in the genus *Yersinia* but its classification is controversial. *Y. pestis* and *Y. pseudotuberculosis* share greater than 90% DNA homology, which suggests that they are different pathotypes of the same species. The current species concept is based on two organisms sharing a DNA-DNA hybridisation value of greater than 70%. *Y. enterocolitica*, on the other hand, is less than 50% related to *Y. pseudotuberculosis* and *Y. pestis*. Classical characteristics of bacteria are based on phenotypic traits such as morphology and physiology, whereas current taxonomy integrates phenotypic, genotypic and phylogenetic information.

Y. enterocolitica subtypes

The species *Y. enterocolitica* is highly heterogeneous and is divided into six biotypes (1A, 1B, 2-5) and more than 50 serotypes according to their biochemical reactions and composition of lipopolysaccharide (LPS) antigens, respectively (Table 1). Members of biotype 1A are mainly isolated from the environment and are considered nonpathogenic, whereas most of the strains belonging to the biotypes 1B, 2, 3, 4 and 5 are human and/or animal pathogens. Human pathogenic *Y. enterocolitica* forms two groups. The mouse-lethal biotype 1B strains, which are more virulent than the other biotypes, were previously referred to as the American strains because they caused a number of outbreaks of yersiniosis in the United States (Table 2). The less virulent (and mouse-nonlethal) group comprises biotypes 2, 3, 4 and 5, and these were named the European strains or non-American strains. The nomenclature is based on the origin of the first isolations, but nowadays either type of strain is found worldwide. At the end of 1980s, a

bioserotype shift occurred in the United States and the *Y. enterocolitica* 4/O:3 strains became the most frequent bioserotype isolated from human yersiniosis cases. Other commonly occurring serotypes are O:9 and O:5,27, especially in northern Europe. Bioserotype 3/O:3 has been reported mostly from Japan and China (Zheng & Xie, 1996; Fukushima *et al.*, 1997).

The most commonly encountered bioserotype isolated from human yersiniosis patients in Sweden is *Y. enterocolitica* biotype 4 serotype O:3, accounting for more than 95% of the notified cases. The studies presented in this thesis therefore primarily focus on *Y. enterocolitica* 4/O:3. (Sometimes in the text only the serotype is given, *i.e. Y. enterocolitica* O:3, and not 4/O:3, because many researchers report only the serotype.)

Table 1. The eleven *Yersinia* spp. and the most commonly encountered *Y. enterocolitica* bio/serotypes (adapted from Robins Browne, 1997)

Species	Serotype
Yersinia enterocolitica	
Biotype 1A……	O:3; O:4; O:5; O:6,30; O:6,31; O:7:8; O:7,13; O:9; O:10; O:14; O:16; O:21; O:22;O:25; O:37; O:41,42; O:46; O:47; O:57; NT
Biotype 1B……..	**O:4,32; O8; O:13a,13b**; O:16; **O:18; O:20; O:21**; O:25; O:41;42; NT
Biotype 2……	**O:5,27; O:9;** O:27
Biotype 3……	**O:1,2,3; O:3; O:5,27;O:9**
Biotype 4……	**O:3**
Biotype 5……	**O:2,3**
Y. frederiksenii	O:3; O:9,71; O:16; O:35; O:38; O:44; NT
Y. kristensenii	O:3; O:9; O:11; O:12,25; O:12,26; O:16; O:16,29; O:28,50; O:46; O:52; O:59; O:61; NT
Y. intermedia	O:3; O:17; O:21,46; O:35; O:37; O:40; O:48; O:52; O:55; NT
Y. bercovieri	O:8; O:10; O:58,16; NT
Y. mollaretii	O:3; O:6,30; O:7,13; O:59; O:62,22; NT
Y. rohdei	
Y. aldovae	
Y. pseudotuberculosis	
Y. pestis	
Y. ruckeri	

Bio/serotypes considered primary pathogens are in boldface
Biotype 1B: serotypes in boldface are named 'American' strains
Biotype 2-5: serotypes in boldface are named 'European' strains
NT: not typable

Yersiniosis

Yersiniosis is an infectious disease caused by enteric bacteria of the genus *Yersinia*. Cells of *Y. enterocolitica* enter the gastrointestinal tract after ingestion of contaminated food or water (the occurrence of toxins preformed in the food is unclear). Cells that survive passage through the stomach acid adhere to the mucosal cells in Peyer's patches (gut associated lymphoid tissue). The infective dose for humans is not known. The adhering cells are taken up by the epithelial cells, from which they are released into the *lamina propria,* where they invade

phagocytic cells and multiply extracellularly, producing a local inflammatory response. Damage to the absorptive epithelial cells results in malabsorption and fluid loss characterised by diarrhoea. The role of the heat-stable enterotoxin in the genesis of diarrhoea is unclear (Adams & Moss, 1995; Robins Browne, 1997).

Acute gastroenteritis or enterocolitis is the most frequent clinical form of the infection, most often resulting in self-limiting diarrhoea (Cover & Aber, 1989). Although most yersiniosis cases are mild infections, there can arise a variety of complications largely determined by host factors, especially age and immune status of the patient. In children younger than 5 years, the symptoms are predominantly those of enterocolitis (diarrhoea with blood-streaked stools, fever, abdominal pain and vomiting) (Ehara *et al.,* 2000). Illness in infants can last 3-28 days (Lee *et al.,* 1990). In children older than 5 years and young adults, yersiniosis often presents as a pseudoappendicular syndrome including symptoms such as fever, abdominal pain and tenderness of the right lower quadrant (Robins Browne, 1997). Adults usually present with nonspecific abdominal pain and diarrhoea. A sore throat is a frequent accompaniment and may dominate the clinical picture in older patients (Tacket *et al.,* 1984). A large variety of immunological complications may follow the acute infection, including reactive arthritis, erythema nodosum, iridocyclitis, glomerulonephritis, carditis and thyroiditis (Cover & Aber, 1989). Reactive arthritis is the most widely recognized and people who are human lymphocyte antigen (HLA)-B27 positive are especially at risk. Reactive arthritis typically appears 1 to 3 weeks after infection and normally continues for up to 3 months, although some patients may have symptoms that persist for several years (Leirisalo-Repo, Skylv & Kousa, 1987; Herrlinger & Asmussen, 1992).

Bacteraemia is a rare complication except in immunocompromised patients and in patients with iron overload (Tacket *et al.,* 1984). Bacteraemia during blood transfusion is uncommon but may occur (Bottone, 1997). The probable cause is blood donors with low-grade subclinical bacteraemia. Factors that contribute to the occurrence are the ability of the bacterium to multiply at 4 ºC and to utilize iron from the blood cells. Bottone (1999) reviewed 27 out of 49 cases of transfusion-associated *Y. enterocolitica* bacteraemia reported between the years 1975 and 1994. Only O:3, O:9 and O:5,27 were recovered from these cases. These are the most commonly encountered serotypes isolated from yersiniosis patients, especially in northern Europe. A case fatality rate of 30-60% is reported for yersinia bacteraemia, but absence of symptoms in some blood donors may suggest low-grade bacteraemia without serious consequences (Robins Browne, 1997).

Table 2. Selected foodborne outbreaks of *Y. enterocolitica* infections

Location	Year [a]	No of cases	Bio-/Serotype	Source/Vehicle	Reference
Japan	1973	544	O:3	Not identified	Asakawa *et al.,* 1973
Finland	1973	7	O:9	Hospital patients	Toivanen *et al.,* 1973
North Carolina, US	1973	16	O:8	Dog[b]	Gutman *et al.,* 1973
Japan	1972	198	O:3	Not identified	Zen-Yoji & Maruyama, 1972
Czechoslovakia	1975	15	O:3	Not identified	Olsovsky *et al.,* 1975
Canada	1976	138	O:5,27	Raw milk[b]	Kasatiya, 1976
New York, US	1976	222	O:8	Flavoured milk	Black *et al.,* 1978
Japan	1980	1.051	O:3	Milk	Maruyama, 1987
New York, US	1981	239	O:8	Pwdr. milk, chow mein	Shayegani *et al.,* 1983
Washington, US	1981	50	O:8	Tofu, spring water	Tacket *et al.,* 1985
Pennsylvania, US	1982	16	O:8	Bean sprouts, w. water	Aber, 1982
Southern US	1982	172	O:13a, O:13b, O:18	Pasteurised milk[b]	Tacket *et al.,* 1984
Hungary	1983	8	O.3	Pork cheese	Marjai *et al.,* 1987
Canada	1984	2	4/O:3	Well water	Thompson & Gravel, 1986
Australia	1987-8	11	O:3; O:6,30	Not identified	Butt *et al.,* 1991
Georgia, US	1988	15	O:3 (14); O:1,2,3 (1)	Pork chitterlings	Lee *et al.,* 1990
Sweden	1988	61	O:3	Milk, cream[b]	Alsterlund *et al.,* 1995
Vermont, US	1995	10	O:8	Pasteurised milk[b]	Ackers *et al.,* 2000

[a] Outbreak year or year of publication

[b] The pathogen was not isolated from the suspected source

Human incidence

Y. enterocolitica was first recognised as a human pathogen in the 1930s (Bottone, 1999). During the 1980s, several countries in Europe reported a dramatic increase in the number of recovered human cases (Cover & Aber, 1989). In the beginning of the 1990s, the diagnosed yersiniosis cases in Sweden reached numbers exceeding 1000 and in 1996 it was classified a notifiable disease. Laboratory-confirmed cases and cases reported by physicians are collected separately and are matched before recorded. The incidence data for yersiniosis in Sweden for 1997-2004 are given in Table 3. Yersiniosis notifications by month for January 1997 to July 2004 are shown graphically in Figure 1. Peak incidence in the reported cases is observed in late summer.

Table 3. Incidence data for yersiniosis in Sweden, 1997-2004
(The Swedish Institute for Infectious Disease Control, SMI)

Year	Number of cases /Rate per 100 000 inhabitants
1997	752/6.5
1998	640/7.2
1999	549/6.1
2000	632/7.1
2001	579/6.5
2002	610/6.8
2003	713/7.9
2004	811/9.0

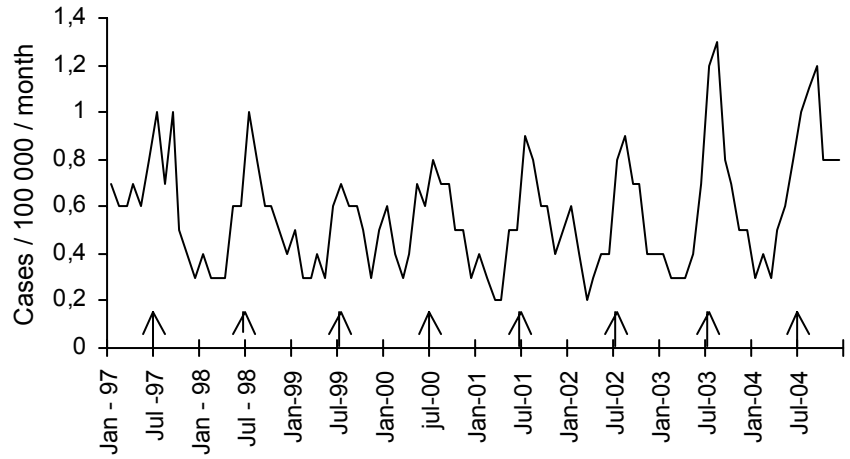

Figure 1. Yersiniosis notifications in Sweden by month, Jan 1997-Jul 2004
(The Swedish Institute for Infectious Disease Control, SMI)

Y. enterocolitica serotype O:3 accounted for more than 95% of the reported human cases in Sweden during the years 1996 to 2004 (Marika Hjertqvist, SMI, pers. comm.). Only a few isolates of O:9 and O:5,27 are reported annually. The

majority of the infections are domestically acquired, in 2004 approx. 75%. The age distribution shows a peak between 0 to 4 years, comprising 28% of the cases reported in 2004. Boys were more frequently notified in the age up to 4 years, whereas the gender distribution was about equal in the other age groups.

The proportion of reported infections with *Y. pseudotuberculosis* is low in Sweden. During the period from 1996 to 2004, only approx. 1-2 yersiniosis cases annually were recorded as being caused by *Y. pseudotuberculosis* (Marika Hjertqvist, SMI, pers. comm.).

Within Europe, 9399 cases of human yersiniosis were reported in 2003 (data collected from 11 EU countries plus Norway), corresponding to an incidence of 4 per 100,000 inhabitants (Anon., 2003b). In the United States, the Center for Disease Control and Prevention (CDC) estimates about 17,000 annual cases, *i.e.* an incidence of 6 per 100,000 inhabitants (www.cdc.gov).

