Treponema spp. in Porcine Skin Ulcers

Clinical Aspects

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Cover: The apocalypse is near, or *Treponema pedis* (red) in a sow shoulder ulcer. (photo: Tim K. Jensen)

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Abstract

The hypothesis tested in this work is that bacteria of genus *Treponema* play a main role when shoulder ulcers and ear necrosis occur in an infectious or severe form, and perhaps also in other skin conditions in the pig. Samples were collected from pigs in 19 Swedish herds 2010-2011. The sampled skin lesions included 52 shoulder ulcers, 57 ear necroses, 4 facial necroses and 5 other skin ulcers. Occurrence of spirochetes was detected by phase contrast microscopy, Warthin-Starry silver staining, PCR and Fluorescent *In Situ* Hybridization (FISH). Treponemal diversity was investigated by sequencing of 16S-23S rRNA intergenic spacer region 2 (ISR2) and high-throughput sequencing (HTS) of a part of the 16S rRNA gene. Culturing and characterization of treponemes by biochemical analyses, testing of antimicrobial susceptibility and fingerprinting by random amplified polymorphic DNA (RAPD) were carried out. A challenge study was performed to test if *Treponema pedis* induced skin lesions. Serological response towards TPE0673, a *T. pedis* protein, was tested with ELISA.

Spirochetes were found in all types of skin ulcers and in all herds. The occurrence of *Treponema* spp. detected by PCR was 52% in shoulder ulcers, 46% in ear necrosis and 9.7% in gingiva. Treponemes were identified in 69% of the shoulder ulcers and in 59% of the ear necroses by FISH. A phylogenetic tree revealed a great variability of treponemes. Three main phylotypes were identified; *T. pedis*, *Treponema parvum* and one phylotype without designation. Twelve isolates of *T. pedis*, *T. parvum*, and one phylotype most similar to *Treponema* sp. OMZ 840 were obtained. All except two had unique RAPD fingerprints. Biochemical tests could not differentiate between the isolates and they were generally susceptible to tested antimicrobials. By FISH, treponemes were visualized deep in the ulcers and a predominance of *T. pedis* was noted, and confirmed by HTS. Challenged pigs did not develop any lesions or IgG response towards the *T. pedis* protein. Most sows with shoulder ulcers showed a strong, and most cases of ear necrosis a weak IgG response towards TPE0673. In conclusion, *Treponema* spp. are frequently abundant in ear necroses and shoulder ulcers in pigs. Identical phylotypes and ISR2 sequences from ulcers and gingiva indicate spreading from mouth to ulcer. A broad diversity of phylotypes was revealed, but the predominance of *T. pedis* suggests specific importance of this species. Our results point towards an important role of treponemes in chronic and severe skin ulcers in pigs.

Keywords: Treponema, skin ulcer, shoulder ulcer, ear necrosis, facial necrosis, pig

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Dedication

To my friends and family.

Med läsning öd ej tiden bort - Vårt kön så föga det behöfver. Och skall du läsa, gör det kort. Att såsen ej må fräsa öfver!

Anna Maria Lenngren

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List of Publications

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

I Karlsson, F., Svartström, O., Belák, K., Fellström, C. & Pringle, M. (2013). Occurrence of *Treponema* spp. in porcine skin ulcers and gingiva. *Veterinary Microbiology* 165(3-4), 402-409.

II Svartström, O., Karlsson, F., Fellström, C. & Pringle, M. (2013). Characterization of *Treponema* spp. isolates from pigs with ear necrosis and shoulder ulcers. *Veterinary Microbiology* 166(3-4), 617-623.

III Karlsson, F., Klitgaard, K. & Jensen, T. K. (2014). Identification of *Treponema pedis* as the predominant *Treponema* species in porcine skin ulcers by Fluorescence *In Situ* Hybridization and high-throughput sequencing. *Veterinary Microbiology*. DOI: 10.1016/j.vetmic.2014.03.019.

IV Karlsson F., Backhans A., Fellström C. & Rosander A. (2014). A *Treponema pedis* challenge model in pigs and serological response to IdeT homologue TPE0673. *Manuscript.*

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

The contribution of Frida Karlsson to the papers included in this thesis was as follows:

I Frida Karlsson and Olov Svartström performed the experiments, analyzed the results and wrote the manuscript under supervision of Märit Pringle and Claes Fellström. Katinka Belák performed the histopathological investigations.

