

Enteropathogenic *Yersinia* spp. and
Salmonella spp. in Swedish wild boars
The presence and molecular epidemiology

Axel Sannö

*Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala*

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2018

Acta Universitatis agriculturae Sueciae

2018:46

Cover: Illustration of a wild boar (*Sus scrofa*) sow and piglet by
Karin Olofsson and Per Sannö

ISSN 1652-6880

ISBN (print version) 978-91-7760-232-3

ISBN (electronic version) 978-91-7760-233-0

© 2018 Axel Sannö, Uppsala

Print: SLU Service/Repro, Uppsala 2018

Enteropathogenic *Yersinia* spp. and *Salmonella* spp. in Swedish wild boars. The presence and molecular epidemiology.

Abstract

Wild boars are reported as carriers of several zoonotic agents. The aims of this thesis was to investigate the presence of the foodborne enteropathogens *Salmonella* spp., *Yersinia* (*Y.*) spp. and *E. coli* O157:H7 in Swedish wild boars, the influence of potential risk factors presumably associated with their occurrence, the presence of *Yersinia* spp. and *Salmonella* spp. in minced meat, and to establish a method for molecular epidemiological studies (MLVA).

The thesis includes studies on lymphatic tissue and faeces from 178 wild boars and 32 samples of wild-boar minced meat. MLVA was evaluated on 254 isolates of *Y. enterocolitica* from several sources. Further, the wild boar populations were characterized with respect to four factors that possibly may be associated with the presence of enteropathogens in the wild boar.

A PCR-based protocol for the detection of enteropathogenic *Yersinia* spp. and *Salmonella* spp. was developed and evaluated. The protocol offered the possibility to obtain molecular epidemiological data of *Yersinia* spp. by MLVA on enrichment broths.

In total, 91 (51.1%) of the sampled wild boars carried the enteropathogens investigated and 46, 37 and 32 animals were PCR-positive for the presence of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Salmonella* spp., respectively. The subsequent cultivation yielded a total of 28 isolates of *Salmonella* spp., 10 isolates of *Y. enterocolitica* and 13 isolates of *Y. pseudotuberculosis*. None of the investigated risk factors were associated with the presence of these enteropathogens. However, a strong association between high levels of artificial feeding and high population densities was seen and the practice of leaving slaughter waste out in nature was found to be common among the hunters. In the new protocol, a positive selection for motile bacteria was used and seemed favourable in the detection of *Y. enterocolitica* and *Salmonella* spp. but not in the detection of *Y. pseudotuberculosis*. Further, MLVA performed directly on the enrichment broth seemed promising. MLVA on the isolates demonstrated a close resemblance between two human isolates and those obtained from domestic pigs whereas one rodent isolate was identical to that from pigs in the same farm.

In conclusion, human enteropathogens are present in a majority of the Swedish wild boars which may be of concern for the public health. The findings warrants further surveillance on the enteropathogens present and the new protocol presented within this thesis may be useful to obtain molecular epidemiological data for future reference in e.g. outbreak investigations.

Keywords: Wild boar, *Salmonella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, zoonosis, MLVA, molecular epidemiology, source attribution.

Author's address: Axel Sannö, SLU, Department of Clinical Sciences,
P.O. Box 7054, 750 07 Uppsala, Sweden
E-mail: axel.sanno@slu.se

Dedication

Till farsan

Contents

List of publications	7
Abbreviations	9
1 Introduction	11
1.1 The Swedish wild boar population	11
1.2 Slaughter of hunted wild boar	12
1.2.1 Risks associated with handling and consumption of wild boar meat	12
1.3 Foodborne pathogens in wild boar and wild boar meat	14
1.4 <i>Yersinia</i> spp.	14
1.4.1 <i>Yersinia pseudotuberculosis</i>	15
1.4.2 <i>Yersinia enterocolitica</i>	16
1.4.3 Enteropathogenic <i>Yersinia</i> spp. in wild boar and wild boar meat	17
1.5 <i>Salmonella</i> spp.	21
1.5.1 <i>Salmonella bongori</i>	21
1.5.2 <i>Salmonella enterica</i>	22
1.5.3 <i>Salmonella</i> spp. in wild boar and wild boar meat	23
1.6 Cultivation	26
1.6.1 <i>Yersinia</i> spp.	26
1.6.2 <i>Salmonella</i> spp.	27
1.7 Molecular epidemiology of <i>Salmonella</i> spp. and enteropathogenic <i>Yersinia</i> spp.	27
1.7.1 Pulse Field Gel Electrophoresis (PFGE)	28
1.7.2 Multi Locus Sequencing Typing (MLST)	28
1.7.3 Multi Locus Variable tandem repeats Analysis (MLVA)	29
2 Hypothesis and aims	31
3 Considerations on Materials and Methods	33
3.1 Study design	33
3.1.1 Sampling	34
3.2 Development of the laboratory protocol	36
3.3 Evaluation of the final protocol	38

4	Results and discussion	41
4.1	Food-borne pathogens in Swedish wild boars	41
	4.1.1 Implications for public health	43
	4.1.2 Risk factors for the presence of enteropathogens in wild boar	43
4.2	Evaluation of the new protocol	45
	4.2.1 The positive selection for motile bacteria	45
	4.2.2 Detection of enteropathogenic <i>Y. enterocolitica</i> using PCR	46
4.3	MLVA as a tool for epidemiological studies of enteropathogenic <i>Yersinia</i> spp.	47
5	Concluding remarks and future perspectives	51
	References	53
	Popular science summary	65
	Populärvetenskaplig sammanfattning	67
	Acknowledgements	69

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Sannö A., Aspán A., Hestvik G. & Jacobson M. (2014). Presence of *Salmonella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Escherichia coli* O157:H7 in wild boars. *Epidemiology & Infection*, 142 (12), pp. 2542-2547.
- II Sannö A., Jacobson M., Sterner S., Thisted-Lambertz S. & Aspán A. (2018). The development of a screening protocol for *Salmonella* spp. and enteropathogenic *Yersinia* spp. in samples from wild boar (*Sus scrofa*) also generating MLVA–data for *Y. enterocolitica* and *Y. pseudotuberculosis*. *Journal of Microbiological Methods*, 150, pp. 32-38.
- III Sannö A., Rosendal T., Aspán A., Backhans A. & Jacobson M. (2018). Distribution of enteropathogenic *Yersinia* spp. and *Salmonella* spp. in the Swedish wild boar population, and assessment of risk factors that may affect the prevalence of these zoonotic agents. *Acta Veterinaria Scandinavica*, 60 (40).
- IV Sannö A., Rosendal T., Aspán A., Backhans A. & Jacobson M. Comparison of Multiple-Locus Variable number tandem repeat Analysis profiles of enteropathogenic *Yersinia* spp. obtained from humans, domestic pigs, wild boars, rodents, red fox, pork, dog food and jack daws. (In manuscript)

Papers I-III are reproduced with the permission of the publishers.

Additional studies relevant to the thesis:

Råsbäck T., Rosendal T., Stampe M., Sannö A., Aspán A., Järnevi K. & Lahti E. (2018). Prevalence of human pathogenic *Yersinia enterocolitica* in Swedish pig farms. *Acta. Vet. Scand.*, 60 (39).

Hällbom, Hampus (2015). Förekomsten av *Yersinia* spp. och *Salmonella* spp. hos svenska jakthundar – Presence of *Yersinia* spp. and *Salmonella* spp. in Swedish hunting dogs. *Master Thesis, Swedish university of Agricultural Sciences, veterinary program.*

(Assistant supervisor)

Sterner, Sandra (2017). Smittspårning av *Yersinia enterocolitica* och *Yersinia pseudotuberculosis* med Multilocus Variable-Number Tandem-Repeat Analysis – Source attribution of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* with Multilocus Variable-Number Tandem-Repeat Analysis. *Master Thesis, University of Örebro, School of Health Sciences*

(Assistant supervisor)

Abbreviations

BG –Brilliant Green
BHI –Brain Heart Infusion
BPW –Buffered Peptone Water
CIN –Cefsulodin Irgasan, Novobiocin
CT-SMAC –MacConkey Agar with Sorbitol, Cefixime and Tellurite
DNA –Deoxyribonucleic Acid
EC –European Commission
EHEC –Enterohaemorrhagic *Escherichia coli*
HUS –Hemolytic-uremic syndrome
KOH –potassium hydroxide
ISO –International Organization for Standardization
MLVA –Multiple-Locus Variable number tandem repeat Analysis
PCR –Polymerase Chain Reaction
PFGE –Pulsed Field Gel Electrophoresis
pYP –Plasmid for *Yersinia* Virulence
SVA –National Veterinary Institute
SLU –Swedish University of Agricultural Sciences
XLD –Xylose Lysine Deoxycholate

1 Introduction

Wild boar populations are increasing all over the globe and so is the interest for wild-boar meat. This is however not without problems and damage to cultivated land, spread of epizootic pathogens to domestic animals, and wild boars finding new habitats in urban settings are all grounds for conflict between human interests and the wild-boar populations (Navarro-Gonzalez *et al.*, 2013a; Laurent & J., 2003). Although the meat from wild boars are attributed with many desirable quality traits (Sales & Kotrba, 2013), several studies also report on zoonotic agents present in wild boars and wild boar meat (Ruiz-Fons, 2017). The presented thesis focuses on three important foodborne zoonotic pathogens, *i.e.* *Salmonella* spp., *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, their presence in the Swedish wild boar population, and the development of suitable methods to detect the presence and obtain data that are useful for molecular epidemiological investigations.

1.1 The Swedish wild boar population

Besides a local population on the island of “Öland”, present during the 1500-1600 century and between 1723 to 1752, which was kept solely for hunting by the king, the wild boar has likely not been present in the wild in Sweden since 4 000 - 2 000 B.C. (Jonsson, 1986). In the 1940ies, a small number of wild boars escaped from hunting pens, but were hunted to extinction. The current Swedish population stems from several escapees from hunting and meat production enclosures in the 1970ies and in 1986, the Swedish parliament decided that the wild boar once again had a place in the Swedish fauna¹. The scattered population grew slowly during the late -80ies and the early -90ies, however, after the millennium shift the growth rate accelerated and during the following decade,

¹ Swedish hunting regulation 1987:905

the annual growth of the population amounted to 30%. By 2010, the national population was estimated to 100 000 animals (Anonymous, 2018). The annual growth potential of the population has been estimated to 48% and the main regulator of the population is hunting (Massei *et al.*, 2015). The annual harvest of wild boars during the past two years has amounted to 100 000 animals and an additional 5 000-6 000 wild boars are killed in collisions with vehicles². However, the size of the total population is difficult to estimate since there are no well-documented methods available.

1.2 Slaughter of hunted wild boar

The majority of the hunted wild boars are slaughtered and consumed in the homes of the hunters. According to the European legislation (EC No 853/2004), no wild boars or part thereof are to be let out on the market without having passed through a wild-game handling establishment and a veterinary inspection including sampling for the presence of *Trichinella* spp. The Swedish board of agriculture reported that in 2012, only 15% of the harvested wild boars were passing through a wild-game handling establishment and hence let out on the open market (Anonymous, 2013). Slaughter waste generated in these establishments are to be handled and disposed of according to EC No 1069/2009. Wild boars can also be eviscerated and delivered to wild-game handling establishments provided that a specially trained person have examined the offal and filled out a certificate stating the freedom from pathological lesions (EC No 853/2004). However, slaughter-waste from wild-game shot by private hunters, including wild boars, are allowed to be disposed of and left out in nature.

1.2.1 Risks associated with handling and consumption of wild boar meat

There are several pathogens associated with wild boars and wild-boar meat (Ruiz-Fons, 2017). In healthy wild boars, bacteria or viruses are not present in the muscles of the animal. However, pathogenic parasites *e.g.* *Trichinella* spp. and *Toxoplasma gondii* that are frequently reported in wild boar may be dispersed in the muscle tissue also in the apparently healthy wild boar (Dubey, 2009; Pozio, 2007). Tests for the presence of *Trichinella* spp. in meat samples is available (Gajadhar *et al.*, 2009). Most hunters comply with the recommendation to test all wild boars aimed for private consumption for the presence of

² National statistics of traffic accidents involving wild game (in Swedish only) Available at: <https://www.viltolycka.se>

Trichinella spp. and in Sweden, less than 0.1% are tested positive each year³. A recent serological study on the Swedish wild boar population showed that the prevalence of *T. gondii* varied between 38 and 72% in different years (Wallander *et al.*, 2015), similar to findings in other parts of Europe. If meat with *Toxoplasma gondii* are frozen below -12°C these are however rendered uninfectious (Kotula *et al.*, 1991). Bacteria and viruses can be carried by wild boars in internal organs such as the tonsils, lymphatic tissue, and in the oropharynx, as well as in the intestinal content. Thus, any injury inflicted to these organs during hunting or evisceration and slaughter may result in contamination of the carcasses (Avagnina *et al.*, 2012). Cold storage of the carcass and the meat may prevent most pathogens from multiplying. However, the psychotropic properties of *e.g.* enteropathogenic *Yersinia* spp. allows the bacteria to grow at temperatures as low as below 0°C. Bacteria will therefore multiply also during cold storage. Provided that the temperatures exceed 70°C, most pathogens present on the surface are killed during preparation of the meat. However, bacteria present on the surface of the meat may still pose a risk for cross-contamination during slaughter, cutting and the further processing in the kitchen with the spread of bacteria to *e.g.* raw vegetables or other produce that are not heated before consumption, hence constituting a risk for infection (Gorman *et al.*, 2002). In this respect, minced meat poses a particular risk because of the increased processing and extended surface.

An additional risk in the handling of wild boar and wild-boar meat is the introduction of pathogenic bacteria into the blood-stream through wounds. Of particular concern are *Streptococcus (S.) suis* and *Erysipelothrix (E.) rhusiopathiae*, common pathogens present also in the conventional pig. There are 35 different serotypes of *S. suis* described. Serotypes 2, 5, and 14 regularly cause disease in humans and may also cause serious illness and death (Fernández-Aguilar *et al.*, 2018; Goyette-Desjardins *et al.*, 2014). *S. suis* are present in the Swedish wild boar, although to what extent and of which serotypes are yet to be investigated⁴. In Sweden, three cases of disease have been described, one concerning a 63-year old man becoming seriously ill after having slaughtered a wild boar and cut himself during the process (Dalsjö *et al.*, 2014). Infection with *E. rhusiopathiae* is considered to be related to people working with animals, slaughter and other risk occupations. The infection most commonly manifests as an acute localised cutaneous infection, however

³ National Veterinary Institute (SVA) Available at: <http://www.sva.se/analyser-och-produkter/referenslaboratorium/referenslaboratorium-och-nrl/trikinfynd-tabell> [2018-08-01]

⁴ Anna Werinder personal communication 2018-06-28

systemic infections and endocarditis may occur with a mortality rate that may approach 38% (Brooke & Riley, 1999).

1.3 Foodborne pathogens in wild boar and wild boar meat

A number of other foodborne zoonotic viruses, parasites and bacteria have been demonstrated in the wild boar and in wild boar meat. Hepatitis E have been found in Swedish wild boars (Widén *et al.*, 2010) as well as in wild boars from several other European countries (Oliveira-Filho *et al.*, 2014) and in Japan, links to human cases have been established (Masuda *et al.*, 2005). *Campylobacter* spp. is the most common cause of food-borne disease in humans. *Campylobacter* spp. have been detected in faecal samples from Swedish (Wahlström *et al.*, 2003) as well as from wild boars in several other countries (Díaz-Sánchez *et al.*, 2013; Sasaki *et al.*, 2013; Jay-Russell *et al.*, 2012), but also in the tonsils (Jay-Russell *et al.*, 2012), and on the carcasses (Atanassova *et al.*, 2008).

Escherichia coli carrying the enterotoxin genes *stx1* and *stx2* is known to be associated with disease in humans including the serious condition HUS⁵, and is frequently detected in wild boars. Rarely, also EHEC O157:H7 carrying the *eae* gene have been detected. A large outbreak in North America linked to baby spinach contaminated with *E. coli* O157:H7 have been reported and feral swine shedding the bacteria in the field was the suspected source of contamination (Jay *et al.*, 2007).

Listeria monocytogenes is not commonly detected in wild boars, however, isolates have been reported from both tonsils and faeces (Wacheck *et al.*, 2010) as well as on the carcasses (Avagnina *et al.*, 2012; Atanassova *et al.*, 2008) and in retail meat samples (Kanai *et al.*, 1997). There are few cases of human listeriosis reported annually in Europe (2 206 cases in 2015) but with a mortality rate of 270 deaths out of the 1 524 cases with known outcome in 2015, it is by far the most deadly food-borne disease with a case fatality rate of 17.7% (European Food Safety *et al.*, 2016).

1.4 *Yersinia* spp.

The causative agent of the bubonic plague, in 1944 given the name *Yersinia* (*Y.*) *pestis*, was first discovered in 1894 by the bacteriologist Alexandre Yersin at the Pasteure Institute (van Loghem, 1944). A few years earlier (1883) *Y.*

⁵ HUS –Hemolytic-uremic syndrome

pseudotuberculosis had been described, however, it was not until 1974 that it was given the current name and classification (Carniel *et al.*, 2006). In 1934, a bacterium isolated from a facial abscess of a 53-year-old farm worker was five years later linked to and described together with isolates from patients with enteric disease, and given the name *Bacillus enterocoliticum* (Bottone, 1999). The bacterium was then reclassified by Fredriksen in 1964 and designated as *Y. enterocolitica* (Carniel *et al.*, 2006). The genus *Yersinia* within the family of Enterobacteriaceae are Gram-negative coccoid rods and facultatively anaerobic bacteria. The genus today consists of 18 species, some of which are pathogenic to humans (McNally *et al.*, 2016). The species include *Y. pestis* and the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica*, causing yersiniosis (McNally *et al.*, 2016).