Sporadic cases and outbreaks

On a worldwide basis, outbreaks of yersiniosis are an uncommon event and the information from them is not strong. A number of outbreaks occurred from 1973 to 1995 in the United States (Table 2). These outbreaks mostly involved strains belonging to bioserotype 1B, a biotype not commonly isolated from humans after the 1980s and believed to have a quite different epidemiology from that of the commonly isolated strains from humans (especially 4/O:3 strains). In Japan, several presumed foodborne outbreaks have been reported, with *Y. enterocolitica* 4/O:3 as the causative agent. However, the sources of these infections were not identified. Two outbreaks, one in the United States (Lee *et al.*, 1990) and one in Hungary (Marjai *et al.,* 1987) involved bioserotype 4/O:3 as the responsible agent. In both cases consumption and/or preparation of chitterlings (the intestines of young pigs) were involved. Furthermore, bioserotype 4/O:3 was isolated from well water in a small family outbreak in Canada in 1984 (Thompson & Gravel, 1986).

With only few exceptions, all the yersiniosis cases in Sweden are reported as sporadic cases, with serotype O:3 strains responsible for the infections and with the source of the infections unsolved.

Five outbreaks have occurred during the years 1980 to 2002, with the pathogen not traced in any of them. The data reported for two of the outbreaks indicate that consumption of milk and brawn, respectively, may have been the source of the infection (www.smittskyddsinstitutet.se, in Swedish).

The virulence plasmid

The genome of fully virulent *Y. enterocolitica* isolates consists of a single chromosome of about 4.6×10^6 bp, with a G+C content of 47.3 % (*Y. enterocolitica* 1B/O:8, strain 8081) and a 68 kb virulence plasmid (pYV), *i.e.* plasmid for yersinia virulence (Parkhill, 2002). A functional virulence plasmid is absolutely required for mouse pathogenicity. The virulence plasmid carries genes encoding

the type III secretion system. At least 11 important virulence-associated proteins termed Yops (for *Yersinia* outer membrane proteins) are involved. (However, they are actually secreted proteins.) The regulator *Vir*F, a transcriptional activator, controls most of the genes involved in Yop synthesis and secretion. Through the Yop system, extracellular bacteria in close contact with a eukaryotic cell deliver proteins into the cytosol of this cell, and the substances act collectively to overcome the nonspecific immune defences of the host. In addition, the virulence plasmid carries genes for the production of YadA, a true outer membrane protein. YadA acts as an adhesin and may contribute to bacterial invasion. If plasmid-bearing cells are grown at 37 ºC in media containing low concentrations of Ca^{2+}, growth ceases after one or two generations and instead a massive production of the Yop proteins occurs. This is known as the low calcium response. On the other hand, if plasmid-bearing *Y. enterocolitica* is subcultured *in vitro* at 37 ºC (or above 32 ºC) or stored over time, the virulence plasmid may be lost (Robins Browne, 1997).

Biochemical characteristics and detection

The genus *Yersinia* contains oxidase-negative, catalase-positive, gram-negative and rod-shaped facultative anaerobes that ferment glucose under production of acid. The phenotypic characteristics of *Y. enterocolitica* are often temperature-dependent, being more active biochemically at 25 ºC than at 37 ºC (Bottone, 1997). Presumptive *Y. enterocolitica* colonies grown on CIN agar can be identified by inoculation on Kligler iron agar followed by a test for urease production (Devenish & Schiemann, 1981). The species *Y. enterocolitica* can be identified by a positive result for fermentation of sucrose and negative reactions for rhamnose and melibiose (Schiemann, 1989). Commercial rapid tests for the identification are available (Arnold *et al.,* 2001). Differentiation between pathogenic and nonpathogenic strains by serotyping according to the bacterial O-antigens and a biotyping schema has become most useful. This is convenient because pathogenic strains are mainly associated with only a few bioserotype combinations (Table 1). The biotyping scheme proposed by Wauters, Kandolo & Janssens (1987) has been widely used. Aleksic (1995) showed that 76 O-serotypes with 293 other serovars could be distinguished in *Y. enterocolitica* and other *Yersinia* species, including both pathogenic and nonpathogenic strains, by means of their O- and H-antigens. Phage typing has also been utilised (Schiemann, 1989). However, these tests are laborious and require experienced personnel, and therefore complete biochemical testing is only performed at specialist or reference laboratories. Furthermore, a number of different *in vitro* biochemical tests have been proposed to predict the virulence of the organism. These tests include autoagglutination (Skurnik *et al.,* 1984), calcium dependence (Gemski, Lazere & Casey, 1980; Bhaduri *et al.*, 1990), resistance to human serum, binding of Congo red dye (Prpic, Robins-Browne & Davey, 1983; Riley & Toma, 1989) and pyrazinamidase testing (Kandolo & Wauters, 1985). The two latter tests indicate virulence through chromosomally encoded properties based on features correlated to the ability to carry the plasmid but not to the presence of the plasmid. All biochemical tests depend on the expression of the gene during the test and some of them also require presence of the plasmid. The sensitivity and specificity of some of these tests have been

questioned (Prpic, Robins-Browne & Davey, 1985; Farmer *et al.*, 1992), and for reliable results a combination of several tests has been recommended (Noble *et al.*, 1987; Farmer *et al.*, 1992).

A description of pathogenic *Y. enterocolitica* (4/O:3)

Presence in the environment (including water)

Y. enterocolitica and other *Yersinia* species are ubiquitous in the natural environment and can be isolated from water and soil (Kapperud, 1991). However, the vast majority of the isolates lack the classical markers of bacterial virulence and are considered nonpathogenic. The possibility for the microorganism to survive in this type of environment has been investigated. Chao, Ding & Chen (1988) showed that *Y. enterocolitica* could survive in soil and water systems, especially at low environmental temperatures. Karapinar & Gonul (1991) found that a mixed culture of nonpathogenic and pathogenic strains (including O:3 and O:9) held at 4 ºC were recovered after 56 weeks incubation in sterile spring water. In a study carried out in Australia, Sandery, Stinear & Kaucner (1996) tested 251 water samples by nested PCR. Eleven samples from 4 separate locations tested positive. One of the PCR-positive results was confirmed by culture. Some waterborne cases/outbreaks caused by the bacterium have been reported from North America (Aber, 1982; Tacket *et al.*, 1985). The isolated strains belonged to bioserotype 1B/O:8, a bioserotype not commonly isolated in the United States after the 1980s. Bioserotype 4/O:3 was isolated from well water in a small family outbreak of gastroenteritis in Ontario, Canada (Thompson & Gravel, 1986). Furthermore, in a case-control study performed on sporadic cases of yersiniosis in Norway between 1988 and 1990, patients were more likely than controls to report drinking untreated water during the 2 weeks before onset of illness (Ostroff *et al.,* 1994).

Presence in animals

Animals, especially domestic animals, have been suspected as transmitters of *Y. enterocolitica* to humans. Published reports are contradictory in this regard. The Member States of the EU report annually to the EU-Commission on the zoonose situation in their country. However, *Y. enterocolitica* infection in animals is not notifiable, and reports on its prevalence are for the most part results from research projects obtained from institutions or authorities in the member countries. Various studies have investigated wild and farm animals for the presence of pathogenic strains of *Y. enterocolitica*, and only a few have been recovered (Fantasia *et al.,* 1993; Cork *et al.,* 1995; Suzuki *et al.,* 1995; Busato *et al.,* 1999; McNally *et al.,* 2004). Shayegani *et al.,* (1986) examined faecal specimens from 1,426 animals including mammals, birds, reptiles, fish and invertebrates throughout New York State. Eleven human pathogenic strains were isolated, including bioserotype 1B/O:8 (2), 4/O:3 (1) and O:5,27 (8). In various studies examining healthy domestic animals and animals with acute enteritis, including cattle, sheep, goats, deer, calves, broilers, hens, turkeys and ducks, only a few pathogenic isolates have been recovered, and they differed from those usually associated with human

infections (Slee & Skilbeck, 1992; Fantasia *et al.,* 1993; Busato *et al.,* 1999). A national survey in Great Britain between 1999 and 2000 investigated the relationship between livestock (cattle, sheep and pigs) carriage of pathogenic *Y. enterocolitica* and isolates from human cases of yersiniosis during the same period (McNally *et al.,* 2004). Bioserotypes recognised as human pathogens were recovered from all three groups of animals. The main bioserotype found in the animals 3/O:5,27 (35% of sheep, 22% of pigs and 4% of cattle) was not detected in any of the human isolates. The major pathogenic bioserotype isolated from humans (3/O:9) was only isolated from pigs. In a study from New Zealand designed to identify risk factors for sporadic human yersiniosis, contact with farm animals was cited as the primary risk factor (Wright, 1996). In the summarised notification for 2004, the Swedish Institute for Infectious Disease Control reported that out of 818 notified yersiniosis cases where information on contact animal was included, only 2% reported contact with animals.

Dogs and cats occasionally harbour *Y. enterocolitica* 4/O:3 (Szita *et al.,* 1980; Fantasia *et al.,* 1993; Fenwick, Madie & Wilks, 1994). Fredriksson-Ahomaa, Korte & Korkeala (2001) showed that raw pork was an important source of yersiniosis in dogs and cats. Furthermore, Fenwick, Madie & Wilks (1994) observed that *Y. enterocolitica* 4/O:3 could readily be transmitted between dogs. The infected dogs showed no clinical signs of infection. The duration of faecal shedding by the dogs varied between 7 and 23 days. These findings suggest that dogs can act as a potential source of the infection to humans.

Pigs are healthy carriers of *Y. enterocolitica* 4/O:3 and are the only animal species from which the bacterium can be isolated frequently (Robins Browne, 1997). Based on results from several studies, it can be concluded that *Y. enterocolitica* 4/O:3 is present in the pig population in many countries in the world. The prevalence often varies herd-wise (Fredriksson-Ahomaa, 2001). In a German study, Gurtler *et al.,* (2005) found that the prevalence ranged between 0 and 65% in fattening pig herds and was present in 39% of pig tonsils at the abattoir. Almost all isolates were *Y. enterocolitica* 4/O:3, only one strain was identified as O:9. In Denmark and Norway, the frequency of *Y. enterocolitica* O:3 at herd level has been found to be 64% and 70% respectively (Lund-Sörensen, 1996; Skjerve *et al.,* 1998). Korte *et al.,*. (2004) found that the prevalence in fattening pigs in Finland, investigated in five slaughterhouses, increased from 33% to 64% between 1995 and 1999. In a study performed in Sweden between 1997 and 1998, the frequency at herd level was 67% (Elisabeth Borch, SIK, pers. comm.).

Presence in food

The primary route of human infection is most probably ingestion of food. Based on the information on domestic cases notified by clinicians for 2004 in Sweden, the Swedish Institute for Infectious Disease Control reported that 75% of the yersiniosis cases were suspected to be food- or waterborne infections. However, pathogenic strains of *Y. enterocolitica* are difficult to isolate from food. In several studies, high frequencies of nonpathogenic strains have been isolated from food, whereas pathogenic strains are only occasionally recovered (De Boer, 1995;

Velazquez, Escudero & Stefanini de Guzman, 1995; Logue *et al.,* 1996). In studies from different countries, various ready-to-eat products have been investigated including, fresh salad, whole and sliced vegetables, sandwiches, milk, dairy products, desserts and soft cheese (Hudson *et al.,* 1992; Walker & Brooks, 1993; Tassinari, Franco & Landgraf, 1994). Only a few pathogenic strains were isolated from these foods. In a study carried out in Finland, Fredriksson-Ahomaa *et al.,* (2001) tested 200 samples of raw fish, 43 samples of raw chicken and 101 samples of lettuce by nested PCR targeting the *yad*A gene. Three PCR-positive results were obtained, all recovered from the lettuce samples. Logue *et al.,* (1996) investigated Irish meat and meat products and isolated pathogenic serotypes of *Y. enterocolitica* from 10% of samples of cooked ham (n=20), corned beef (n=40) and pork sausage (n=20). Wang, Cao & Cerniglia (1997) examined seafood and found that none of the samples tested positive for pathogenic *Y. enterocolitica* by PCR. In an Australian study, Szabo, Scurrah & Burrows (2000) examined 120 samples of minimally processed lettuce collected over an 8-month period and isolated 71 *Y. enterocolitica* strains, all belonging to nonpathogenic serotypes. Walker & Brooks (1993) examined bottled water and isolated only nonpathogenic strains.