II Olov Svartström and Frida Karlsson performed the experiments, analyzed the results and wrote the manuscript under supervision of Claes Fellström and Märit Pringle.

III Frida Karlsson performed the investigations by FISH under supervision of Tim K. Jensen. Tim K. Jensen performed the histopathological analyses. Kirstine Klitgaard designed the probes and performed the high-throughput sequencing analysis. Frida Karlsson wrote the manuscript with Kirstine Klitgaard and Tim K. Jensen as co-authors.

IV Frida Karlsson planned and performed the experiments under supervision of Claes Fellström and Annette Backhans. Anna Rosander performed the ELISA. Frida Karlsson wrote the manuscript with Annette Backhans, Anna Rosander and Claes Fellström as co-authors.

Abbreviations

16S rRNA	16S ribosomal RNA
23S rRNA	23S ribosomal RNA
BDD	Bovine digital dermatitis
CODD	Contagious ovine digital dermatitis
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
FAA	Fastidious anaerobe agar
FISH	Fluorescence *In Situ* Hybridization
HE	Hematoxylin and eosin staining
HTS	High-throughput sequencing
IgG	Immunoglobuline G
ISR2	16S-23S rRNA intergenic spacer region 2
MIC	Minimum inhibitory concentration
PCM	Phase contrast microscopy
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA
rDNA	DNA coding for a ribosomal RNA gene
RNA	Ribonucleic acid
sp.	Species (singular)
spp.	Species (plural)
subsp.	Subspecies
TPP	Thiamine pyrophosphate
tRNA	Transfer RNA
UMD	Ulcerative mammary dermatitis
W-S	Warthin-Starry silver staining

1 Introduction

This thesis concerns a new, yet old, research area, namely bacteria of genus *Treponema* in necrotic skin ulcers of pigs.

1.1 Spirochetes in porcine skin ulcers

In the beginning of the last century, a number of reports on certain skin problems in pigs were published. The typical appearance of these skin problems were non healing lesions with a necrotic centre, and they could be situated almost anywhere on the body. In scrapings or smears from these ulcers, spiral shaped bacteria (spirochetes) could be identified in the microscope. The syndrome was named ulcerative granuloma (Hindmarsch, 1937), ulcerative dermatitis (Gill, 1929) or spirochetal wound infection (Neitz & Canham, 1930).

Sydney Dodd was the first to describe spirochetes in cutaneous lesions (Dodd, 1906). The skin lesions he studied were superficial ulcers situated behind the ear, on the head or generally spread on the rest of body. Spirochetes were found in scrapings from the lesions, but not from intact skin. He also showed that the disease could be transmitted through contact between pigs. In one case he managed to reproduce disease through inoculation of scraping material from a pig with lesions containing spirochetes to scarified skin of a healthy pig.

After this, reports of similar kind followed where spirochetes were found in ulcers on the head, jaw, shoulder, elbow, knee, feet, scrotum and body (Neitz & Canham, 1930; Gill, 1929; Schmid, 1925; Nomi & Matsuo, 1922; Gilruth, 1910; Cleland, 1908). Other microorganisms occurring in the lesions were cocci, bacilli, fusiform bacilli and spore forming bacteria (Neitz & Canham, 1930; Nomi & Matsuo, 1922; Gilruth, 1910; Cleland, 1908). Although most writers at that time seemed to hold spirochetes responsible for the skin lesions, Gill was doubtful whether spirochetes were the actual cause of the disease

(Gill, 1929). He pointed out that even though spirochetes were often found in large numbers, especially deep within the necrotized tissue, other microorganisms were also present. He expressed the hypothesis that perhaps the spirochetes were only saphrophytes taking advantage of a lowered host resistance caused by other factors.

New transmission experiments were performed, but few were completely successful (Hindmarsch, 1937; Gill, 1929; Gilruth, 1910). However in 1955, Osborne and Ensor managed to repeat in four cases what Dodd had done, by transmitting material containing spirochetes from pigs with skin ulcers to healthy animals and causing both foot-rot and necrotic ulcers (Osborne & Ensor, 1955). They noted that they only succeeded in cases where they injected material from fresh ulcers containing motile spirochetes.