Initially, media used for other enteric pathogens, such as sheep blood agar with tryptose, MacConkey's agar and salmonella-shigella-agar, was used in the cultivation (Delorme *et al.*, 1974). The introduction in 1975 of cold enrichment improved the isolation (Eiss, 1975) and several different protocols including 2-4 weeks of cold enrichment have been proposed and evaluated, but no common standard has been established (Fredriksson-Ahomaa & Korkeala, 2003; Nesbakken *et al.*, 1991). In 1979, Schemmann developed the cefsulodin-irgasan-novobiocin (CIN) agar, a selective medium aimed for isolation of *Y. enterocolitica* (Schemmann, 1979), which is still today the most commonly used selective media for cultivation of enteropathogenic *Yersinia* spp.

Polymerase Chain Reaction (PCR) for the detection of enteropathogenic *Yersinia* spp. was developed in the early nineties and already in these first studies, the *ail*-gene in *Y. enterocolitica* was the selected target (Nakajima *et al.*, 1992). Since then, PCR-protocols for other target genes have been developed, often in duplex or multiplex PCRs also targeting the *ail*-gene, further substantiating the association to pathogenicity (Petsios *et al.*, 2016).

1.4.1 *Yersinia pseudotuberculosis*

The name of this species derives from its ability to cause tuberculosis-like lesions in several animal species such as hares, deer and non-human primates with granulomas in the spleen, liver and lymph nodes (Fredriksson-Ahomaa, 2015). In humans, different strains can cause a variety of symptoms including anything from self-limiting diarrhoea and abdominal pain to mesenteric lymphadenitis with mesenteric granulomas mimicking appendicitis (Galindo *et al.*, 2011) and the severe Far East scarlet-like fever or Izumi-fever (Amphlett,

2016). Sequelae following yersiniosis includes erythematous nodosus and reactive arthritis.

Y. pseudotuberculosis is divided into ten serotypes and 15 biotypes, all considered to be pathogenic (Fredriksson-Ahomaa, 2015). Infection usually occurs via the gastro-intestinal route following ingestion of contaminated food and is facilitated by bacterial attachment and invasion of the intestinal cells, encoded by the attachment and invasion gene (*ail*-gene) present in all pathogenic *Yersinia* (Lambertz *et al.*, 2008a). The propagation of the infection and avoidance of the host immune response is regulated mainly by genes located on the 70-Kb “plasmid for *Yersinia* virulence” (pYV; Bancercz-Kisiel *et al.*, 2018).

In Europe, a few sporadic cases of yersiniosis caused by *Y. pseudotuberculosis* are reported annually, however larger outbreaks have occurred and is often associated with raw vegetables. In Finland, outbreaks affecting several hundred children occurred following the consumption of contaminated raw-grated carrots (Rimhanen-Finne *et al.*, 2009; Jalava *et al.*, 2006). The source of the contamination was never detected but shrews were suspected (Kangas *et al.*, 2008). Outdoor-raised pigs have been shown to carry *Y. pseudotuberculosis* more frequently than indoor-raised pigs (Laukkanen *et al.*, 2008) and in wild-life such as rodents and wild birds, *Y. pseudotuberculosis* are commonly isolated (Backhans *et al.*, 2011; Niskanen *et al.*, 2003).

1.4.2 *Yersinia enterocolitica*

Y. enterocolitica is a very heterogeneous species that includes six different biotypes, differentiated by biochemical reactions, and 76 serotypes based on the lipopolysaccharide O-antigen present on the cell surface (Nesbakken, 2015). The biotype 1A is generally considered as non-pathogenic (Fredriksson-Ahomaa, 2017), although the opinions differ regarding the pathogenicity (Platt-Samoraj *et al.*, 2017; Sihvonen *et al.*, 2011a; Tennant *et al.*, 2003). The biotype 1B is highly pathogenic, whereas the biotypes 2-5 is considered as being of low pathogenicity. The serotype is also related to the pathogenic properties of the bacteria with O:3, O:8, O:9 and O:5,27 being the serotypes most commonly associated with human disease (Fredriksson-Ahomaa, 2017). In Europe, the bioserotype 4/O:3 dominates while in North America, the bioserotype 1B/O:8 has been most commonly associated with human disease. In recent years however, 4/O:3 has been introduced into North America and is now regarded as the most common bioserotype (Bottone, 1999).

In 2014, the annual notification rate for yersiniosis in Europe was 1.92 cases per 100 000 population, making it the third most common food-borne disease

next to *Campylobacter*-infection and salmonellosis (Anonymous, 2015). Children aged 0-5 years are affected to a higher degree as compared to adolescents and adults. The main symptoms include self-limiting diarrhoea and vomiting, abdominal pain, sometimes mimicking appendicitis, and sequelae such as reactive arthritis and erythema nodosum may occur (Galindo *et al.*, 2011).

The most commonly identified reservoir of *Y. enterocolitica* 4/O:3 is the domestic pig and several epidemiological studies have identified the consumption of raw or undercooked pork as an important risk factor for contracting yersiniosis (Fredriksson-Ahoma *et al.*, 2006b). Other reservoirs include wild birds, rodents, wild boars, sheep, dogs, and cat (Stamm *et al.*, 2013; Wacheck *et al.*, 2010; McNally *et al.*, 2004; Niskanen *et al.*, 2003).

1.4.3 Enteropathogenic *Yersinia* spp. in wild boar and wild boar meat

Yersinia spp. are host-adopted to pigs and present in domestic pigs throughout the world (Bottone, 1999). They are also commonly found in wild boars and feral pigs. Table 1 present studies describing the detection of enteropathogenic *Yersinia* spp., including those detailed in paper I and III. However, not all studies, *e.g.* studies based solely on serology and PCR-analysis, have fully elucidated the pathogenic properties of the bacteria. Recently, Platt-Samoraj (2017) investigated rectal swabs from 434 hunted wild boars for the presence of *Y. enterocolitica* and found 18 (4.3%) strains of the biotype A1 that all harboured both the *ail*- and the *ystB* genes (Ramamurthy *et al.*, 1997). The pathogenic properties of these biotype 1A strains are yet to be investigated.

Identified human cases also need to be linked to these findings. Although several studies have demonstrated the presence of enteropathogenic *Yersinia* spp. in the wild boar, reports establishing a link to human disease are still missing. However, these findings may still be of concern since carcasses and meat can become contaminated during slaughter, thus constituting a risk for further transmission to happen through consumption of wild-boar meat.

Table 1. *A summary of previous studies reporting the presence of Yersinia spp. in wild boars*

Country (year)	Sample type	No of samples (individuals)	Method	No of positive samples (%)	No of positive individuals (%)	Reference
Switzerland (2007-08)	Tonsils	153 (153)	PCR (<i>ail</i> -gene)	53 (35.0) ¹	53 (35.0)	(Wacheck <i>et al.</i> , 2010)
			PCR (<i>ail</i> -gene)	30 (19.6) ²	30 (19.3)	
			Cultivation	14 ¹	N.s.	
			Cultivation	4 ²	N.s.	
	Faeces	73 (73)	PCR (<i>ail</i> -gene)	4 (5.0) ¹	4 (5.0) ¹	
			Cultivation	1 (1.0) ¹	N.s.	
Spain (2010-12)	Serum	490 (490)	Serology	257 (52.2)		(Arrausi-Subiza <i>et al.</i> , 2016)
	Tonsils	72 (72)	PCR (<i>ail</i> -gene)	37 (51.4) ¹		
			PCR (<i>ail</i> - and <i>wzz</i> -gene)	18 (25.0) ²		
Poland (2012-13)	Rectal swabs	302 (151)	Cultivation	1 (0.3) ¹	1 (0.7) ¹	(Bancerz-Kisiel <i>et al.</i> , 2015)
Sweden (2010-2011)	Tonsils	175 (88)	PCR (<i>ail</i> -gene)	19 (10.9) ¹		(Sannö <i>et al.</i> , 2014; paper I)
			PCR (<i>ail</i> -gene)	19 (10.9) ²		
	Ileocaecal lymph nodes	56 (56)	PCR (<i>ail</i> -gene)	4 (7.1) ¹		
			PCR (<i>ail</i> -gene)	1 (1.8) ²		
	Faeces	88 (88)	PCR (<i>ail</i> -gene)	1 (1.1) ¹		
			PCR (<i>ail</i> -gene)	1 (1.1) ²		
	PCR-positive Buffered Peptone-Water broths	24(18) ¹	Cultivation	1(4.2) ¹		
	21(17) ²	Cultivation	5(23.8) ²			
Sweden (2014-2016)	Tonsils	136 (90)	PCR (<i>ail</i> -gene)	19 (14.0) ¹		(Sannö <i>et al.</i> 2018; paper III)
			PCR (<i>ail</i> -gene)	20 (14.7) ²		
	Mesenteric lymph nodes	90 (90)	PCR (<i>ail</i> -gene)	6 (6.7) ¹		
			PCR (<i>ail</i> -gene)	4 (4.4) ²		

Country (year)	Sample type	No of samples (individuals)	Method	No of positive samples (%)	No of positive individuals (%)	Reference
	Faeces	90 (90)	PCR (<i>ail</i> -gene)	4 (4.4) ¹		
			PCR (<i>ail</i> -gene)	2 (2.2) ²		
	PCR-positive Brain-Heart	36(28) ¹	Cultivation	10(27.8) ¹		
	Infusion broths	26(20) ²	Cultivation	2(7.7) ²		
Germany (1995-96)	Serum	763	Serology	478 (62.6)	478 (62.6)	(Al Dahouk <i>et al.</i> , 2005)
Bulgaria (1998-1999)	Heart	2 (2)	Cultivation	1 ²	1 (50)	(Nikolova <i>et al.</i> , 2001)
	Spleen	2 (2)	Cultivation	2 ²	2 (100)	
	Liver	2 (2)	Cultivation	2 ²	2 (100)	
	Kidney	2 (2)	Cultivation	1 ¹	1 (50)	
Italy (2006)	Diseased/dead animals	5 (5)	Cultivation	4 (80) ¹	4 (80)	(Magnino <i>et al.</i> , 2011)
Italy (2008-10)	Carcass swabs	251 (251)	Cultivation	3 (1.2) ^{1,3}	3 (1.2)	(Avagnina <i>et al.</i> , 2012)
Japan (1993-94)	Meat from retail	93 (N.s.)	Cultivation	36 (38.7) ^{1,4}		(Kanai <i>et al.</i> , 1997)
Japan (1994-95)	Faeces	131 (131)	Cultivation	41 (31.0) ^{1,4}		(Hayashidani <i>et al.</i> , 2002)
			Cultivation	5 (4.0) ²		
Czech Republic (2013-14)	Meat juice	135 (135)	Serology	89 (65.9)		(Alena <i>et al.</i> , 2016)

1. *Y. enterocolitica*

2. *Y. pseudotuberculosis*

3. PCR-confirmation detected the *inv*-gene but not the *ail*- or *yst*-genes

4. No pathogenic strains detected

N.s. Not stated

1.5 *Salmonella* spp.

The genus *Salmonella* is named after the American veterinarian Salmon who together with his colleague Smith in 1886 isolated what was later identified as *S. Choleraesuis* from pigs thought to suffer from swine fever. In 1880, a bacteria isolated from a deceased patient suffering from typhoid fever was assigned to the same genus (Wray & Wray, 2000). In 1896, the bacteriologists M. von Gruber and H. Durham made the first discoveries (Durham, 1901) of agglutination of bacteria together with antibodies that later led to the development of the slide-agglutination test used to serotype *Salmonella* spp., known as the Kauffman-White classification (White, 1926) that is still being used today (Grimont & Weill, 2007).

The genus *Salmonella* is included within the family of Enterobacteriaceae and are Gram-negative rods, facultatively anaerobic, and predominantly motile. Two main species are included in the genus, *i.e.* *Salmonella bongori* and *Salmonella enterica*, the latter being subdivided into six subspecies (Brenner *et al.*, 2000). These subspecies are further subdivided into more than 2 500 serovars according to the Kauffman–White classification utilising the lipopolysaccharide O-antigen and the flagellar H-antigen located on the bacterial surface (Issenhuth-Jeanjean *et al.*, 2014). The most common symptoms of salmonellosis in humans include enteritis, diarrhoea, abdominal pain, and fever, referred to as non-typhoid salmonellosis (NTS), and is associated with “classical” food poisoning. A severe systemic disease with an estimated 21.7 million cases annually and a conservatively calculated case-fatality rate of 1% is caused by specific serotypes that are adopted and spread between humans or non-human primates and is referred to as typhoid or paratyphoid salmonellosis (Crump *et al.*, 2004)

1.5.1 *Salmonella bongori*

This microbe is mainly associated with cold-blooded animals and regarded as part of the normal microbiota in reptiles, but has also recently been isolated from wild birds (Foti *et al.*, 2009). Salmonellosis caused by *S. bongori* have been reported in dogs as well as in humans and causes the non-typhoid form of the disease mainly in children younger than three years of age (Giammanco *et al.*, 2002).

1.5.2 *Salmonella enterica*

The six subspecies of *S. enterica* includes *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*. Almost all isolates from humans and mammals belong to *S. enterica* subsp. *enterica*, while the remaining subspecies are most commonly found in reptiles and other cold-blooded animals as well as in environmental and water samples (Brenner *et al.*, 2000). The various serotypes of *Salmonella* spp. are usually host-adopted and specific serotypes will cause serious disease in one or a few host species, however, other hosts may be asymptomatic carriers or display moderate signs of disease (Uzzau *et al.*, 2001). Salmonellosis is most commonly caused by serovars belonging to *S. enterica* subsp. *enterica*, although serovars belonging to other subspecies are also infrequently associated with disease (Aleksic *et al.*, 1996).

The two serovars most commonly isolated from patients with salmonellosis in Europe are *S. Enteritidis* and *S. Typhimurium*, accounting for 44.4% and 17.4% of the reported cases in 2014, respectively. Egg, products thereof, and poultry meat are regarded as the most common sources of salmonellosis in Europe (Anonymous, 2015).

In 2014, the annual notification rate for salmonellosis in Europe was 23.4 cases per 100 000 population (Anonymous, 2015). According to estimations by the World Health Organization (2010), foodborne non-typhoid *Salmonella* caused the most Disability Adjusted Life Years (DALYS) of all foodborne pathogens with 4 067 929 DALYs (2 486 092–6 271 290)⁶ and worldwide, 78 707 591 (31 843 647– 211 154 682)³ people suffered from salmonellosis resulting in 59 153 (36 341–89 045)³ deaths (WHO, 2015).

⁶ 95% uncertainty intervals

1.5.3 *Salmonella* spp. in wild boar and wild boar meat

The host-adopted serovar *S. Cholerasuis* have the potential to cause severe disease and death in pigs, including the wild boar (Methner *et al.*, 2010), while other serovars such as *S. Typhimurium*, *S. Derby* and *S. Rissen* are usually asymptotically carried by the pig and is thus mainly of concern for the public health (EFSA, 2016). A large variety of *Salmonella* spp. serovars that are known to be capable of causing disease in humans have been reported in wild boars (Table 2). Serovars being pathogenic to humans can be carried by pigs and wild boars and the carcass or the meat can become contaminated by unprofessional shooting, spoilage of intestinal content during evisceration, or transfer of bacteria from the oropharynx to other parts of the carcass or the meat (Avagnina *et al.*, 2012). *Salmonella* spp. has a limited ability to grow in temperatures below 10°C and the bacteria are killed at temperatures above 70°C (Silva & Gibbs, 2012). Hence, the importance of proper handling at slaughter and proper preparation of the wild boar meat should not be underestimated. Similarly to enteropathogenic *Yersinia* spp., there are no reported human cases of salmonellosis that has been linked to the consumption of wild boar meat.

Table 2. *A summary of previous studies reporting the presence of Salmonella spp. in wild boar and feral pigs*

Country (year)	Sample type	No of samples (individuals)	Method	No of positive samples (%)	No of serovars detected	No of positive individuals (%)	Reference
Italy (2007-10)	Intestinal content	1 313 (1 313)	Cultivation	326 (24.8)	30 ¹	326 (24.8)	(Chiari <i>et al.</i> , 2013)
Italy (2006-09)	Intestinal content	2 356 (2 356)	Cultivation	441 (18.7)	N.s. >7	441 (18.7)	(Magnino <i>et al.</i> , 2011)
USA, Texas (2013-15)	Faeces	442 (442)	Cultivation	194 (43.9)	27	194 (43.9)	(Cummings <i>et al.</i> , 2016)
Portugal (2005-06)	Faeces	77 (77)	Cultivation	17 (22.1)	2	17 (22.1)	(Vieira-Pinto <i>et al.</i> , 2011a)
Switzerland (2007-08)	Tonsils	153 (153)	PCR Cultivation	19 (12.4) 8 (5.2)	3	19 (12.4)	(Wacheck <i>et al.</i> , 2010)
Sweden (2010-2011)	Tonsils Ileocaecal lymph nodes Faeces PCR-positive Buffered Peptone-Water broths	175 (88) 56 (56) 88 (88) 11 (9)	PCR PCR PCR Cultivation	9 (5.1) 1 (1.8) 1 (1.1) 6 (54.5)	3		(Sannö <i>et al.</i> , 2014; Paper I)
Sweden (2014-2016)	Tonsils Mesenteric lymph nodes Faeces PCR-positive Brain-Heart-Infusion broths	136 (90) 90 (90) 90 (90) 45 (36)	PCR PCR PCR Cultivation	19 (14.0) 9 (10.0) 7 (7.8) 20 (44.4)	6		(Sannö <i>et al.</i> 2018; paper III)
Japan (1993-94)	Meat samples	93	Cultivation	2 (2.2)	1	2 (2.2)	(Kanai <i>et al.</i> , 1997)

Country (year)	Sample type	No of samples (individuals)	Method	No of positive samples (%)	No of serovars detected	No of positive individuals (%)	Reference
Spain (2009-11)	Carcass swabs Faeces	310 301 (333)	Cultivation	4 (1.3) 1 (0.3)	4	4 (1.2)	(Díaz-Sánchez <i>et al.</i> , 2013)
Germany (2006-08)	Diseased animals	N.s. (118)	Cultivation	24 (N.s.)	1 ²	24 (20.3)	(Methner <i>et al.</i> , 2010)
Spain (2007-11)	Faeces	214 (214)	Cultivation	66 (30.8)	27	66 (30.8)	(Navarro-Gonzalez <i>et al.</i> , 2012)
Spain (2010-11)	Faeces	40 (40)	Cultivation	2 (5.0)	2	2 (5.0)	(Navarro-Gonzalez <i>et al.</i> , 2013a)
USA, North Carolina (2007-09)	Faeces	161 (161)	Cultivation	8 (5.0)	7	8 (5.0)	(Thakur <i>et al.</i> , 2011)
Australia (2010)	Faeces Mesenteric lymph nodes	651 (651) 493 (493)	Cultivation	204 (31.3) 65 (13.2)	29 24	231 (35.5)	(Ward <i>et al.</i> , 2013)

1. Including *S. Choleraesuis*

2. Only *S. Choleraesuis* isolated and characterized further

N.s.= not stated

1.6 Cultivation

1.6.1 *Yersinia* spp.