Raw and pasteurised milk have been examined in several studies because outbreaks over a number of years in the United States were traced to milk (Table 2). With the exception of a few isolates of O:5,27, none of the strains isolated in these studies were identified as pathogenic (Walker & Gilmour, 1986; Schiemann, 1989; Rea, Cogan & Tobin, 1992; Tassinari, Franco & Landgraf, 1994; Ramesh *et al.*, 2002).

Pork is likely to be an important vehicle of the infection to humans because pigs are the only animals consumed that frequently harbour the same bioserotype of *Y. enterocolitica* as is isolated from human yersiniosis cases. However, the problem is that only few pathogenic strains have been recovered from pork or pork products (Fredriksson-Ahomaa & Korkeala, 2003). Selected studies on the occurrence of pathogenic strains in this type of food are listed in Table 4. From outbreaks and case-control studies, there are other indications that pork is involved in the transmission of the pathogen to humans. Two outbreaks have been traced to ingestion and/or handling of contaminated pork. In one outbreak, home prepared 'pork cheese' (a sausage variant) was identified as the source of the infection (Marjai *et al.,* 1987). Preparation of pork chitterlings (a dish made from pig intestines) was the source of the infection in the other outbreak (Lee *et al.*, 1990). Both outbreaks involved *Y. enterocolitica* 4/O:3 as the causative agent. In a case-control study performed in Belgium, *Y. enterocolitica* O:3 infection was strongly associated with eating raw pork during the two weeks before illness (Tauxe *et al.,* 1987). Likewise, in a case-control study in Norway, patients with yersiniosis reported having eaten significantly more pork items and sausage in the two weeks before onset of the illness than their matched controls (Ostroff *et al.*, 1994). In a case-control study in New Zealand on potential food vehicles, only pork had a significantly higher rate among cases than controls (Satterthwaite *et al.,* 1999).

It is important to be cautious about the information given in the section above because the isolation of strains of pathogenic *Y. enterocolitica* from food is

difficult. The majority of the studies referred to used traditional culture procedures and no plating medium has been found to be sufficiently discriminatory to allow the recovery. Similar difficulties are encountered with the enrichment medium (Kapperud, 1991; Nesbakken *et al.,* 1991).

As a hazard in food

Y. enterocolitica is a psychotrophic bacterium, which means that it has the ability to grow at temperatures approaching 0 ºC. The growth range is between 0 and 44 ºC, and optimum is approx. 28 ºC. The bacterium is susceptible to heat. There is a 90% reduction in number of organisms when treated at 55 ºC for approx. 2 min, at 60 ºC for approx. 0.5 min, at 65 ºC for approx. 2 s (Lake, Hudson & Cressey, 2004). Pasteurisation at 71.8 ºC for 18 s or hot water (80 ºC) applied on surface-contaminated meat for 10-20 s reduce bacterial viability by at least 99.9% (Robins Browne, 1997). *Y. enterocolitica* readily withstands freezing and can survive in frozen foods for extended periods of time even after repeated freezing and thawing.

The bacterium is a facultative anaerobe, which means that it has the ability to multiply both in air and in the absence of air. Kleinlein & Untermann (1990) showed that *Y. enterocolitica* numbers in minced meat increased by 1 log at 1 ºC and 3.5 log at 4 ºC within 14 days under normal atmospheric conditions. At 10 and 15 ºC, there was about a 5-log increase in cell numbers within 5 days. On the other hand, growth was inhibited in a 20% CO_2:80% O_2 atmosphere at 1 and 4 ºC but not at 10 or 15 ºC. Under vacuum the pathogen was found to grow on sliced roast beef at 3 ºC to maxinum numbers at the end of the product storage life of 3 weeks (Hudson, Mott & Penney, 1994). Furthermore, Bodnaruk & Draughon (1998) showed that virulence of *Y. enterocolitica* was maintained in 25 to 35% of isolates following storage for 30 days at 4 ºC in vacuum- and CO_2-packaged meats and was not affected by the pH of pork loin. The fact that the bacterium can grow in vacuum packaging at low temperatures makes it particularly significant when considering food safety.

Y. enterocolitica can grow over a pH range from 4 to 10, with an optimum at pH 7.6. The tolerance to acid depends on the acidulant (Brocklehurst & Lund, 1990; el-Ziney, De Meyer & Debevere, 1995). At a given pH below that allowing growth, survival is greater at lower temperatures (Little & Knochel, 1994). The bacterium can grow in up to 5% NaCl (a_w=0.945), whereas between 5 and 7% NaCl inhibits growth (Lake, Hudson & Cressey, 2004).

It can be assumed that many types of food can harbour pathogenic *Y. enterocolitica*, and if the food is eaten without thermal treatment or with only mild cooking, or if food is contaminated by the bacterium after preparation, opportunities exist for it to multiply and reach high numbers.

Table 4. Selected studies on the occurrence of pathogenic *Y. enterocolitica* in raw pork detected by PCR and/or traditional culture procedures

Type of food	No of samples	Real-time PCR	Conventional PCR	Culture method	Reference; country
Culture methods:					
Minced pork	125	NT	NT	4.6%	Fukushima, 1985; Japan
Minced pork	46	NT	NT	0	Asplund *et al.,* 1990; Finland
Minced pork	400	NT	NT	1%	De Boer & Nouws, 1991; The Netherlands
Minced pork	20	NT	NT	0	Bhaduri & Cottrell, 1997; USA
Pork products	91	NT	NT	1%	Schiemann, 1980; Canada
Pork (unspecified)	58	NT	NT	0	Asplund *et al.*, 1990; Finland
Pork (unspecified)	14	NT	NT	7%	Tassinari, Franco & Landgraf, 1994; Brazil
Pork (unspecified)	90	NT	NT	0%	De Guisti *et al.*, 1995; Italy
Pork (unspecified)	20	NT	NT	20%	Logue *et al.*, 1996; Ireland
Pork head meat	10	NT	NT	0	Bhaduri, Cottrell & Pickard, 1997; USA
Pork (unspecified)	1278	NT	NT	3%	Fukushima *et al.*, 1997; Japan
Pork tongue	37	NT	NT	30%	Schiemann, 1980; Canada
Pork tongue	40	NT	NT	20%	De Boer & Nouws, 1991; The Netherlands
Pork tongues	30	NT	NT	37%	Bhaduri, Cottrell & Pickard, 1997; USA
Pork tongues	17	NT	NT	41%	Bhaduri & Cottrell, 1997; USA
PCR and culture methods:					
Minced pork	100	35%	NT	35%	Vishnubhatla *et al.*, 2000; USA
Minced pork	350	38%	10%	0%	Boyapalle *et al.*, 2001; USA
Minced pork	255	NT	25%	2%	Fredriksson-Ahomaa, Hielm & Korkeala, 1999; Finland
Pork chops	9	NT	33%	0%	Johannessen, Kapperud & Kruse, 2000; Norway
Pork tongue	51	NT	92%	78%	Fredriksson-Ahomaa, Hielm & Korkeala, 1999; Finland
Chitterlings	350	79%	27%	8%	Boyapalle *et al.*, 2001; USA

NT=not tested

Detection of pathogenic *Y. enterocolitica* in food

Conventional culture methods

Several reference methods for the detection of *Y. enterocolitica* in food exist, proposed by different national and international organisations. Thus, there is no consensus regarding a standard method. In the Nordic countries the methods proposed by MNKL (Nordic Committee on Food Analysis) and ISO (International Organization of Standardization) are the most commonly used. The NMKL method (NMKL 117, 1996) is based on cold enrichment (PBS) at 4 ºC for 21 days, including a selective enrichment (MRB) after 4 days and subculture on a selective agar medium (CIN) four times during the enrichment process. The ISO method (ISO 10273:2004) developed for food and animal feedstuffs is based on two parallel enrichments: a nonselective (PBS) enrichment for 48-72 h and a selective (ITC) enrichment for 48 h, followed by plating on the selective agars CIN and SSDC, respectively. In addition, a fraction of the PBS enrichment is briefly treated with KOH before being spread on a second CIN agar plate. The KOH treatment is included to decrease the background flora. Recently, NMKL proposed a new NMKL 117 method by an approach to the ISO method (NMK 117, 2003). This method has been developed especially for recovery of the European pathogenic serotypes O:3 and O:9 in all types of food. In the United States, the Food Safety and Inspection Service at the US Department of Agriculture (USDA/FSIS) proposes three procedures for the detection as there is no universal enrichment scheme for the three most important serotypes in the United States (O:3, O:8 and O:5,27). In order to improve the chances of recovery, all three procedures are used in parallel. Procedure 1 uses ITC as selective enrichment for the recovery of serotype O:3 strains, and is based on a 48-h incubation and a subsequent plating on CIN, SSDC and on an additional CIN agar plate after a KOH treatment of the enrichment. If no typical colonies appear, an additional 12-h incubation of the ITC broth is performed. This is followed by similar plating performed after the 48-h of incubation (CIN, SSDC and KOH treatment plus CIN), as mentioned above. Procedure 2 uses TSB/BOS, permitting recovery of serotype O:8. A 12-h enrichment is followed by plating on SSDC, CIN and KOH treatment plus CIN. If no suspected colonies appear, broth incubation is continued for another 48 h. Finally, procedure 3 is a PBS cold enrichment lasting 14 days followed by plating on CIN and KOH-treatment plus CIN. The latter procedure has been shown to promote recovery of serotype O:5,27.

PCR-based methods

For many organisms, traditional culture methods for detection and identification of bacteria take approx. 3-5 days or more. Adding PCR to a culture method can reduce the analysis process to 1-3 days. In addition, besides being a rapid and sensitive technique, PCR can be used to identify isolates and to easily separate pathogenic from nonpathogenic strains within the same species. A PCR-based method often comprises the following steps: enrichment in a nonselective broth, pre-PCR sample treatment, PCR analysis, detection of the PCR product and, sometimes in parallel, isolation of colonies. (Sometimes PCR is performed on the nonenriched homogenate.)

PCR can be applied on colonies, either in pure form or in mixed cultures, to characterise strains that have already been isolated by traditional culture methods (Table 5). PCR can also be used to shorten the time needed for traditional methods by being applied either directly on the nonenriched homogenate, or on the enriched homogenate (Table 6). A major goal for food microbiologists using PCR is to be able to apply the technique for direct detection of bacteria in the samples. However, it is difficult to reach this goal and it has so far been applied only to a limited extent (Table 6), since inhibitory substances present in food may interfere with the amplification and need to be efficiently removed (Rossen *et al.,* 1992), and the ability of PCR to detect viable and nonviable bacteria without distinction is also a problem. Analysis directly on the nonenriched homogenate is applied in one of the studies in this thesis (Paper V).

Table 5. PCR assays developed for detection of *Y. enterocolitica* performed on colonies

Reference	Pre-PCR sample treatment	PCR variant; target gene (Reference) [a]
Ibrahim, Liesack & Stackebrandt, 1992	DNA extract kit Isogen Kit™ [b]	Multiplex PCR; *yst* and 16S rDNA Universal
Kwaga, Iversen & Misra, 1992	DNA or crude lysates	Multiplex PCR; *ail*
Nakajima *et al*., 1992	3x freeze-thawed, Prot. K	Multiplex PCR; *ail*, *inv* and *vir*F (Wren & Tabaqchali, 1990)
Koeppel *et al.*, 1993	Lysozyme, Prot. K	Single PCR; *vir*F (Wren & Tabaqchali, 1990)
Bhaduri & Cottrell, 1997 Bhaduri, Cottrell & Pickard, 1997	DNA extract kit G-Nome™ [c]	Multiplex PCR; *ail* (Nakajima *et al*., 1992) and *vir*F (Wren & Tabaqchali, 1990)
Harnett, Lin & Krishnan, 1996	Boiling and Lysozyme, Prot. K, phenol/chloroform	Multiplex PCR; *ail, virF* and *yst*
Sandery, Stinear & Kaucner, 1996	InstaGene™ [d]	Single PCR; *ail* (Kwaga *et al*., 1992)
Weynants *et al.*, 1996	3x freeze-thawed, Prot. K	Multiplex PCR; *rfb*C, *ail*, *inv* (Nakajima *et al*., 1992) and *vir*F (Wren & Tabaqchali, 1990)
Ibrahim *et al.*, 1997	DNA extract kit Isogen Kit ™ [b]	Single PCR; *yst*
Neubauer *et al*., 2000a	Lysis buffer, Prot. K	Single PCR; *Yad*A (Blais & Phillippe, 1995)
Gierczynski, Jagielski & Rastawicki, 2001	Triton X-100 and Prot. K	Nested PCR; *ail* (Feng *et al*., 1992; Gierczynski *et al*., 2000)
Wannet *et al.*, 2001	QiaAmp Tissue kit™ [e]	Multiplex PCR; *ail* and 16S rRNA gene (Neubauer *et al*., 1999)

