Wild Rodents as Carriers of Potential Pathogens to Pigs, Chickens and Humans

With special emphasis on *Brachyspira* spp. and *Yersinia enterocolitica*

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Abstract

The aim of this thesis was to investigate the specific risks that rodents constitute for proliferation of pathogens and transmission of those to farm animals, and indirectly to humans. Rodents were captured in pig and chicken flocks, in wastewater treatment plants and other urban environments.

The enteric pig pathogens *Brachyspira hyodysenteriae* and *B. pilosicoli* and the chicken pathogen *B. intermedia* were detected. Fingerprinting by Random Amplified Polymorphic DNA and Pulsed Field Gel Electrophoresis indicated cross-species transmission of *B. pilosicoli*, *B. intermedia*, *B. innocens*, and *B. murdochii* between rodents and farm animals. A phylogeny of murine brachyspiras was established. Three new genetic rodent variants of *Brachyspira* spp. were discovered, for which the provisional names '*B. rattus'*, '*B. muridarum'* and '*B. muris'* were suggested. *Lawsonia intracellularis* and encephalomyocarditis virus (EMCV) were detected in rodents trapped on pig farms. The clinical significance of leptospirosis in Sweden is reportedly minor. However, the detection of pathogenic leptospiras in mice, rats and a water vole indicated that rodents constitute a potential hazard to pigs and humans. Campylobacteriosis, salmonellosis and yersiniosis are the most frequently reported zoonosis in Europe. Rodents in the study carried *C. jejuni*, *C. coli* and *C. upsaliensis*. Identical isolates of the human pathogen *Yersinia enterocolitica* bioserotype 4/O:3 were isolated both from rodents and pigs on the same farm, indicating cross-species transmission. *Salmonella enterica* could not be detected by the applied real-time PCR, indicating a low sensitivity of this test. No zoonotic variants of *Giardia* spp. or *Cryptosporidium* spp. were detected. All samples were tested negative for *Trichinella* spp. indicating that trichinellosis is not a widespread infection in wild rodents in Sweden. No rodents were seropositive to *Toxoplasma gondii*.

In conclusion, the results show that rodents could be a risk for the transmission of the pig pathogens *Lawsonia intracellularis, Brachyspira hyodysenteriae, B. pilosicoli*, pathogenic *Leptospira* spp. and EMCV, and zoonotic *Campylobacter* species and *Yersinia enterocolitica* 4/O:3 in Sweden.

Keywords: rodent, pig, chicken, *Brachyspira* spp., *Yersinia enterocolitica*

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Dedication

To my family

”There are thing we know that we know. There are known unknowns. That is to say there are things that we now know we don't know. But there are also unknown unknowns. There are things we don't know we don't know. So when we do the best we can and we pull all this information together, and we then say well that's basically what we see as the situation, that is really only the known knowns and the known unknowns”

Donald H. Rumsfeld

Contents

List of Publications

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

I Backhans, A., Johansson, K.E., Fellström, C. (2010). Phenotypic and molecular characterization of *Brachyspira* spp. isolated from wild rodents. *Environmental Microbiology Reports* 2 (6), 720-727.

II Backhans, A., Fellström, C., Thisted Lambertz, S. Occurrence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in small wild rodents. *Epidemiology and Infection*, doi:10.1017/S0950268810002463 (In Press)

III Backhans, A., Jansson D.S., Aspàn A., Fellström, C. (2011). Typing of *Brachyspira* spp. from rodents, pigs and chickens on Swedish farms. *Veterinary Microbiology*, doi: 10.1016/j.vetmic.2011.03.023 (In Press)

IV Backhans, A, Jacobsson, M., Hansson, I., Lebbad, M., Thisted Lambertz, S., Gammelgård, E., Saager, M., Akande, O., Fellström, C. (2011). Presence of several pathogens in wild rodents caught on Swedish pig and chicken farms. (Manuscript)

Abbreviations

DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
HIS	Human intestinal spirochaetosis
IFAT	Indirect immunofluorescent antibody test
ILAT	Indirect latex agglutination test
MAT	Microscopic agglutination test
PCR	Polymerase chain reaction
PCS	Porcine colonic spirochaetosis
PFGE	Pulsed field gel electrophoresis
RAPD	Rapid amplified polymorphic DNA
rRNA	Ribosomal ribonucleic acid
SD	Swine dysentery
sp.	species (singularis)
sp.nov.	species novum (new species)
spp.	species (pluralis)
Superscript T	Type strain for a species
YE	*Yersinia enterocolitica*

1 Introduction

This thesis examines the occurrence of various pathogens in rodents caught on pig and chicken farms. First, a brief description is given of the three rodent species that frequently inhabit farms. The pathogens involved are also described, with special emphasis on *Brachyspira* spp. and *Yersinia enterocolitica*, to provide important background information for this study.

1.1 Commensal rodents

The order Rodentia (L. *rodere*, to gnaw) constitutes the most successful mammalian group in terms of the number of species and individuals (Hanney, 1975). The house mouse, *Mus (M.) musculus (m.),* originated from Asia, from where it has spread over the world as a commensal to humans (L. *cum mensa*, sharing a table), along with the development of agriculture which provided shelter and supplies of food. It is an underground dweller that weighs 12-30 grams, eats vegetables or any available food, and is active at any hour of the day. It manages well without water for a substantial time and can adapt to temperatures down to -10°C (Hanney, 1975). Its home range is less than 10 square metres and daily movement of an individual mouse is only a few square or cubic metres. They reproduce throughout the year under favourable conditions and a female can produce up to 10-14 litters, each containing 3-12 puppies per year (Nowak, 1999).

The brown rat, syn. Norwegian rat, (*Rattus norvegicus*), is believed to have originated from northern China and spread to the rest of the world by following humans as a commensal. In Europe it was recognised first in the eighteenth century. It is an underground dweller generally found on lower floors, basements and cellars of buildings (Hanney, 1975). Body weight is usually 200-400 grams. The brown rat lives in territorial colonies with population densities on farms of 50-300 individuals. It is omnivorous and

has a normal home range of 25-150 metres in diameter, but can move 3 km away and back in one night. Like the commensal house mouse, it can breed year-round (Nowak, 1999).

The yellow-necked mouse (*Apodemus flavicollis*) lives in cultivated areas and forests, but may move indoors during the cold season, if available. It is a good climber and jumper. Its home-range is 180 metres in diameter and it weighs 15-50 grams. The feed includes grains, seeds, berries and insects (Nowak, 1999).

1.2 Rodent control

Rodent control can be divided into three types of methods: preventioning, trapping, and poisoning. Prevention methods obstruct the establishment of rodent population by scaring them off (by use of a cat or other predator), building physical barriers, and keeping feed in rodent-proof containers (Hygnstrom *et al.*, 1994). Traps either kill the rodent, or capture it alive. Among the vast number of types of traps available, some are more inhumane than others, e.g. glue traps. Various poisons have been used, and today anticoagulant compounds are the most common. The brown rat is especially difficult to trap and poison due to its avoidance of unfamiliar objects and food (neophobia) (Brunton *et al.*, 1993; Mitchell, 1976). However, widespread use of poisons has led to the development of resistance to warfarin and diphacinone (Heiberg, 2009; Pelz *et al.*, 2005; Brunton *et al.*, 1993). Also, immuno-contraceptive vaccines have been developed for use in mice (Hardy *et al.*, 2006; Gao & Short, 1993).

1.3 Rodents as disease carriers

The rodent can cause large problems due to destruction and contamination of food, and also by the spread of various diseases. Several studies have focused on rodents as possible carriers of various pathogens. Some of these are summarized in Table 1.

Table 1. Examples of studies on rodents as carriers of pathogens.

Pathogen surveilled	Rodent species	Country	Location	Detection rate	Detection method	Reference
Campylobacter spp.	*R. norvegicus*	ns	organic farms	1/8	culture	(Meerburg *et al.*, 2006)
	M. musculus			8/83		
Cryptosporidium parvum	*R. norvegicus*	UK	farms	105/438	IFAT	(Quy *et al.*, 1999)
Cryptosporidium parvum	*R. norvegicus*	UK	rural	46/73	modified Ziehl-Nielsen	(Webster & MacDonald, 1995)
Erysipelothrix rhusiopathiae	*R. norvegicus*	Sweden	ns	17/257	culture	(Hülphers & Henricson, 1943)
Lawsonia intracellularis	Rats	Australia	pig farms	140/327	real-time PCR	(Collins *et al.*, 2011)
Lawsonia intracellularis	*R. norvegicus*	Czech Republic	pig farms	1/6	nested PCR	(Friedman *et al.*, 2008)
	M. musculus			91/213		
	A. agrarius			8/51		
	A. flavicollis			3/12		
	Microtus arvalis			3/9		
Pathogenic *Leptospira*	*R. norvegicus*	France	feral	4/26	PCR	(Aviat *et al.*, 2009)
	Myocaster coypu			14/428		
	Ondatra zibethicus			3/19		
Leptospira interrogans serovar Copenhageni	*R. norvegicus*	Brazil	urban	52/62	PCR	(Faria *et al.*, 2008)
Leptospira spp.	*R. norvegicus*	UK	rural	37/259	MAT, ELISA, cultivation	(Webster *et al.*, 1995)

Salmonella spp.	*R. norvegicus*	Sweden	ns	48/186	culture	(Hülphers & Henricson, 1943)
Salmonella Enteritidis	*R. rattus*	Japan	layer farms	113/851	culture	(Lapuz *et al.*, 2008)
Salmonella Infantis				158/851		
Salmonella Livingstone	*R. norvegicus*	ns	organic farms	0	culture	(Meerburg *et al.*, 2006)
	M. musculus			1/83		
Salmonella spp.	*M. musculus domesticus*	UK	mixed farms	0/341	culture	(Pocock *et al.*, 2001)
Salmonella Enteritidis	*M. musculus*	USA	poultry farms	168/713	culture	(Henzler & Opitz, 1992)
Trichinella spp.	*R. norvegicus*	Sweden	ns	Neg	microscopic examination	(Hülphers & Henricson, 1943)
Trichinella spp..	*R. norvegicus*	Finland	waste disposal sites	142/767	HCl-pepsin method	(Mikkonen *et al.*, 2005)
Trichinella spiralis	*R. norvegicus*	Croatia	pig farms	18/2287	ns	(Stojcevic *et al.*, 2004)
Toxoplasma gondii	*R. rattus*	Netherlands	organic farms	4/39	TaqMan PCR	(Kijlstra *et al.*, 2008)
	M. musculus			2/31		
	A. sylvaticus			1/7		
	Crocidura russula			3/22		
	Microtus arvalis			1/1		
	Clethrionomys glareolus			1/1		
Toxoplasma gondii	*M. musculus*	UK	households	117/200	nested PCR	(Murphy *et al.*, 2008)
				2/190	serology	
Toxoplasma gondii	*R. norvegicus*	Grenada	ns	2/238	serology (MAT)	(Dubey *et al.*, 2006)

Toxoplasma gondii	*R. norvegicus*	USA	pig farms	0/9	serology (MAT)	(Smith *et al.*, 1992)
	M. musculus			2/588		
Toxoplasma gondii	*R. norvegicus*	UK	rural		ILAT	(Webster, 1994)
					ELISA	
Yersinia spp. biotype 1A	*M. musculus*	UK	mixed farms	21/354	culture	(Pocock *et al.*, 2001)
Yersinia pseudotuberculosis				1/354		
Yersinia enterocolitica O8	small wild rodents	Japan	ns	6/223	culture	(Iinuma *et al.*, 1992)
Yersinia enterocolitica O3				1/223		
Yersinia pseudotuberculosis	*R. norvegicus*	Japan	barn	8/270	culture	(Kaneko *et al.*, 1979)
	R. rattus					
Yersinia enterocolitica O:3, *Yersinia pseudotuberculosis*	*R. rattus*	Czechoslovakia	pig houses	16/178	culture	(Aldova *et al.*, 1977)
	R. norvegicus					
Yersinia enterocolitica O:3	*R. rattus*	Czechoslovakia	pig houses	5/36	culture	(Pokorna & Aldova, 1977)
Yersinia enterocolitica biotypes 1, 2 serotypes 1, 3-7, 16	*Clethrionomys glareolus*	Scandinavia	nature	6/56	culture	(Kapperud, 1975)
	Clethrionomys rufocanus			3/53		
	Microtus oeconumus			3/10		
	M. agrestis			3/5		
Yersinia enterocolitica 4/O:3	*R. norvegicus*	Japan	slaughter-house, barn,zoo	2/270	culture	(Kaneko *et al.*, 1978)
	R. rattus					

R. = Rattus, M. = Mus, A. = Apodemus, ns = not specified

1.4 A selection of pathogens

1.4.1 *Brachyspira* spp.