In the current ISO-standard (Anonymous, 2017) the following description of suspected colonies of *Y. enterocolitica* on CIN-agar, following 24 h of incubation at 30°C is given: “On CIN agar, pathogenic *Yersinia enterocolitica* appears as small (approximately 1 mm or under) circular, smooth colonies with entire edge. The colonies have a small, deep red sharp bordered centre (“bull’s eye”). The surrounding rim is translucent or transparent and, when examined with obliquely transmitted light, non-iridescent and finely granular”. Colony identification is recommended to be performed by the aid of a stereomicroscope. Suspected colonies of *Y. pseudotuberculosis* following 24 h of incubation at 30°C are seen as needlepoint colonies and incubation for additionally 24 h is recommended before further confirmation is done. Recently, a chromogenic agar has been developed (Weagant, 2008) that is more specific in detecting the bioserotype 4/O:3 as compared to the CIN-agar, whereas *Y. pseudotuberculosis* will not grow. The selective agars are most commonly used following pre-enrichment in selective broths such as Peptone, Sorbitol and Bile salts (PSB), Irgasan™-Ticarillin-potassium Chlorate (ITC), or modified Rappaport broth that commonly are incubated at +4°C for up to 2 weeks to utilise the psychrophile properties of *Yersinia* spp. Before sub-cultivation on the agar plates, it is recommended to dissolve a portion of the broth in potassium hydroxide (KOH) to further eliminate contaminant flora. In stool samples from patients with yersiniosis, however, direct plating on CIN-agar may be successful due to the high numbers of bacteria present. The need for cold enrichment before the subsequent culture on selective agar-plates makes cultivation for enteropathogenic *Yersinia* spp. time-consuming. Because of the high number of “false-positive” colonies present on the CIN-agar plates, a large number of colonies must be subject to confirmation, further increasing the workload. The lack in both specificity and sensitivity is particularly evident when cultivation is combined with PCR-detection that has a higher specificity and sensitivity in the detection of enteropathogenic *Yersinia* spp. (Fredriksson-Ahomaa & Korkeala, 2003)

1.6.2 *Salmonella* spp.

In the cultivation of *Salmonella* spp., the motile properties of the bacteria is commonly utilised in the protocols used for the detection. In the current ISO-standard (Anonymous, 2002), the modified semisolid Rappaport-Vassiliadis agar (MSRV-agar) is used. This agar is a development from the first publication utilising the motility of *Salmonella* spp. (Stuart & Pivnick, 1965). Pre-enrichment in Buffered Peptone-Water (BPW) broth is performed before a small drop of the broth is inoculated in the semisolid media, incubated at 41°C, and inspected after 24 and 48 h for the presence of a halo around the site of inoculation. The outer rim of the halo, when present, is then streaked onto solid media before confirmation. In the ISO-standard, xylose-lysine-deoxycholate agar (XLD) and brilliant-green agar (BG) are used, however, other media as well as chromogenic agar have also been utilised. The ISO-standard has a good specificity and sensitivity in the analysis of faeces and mesenteric lymph nodes (Mainar-Jaime *et al.*, 2013; Eriksson & Aspan, 2007), and is a rapid method that is less dependent on the skills of the laboratory personnel.

1.7 Molecular epidemiology of *Salmonella* spp. and enteropathogenic *Yersinia* spp.

In order to determine the source of a food-borne disease, several molecular-epidemiological methods have been developed. The aim of these methods are to determine if the pathogenic bacteria isolated from a patient suffering from a suspected food-borne infection is of a similar clone as a bacteria isolated from food items suspected to have caused the illness. With the use of the Kauffman-White classification, pure culture of *Salmonella* spp. are classified based on the lipopolysaccharide O- and H-antigens present on the cell surface. In a saline solution, the bacteria agglutinates with antibodies of known specificity for the different serotypes. This comparison between isolates of different origin is not as specific as the more recently developed DNA-based methods but has proven useful in surveys and epidemiological studies on salmonellosis (Wattiau *et al.*, 2011; Herikstad *et al.*, 2002).

Similarly, slide-agglutination tests are used in the serotyping of *Y. enterocolitica* and *Y. pseudotuberculosis*, subdividing the former into 60 and the latter into 21 different serotypes (Bottone, 1997). In addition, *Y. enterocolitica* is subdivided by biotyping based on biochemical reactions into 6 different biotypes (Bottone, 1997). Unlike *Salmonella* spp., serotyping is of limited value in source-attribution and outbreak investigations of *Y. enterocolitica* since only a few serotypes are considered to be pathogenic.

Recently, other methods based on sequencing of genes or gene fragments have been developed and can roughly be divided in whole-genome sequencing (WGS) and locus-based sequencing, the latter comparing selected parts of the genome or specific genes.

1.7.1 Pulse Field Gel Electrophoresis (PFGE)

In 1971, the use of restriction enzymes to cleave double-stranded viral DNA into fragments that were separated by size when migrating through a gel by the aid of electrical charge, known as gel-electrophoresis, was described (Danna & Nathans, 1971). Later, alternate directions of the electrical charge was introduced (Schwartz & Cantor, 1984), known as Pulsed Field Gel Electrophoresis. This method facilitated the separation of larger DNA fragments (>20Kb) and was further developed during the 1970- and 1980-ies. Before the introduction of gene-sequencing methods, PFGE was considered as the Gold Standard for molecular epidemiological investigations of several pathogens including enteropathogenic *Yersinia* spp. and *Salmonella* spp. (Wattiau *et al.*, 2011; Foley *et al.*, 2009; Iteman *et al.*, 1996). The most commonly used restriction enzymes for analysis of *Y. enterocolitica* are *NotI* and *XbaI* (Fredriksson-Ahomaa *et al.*, 2006a) while for *Y. pseudotuberculosis*, *NotI* and *SpeI* are used (Magistrali *et al.*, 2015). The resulting gel-banding-patterns have been difficult to interpret due to the presence of a large number of bands. However, with the aid of computerised image analysis the method has proven useful in molecular epidemiology investigations and source attribution studies (Carniel *et al.*, 2006). Results have also been possible to compare between laboratories and common databases for comparison of gel-banding-patterns obtained from *Escherichia coli* O157:H7, *Salmonella* spp. and *Shigella* spp. are available (Ribot *et al.*, 2006; <https://www.cdc.gov/pulsenet/index.html>) The method is however labour-intensive and time-consuming, thus hampering the analysis of large sample sets and the use in outbreak investigations when there is a need for rapid analysis.

1.7.2 Multi Locus Sequencing Typing (MLST)

MLST is a sequence-based method analysing so-called housekeeping genes within the genome (Maiden *et al.*, 1998). Depending on the desired discriminatory power of the analysis, a number of these genes are selected. The conserved parts of the housekeeping genes are sequenced and compared between strains. Seven genes have been selected in the analysis of both *Salmonella* spp. and *Yersinia* spp. In each strain analysed the sequences of each

locus, *i. e.* an allele, are determined. In the first strain analysed, the sequences of each locus was entered into a database and each allele was designated with number 1. When subsequent strains are analysed and entered into the database, the sequence for each locus is compared with the first strain as well as all other previously analysed strains. Each novel sequence of a locus is given a consecutive allele number. If the sequence has been identified previously, that locus is given the corresponding allele number. The result is a seven-digit code for each strain, called a “Sequence type” (ST), and each new code is numbered consecutively starting with ST1 designated for the first strain entered.

Common protocols and associated databases for MLST analysis have been developed for *Salmonella* spp. (Achtman *et al.*, 2012), *Y. pseudotuberculosis* (Laukkanen-Ninios *et al.*, 2011; Achtman *et al.*, 1999) and for the whole genus of *Yersinia* (Hall *et al.*, 2015) where isolates are identified to the species or subspecies level. Further, isolates of *Y. enterocolitica* are grouped together based on their different pathogenic properties. The MLST-data generated is mainly used to gain deeper insight into the evolution of different lineages of the pathogen investigated, *e.g.* *Y. pestis* have been shown to be a distinct sub-clone of *Y. pseudotuberculosis* (Achtman *et al.*, 1999).

1.7.3 Multi Locus Variable tandem repeats Analysis (MLVA)

Similarly to MLST, MLVA focuses on specific loci within the bacterial genome and the method was first developed to distinguish strains of the highly monomorphic bacteria *Bacillus anthracis* (Keim *et al.*, 2000). Within the loci investigated there are tandem repeats of specific basepair-sequences commonly consisting of 5-15 basepairs, *i.e.* the same short sequence of basepairs is repeated a variable number of times with no other sequences in-between. Mutations that occur over time will delete or add a whole repeat, thus the size of the locus will vary between different strains of a bacterium depending on how many repeats that are present, and depending on the size of these repeats. PCR-primers are designed to correspond to conserved regions flanking the locus containing the tandem repeats (Fig. 1). Hence, flanking regions of known sizes will be subtracted from the size of the resulting PCR-fragments obtained and the size of the remaining fragment is then dividable by the size of the tandem repeat, giving the number of repeats present in the locus.

$$\frac{\text{Size of PCR-product} - \text{Size of flanking regions}}{\text{Size of tandem repeat}} = \text{Number of repeats}$$

In the analysis of *Y. enterocolitica* and *Y. pseudotuberculosis*, six respective seven tandem-repeat loci are investigated. When the number of repeats are determined for each locus, a six- or seven-digit code is obtained that is then compared between the strains. The main difference between MLST and MLVA is that while the former analyses the sequence of each locus, the latter is only focusing on the size of the locus. The MLVA has a higher discriminatory power as compared to MLST. The mutation rate of the different loci analysed by MLVA can vary and may depend on the selection pressure put on the specific strain analysed (Noller *et al.*, 2006). Hence, MLVA are mainly useful when performing outbreak investigations and source attribution studies (Malorny *et al.*, 2008) and has become the Gold Standard for such investigations in *Salmonella enterica* subsp. *enterica* and *Salmonella enterica* serovar Typhimurium. Common protocols and databases have been developed, proving useful tools *e.g.* in outbreak investigations of human salmonellosis (Larsson *et al.*, 2013). Similarly, protocols have been developed for *Y. pseudotuberculosis* (Halkilahti *et al.*, 2013) and *Y. enterocolitica* (Sihvonen *et al.*, 2011b; Gierczynski *et al.*, 2007). However, these protocols have yet to be implemented on a broad scale and have thus far only been used for research purposes.

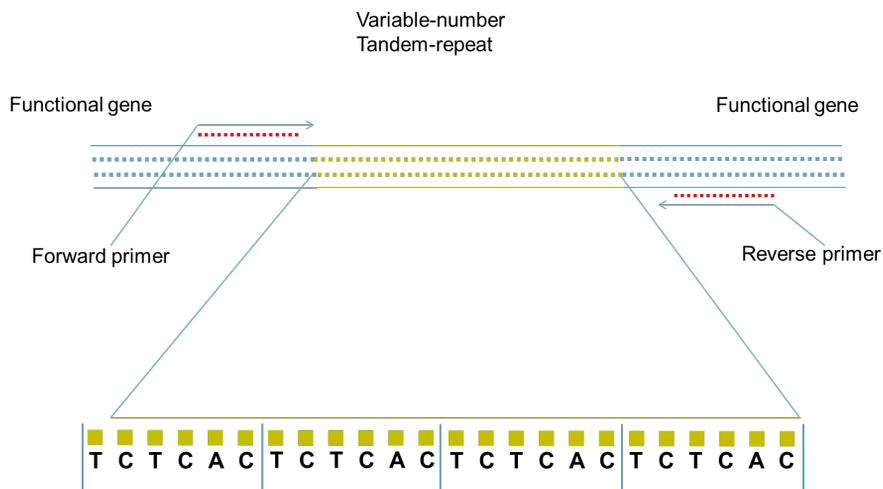


Figure 1. Schematic illustration of MLVA. A tandem repeat sequence in-between functional genes is amplified and the size of the resulting fragment is then determined using capillary gel electrophoresis.

2 Hypothesis and aims

The main hypothesis of the present study is that the Swedish wild boar population could act as a reservoir for enteropathogenic *Yersinia* spp., *Salmonella* spp., and *E. coli* O157:H7. Further, differences in population characteristics will influence the presence of these enteropathogens in the local population of wild boars.

The overall aim of this thesis has been to investigate the presence and distribution of *Salmonella* spp. and enteropathogenic *Yersinia* spp. in Swedish free-living wild boars (*Sus scrofa*) and the influence of potential risk factors that presumably are associated with their occurrence. More specific aims were:

- To investigate the presence of enteropathogenic *Yersinia* spp., *E. coli* O157:H7 and *Salmonella* spp. in Swedish wild boars. (Paper I and III)
- To develop and evaluate a protocol for the diagnosis of *Salmonella* spp., *Y. pseudotuberculosis* and *Y. enterocolitica* that is robust, with a reliable performance and that can be used for large sample sizes. The protocol should also be able to generate data that can be used for epidemiological investigations. (Paper II)
- To investigate if factors such as population density, level of artificial feeding, time since establishment of a given population, and the handling of animal by-products from slaughtered animals could influence the presence of *Salmonella* spp., *Y. pseudotuberculosis* and *Y. enterocolitica* in the wild boar. (Paper III)
- To compile MLVA-profiles and compare isolates of *Y. enterocolitica* and *Y. pseudotuberculosis* from different sources by MLVA and investigate to what extent human enteropathogens are present in wild-boar minced meat. (Paper IV)



Photo by Mikael Grennard

3 Considerations on Materials and Methods

The detailed descriptions of the methods used for analysis of collected samples are presented in paper I-III. In the following section, additional aspects on the material and methods are discussed.

3.1 Study design

The first study (Paper I) relied on the acquisition of samples from a study designed and performed at the section for Pathology, National Veterinary Institute (SVA), Uppsala. The aim of that study was to evaluate live traps for wild boars with respect to the possible stress and injuries inflicted to the animals during captivity. Two hunting estates and their professional hunting managers were recruited to perform the practical parts of the study. All trapped animals were euthanized within the traps and transported to SVA. Hence, we did not have any influence on the study design. Some traps were designed to capture several wild boars simultaneously and on several occasions, more than one animal was captured together. Pigs from the same populations have most likely been subjected to the same risks of acquiring pathogens, potentially influencing the interpretation of the results. Further, some methodological concerns were identified and addressed in study II.

In order to get a more representative sample of the Swedish wild boar population, the aim of the third study was to obtain samples from a larger variety of populations and a larger geographical area. Further, the aim was to investigate presumptive risk factors associated with the presence of enteropathogens in Swedish wild boars and hence the desired population characteristics were formulated in Paper III. The plan was to sample animals from populations exposed to these risk factors and compare to the results from analysis of samples

obtained from populations not being exposed. The Swedish Hunters Association and SVA has organized a network of coordinators for sampling of wildlife and for dissemination of information. Within this network, one coordinator is appointed in each of the 21 counties. The wild boars are today only present in the southern parts of Sweden and the coordinators in these southern counties were contacted by phone. The study design was described and the coordinators were requested to aid in the distribution of sampling material and instructions to hunters within their county. Similarly, informal contacts were also made with the hunters. In total, 220 sampling kits, sampling instructions, and a questionnaire was sent out. However, samples were only achieved from 47 animals, representing ten out of the thirteen counties.

Paper IV also included analyses of minced wild-boar meat from private hunters and wild-game handling establishments. In study III, participating hunters was also requested to submit samples of minced meat, either from the wild boars included in the study or from minced meat in their freezer from previously shot wild boars. The national organization for the wild-game handling establishments was contacted to include anonymous samples. Eight establishments, handling more than one tonne of wild-boar meat per year and certified to produce and sell wild-boar minced meat, agreed to participate in the study. Despite several reminders by e-mail and phone, only one establishment returned one set of four samples in accordance with the given instructions. Due to the poor compliance, samples were also obtained indirectly from five establishments by purchasing frozen packages of minced wild-boar meat available on the open market.

3.1.1 Sampling

The sampling of wild boars in study I was performed by an experienced pathologist at SVA. Hence, the quality of the samples obtained was high. During the course of the study, a farm with outdoor-raised pigs were infected with *Salmonella* Derby and therefore, the hunters in the area were contacted and requested to collect samples from shot wild boars. A brief, written sampling instruction including illustrations on the location of the mesenteric lymph nodes and on the tonsils was constructed (Fig. 2). The instructions, alongside material for storage and shipment of the samples, were sent to the hunters. Samples of a high quality were returned from four wild boars. The hunters reported that there was no problems in identifying the tissue structures requested. In preparation for the second sampling (paper III), these instructions were revised slightly and supplemented with a short description on the project and a short questionnaire

including four questions on population characteristics, and information on the type of populations targeted (detailed in paper III).

In the sampling from the eight wild-game handling establishments, each establishment was assigned a number 1-8 and sampling instructions, alongside an envelope addressed to the lab, was distributed. The principal investigator was blinded to the identity of the establishments. A maximum of four samples of 50 g of freshly-ground minced meat per establishment, collected at one-week intervals using a clean glove, were to be obtained. The samples were frozen immediately and sent to the laboratory after completion of the sampling.

Additional samples of wild-boar minced meat was purchased in grocery stores and local farm shops. Packages of frozen minced wild boar meat (500 g to 1kg) was obtained and if more than one package was bought on the same occasion, the labelling of the packages was scrutinised to ascertain the selection of different batches based on the dates stated. A subsample of 30-50 g was included in the analysis.