[a] Refers to where the primers were first published
[b] Perkin Elmer, Melbourne, Australia; [c] BIO 101 La Jolla, CA, USA; [d] BioRad Laboratories, UK; [e] Qiagen Inc. Leusden, The Netherlands
Prot. K, Proteinase-K; PCR-optim., PCR optimation

Table 6. PCR-based methods developed for detection of pathogenic *Y. enterocolitica* performed on enriched homogenate

Reference	Sample	Medium[a] / pre-PCR sample treatment	PCR variant; target gene (reference)[b]
Kapperud *et al.,* 1993	A: Minced meat, minced beef, pork chops A: Water	Nonselective /IMS or Proteinase K; Filter, overnight nonselective/As above;	Nested PCR; *yad*A
Rasmussen *et al.,* 1995	N: Pig tonsil (swabs)	7-10 days nonselective 4 ºC /IMS;	Single PCR; *inv* (Rasmussen *et al*., 1994)
Sandery, Stinear & Kaucner, 1996	A: Water N: Water	Filter, 48-h nonselective /InstaGene[c]	Semi-nested PCR; *ail* (Kwaga *et al*., 1992)
Wang, Cao & Cerniglia, 1997	N: Soft cheese, seafood	Nonselective /3x washing, Triton-X-100;	Multiplex PCR; (13 primer sets) *yst* (Ibrahim *et al*., 1992)
Bhaduri & Cottrell, 1997	A: Pork chops, ground pork, cheese and zucchini (swabs) N: Pig tongues (swabs)	Nonselective and 48-h selective /Proteinase K;	Multiplex PCR; *ail* (Nakajima *et al*., 1992) *virF* (Wren & Tabaqchali, 1990)
Lantz *et al.,* 1998	A: Minced pork, pork leg	6-8 h, nonselective and selective / XTRAX™ [d] or Aqueous two-phase or Buoyant density centrifugation (BDC)	Multiplex PCR; *yad*A and 16S rRNA
Fredriksson-Ahomaa, Hielm & Korkeala, 1999, 2000	N: Minced meat, pig tonsils (1999); surface, environment (swabs), pig offal (2000); N: Pork samples	Nonselective /Proteinase K; (see Kapperud *et al*., 1993) (Ditto)	Nested PCR; *yad*A (Kapperud *et al*., 1993) (Ditto)
Johannessen, Kapperud & Kruse, 2000 Waage *et al.,* 1999	A: Water	(Ditto)	(Ditto)
Vishnubhatla *et al.*, 2000	A: Minced pork N: Minced pork	Nonselective /Prepman™ reagent[e]	Real-time PCR; *yst* (Ibrahim *et al*., 1997)
Jourdan, Johnson & Wesley, 2000	A: Pork	0, 12 h and 24-h selective /Prepman™ reagent[e]	Real-time PCR; *ail*
Boyapalle *et al.*, 2001	A: Minced pork N: Pork products	48-h selective /Guanidine-silica particle extraction[f] alt. Prepman™ reagent[e]	Multiplex PCR; *ail* (Feng, Kearsler & Hill, 1992) and *yad* A and real-time PCR; *ail*
Ramesh *et al*., 2002	A: Milk N: Raw milk	48-h nonselective /DNA extraction	Multiplex PCR; *ail* (Fenwick & Murray, 1991)

[a] Overnight enrichment is used if no incubation time is mentioned; [b] Refers to where the primers were first published;
[c] BioRad Laboratories, UK; [d] XTRAX™, Gull Laboratories, Inc., UT, USA; [e]Applied Biosystems, Foster city, Calif., USA; [f] Carter & Milton, 1993;
A, artificially inoculated food; N, naturally contaminated food
IMS, immunomagnetic separation

PCR targets

Published PCR primer pairs that target different genes commonly used as targets for the detection of pathogenic *Y. enterocolitica* are listed in Table 7. Virulence genes are the most frequently used and they are excellent targets because they indicate the potential for virulence and define the pathogenic group at the same time as the presence of the organism. Virulence in *Y. enterocolitica* results from a complex interplay between a series of plasmid- and chromosome-located genes. Some of the chromosome-located genes used as PCR targets are: the *ail* gene for the attachment invasion locus; the invasin gene (*inv*); the Yersinia heat stable enterotoxin gene (*yst*) and the *rfb*C gene located within the rfb cluster responsible for the biosynthesis of the O-side chain of *Y. enterocolitica* O:3. Commonly used targets located on the virulence plasmid are the yersinia adhesin gene (yadA) and the *vir*F gene, a transcriptional activator for many yersinia outer membrane proteins (Wren & Tabaqchali, 1990; Kapperud *et al.,* 1993). However, PCR targets located on the virulence plasmid must be considered unsuitable as targets for detection, because the plasmid is unstable and easily lost during laboratory treatment (Blais & Phillippe, 1995; Li, Bhaduri & Magee, 1998).

It is important to be aware of the sequence variation that exists within these genes, which may lead to lack of sensitivity and/or specificity of the selected primers. Although the *ail* gene is largely restricted to pathogenic serotypes of *Y. enterocolitica*, a variant of it has been detected, albeit at rare frequencies, in 1A strains (Grant, Bennett-Wood & Robins-Browne, 1998). Thus, primers directed towards the ail gene may also amplify DNA from some of these strains (Paper IV). *Y. pseudotuberculosis* strains also harbour a homologous *ail* locus (Miller *et al.,* 1989). A homologous *inv* locus is present in all *Yersinia* spp. strains, but the protein is not expressed in the environmental isolates (Robins Browne, 1997). A homologous *yst* locus has been found in some isolates of *Y. kristensenii* and the gene differs in the American and European strains of *Y. enterocolitica* (Delor *et al.,* 1990; Ibrahim *et al.,* 1997).

Recently, other optional PCR targets apart from virulence genes have been suggested. For example the 16S rRNA gene has been selected for genus-, species- and group-specific identification (Ibrahim *et al.,* 1997; Trebesius *et al.,* 1998; Neubauer *et al.,* 2000b; Wannet *et al.,* 2001). ERIC-element (Enterobacterial Repetitive Intergenic Consensus sequences) has been used to simultaneously detect *Y. enterocolitica* and discriminate between the pathogenic and nonpathogenic strains (Aarts *et al.,* 2001).

Table 7. Commonly used PCR assays developed for detection of strains of pathogenic Y. enterocolitica, all targeting different locations within the respective gene

	Target gene	Reference
Chromosome	*ail*	Fenwick & Murray, 1991 Feng, Keasler & Hill, 1992 Kwaga, Iversen & Misra, 1992 Nakajima *et al.,* 1992 Blais & Phillippe, 1995 Harnett, Lin & Krishnan, 1996 Thisted Lambertz *et al.,* 1996 [a)] Jourdan, Johnson & Wesley, 2000 Boyapalle *et al.,* 2001 Wannet *et al.,* 2001
	inv	Rasmussen *et al.,* 1994
	yst	Ibrahim, Liesack & Stackebrandt, 1992 Ibrahim *et al.,* 1997 Harnett, Lin & Krishnan, 1996
	*rfb*C	Weynants *et al.,* 1996
	16S rRNA	Lantz *et al.,* 1998 Trebesius *et al.,* 1998 Neubauer *et al.,* 1999
Plasmid	*vir*F	Wren & Tabaqchali, 1990 Thisted Lambertz *et al.,* 1996
	*yad*A	Kapperud *et al.,* 1993 [a)] Blais & Phillippe, 1995 Lantz *et al.,* 1998 Neubauer *et al.,* 2000a [a)] Boyapalle *et al.,* 2001

[a)] Detects *Y. enterocolitica* and *Y. pseudotuberculosis*

Validation of PCR methods

In recent years, a number of PCR methods have been developed for the detection of pathogenic *Y. enterocolitica* applied either on colonies (Table 5) or on homogenates (Table 6). If a PCR method is used in only one laboratory and for a single matrix, the laboratory may conduct only a smaller in-house evaluation of the method. On the other hand, if the method is developed for use by other laboratories, a more comprehensive evaluation must be undertaken.

In 1999, the European Commission approved a research project entitled FOOD-PCR with the aim of validating and standardising the use of PCR-based methods for detection of the five major foodborne pathogens: thermophilic *Campylobacter*, *E. coli* O157, *Y. enterocolitica*, *Listeria monocytogenes* and *Salmonella* spp. (Hoorfar & Cook, 2003). A consortium of 35 institutes, companies and

universities from 14 EU-countries and 7 associated States worked in the project. The project group proposed the process of developing a standardised PCR-based method to take place in three phases. In brief, Phase 1 included selection of promising candidate primers and testing them for selectivity against a list of reference strains. One of the PCR assays was then selected and optimised and taken forward into the next phase. In Phase 2, a collaborative trial was conducted to test the repeatability and the robustness of the PCR assay (including amplification and detection). This phase itself was conducted in two steps. In the first step, the organising laboratory supplied all reagents whereas in the second step the participants used their own reagents. Finally, in Phase 3, the complete PCR-based method (including sample treatment, amplification and detection) was intended to be tested on food samples by comparison with the conventional culture method and in a collaborative trial.

The group also suggested a set of specific definitions of validation parameters and test controls. These were partly based on international working papers such as the MicroVal protocol (Anon., 2003a). In collaboration with European Committee on Standardisation (CEN), the project group prepared overall guidelines on General requirements and definitions (Anon., 2004a), sample preparation (Anon., 2004b), amplification and detection (Anon., 2004c), and a guideline on performance testing of thermal cyclers (Anon., 2004d).

Aims

The overall aim of the present thesis was to develop a PCR-based method for detection of pathogenic *Y. enterocolitica* in pork and to identify isolates of potential pathogenic *Y. enterocolitica.*

The specific aims were to:

- Develop a PCR (*ail*) assay, including examining the selectivity of the selected primers, and a pre-PCR sample treatment method
- Construct an internal amplification control
- Develop a PCR (*vir*F) assay for determination of the virulence potential of strains
- Evaluate the complete PCR-based method with artificially and naturally contaminated samples
- Compare the performance of the developed method with a conventional culture method
- Develop a multiplex PCR assay to identify four virulence factors in the strains and simultaneously identify the pathogenic *Y. enterocolitica* strains, specifically *Y. enterocolitica* 4/O:3 and *Y. pseudotuberculosis*

Materials and methods

Materials and methods used in this thesis are described in detail in each paper I-V. Here, only general comments are made. Two collaborative trials (data not published) are presented, and the techniques used are described.

Techniques used (Papers I-V)

Polymerase chain reaction, PCR

PCR-based methods are used increasingly in food microbiology because they offer a rapid, sensitive and specific detection and identification of pathogenic microorganisms. PCR is an enzymatic amplification of a specific region of the DNA molecule (Saiki *et al.,* 1988). The borders of the region must be known because in order to carry out the amplification, two specific oligonucleotides (primers) must be annealed to the DNA molecule, one to each strand of the denatured double helix. This delimits the region that will be amplified. A key enzyme, a thermostable DNA polymerase, carries out the amplification. The reaction cycle consists of the three steps: denaturation of the double-stranded DNA, annealing of primers to the single DNA strands and extension of the primers with the DNA polymerase. The detection of the PCR product is possible through gel electrophoresis, ethidium bromide staining and visual examination under ultraviolet light.

Variants of PCR assays

Single PCR is the most commonly used variant of PCR. It is carried out in one step and uses one pair of primers to amplify a chosen target DNA sequence. Nested PCR is two PCR analyses in succession. Two pairs of PCR primers are used to amplify the same target sequence. The first pair amplifies the target sequence as seen in any PCR experiment and the second pair of primers (nested primers) bind within the first PCR product and produce a second PCR product that is shorter than the first one. The logic behind this strategy is that if the wrong sequence were amplified by mistake in the first round, the probability is very low that it would also be amplified a second time by a second pair of primers. In addition, nested PCR offers an extreme increase in sensitivity over the primary PCR.

A multiplex PCR consists of multiple sets of primers in the same PCR assay to amplify different target sequences of different sizes, either on the same bacterial genome or on different bacterial genomes. Usually 2-4 primer sets are used. Multiplex PCRs have been developed both to characterize *Y. enterocolitica* strains with respect to various virulence properties (Weynants *et al.*, 1996), and to simultaneously detect different pathogens (including *Y. enterocolitica*) in the same PCR reaction tube (Ramesh *et al.*, 2002). In an extreme case, a PCR mix was developed targeting 13 different foodborne pathogens in the same reaction, including *Y. enterocolitica* and *Y. pseudotuberculosis* (Wang, Cao & Cerniglia, 1997).