The genus *Brachyspira* constitutes spirochaetal, *i.e.* helically coiled, oxygen-tolerant anaerobic bacteria that are found in the intestines of many species of mammals and birds. At present, the genus comprises seven valid species and six provisionally proposed species, which are listed in Table 2.

Table 2. *Valid and proposed* Brachyspira spp.*, their described host range, prior to the present studies, and pathogenicity*

Brachyspira sp.	Reference	Host range	Pathogenic to
Brachyspira hyodysenteriae	(Harris *et al.*, 1972)	pig, rhea, mallard, rat, mouse, laying hen	pig, rhea
Brachyspira pilosicoli	(Trott *et al.*, 1996b)	pig, dog, chicken, mouse, macaque, horse, humans	pig, dog, macaque
Brachyspira intermedia	(Stanton et al., 1997)	pig, chicken, mallard, dog	chicken
Brachyspira innocens	(Kinyon & Harris, 1979)	pig, chicken, dog	not verified
Brachyspira murdochii	(Stanton et al., 1997)	pig, rat	not verified
Brachyspira aalborgii	(Hovind Hougen *et al.*, 1982)	humans, macaque	not verified
Brachyspira alvinipulli	(Stanton *et al.*, 1998)	chicken	chicken
'Brachyspira canis'	(Duhamel *et al.*, 1998b)	dog	dog
'Brachyspira pulli'	(Stephens & Hampson, 1999)	chicken, dog	not verified
'Brachyspira christiani'	(Jensen *et al.*, 2001)	humans	not verified
'Brachyspira suanatina'	(Råsbäck *et al.*, 2007a)	pig, mallard	pig
'Brachyspira ibaraki'	(Tachibana *et al.*, 2002)	humans	not verified
'Brachyspira corvi'	(Jansson et al., 2008a)	corvids	unknown

Clinical relevance

Pigs

Brachyspira hyodysenteriae is the aetiological agent of swine dysentery (SD) (Harris *et al.*, 1972; Taylor & Alexander, 1971), a pig disease that causes mucohaemorrhagic diarrhoea. All age groups of pigs except for newborns can be affected. In outbreaks of SD, morbidity and mortality may reach 90 and 30% respectively, whereas in herds where SD is endemic clinical signs can sometimes be absent (Hampson *et al.*, 2006a). The increasing resistance to antimicrobials used to treat SD poses a threat to effective treatment and eradication of SD from pig herds (Ohya & Sueyoshi, 2010; Hidalgo *et al.*, 2009; Lobova *et al.*, 2004). Recently, a novel *Brachyspira* was isolated from pigs with SD-like symptoms. It has been given the proposed name '*B. suanatina*' due to its known hosts, *i.e.* pigs and mallards (Råsbäck *et al.*, 2007a).

Brachyspira pilosicoli causes a milder colitis referred to as porcine intestinal spirochaetosis (PIS) (Trott *et al.*, 1996b) or porcine colonic spirochaetosis (PCS) (Girard *et al.*, 1995). Weaners and growers are affected with watery diarrhoea or porridge-like faeces, sometimes with mucus, resulting in reduced growth rate. Not all individuals are affected and subclinical infections, which can result in reduced growth rate, are common (Hampson & Duhamel, 2006). Histologically, PCS is characterised by the formation of a 'false brush border', consisting of large amounts of spirochaetes attached by one end to the epithelium (Taylor *et al.*, 1980), however some studies have indicated that the colonisation of *B. pilosicoli* strains not always is associated with this end-on attachment (Thomson *et al.*, 1998; Thomson *et al.*, 1997).

Brachyspira innocens (Kinyon & Harris, 1979), *B. intermedia* and *B. murdochii* (Stanton et al., 1997) are common in pigs and are generally regarded as commensals. However, some strains have been described as mildly pathogenic to pigs (Jensen *et al.*, 2010; Komarek *et al.*, 2009; Weissenböck *et al.*, 2005; Neef *et al.*, 1994).

Brachyspira species are common in pig herds. In a survey in Sweden, 80% of piglet-producing herds tested positive for *Brachyspira* spp. Herd prevalence was 32% for *B. pilosicoli*, 14% for *B. intermedia*, 78% for *B. innocens/murdochii*, and all tested herds were negative for *B. hyodysenteriae* (Jacobson *et al.*, 2005). The frequency of *B. hyodysenteriae* in Swedish fattening herds is unknown, but it has become a rare disease (Råsbäck *et al.*, 2009). A similar situation exists in Finland (Heinonen *et al.*, 2000), whereas in southern Europe there is a higher prevalence of *B. hyodysenteriae* and a lower prevalence of *B. pilosicoli* (Carvajal *et al.*, 2006; Merialdi *et al.*, 2003).

Birds

In chicken, *Brachyspira* spp. colonisation is sometimes referred to as avian intestinal spirochaetosis (AIS). *Brachyspira alvinipulli* (Stanton *et al.*, 1998; Swayne *et al.*, 1995), *B. pilosicoli* (Stephens & Hampson, 2002) and *B. intermedia* (Hampson & McLaren, 1999; Stanton *et al.*, 1997) are associated with egg production losses and signs of disease, whereas *B. innocens*, *B. murdochii* and '*B. pulli*' are presumed to be apathogenic (Jansson *et al.*, 2008b; Stephens *et al.*, 2005). The importance of *B. hyodysenteriae* is unclear (Feberwee *et al.*, 2008). A study of 92 Swedish laying hen farms showed a farm prevalence of *Brachyspira* spp. of 27-35% on conventional farms, depending on housing system, and 71% on organic farms. *Brachyspira intermedia*, *B. alvinipulli*, '*B. pulli*', *B. innocens*, *B. murdochii* and isolates of unknown species affiliation were identified (Jansson *et al.*, 2008b).

Concerning other birds, *Brachyspira hyodysenteriae* has been reported as a cause of necrotising typhlocolitis in reas (*Rhea americana*) and ducks whereas it seems to be apathogenic in mallards (*Anas platyrhynchos*) (Glávits *et al.*, 2011; Jansson *et al.*, 2004; Buckles *et al.*, 1997). Mallards can also harbour '*B. suanatina*'. "*Brachyspira corvi*" has been described as a commensal *Brachyspira* sp. in corvids (Jansson *et al.*, 2008a) and an unknown *Brachyspira* was recently isolated from an Antarctic snowy sheathbill (Jansson *et al.*, 2009).

Other animals and humans

In dogs, intestinal spirochaetes are common findings. '*Brachyspira canis'* sp. nov. (Duhamel *et al.*, 1998b) is thought of as a commensal, whereas some studies indicate that *B. pilosicoli* is a pathogen (Hidalgo *et al.*, 2010b; Oxberry & Hampson, 2003; Fellström *et al.*, 2001; Duhamel *et al.*, 1998b). In horses spirochaetes have been associated with chronic diarrhoea and *B. pilosicoli* and *B. innocens* have been isolated from weanlings (Hampson *et al.*, 2006b; Shibahara *et al.*, 2005; Shibahara *et al.*, 2002). In macaques *B. pilosicoli* causes colitis very similar to ulcerative colitis in humans (Duhamel *et al.*, 1997).

Human intestinal spirochaetosis (HIS) is usually associated with any of the two species *B. aalborgii* and *B. pilosicoli* (Tompkins *et al.*, 1986; Hovind Hougen *et al.*, 1982). *Brachyspira aalborgii* has only been isolated from humans and non-human primates (Duhamel *et al.*, 2003; Duhamel *et al.*, 1997). In Western countries the prevalence of in healthy populations varies between 5.6-7.9%, and is the dominating cause of HIS (Brooke *et al.*, 2006), whereas *Brachyspira pilosicoli* is more common in developing countries

(Margawani *et al.*, 2004; Trott *et al.*, 1997). The clinical relevance of HIS is uncertain (Sato *et al.*, 2010; Gad *et al.*, 1977; Lee *et al.*, 1971).

Epidemiology

Transmission of *Brachyspira* spp. occurs via the faecal-oral route from infected animals. Asymptomatic carriers can secrete *B. hyodysenteriae* for several months after recovering from the disease. The bacteria can survive in soil and faeces for up to two months, which makes the environment a possible reservoir (Boye *et al.*, 2001). Humans can spread bacteria via equipment, and other animals such as dogs, wild birds and rodents can act as carriers (Råsbäck *et al.*, 2007b; Fellström *et al.*, 2004; Trott *et al.*, 1996a; Koopman *et al.*, 1993). There seems to be no association between *B. pilosicoli* colonisation in humans and pig contact (Jacobson *et al.*, 2007; Trott *et al.*, 1998; Trott *et al.*, 1997). However, there are indications of cross-species transmission of *B. pilosicoli* between dog and man (Trott *et al.*, 1998; Lee & Hampson, 1994; Koopman *et al.*, 1993).

Brachyspira *spp. in rodents*

Intestinal spiral-shaped bacteria have been observed microscopically in both laboratory and wild-caught rodents of which some showed the morphological characteristics of *Brachyspira* spp. (Lee & Phillips, 1978; Davis *et al.*, 1973; Savage *et al.*, 1971). Isolates designated as *B. hyodysenteriae* have been detected in both wild and laboratory rodents (Blaha, 1983; Joens & Kinyon, 1982). Experimentally, *B. hyodysenteriae* has been shown to effectively spread between laboratory mice and pigs (Joens, 1980). Furthermore spirochaetes isolated from wild and laboratory rats produced clinical signs of swine dysentery, after three passages, in SPF-pigs (Blaha, 1983). However, the species designation of these isolates is uncertain. Later on, isolates of *B. hyodysenteriae* of porcine genotypes were isolated from rats and mice caught in pig herds (Fellström *et al.*, 2004; Trott *et al.*, 1996a). *Brachyspira pilosicoli* has also been isolated from wild mice caught in pig herds (Fellström *et al.*, 2004). It is unknown whether *B. hyodysenteriae* and *B. pilosicoli* are actually pathogenic, or even infective, to wild rodents, or whether the rodents are just accidental hosts.

Laboratory mice are useful in animal models of swine dysentery, and develop lesions similar to those described in the pig (Hutto *et al.*, 1998; Nibbelink & Wannemuehler, 1991; Joens *et al.*, 1981; Joens & Glock, 1979). Mice have also been used as a model for intestinal spirochetosis (Jamshidian *et al.*, 2004; Sacco *et al.*, 1997).