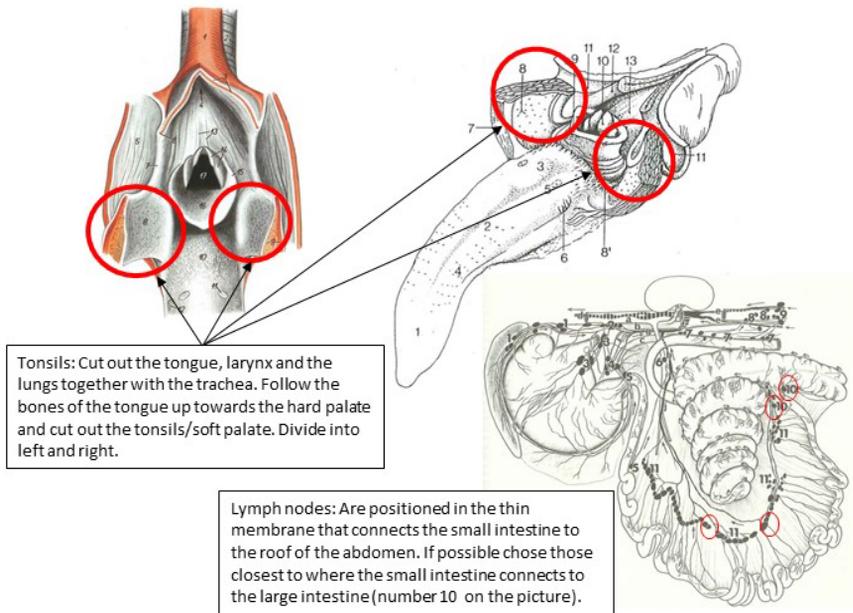


Figure 2. The illustration included in the sampling instructions sent out to the hunters. (The text within the boxes are translated from the original Swedish language; Dyce *et al.*, 2009; Popesko, 2007)

3.2 Development of the laboratory protocol

In the analyses in study I, *Salmonella* spp. and enteropathogenic *Yersinia* spp. was chosen as targets based on previous experiences from other European countries (Wacheck *et al.*, 2010). *E. coli* O157:H7 was included because a PCR-positive faecal sample had been found in a previous Swedish study (Wahlström *et al.*, 2003). Since all samples were frozen before analysis to facilitate batch-wise handling at the laboratory, *Campylobacter* spp. was not included in the analyses (Georgsson *et al.*, 2006).

In order to create a laboratory protocol adopted to detect all three pathogens, a non-specific broth (BPW) was chosen for the pre-enrichment, followed by cultivation on selective media for each of the pathogens. To detect *Salmonella* spp., XLD and BG agar was selected, and in the cultivation of enteropathogenic *Yersinia* spp. and *E. coli* O157:H7, CIN- respective CT-SMAC⁷-agar was selected. In order to increase the possibilities to obtain isolates for the subsequent analyses and to reduce the workload, PCR on the pre-enrichment broth was utilized to detect positive samples to be included in the subsequent cultivation. The method used for cultivation of *Salmonella* spp. is described in Paper II, Appendix A. Isolates of *Y. pseudotuberculosis* was possible to obtain from eight of the PCR-positive samples by cultivation. However, the cultivation of *Y. enterocolitica* was unsuccessful, despite the use of two different protocols in the attempts to increase the sensitivity, *i.e.* NMKL-117 (Thisted *et al.*, 1996) and the recently revised ISO-standard (Anonymous, 2017). In the PCR-analysis, bacterial colonies from the selective plates suspended in ddH₂O was used and DNA was extracted by boiling. Due to the crude DNA-extraction method used and the amount of bacterial DNA obtained, some problems with inhibition occurred. A 1:10 dilution of the template resolved these problems. Thereafter, the protocol used in Paper I was scrutinised and revised based on the experiences made by Råsbäck *et al.* (2018) regarding selection of *Y. enterocolitica* based on motility. Further, based on in-house evaluations performed at SVA, an alternative Master-mix was identified. In order to increase the recovery rate following cultivation, the bacterial colonies harvested from the selective agar plates were dissolved in an enrichment broth (BHI) instead of ddH₂O, and used as the substrate in the subsequent MLVA and confirmative cultivation. In the first study, no positive samples for *E. coli* O157:H7 was obtained. It has previously been demonstrated that pigs will only be transient carriers of this pathogen (Eriksson *et al.*, 2003). Hence, this analysis was excluded in the subsequent studies. The PCR-target genes remained otherwise

⁷ MacConkey Agar with Sorbitol, Cefixime and Tellurite (CT-SMAC)

the same and will be discussed further in the “Results and Discussions” section.

The final protocol is presented in Fig 3 and in Paper II that also includes the evaluation of the protocol.

To perform outbreak investigations, studies on source attributions and other epidemiological studies on pathogenic bacteria, additional information on the bacterial strains is needed. Today, Whole Genome Sequencing or sequencing of selected parts of the genome is becoming increasingly common. Most of these methods require pure-culture isolates of the bacteria. In the case of enteropathogenic *Yersinia* spp., this has become a severe hindrance. Although cultivation is commonly used in the diagnosis of yersiniosis, samples from possible sources of infection or natural reservoirs may not generate isolates with the same ease (Fredriksson-Ahomaa & Korkeala, 2003). PCR-analysis may demonstrate the presence of enteropathogenic *Yersinia* spp., while cultivation of the same sample will be unsuccessful. The PCR-based method MLVA offers the possibility to obtain molecular epidemiological data without the need for an isolate. Previously, an eight-loci MLVA-protocol for analysis of *Legionella pneumophila* have been used to generate profiles of DNA extracted from drinking water, where isolation of the bacteria had been unsuccessful (Kahlisch *et al.*, 2010). Further, food samples spiked with *Listeria monocytogenes* generated MLVA-data by analysis of DNA extracted direct from the pre-enrichment broth (Chen *et al.*, 2011), and in samples from patients suffering from *Mycoplasma pneumoniae*, MLVA-data was obtained from all (n=54) PCR-positive samples (Dumke & Jacobs, 2011). This has however previously not been used in the analyses of enteropathogenic *Yersinia* spp.

Enrichment broths (BHI) positive by PCR for the presence of *Y. enterocolitica* and/or *Y. pseudotuberculosis* was initially subjected to MLVA using DNA extracted by InstaGene[®] Matrix, however the results were not satisfactory. BHI stored at -80°C was then thawed and DNA was extracted using the method in the MLVA-protocol by (Halkilahti *et al.*, 2013; GeneJET Genomic DNA Purification Kit Fermentas, St. Leon-Rot, Germany). The subsequent MLVA generated results of a better quality.

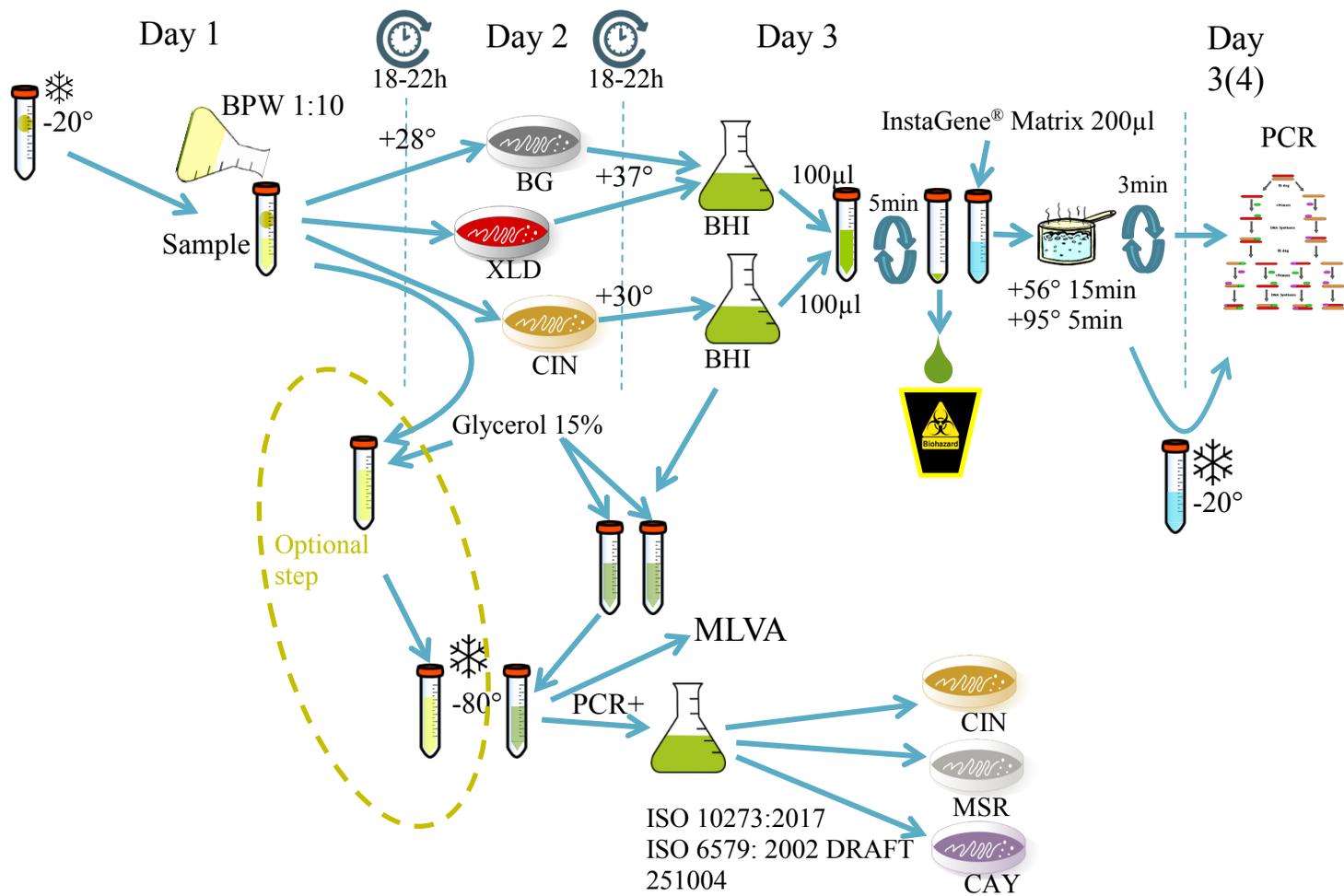
3.3 Evaluation of the final protocol

In the evaluation of the new protocol, we focused on the novel aspects of the protocol *i.e.* selection of motile bacteria and the MLVA performed directly on the enrichment broth, by comparison to the current ISO-standards for cultivation of *Salmonella* spp. and *Y. enterocolitica*.

Apart from the study by (Råsbäck *et al.*, 2018), selection for motility has, to the best of our knowledge, not been used previously in the cultivation of enteropathogenic *Yersinia* spp. This is, however, commonly used in the cultivation of *Salmonella* spp. and also included in the current ISO-standard. The different sample matrices (tonsils, mesenteric lymph nodes, faeces, and tissue from the oropharynx) was minced, vortexed in the primary enrichment broth (BPW), and incubated at 28°C, since all enteropathogenic *Yersinia* spp. are stated to be motile at temperatures below 30°C but not at 37°C (Bottone, 2015). However, according to the Pasteure institute this is apparently not true for all strains of *Y. pseudotuberculosis* (Savin *et al.*, 2014). Hence, the introduced selection step for motility was also evaluated. Following incubation of the enrichment broth, the particles had most commonly sedimented to the bottom of the tubes. An inoculum was collected from the surface of the broth and streaked on selective agar plates. In order to detect the presence of enteropathogenic *Yersinia* spp. and *Salmonella* spp. deeper down in the tubes or adherent to the matrix, the tubes were vortexed, aliquots of 2 mL were collected, and frozen in -80°C for later analysis. The aliquots were thawed and a template for PCR-analysis was prepared using InstaGen® Matrix. The PCR-analysis was then performed in accordance with the new protocol.

The evaluation of the MLVA performed directly on the enrichment broth was done by comparison of the putative profiles obtained in the analysis of the enrichment broth (BHI) to those obtained after analysis of the ten isolates recovered following cultivation of the PCR-positive samples. The comparison was however, hampered by the fact that mainly isolates of bioserotype 1A:O14 and 3:O15 was recovered, since the MLVA protocol is not developed to generate complete profiles for these bioserotypes (Sihvonen *et al.*, 2011b).

Figure 3. Schematic presentation of the laboratory protocol developed and evaluated in Paper II.



4 Results and discussion

4.1 Food-borne pathogens in Swedish wild boars

In the present thesis, we have demonstrated that *Salmonella* spp., *Y. pseudotuberculosis* and *Y. enterocolitica* with the potential to cause disease in humans, are common findings in the Swedish wild boar. In total, 178 wild boars have been sampled and analysed in two different studies. Of these, 91 animals carried the zoonotic enteropathogens investigated, whereof 46 were PCR-positive for the presence of *Y. enterocolitica*, 37 were PCR-positive for the presence of *Y. pseudotuberculosis* and 32 were PCR-positive for the presence of *Salmonella* spp. From these samples, a total of 10 isolates of *Y. enterocolitica*, 13 isolates of *Y. pseudotuberculosis*, and 28 isolates of *Salmonella* spp. were obtained, thus substantiating the difficulties to cultivate *Yersinia* spp. Out of the 28 isolates of *Salmonella* spp., ten belonged to serovars that has previously been confirmed to be associated with disease in humans, a further nine belonged to *Salmonella* subsp. *enterica* that possibly are associated with disease in humans, while the remaining nine isolates belonged to *Salmonella* subsp. *diarizonae* that has unknown pathogenic properties and are commonly associated with reptiles (Aleksic *et al.*, 1996). One of the ten isolates of *Y. enterocolitica* belonged to the bioserotype 4/O:3, the bioserotype most commonly associated with yersiniosis, and one isolate belonged to the biotype 1B that is considered to be highly pathogenic (Fredriksson-Ahomaa, 2017). A further four isolates belonged to the pathogenic biotype 3, however the serotype was determined as O:15 that is not associated with yersiniosis, and four isolates were of bioserotype 1A/O:14 that is not considered as being pathogenic. However, all isolates were PCR-positive for the presence of the *ail*-gene that is highly associated with pathogenicity (Miller *et al.*, 1989) and thus the pathogenic potential of these eight isolates is yet to be determined. In *Y. pseudotuberculosis*, all serotypes are considered pathogenic to humans. These findings are in line with previous

reports from other parts of Europe (Table 1 & 2; Arrausi-Subiza *et al.*, 2016; Chiari *et al.*, 2013; Navarro-Gonzalez *et al.*, 2012; Vieira-Pinto *et al.*, 2011a; Wacheck *et al.*, 2010), indicating that wild boars may be a common source of these pathogens.

The reported prevalences of various pathogenic microorganisms present in different wild boar populations vary greatly (Ruiz-Fons, 2017) both with respect to species and regarding the presence of pathogenic and non-pathogenic strains. This variation poses a great challenge in the evaluation of clinical outbreaks. Further, although several studies have demonstrated the presence of *Salmonella* spp. and enteropathogenic *Yersinia* spp. in wild boars, evidence is still missing that link those findings to human disease. A prerequisite to establish this link is to acquire molecular-epidemiological data that can also be used as a reference in outbreak investigations.

All serovars of *Salmonella* spp. have the potential to cause disease in humans (Aleksic *et al.*, 1996). However, only two-thirds of the serovars isolated in our studies have previously been associated with salmonellosis, showing the importance of further subtyping of obtained isolates through whole-genome sequencing and/or MLST. Wild boars can then be dismissed or confirmed as the source of salmonellosis in *e.g.* outbreak investigations.

Y. enterocolitica is a highly heterogenic species. Only a few bioserotypes are confirmed to be associated with yersiniosis, while others are only rarely isolated or not associated with human disease, making the diagnostics and evaluation of obtained isolates challenging (Fredriksson-Ahomaa *et al.*, 2018). The pathogenic properties of the ten isolates of *Y. enterocolitica* obtained in the evaluation of the protocol (Paper II) have not been evaluated further. Hence, the *Y. enterocolitica* present in Swedish wild boars comprises both pathogenic and presumably non-pathogenic strains, although all carried the *ail*-gene that is associated with pathogenicity.

The results from the extended investigations presented in Table 3 indicate that more than half of the Swedish wild boars may carry *Y. pseudotuberculosis*. All strains of this pathogen is considered to be pathogenic and although *Y. pseudotuberculosis* are rarely isolated in cases of yersiniosis, outbreaks have occurred and been linked to raw vegetables contaminated somewhere in the production-chain between the field and the kitchen (Kangas *et al.*, 2008). With a growing wild-boar population, similar contamination of vegetables in the field, directly or through contaminated irrigation water, may become more common also with respect to *Salmonella* spp. and *Y. enterocolitica*.

4.1.1 Implications for public health

With zoonotic pathogens present in the intestinal content, lymph nodes and the oropharynx of wild boars, the correct handling during hunting, slaughter, cutting and preparations in the kitchen are all important to prevent infection in humans. With more than 100 000 wild boars of an estimated slaughter weight of 30 kg shot annually (Anonymous, 2018), an estimated 2 000-2 500 tonnes of wild-boar meat may be available for consumption each year. In study IV, *Y. enterocolitica* was demonstrated to be present in wild-boar minced meat obtained from both private hunters and wild-game handling establishments, thus demonstrating the difficulties in preventing contamination of the meat.

The risks are however difficult to assess. Within the food sector, a large emphasis is laid on preventing the occurrence of pathogens within the farm-to-fork food chain. The Swedish legislation focuses on keeping the primary production free from *Salmonella* spp. to reduce the risk for contamination of meat and equipment in the abattoir. To prevent contamination of meat with enteropathogenic *Yersinia* spp. at slaughter, certain control points are implemented (Borch *et al.*, 1996) and supervised both by the abattoirs and by the National Food Agency. The majority of the wild-boar meat is handled and consumed by the hunters and their families themselves and such control points is thus lacking. In the present study, four out of 20 samples of wild-boar minced meat obtained from hunters, were shown to be positive for the presence of *Y. enterocolitica* by PCR. The hunters' knowledge on how to handle and slaughter wild boars to prevent contamination of the meat, how the meat is handled in the kitchen and the handling of the slaughter waste, are factors that all may affect the risk. Further, dogs and cats with access to slaughter waste or contaminated meat may become infected or act as asymptomatic carriers, hence constituting a risk for spread to *e.g.* children in close contact with these pets (Fredriksson-Ahoma *et al.*, 2001).