In the seventies, Blandford *et al.* (1972) and Harcourt (1973) described the first cases of suspected spirochetosis in pigs in England. These reports were case descriptions of pigs suffering from ear ulcers, lesions on the feet or in the mouth, and where spirochetes could be confirmed by microscopy.

Until recently, the spirochetes occurring in these varying types of porcine skin ulcers had only been described microscopically. They were called *Spirochaeta suilla* (Neitz & Canham, 1930; Gill, 1929) although Gill suggested calling them *Treponema*. Other species names mentioned in the literature are *Borrelia suis* or *Borrelia suilla* (Cameron, 2012; Mullowney & Hall, 1984; Penny *et al.*, 1971). No attempts to culture these spirochetes had been described, and the bacteria had therefore not been isolated or further characterized.

In 2008, during outbreaks of ear necrosis in two organic pig herds in Sweden, our group was able to isolate and characterize spirochetes of genus *Treponema* both from ear necrosis and gingiva (Pringle *et al.*, 2009). Later, isolation from a sow shoulder ulcer in another herd was also successful (Pringle & Fellström, 2010). These findings laid the foundations for further studies, described in this thesis.

1.2 *Treponema* spp.

Genus *Treponema* (etymology Gr. *trepô* ‘to turn’ and *nema* ‘a thread’) (Norris *et al.*, 2011) belongs to the family *Spirochaetaceae* within the phylum *Spirochaetes* of the domain *Bacteria.* Spirochetes are considered representing a monophyletic group (Paster & Dewhirst, 2006). This means that they originate from a single ancient ancestor. This has been demonstrated based on phenotype and 16S rRNA sequence analysis (Paster & Dewhirst, 2000; Paster *et al.*, 1991), as well as whole genome sequence analysis (Gupta *et al.*, 2013)

1.2.1 Morphology

As the name implies, bacteria of genus *Treponema* are long, up to 20 µm, thin, 0.1-0.7µm in diameter, and with a spiral or wave-like shape (Norris *et al.*, 2011). A picture of a treponemes viewed in a phase contrast microscope is shown in figure 1. *Treponema* bacteria have an outer and an inner membrane. The outer membrane resembles that of Gram-negative bacteria, consisting of lipids, proteins and carbohydrates, but spirochetes do not stain well with Gram stain.

Located between the membranes, in the periplasmic space, are the periplasmic flagella. These may vary in number between species. The flagella are attached at the end of the bacterium, stretch along the bacterial cylinder and give rise to a rotating movement. Treponemes are therefore motile.

1.2.2 Metabolism

The metabolism varies between species. Naturally, more is known about the requirements of cultivated than not yet cultivated species. The today cultivated species are considered strict anaerobes (Norris *et al.*, 2011). Some use carbohydrates to gain energy; others use fermentation of amino acids. Their requirements of fatty acids also vary, but all cultivated species either need long-chain fatty acids present in serum, or short-chain fatty acids available in rumen fluid. Some species also require thiamine pyrophosphate (TPP). The pathogens within the *Treponema pallidum, Treponema carateum* and *Treponema paraluiscuniculi* group cannot yet be continuously cultured *in vitro*. The most common way to multiply these treponemes is to inoculate testes of rabbits.

1.2.3 Antimicrobial susceptibility

In their studies in the seventies, Abramson & Smibert performed extensive antimicrobial susceptibility tests on seventeen treponemal strains (Abramson & Smibert, 1971). Included species were *Treponema phagedenis*, *Treponema refringens*, *Treponema denticola* and *Treponema vincentii*, as well as unnamed isolates from human oral cavity and pig faeces. In recent years, antimicrobial susceptibility of treponemes isolated from bovine digital dermatitis (BDD) in Sweden, UK and Japan have been reported (Evans *et al.*, 2012; Yano *et al.*, 2010a; Evans *et al.*, 2009a; Pringle *et al.*, 2008). Species represented in these studies were *T. medium/T. vincentii-like, T. phagedenis*-like and *Treponema pedis* (formerly called *T. denticola/T. putidum*-like). The minimum inhibitory concentrations (MICs) of a selection of agents tested in the more recent studies are presented in table 1. In all of the studies different types of broth dilution methods were used, and the antimicrobials tested differed between the studies,

as well as the range of concentrations and the incubation time used. It is therefore not possible to fully compare the studies. As pointed out in several of the studies, no standardised method for antimicrobial susceptibility testing of treponemes has been established (Evans *et al.*, 2012; Yano *et al.*, 2010a; Evans *et al.*, 2009a).