1.4.2 *Lawsonia intracellularis*

The intracellular bacterium *Lawsonia intracellularis* (McOrist *et al.*, 1995) is the cause of porcine proliferative enteropathy (PPE), common in weaning pigs (Jacobson *et al.*, 2003b; McOrist *et al.*, 1993). The clinical appearance is similar to that of intestinal spirochaetosis, with diarrhoea and retarded growth, but the pathological changes are located to the ileum rather than the colon. The infection can be subclinical, and there is also an acute form, proliferative haemorrhagic enteropathy (PHE), which causes sudden death and intestinal haemorrhage (McOrist & Gebhart, 2006). In Sweden, 48% of piglet-producing herds were infected with *Lawsonia intracellularis* (Jacobson *et al.*, 2005). In other European countries, reported herd prevalence varies between 15 and 93.7% (Stege *et al.*, 2000; Thomson *et al.*, 1998). *Lawsonia intracellularis* has been detected in a number of animal species other than the pig, *i.e.* hamster, deer, ostrich, ferret, horse and rabbit (Duhamel *et al.*, 1998a; Frank *et al.*, 1998; Cooper *et al.*, 1997; Drolet *et al.*, 1996). Rodents have been implicated as possible reservoirs for the bacteria (Friedman *et al.*, 2008), and recently a study showed that infected rats shed large numbers of bacteria in their faeces for up to three weeks (Collins & Love, 2007).

1.4.3 *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

Yersiniosis in humans is caused by pathogenic bioserotypes of *Y. enterocolitica* (YE) and *Y. pseudotuberculosis*.

The species *Yersinia enterocolitica* can be further subdivided into subspecies *Y. enterocolitica* subsp. *palearctica* and *enterocolitica*, which comprise the European and American pathogenic bioserotypes, (Neubauer *et al.*, 2000), and possibly a third subspecies (Howard *et al.*, 2006). Biochemical reactions divide strains in biotypes 1A, 1B and 2-6 (Wauters *et al.*, 1987). Biotype 1A comprises mostly non-pathogenic strains, 1B highly pathogenic strains, and types 2-6 weakly pathogenic strains.

Virulence is associated with a 68 kb virulence plasmid (*p*YV, plasmid for *Yersinia* virulence) (Gemski *et al.*, 1980), on which virulence genes such as the yersinia adhesin gene (*yad*A) and the *vir*F gene, are located. Chromosomal genes involved in virulence include the attachment and invasion locus (*ail*), the invasion gene (*inv*) and the *Yersinia* heat stable enterotoxin gene (*yst*) (Delor *et al.*, 1990; Miller *et al.*, 1989; Miller & Falkow, 1988).

Yersinia pseudotuberculosis can be divided into four biotypes (Tsubokura & Aleksić, 1995) and several serotypes of which O:1 and O:3 have been reported in outbreaks of human disease in Finland (Jalava *et al.*, 2006; Jalava *et al.*, 2004; Nuorti *et al.*, 2004) and O:1 in France (Vincent *et al.*, 2008).

Yersinia pseudotuberculosis strains are generally pathogenic, and virulence is associated with the virulence plasmid pYV.

Zoonotic relevance

Yersiniosis is the third most frequently reported zoonosis in Europe (Anon, 2009a). In Sweden, 281 cases of yersiniosis were reported in 2010, of which 78% were domestic (Smittskyddsinstitutet, 2011). The majority of cases involved children under the age of four. There has been a decrease in number of cases since 2004, for unknown reasons (Anon, 2009b). Besides gastrointestinal illness of varying severity, immune-mediated secondary complications such as arthritis, glomerulonephritis, myocarditis, erythema nodosum and Reiter's syndrome are reported (Bottone, 1997). The majority of human cases are caused by 4/O:3 (Fredriksson-Ahomaa *et al.*, 1999). *Yersinia pseudotuberculosis* is not nearly as common a cause of yersiniosis, but occasional outbreaks occur, especially in the northern hemisphere (Rimhanen-Finne et al., 2008; Jalava et al., 2006; Nuorti et al., 2004; Press et al., 2001; Tertti et al., 1984).

Yersinia *spp. in pigs*

The reservoir of human pathogenic *Yersinia enterocolitica* is the domestic pig (Hurvell *et al.*, 1979; Wauters, 1979), from which strains identical to strains from human cases have been isolated (Fredriksson-Ahomaa *et al.*, 2006; Fredriksson-Ahomaa *et al.*, 2001). Eating pork and pork products are risk factors for acquiring YE infection (Boqvist *et al.*, 2009; Ostroff *et al.*, 1994; Tauxe *et al.*, 1987). Bioserotype 4/O:3 dominates in pigs in European countries (Terentjeva & Bērziņs, 2010; Fredriksson-Ahomaa et al., 2007) except in England where 2/O:9 and 2/O:5 are more common. The number of reported cases of human yersiniosis are also fewer in England than in other European countries (Ortiz Martínez *et al.*, 2010). The prevalence in European pigs at slaughter varies between 34 and 70% (Terentjeva & Bērziņs, 2010; Bucher *et al.*, 2008; Fredriksson-Ahomaa *et al.*, 2007). In some studies, there was a higher prevalence in fattening farms than integrated systems (Skjerve *et al.*, 1998), while in others there were no differences (Virtanen *et al.*, 2011). In Sweden, 16% of pigs test positive for pathogenic *Y. enterocolitica* at slaughter (Lindblad *et al.*, 2007). In a Finnish study, 4% of fattening pigs harboured *Y. pseudotuberculosis* bioserotype 2/O:3 (Niskanen *et al.*, 2002), and in England, 18% were positive (Ortiz Martínez *et al.*, 2010).

Epidemiology

The epidemiology of YE on farm level is not fully understood. Piglets less than eight weeks old are generally not colonised by YE, but the prevalence increases with age (Bowman *et al.*, 2007; Gurtler *et al.*, 2005; Fukushima *et al.*, 1983). Infected pigs excrete bacteria in feces 30-70 days after inoculation, and at the time of slaughter bacteria can often be detected in the tonsils (Nielsen *et al.*, 1996; Nesbakken, 1988). In one study gestating sows were shown to have a high prevalence of YE, but at farrowing, there was no detection of bacteria (Bowman *et al.*, 2007). The suggested source of infection is other growing pigs instead of the sow, via infected faeces and pen floors (Bowman *et al.*, 2007; Fukushima *et al.*, 1983). On positive farms, YE can be found also in the environment, *e.g.* in passages, on shovels, pipings etc (Pilon *et al.*, 2000). On contaminated floors, YE can survive for at least three weeks (Fukushima *et al.*, 1983).

Yersinia pseudotuberculosis appears to circulate between animals and the environment in wild birds (Niskanen *et al.*, 2003; Fukushima & Gomyoda, 1991; Hamasaki *et al.*, 1989), various free-living mammals such as deer, hare, marten and racoon dog (Fukushima & Gomyoda, 1991) and water (Fukushima *et al.*, 1988). In Finland, recent outbreaks of *Y. pseudotuberculosis* were traced to carrots and iceberg lettuce (Rimhanen-Finne *et al.*, 2008; Jalava *et al.*, 2006; Nuorti *et al.*, 2004). *Yersinia pseudotuberculosis* have also been isolated from pigs in Finland, Latvia, Japan and England (Ortiz Martínez *et al.*, 2010; Terentjeva & Bērziņs, 2010; Laukkanen *et al.*, 2008; Niskanen *et al.*, 2008; Smith *et al.*, 2006; Niskanen *et al.*, 2002; Shiozawa *et al.*, 1988) and from wild boars in Switzerland and Japan (Fredriksson-Ahomaa *et al.*, 2009; Hayashidani *et al.*, 2002).

Yersinia enterocolitica and Y. pseudotuberculosis in rodents

Kapperud 1975 found *Y. enterocolitica* in about 8% of wild rodents in Scandinavia, however of no human pathogenic biotypes (Kapperud, 1975). Later, serotype O:3 was isolated from black rats (*Rattus rattus*) in pig houses (Aldova *et al.*, 1977; Pokorna & Aldova, 1977), and 4/O:3 from brown rats caught in a slaughterhouse (Kaneko *et al.*, 1978). House mice on farms are colonised mainly by YE serogroup 1A (Pocock *et al.*, 2001). In Japan, *Y. pseudotuberculosis* has been found in mice and moles and in barn rats (Fukushima *et al.*, 1990; Kaneko *et al.*, 1979).

1.4.4 *Campylobacter* spp. and *Salmonella enterica*

Campylobacter spp. is the most commonly reported zoonotic disease in humans in the EU. In Sweden, 7,000 cases are reported yearly, of which

the majority are domestic cases (Smittskyddsinstitutet, 2011). Poultry meat is the main source, together with meat from other sources (Anon, 2010). Occurrence of rodents is a risk factor for high *Campylobacter* prevalence in broiler chicken flocks (Berndtson *et al.*, 1996; Kapperud *et al.*, 1993).

Salmonella is rare in chicken and pigs in Sweden. During 2009 it was only detected as new infections in four broiler flocks, three laying hen flocks and three pig herds (Anon, 2009b). Still, 4,000 human cases of salmonellosis are reported each year however 85% of those were infected abroad (www.smittskyddsinstitutet.se).

1.4.5 *Leptospira* spp.

Leptospirosis in humans can in severe cases lead to icteric leptospirosis with renal failure (Levett, 2001). *L. interrogans* causes most human infections (Anon, 2010). Animals of different species, including rodents, act as maintenance hosts for different serovars (Ellis, 2006). In Sweden, four cases of human leptospirosis were reported in 2010, none of them domestic (Smittskyddsinstitutet, 2011). Leptospirosis in pigs has been associated with reproductive failure (Bolin *et al.*, 1991) but the clinical relevance of *Leptospira* serovar Bratislava present in Swedish pigs remains unclear (Swedberg & Eliasson-Selling, 2006; Sandstedt & Engvall, 1985).

1.4.6 Encephalomyocarditis virus

Encephalomyocarditis virus (EMCV) is a Cardiovirus of the Picornaviridae that in growing pigs causes acute myocarditis and sudden deaths (Billinis *et al.*, 2004; Koenen *et al.*, 1999; Murnane *et al.*, 1960). In sows, it causes reproductive problems with abortions and dead and weak piglets (Koenen *et al.*, 1994; Dea *et al.*, 1991). Outbreaks occur mainly in clusters in certain areas, which in Europe have been located in Belgium, Italy, Greece and Cyprus (Maurice *et al.*, 2005). In Sweden, no clinical outbreaks have been reported, but in one study 16.8% of slaughter pigs were seropositive (Widén, 1994). The epidemiology is inconclusive, but wild rodents are considered a natural reservoir for EMCV (Spyrou *et al.*, 2004a), from which the virus is shed in faeces (Psalla *et al.*, 2006b; Psalla *et al.*, 2006a; Spyrou *et al.*, 2004b). In a few cases, EMCV has been suspected of causing disease in humans (Oberste *et al.*, 2009; Tesh, 1978) and seroprevalence was found to be high in veterinarians, farmers, abattoir workers and especially hunters (Deutz *et al.*, 2003).

Giardia and *Cryptosporidium* spp.

Giardiosis and Cryptosporidiosis are common gastrointestinal infections in humans with worldwide distribution. Outbreaks can often be traced to water, or to food, or contact infection. The infectious dose is small for both (Plutzer *et al.*, 2010; Smith *et al.*, 2006; Cacciò *et al.*, 2005). *Giardia lamblia* (syn. *intestinalis, duodenalis*) is an intestinal flagellate that can be divided into six assemblages A-G, of which assemblages A and B are zoonotic genotypes. C and D are dog genotypes, and E are livestock, F cat and G rat genotypes (Thompson, 2004). *Giardia* is commonly found in pigs but has no association to disease (Xiao *et al.*, 1994).