Thus, information to the hunters is key in preventing the risks associated with the presence of zoonotic pathogens in wild boar.

4.1.2 Risk factors for the presence of enteropathogens in wild boar

In order to recommend changes in management routines to hunters and governments, an association between a suspected risk factor and the presence of enteropathogens need to be established. In study III, no associations were noted between the risk factors examined and the prevalence of the enteropathogens investigated. Given the number of samples obtained, the difference in prevalences of a certain pathogen should have been at least 23% to be able to

identify a specific risk factor. Possibly, a larger sample size could have identified smaller differences. Since a strong correlation between high population density and high levels of artificial feeding was identified, these risk factors are difficult to evaluate separately. In a dense population, a higher number of interactions between individuals will occur and at artificial feeding places, infected faecal matter from wild boars but also from other scavengers such as rodents and birds can contaminate the feed and hence constitute a risk. Our goal was to obtain 50 samples from wild boars in areas only using artificial feeding to a limited extent. However, only eleven samples were submitted from areas with less than three feeding places per 10 km² and only six samples came from areas shooting less than five wild boars per 10 km² annually. Thus, to elucidate the possible importance of artificial feeding in the transmission of foodborne diseases further studies are needed, focusing on sampling areas with high-density populations and limited amount of feeding and vice versa, if such areas exist.

The practice of leaving slaughter waste out in nature was common. In the present study it was not possible to establish an association between this practice and the presence of the pathogens investigated. However, since the slaughter waste includes the main predilection sites for these pathogens, the habit of leaving slaughter waste out in the forest could be discussed. Wild-life, such as corvid birds and the red fox, with access to slaughter waste could spread pathogens over larger areas. Hence, human enteropathogens from wild-boar slaughter waste may be present also in other parts of the ecosystem. It would thus be interesting to investigate to what extent other species may act as reservoirs for various enteropathogens.

Other risk factors may exist that could be unrelated to the population characteristics of the wild boars such as densities of scavenger-, rodent or wild-bird populations, closeness to farms and other human infrastructure such as landfills, cities or recreational areas. To investigate this, more detailed information on the area wherefrom the wild boars are sampled is needed.

Despite that no risk factors could be identified in the present study, some recommendations can still be made: Feeding places may act as “hot-spots” for disease transmission (Brook *et al.*, 2013; Navarro-Gonzalez *et al.*, 2013b) and overabundance of animals in an area will increase the risk of disease (Gortázar *et al.*, 2006). Hence, reducing the number of feeding places, decreasing the population densities, and dispose the slaughter waste in a way that render it unavailable to wild-life are all recommendable management strategies.

4.2 Evaluation of the new protocol

In the evaluation (Paper II), the pre-enrichment broths (BPW) from 54 individual wild boars were analysed by PCR and 11 individuals were demonstrated to be positive for the presence of *Salmonella* spp., 16 were positive for *Y. enterocolitica* and 31 were positive for *Y. pseudotuberculosis*. Table 3 summarizes the results for these 54 individuals and for all 90 individuals analysed by the new protocol.

Table 3. The number (n) and percentage (%) of samples from 54 positive wild boars analysed by PCR on the primary enrichment broth (Buffered Peptone Water, BPW) in the evaluation of the new protocol, and the results of samples from all 90 individuals analysed by PCR on the second enrichment broth (Brain Heart Infusion broth, BHI) in the new protocol

	54 individuals analysed by PCR on BPW	90 individuals analysed by PCR on BHI
	n (%)	n (%)
<i>Salmonella</i> spp.	11 (20.4)	24 (26.7)
<i>Y. enterocolitica</i>	16 (29.6)	28 (31.1)
<i>Y. pseudotuberculosis</i>	31 (57.4)	20 (22.2)

Analysis of the second enrichment broth improved the detection rate of *Salmonella* spp. and *Y. enterocolitica* slightly as compared to analysis of the primary enrichment broth. However, the results from the analysis of the primary enrichment broth for the presence of *Y. pseudotuberculosis* indicate that the prevalence of this pathogen in wild boars is underestimated (Table 3).

Thus, *Y. pseudotuberculosis* may actually be the most widespread pathogen of those investigated within the Swedish wild-boar population.

4.2.1 The positive selection for motile bacteria

Motility is associated with pathogenicity in several bacterial species (Moens & Vanderleyden, 1996) and in *Y. enterocolitica*, motility is required for the invasion of epithelial cells (Young *et al.*, 2000). Motility has also been observed in the presumably non-pathogenic biotype 1A strains that were able to invade epithelial cells *in vitro*. However, when a non-motile mutant of this strain was constructed, the ability to invade and survive in cells was inhibited (McNally *et al.*, 2007).

In the evaluation of the protocol, positive selection for motility was used. This is a common practice in the routine diagnostics of *Salmonella* spp., but has not previously been applied in the cultivation of enteropathogenic *Yersinia* spp. It is however stated that all pathogenic *Yersinia*, except *Y. pestis*, are motile at

temperatures below 30°C, but not at 37°C (Bottone, 2015). The results detailed in Paper II however indicates the presence of *Y. pseudotuberculosis* strains that are incapable of reaching the surface of the broth following 20 h of incubation. It is possible that these strains lack motile properties but it is also possible that the concentration in the surface layer of the broth is lower because these bacteria grow at a slower rate than e.g. *Y. enterocolitica* and *Salmonella* spp. This may be a disadvantage in the subsequent cultivation on the selective media (CIN). The latter scenario seems however less likely since in Paper I, not including any positive selection for motility, similar detection rates of *Y. pseudotuberculosis* and *Y. enterocolitica* was noted whereas more *Y. enterocolitica* was detected in study III.

In the evaluation of the protocol, the selection for motility was shown to increase the detection rate of *ail*-positive *Y. enterocolitica* slightly, while the detection of *Salmonella* spp. before and after this selection was similar (Paper II). In addition, the concentration of bacteria might also increase, however further studies are needed in this respect since the cultivation on selective media will also influence the concentration. In conclusion, the results obtained in study II as well as those by Råsbäck et al. (2018) indicates that selection for motility in the cultivation of *Y. enterocolitica* may be beneficial.

4.2.2 Detection of enteropathogenic *Y. enterocolitica* using PCR

In the presented studies, the *ail*-gene was used as a target in the PCR-analysis. This target is used in the ISO-standard for PCR-detection of *Y. enterocolitica* and in several other studies investigating the presence of this pathogen in wild boars (Table 1), domestic pigs, and food products (Dziedzinska et al., 2018; Wacheck et al., 2010; Lambertz et al., 2008b; Wesley et al., 2008). The *ail*-gene is present in all pathogenic *Y. enterocolitica* but has also been demonstrated in several strains of biotype 1A (Platt-Samoraj et al., 2017; Sihvonen et al., 2011a). Differences in pathogenic properties have been demonstrated between biotype 1A strains isolated from clinical and from environmental samples, however, the associated genes has not yet been identified (Tennant et al., 2003).

The possible occurrence of false-positive and false-negative results must always be considered in the interpretation of the results. The sensitivity and specificity of the primers and probe used in this thesis has been evaluated previously (Arrausi-Subiza et al., 2014; Lambertz et al., 2008b) and the *ail*-gene as a target for PCR to detect pathogenic *Y. enterocolitica* has been further evaluated in several studies (Mäde et al., 2008; Boyapalle et al., 2001; Feng et

al., 1992; Miller *et al.*, 1989). Despite this, it is still possible that highly contaminated samples such as faeces and tonsils may generate false positive results due to the amplification of genes of high similarity that may be present in *e.g.* commensal bacteria. False negative results caused by amplification failure most often occur when inhibitory substances are present. Therefore, an Internal Positive Control (IPC) should be included in the PCR to detect the presence of inhibition.

Several other genes associated with pathogenicity has been identified and used as PCR-targets (Petsios *et al.*, 2016), both genes present on the chromosome, and on the virulence plasmid pYV. The pYV is present in all pathogenic *Yersinia* spp. and is highly associated with virulence, however, the plasmid may be lost during cultivation (Blais & Phillippe, 1995) and hence identification of pathogenic strains only based on the presence of the plasmid may be insufficient.

The inclusion of a second or even a third PCR-target in addition to the *ail*-gene in the PCR-detection step would most likely increase the specificity of the protocol, focusing the subsequent MLVA on samples more likely to contain pathogenic strains of *Y. enterocolitica*. The genes *ystA*, (Delor *et al.*, 1990) encoding a heat-stable enterotoxin and present on the chromosome, and *yadA* (El Tahir & Skurnik, 2001) that is an adhesin gene present on the pYV, could serve as suitable targets. Several protocols have been published analysing these genes in multiplex PCR (Weynants *et al.*, 1996). Possibly, genes associated with motility could also act as suitable targets.

4.3 MLVA as a tool for epidemiological studies of enteropathogenic *Yersinia* spp.

In Sweden, no previous studies have investigated the presence of *Salmonella* spp. or enteropathogenic *Yersinia* spp. in wild boars, hence no previous results have been available for comparison to strains isolated from humans. A continuous surveillance in wild boars would aid in outbreak investigations and source-attribution studies. The information obtained should be easily accessed and contain as much information as possible on the strains and the source of origin. The new protocol presented in Paper II, Appendix A, offers a time-efficient protocol, suitable for analysis of a large number of frozen samples that is desirable in the screenings of wild-life. Also, faecal samples could be utilised, excluding the need to obtain samples from carcasses, which may be favourable *e.g.* in areas with low population densities or in pens where wild boars are kept

for the training of hunting dogs. However, it should be noted that fewer positive faecal samples were detected in study I and III as compared to those obtained from tonsil samples, thus lowering the sensitivity in the detection of positive animals. Further, the protocol has proven to be more sensitive in detecting and obtaining *Salmonella* spp. isolates as compared to the routine-diagnostic methods employed at e.g. SVA, and also generates molecular epidemiological data from samples PCR-positive for enteropathogenic *Yersinia* spp. The use of this new protocol in the screenings of Swedish wild boars, domestic pigs, and other possible sources of enteric pathogens could contribute to a national database with information that may be used as a reference in outbreak investigations and source-attribution studies.

One biotype 1B strain was obtained and the MLVA-profile of this strain was identified both in the analysis of the enrichment broth and in the analysis of the isolate, yielding identical profiles. In addition, one isolate of the bioserotype 4/O:3 was obtained that yielded an unusual MLVA-profile with no repeats for locus V2A (Paper IV). The presence of this MLVA-profile was also clearly indicated in the analysis of the enrichment broth, however some slight differences was noted in two loci that generated two PCR-fragments each. In the analysis of the 1A isolates, the loci V2A, V4, V5, V6, and V7 generated similar profiles as indicated in the analysis of the enrichment broth. In locus V2A, fragments corresponding to 8 and 11 repeats was also present in the analysis of the enrichment broth. Three repeats was indicated for locus V9 in the analysis of the enrichment broth, however, no PCR-fragment for the V9 locus was present in the analysis of the isolate. Thus, with the exception of the “false” 3-repeat fragment in locus V9, the profiles obtained in the analysis of the enrichment broth and those obtained in the analysis of the isolate corresponded well. The additional PCR-fragments present in the analysis of the enrichment broth may indicate that more than one strain of the pathogen is present in the sample, as previously demonstrated (Bancerz-Kisiel *et al.*, 2015). Thus, the usefulness of the presented protocol is further supported since the difficulties in cultivation usually excludes the identification of several strains. However, as discussed above, the additional fragments could also be a result of unspecific amplifications of genes present in commensal bacteria. To investigate this further, samples spiked with a mixture of strains should be analysed, as well as samples negative for the target pathogens.

MLVA performed on isolates of *Y. enterocolitica* 4/O:3 and 2/O:9 have previously been used successfully to both confirm and refute common sources in outbreaks of yersiniosis (Sihvonen *et al.*, 2011b), proving the usefulness of the method in these investigations. In the present study, possible links between

human isolates and domestic pigs was indicated, as well as links between human isolates obtained in different years. Further, the importance of rodents in the on-farm epidemiology of *Y. enterocolitica* was indicated as a similar MLVA profile was obtained in both rodent- and pig samples from the same farm. The possibility to acquire molecular epidemiological data quickly and without the need to obtain isolates of the pathogen of interest offers new possibilities. Enteropathogenic *Yersinia* spp. are difficult to cultivate and obtaining the MLVA-data without previous isolation of the bacteria may be a way to circumvent this problem. The attempts to acquire MLVA-data directly from the enrichment broth was not included in the original study plan and hence, due to time and budget constraints, only comparison with those isolates obtained after cultivation was possible. Further evaluation, using spiked samples or samples including known bioserotypes of enteropathogenic *Yersinia* spp. would have been beneficial. In addition, sequencing of PCR-products of unexpected sizes would also be desirable. The technique is however promising, and warrants further studies.

5 Concluding remarks and future perspectives

Enteropathogenic *Yersinia* spp. and *Salmonella* spp. are commonly detected in the Swedish wild boar population. It was however not possible to demonstrate the presence of *E. coli* O157:H7. Further, none of the investigated population characteristics investigated could be identified as a risk factor for the presence of enteropathogens in the local populations of wild boar.

- In total, the enteropathogens studied was demonstrated in approximately 50% of the 178 wild boars investigated and 46 individuals were PCR-positive for *Y. enterocolitica*, 37 were positive for *Y. pseudotuberculosis* and 32 were PCR-positive for *Salmonella* spp.
- A new protocol was developed that utilised frozen samples to enable the batch-wise analysis of large sample sizes. The protocol generated MLVA-data for enteropathogenic *Yersinia* spp. by analysis of enrichment broth without the need for isolation of the bacteria and within four working days. The obtained MLVA-profiles could be used for epidemiological investigations, and the results were confirmed by analysis of isolates obtained from the same samples.
- No associations were found between the presence of these enteropathogens and the potential risk factors; population density, level of artificial feeding, time since establishment of the given population, and the handling of animal by-products from slaughtered animals. A strong correlation between high levels of artificial feeding and high population densities was seen.
- A total of 254 isolates of *Y. enterocolitica* from pigs, humans, pork, rodents, wild boars, and dog feed were analysed by MLVA, resulting in 145 different MLVA-profiles. Similar MLVA-profiles were detected in a few isolates from pigs and humans, as well as in pig and rodent samples

from the same farm. Further, 13 isolates of *Y. pseudotuberculosis* generated seven unique MLVA-profiles that were similar to the MLVA-profiles obtained in the analysis of the enrichment broth. *Y. enterocolitica* was demonstrated in ten out of 32 samples of wild-boar minced meat but *Salmonella* spp. and *Y. pseudotuberculosis* was not detected.

Based on the findings in this thesis, information to the hunters about the risks associated with enteropathogens present in wild boars and how to reduce these risks is of great importance. Additional studies on possible risk factors, in particular the use of artificial feeding places and the handling of the slaughter waste related to the presence of presumptive zoonotic pathogens, should be performed. Further, continuous surveillance of various possible sources of human enteropathogens and storage of data in national, easily accessible, databases would be useful to gain further knowledge on the importance of these pathogens. An easily adopted, time- and cost-effective protocol is needed. Within this thesis, a suggestion for such a protocol is presented. The MLVA-protocol for enteropathogenic *Yersinia* spp. needs to be implemented in diagnostic laboratories in a standardised manner, as well as a system for easy storage and accessibility of these data.

The new protocol should be developed and evaluated further, as follows:

- Inclusion of at least a second PCR-target for *Y. enterocolitica* to increase the specificity towards bioserotypes known to be pathogenic to humans
- Further development of the cultivation of PCR-positive enrichment broths (BHI) to improve the isolation rate, possibly utilising cold-enrichment as part of the cultivation.
- Evaluation of the MLVA on the enrichment broth by analysing samples spiked with known bioserotypes of *Y. enterocolitica*.
- Sequencing of PCR-products of unexpected sizes obtained in the MLVA
- Evaluation of the detection limits both by PCR, MLVA, and cultivation, using samples spiked with known concentrations of the three pathogens investigated.
- Inclusion of a step within the protocol to identify non-motile *Y. pseudotuberculosis*.

References

- Achtman, M., Wain, J., Weill, F.-X., Nair, S., Zhou, Z., Sangal, V., Krauland, M.G., Hale, J.L., Harbottle, H., Uesbeck, A., Dougan, G., Harrison, L.H., Brisse, S. & the, S.e.M.s.g. (2012). Multilocus Sequence Typing as a replacement for serotyping in *Salmonella enterica*. *PLOS Pathogens*, 8(6), p. e1002776.
- Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A. & Carniel, E. (1999). *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proceedings of the National Academy of Sciences*, 96(24), pp. 14043-14048.
- Al Dahouk, S., Nöckler, K., Tomaso, H., Spletstoesser, W.D., Jungersen, G., Riber, U., Petry, T., Hoffmann, D., Scholz, H.C., Hensel, A. & Neubauer, H. (2005). Seroprevalence of brucellosis, tularemia, and yersiniosis in wild boars (*Sus scrofa*) from North-Eastern Germany. *Journal of Veterinary Medicine, Series B*, 52(10), pp. 444-455.
- Aleksic, S., Heinzerling, F. & Bockemühl, J. (1996). Human infection caused by Salmonellae of subspecies II to VI in Germany, 1977–1992. *Zentralblatt für Bakteriologie*, 283(3), pp. 391-398.
- Alena, L., Vladimir, B. & Jiri, L. (2016). Serological prevalence of enteropathogenic *Yersinia* spp. in pigs and wild boars from different production systems in the Moravian region, Czech Republic. *Foodborne Pathogens and Disease*, 13(5), pp. 275-279.
- Amphlett, A. (2016). Far East scarlet-like fever: a review of the epidemiology, symptomatology, and role of superantigenic toxin: *Yersinia pseudotuberculosis*-derived mitogen A. *Open Forum Infectious Diseases*, 3(1), pp. ofv202-ofv202.
- Anonymous (2002). ISO 6579:2002 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp. Geneva: International Organization for Standardization (ISO) p. 27.
- Anonymous (2013). *Swedish Board of Agriculture, Från skog till krog – Vilka hinder motverkar mer vildsvinskött på marknaden? (Swedish only)*28): Swedish Board of Agriculture.
- Anonymous (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal*, 13(12), p. 4329.
- Anonymous (2017). EN ISO 10273 Microbiology of the food chain -Horizontal method for the detection of pathogenic *Yersinia enterocolitica*. Geneva, Switzerland: International Organization for Standardization.