Pulsed-field gel electrophoresis, PFGE

Pulsed-field gel electrophoresis provides the possibility to determine the relatedness of two bacterial isolates of the same species. The bacterial genome is cut into fragments by an enzyme and an electric field of hexagonal electrodes separates the fragments based on differences in size. First, intact bacterial cells are embedded in agarose and then the cells are lysed. DNA is subsequently digested with a rare cutting restriction endonuclease, ending up with a number of DNA fragments. Typically (and ideally) a small number of between 15 and 20 fragments will resolve during the following gel electrophoresis. The polarity of the current is changed at predetermined intervals to force the fragments to migrate in the gel to yield a unique banding pattern.

Buoyant density centrifugation, BDC

Techniques based on differences in density or particle size during centrifugation in a gradient medium have proven useful for separating PCR inhibitors in food from microorganisms and simultaneously concentrating the latter prior to PCR (Lindqvist, Norling & Thisted Lambertz, 1997). Percoll® (Amersham Biotech Pharmacia, Uppsala, Sweden) or BactXtractor™ (QRAB, Bålsta, Sweden) is used as the gradient medium. Percoll® consists of colloidal silica particles coated with polyvinylpyrrolidone (PVP) with diameters between 10-30 nm forming gradients in the range of 1.0-1.3 g ml^{-1}, which covers the cell densities of many microorganisms (Anon., 1995). It has advantageous properties such as not affecting the viability of the cells and possessing physiological ionic strength and pH. In Paper III, a gradient medium with silanised silica instead of PVP BactXtractor™ was used. The density gradient properties were similar to Percoll®.

Collaborative trials (data not published)

– Within the European Union research project FOOD-PCR

In 2002, Phases 1 and 2 of the three phases proposed by the research project group FOOD-PCR for development of a PCR-based method for detection of pathogenic *Y. enterocolitica* in pork were carried out.

In Phase 1, laboratories with expertise in working with the pathogen cooperated in surveying for suitable PCR primers. Three pairs, all previously published (Ibrahim *et al.*, 1997; Lantz *et al.*, 1998; Thisted Lambertz *et al.*, 2000), were chosen as candidate primers for further evaluation. The three primer sets were tested in parallel to evaluate their selectivity on a list of 50 representative strains. The testing was conducted at the Department of Applied Microbiology, Lund Institute of Technology, Sweden. The *ail* PCR primers (9A and 10A) were chosen among the three to be used in the next round of the standardisation process. The next step comprised a more extensive selectivity test and determination of the detection limit.

The *ail* primers 9A and 10A were tested on a list of 175 strains: 151 *Yersinia* spp. strains (of which 117 were target strains and 34 other *Yersinia* serotypes or *Yersinia* spp.) and 24 non-*Yersinia* spp. The *Y. enterocolitica* strains represented several serotypes. The non-yersinia strains were either closely related to, or encountered in, the same foods as the pathogenic strains. The tests were carried out at the National Food Administration in Sweden.

The detection limit was determined in terms of the relative frequency of positive PCR responses at various concentrations of the target cell (Knutsson *et al.,* 2002). Briefly, *Y. enterocolitica* strain SLV-408 was grown in BHI-broth to exponential phase and the cell number was determined by use of a Bürker-chamber and by plate count. A ten-fold dilution series was prepared in double distilled water and five PCR analyses were performed on each dilution. The number of positive signals obtained was expressed as a percentage, *e.g.* 3 positive signals from 5 reactions = 60%, and plotted against the number of cells contained in the reaction as calculated from the dilution factor of the original count. The procedure was repeated five times.

In Phase 2, a collaborative trial was conducted. The objective was to evaluate the repeatability and the robustness of the *ail* PCR assay (including amplification and detection). Fourteen European laboratories (in Austria, Czech Republic, Denmark (2 laboratories), France, Germany (2 laboratories), Greece, The Netherlands, Norway, Slovakia (2 laboratories), Sweden, and the United Kingdom) participated in the trials. Each laboratory received 18 blind coded identical DNA (20 pg $\mu l^{-1} \approx 4x10^4$ copies per reaction) samples including extracts from 7 strains of pathogenic *Y. enterocolitica*, 2 strains of nonpathogenic *Y. enterocolitica*, 6 strains of nontarget *Yersinia* spp. and 3 non-*Yersinia* strains. The trial was performed in two rounds. First the assay was tested on the 18 strains with reagents supplied by the trial organiser. Second, the assay was tested on the same 18 strains in a new round in which the participants used their own reagents. Each participant received a method procedure and a test report in which to record the results and return them to the trial leader. Each participant received sufficient reagents to perform the PCR analysis in triplicate on each sample in each step.

Phase 3 should have involved 10-12 partners from the EU project group in a collaborative trial validating the complete PCR method, but this phase has not yet been performed. (In 1997 the complete PCR method was evaluated in a collaborative trial performed within the Nordic countries and funded by the Nordic Committee on Food Analysis, NMKL. In this trial the method was compared with another PCR-based method, see below.)

– *Within the Nordic Committee on Food Analysis, NMKL*

In 1997, eight laboratories in Scandinavia (Sweden, Norway, Denmark and Finland) took part in a collaborative trial in which the complete PCR-based method (Paper III; Method 1 here) was compared with a PCR-based method for detection of pathogenic *Y. enterocolitica* in food developed by Kapperud *et al*. (1993). The latter method (Method 2) included a proteinase-K treatment prior to a nested PCR performed both on the nonenriched and the enriched sample. The

primers were directed towards a target on the virulence plasmid, *yad*A. As is described in Paper III, the proposed PCR-based method (Method 1) examined enriched samples only, and used a buoyant density centrifugation followed by a short NaOH-treatment as sample treatment prior to a single PCR (primers 9A and 10A). PCR was performed both on the enrichments and on cells isolated on CIN agar. Each laboratory received a set of instructions, reporting sheet and, within a week, reagents and samples for analysis. Two samples were sent prior to the trial as a test. In the trial the laboratories received six samples each on two different occasions with different strains used for inoculation. Two food matrices, brawn and unprocessed beef cuts, were each inoculated with *Y. enterocolitica* at levels of 0, 10 and 100 cfu per gram.

General comments on Materials and methods

Bacterial strains

Y. enterocolitica strain SLV-408, CCUG 45643, originally isolated from frozen raw dog feed (pig meat) was used as the reference strain in Papers I-III and V. The strain identity was confirmed at the Culture Collection University of Gothenburg, Sweden, and is commercially available. All strains used in the studies in this thesis are stored in the NFA Culture Collection.

Sample treatment prior to PCR

When PCR was used on pure or mixed colonies (Papers III and IV) a short sample treatment with NaOH-treatment (0.08M NaOH, heating at 75 ºC for 10 min, neutralisation) was applied prior to the PCR. Rossen *et al*. (1992) recommended a NaOH-treatment prior to PCR, to inactivate proteinases which could otherwise destroy or reduce the activity of the DNA polymerase. Furthermore, Bourke *et al*. (1998) reported that when DNA is held single stranded under alkaline conditions, the affinity of the inhibitory agents for the DNA immediately before PCR is reduced.

When PCR was used on the enriched or nonenriched homogenates (Papers III, IV and V) buoyant density centrifugation (BDC) was used as sample treatment prior to the NaOH treatment and PCR. Two similar gradient media were used: BactXtractor™ (Paper III) and Percoll® (Paper IV and V). In contrast to Percoll®, BactXtractor™ can be autoclaved in the presence of saline. This is an advantage when preparing the medium, but it is more expensive to purchase. Instead of autoclaving, Percoll® can be sterile filtered. For example, a 100% Percoll solution is prepared by mixing 100 ml Percoll® with 0.85 gram of NaCl followed by sterile filtering. The solution is stored at approx. 8 ºC or below.

Bovine serum albumin, BSA

Bovine serum albumin was added to the PCR mixture in all the studies (Papers I-V). BSA is a protein with certain binding properties that has been shown to reduce the effect of many inhibitory substances present in complex samples such as food

(Kreader, 1996). When PCR analysis was performed on colonies, BSA was sometimes replaced by double distilled water.

Internal amplification control, IAC (Papers II, III and V)

We found it problematic to keep the IAC stored at – 20 ºC at low concentrations (25-50 copies per µl) in 10 mM Tris-EDTA buffer or in double distilled water, since the signal weakened within 2-3 weeks. When instead the IAC was diluted (25 copies per µl) in *E. coli* DNA (20 µg ml^{-1}), and stored at – 70 ºC (in aliquots of 10 µl), no decrease in the intensity of the PCR signal was observable after nine months of storage.

Digest of the ail PCR product (data not published)

A (*Tsp*) restriction enzyme digest of the *ail* PCR products was used to distinguish products originating from *Y. enterocolitica* and *Y. pseudotuberculosis*. The enzyme was tested on amplified products from 10 reference strains. These consisted of five pathogenic *Y. enterocolitica* (Y.e) strains (Y.e O:18 IP-846; Y.e O:20 IP-845; Y.e O:21 IP1110; Y.e O:1,2,3 IP-64; Y.e O:2,3 IP-178); two nonpathogenic *Y. enterocolitica* strains (Y.e O:5 1A IP-124, Y.e O:8 1A IP-1105) and three *Y. pseudotuberculosis* strains (Y.p O:3 0071; Y.p O:2A 0069, Y.p O:1B 0068). Briefly, 3 µl NE Buffer 1, 1 µl *Tsp* enzyme (=10U) (New England BioLabs, UK), 5 µl PCR product and 21 µl of double distilled water were mixed. Samples were incubated at 65 ºC for 1-2 h. Immediately after the incubation the enzyme activity was inactivated by mixing 10 µl of the cleaved product with 1.5 µl 0.5 M EDTA, pH 8, and 1.5 µl LB (gel loading buffer). Bands were visualised after running electrophoresis on 13µl, loaded on a 2% agarose gel.

Terms

A standardised PCR method is a method that constantly gives the same results for a given sample when analysis is repeated several times and when performed by different analysts in different laboratories (Malorny *et al.,* 2003).

*Analytical accuracy – relates to P*CR *detection on colonies or DNA*

Analytical accuracy is a measure of the selectivity of the primers, which means the degree of response from target and nontarget microorganisms. It comprises the two terms (Anon., 2003a; Malorny *et al.*, 2003):

1) *Inclusivity,* detection of the target-pathogen from a wide range of strains.
2) *Exclusivity,* lack of response from a relevant range of closely related nontarget strains.

Diagnostic accuracy – relates to PCR detection in the presence of a biological matrix

A high degree of *diagnostic accuracy* means that a method detects, truly and precisely, the target microorganism in the presence of a biological matrix without interference from nontarget components. Diagnostic accuracy is used to evaluate the closeness of agreement between results of a PCR-based method and the accepted reference culture method. It comprises the two terms (Anon., 2003a; Malorny *et al.*, 2003):

1) *Specificity,* a measure of the degree to which the method is affected by nontarget components in the biological matrix (these tests may result in false-positive responses).
2) *Sensitivity,* a measure of the degree to which the method detects the target pathogen in the biological matrix (these tests may result in false-negative responses).

High robustness of a method means tolerance to a range of physical and chemical parameters. It is measured in terms of its reproducibility by other laboratories when different batches and brands of reagents, thermal cyclers and equipments are used.

Results

Here results from all papers I-V are briefly summarised, and results from two collaborative trials are described (data not published).

Development of two PCR assays

The ail *PCR assay (Papers I and III)*

A *ail* PCR assay was developed for detection of strains of pathogenic *Yersinia* spp., including the pathogenic serotypes of *Y. enterocolitica*, and *Y. pseudotuberculosis*. The selected primers targeted the chromosomally located *ail* gene. The PCR assay included both single and nested PCR primers. Only the outer primer pair was designed here. Wren & Tabaqchali (1990) designed the inner primer pair.

Evaluation of the ail PCR assay (Paper I)

In the evaluation of the *ail* PCR assay on pig tonsils, pathogenic *Y. enterocolitica* was detected by both PCR and a traditional culture method (NMKL-117) in five out of six samples analysed. The pathogen was detected by PCR in all five steps within the NMKL-117 method. Nested PCR was needed in almost all steps; an exception was after eight days of cold enrichment, where a single PCR was sufficient. No special sample treatment was applied prior to the PCR analysis; only a short NaOH-treatment was used. Strains of pathogenic *Y. enterocolitica* were isolated from only three of the samples on all sampling occasions. The pathogenic *Y. enterocolitica* strains had colony sizes of about 1-1.5 mm in diameter and not 2-4 mm as stated in the NMKL-method.