Two of the currently 19 known species (Fayer, 2010) of *Cryptosporidium*, *C. hominis* and *C. parvum,* can cause diarrhea in humans. *C. hominis* is restricted to humans whereas *C. parvum* is zoonotic. In Sweden a large outbreak of cryptosporidiosis in 2010 caused an increase in the number of cases to 392 (159 cases in 2009), of which 69% were domestic. The cause was identified as being *C. hominis* (Smittskyddsinstitutet, 2011). In piglets, *Cryptosporidium suis* may be associated with diarrhoea (Hamnes *et al.*, 2007).

Toxoplasma gondii and *Trichinella* spp.

Toxoplasma gondii is a coccidium that infects all warm-blooded animals (Tenter *et al.*, 2000). Congenital toxoplasmosis causes CNS and ocular disease in the foetus when the mother becomes infected during pregnancy (Jones *et al.*, 2003). In Sweden, antibodies against *T. gondii* have been found in moose, roe deer, free-ranging Eurasian lynx and pigs (Malmsten *et al.*, 2010; Ryser-Degiorgis *et al.*, 2006; Lundén *et al.*, 2002). However, congenital toxoplasmosis in humans is rare (Evengård *et al.*, 2001). Seroprevalence of *T. gondii* antibodies is considerably higher in pigs in outdoor systems than in conventional indoor systems (van der Giessen *et al.*, 2007; Kijlstra *et al.*, 2004). Rodent are often infected and may play a role in the transmission of *T. gondii* (Kijlstra *et al.*, 2008; Murphy *et al.*, 2008; Hughes *et al.*, 2006; Marshall *et al.*, 2004; Dubey & Frenkel, 1998).

Nematodes of *Trichinella* spp. are all pathogenic to humans. *T. spiralis,* which is adapted to domestic and wild pigs, is the most common cause of human infection (Gottstein *et al.*, 2009). In Sweden, the last case of trichinosis in pigs was recorded in 1994. In 2009 the prevalence in wild boars was only 0.004%, whereas a larger proportion of wolves, lynx and wolverines were infected, primarily by *T. nativa* (Anon, 2009b). In Europe, *T. britova* has become more widespread in sylvatic carnivores, whereas *T.*

spiralis dominates in domestic pigs and wild boars (Pozio *et al.*, 2009). Romania is an example of a European country where trichinellosis has become a serious health problem in later years (Neghina *et al.*, 2010).

2 Aims

The general aim of this thesis was to investigate the specific risks that rodents constitute for proliferation of pathogens and transmission of those, primarily to pig herds and chicken flocks, and indirectly to humans. The specific objectives of the project were:

- To describe the species of small rodents caught on pig and chicken farms (Paper IV). The hypothesis was that house mouse (*Mus musculus*), wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*A. flavicollis*), bank vole (*Myodes glareolus*) and brown rat (*Rattus norvegicus*) are common in and around farms.

- To look for a selection of potential pathogens in rodents, with the hypothesis that those are prevalent in rodents (Paper IV).

- To study the biodiversity of murine *Brachyspira* spp. (Paper I).

- To compare *Brachyspira* spp. isolated from wild rodents with isolates from pigs and chicken, with the hypothesis that cross-species transmission occurs between rodents and pigs and chickens (Paper III).

- To study the occurrence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild rodents and to compare the isolates obtained with isolates from pigs, with the hypothesis that rodents are reservoirs for these zoonotic agents (Paper II).

3 Considerations on Materials and Methods

3.1 Locations and capture of rodents

Most of the trapping locations were situated in the Mälardalen region, within 2-3 hours drive from Uppsala. In addition, four pig farms; satellite units and the central unit of a large sow pool located in Småland and Halland, were included for a 24 h trapping effort with >100 traps at each location. The goal was to capture 10 rodents in each of 20 selected pig and laying hen farms and 50 rodents at sewage treatment plants. However, that goal could not be achieved due to unforeseen difficulties. Judging from faecal droppings and burrows, rodents were common inhabitants on all the farms visited. However, traps could not always be placed at the most strategic points in order to avoid disturbing daily work *etc.* In addition, active pest control was applied on all farms visited, usually managed by a control company that regularly replenished bait stations with rat poison. It should be mentioned however, that despite intense rodent control, three of the pig farms experienced problems with large amount of rodents. Finally, the conditions differed regarding *e.g.* free access to the farm for trapping, tidiness on the farm and the presence of cats, all of which probably affected the trapping result. On the sewage treatment plants, there were considerably fewer signs of rodents than on the farms.

Both live and snap traps were used for trapping. The sizes of traps used depended on the rodent species expected, based on the faecal droppings found at the locations. Different kinds of droppings were seldom observed at the same location, indicating that only one rodent species was present. Trapping with live traps was very time-consuming, especially for the trapping of brown rats. Eventually, 5-10 snap traps of appropriate sizes

attached to ~1 m long wooden boards were found to be the most effective trapping method, especially for mice. The boards could easily be moved around when needed. Nevertheless, it often took several weeks to trap around 10 rodents at one location and on eight of the 16 pig farms, one of the two sewage plants and on the waste disposal site, no rodents were trapped (Table 3).

Table 3. *Description of locations visited for trapping, number of rodents captured and comments regarding trapping conditions.*

No	Location description	Rodents captured	Comments
1	Combined pig herd, pasture	12	Rodents caught near feeding place and local slaughter house at the farm
2	Combined pig herd	12	Rodents caught in feed storage room
3	Combined pig herd	0	Medium amount of droppings
4	Combined pig herd	0	Large amount of droppings, cats present
5	Combined pig herd	0	Medium amount of droppings
6	Combined pig herd	18	Rats trapped in manure culvert, mice in feed storage room
7	Combined pig herd	23	Large amount of droppings
8	Combined pig herd	0	Medium amount of droppings, cats present
9	Fattening herd	21	Large population in feed storage room
10	Fattening herd	0	Medium amount of droppings
11	Fattening herd	11+14[1]	Large amounts of droppings, repeated rat infestations
12	Fattening herd	1	
13	Satellite unit	1	Medium amount of droppings, cats present
14	Satellite, central units	0	Sparse amount of droppings
15	Satellite unit	4	Small farm
16	Satellite	0	Small amount of droppings
17	Pullet-rearing herd	17+15[1]	Mice caught in food storage room and corridor
18	Laying hens, free range, pasture	6	Large amounts of droppings on egg band and storage rooms, mice trapped in storage room
19	Laying hens, conventional	12	Medium-large amounts of droppings, mice trapped in feed storage rooms
20	Laying hens, free range	2	Mice captured in storage room
21	Laying hens	7	Traps set for several weeks
22	STP	7	Reported presence of rodents
23	STP	0	Difficult to place traps
24	Waste disposal site	0	Difficult to place traps
25	Mill	7	Traps set for several weeks
26	Pond	8	Rats were donated by the municipal game warden
27	Veterinary clinic	7	Medium amounts of droppings
28	Supermarket	2	Pest control company did the trapping

STP=Sewage treatment plant, droppings=faecal droppings, [1]Rodents were trapped during two different years; the numbers refers to the rodents captured each year.

3.2 Rodent identification and sample collection

Species identification of the rodents was performed with an identification key based on phenotypic characteristics including weight and length (body, tail and hind-paw) (Siivonen, 1968). A detailed description of how samples were collected from the rodents can be found in Paper **IV** and a schematic figure of the procedure is outlined below (Fig. 1). The pathogens, the tissues used for analyses and the methods used for detection are listed in Table 4.

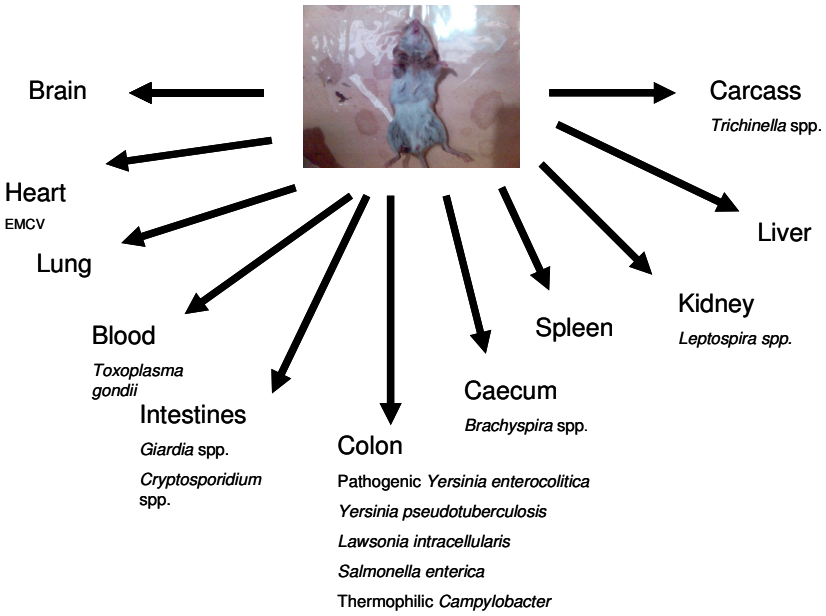

Figure 1. A tissue bank was built up from the rodents: brain, heart, lung, spleen, liver, kidney and parts of the intestines were cut into two pieces of which one part was frozen at -80°C and the other was fixed in formalin. Some of the tissues were used for the detection of pathogens described in this thesis.

Table 4. *Pathogens, target tissue for detection, methods used for detection and further characterisation.*

Pathogen	Tissue	Detection method	Further characterisation
Brachyspira spp.	Caecal sample	Culture (Fellström *et al.*, 1999; Hovind Hougen *et al.*, 1982)	Phenotypic characterisation, species-specific PCRs, RAPD, PFGE
Campylobacter spp.	Colon sample	mCCDA, CAT, 48 ± 2 h, 41.5 ± 1°C ISO 10272-1:2006 (Anon, 2006),	Multiplex PCRs
Cryptosporidium spp.	Intestines	Fluorescein-labeled direct immunofluorescence kit, Aqua-Glo™G/C	Sequencing the ssuRNA gene (Xiao *et al.*, 2000)
EMCV	Heart	Reverse transcriptase PCR targeting the 5´-UTR region (Denis *et al.*, 2006)	Sequencing of PCR products
Giardia spp.	Intestines	Fluorescein-labeled direct immunofluorescence kit, Aqua-Glo™G/C	Sequencing the ssrRNA locus (Lebbad *et al.*, 2010)
Lawsonia intracellularis	Colon	PCR (Jones *et al.*, 1993)	Sequencing of PCR products
Leptospira spp.	Kidney	PCR targeting the *hap*1 gene (Branger *et al.*, 2005)	Sequencing of PCR products
Salmonella spp.	Colon	Real-time PCR targeting the chromosomal *inv* gene (Hoorfar *et al.*, 2000)	n.a
Trichinella spiralis	Whole carcasses from mice, approximately 25 g of muscle tissue from rats	Magnetic stirrer digestion method EC 2075/2005.	n.a
Toxoplasma gondii	Serum	Direct agglutination test Toxo-Screen DA (BioMerieux, France)	n.a
Yersinia spp.	Colon	Culture (Schiemann, 1979), Real-time PCR	Bioserotyping, RAPD, PFGE

n.a= not applicable

3.3 Zoonotic pathogens in pigs and chicken

Food derived from farm animals, or the animal itself can be the source of infections to humans; sometimes the causing agent can cause disease also in the animal, but usually they are unaffected. To lower the prevalence in farm animals and reduce the number of human cases, specific knowledge of the

epidemiology of these agents on farms is needed. All pathogens in Table 5 have previously been detected in rodents, however most of these studies were carried out at different types of locations, and sometimes characterization was insufficient. The occurrence in Swedish rodents was unknown prior to these studies.