- Anonymous Swedish Association for Hunting and Wildlife Management Available at: <https://jagareforbundet.se/vilt/vildsvinsbarometern/> [2018-05-31].
- Arrausi-Subiza, M., Gerrikagoitia, X., Alvarez, V., Ibabe, J.C. & Barral, M. (2016). Prevalence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in the Basque Country, northern Spain. *Acta Vet Scand*, 58(1), p. 4.
- Arrausi-Subiza, M., Ibabe, J.C., Atxaerandio, R., Juste, R.A. & Barral, M. (2014). Evaluation of different enrichment methods for pathogenic *Yersinia* species detection by real time PCR. *BMC Veterinary Research*, 10(1), p. 192.
- Atanassova, V., Apelt, J., Reich, F. & Klein, G. (2008). Microbiological quality of freshly shot game in Germany. *Meat Science*, 78(4), pp. 414-419.
- Avagnina, A., Nucera, D., Grassi, M.A., Ferroglio, E., Dalmaso, A. & Civera, T. (2012). The microbiological conditions of carcasses from large game animals in Italy. *Meat Science*, 91(3), pp. 266-271.
- Backhans, A., Fellström, C. & Lambertz, S.T. (2011). Occurrence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in small wild rodents. *Epidemiology & Infection*, 139(08), pp. 1230-1238.
- Bancerz-Kisiel, A., Pieczywek, M., Łada, P. & Szweda, W. (2018). The most important virulence markers of *Yersinia enterocolitica* and their role during infection. *Genes*, 9(5), p. 235.
- Bancerz-Kisiel, A., Platt-Samoraj, A., Szczerba-Turek, A., Szczylo, K. & Szweda, W. (2015). The first pathogenic *Yersinia enterocolitica* bioserotype 4/O:3 strain isolated from a hunted wild boar (*Sus scrofa*) in Poland. *Epidemiol Infect*, 143(13), pp. 2758-65.
- Blais, B.W. & Phillippe, L.M. (1995). Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica*. *Food Control*, 6(4), pp. 211-214.
- Borch, E., Nesbakken, T. & Christensen, H. (1996). Hazard identification in swine slaughter with respect to foodborne bacteria. *International Journal of Food Microbiology*, 30(1), pp. 9-25.
- Bottone, E.J. (1997). *Yersinia enterocolitica*: the charisma continues. *Clin Microbiol Rev*, 10.
- Bottone, E.J. (1999). *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microbes and Infection*, 1(4), pp. 323-333.
- Bottone, E.J., Bercovier, H. and Mollaret, H. H. (2015). *Yersinia*. In: *Bergey's Manual of Systematics of Archaea and Bacteria*. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118960608.gbm01174>.
- Boyapalle, S., Wesley, I.V., Hurd, H.S. & Reddy, P.G. (2001). Comparison of Culture, Multiplex, and 5' Nuclease Polymerase Chain Reaction Assays for the Rapid Detection of *Yersinia enterocolitica* in Swine and Pork Products. *Journal of Food Protection*, 64(9), pp. 1352-1361.
- Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R. & Swaminathan, B. (2000). Salmonella Nomenclature. *Journal of Clinical Microbiology*, 38(7), pp. 2465-2467.
- Brook, R.K., Wal, E.V., van Beest, F.M. & McLachlan, S.M. (2013). Evaluating use of cattle winter feeding areas by elk and white-tailed deer: Implications for managing bovine tuberculosis transmission risk from the ground up. *Preventive Veterinary Medicine*, 108(2), pp. 137-147.

- Brooke, C.J. & Riley, T.V. (1999). *Erysipelothrix rhusiopathiae*: bacteriology, epidemiology and clinical manifestations of an occupational pathogen. *Journal of Medical Microbiology*, 48(9), pp. 789-799.
- Carniel, E., Autenrieth, I., Cornelis, G., Fukushima, H., Guinet, F., Isberg, R., Pham, J., Prentice, M., Simonet, M., Skurnik, M. & Wauters, G. (2006). *Y. enterocolitica* and *Y. pseudotuberculosis*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds) *The Prokaryotes: Volume 6: Proteobacteria: Gamma Subclass*. New York, NY: Springer New York, pp. 270-398. https://doi.org/10.1007/0-387-30746-X_13.
- Chen, S., Li, J., Saleh-Lakha, S., Allen, V. & Odumeru, J. (2011). Multiple-locus variable number of tandem repeat analysis (MLVA) of *Listeria monocytogenes* directly in food samples. *International Journal of Food Microbiology*, 148(1), pp. 8-14.
- Chiari, M., Zanoni, M., Tagliabue, S., Lavazza, A. & Alborali, L. (2013). Salmonella serotypes in wild boars (*Sus scrofa*) hunted in northern Italy. *Acta veterinaria Scandinavica*, 55(1), p. 42.
- Crump, J.A., Luby, S.P. & Mintz, E.D. (2004). The global burden of typhoid fever. *Bulletin of the World Health Organization*, 82, pp. 346-353.
- Cummings, K.J., Rodriguez-Rivera, L.D., Grigar, M.K., Rankin, S.C., Mesenbrink, B.T., Leland, B.R. & Bodenchuk, M.J. (2016). Prevalence and characterization of *Salmonella* isolated from feral pigs throughout Texas. *Zoonoses and Public Health*, 63(6), pp. 436-441.
- Dalsjö, A., Nilsson, A.C. & Ramussen, M. (2014). Complicated infection caused by *Streptococcus suis* serotype 14 transmitted from a wild boar. *JMM Case Reports*, 1(3).
- Danna, K. & Nathans, D. (1971). Specific cleavage of Simian virus 40 DNA by restriction endonuclease of *Hemophilus Influenzae*. *Proceedings of the National Academy of Sciences of the United States of America*, 68(12), pp. 2913-2917.
- Delor, I., Kaeckenbeeck, A., Wauters, G. & Cornelis, G.R. (1990). Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and nonpathogenic yersiniae. *Infection and Immunity*, 58(9), pp. 2983-2988.
- Delorme, J., Laverdière, M., Martineau, B. & Lafleur, L. (1974). Yersiniosis in children. *Canadian Medical Association Journal*, 110(3), pp. 281-284.
- Díaz-Sánchez, S., Sánchez, S., Herrera-León, S., Porrero, C., Blanco, J., Dahbi, G., Blanco, J.E., Mora, A., Mateo, R., Hanning, I. & Vidal, D. (2013). Prevalence of Shiga toxin-producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. in large game animals intended for consumption: Relationship with management practices and livestock influence. *Veterinary Microbiology*, 163(3), pp. 274-281.
- Dubey, J.P. (2009). Toxoplasmosis in pigs—The last 20 years. *Veterinary Parasitology*, 164(2), pp. 89-103.
- Dumke, R. & Jacobs, E. (2011). Culture-independent multi-locus variable-number tandem-repeat analysis (MLVA) of *Mycoplasma pneumoniae*. *Journal of Microbiological Methods*, 86(3), pp. 393-396.
- Durham, H.E. (1901). Some theoretical considerations upon the nature of agglutinins, together with further observations upon *Bacillus typhi abdominalis*, *Bacillus enteritidis*, *Bacillus coli communis*, *Bacillus lactis aerogenes*, and some other *Bacilli* of allied character. *The Journal of Experimental Medicine*, 5(4), pp. 353-388.

- Dyce, K.M., Sack, W.O. & Wensing, C.J.G. (2009). *Textbook of Veterinary Anatomy-E-Book*: Elsevier Health Sciences.
- Dziedzinska, R., Moravkova, M., Hrady, J., Slana, I., Vlkova, H., Kunstovna, H. & Vasickova, P. (2018). Occurrence of selected viral, bacterial and protozoan pathogens in fresh juices and smoothies in Prague, Czech Republic. *Food Control*, 93, pp. 310-314.
- EFSA (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal*, 14(12).
- Eiss, J. (1975). Selective culturing of *Yersinia enterocolitica* at a low temperature. *Scandinavian journal of infectious diseases*, 7(4), pp. 249-251.
- El Tahir, Y. & Skurnik, M. (2001). *YadA*, the multifaceted *Yersinia* adhesin. *International Journal of Medical Microbiology*, 291(3), pp. 209-218.
- Eriksson, E. & Aspan, A. (2007). Comparison of culture, ELISA and PCR techniques for salmonella detection in faecal samples for cattle, pig and poultry. *BMC Veterinary Research*, 3(1), pp. 1-19.
- Eriksson, E., Nerbrink, E., Borch, E., Aspan, A. & Gunnarsson, A. (2003). Verocytotoxin-producing *Escherichia coli* O157:H7 in the Swedish pig population. *Veterinary Record*, 152(23), pp. 712-717.
- European Food Safety, A., European Centre for Disease, P. & Control (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal*, 14(12), pp. e04634-n/a.
- Feng, P., Keasler, S. & Hill, W. (1992). Direct identification of *Yersinia enterocolitica* in blood by polymerase chain reaction amplification. *Transfusion*, 32(9), pp. 850-854.
- Fernández-Aguilar, X., Gottschalk, M., Aragon, V., Càmara, J., Ardanuy, C., Velarde, R., Galofré-Milà, N., Castillo-Contreras, R., López-Olvera, J.R., Mentaberre, G., Colom-Cadena, A., Lavín, S. & Cabezón, O. (2018). Urban wild boars and risk for zoonotic *Streptococcus suis*, Spain. *Emerging infectious diseases*, 24(6), pp. 1083-1086.
- Foley, S.L., Lynne, A.M. & Nayak, R. (2009). Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infection, Genetics and Evolution*, 9(4), pp. 430-440.
- Foti, M., Daidone, A., Aleo, A., Pizzimenti, A., Giacobello, C. & Mammina, C. (2009). *Salmonella bongori* 48:z(35):- in migratory birds, Italy. *Emerging infectious diseases*, 15(3), pp. 502-503.
- Fredriksson-Ahomaa, M. (2015). Enteropathogenic *Yersinia* spp. In: Sing, A. (ed. *Zoonoses - Infections Affecting Humans and Animals: Focus on Public Health Aspects*. Dordrecht: Springer Netherlands, pp. 213-234. https://doi.org/10.1007/978-94-017-9457-2_8.
- Fredriksson-Ahomaa, M. (2017). Chapter 9 - *Yersinia enterocolitica*. In: *Foodborne Diseases (Third edition)* Academic Press, pp. 223-233.
- Fredriksson-Ahomaa, M., Joutsen, S. & Laukkanen-Ninios, R. (2018). Identification of *Yersinia* at the species and subspecies levels is challenging. *Current Clinical Microbiology Reports*.
- Fredriksson-Ahomaa, M. & Korkeala, H. (2003). Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin Microbiol Rev*, 16(2), pp. 220-9.

- Fredriksson-Ahomaa, M., Korte, T. & Korkeala, H. (2001). Transmission of *Yersinia enterocolitica* 4/O:3 to pets via contaminated pork. *Letters in Applied Microbiology*, 32(6), pp. 375-378.
- Fredriksson-Ahomaa, M., Stolle, A. & Korkeala, H. (2006a). Molecular epidemiology of *Yersinia enterocolitica* infections. *FEMS Immunology & Medical Microbiology*, 47(3), pp. 315-329.
- Fredriksson-Ahomaa, M., Stolle, A., Siitonen, A. & Korkeala, H. (2006b). Sporadic human *Yersinia enterocolitica* infections caused by bioserotype 4/O:3 originate mainly from pigs. *Journal of Medical Microbiology*, 55(6), pp. 747-749.
- Gajadhar, A.A., Pozio, E., Ray Gamble, H., Nöckler, K., Maddox-Hyttel, C., Forbes, L.B., Vallée, I., Rossi, P., Marinculic, A. & Boireau, P. (2009). *Trichinella* diagnostics and control: Mandatory and best practices for ensuring food safety. *Veterinary Parasitology*, 159(3), pp. 197-205.
- Galindo, C.L., Rosenzweig, J.A., Kirtley, M.L. & Chopra, A.K. (2011). Pathogenesis of *Y. enterocolitica* and *Y. pseudotuberculosis* in human yersiniosis. *Journal of Pathogens*, 2011, p. 16.
- Georgsson, F., Þorkelsson, Á.E., Geirsdóttir, M., Reiersen, J. & Stern, N.J. (2006). The influence of freezing and duration of storage on *Campylobacter* and indicator bacteria in broiler carcasses. *Food Microbiology*, 23(7), pp. 677-683.
- Giammanco, G.M., Pignato, S., Mammina, C., Grimont, F., Grimont, P.A.D., Nastasi, A. & Giammanco, G. (2002). Persistent endemicity of *Salmonella bongori* 48:z(35):– in Southern Italy: molecular characterization of human, animal, and environmental isolates. *Journal of Clinical Microbiology*, 40(9), pp. 3502-3505.
- Gierczynski, R., Golubov, A., Neubauer, H., Pham, J.N. & Rakin, A. (2007). Development of multiple-locus variable-number tandem-repeat analysis for *Yersinia enterocolitica* subsp *palaearctica* and its application to bioserogroup 4/O3 subtyping. *Journal of Clinical Microbiology*, 45(8), pp. 2508-2515.
- Gorman, R., Bloomfield, S. & Adley, C.C. (2002). A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland. *International Journal of Food Microbiology*, 76(1), pp. 143-150.
- Gortázar, C., Acevedo, P., Ruiz-Fons, F. & Vicente, J. (2006). Disease risks and overabundance of game species. *European Journal of Wildlife Research*, 52(2), pp. 81-87.
- Goyette-Desjardins, G., Auger, J.-P., Xu, J., Segura, M. & Gottschalk, M. (2014). *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent—an update on the worldwide distribution based on serotyping and sequence typing. *Emerging Microbes & Infections*, 3, p. e45.
- Grimont, P.A. & Weill, F.-X. (2007). Antigenic formulae of the *Salmonella* serovars. *WHO collaborating centre for reference and research on Salmonella*, 9, pp. 1-166.
- Halkilahti, J., Haukka, K. & Siitonen, A. (2013). Genotyping of outbreak-associated and sporadic *Yersinia pseudotuberculosis* strains by novel multilocus variable-number tandem repeat analysis (MLVA). *J Microbiol Methods*, 95(2), pp. 245-50.
- Hall, M., Chattaway, M.A., Reuter, S., Savin, C., Strauch, E., Carniel, E., Connor, T., Van Damme, I., Rajakaruna, L., Rajendram, D., Jenkins, C., Thomson, N.R. & McNally, A. (2015). Use of whole-genus genome sequence data to develop a multilocus sequence typing

- tool that accurately identifies *Yersinia* isolates to the species and subspecies levels. *J Clin Microbiol*, 53(1), pp. 35-42.
- Hayashidani, H., Kanzaki, N., Kaneko, Y., Okatani, A., Taniguchi, T., Kaneko, K. & Ogawa, M. (2002). Occurrence of yersiniosis and listeriosis in wild boars in Japan. *Journal of Wildlife Diseases*, 38(1), pp. 202-205.
- Herikstad, H., Motarjemi, Y. & Tauxe, R.V. (2002). *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiology and Infection*, 129(1), pp. 1-8.
- Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., De Pinna, E., Nair, S., Fields, P.I. & Weill, F.-X. (2014). Supplement 2008–2010 (no. 48) to the White–Kauffmann–Le Minor scheme. *Research in microbiology*, 165(7), pp. 526-530.
- Iteman, I., Guiyoule, A. & Carniel, E. (1996). Comparison of three molecular methods for typing and subtyping pathogenic *Yersinia enterocolitica* strains. *Journal of Medical Microbiology*, 45(1), pp. 48-56.
- Jalava, K., Hakkinen, M., Valkonen, M., Nakari, U.-M., Palo, T., Hallanvuori, S., Ollgren, J., Siitonen, A. & Nuorti, J.P. (2006). An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis*. *The Journal of Infectious Diseases*, 194(9), pp. 1209-1216.
- Jay-Russell, M.T., Bates, A., Harden, L., Miller, W.G. & Mandrell, R.E. (2012). Isolation of *Campylobacter* from feral swine (*Sus scrofa*) on the ranch associated with the 2006 *Escherichia coli* O157:H7 spinach outbreak investigation in California. *Zoonoses and Public Health*, 59(5), pp. 314-319.
- Jay, M.T., Cooley, M., Carychao, D., Wiscomb, G.W., Sweitzer, R.A., Crawford-Miksza, L., Farrar, J.A., Lau, D.K., O'Connell, J., Millington, A., Asmundson, R.V., Atwill, E.R. & Mandrell, R.E. (2007). *Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast. *Emerging infectious diseases*, 13(12), pp. 1908-11.
- Jonsson, L. (1986). From wild boar to domestic pig -a reassessment of neolithic swine of northwestern Europe. *Nordic Late Quaternary Biology and Ecology*, 24, pp. 125-129.
- Kahlisch, L., Henne, K., Draheim, J., Brettar, I. & Höfle, M.G. (2010). High-resolution in situ genotyping of *Legionella pneumophila* populations in drinking water by Multiple-Locus Variable-number tandem-repeat Analysis using environmental DNA. *Applied and Environmental Microbiology*, 76(18), pp. 6186-6195.
- Kanai, Y., HAYASHIDANI, H., Kaneko, K.-I., Ogawa, M., Takahashi, T. & Nakamura, M. (1997). Occurrence of zoonotic bacteria in retail game meat in Japan with special reference to *Erysipelothrix*. *Journal of Food Protection*, 60(3), pp. 328-331.
- Kangas, S., Takkinen, J., Hakkinen, M., Nakari, U.-M., Johansson, T. & Henttonen, H. (2008). *Yersinia pseudotuberculosis* O:1 traced to raw carrots. *Emerging infectious diseases*, 14(12), pp. 1959-1961.
- Keim, P., Price, L., Klevytska, A., Smith, K., Schupp, J., Okinaka, R., Jackson, P. & Hugh-Jones, M. (2000). Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *Journal of bacteriology*, 182(10), pp. 2928-2936.
- Kotula, A.W., Dubey, J.P., Sharar, A.K., Andrews, C.D., Shen, S.K. & Lindsay, D.S. (1991). Effect of freezing on infectivity of *Toxoplasma gondii* tissue cysts in pork. *Journal of Food Protection*, 54(9), pp. 687-690.