Stanton & Canale-Parola found that spirochetes present in the bovine rumen were resistant to rifampicin (1-50 μg/ml) and used rifampicin as an additive to selective media to isolate spirochetes (Stanton & Canale-Parola, 1979). They also reported that human oral spirochetes, free-living spirochetes and porcine intestinal spirochetes were resistant to rifampicin (Leschine & Canale-Parola, 1980; Stanton & Canale-Parola, 1979). This rifampicin resistance has been confirmed in all treponemal species tested so far, and is caused by a difference in the RNA polymerase of the bacterium (Stamm *et al.*, 2001). Resistance to macrolides has been described in strains of *T. pallidum* subsp. *pallidum* and is caused by a point mutation in the genes for 23S rRNA (Stamm, 2010; Stamm & Bergen, 2000).

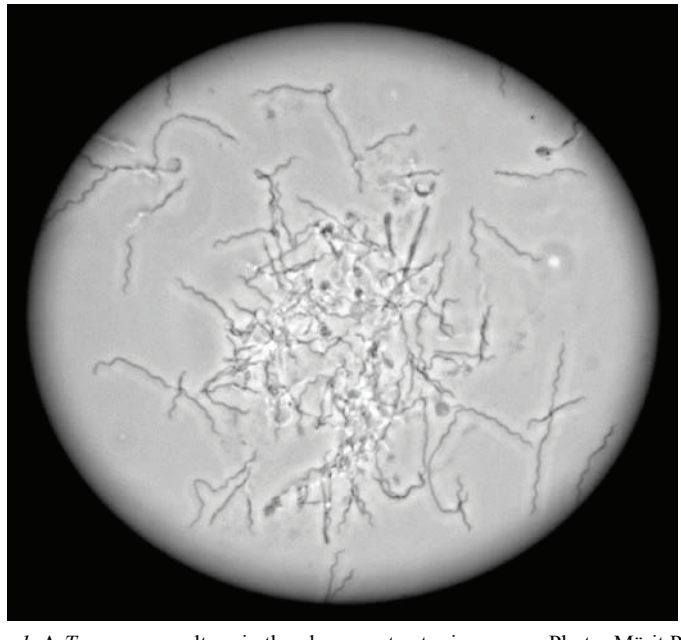

Figure 1. A *Treponema* culture in the phase contrast microscope. Photo: Märit Pringle

Table 1. *Minimum inhibitory concentrations of 15 antimicrobial agents for* Treponema *spp.*

Reference	Species	Origin	N[1]	MIC (μg/ml) of antimicrobial agents[2]														
				Pc	Amp	Amox	Tia	Val	Ery	Tyl	Aivl/ Tylv	Azi	Linco	Doxy	Oxy	Rif	Trim	Sulf
Pringle et al., 2008	*T. phagedenis* -like	BDD[3]	7	Nt[4]	Nt	Nt	0.25-1	0.063 -0.25	Nt	≤0.5	≤0.25	Nt	>4	0.031 -0.25	Nt	Nt	Nt	Nt
Evans et al., 2009	*T. medium/ T. vincentii*-like	BDD	6	0.0469	0.0938	Nt	Nt	Nt	0.0059-0.0117	Nt	Nt	Nt	6-12	Nt	0.375	Nt	Nt	Nt
"	*T. phagedenis -like*	BDD	8	0.0117-0.0235	0.0469-0.1875	Nt	Nt	Nt	0.0235-0.1875	Nt	Nt	Nt	12-24	Nt	0.1875 -1.5	Nt	Nt	Nt
"	*T. pedis*	BDD/ CODD[5]	5	0.0235-0.0469	0.1875-0.375	Nt	Nt	Nt	0.017-0.0235	Nt	Nt	Nt	0.75-6	Nt	0.375-0.75	Nt	Nt	Nt
Yano et al., 2009	*T. phagedenis -like*	BDD	23	<0.06	<0.06	Nt	Nt	Nt	<0.06	Nt	Nt	Nt	4-16	Nt	0.06-1	>128	>128	>128
Evans et al., 2012	*T. phagedenis -like*	BDD	6	Nt	Nt	0.1875-0.75	Nt	Nt	Nt	Nt	Nt	0.0234-0.0469	Nt	Nt	Nt	Nt	96	Nt
"	*T. pedis*	BDD/ CODD	6	Nt	Nt	0.1875-0.375	Nt	Nt	Nt	Nt	Nt	0.0234-0.0469	Nt	Nt	Nt	Nt	96-192	Nt