The detection limit of the ail PCR assay (data not published)

The detection limit of the *ail* PCR assay was determined in terms of the detection probability. The analysis range was 10^1-10^4 cfu ml^{-1}. The number of positive signals was expressed as a percentage, *e.g.* 3 positive signals from 5 reactions testing a certain number of cells in the reaction means 60%. It was estimated that the detection probability at a cell concentration of 45 cells per reaction was 100%. Furthermore, the detection probability at a cell concentration of 25 cells per PCR reaction was 75%, and at 15 cells per reaction it was 50%. The analysis was performed in double distilled water.

In addition, the detection limit was determined by a traditional set-up, *i.e.* testing duplicates instead of 5 reactions of different concentrations ($5x10^1$ to $5x10^3$ cfu ml^{-1}) of a serial dilution (Paper III). By this method the detection limit was determined to 25 cfu per PCR reaction volume of 10 µl. This number of bacteria of 25 cfu per PCR reaction corresponded to a detection limit of 200 cfu per ml obtained with the sample treatment (BDC) applied prior to PCR when the analysis was performed in PBS, *i.e.* with no food matrix present. Without the BDC step, the detection limit was $2.5x10^3$ cfu ml^{-1}.

Digest of the ail *PCR product (data not published)*

Selective restriction enzyme (*Tsp*) cleavage of the *ail* PCR products was used to identify the species as either pathogenic *Y. enterocolitica* or *Y. pseudotuberculosis*. A *Tsp* digest of the *ail* PCR products originating from pathogenic *Y. enterocolitica* produced one fragment when visualised on a gel, whereas the *ail* PCR products originating from *Y. pseudotuberculosis* produced two fragments.

The vir*F PCR assay (Papers I and III)*

A *vir*F PCR assay was used to determine the virulence potential of the detected pathogenic *Yersinia* bacteria. The primers target the *vir*F gene that is situated on the virulence plasmid. The assay included both single and nested PCR primers. Only the outer primer pair was designed here. Fenwick & Murray (1991) designed the inner primer pair.

Evaluation of the vir*F PCR assay (Paper III)*

Samples of pig tongue, head meat and minced meat were first screened by the *ail* PCR assay. Three *ail* PCR-positive samples of each of the three pork items were analysed for detection of the virulence plasmid to evaluate the *vir*F PCR assay. Of the nine *ail* PCR-positive strains tested, four were found fully virulent, *i.e.* they were PCR-positive for the *vir*F gene.

Development of the PCR-based method

The pre-PCR sample treatment, BDC (Paper III)

A sample treatment based on buoyant density centrifugation (BDC) was optimised for use prior to the *ail* PCR assay. The buoyant densities (BD) of the pathogenic *Y. enterocolitica* strains, the nonpathogenic strains and the *Y. pseudotuberculosis* strains were determined as ranging from 1.084 to 1.098 g ml^{-1}. The BD of the foods tested (brawn, beef and minced pork homogenate) were lower and ranged from 1.060-1.068 g ml^{-1}. Based on the determination of the BD, the optimal concentration of the density medium Percoll® (or BactXtractor) for best separation of bacteria and food (pork) was achieved in a 1.5-ml Eppendorf tube when 900 µl of the overnight enrichment was layered on top of 600 µl of 50% SIM (stock isotonic solution of the density medium), which corresponded to 1.077 g ml^{-1}, and finally centrifuged at 16 200 x *g* for 30 s. During the BDC the bacteria were separated from the food particles and concentrated in a volume of 50 µl.

Detection limit of the PCR-based method – determined in various artificially inoculated food samples (Papers III and V)

The same strain (SLV-408) was used for inoculation in two studies. Pure cultures were used in the study in Paper III. To somewhat mimic the situation for a natural contaminant as proposed by Rijpens *et al*. (2002), freeze-dried bacteria, *i.e*. bacteria exposed to stress, were used in the study in Paper V.

In Paper III, the PCR method yielded a detection limit of 2.5 x 10^2 and >5 x 10^4 cfu ml^{-1} for the brawn and beef samples, respectively, when a single PCR was used. By use of nested PCR, a detection limit of 60 and 125 cfu ml^{-1}, respectively, was obtained. The samples were tested on the nonenriched homogenates. When the PCR analysis was performed on enriched homogenates (incubation time up to 18 h), an initial number of 10 cfu or less per gram of brawn, raw beef or minced meat was detectable by single PCR. After 18 h of enrichment the aerobic plate count was estimated at 1.7 x 10^6, 6.2 x 10^9 and 7.9 x 10^9 cfu per gram of brawn, raw beef and minced pork, respectively. Culturable pathogenic *Yersinia* spp. were detected in all the three inoculated samples within 18 h of enrichment.

In Paper V, the method yielded a detection limit of 2.5 x 10^2 cfu ml^{-1} by single PCR for the two food samples tested, minced beef and cold-smoked pork sausage, when applied on nonenriched homogenates. By use of nested PCR, a detection limit of 25 cfu ml^{-1} was obtained. When the PCR was performed on the enriched homogenates, an initial inoculum of 25 cfu or less per gram was detected by single PCR for both food types.

Detection in naturally contaminated pork samples (Papers IV and V)

In all, 118 pork meat samples were collected and analysed (Paper IV). Approx. 10% (9 out of 91) of the raw pork products and none of 27 samples of ready-to-eat products tested positive for pathogenic *Y. enterocolitica*. Only single PCR was used. The PCR-positive raw pork samples were: loin of pork, fillet of pork, pork chop, ham and minced meat. Isolates of *Y. enterocolitica* 4/O:3 were recovered from six of the PCR-positive samples.

In Paper V, 100 minced pork samples and 97 samples of cold-smoked pork sausages were analysed. The pathogen was detected in 35% of the minced pork samples by PCR, and colonies were isolated from 5%. Approx. 11% of the cold-smoked pork sausage samples tested positive in PCR. No colonies were isolated.

Collaborative trials (data not published)

– Within the framework of the European Union research project FOOD-PCR, conducted in 2002

Phase 1. Three sets of primers were chosen for comparison in the first evaluation. Along with the primer set targeted against the *ail* gene, one set targeted the *yst* gene (Ibrahim *et al.,* 1997), and the last set simultaneously targeted the 16S rRNA and the *yad*A sequences, being a multiplex PCR (Lantz *et al.,* 1998). Of the three sets, the *ail* primers were selected for further evaluation because they performed well in the initial selectivity test, they targeted a well-studied virulence-encoding gene located on the chromosome and they were not included in a multiplex PCR. The next step included an in-house selectivity test on a list of 175 strains. The assay yielded 100% inclusivity and 94% exclusivity (Terms, Material and Methods). Three strains generally considered as nonpathogenic produced weak PCR products at the same size as the target product. These strains, designated *Y.*

kristensenii, 2207, *Y. enterocolitica* 1A, 2194, and *Y. frederiksenii*, 2208, were sent to an expert laboratory for species confirmation (Culture Collection University of Gothenburg (CCUG), Sweden, devoted to classification of bacteria (http://ftp.ccug.gu.se). Two strains, 2194 and 2208, were classified as *Y. enterocolitica* nontypable and strain 2207 was reclassified as *Y. kristensenii*.

Phase 2a, Trial 1. The objective was to evaluate the repeatability and the robustness of the *ail* PCR assay. Fourteen European laboratories tested 7 predicted PCR-positive samples and 11 predicted PCR-negative samples. Twelve participants performed the test in triplicate and 2 in duplicate only. The participating laboratories correctly classified 278 out of a total of 280 samples, all expected to be PCR-positive, corresponding to an inclusivity of 99.3%. Thus, 2 false-negatives were reported. Furthermore, 435 out of a total of 440 analysed samples all expected to be PCR-negative, corresponding to an exclusivity of 98.9%, were correctly classified. Thus, 5 were false-positives. Five of the samples expected to be PCR-negative were reported as giving weak signals. The cause was suggested to be carryover contamination from positive samples. Furthermore, one laboratory reported one of the target strains as PCR-negative, and thus only 2 out of the 3 replicates tested positive for that sample and laboratory. This trial established that the *ail* PCR assay was just as reproducible between laboratories as it was repeatable within a laboratory.

Phase 2b. In Phase 2a only one polymerase, Ampli Taq™ (Applied Biosystems), was trialled by all participants. In 2b they used their polymerase of choice. There were 6 reported polymerases used, but two partners did not return any information as to what they used: Tth (Roche); Platinum Taq (Gibco); Thermoprime Plus (ABgene) and Faststart Taq (Roche). The inclusivity and exclusivity values obtained in Phase 2b were greater than 90%. This indicates that a variety of polymerases may be successfully used in the assay, although it is strongly recommended that end users who wish to employ another polymerase consequently validate the selectivity of the assay against an appropriate selection of target and nontarget strains.

– Within the framework of the Nordic Committee on Food Analysis (NMKL), conducted in 1997

Trial 2. When analysed by Method 1 (Paper III), approx. 98% (63 out of 64) of the inoculated samples were reported as positives, while 88 % (28/32) of the noninoculated samples were reported as negatives. Thus four false positives were reported. The cause of the false positives was suggested to be carryover contamination from positive samples. The method targeted a chromosome-located gene. In addition to the PCR analysis, this method also included a step of streaking bacteria onto CIN agar plates to ensure detection of viable cells. Viable cells were recorded in 84% (54 out of 64) of the inoculated samples. (The method procedures have been published by the Nordic Committee on Food Analysis as NMKL-163 A and B for Method 1 and 2, respectively, www.nmkl.org.)

Of a total of 64 inoculated food samples sent to the eight participating laboratories, approx. 83% (53 out of 64) of the inoculated samples were reported as positives and 100% of the noninoculated as negatives when analysed by Method 2 (Kapperud *et al.,* 1993). The 53 positives were distributed between the two trial rounds, with 22 positives in the first round and 31 in the second round. The lower sensitivity obtained in the first round was due to the strain used for inoculation – a strain that produced low numbers (20-30%) of plasmid-harbouring cells when grown on BHI agar. The primers targeted a gene located on the virulence plasmid. In the second round, a strain was used that generated 80-90% plasmid bearing cells when grown on BHI agar.

Discussion

The primary transmission route of pathogenic *Y. enterocolitica* to humans is considered to be via contaminated food. This thesis concerns detection of the bacterium in pork, as the pig is the only animal used for human food production that regularly harbours the pathogen (Lake, Hudson & Cressey, 2004). One of the reasons is that pigs are recognised as the principal reservoir for the bacterium. However, whereas the pathogen can readily be isolated from the oral cavity and intestines of pigs, there is a lack of data regarding its occurrence in pork. This is due to methodological problems because no plating medium has yet been found to be sufficiently discriminatory or sensitive to allow the recovery of the bacterium by culture from pork. Only occasionally is the bacterium isolated from pork. Recent studies have shown that it is possible to detect the presence of the pathogen in pork at much higher frequencies by PCR than by culture methods. However, before a PCR method can be reliably applied for food analyses, it needs to be evaluated. Criteria to validate and standardise PCR-based methods for detection of foodborne pathogens, proposed by the European Union funded project group FOOD-PCR, were applied to the PCR method developed in this thesis.

Difficulties in isolation of strains of pathogenic *Y. enterocolitica*

Isolation of colonies of *Y. enterocolitica* is necessary for two reasons. One is that it enables epidemiological investigations, the other that isolated colonies confirm the viability of the cells detected by PCR. It has been suggested that the lack of recovery of the bacterium by culture methods stems from (i) low numbers of the pathogen present in the food, (ii) competition between the pathogen and other microorganisms during growth in liquid medium, or (iii) lack of selectivity of the solid medium, or maybe a combination of these factors (Kapperud, 1991; Fredriksson-Ahomaa & Korkeala, 2003; Lake, Hudson & Cressey, 2004).