- Lambertz, S.T., Nilsson, C. & Hallanvuoto, S. (2008a). TaqMan-based real-time PCR method for detection of *Yersinia pseudotuberculosis* in food. *Appl Environ Microbiol*, 74(20), pp. 6465-9.
- Lambertz, S.T., Nilsson, C., Hallanvuoto, S. & Lindblad, M. (2008b). Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Appl Environ Microbiol*, 74(19), pp. 6060-7.
- Larsson, J.T., Torpdahl, M., MLVA working group, C. & Møller Nielsen, E. (2013). Proof-of-concept study for successful inter-laboratory comparison of MLVA results. *Eurosurveillance*, 18(35), p. 20566.
- Laukkanen-Ninios, R., Didelot, X., Jolley, K.A., Morelli, G., Sangal, V., Kristo, P., Brehony, C., Imori, P.F., Fukushima, H., Siitonen, A., Tseneva, G., Voskressenskaya, E., Falcao, J.P., Korkeala, H., Maiden, M.C., Mazzoni, C., Carniel, E., Skurnik, M. & Achtman, M. (2011). Population structure of the *Yersinia pseudotuberculosis* complex according to multilocus sequence typing. *Environ Microbiol*, 13(12), pp. 3114-27.
- Laukkanen, R., Martinez, P.O., Siekkinen, K.-M., Ranta, J., Maijala, R. & Korkeala, H. (2008). Transmission of *Yersinia pseudotuberculosis* in the pork production chain from farm to slaughterhouse. *Appl. Environ. Microbiol.*, 74(17), pp. 5444-5450.
- Laurent, S. & J., R.T. (2003). Diet of wild boar *Sus scrofa* in Western Europe, with particular reference to consumption of agricultural crops. *Mammal Review*, 33(1), pp. 43-56.
- Magistrali, C.F., Cucco, L., Pezzotti, G., Farneti, S., Cambiotti, V., Catania, S., Prati, P., Fabbri, M., Lollai, S., Mangili, P., Sebastiani, C., Bano, L., Dionisi, A.M. & Luzzi, I. (2015). Characterisation of *Yersinia pseudotuberculosis* isolated from animals with yersiniosis during 1996–2013 indicates the presence of pathogenic and Far Eastern strains in Italy. *Veterinary Microbiology*, 180(1–2), pp. 161-166.
- Magnino, S., Frasnelli, M., Fabbri, M., Bianchi, A., Zanoni, M.G., Merialdi, G., Pacciarini, M.L. & Gaffuri, A. (2011). The monitoring of selected zoonotic diseases of wildlife in Lombardy and Emilia-Romagna, northern Italy. In: Paulsen, P., Bauer, A., Vodnansky, M., Winkelmayr, R. & Smulders, F.J.M. (eds) *Game meat hygiene in focus* Wageningen Academic Publishers, pp. 223-244.
- Maiden, M.C.J., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. & Spratt, B.G. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences*, 95(6), pp. 3140-3145.
- Mainar-Jaime, R.C., Andrés, S., Vico, J.P., San Román, B., Garrido, V. & Grilló, M.J. (2013). Sensitivity of the ISO 6579:2002/Amd 1:2007 standard method for detection of *Salmonella* spp. on mesenteric lymph nodes from slaughter pigs. *Journal of Clinical Microbiology*, 51(1), pp. 89-94.
- Malorny, B., Junker, E. & Helmuth, R. (2008). Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. *BMC Microbiology*, 8(1), p. 84.
- Massei, G., Kindberg, J., Licoppe, A., Gačić, D., Šprem, N., Kamler, J., Baubet, E., Hohmann, U., Monaco, A., Ozoliņš, J., Cellina, S., Podgórski, T., Fonseca, C., Markov, N., Pokorny, B.,

- Rosell, C. & Náhlik, A. (2015). Wild boar populations up, numbers of hunters down? A review of trends and implications for Europe. *Pest Management Science*, 71(4), pp. 492-500.
- Masuda, J.-I., Yano, K., Tamada, Y., Takii, Y., Ito, M., Omagari, K. & Kohno, S. (2005). Acute hepatitis E of a man who consumed wild boar meat prior to the onset of illness in Nagasaki, Japan. *Hepatology Research*, 31(3), pp. 178-183.
- McNally, A., Cheasty, T., Fearnley, C., Dalziel, R.W., Paiba, G.A., Manning, G. & Newell, D.G. (2004). Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999–2000. *Letters in Applied Microbiology*, 39(1), pp. 103-108.
- McNally, A., La Ragione, R.M., Best, A., Manning, G. & Newell, D.G. (2007). An aflagellate mutant *Yersinia enterocolitica* biotype 1A strain displays altered invasion of epithelial cells, persistence in macrophages, and cytokine secretion profiles in vitro. *Microbiology*, 153(5), pp. 1339-1349.
- McNally, A., Thomson, N.R., Reuter, S. & Wren, B.W. (2016). "Add, stir and reduce": *Yersinia* spp. as model bacteria for pathogen evolution. *Nature Reviews Microbiology*, 14, p. 177.
- Methner, U., Heller, M. & Bocklisch, H. (2010). *Salmonella enterica* subspecies *enterica* serovar Choleraesuis in a wild boar population in Germany. *European Journal of Wildlife Research*, 56(4), pp. 493-502.
- Miller, V.L., Farmer, J.J., Hill, W.E. & Falkow, S. (1989). The ail locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infection and Immunity*, 57(1), pp. 121-131.
- Moens, S. & Vanderleyden, J. (1996). Functions of Bacterial Flagella. *Critical Reviews in Microbiology*, 22(2), pp. 67-100.
- Mäde, D., Reiting, R., Strauch, E., Ketteritzsch, K. & Wicke, A. (2008). A real-time PCR for detection of pathogenic *Yersinia enterocolitica* in food combined with an Universal Internal Amplification control system. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, 3(2), pp. 141-151.
- Nakajima, H., Inoue, M., Mori, T., Itoh, K., Arakawa, E. & Watanabe, H. (1992). Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *Journal of Clinical Microbiology*, 30(9), pp. 2484-2486.
- Navarro-Gonzalez, N., Casas-Díaz, E., Porrero, C.M., Mateos, A., Dominguez, L., Lavín, S. & Serrano, E. (2013a). Food-borne zoonotic pathogens and antimicrobial resistance of indicator bacteria in urban wild boars in Barcelona, Spain. *Veterinary Microbiology*, 167(3–4), pp. 686-689.
- Navarro-Gonzalez, N., Fernández-Llario, P., Pérez-Martín, J.E., Mentaberre, G., López-Martín, J.M., Lavín, S. & Serrano, E. (2013b). Supplemental feeding drives endoparasite infection in wild boar in Western Spain. *Veterinary Parasitology*, 196(1), pp. 114-123.
- Navarro-Gonzalez, N., Mentaberre, G., Porrero, C.M., Serrano, E., Mateos, A., Lopez-Martin, J.M., Lavin, S. & Dominguez, L. (2012). Effect of cattle on *Salmonella* carriage, diversity and antimicrobial resistance in free-ranging wild boar (*Sus scrofa*) in northeastern Spain. *PLoS ONE*, 7(12), p. e51614.

- Nesbakken, T. (2015). 2 - Update on *Yersinia* as a foodborne pathogen: analysis and control In: Sofos, J. (ed. *Advances in Microbial Food Safety*. Oxford: Woodhead Publishing, pp. 33-58.
- Nesbakken, T., Kapperud, G., Dommarsnes, K., Skurnik, M. & Hornes, E. (1991). Comparative study of a DNA hybridization method and two isolation procedures for detection of *Yersinia enterocolitica* O:3 in naturally contaminated pork products. *Applied and Environmental Microbiology*, 57(2), pp. 389-394.
- Nikolova, S., Tzvetkov, Y., Najdenski, H. & Vesselinova, A. (2001). Isolation of pathogenic *Yersiniae* from wild animals in Bulgaria. *Journal of Veterinary Medicine, Series B*, 48(3), pp. 203-209.
- Niskanen, T., Waldenström, J., Fredriksson-Ahomaa, M., Olsen, B. & Korkeala, H. (2003). *virF*-Positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* found in migratory birds in Sweden. *Applied and Environmental Microbiology*, 69(8), pp. 4670-4675.
- Noller, A.C., McEllistrem, M.C., Shutt, K.A. & Harrison, L.H. (2006). Locus-specific mutational events in a Multilocus Variable-number tandem repeat Analysis of *Escherichia coli* O157:H7. *Journal of Clinical Microbiology*, 44(2), pp. 374-377.
- Oliveira-Filho, E.F., Bank-Wolf, B.R., Thiel, H.-J. & König, M. (2014). Phylogenetic analysis of hepatitis E virus in domestic swine and wild boar in Germany. *Veterinary Microbiology*, 174(1), pp. 233-238.
- Petsios, S., Fredriksson-Ahomaa, M., Sakkas, H. & Papadopoulou, C. (2016). Conventional and molecular methods used in the detection and subtyping of *Yersinia enterocolitica* in food. *International Journal of Food Microbiology*, 237, pp. 55-72.
- Platt-Samoraj, A., Syczłyo, K., Szczerba-Turek, A., Bancercz-Kisiel, A., Jabłoński, A., Łabuć, S., Pajdak, J., Oshakbaeva, N. & Szwed, W. (2017). Presence of *ail* and *ystB* genes in *Yersinia enterocolitica* biotype 1A isolates from game animals in Poland. *The Veterinary Journal*, 221, pp. 11-13.
- Popesko, P. (2007). *Atlas der topographischen Anatomie der Haustiere*: Georg Thieme Verlag.
- Pozio, E. (2007). World distribution of *Trichinella* spp. infections in animals and humans. *Veterinary Parasitology*, 149(1), pp. 3-21.
- Ramamurthy, T., Yoshino, K.-i., Huang, X., Balakrish Nair, G., Carniel, E., Maruyama, T., Fukushima, H. & Takeda, T. (1997). The novel heat-stable enterotoxin subtype gene (*ystB*) of *Yersinia enterocolitica*: nucleotide sequence and distribution of the *yst*-genes. *Microbial Pathogenesis*, 23(4), pp. 189-200.
- Ribot, D.E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B. & Barrett, T.J. (2006). Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease*, 3(1), pp. 59-67.
- Rimhanen-Finne, R., Niskanen, T., Hallanvuo, S., Makary, P., Haukka, K., Pajunen, S., Siitonen, A., Ristolainen, R., Pöyry, H., Ollgren, J. & Kuusi, M. (2009). *Yersinia pseudotuberculosis* causing a large outbreak associated with carrots in Finland, 2006. *Epidemiology & Infection*, 137(Special Issue 03), pp. 342-347.
- Ruiz-Fons, F. (2017). A review of the current status of relevant zoonotic pathogens in wild swine (*Sus scrofa*) populations: changes modulating the risk of transmission to humans. *Transbound Emerg Dis*, 64(1), pp. 68-88.

- Råsback, T., Rosendal, T., Stampe, M., Sannö, A., Aspán, A., Järnevi, K. & Lahti, E.T. (2018). Prevalence of human pathogenic *Yersinia enterocolitica* in Swedish pig farms. *Acta veterinaria Scandinavica*, 60(1), p. 39.
- Sales, J. & Kotrba, R. (2013). Meat from wild boar (*Sus scrofa* L.): A review. *Meat Science*, 94(2), pp. 187-201.
- Sannö, A., Aspan, A., Hestvik, G. & Jacobson, M. (2014). Presence of *Salmonella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Escherichia coli* O157:H7 in wild boars. *Epidemiol Infect*, 142(12), pp. 2542-7.
- Sasaki, Y., Goshima, T., Mori, T., Murakami, M., Haruna, M., Ito, K. & Yamada, Y. (2013). Prevalence and antimicrobial susceptibility of foodborne bacteria in wild boars (*Sus scrofa*) and wild deer (*Cervus nippon*) in Japan. *Foodborne Pathogens and Disease*, 10(11), pp. 985-991.
- Savin, C., Martin, L., Bouchier, C., Filali, S., Chenau, J., Zhou, Z., Becher, F., Fukushima, H., Thomson, N.R., Scholz, H.C. & Carniel, E. (2014). The *Yersinia pseudotuberculosis* complex: characterization and delineation of a new species, *Yersinia wautersii*. *Int J Med Microbiol*, 304(3-4), pp. 452-63.
- Schiemann, D.A. (1979). Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Canadian Journal of Microbiology*, 25(11), pp. 1298-1304.
- Schwartz, D.C. & Cantor, C.R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, 37(1), pp. 67-75.
- Sihvonen, L.M., Hallanvuoto, S., Haukka, K., Skurnik, M. & Siitonen, A. (2011a). The *ail*-gene is present in some *Yersinia enterocolitica* biotype 1A strains. *Foodborne Pathog Dis*, 8(3), pp. 455-7.
- Sihvonen, L.M., Toivonen, S., Haukka, K., Kuusi, M., Skurnik, M. & Siitonen, A. (2011b). Multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, and antimicrobial susceptibility patterns in discrimination of sporadic and outbreak-related strains of *Yersinia enterocolitica*. *BMC Microbiol*, 11(1), p. 42.
- Silva, F.V.M. & Gibbs, P.A. (2012). Thermal pasteurization requirements for the inactivation of *Salmonella* in foods. *Food Research International*, 45(2), pp. 695-699.
- Stamm, I., Hailer, M., Depner, B., Kopp, P.A. & Rau, J. (2013). *Yersinia enterocolitica* in diagnostic fecal samples from European dogs and cats: identification by Fourier Transform Infrared Spectroscopy and Matrix-assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. *Journal of Clinical Microbiology*, 51(3), pp. 887-893.
- Stuart, P.F. & Pivnick, H. (1965). Isolation of *Salmonellae* by selective motility systems. *Applied Microbiology*, 13(3), pp. 365-372.
- Tennant, S.M., Grant, T.H. & Robins-Browne, R.M. (2003). Pathogenicity of *Yersinia enterocolitica* biotype 1A. *FEMS Immunology & Medical Microbiology*, 38(2), pp. 127-137.
- Thakur, S., Sandfoss, M., Kennedy-Stoskopf, S. & DePerno, C.S. (2011). Detection of *Clostridium difficile* and *Salmonella* in feral swine population in North Carolina. *Journal of Wildlife Diseases*, 47(3), pp. 774-776.
- Thisted, L.S., A., B.P., A., N., P., N. & M.-L., D.T. (1996). A comparison between a PCR method and a conventional culture method for detecting pathogenic *Yersinia enterocolitica* in food. *Journal of Applied Bacteriology*, 81(3), pp. 303-308.

- Uzzau, S., Brown, D.J., Wallis, T., Rubino, S., Leori, G., Bernard, S., CasadesÚS, J., Platt, D.J. & Olsen, J.E. (2001). Host adapted serotypes of *Salmonella enterica*. *Epidemiology and Infection*, 125(2), pp. 229-255.
- Wacheck, S., Fredriksson-Ahomaa, M., Konig, M., Stolle, A. & Stephan, R. (2010). Wild boars as an important reservoir for foodborne pathogens. *Foodborne Pathogens and Disease*, 7(3), pp. 307-312.
- Wahlström, H., Tysén, E., Olsson Engvall, E., Brändstrom, B., Eriksson, E., Mörner, T. & Vågsholm, I. (2003). Survey of *Campylobacter* species, VTEC 01 57 and *Salmonella* species in Swedish wildlife. *Veterinary Record*, 153(3), pp. 74-80.
- Wallander, C., Frossling, J., Vågsholm, I., Uggla, A. & Lunden, A. (2015). *Toxoplasma gondii* seroprevalence in wild boars (*Sus scrofa*) in Sweden and evaluation of ELISA test performance. *Epidemiol Infect*, 143(9), pp. 1913-21.
- van Loghem, J.J. (1944). The classification of the plague-bacillus. *Antonie van Leeuwenhoek*, 10(1), pp. 15-16.
- Ward, M.P., Cowled, B.D., Galea, F., Garner, M.G., Laffan, S.W., Marsh, I., Negus, K., Sarre, S.D. & Woolnough, A.P. (2013). *Salmonella* infection in a remote, isolated wild pig population. *Veterinary Microbiology*, 162(2), pp. 921-929.
- Wattiau, P., Boland, C. & Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. *enterica* Subtyping: Gold Standards and Alternatives. *Applied and Environmental Microbiology*, 77(22), pp. 7877-7885.
- Weagant, S.D. (2008). A new chromogenic agar medium for detection of potentially virulent *Yersinia enterocolitica*. *Journal of Microbiological Methods*, 72(2), pp. 185-190.
- Wesley, I.V., Bhaduri, S. & Bush, E. (2008). Prevalence of *Yersinia enterocolitica* in market weight hogs in the United States. *Journal of Food Protection*, 71(6), pp. 1162-1168.
- Weynants, V., Jadot, V., Denoel, P.A., Tibor, A. & Letesson, J.J. (1996). Detection of *Yersinia enterocolitica* serogroup O:3 by a PCR method. *Journal of Clinical Microbiology*, 34(5), pp. 1224-7.
- White, P.B. (1926). Further Studies of the *Salmonella* Group. *Further Studies of the Salmonella Group*.(103).
- WHO (2015). *WHO estimates of the global burden of foodborne diseases*: World health organization.
- Widén, F., Sundqvist, L., Matyi-Toth, A., Metreveli, G., Belák, S., Hallgren, G. & Norder, H. (2010). Molecular epidemiology of hepatitis E virus in humans, pigs and wild boars in Sweden. *Epidemiology and Infection*, 139(3), pp. 361-371.
- Vieira-Pinto, M., Morais, L., Caleja, C., Themudo, P., Torres, C., Igrejas, G., Poeta, P. & Martins, C. (2011a). *Salmonella* sp. in Game (*Sus scrofa* and *Oryctolagus cuniculus*). *Foodborne Pathogens and Disease*, 8(6), pp. 739-740.
- Vieira-Pinto, M., Morais, L., Caleja, C., Themudo, P., Torres, C., Igrejas, G., Poeta, P. & Martins, C. (2011b). *Salmonella* sp. in game (*Sus scrofa* and *Oryctolagus cuniculus*). *Foodborne Pathog Dis*, 8(6), pp. 739-40.
- Wray, C. & Wray, A. (2000). *Salmonella in domestic animals*: Cabi.
- Young, G.M., Badger, J.L. & Miller, V.L. (2000). Motility is required to initiate host cell invasion by *Yersinia enterocolitica*. *Infection and Immunity*, 68(7), pp. 4323-4326.