[1.] Number of tested isolates. [2.] Penicillin, Ampicillin, Amoxicillin, Tiamulin, Valnemulin, Erythromycin, Tylosin, Aivlosin/Tylvalosin, Azithromycin, Lincomycin, Doxycycline, Oxytetracycline, Rifampicin, Trimethoprim, Sulfamethoxazole. [3.] Bovine digital dermatitis [4.] Not tested [5.] Contagious ovine digital dermatitis

1.2.4 Isolation

Bacteria of genus *Treponema* are extremely fastidious. Not all species are considered cultivable, but several species may be isolated using either a selective medium or a membrane filter. The following text is an attempt to make a short summary of isolation methods reviewed in Manual of Clinical Microbiology (Smibert, 1991).

For growth of most treponemes a basic medium supplemented with serum, short-chained fatty acids, TTP, glucose and pectin is recommended. To this medium rifampicin (2µg/ml) or rifampicin-polymyxin (2µg/ml-800U/ml) is added. The culture medium may be liquid or semisolid. Samples are inoculated and cultures incubated anaerobically at 37ºC for 7-14 days. After one week of culturing it is recommended to check the cultures for presence of treponemes by using a dark field microscope. If treponemes are present, the culture is inoculated to fresh selective medium. Separation of treponemes from contaminating bacteria can be performed by using a membrane filter with pore size 0.2-0.3µm. The procedure and equipment is described in detail in Manual of Clinical Microbiology (Smibert, 1991).

The membrane filter may also be used directly. In this case the filter is placed on selective agar medium supplied with rifampicin or rifampicin-polymyxin. The culture is added to the filter and the agar plate incubated anaerobically for 7-14 days. The filter is removed and a plug of agar examined using dark field microscopy. If treponemal growth is confirmed, a plug of agar is removed with a Pasteur pipette and moved to selective medium.

For isolation of single colonies, two to three day old cultures are streaked onto selective agar medium. The agar plates may be prereduced to ensure anaerobic conditions. The plates are incubated anaerobically at 37ºC for 7-14 days. Successfully grown treponemes appear as white, hazy colonies growing in the agar. These may be moved to selective broth or semisolid medium by using a Pasteur pipette. Smibert emphasizes that soft agar medium is required, as the bacteria actually do not grow on the surface of the agar but in the agar.

The above description should be regarded as a basic overview on *Treponema* culturing. Many research groups have used modified versions of these methods according to their own experience (Yano *et al.*, 2009; Evans *et al.*, 2008; Pringle *et al.*, 2008; Demirkan *et al.*, 1999; Schrank *et al.*, 1999; Walker *et al.*, 1995).

1.2.5 Detection methods

Culture independent methods are important tools to detect treponemes, as cultivation is extremely difficult. Old, well-established methods are used in parallel with modern, molecular approaches.

As mentioned earlier, bacteria of genus *Treponema* do not stain well with Gram stain. In studies of various necrotic skin ulcers in pigs, Fuchsin, or Giemsa are examples of stainings used to detect spirochetes in smears (Osborne & Ensor, 1955; Neitz & Canham, 1930; Nomi & Matsuo, 1922; Gilruth, 1910; Dodd, 1906). In Bergey's Manual of Systematic Bacteriology Giemsa is however reported to stain treponemes poorly (Norris *et al.*, 2011).

Direct identification of *Treponema* spp. without staining may be performed by dark field microscopy or phase contrast microscopy (Norris *et al.*, 2011; Smibert, 1991). The bacteria are identified based on size, morphology and motility. The method gives an immediate overview of treponemal presence in a sample and a trained eye may well differentiate treponemes from other spirochetes, even though species identification not is possible.

A common method to visualize the bacteria in tissue samples is by silver-staining; Warthin-Starry staining (Harcourt, 1973), modified Steiner staining (Read *et al.*, 1992), Levaditis method or Fontana's method are some examples (Osborne & Ensor, 1955; Gill, 1929; Noguchi, 1911). In Warthin-Starry silver staining (W-S) the treponemes are visible as black or brown spirals in the tissue. Spirochetes are differed from other bacteria by their characteristic morphology, but treponemes cannot be differed from other spirochetes, although habitat may give some clue. Another technique that have been used to detect *Treponema* spp. in sections is immunohistochemistry, often to confirm findings made by silver staining (Park *et al.*, 2013; Cruz *et al.*, 2005; Demirkan *et al.*, 1998).