As a consequence, only a few publications can be found in the literature in which the numbers of the pathogen present in food have been reported. Nesbakken *et al*. (1991) estimated the number of cells in eight samples of raw pork sausage with a colony hybridisation method and found it to vary between 50 and 2,500 cfu *Y. enterocolitica* per gram (average: 439 cfu g^{-1}). Furthermore, in three samples of raw pork cuts, the count ranged between 50 and 300 cfu *Y. enterocolitica* per gram (average: 200 cfu g^{-1}). Nortjé *et al*. (1999) isolated *Y. enterocolitica* from one sample of Vienna sausage at a count of 260 cfu per gram, and from two samples of ham at counts of 260 and 2,730 cfu per gram. In Paper V, where samples of naturally contaminated cold-smoked sausages were analysed, approx. 9% became positive by nested PCR. The analysis was performed on the nonenriched homogenate. A rough estimate of the level of *Y. enterocolitica* in these samples can be made by means of the detection limit obtained by use of artificially inoculated samples (Paper V). The detection limit for a single PCR was established to approx. 2.5 x 10^3 cfu per gram of food. Thus, as the PCR-positive cold-smoked pork sausage samples were detected only by nested PCR the number of the target bacterium most probably was less than 2.5 x 10^3 cfu per gram of

food. However, a PCR-positive signal could have failed to appear due to PCR inhibiting food substances present in the sample. The amplification control (AC) was added to the food matrices to monitor for such false negatives. The AC, which was added at a rate of 100 copies per PCR reaction tube, was amplified by single PCR showing that no inhibition occurred at that level. In the study in Paper III it was found that 25 cfu per PCR reaction tube, corresponding to 200 cfu per ml of the original sample, was enough to produce a single PCR positive signal. Provided that the same PCR amplification efficiency can be assumed for the AC and the target it could be calculated that 100 copies of the AC corresponded to 800 cfu per ml of the original sample, or 8 x 10^3 per gram of food. Thus it can be concluded that the number of the target bacterium most probably was less than 10^3-10^4 cfu per gram of food. This estimate is of the same magnitude as that obtained in the two other studies (Nesbakken *et al.*, 1991; Nortjé, 1999). It seems that *Y. enterocolitica* is found in retail pork products in numbers less than 10^3-10^4 cfu per gram. However, these data derive from only a few studies and it can be concluded that quantitative data on these types of products are significantly lacking.

Various researchers have reported problems in getting pathogenic *Y. enterocolitica* to grow in liquid media (Fredriksson-Ahomaa & Korkeala, 2003; Lake, Hudson & Cressey, 2004). Fukushima & Gomyoda (1986) showed that growth of a strain of *Y. enterocolitica* O:3 was suppressed in mixed cultures with *Yersinia*-related species, especially when competitors were initially present at 10^2-10^3 times the amount of the pathogen. Likewise, other members of *Enterobacteriaceae* were inhibitory to the pathogenic strain under similar conditions. This is consistent with results obtained in Paper III, in which it was shown (by PCR) that growth of a strain of *Y. enterocolitica* 4/O:3 was negatively affected by the background flora. The detection limit was dependent on the initial ratio of the pathogen to the level of background flora. Moreover, Strauch *et al*. (2001) showed that a strain of *Y. enterocolitica* biotype 1A (isolated from food) was able to inhibit the growth of pathogenic strains of *Y. enterocolitica*, serotype O:3, O:9 and O:5,27, due to the production of a bacteriocin. Bacteriocins are antibacterial substances produced by various species of bacteria that are usually active against closely related organisms. Strains of *Y. frederiksenii*, *Y. kristensenii* and *Y. intermedia* have also been demonstrated to produce bacteriocin-like substances (Calvo *et al.,* 1986; Toora, 1995). Both biotype 1A strains and the species mentioned are frequently isolated from food (Walker & Gilmour, 1986; Hudson *et al.*, 1992; Tassinari, Franco & Landgraf, 1994; De Boer, 1995; Logue *et al.*, 1996).

In several studies, it has been found that CIN agar is the most selective solid medium available for detection of pathogenic *Y. enterocolitica* (Head, Whitty & Ratnam, 1982; Schiemann, 1983; Walker & Gilmour, 1986; Aldova *et al.*, 1990; Cox *et al.*, 1990). It is well documented that on CIN agar, the colony morphology of nonpathogenic *Y. enterocolitica* variants and other bacteria commonly encountered on meat resembles that of pathogenic *Y. enterocolitica*, rendering selection of the relevant colonies very difficult (Devenish & Schiemann, 1981; Kapperud, 1991; Fredriksson-Ahomaa & Korkeala, 2003). In Paper III, 10 cfu per gram of a strain of pathogenic *Y. enterocolitica* 4/O:3 was inoculated into brawn, beef and minced meat, all with different initial numbers of the background

microflora. It was possible to locate individual colonies on CIN agar only from the brawn samples. The CIN agar plates were overgrown by other bacteria than that of interest on the plated samples of raw beef and minced meat.

Buoyant density centrifugation (BDC) as pre-PCR sample treatment (Paper III)

When PCR is used to identify pure cultures, problems are rarely encountered and PCR can be applied after a short sample pre-treatment. However, when PCR is applied on enriched or nonenriched homogenates, potential inhibition by media and/or food components must be considered (Rossen *et al.*, 1992). To a great extent, the sensitivity of a PCR method is determined by the efficiency of the sample treatment prior to the PCR (Lantz, 1998). In Paper III, a sample treatment based on buoyant density centrifugation (BDC) was developed. The BDC was found to remove PCR-inhibiting food substances and to concentrate the bacteria. Ideally, a PCR method should include a pre-treatment step that concentrates the target organism (Rijpens & Herman, 2002). This is especially important in the detection of a microorganism such as pathogenic *Y. enterocolitica* that is difficult to culture. In the extreme, it was possible to concentrate a volume of 10 ml homogenate with the DNeasy-tissue kit (Qiagen, GmbH, Hilden, Germany) used as sample treatment prior to the PCR (Paper V).

In the study in Paper V, the BDC was compared with two other sample treatments. All three were tested by the same *ail* PCR assay. The results showed that BDC and the DNeasy-Tissue-kit performed equally well by achieving the same low detection limit when the analysis was conducted on the enriched homogenates, whereas the commercial kit was superior when the analysis was performed on the nonenriched homogenates. Prepman™ reagent (Applied Biosystems) was the least efficient sample treatment (Paper V). Jourdan, Johnson & Wesley, (2000), on the other hand, obtained a low detection limit (approx. 10 cfu ml^{-1}) by real-time PCR using the Prepman™ reagent as sample pretreatment. Furthermore, Lantz *et al.* (1998) also compared three sample treatments and found that BDC when applied on the enriched homogenates achieved the lowest detection level. The fact that the sample treatment steps in these three studies were tested by different PCR assays makes comparison very difficult. An additional complication is that different types of food differ in their composition, which can also influence the performance. These examples illustrate the importance of testing various sample treatments in combination with the selected PCR assay and the specific food item before conducting a survey in order to obtain the lowest detection limit.

The *ail* gene as the target molecule for detection (Papers I-V)

The PCR assay developed for detection in this thesis uses a primer set that targets the chromosome-located *ail* gene (Papers I-V). The gene was found uniquely in strains of *Yersinia* spp. associated with pathogenicity in humans (Miller *et al.*, 1989). At least two variants of the *ail* gene exist in *Y. enterocolitica*, referred to as American and European strains (Beer & Miller, 1992). A variant is also present in *Y. pseudotuberculosis*. As a consequence, it is possible to construct a PCR assay

targeting locations within the *ail* gene specific for one or more of these strains (Table 7). As shown in Paper IV, the *ail* PCR assay developed in this thesis utilises sequence similarities within the variants of the *ail* gene, allowing for simultaneous detection of all three variants. A number of other PCR assays based on the *ail* gene, and on alternative genes, as target molecules for the detection are listed in Table 7. The PCR assays targeting the *ail* gene (except the *ail* PCR assay developed here) restrict detection to the pathogenic strains of *Y. enterocolitica*. Thus, none of the other PCR assays amplifies fragments of the *ail* gene present in *Y. pseudotuberculosis*. We found it beneficial to detect all the foodborne pathogenic *Yersinia* spp. simultaneously, by the same PCR method. After detection, the species, either *Y. enterocolitica* or *Y. pseudotuberculosis*, could be revealed by use of the multiplex PCR, if colonies were available (Paper IV), or by enzyme cleavage of the PCR product, if colonies were not available (see Results).

The results presented in Paper IV indicate that the *ail* gene may be a suitable target molecule for the detection because all pathogenic *Yersinia* spp. strains tested yielded a positive PCR signal when analysed by the *ail* PCR assay. In that, our results are in agreement with those of others who have utilised the *ail* gene as the target gene for detection (Blais & Phillippe, 1995; Weynants *et al.*, 1996; Wannet *et al.*, 2001). Furthermore, despite significant similarities to the Ail protein occurring in bacteria such as *Salmonella* Typhimurium and *Enterobacter cloacae*, no cross-reactions have been reported (Miller, 1992). Another important aspect to consider when choosing an appropriate target gene is the stability of the gene sequence. In a study of a sequence of the *ail* gene amplified from a number of clinical O:3 strains collected in Poland, Gierczynski *et al*. (2001) found that the sequence was highly conserved. Compared to the published *ail* sequence (Genbank AJ605740), eight PCR-amplified *ail* fragments sequenced in the study in Paper V were essentially identical, supporting the assumption that the *ail* gene sequence may be highly conserved.

Genes encoding different virulence factors in strains of *Y. enterocolitica* are located on the chromosome and on the virulence plasmid. Although a large number of virulence genes suitable as PCR target molecules are located on the plasmid, they are not considered appropriate due to the risk of losing them during laboratory procedures (Rijpens & Herman, 2002). In Paper IV, only 80% (*i.e*. 79 out of 98) pathogenic *Y. enterocolitica* strains examined harboured the plasmid, as indicated by a PCR-positive signal for the *vir*F gene. By contrast, all 98 strains were positive with the chromosomal *ail* gene as the PCR-target molecule. On the other hand, in Paper V seven out of seven strains analysed immediately after being isolated from food harboured the plasmid. Blais & Phillippe (1995) showed that repeated subculture at 37 °C resulted in an increased frequency of plasmid negative colonies. They detected 45 out of 45 isolates with the chromosome-located *ail* gene as target molecule but only 39 of the same isolates with the plasmid located *yad*A gene as target molecule. However, even if the plasmid is not a suitable target for PCR detection, it may sometimes be important to verify its presence because only strains that harbour both the chromosome- and plasmid-borne virulence genes are considered fully virulent.

Steps towards a standardised PCR-based method (Papers II-V)

Among the criteria considered for a standardised PCR-based method set by the European Union research project group FOOD-PCR were: high accuracy and robustness, the detection limit determined in terms of the detection probability, inclusion of an internal amplification control, low carryover contamination risk, accessible and user-friendly protocols for application and interpretation of the results. The method should also be validated against a reference method testing artificially and/or naturally contaminated food samples, and the method should be evaluated through a collaborative trial.

The detection limit of a PCR assay is defined as the lowest number of the target that can be reproducibly detected by the PCR itself (amplification and detection) and therefore it is performed on bacterial cells or DNA. The detection limit of the *ail* PCR assay developed in this thesis was determined to 45 bacterial cells per PCR reaction. A PCR assay developed for detection in food should detect at least 10-100 copies to enable detection of 10^3-10^4cells per ml of an enriched sample (Rijpens & Herman, 2002; Malorny *et al.*, 2003).

The detection limit of a PCR method is defined as the lowest number of the target that can be reproducibly detected by the complete PCR method (sample treatment, amplification and detection) and therefore must be tested on food matrices. Approx. 10 cfu *Y. enterocolitica* per gram of food was detected by single PCR when artificially inoculated samples were analysed after enrichment (Papers III and V). The result was valid for both pure cultures and stressed bacteria used for inoculation. However, when naturally contaminated samples were analysed, the majority required the sensitivity of a nested PCR to be detected (Paper V). This underlines the importance of using naturally contaminated food samples or relevant alternatives, *e.g*. testing a lower contamination level than 10 cfu per gram, when evaluating methods for detection of pathogenic *Y. enterocolitica* in food.

In the study presented in Paper II, an internal amplification control (IAC) was developed. The same primer recognition sites flanked the IAC as the target DNA. This construction may imply a risk for competition between the target DNA and the IAC for the PCR reaction components. This may lead to false-negative PCR results. This is especially a problem in samples containing the target organism in low numbers (Rijpens & Herman, 2002). Pathogenic *Y. enterocolitica* is most probably present in food in low numbers (Fredriksson-Ahomaa & Korkeala, 2003). The risk of competition can be decreased by use of low copy numbers of the IAC (Malorny *et al.*, 2003). In addition, the IAC can be used in a separate PCR tube tested in parallel with the sample (Paper V). Thus, rather than using it as an internal amplification control, the IAC can be used as an amplification control (AC).

It must be emphasised that when performing nested PCR analysis, particular care is required to avoid cross-contamination (Belák & Ballagi-Pordány, 1993). Among a large number of precautions that can be taken, we find it particularly important to mention that no positive control strain was handled in the laboratory throughout the test periods. Instead, we used the amplification control (AC) with the

advantage of producing a PCR product distinguishable from a true positive result (Paper V).