Table 5. *Zoonotic agents in study **II** and **IV**, the main source of infection to humans, ability to cause disease in pigs, and previous reports of occurrence in rodents.*

Zoonotic agent	Main source	Occurs in pigs	Disease in pigs	Occurs in rodents	References
Campylobacter spp.	Poultry meat	Yes	No	Yes	(Anon, 2010; Meerburg *et al.*, 2006; Boes *et al.*, 2005; Jacobson *et al.*, 2003b; Nesbakken *et al.*, 2003)
Salmonella enterica	Egg, poultry meat	Yes	Diarrhoea, to systemic	Yes	(Anon, 2010; Griffith *et al.*, 2006)
Yersinia enterocolitica 4/O:3	Pork products	Yes	Disputable	Yes	(Schiemann, 1988; Tauxe *et al.*, 1987; Hurvell *et al.*, 1979; Wauters, 1979; Kaneko *et al.*, 1978)
Yersinia pseudotuberculosis	Carrots, iceberg lettuce	Yes	Disputable	Yes	(Hallanvuo, 2009; Laukkanen *et al.*, 2008; Rimhanen-Finne *et al.*, 2008; Neef & Lysons, 1994; Kaneko *et al.*, 1979)
Giardia	Water	Yes	No	Yes	(Langkjaer *et al.*, 2007; Zintl *et al.*, 2007; Xiao *et al.*, 2006; Thompson, 2004)
Cryptosporidium	Water	Yes	Diarrhoea in piglets	Yes	(Hamnes *et al.*, 2007; Zintl *et al.*, 2007; Xiao *et al.*, 1994)
Leptospira spp.	Varies	Yes	Reproductive disorder	Yes	(Ellis, 2006)
Toxoplasma gondii	Under-cooked meat, cat feces, soil	Yes	Reproductive disorder	Yes	(Dubey, 2009; Birgisdóttir *et al.*, 2006; Dubey & Frenkel, 1998; Kapperud *et al.*, 1996)
Trichinella spp.	Under-cooked meat	Yes	No	Yes	(Gottstein *et al.*, 2009)

Campylobacter spp., *Salmonella enteritidis* and *Yersinia enterocolitica* 4/O:3 are major foodborne pathogens within EU (Anon, 2010) and also in Sweden. *Giardia* spp., *Cryptosporidium* spp. are not as common but human cases occur

regularly. Figure 2 shows the number of reported human domestic cases of the above mentioned pathogens in Sweden 2010.

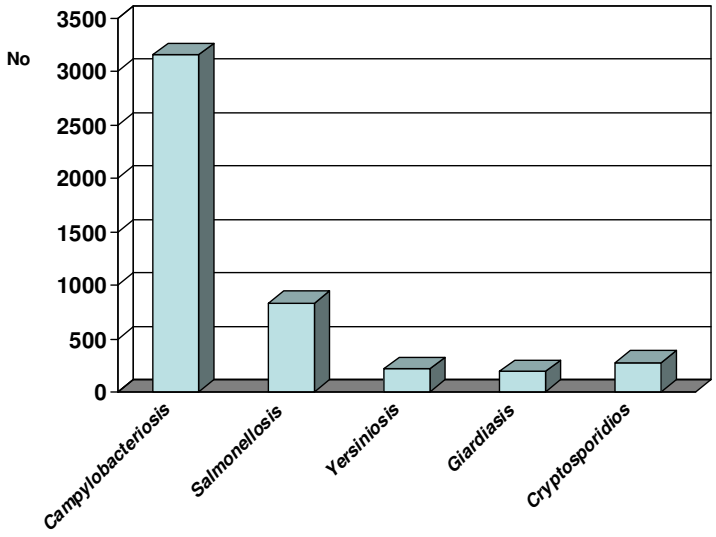

Figure 2. The number of reported domestic cases in 2010 of zoonosis included in this study. Figure based on statistics from the Swedish Institute for Communicable Disease Control at www.smittskyddsinstitutet.se.

Leptospira spp., *Toxoplasma gondii* and *Trichinella* spp. can cause serious zoonotic infections, although are not as important in terms of reported cases, at least not in Sweden. However, a reservoir in the wild fauna could constitute a risk for the transmission to pigs, which make the rodent interesting as a carrier between the wild fauna and farm animals.

3.4 Detection of bacteria

Detailed descriptions of the methods used for cultivation and characterisation of *Brachyspira* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Campylobacter* spp. and the detection of *Lawsonia intracellularis*, *Salmonella enterica* and *Leptospira* spp. by PCR are given in Papers **I-IV**, and are summarised with references in Table 4. Here the methods are described more generally and specific problems are discussed.

3.4.1 *Brachyspira* spp.

Cultivation

In Paper **I** two kinds of selective agar plates were used that differed in the type and concentration of antibiotics: Medium I (Fellström *et al.*, 1995) is generally used in routine diagnostics of *Brachyspira* spp. at the National Veterinary Institute while Medium II was developed for the isolation of *Brachyspira aalborgi* (Hovind Hougen *et al.*, 1982). Samples were cultivated on both of these media and incubated at 42 and 37 °C respectively. The reason for using two kinds of media was to enable growth of any possible known *Brachyspira* sp. *Brachyspira aalborgii* is very slow growing, and with that in mind, the samples plated on Medium II were incubated for up to four weeks although they were read at least once a week.

Isolate characterisation

The isolates were characterised with biochemical classification, by a set of tests: degree of haemolysis, spot-indole, hippurate, alpha-galactosidase and beta-glucosidase. This classification divides isolates into groups I-IV, corresponding to: I *Brachyspira hyodysenteriae*, II *B. intermedia*, IIIa *B. murdochii*, IIIbc *B. innocens*, IV *B. pilosicoli* (Fellström *et al.*, 1999). The classification was developed for porcine strains for which this species designation correlates well with PCR analysis and 16S rRNA gene sequencing. The system has also proven useful for the identification of isolates from laying hens as a tool of preliminary characterisation (Jansson *et al.*, 2008b). However, the classification only includes certain phenotypes and a number of isolates from non-porcine animal species cannot be classified in any of groups I-IV.

In Paper **I**, instead of using the group I-IV classification, isolates were given a biochemical profile based on the biochemical tests, but instead of +/- they were given the numbers 0-2 (Table 6). The biochemical profile obtained can be applied on all *Brachyspira* isolates and the use of numbers might be easier to handle in databases. Other tests can be added to the profile when needed.

Table 6. Alternative classification of Brachyspira *spp. with numbers instead of +/-*

Haemolysis	Indole production	Hippurate Hydrolysis	α-galactosidase activity	β-glucosidase activity
s strong	0 negative	0 negative	0 negative	0 negative
w weak	1 weakly positive	1 weakly positive	1 weakly positive	1 weakly positive
n none	2 strongly positive	2 strongly positive	2 strongly positive	2 strongly positive

Rodent isolates were also characterised by various species-specific PCRs (Table 7). DNA was extracted from bacteria by boiling for 10 min and then collected in the supernatant after centrifugation at 6,000 rpm for 4 min. Dilutions of 1/10 were always used for PCR and later for RAPD. The following type strains were used as positive controls added to each run: B78^T (*B. hyodysenteriae*), PWS/A^T (*B. intermedia*), B256^T (*B. innocens*) and P43/6/78^T (*B. pilosicoli*). SuperQ water was used as a negative control.

Table 7. PCR systems used in study I for the designation of Brachyspira *spp.*

Species	PCR target	Primers	Reference
B. hyodysenteriae	23S rRNA gene	F: 5´-ggcggtaagtgatgtacttgca-3´ R: 5´-cttctttaaggttgaggctgg-3´	(Leser *et al.*, 1997)
B. hyodysenteriae	tlyA gene	F: 5´-gcagatctaaagcacaggat-3´ R: 5´-gccttttgaaacatcacctc-3´	(Råsbäck *et al.*, 2006)
B. pilosicoli	16S rRNA	F: 5´-cataagtagagtagaggaaagttttt-3´ R: 5´-ctcgacattactcggtagcaacag-3´	(Råsbäck *et al.*, 2006)
B. intermedia	23S rRNA gene	F: 5´-ggcggtaagtgatgtacttaca-3´ R: 5´-t.gctttaaggttgaggctgg-3´	(Leser *et al.*, 1997)
B. intermedia	nox gene	F: 5´-agagtttgaagacacttatgac-3´ R: 5´-ataaacatcaggatctttgc-3´	(Jansson *et al.*, 2008b; Phillips *et al.*, 2005; Atyeo *et al.*, 1999b)
B. innocens/ *B. murdochii*	nox gene	F: 5´-cctgaaagtttaaaagctg-3´ R: 5´-cgatgtattcttcttttcc-3´	(Atyeo *et al.*, 1999b)

Phylogenetic analysis

The 16S rRNA gene, involved in the biosynthesis of proteins, is a valuable target for analysis of the evolutionary relationship between bacteria (Fox *et al.*, 1977; Woese & Fox, 1977). In Paper **I** the 16S rRNA gene of 34

rodent isolates was sequenced. The selection of isolates aimed to I) include all phenotypes obtained, and II) include isolates with unexpected PCR results. The sequencing was performed by capillary electrophoresis with a ABI Prism 3100 genetic analyser, and almost complete sequences were aligned manually to sequences of type, reference and field strains of valid and proposed *Brachyspira* spp. from different hosts. A distance matrix based on 1452 nucleotides was used to construct an evolutionary tree by the neighbour-joining method (Johansson *et al.*, 2004; Pettersson *et al.*, 1996).

3.4.2 *Yersinia enterocolitica* and *Y. pseudotuberculosis*

In other studies on YE in rodents the intestines have been the target for detection. However, the sample size has differed from the whole intestines (Kaneko *et al.*, 1978; Aldova *et al.*, 1977), a 40 mm section of the large intestine (Pocock *et al.*, 2001) to only the rectal contents (Iinuma *et al.*, 1992) and 'extracted faeces' (Kapperud, 1975). One concern in Paper **II** was that there would be too small numbers of any presumtive YE to be detected by culture, since the colon samples available eventually were too small (0.5-1.5 cm). Lymph nodes from the throat were added to the analysis from some of the rodents, but none of these were positive. One other concern was that the freezing to -80 °C of some of the samples would further reduce the number. Therefore, two methods were used: cultivation and real-time PCR (TaqMan PCR). Isolates were needed for further characterisation, and PCR was used both as a screening method for all rodents samples, and for confirmation of pathogenicity of the isolates obtained for further characterisation. The procedure used for the detection of YE and YP is summarised in Figure 3.