Popular science summary

During the past 20 years, the wild boar populations have increased rapidly and spread to new areas in Sweden similarly to other parts of the world. Accordingly, the conflicts with human activities are increasing, but so is the hunting activities and the consumption of wild boar meat. However, several studies report that wild boars may carry microorganisms with the potential to cause serious disease in humans and domestic animals.

This thesis focuses on the three important foodborne pathogens *Salmonella* spp., *Yersinia (Y.) enterocolitica* and *Y. pseudotuberculosis* and their occurrence in Swedish wild boars. In addition, the presence of *E. coli* O157:H7 was investigated. Potential risk factors possibly associated with this occurrence were investigated, and a new protocol for the detection of these pathogens have been developed that may also aid in investigations on disease outbreaks caused by *Yersinia* spp. in humans. To investigate to what extent these pathogens are found in products from wild boars, minced wild-boar meat was investigated.

Samples from 178 wild boars were obtained from hunting estates and private hunters. Twenty samples of wild-boar minced meat was obtained from hunters and an additional twelve packages of minced meat was purchased in grocery stores or obtained directly from wild-game handling establishments.

In the evaluation of the new protocol, 354 samples from 90 wild boars were included. The method Multi Locus Variable tandem repeats Analysis (MLVA) was used to analyse parts (locus) of the bacterial DNA, resulting in a numerical profile. Closely related bacteria will have similar MLVA-profiles. The new protocol offers the possibility to perform the MLVA without the need for pure cultures of the bacteria, thus increasing the detection rate.

In total, 91 individuals (51%) were shown to carry DNA from at least one of the pathogens investigated. DNA from *Y. enterocolitica* was found in 46 (25.8%) individuals, *Y. pseudotuberculosis* DNA in 37 (20.8%) animals and in 32 (18.0%) individuals, DNA from *Salmonella* spp. was found. Further, nine different subtypes of *Salmonella* spp. were obtained. In the wild-boar minced

meat submitted by hunters, *Y. enterocolitica* DNA was present in four samples and in the samples from grocery stores and wild-game handling establishments, DNA was present in six samples. DNA from *Salmonella* spp. and *Y. pseudotuberculosis* was not present.

Further, potential risk factors associated with the presence of these pathogens were investigated. Information on the characteristics of the local wild-boar populations were gathered and analysed statistically. The factors included population density, frequency of artificial feeding, time since establishment of the local population, and handling of the slaughter waste. None of the investigated factors were identified as a risk. However, a clear correlation was seen between a high population density and high levels of artificial feeding. Further, two-thirds of the hunters reported leaving the slaughter-waste out in nature, available for other wild-life.

In the MLVA of *Y. enterocolitica* performed according to the new protocol, complete profiles were obtained from 15 out of 36 samples. From additionally 15 samples, complete profiles were obtained, but in a few positions, several digits were obtained, indicating that more than one strain of the bacteria might be present. In the MLVA of *Y. pseudotuberculosis*, eight complete profiles was obtained while in four samples, several alternative profiles were found. In the remaining samples, the results were inconclusive.

The new protocol was shown to be well suited to detect *Salmonella* spp. and *Y. enterocolitica* but was less suited to detect *Y. pseudotuberculosis* and further development is needed. Further, MLVA-profiles can be obtained already within four working days and used in outbreak investigations.

The results from the studies clearly shows that foodborne pathogens are commonly found in the Swedish wild-boar population, mainly present in the throat region and the gastrointestinal tract of the wild boars. The hygiene at slaughter is hence important to prevent contamination of the carcass and the meat, including the processing of minced meat. The majority of the wild boars shot in Sweden are handled and consumed by the hunters themselves. Hence, it is important to inform about the associated risks.

Additional studies are needed to identify potential risk factors associated with the presence of foodborne pathogens in wild boars, to optimize recommendations to hunters and governments on how to reduce the transmission of pathogens. Based on findings in previous studies, some recommendations may still be made: Feeding places may act as “hot-spots” and overabundance of animals in an area will increase the risks for disease transmission. Hence, reducing the number of feeding places, decreasing the population densities, and dispose the slaughter waste in a way that render it unavailable to wild-life are all recommendable management strategies.

Populärvetenskaplig sammanfattning

Under de senaste 20 åren har vildsvinstammen vuxit kraftigt och spridit sig till nya områden både i Sverige och i övriga världen. Som en följd av detta ökar konflikterna med mänsklig aktivitet, men de växande stammarna medför också att allt fler människor jagar och äter vildsvinskött. Flera studier rapporterar dock att vildsvin kan bära på mikroorganismer som kan orsaka sjukdom hos människor.

Denna avhandling har fokuserat på tre viktiga matförgiftningsbakterier, *Salmonella* spp., *Yersinia (Y.) enterocolitica* och *Y. pseudotuberculosis*, och deras förekomst hos svenska vildsvin. Förekomsten av *E. coli* O157:H7, som kan orsaka EHEC, har också undersökts. Eventuella riskfaktorer associerade med förekomsten av dessa bakterier har utvärderats och ett nytt protokoll för att påvisa bakterierna har utvecklats. Protokollet kan även användas vid smittspårning av sjukdomen yersinios hos människa. För att undersöka om dessa matförgiftningsbakterier återfinns i produkter från vildsvin har även vildsvinsfärs undersökts.

I studien har ingått prover från 178 vildsvin från både jaktgods och privata jägare. Tjugo prover från vildsvinsfärs erhöles från jägare och ytterligare 12 konsumentförpackningar med färs köptes in från dagligvaruhandel eller direkt från vilthanteringsanläggningarna.

Totalt 354 prover från 90 vildsvin användes för att utvärdera det nya protokollet. Metoden Multi Locus Variable tandem repeats Analysis (MLVA) användes för att analysera delar (locus) av bakteriernas DNA. Analysen genererar en sifferkod där likartade bakterier har samma eller likartade koder, medan bakterier som är obesläktade får annorlunda MLVA-profiler. Protokollet möjliggör att MLVA kan genomföras utan att de svårödlade bakterierna måste renodlas.

Totalt hittades DNA från minst en av de undersökta bakterierna hos 91 djur (51%), varav 46 (25.8%) individer bar på DNA från *Y. enterocolitica*, 37 (20.8%) bar på *Y. pseudotuberculosis* DNA och 32 (18.0%) individer bar på

DNA från *Salmonella* spp. Totalt påvisades nio olika typer av *Salmonella* spp. I den vildsvinsfärs som erhöles från jägare påvisades förekomst av *Y. enterocolitica* DNA i fyra prover och i proverna från dagligvaruhandeln och vilthanteringsanläggningarna var sex prover positiva. *Salmonella* spp. och *Y. pseudotuberculosis*-DNA återfanns inte.

De undersökta riskfaktorerna inkluderade populationstäthet, utfodringsintensitet, tid sedan etablering av den lokala populationen och hantering av slaktavfallet från skjutna vildsvin. Resultaten analyserades statistiskt. Ingen av faktorerna kunde visas utgöra en risk men ett klart samband sågs mellan hög populationstäthet och hög utfodringsintensitet. Vidare rapporterade drygt två-tredjedelar av tillfrågade jägare att de regelmässigt lämnade slaktavfall ute i naturen.

MLVA resulterade i kompletta profiler från 15 av totalt 36 prover och från ytterligare 15 prover erhöles flera alternativa profiler vilket tyder på att mer än en bakteriestam fanns i provet. Vid analys med MLVA av de 26 prover som var positiva för *Y. pseudotuberculosis* erhöles åtta kompletta profiler, medan fyra prover hade flera alternativa profiler. I 14 prov saknades vissa data.

Det nya protokollet visade sig vara väl anpassat för att påvisa förekomst av *Salmonella* spp. och *Y. enterocolitica* medan det var mindre lämpligt för att påvisa *Y. pseudotuberculosis*. Vidare utvecklingsarbete krävs därför.

Resultaten visar att matförgiftningsbakterier är vanligt förekommande i den svenska vildsvins-populationen. Vid analys enligt det nya protokollet kan data i form av MLVA-profiler erhållas redan inom fyra arbetsdagar vilket kan vara mycket användbart vid smittspårning. Matförgiftningsbakterier återfinns främst i svalget men även i magtarm-kanalen hos vildsvin. Hygienen vid slakt är därför viktig för att undvika förorening av slaktkropp och kött. Vid undersökningen av vildsvinsfärs återfanns DNA från *Y. enterocolitica* vilket indikerar en risk för de som hanterar och konsumerar färsen. Merparten av de vildsvin som skjuts i Sverige hanteras och konsumeras av jägarna själva och det är därför viktigt att nå ut med information om detta.

Mer omfattande undersökningar behöver genomföras avseende de faktorer som kan påverka risken för förekomst av matförgiftningsbakterier hos vildsvin, för att kunna ge rekommendationer till jägare och myndigheter om hur förekomst och spridning skall kunna minskas.

Baserat på tidigare studier kan vissa rekommendationer ändå ges redan nu: Foderplatser kan fungera som "hot-spots" för spridning av sjukdomar och inom täta populationer ökar risken för smittspridning. Därför kan färre foderplatser, minskade populationer och korrekt hantering av slaktavfall för att minska tillgängligheten för andra djur mycket väl rekommenderas.

Acknowledgements

The work in this thesis was generously supported by funding from the **Swedish Environmental Protection Agency** with means from **Viltvårdsfonden, Ivar and Elsa's foundation** and the **CF Lundströms foundation** through **KSLA**.

The sampling of wild boars and other wild life has been essential for the work in this thesis. Thank you all **hunters, colleagues, wild game handling establishment owners and workers, field assistants** and **farmers** for your time and effort put into the sampling. Thank you for showing interest in my work, questioning my research and for sharing your experiences.

The main work within this thesis was carried out at the Department of Clinical Sciences, **Swedish University of Agricultural Sciences (SLU)** and the **National Veterinary Institute (SVA)**, Uppsala, Sweden. The laboratory work was performed at the Department of Biomedical Sciences and Veterinary Public Health (SLU), at the Science Department at the National Food Agency and at the Department of Microbiology (SVA).

During the eight years that it has taken me to complete the work within this thesis many people have been of great help. I wish to thank you all from the bottom of my heart. Firstly, I would especially like to express my gratitude to my supervisor group:

Magdalena Jacobson, my main supervisor who has been a great help from the start. You have been so generous with your scientific knowledge and experience, with your time to listen to crazy, over-optimistic ideas, and also with valuable advice on everything big and small in life. I admire your calm and your patience and I sincerely hope that we can have further collaborations in the future. Thank you for putting up with me and for guiding me through this work.

Annette Backhans, my co-supervisor and office roommate. Always willing to share a warming smile and a cynical comment simultaneously. Your strength and perseverance is an inspiration and your support has been much appreciated.

Anna Aspán, co-supervisor and PCR-genius. I don't know how to thank you enough for all the help in the lab. Always willing to answer stupid or complicated questions, finding additional lab-equipment when I have ran out and forgotten to order more, you have been there with a smile. Without your help there wouldn't have been any results to analyse at all. It has been a pleasure.

Thomas Rosendal, the latest addition to my supervisor group which has been crucial to me due to my close to non-existent comprehension of statistics and epidemiology. I have learnt so much from you in a short amount of time and I'm forever grateful for the work you have put into the last part of my work.

Dolores Gavier-Widén, thank you for taking me in as a wild-life pathologist at SVA and allowing me to become a part of your fantastic team during a short period of time. Thank you for securing that I had employment enough to be able to be accepted as a PhD-student and allowing me to participate in fantastic international conferences which was hugely rewarding.

Susanne Thisted-Lambertz, thank you for sharing your vast knowledge on Yersinia with me during my efforts to try and cultivate these elusive little bacteria. Your kindness and patience have been much appreciated during my struggle in the lab and your comments on my work and my methods have been extremely valuable.

Secondly, there are so many colleagues, friends and family that have made this thesis possible and that deserve a special mentioning:

Claes Fellström, professor emeritus and former head of the section for swine medicine at KV, SLU. In being almost a caricature of a professor with your absentmindedness coupled with a deep knowledge and understanding for swine medicine and research you have offered so much laughter and good advice on just about anything. Thank you for making me a part of the swine medicine group nine years ago, it has truly been a great time.

Marie Sterning, your organization skills and your good mood has been essential in keeping the wonderful swine medicine group together. Your dedication to teaching and the veterinary program is impressive, I appreciated this as a student but even more so as a colleague. You are doing such an important work and I hope you receive and feel the appreciation you truly deserve from students and colleagues alike.

Frida and Jenny, colleagues and former PhD-students at the section for swine medicine at KV, SLU. You two are a hard act to follow, both excelling during your PhD studies, performing impressive research and writing on a level impossible to reach up to. It has been a delight to work alongside you and I wish you all the best of luck in your future endeavours, although you most likely will not need it, you will both continue to excel at everything that is put before you. Thank you for being fantastic colleagues and even better friends.

Malin, the “**Chief of fika**”, thank you for all your support and your friendship, for making sure we all take the time to relax from the responsibilities and enjoy a cup of coffee at least once a day. I have truly appreciated our chats during dog-walks and fika, I will miss seeing you on a daily basis.

All other PhD student, past and present, at the Department of Clinical Sciences and at the Department of Biomedical Sciences and Veterinary Public Health (SLU). **Elin G, Anna W, Oskar, Camilla, Viktor, Maria, Elin M, Emelie, Hanna, Lena, Olov, Malin, Sanna** and many others, thank you and the very best of luck in the future.

The pig group at SVA, **Per, Mate, Marie, Julia, Maja and Sigge**, thank you for all the cooperation, all the wonderful meetings at Ekenäs and in Uppsala and for all the funny and interesting conferences where you have been excellent traveling companions.

To all the hard working people at the Department of Wild-life Pathology at SVA, past and present, **Erik, Caroline, Henrik, Gete, Lena, Aleksija, Karin, Arne, Ewa and Jessica**, thank you for all the things you taught me during my time with you and for supplying excellent samples for my first study. Thanks also to all others at the Department of Pathology, SVA for making my time there joyful and educational.

Present and former colleagues at the Department of Clinical Sciences, thank you for almost nine years of both fruitful discussions and everyday nonsense talk by the coffee machine, both equally important for an enjoyable work place. Keep up the good work!

To all the colleagues at **Gård & Djurhälsan** and especially **Maria, Lena, Kaisa, Thea, Jeanette** and **Petra**, thank you! I am sorry for my lack of planning skills and poor communication at times, I can only promise to try and do better in the future.

Robert and **Jani**, thank you both for introducing me into the wonderful world of MLVA, supporting me and putting up with all the questions. **Katarina, Agneta** and **Jenny** at the microbiology lab at SVA, thank you for all the help with all things related to my bacteria.

My master thesis students **Hampus** and **Sandra**, thank you for allowing me to supervise you during your projects. They have both been important parts of my thesis and I have learnt so much when teaching you and through you questions.

A special thanks to **Lise-Lotte**, but also to all other at the microbiology lab at the Department of Biomedical Sciences and Veterinary Public Health (SLU).

Likewise, a big thank you to all at the Department of Microbiology at SVA and at the Department of Microbiology at the National Food Agency for technical assistance and scientific advice in different parts of my laboratory work.

Thank you **Arja** at the National Food Agency for valuable advice and contacts with wild game handling establishments in preparation for my last study and to **Fredrik** for really trying to convince the different establishments to submit samples, sorry for any inconvenience.

Thank you **Mikael** for helping to spread the results of my research to the hunting community, for many fun and exciting hunts and for all the excellent photos that have been very useful in presentations and in the thesis.

My good friend **Karlsson**, thank you for the sample of minced meat, sorry it was positive, but most of all, thank you for the constant stream of entertaining, interesting and quite often hilarious chat-messages that always helps me through the day.

To all other dear friends spread out across the country, the **Malmstens, Schwans, Peppers, Törnqvists, Bäckgrens, Nilsson-Nilssons, Näslunds, Lundins, Brandts, Nordström-Lindbergs Pedersen-Björkqvists, Hanna, Markus, Sara** and many, many others, although we don't see each other as often as I would like, your support has been crucial for me to survive my PhD. Thank You all!

Christina and Greger, thank you for your wonderful daughter, for being fantastic grandparents and for welcoming me into your family and your home with open arms and lots of love.

The **Hultqvist** family, I am in awe over all that you have overcome. You are an inspiration in showing that all obstacles can be overcome with love and perseverance. Your home always feels welcoming as you are welcome in ours.

My immediate family, **Yvonne, Anna and Oskar**, I love you all and I am forever grateful for everything you have done for me, the love and support you have given, I will do everything in my power to support and love you as much in return. My ever growing extended family, **Stefan, Andreas, Ellinor, Patrik, Josefin, Rebecka, Anton** and **all the kids**, present and those to come. I am glad to call you family and fortunate to have you in my life.

Karin, you make me strive to become a better version of myself in every way, every day. Your love, kindness and compassion is always an inspiration. I would be lost without you, in so many ways. You are forever in my heart, I love you and adore you, plenty.

Pelle and Ilse, the light of my life and my inspiration for everything. If I could, and I will certainly try, I would spend every waking hour with you two, to enjoy your smiles, wipe your tears and share your laughter. Love you to bits!

To all other family, friends and colleagues that I have failed to mention above, **Thank you!**