A widely used molecular method for detection of *Treponema* spp. in tissue samples is PCR amplification of 16S rDNA, and identification of phylotypes through sequence homology (Choi *et al.*, 1997; Collighan & Woodward, 1997; Rijpkema *et al.*, 1997; Choi *et al.*, 1994). This method has naturally also been used for identification of isolates (Walker *et al.*, 1998). Other regions commonly used for identification and phylotyping are the 16S-23S rRNA intergenic spacer region 2 (Pringle & Fellström, 2010; Sayers *et al.*, 2009; Stamm *et al.*, 2009; Pringle *et al.*, 2008; Stamm *et al.*, 2002) or a flagellin gene, *flab2* (Brandt *et al.*, 2011; Evans *et al.*, 2009b; Evans *et al.*, 2008; Demirkan *et al.*, 2001).

Probes directed against 16S rRNA have been used to detect *Treponema* spp. by dot-blot analysis or Fluorescence *In Situ* Hybridization (FISH) in samples from BDD (Nordhoff *et al.*, 2008; Choi *et al.*, 1997), but also to visualize the

treponemes in sections by FISH (Klitgaard *et al.*, 2013; Rasmussen *et al.*, 2012; Klitgaard *et al.*, 2008; Nordhoff *et al.*, 2008; Moter *et al.*, 1998).

With the arrival of efficient and affordable massive parallel sequencing techniques new possibilities to explore bacterial presence and diversity in various types of samples have developed. This technique was recently used in a study specifically on *Treponema* spp. in BDD, where the results of phylotype occurrences and abundances were in accordance with results from FISH (Klitgaard *et al.*, 2013).

1.2.6 Habitat

Bacteria of genus *Treponema* are found in a broad range of different hosts and environments. The human pathogens may be found in lesions in skin and mucus membranes or if systemic infection occurs, in internal organs (Norris *et al.*, 2011). Skin-associated, non-pathogenic spirochetes have been found on human genital organs. Oral treponemes are present in dental plaque and in gingiva of humans, dogs and cats (Dewhirst *et al.*, 2012; Dewhirst *et al.*, 2010; Valdez *et al.*, 2000). Treponemes have also been associated with claw disease in cattle and sheep (Naylor *et al.*, 1998; Walker *et al.*, 1995), and other treponemes have been identified in bovine rumen (Stanton & Canale-Parola, 1979). In pigs, treponemes are known to occur in the gastrointestinal tract and in faeces (Nordhoff *et al.*, 2005; Cwyk & Canale-Parola, 1979). Recently, other treponemal species were isolated from ear necrosis, shoulder ulcers and gingiva of pigs (Pringle & Fellström, 2010; Pringle *et al.*, 2009).

Interestingly, treponemes are not only found in mammals, but are also present in the termite gut (Breznak, 2006). *Treponema* spp. are generally host-associated, but there are some examples of free-living treponemes. There are hypotheses that these free spirits may either be descendants or precursors of host-associated treponemes (Paster *et al.*, 1991).

1.3 *Treponema* spp. associated with disease in non-porcine animal species

This chapter focuses on diseases where a polymicrobial or polytreponemal etiology has been suggested, i.e. syphilis and endemic treponematosis (yaws, bejel, pinta) in humans or rabbit venereal syphilis will not be discussed.

1.3.1 Bovine digital dermatitis

Bacteria of genus *Treponema* are associated with bovine digital dermatitis (BDD). This infectious claw disease which causes lameness in cattle is also called papillomatous digital dermatitis (PDD) (Walker *et al.*, 1995), digital

dermatitis (Blowey & Sharp, 1988) or interdigital papillomatosis (Read *et al.*, 1992).

Spirochetes in cases of claw disease in cattle were reported as early as 1964 from USA (Gupta *et al.*, 1964), but the disease BDD was first described in Italy (Cheli & Mortarello, 1974). Also in this report were spirochetes mentioned, and when the disease was first reported in the UK, spirochetes were again observed (Blowey & Sharp, 1988). Since then the disease has emerged and is now recognised world-wide. The first herd outbreak in Sweden was reported in 2005 (Hillström & Bergsten, 2005).