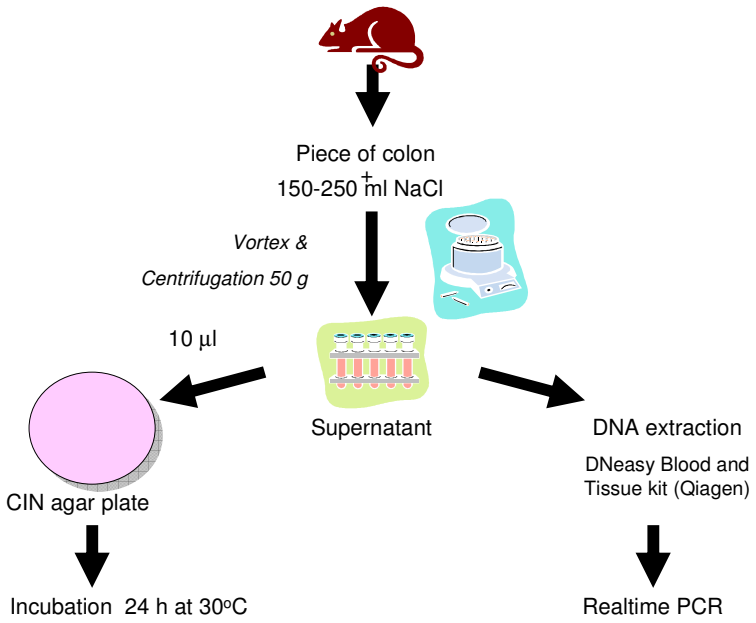

Figure 3. The treatment of samples and simultaneous detection of YE and YP using both culture and real-time PCR.

Cultivation

A large number of presumptive 'bull's eye' colonies were subcultured and stored at -80 °C. For the untrained eye, it is difficult to differentiate YE colonies from some other species of *Enterobacteriaceae* (Head *et al.*, 1982). The use of real-time PCR to detect pathogenic isolates for further characterisation by bioserotyping reduced the workload otherwise needed.

Real-time PCR

A TaqMan protocol for the detection of the pathogenic *ail* gene of YE (Thisted Lambertz *et al.*, 2008b) was used for screening of the samples and on all presumptive colonies. A TaqMan method for detecting *Yersinia pseudotuberculosis* (Thisted Lambertz et al., 2008a) was also applied. Bacterial DNA from colon and lymph nodes was extracted with Qiagen DNeasy® Blood and Tissue kit. 96-well plates were used, and to reduce the risk of DNA carry-over between wells, no more than 50 samples were analysed in each run and samples were added to every second row. Positive controls (Strain SLV-408) were always loaded last, and always in the right lower

corner. Several negative controls (sterile distilled water) were distributed evenly on the plate.

Characterisation

The isolates that were positive in the real-time PCR were further characterised by biotyping (Wauters *et al.*, 1987), serotyping and detection of the virulence plasmid (Bhaduri *et al.*, 1990). Strain SLV-408 was used as a positive control strain for all these tests.

3.4.3 Other bacteria

In Paper **IV**, *Campylobacter* spp. were isolated and characterised to species level, whereas *Lawsonia intracellularis, Leptospira* spp. and *Salmonella enterica* were detected by PCRs (Table 4). Bacterial DNA from the colon for the detection of *Lawsonia intracellularis* and *Salmonella enterica* was already extracted by Qiagen DNeasy® Blood and Tissue kit and the same kit was used with rodent kidneys for *Leptospira* detection. The analysis for *Campylobacter* spp. was carried out at the Department of Bacteriology, National Veterinary Institute. Samples were taken from the colon by two methods: either transferred to Cary-Blair transport medium (Cary & Blair, 1964) or to a swab in Amies medium. As we experienced problems with mould using the first method, the second was used for the rest of the samples. Two types of media were used: mCCDA and CAT with incubation at 41.5 ± 1°C in a microaerophilic atmosphere for 48 ± 2 h.

In the *Lawsonia intracellularis* PCR, an internal control mimic (Jacobson *et al.*, 2003a) was added to each sample to detect inhibition. For undiluted samples, 70% of the PCR reactions were inhibited. Diluting those samples 1/10 reduced inhibition to 7%. A few remaining samples were further diluted to 1/100, which eliminated inhibition, but no additional positive samples were found. Despite the use of a commercial kit, there were obviously many inhibitory substances present in the undiluted samples. As a consequence, 1/10 dilutions of the same samples were used in the real-time PCR for the detection of *Salmonella enterica* (Hoorfar *et al.*, 2000). For a small selection of rodent samples, culturing of samples was carried out at the Department of Bacteriology, National Veterinary Institute. These rodents were caught on a laying hen farm with an ongoing outbreak of *Salmonella* Typhimurium.

Leptospira spp. were analysed in rodent samples (n=133) selected from seven pig herds and four other non-pig related locations in a student project. Two

different methods were applied to detect pathogenic *Leptospira*, one targeting the hap-1 gene (Branger et al., 2005), and one targeting *ligA* and *B* genes (Palaniappan *et al.*, 2005). Positive rodent kidney samples were only found by the hap-1 method, whereas the *lig* PCR yielded unspecific bandings. The reason for the failure of the second PCR could be inhibition, which was not tested for, or reduced DNA concentration due to repeted freezing and thawing of samples. In fact, Branger *et al.*, 2005 concluded that the use of frozen samples reduced the sensitivity significantly for their method. Sequencing of positive amplicons revealed that, awkwardly, a 240 bp part of the 9th mouse chromosome had been amplified in some of the samples. On the electrophoresis gel they looked the same size as the 262 bp amplicon. Thus, an appropriate size ladder should be used, and the gels run for enough time to separate fragments, especially when analysing mouse samples.

3.5 Detection of parasites and encephalomyocarditis virus

In Paper **IV**, the analyses for *Giardia* spp., *Cryptosporidium* spp., *Toxoplasma gondii*, *Trichinella spiralis* and EMCV were carried out by standard methods at the Dept of Parasitology and Virology, National Veterinary Institute. Further characterisation of some of the *Giardia* spp. and *Cryptosporidium* spp. isolates was carried out at the Dept of Parasitology, Mycology and Environmental Microbiology, Swedish Institute for Infectious Disease Control, Solna, Sweden. Unfortunately, the parasitological tests could not be performed immediately at the beginning of the project and since the tests, except for *Toxoplasma gondii* serology, depend on fresh material, the number of samples analysed was lower than in the other analyses.

Blood for *Toxoplasma gondii* serology was collected from cardiac and lung vessels of the rodents and it was difficult to retrieve a large enough volume to spin down to get serum, especially from mice. For the majority of the rodents, blood was instead saved in dry form on a filter paper that could be stored at room temperature.

For the detection of EMCV, cardiac tissue was chosen as the target. In experimental studies on rats and mice, however, the virus has also been detected in faeces and in internal organs (Psalla *et al.*, 2006b; Psalla *et al.*, 2006a). It could be argued that for the study of rodents as carriers of EMCV, faeces might have been more interesting to study, since faecal contamination of the environment is thought to be the transmission route

(Murnane *et al.*, 1960). However, there were no intestinal samples available for RNA extraction, since they had been used for DNA extraction.

3.6 Molecular epidemiology

In Paper **I**, rapid amplified polymorphic DNA (RAPD) was used as a tool to identify unique *Brachyspira* isolates for further characterisation. In Papers **II** and **III**, RAPD and pulsed field gel electrophoresis (PFGE) were used to compare *Brachyspira* spp. and YE rodent isolates with isolates from pigs and chicken (Paper **III**) and from pigs (Paper **II**) on the same farms.

RAPD

RAPD is a relatively cheap and fast PCR-based method, useful for fingerprinting of *Brachyspira* spp. (Hidalgo *et al.*, 2010a; Jansson *et al.*, 2008a; Råsbäck *et al.*, 2007a; Dugourd *et al.*, 1996) with good discriminatory power (Fellström *et al.*, 2008). RAPD has also been used for YE (Blixt *et al.*, 2003; Gray *et al.*, 2001; Leal *et al.*, 1999; Odinot *et al.*, 1995), but the discriminatory power is less than with PFGE (Blixt *et al.*, 2003). The main disadvantage with RAPD is the lack of reproducibility between runs. In Papers **I**, **II** and **III**, a commercial kit, Ready-To-Go RAPD Analysis Beads (Pharmacia Biotech) was used. This kit was easy to use and reduced the workload compared with one other protocol (Jansson *et al.*, 2004) that was also tested for *Brachyspira* spp. (Backhans *et al.*, 2007). The use of the kit reduced the pipetting steps to ensure greater reproducibility. However, especially for isolates with the phenotype of *B. innocens*, it produced bands of varying intensity that were not so easy to interpret.

PFGE

PFGE is still considered the gold standard for the genotyping of many bacteria, but the discriminatory power varies. It has been reported to be useful for *Brachyspira* spp. (Hidalgo *et al.*, 2010a; Jansson *et al.*, 2008a; Råsbäck *et al.*, 2007a; Dugourd *et al.*, 1996) and YE (Laukkanen *et al.*, 2008; Jalava *et al.*, 2006; Thisted Lambertz & Danielsson-Tham, 2005; Fredriksson-Ahomaa *et al.*, 1999; Asplund *et al.*, 1998).

In Paper **III**, PFGE was used to compare selected isolates of *B. hyodysenteriae*, *B. pilosicoli*, *B. intermedia*, *B. murdochii* and *B. innocens* from rodents, pigs and laying hens. Two restriction enzymes *Not*I and *Sal*I were used; but no additional differentiation was achieved for *Sal*I. Two *B. murdochii* isolates from rat and pig, could not be typed by PFGE and despite

repetitive trials with new plugs using different concentrations of bacterial cells, no bands appeared. Similar results have been reported for some strongly haemolytic strains (Råsbäck *et al.*, 2007a; Atyeo *et al.*, 1999a). In Paper **II**, rodent and pig isolates of YE 4/O:3 were typed by a standardised PFGE protocol developed for subtyping of *Escherischia coli* O157:H7, *Salmonella* and *Shigella* (Ribot *et al.*, 2006) by PulseNet laboratories, with slight modifications: less enzyme was used compared with the PulseNet protocol, which was compensated for by extending the incubation time, with good results. The restriction enzymes *Xba*I, *Not*I and *Apa*I were used.

In Paper **II**, in addition to an λ–marker (N03405, New England Biolabs) for fragment size determination, a *Salmonella* serotype Braenderup H9812 was used as a standard for the normalisation of gels (Hunter *et al.*, 2005). In Paper **III** the λ–marker was used, but the bands produced were not always of the best quality. The use of a size standard would have been preferable for an optimal normalisation of the gels.

3.7 Pathology

Rodents positive for pathogenic *Yersinia enterocolitica* (*n*=6), *Y. pseudotuberculosis* (*n*=1), *Lawsonia intracellularis* (*n*=8) and 16 rodents colonised with *Brachyspira* spp. were selected for histopathological examination at the Department of Pathology, National Veterinary Institute. Unfortunately, the intestinal mucosa was disrupted by artefacts from sampling, which obstructed the histopathological evaluation.

3.8 Anti-coagulant resistance

One of the pig farms in this study had experienced problems with large amounts of rats for several years. The problem returned despite the use of anti-coagulants, and warfarin resistance was suspected. From this farm, 20 rats were analysed for a mutation in the VKORC1 gene (Tyr139Cys) which has been associated with warfarin resistance. However, this mutation was not detected in any of the rats.

4 Results and Discussion

4.1 Paper I

Brachyspira spp. were common in the rodent population studied: 83% of rats and 33% of mice were colonised, and these proportions were similar regardless of type of location. Phenotypic characterisation showed that all phenotypes of groups I-IV were represented, plus additional phenotypes that could not be classified to any of these biochemical groups. However, the most common phenotype (35% of isolates) was that of *B. murdochii*, which is in agreement with previous findings (Trott *et al.*, 1996a). Designation of rodent isolates was based on characterisation by biochemical classification, species-specific PCRs and 16S rRNA sequence analysis and referred them to *B. hyodysenteriae*, *B. pilosicoli, B. intermedia, B. murdochii, B. innocens, 'B. canis'* or new provisionally designated species.