Two terms are used to describe the accuracy of a PCR test, one for the PCR assay itself (analytic accuracy) and one for the complete PCR method (diagnostic accuracy). The *ail* PCR assay showed high analytical accuracy by reaching 100% inclusivity and 95% exclusivity for accurate detection of the target and nontarget strains, respectively. However, when evaluating the diagnostic accuracy difficulties were encountered because the bacterium was detected in 35% of the samples by PCR but in none of the samples by the reference culture method ISO 1994 (Paper V). In an earlier study, the method was evaluated against a different PCR-based method. The evaluation was carried out through a collaborative trial. Agreement of the results was obtained in 98% of the inoculated samples and in 88% of the noninoculated samples (Results, Method 1). The corresponding agreement levels for Method 2 were 83% and 100%, respectively. This illustrates the problem encountered when assessing the diagnostic accuracy of a method developed for detection of a bacterium that is difficult to culture with the traditional culture techniques.

The robustness of a method is a measure of its capacity to withstand a range of physical and chemical variables and to tolerate different handling and PCR machines. Results from a collaborative trial performed within the framework of the European Union project group FOOD-PCR indicated high robustness for the PCR-based method developed in this thesis, *i.e*. the PCR results could be confidently reproduced by quite a few other laboratories. The project group took different aspects into consideration when deciding whether to take one or more levels of the bacterium to determine the robustness. To avoid involving too large a number of samples, it was decided to test only one level ($\sim 10^4$ copies per reaction tube). One level meant a total of 108 tests per laboratory. (It included a test for repeatability by triplicate samples of 18 strains to be tested in the same trial round.) However, it can be questioned whether the robustness was tested at an enough low level. Malorny *et al.* (2004) tested a range of parameters for a real-time PCR method by use of only 100 Salmonella genome equivalents. Furthermore, it has been suggested that the robustness of a method should be tested at the levels at which the target organism will be found in the food, or if this is not known (as is the case for *Y. enterocolitica*), at levels 10 to 100 times greater than that of the detection limit (Anon., 2004d). Hence, it is important when adopting a PCR method, although already validated, to ensure that the published detection limit can be reached, and to test this limit in as many well positions in the thermo cycler as possible. For this purpose, the availability of certified DNA reference materials or an appropriate internal amplification control (IAC) could be very helpful.

Detection of pathogenic *Y. enterocolitica* in pork (Papers I-V)

In several studies it has been reported that clearly higher yields of pathogenic *Y. enterocolitica* in minced pork have been detected by PCR than by culture (Fredriksson-Ahomaa & Korkeala, 2003). For example, Boyapalle *et al*. (2001) analysed 350 samples of minced pork collected from four different geographical regions in the United States. Approx. 38% tested positive by PCR, and at the same time none were positive by culture. Fredriksson-Ahomaa, Hielm & Korkeala (1999) analysed 255 samples of minced pork and obtained 25% positives by PCR and only 2% of the same samples by culture. The results obtained in Paper V, when investigating 100 samples of potentially contaminated minced pork, were in agreement with these findings – 35% positives were detected by PCR, whereas none were detected by culture. It can be argued that PCR detects only DNA and that the bacterium itself may be dead in raw pork. However, it has been documented in several studies that *Y. enterocolitica* can survive and grow on raw pork (Kapperud, 1991). Hellmann & Heinrich (1985) reported that viable *Y. enterocolitica* cells were recovered from raw minced pork after inoculation of 2.9-6.6x10^2 cfu *Y. enterocolitica* per gram and storage at 4 °C for 72 h. In another study, Nissen, Maugesten & Lea (2001) showed that *Y. enterocolitica* inoculated at about log 4 cfu per cm^2 on pork reached approx. log 8 cfu per cm^2 after 4 days of storage at 10°C. The background flora grew from about log 5 to log 9 cfu per cm^2 during the same time period. Thus the issue is not that pathogenic *Y. enterocolitica* are unable to survive (or growth) on fresh pork. Instead, it is that the available enrichment and isolation methods are not sufficiently sensitive to detect low numbers of the pathogen among a higher population of other bacteria, especially in the presence of nonpathogenic *Yersinia* spp. which are very common in fresh food (Schiemann, 1989; Kapperud, 1991).

Whereas minced pork has been analysed in several studies, few data are available in the published literature on the occurrence of pathogenic *Y. enterocolitica* in other kinds of pork commonly consumed (Lake, 2004). In Paper IV, samples of various raw pork products (n=91) collected from shops and homes related to yersiniosis patients were analysed, and in Paper V samples of cold-smoked pork sausage (n=97) collected from nine different meat-processing factories were examined. The bacterium was detected in fillet of pork, pork chop, ham and loin of pork (Paper IV), and in 11% of the cold-smoked pork sausages (Paper V). In one study conducted in Norway, 47 samples of sausage meat and 99 samples of pork chop collected from five slaughterhouses and one retail outlet were analysed (Johannessen, Kapperud & Kruse, 2000). The presence of pathogenic *Y. enterocolitica* was detected by PCR in approx. 15% and 26% of these samples, respectively. In another study, Nesbakken *et al*. (1991) examined 12 samples of pork cuts and 33 samples of sausage meat and obtained 5 and 23 positives, respectively. From these data it can be concluded that the bacterium can be detected by PCR in different kinds of raw pork cuts and pork products (other than minced pork) collected in retail stores. Kleemann & Bergann (1996) showed that *Y. enterocolitica* survived (but did not multiply) in pork sausage after smoking and storage. In that study, pathogenic *Y. enterocolitica* (10^4-10^5 cfu g^{-1}) were

inoculated into three kinds of German produced dry sausages prior to smoking (28-30 °C) and either cold storage (3-5 °C) or curing room storage (13-16 °C). The bacterium was re-isolated by direct plating up to 35 d and 20 d, respectively, after the inoculation. Whether or not the bacteria were alive in the studies mentioned above, the fact that the pathogen or DNA from the pathogen was detected (by PCR) in noninoculated samples provides evidence for its existence in this type of food. This may be important information in studies investigating possible food vehicles responsible for the transmission of the bacterium to humans.

It is important to note that the PCR methods with the highest yields of positives referred to above were variants with high sensitivities, either real-time PCR or nested PCR. Nested PCR, which uses two rounds of PCR, has been reported to be 100-1000 times more sensitive than a single PCR (Rijpens *et al.,* 1996; Shariff *et al.,* 2004). In Paper V, naturally contaminated minced pork and cold smoked pork sausage were analysed by both single and nested PCR. The vast majority (90%, *i.e*. 41 of 46) of the positives were detected by nested PCR. These results indicate that sensitivities such as those obtained by nested (or real-time PCR) are required to enable detection of pathogenic *Y. enterocolitica* in naturally contaminated pork and pork products. According to our results (Paper V), a conventional single PCR is clearly not sensitive enough to detect the pathogen in these products.

PCR used for identification of pathogenic *Y. enterocolitica* isolates (Paper IV)

The most widely used biochemical kit for identification of *Y. enterocolitica* and other *Yersinia* spp. is the API 20E system (Biomérieux, Vitek, Inc., Hazelwood, MO, USA). It is a kit for species determination and does not include a test for virulence, so additional biochemical tests correlated with the pathogenicity of the strains are necessary (Paper IV). These tests add to five days more work to the two days of species identification. Although previous reports diverge (Archer *et al.,* 1987), Neubauer *et al*. (1998) found that the API 20E system properly identified pathogenic strains of *Y. enterocolitica*, and that 95% of the nonpathogenic *Y. enterocolitica* strains investigated were identified. The former is consistent with our experiences (data not published). It is also consistent with our experience, especially for strains recovered from food, that the identification of the nonpathogenic isolates is sometimes problematic.

Use of PCR for identification has the advantage of not being dependent on the phenotypic expression and therefore, in addition, biochemically atypical organisms can be identified as readily as typical ones. For example, sucrose-negative isolates have been regarded as nonpathogenic and are therefore not proposed for further testing in the existing reference methods (Anon., 2003c). Recently, some sucrose-negative *Y. enterocolitica* isolates have been reported to harbour virulence characteristics. These strains were readily identified by use of a PCR method developed for detection of pathogenic *Y. enterocolitica* (Fredriksson-Ahomaa *et al.*, 2002). Likewise, Harnett, Lin & Krishnan (1996) reported that one of the phenotypic tests previously found to accurately differentiate pathogenic

from nonpathogenic serotypes, the pyrazinamidaze test generated different results, indicating both virulent and avirulent properties of serotype O:1,2,3. By use of a PCR assay these strains gave an unambiguous result.

Based on experience of the use of PCR for identification (Papers I and IV), and on results from others studies (Weynants *et al.*, 1996; Fredriksson-Ahomaa, Hielm & Korkeala, 1999; Rijpens, 1999-2000; Wannet *et al.*, 2001), it can be concluded that PCR can identify strains of pathogenic *Y. enterocolitica* and distinguish them from pathogenic strains within a couple of hours of work. However, it has been argued that a major limitation of the use of PCR for detection is that showing the presence of only one gene or target sequence does not adequately cover the pathogenic potential of a strain. Thoerner *et al.* (2003) mapped five virulence-associated genes present in the pathogenic strains of *Y. enterocolitica* and used five different PCR assays. As a more efficient system, a number of virulence genes can be detected simultaneously instead by use of a multiplex PCR. In Paper IV four primer pairs were combined in a multiplex PCR. Although multiplex PCR involves a far more complicated reaction system than a normal single PCR, it offers a very efficient identification tool because four virulence factors can be detected by only one PCR analysis.

In conclusion, the evaluation of the PCR-based method developed in this thesis identified the need for a nested PCR, instead of the less sensitive conventional single PCR, to enable detection of pathogenic *Y. enterocolitica* in naturally contaminated raw pork and pork products. The contamination problems usually associated with handling a nested PCR could be overcome by replacing the positive control strain with an amplification control. As the culture methods available today do not allow isolation to confirm the viability of the pathogen in this type of product, the PCR-based method can be used in studies where information about the viability of the bacterium is not of vital importance. It can for example preferably be used in studies trying to identify the critical points during slaughter when contamination of the pork cuts consumed by people occurs (fillet, loin of pork, chop, etc.).

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Sammanfattning

Det senaste decenniet har *Yersinia enterocolitica* orsakat 500-800 rapporterade fall av magsjuka i Sverige årligen. Grisar är det enda livsmedelsproducerande djurslag som regelbundet är bärare av bakterien, varför fläskkött sannolikt är en viktig smittkälla. Tidigare har man bara undantagsvis kunnat isolera bakterien från fläskkött men under senare år har detta kunnat ske med DNA-baserad teknik. I detta avhandlingsarbete har en PCR-metod för påvisning av patogen *Y. enterocolitica* i fläskkött utvecklats och utvärderats.

Den kromosomala genen attachment invasion locus (*ail*), som förekommer i alla sjukdomsframkallande bioserotyper av *Y. enterocolitica*, valdes som mål-DNA i PCR-metoden. *Ail* PCR-testet utvärderades enligt kriterier utformade inom det europeiska forskningsprojektet FOOD-PCR för en standardiserad PCR-baserad metod. I en avprövning med 14 deltagande europeiska laboratorier visade testet hög repeterbarhet och robusthet.

Den kompletta PCR-baserade metoden omfattar ett provbehandlingssteg före *ail* PCR-testet som i sin tur består av antingen en (single) eller två (nested) PCR-analyser samt en internkontroll för att indikera falsk-negativa PCR-resultat. Detektionsgränsen för hela PCR-metoden (single PCR) vid analys av anrikat prov fastställdes till 10 cfu *Y. enterocolitica* eller mindre per gram livsmedel. En ytterligare ökad metodkänslighet, i form av en nested PCR, krävdes dock för detektion av bakterien i naturligt kontaminerat fläskkött. Detta är en viktig kunskap som studien har gett.

Slutligen utvecklades ett multiplex PCR-test bestående av fyra primerpar, alla med en virulensassocierad gen som målmolekyl (*yst*, *rfb*C, *ail* och *vir*F), för karaktärisering av isolerade stammar. Samtidigt som förekomsten av de fyra virulensfaktorerna fastställdes kunde följande grupper identifieras: patogen *Y. enterocolitica* 4/O:3, övriga patogena bioserotyper av *Y. enterocolitica*, *Y. pseudotuberculosis* och icke patogena stammar.

En begränsning i metoden är att man inte har möjlighet att bekräfta bakteriens viabilitet. Det beror på att bakterien inte kan isoleras med nuvarande traditionell odling. PCR-metoden har därför sin tillämpning framför allt där denna information inte är viktig. Detta gäller t.ex. studier för att identifiera kritiska moment under slakten, vilket är betydelsefullt för att minska bakteriens spridning.