The disease is characterized by red, moist circular lesions, sometimes described as "strawberry-like", on the plantar region of the feet, proximal to the interdigital cleft (Hillström & Bergsten, 2005; Read & Walker, 1998b; Read *et al.*, 1992; Blowey & Sharp, 1988). The lesions are more common on the hind feet. Swelling or fever is usually not seen, but lameness is a typical clinical sign and palpation of the lesions is painful. Herd outbreaks of BDD have a sudden onset and the disease tends to spread rapidly among the animals. Clinical cases respond well to topical treatment with oxytetracycline (Laven & Logue, 2006; Blowey & Sharp, 1988) and this is the treatment recommended in Sweden (Alenius *et al.*, 2013). Taken together these characteristics suggest an infectious (bacteriological) cause of the disease.

Various microbial agents have been identified from BDD lesions. A polymicrobial etiology has long been discussed, but the consistent findings of *Treponema* in BDD together with an invasiveness in skin point towards an involvement of *Treponema* in the etiology of the disease (Klitgaard *et al.*, 2013; Santos *et al.*, 2012; Brandt *et al.*, 2011; Yano *et al.*, 2010b; Evans *et al.*, 2008; Nordhoff *et al.*, 2008; Moter *et al.*, 1998; Read *et al.*, 1992).

The first isolation of treponemes, later confirmed as *T. medium*/*T. vincentii*-like, *T. phagedenis*-like and *T. denticola*-like (Stamm *et al.*, 2002; Walker *et al.*, 1998), from BDD was performed from Californian cattle (Walker *et al.*, 1995). Since then, these phylotypes have repeatedly been isolated from cows with BDD (Yano *et al.*, 2009; Evans *et al.*, 2008; Pringle *et al.*, 2008; Trott *et al.*, 2003; Demirkan *et al.*, 1999). A strain belonging to the *T. denticola*/*T. putidum*-like group of treponemes was later proposed as a new species, *T. pedis* (Evans *et al.,* 2009b), and recently it was suggested that *T. phagedenis*-like isolates (Trott *et al.*, 2003) should in fact be designated as *T. phagedenis* (Wilson-Welder *et al.*, 2013). *Treponema brennaborense* has so far only been isolated in BDD-lesions from German cattle (Schrank *et al.*, 1999).

It should be noted that far from all treponemes are cultivable. An increasing number of treponemal phylotypes have been identified by molecular techniques (Klitgaard *et al.*, 2013; Rasmussen *et al.*, 2012; Klitgaard *et al.*,

2008; Choi *et al.*, 1997). The importance of different phylotypes is unknown, although some phylotypes seem to occur more frequently, or reside deeper in the lesions than others (Klitgaard *et al.*, 2013; Santos *et al.*, 2012; Yano *et al.*, 2010b; Nordhoff *et al.*, 2008; Moter *et al.*, 1998).

The evidence of treponemal involvement in the etiology of BDD is mainly circumstantial, but there are reports on reproduction of disease in experimental studies. Successful results have been seen in challenge studies where BDD lesion material has been used (Read & Walker, 1998a; Read & Walker, 1996). In addition, one research group recently induced BDD, or incipient BDD, in an experimental study by inoculation of cattle with either biopsy material from a BDD lesion containing *Treponema* spp. or a pure *Treponema* sp. isolate (Gomez *et al.*, 2012). The results from that study suggest a causative role of *Treponema* spp. in BDD, but the study needs to be repeated on a larger number of animals.

1.3.2 Ulcerative mammary dermatitis

Another skin disorder in cattle is ulcerative mammary dermatitis (UMD). UMD is a necrotic dermatitis located at the cranial junction of the udder with the abdominal wall, or in the median intermammary groove of the udder (Beattie & Taylor, 2000). The lesions in the skin may rapidly progress to severe, deep ulcers with a characteristic, foul smell (Boyer & Singleton, 1998). Sudden increases in herd occurrence of UMD have been described (Beattie & Taylor, 2000; Boyer & Singleton, 1998). The cause of UMD is not well understood, but a necrosis caused by edema around calving has been proposed (Blowey & Edmondson, 1995). In one study from England, a mixed flora of bacteria and fungi, including spirochetes, was present in samples from UMD, but not considered as the initiating cause of the dermatitis (Beattie & Taylor, 2000).