Based on the 16S rRNA sequences analysis, the rodent isolates could be referred to six major clusters: cluster 1 included type, reference or field strains of *B. hyodysenteriae*, *B. suanatina* and *B. intermedia*, cluster 2 *B. innocens/B. murdochii* and *B. pulli*, and cluster 3 *B. pilosicoli*. Clusters 4-6 were separated from type, reference and field strains of previously known *Brachyspira* spp. Cluster 4 isolates originated solely from rats and showed the phenotype of *B. murdochii*, *i.e.* spot-indole negative, hippurate-negative, alpha-galactosidase negative and beta-glucosidase positive. These isolates were specifically recognised because they tested positive in a PCR specific for *B. pilosicoli* (Råsbäck *et al.*, 2006), as well as in two PCR systems developed for specific detection of *B. intermedia* (Jansson *et al.*, 2008b; Phillips *et al.*, 2005; Atyeo *et al.*, 1999b; Leser *et al.*, 1997). In addition, they possessed either a hexa or penta(T) nucleotide segment of the 16S rRNA gene, previously only detected in *B. pilosicoli* (Pettersson *et al.*, 1996) and '*B.*

corvi' (Jansson *et al.*, 2008a). Phylogenetically, this cluster of isolates was separated from *B. pilosicoli* with the closest similarity to the *B. intermedia* type strain. Clusters 5-6 contained isolates that, except for one single isolate, were grown on the '*B. aalborgi*-medium' at 37 °C and had the biochemical profiles 00000 or 10000 (see 3.4.1), not consistent with any known *Brachyspira* spp. The biochemical tests used in these studies could not differentiate between cluster 5 and 6 isolates. However, cluster 5 isolates originated from both rats and mice, whereas cluster 6 isolates all originated from mice (including one yellow-necked mouse).

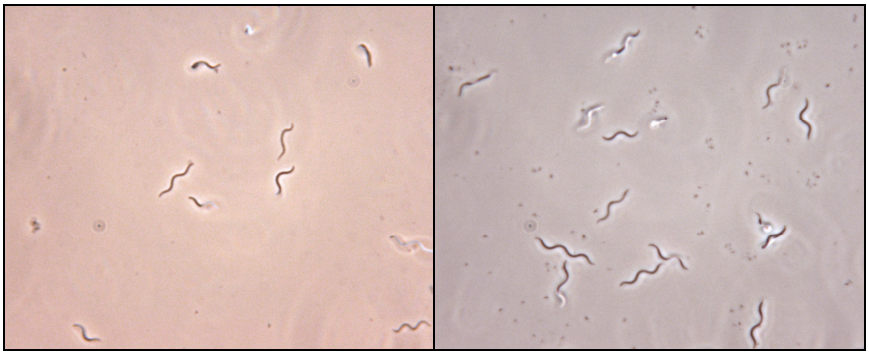

Figure 4. Phase-contrast microscopic pictures of '*Brachyspira muris*' and '*Brachyspira rattus*' (right).

Within the genus *Brachyspira*, the numbers of nucleotide differences in the 16S rRNA gene between species are small compared with other genera (Pettersson *et al.*, 1996; Paster *et al.*, 1991; Stanton *et al.*, 1991). Provisional designations '*Brachyspira rattus'*, '*B. muridarum'* and '*B. muris*' were proposed based on 16S rDNA sequence analysis and referring to their main hosts (rat´ tus. N.L: of the rat, mu.ri.da´rum. N.L: of the mouse-like animals, mu´ris. L: of the mouse). Similarity values calculated indicated that '*B. muris'* and '*B. muridarum*' are genetically closest related to '*B. hyodysenteriae*' (99.0 and 98.9% similarity), and '*B. rattus*' to '*B. intermedia*' (98.3% similarity). These similarity values were comparable to those previously described between recognised *Brachyspira* species, *e.g.* between the type strains of *B. hyodysenteriae* and *B. innocens* (Paster et al., 1991), and therefore, it may be justified to propose that the clusters of isolates described in Paper **I** constitute new species. The designation of new bacterial species requires further phenotypic characterisation, determination of DNA G+C contents,

DNA-DNA hybridisation experiments or whole genome sequencing comparisons (Tindall *et al.*, 2010).

In conclusion, there seems to be a larger genetic diversity within the genus *Brachyspira* isolated from rodents compared with brachyspiras isolated from pigs or dogs. However, a large genetic diversity of brachyspiras has also been described in chicken (Jansson *et al.*, 2008b). The results of this study add to the host range of several *Brachyspira* spp. To the best of the authors knowledge this is the first description of '*Brachyspira canis'* detected in a host other than the dog, of *B. pilosicoli* in rat and of a *'B. suanatina'*-like isolate in mouse. This diversity indicates that cross-species transmission of several *Brachyspira* spp. may occur. The high prevalence (17%) of *B. murdochii* found indicates that this species is commensal in rodents. In addition, '*B. muridarum*' and '*B. muris*' were common, assuming that all isolates with phenotypic profiles 00000 and 10000 can be referred to these proposed species. Considering that those 'species' are very slow-growing and difficult to identify due to sparse growth without haemolysis on blood agar, they might even have been underestimated in the study. So far, it is not known whether these variants have any pathogenic properties, or whether they are capable of colonising other hosts.

4.2 Paper III

There is evidence of cross-species transmission of *B. pilosicoli* between dogs and humans (Trott et al., 1998) and of *B. hyodysenteriae* between rats and pigs (Trott et al., 1996a). Transmission of '*B. suanatina*' between pigs and mallards has also been suggested (Råsbäck *et al.*, 2007a). In Paper **III,** cross-species transmission of *Brachyspira* spp. between rodents and farm animals was investigated. *Brachyspira* isolates from rodents, pigs and laying hens were characterised and compared by RAPD and PFGE. A large proportion of the pigs (74%) and of the rodents (64%) tested *Brachyspira* positive by culture and biochemical tests. *Brachyspira murdochii* was dominant in rodents and *B. pilosicoli* was more common in pigs than in rodents, as can be seen in Figure 5.

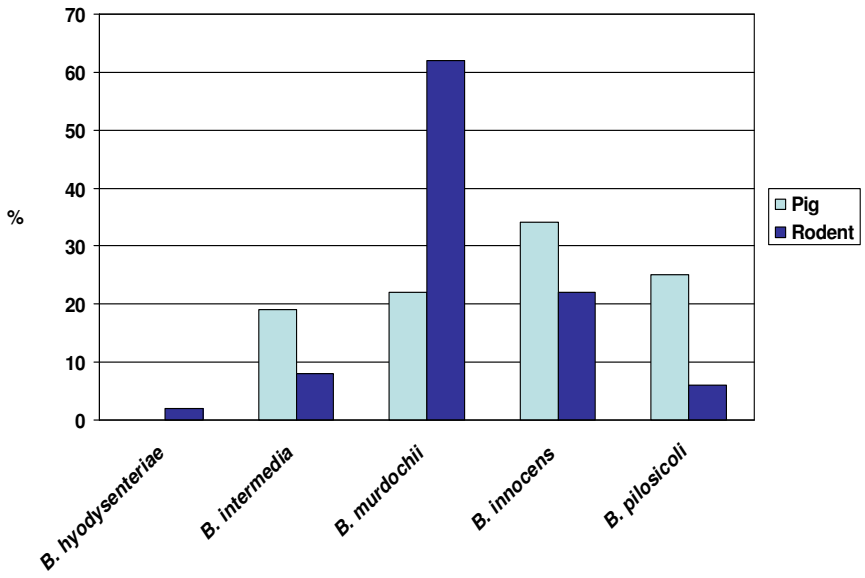

Figure 5. Proportion of different *Brachyspira* spp. in pigs and rodents on farms A-D, shown as a percentage of all *Brachyspira* spp.

Brachyspira hyodysenteriae was not found in any of the pigs, but in one rat on a pig farm. Identical genotypes of *B. pilosicoli*, *B. intermedia*, *B. murdochii* and *B. innocens* were found in rodents and pigs on pig farms, and of *B. murdochii* in rodents and laying hens on chicken farms.

In the *Brachyspira*-negative pig herd, rodents were also free from porcine phenotypes of *Brachyspira*, which suggests an interchange of the intestinal flora between pig and rodent, supporting the hypothesis.

The finding of a rodent *B. hyodysenteriae* isolate in a pig herd not clinically affected by SD was puzzling. This isolate clearly deviated from 37 Swedish, German and Belgian field isolates of *B. hyodysenteriae* from pigs, mallards and mice when PFGE data were compared. Thus, this isolate might be a 'rodent genotype' of *B. hyodysenteriae*. Another rat isolate of *B. hyodysenteriae* clustered with pig strains in PFGE analysis. This finding was perhaps even more surprising, since the isolate was recovered from a rat caught at a bird pond. Mallards were frequent guests in the pond, and might have been the source.

It has not been conclusively shown that *B. hyodysenteriae* from hosts other than the pig have pathogenic properties. One recently suggested virulence marker is the *rfb*BADC gene cluster on the plasmid described as a unique feature of *B. hyodysenteriae* (Wanchanthuek *et al.*, 2010; Bellgard *et*

al., 2009). This set of genes was analysed for by PCR in field strains of *B. hyodysenteriae* from pigs, and in strains from rodents and mallards. The genes *rfb*B, *rfb*A, *rfb*D and *rfb*C were amplified in all *B. hyodysenteriae* strains tested, but amplification of the whole cluster failed in one of the mallard strains. Sequence similarity of the amplified cluster compared with the WA1 plasmid was less for the deviating rat strain (98.2%) than for the other rodent strains and one mallard strain (98.8%).

The indicated presence of this cluster is not enough evidence to suggest virulence, and it is still not known whether the amplified cluster in the rodent isolates is situated on a plasmid or is chromosomal. The fact that the *rfb*BADC gene cluster was present in all field porcine strains of *B. hyodysenteriae* and three rodent strains, but absent from one mallard strain of unknown pathogenicity, needs further attention.

4.3 Paper II

In this study, pathogenic *Yersinia enterocolitica* and *Y. pseudotuberculosis* were detected by real-time PCR (Thisted Lambertz *et al.*, 2008a; Thisted Lambertz *et al.*, 2008b) in colon samples. From the same material, *Y. enterocolitica* isolates of the human pathogenic bioserotype 4/O:3 were obtained. Pigs on three of the farms were also sampled and pathogenic *Yersinia enterocolitica* were detected.

The difficulty in identifying *Y. enterocolitica* among other similar colonies on CIN agar plates could be the reason why nine positive samples were detected by real-time PCR, whereas only five were detected by culture. The use of the direct method, without selective enrichment before plating, probably contributed to the lower sensitivity using culture. The colon samples used were small, especially from the mice, and therefore the possibility cannot be excluded that the result was an underestimation of the true prevalence. Thus, real-time PCR can be recommended as the detection method when screening for pathogenic YE in animals or environmental samples.

Pathogenic *Y. enterocolitica* isolates were obtained from four brown rats and one house mouse caught on three pig farms. Pigs on two of those farms also tested positive for pathogenic *Y. enterocolitica*. On the third farm, the pigs were never sampled. Rodents caught at other locations than pig farms tested negative for pathogenic *Y. enterocolitica*.

RAPD and PFGE analysis failed to differentiate between rodent and pig isolates. RAPD patterns were identical for all isolates. PFGE with the restriction enzyme *Xba*I also yielded identical banding pattern for all isolates.

One rat isolate differed by two bands when the restriction enzyme *Not*I was used, and the use of *Apa*I for a selection of some of the isolates gave similar results to *Not*I. The result was a little surprising, since the farms investigated were situated 20-65 km from each other. Furthermore, one farm, an all in-all out fattening herd, was sampled twice with a one-year interval, and the same PFGE type was isolated in both years from pigs and rodents. However, it can be concluded from the work of others that the discriminatory power of PFGE is rather limited when applied on *Y. enterocolitica* 4/O:3. In recent years, multiple-locus variable-number tandem-repeat analysis (MLVA) has been developed for the typing of 4/O:3 (Gierczynski *et al.*, 2007; de Benito *et al.*, 2004), which is more discriminatory than PFGE (Sihvonen *et al.*, 2011). It could be expected that the use of MLVA would have differentiated the strains further.

The detection rate of *Y. pseudotuberculosis* in rodents was lower than expected (1/190), since rodents are considered one of the reservoirs for this pathogen (Fukushima *et al.*, 1990; Mair, 1973). The pigs in this study also tested negative for *Y. pseudotuberculosis*. In Sweden, outbreaks of *Y. pseudotuberculosis* in hares (*Lepus europaeus*) have been reported (SVA, 2008), but the occurrence of *Y. pseudotuberculosis* in Swedish pigs is unknown and human cases are rare, so the result might just reflect a low prevalence in pigs.

4.4 Paper IV

In Paper **IV,** rodents were shown to be carriers of several animal and human pathogens. The majority of these pathogens have been identified in rodents in other studies (Table 1). However, the findings of *Brachyspira pilosicoli* in rats, and *Campylobacter jejuni* and *Campylobacter upsaliensis* in yellow-necked mice have, to the best of the author's knowledge, not been described before.

Several wild animal species including rodents can harbour *Lawsonia intracellularis* and thereby constitute reservoirs for infections in pigs (Friedman et al., 2008; Dezorzova-Tomanova et al., 2006; Drolet et al., 1996). However, in Paper **IV**, only mice and rats caught on pig farms tested positive for *Lawsonia intracellularis*. As can be seen in Figure 6, 8.6% of rodents on pig farms tested positive. The positive samples originated from three different herds. In a recent study, >70% of rats on endemic pig farms tested positive for *L. intracellularis* (Collins *et al.*, 2011), which is a considerably higher level. However, in that study, a larger proportion of the

intestines was used for analyses. Furthermore, in Paper **IV** the herd prevalence was unknown.

Regarding *Brachyspira hyodysenteriae*, *B. pilosicoli* and *B. intermedia*, these agents were already analysed and discussed in Papers **I** and **III**.

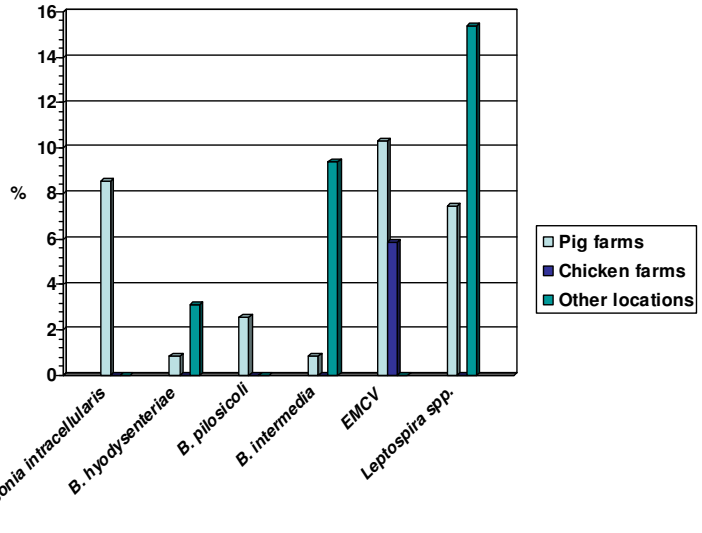

Figure 6. Diagram showing pig and chicken pathogens detected as a percentage of rodents. It should be noted that the numbers of rodents caught at pig farms, chicken farms and other locations differed.

EMCV was detected in both rats and mice caught primarily on pig farms, but also in one mouse from a chicken farm. Positive samples were detected from half the pig farms, and one out of three chicken farms. Outbreaks of EMCV have not been reported in Sweden, but the results showed that EMCV should be considered a possible differential diagnosis in sudden outbreaks of high mortality rates in growing pigs, or reproductive problems when other more common causes have been ruled out.

Leptospira spp. was detected by PCR in 7% of rodents. The positive rodents originated from three farms and from the city pond. Sequencing of the amplicons revealed several different sequences with high similarity to *L. borgpetersenii, L. weili* or *L. interrogans* serovar Copenhageni.

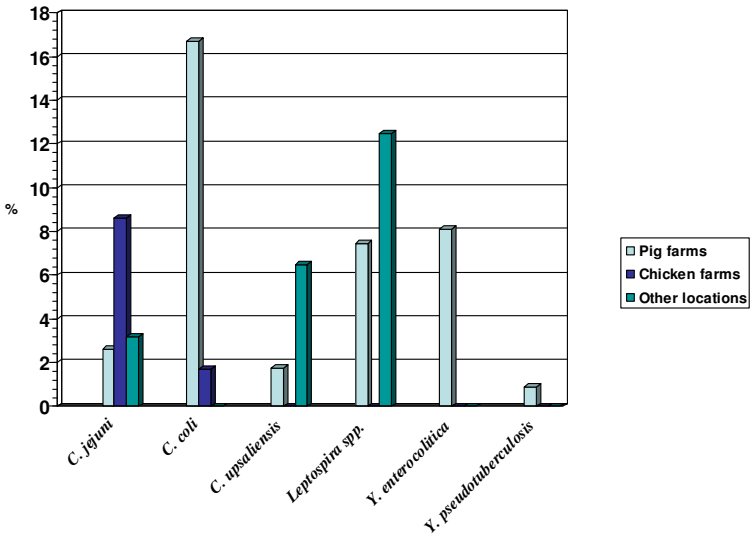

Figure 7. Diagram showing detected human pathogens in percentages of analysed rodents in relation to type of location. It should be noted that the numbers of rodent caught in different locations differed.

Figure 7 shows the percentage of rodents positive for the human pathogens. Human pathogens detected in rodents comprised three species of thermophilic *Campylobacter*, namely *C. jejuni*, *C. coli* and *C. upsaliensis*. Furthermore *Leptospira* (discussed above as a pig pathogen), and *Yersinia enterocolitica* 4/O:3 (discussed in Paper **II**) were detected. Other studies have shown that the occurrence of rodents is a risk factor for high *Campylobacter* prevalence in broiler chicken flocks (Berndtson *et al.*, 1996; Kapperud *et al.*, 1993). *Campylobacter coli* was predominant in pig herds. *Campylobacter upsaliensis* was found on chicken farms and at a sewage treatment plant but only seven animals were caught at that location, all yellow-necked mice.

Yersinia enterocolitica and *Y. pseudotuberculosis* have already been discussed in relation to Paper **II**. *Salmonella* spp. were investigated by PCR. *Salmonella* Typhimurium was isolated from one mouse caught in a laying hen flock affected by *Salmonella*. Only rodent samples from this specific farm were cultured; PCR was negative for all tested samples including this positive sample, which raises the suspicion that PCR was not sensitive enough to detect *Salmonella* spp. in the small-sized samples available.

Giardia and *Cryptosporidium* spp. were common in rodents; 13 and 11% respectively of the rodents were positive. A selection of these was further

characterised, but no types of zoonotic interest were found. Before the development of molecular biology methods capable of genotyping isolates of *Cryptosporidium*, wild animals including rodents were considered carriers of zoonotic *Cryptosporidium* (Appelbee *et al.*, 2005; Quy *et al.*, 1999; Webster & MacDonald, 1995), leading to overestimation of their zoonotic importance. Negative results were obtained from the analysis of *Trichinella* spp. and *Toxoplasma gondii*. The result for *Trichinella* spp. was expected, since Swedish pigs have been free from trichinosis since 1994 (Anon, 2009b). Results for *Toxoplasma gondii* were obtained by serology, commonly used for screening for toxoplasmosis in humans (Petersson *et al.*, 2000) and wildlife (Malmsten *et al.*, 2010; Ryser-Degiorgis *et al.*, 2006). However, several studies have shown that the prevalence in rodents might be underestimated when relying on serology (Dubey & Frenkel, 1998; Dubey *et al.*, 1997). Different serological methods have shown prevalences between 0.8% and 23% in rats (Dubey *et al.*, 2006; Frenkel *et al.*, 1995) and even lower in mice (Dubey *et al.*, 1995; Smith *et al.*, 1992) whereas PCR applied directly on brain tissue showed that 42.2% of rats and 53-59% of mice were infected (Murphy *et al.*, 2008; Hughes *et al.*, 2006; Marshall *et al.*, 2004).

5 Conclusions

- House mice (*Mus musculus*) and brown rats (*Rattus norvegicus*) are the most common rodent species in pig herds. The yellow-necked mouse (*Apodemus flavicollis*) is more of an occasional guest.
- Rodents constitute a risk of spreading diseases to pigs. The results indicate that rodents are carriers of *Brachyspira pilosicoli, Brachyspira hyodysenteriae* and *Lawsonia intracellularis* whereas they are reservoirs of EMCV and *Leptospira* spp. The occurrence of rodent carriers should be taken into account especially when infections are to be eradicated from herds.
- Several of the valid and proposed species of *Brachyspira* spp. were isolated from rodents which reflect the biodiversity within this genus.
- Three phylogenetic groups of *Brachyspira* spp. not previously described were found. These new genetic variants may constitute novel species.
- Evidence was found for cross-species transmission of *Brachyspira pilosicoli*, *B. intermedia*, *B. murdochii* and *B. innocens* between rodents and pigs and of *B. murdochii* between rodents and laying hens.
- A 'rodent genotype' of *Brachyspira hyodysenteriae* was found, deviating from porcine strains and of unknown pathogenic properties.
- Rodents on pig farms carry human pathogenic *Yersinia enterocolitica* 4/O:3, and cross-species transmission of this bacteria between rodents and pigs was indicated.
- Rodents are carriers of *Campylobacter jejuni, C. coli* and *C. upsaliensis*.
- *Toxoplasma gondii* and *Trichinella* spp. were not detected in the rodents investigated in this study.
- *Giardia* and *Cryptosporidium* spp. are common in rodents, but zoonotic types were not found.
- Rodent control should be considered an important measure to provide good bio-safety on farms.

6 Future studies and perspectives

- Rodent strains of *Brachyspira hyodysenteriae* and *B. intermedia* should be useful for comparison when analysing for virulence markers in those species.

- The phylogenetic status of '*B. muris'*, *'B. muridarum'* and *'B. rattus'* should be analysed further.

- Further investigations to determine to which extent wild rodents are carriers of *Salmonella* spp. in Sweden needs to be performed as a separate study in which also wild birds inhabiting the same surroundings should be included.

- The occurrence of thermophilic *Campylobacter* in wild rodents could imply a direct risk of contamination of food. Further analysis of *Campylobacter* in mice caught in households and extended typing of isolates would reveal whether they constitute a source of domestic infection.

- The prevalence of *Toxoplasma gondii* in rodents needs to be analysed further with other methods, for the direct detection of the coccidium in tissue to exclude rodents as a risk for transmission to pigs.

- Prevalence of *Yersinia pseudotuberculosis* in Swedish pigs have not, to the authors knowledge been investigated. For example, the difference in prevalence between conventional and organic farms needs to be analysed.

- The tissue bank collected in this study may be used for investigation of other pathogens of interest, i.e. hepatitis E virus.

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