Specific investigations of *Treponema* spp. in UMD samples have shown they are closely related to treponemes found in BDD (Stamm *et al.*, 2009; Stamm *et al.*, 2003; Keil *et al.*, 2002). An etiologic link between UMD and BDD has therefore been hypothesized (Stamm *et al.*, 2009; Stamm & Trott, 2006; Boyer & Singleton, 1998). Others suggest a polymicrobial etiology is more likely, and that *Treponema* spp. are of less importance in UMD, based on the absence of certain treponemal phylotypes in one study (Evans *et al.*, 2010). A causative relationship between treponemes and UMD has not been shown.

1.3.3 Contagious ovine digital dermatitis

It has long been known that spirochetes may be found in footrot of sheep (Blaizot & Blaizot, 1929). With the advent of a new, severe virulent footrot

clinically different from classic footrot (Harwood *et al.*, 1997), it was hypothesized that spirochetes (and not *Dichelobacter nodosus*) may have a role in the etiology (Demirkan *et al.*, 2001; Collighan *et al.*, 2000). This was based on one report, where spirochetes (together with other bacterial species) were isolated from a lesion, but not *D. nodosus* (Naylor *et al.*, 1998). The hypothesis was later revised, when both spirochetes and *D. nodosus* were identified in cases of classic footrot as well as of CODD (Moore *et al.*, 2005).

Ovine spirochetes from CODD have been affiliated to genus *Treponema* (Demirkan *et al.*, 2001; Collighan *et al.*, 2000). Species that have been isolated are those also isolated from BDD; *T. phagedenis*-like, *T. medium*/T. *vincentii*-like *and T. putidum/T. denticola*-like (Sayers *et al.*, 2009; Evans *et al.*, 2008; Demirkan *et al.*, 2001; Collighan *et al.*, 2000; Naylor *et al.*, 1998; Walker *et al.*, 1995). The findings of the same *Treponema* species from cattle and sheep have suggested that transmission may occur during co-grazing (Sayers *et al.*, 2009; Demirkan *et al.*, 2001; Collighan *et al.*, 2000). The evidence of treponemal association to CODD is however less extensive than those of a link between BDD and *Treponema* spp.

1.3.4 Periodontitis

Periodontitis, an inflammation in the periodontium, is common in the human population. The etiology is considered polymicrobial. Bacteria normally residing in the tooth pockets cause gingivitis and the inflammation eventually leads to destruction of the tooth supporting tissues and alveolar bone loss (Visser & Ellen, 2011). The bacterial community in the oral cavity is very complex. One individual can host approximately 100-300 species (Griffen *et al.*, 2012), and in the human oral microbiome >600 taxonomic units have been identified (Dewhirst *et al.*, 2010). In a study on the canine oral microbiome >350 species were detected (Dewhirst *et al.*, 2012).

Studies on periodontitis have been hampered by the difficulties in studying complex microbial communities (Griffen *et al.*, 2012) but certain species have been associated with disease. These are the “red complex” bacteria *Tannerella forsythia*, *Porphyromonas gingivalis* and *T. denticola* (Holt & Ebersole, 2005; Socransky *et al.*, 1998). A recent study using massive parallel sequencing techniques have confirmed these results and pointed out additional species possibly involved in the etiology (Griffen *et al.*, 2012). Significant differences were shown comparing which phyla, genera and species were more common in individuals with healthy gingivae versus patients with periodontitis. Spirochetes were one of the phyla associated with disease, and it was also

shown that the occurrence of periodontal disease was associated with an increase in microbial diversity.

In the oral cavity, genus *Treponema* is represented by a range of species. In addition to *T. denticola*, *T. vincentii/T. medium* and *Treponema socranskii* are examples of species more common in disease than in health, as well as a number of unnamed and uncultivated treponemes (Griffen *et al.*, 2012). *Treponema* spp. have also been associated with periodontal disease in dogs (Nordhoff *et al.*, 2008).

1.4 Skin ulcers in pigs, general descriptions

1.4.1 Shoulder ulcers

Shoulder ulcers are pressure ulcers developing in the skin over the *tuber spina scapulae* of sows (Fig. 2). The problem is usually seen during the lactation period the weeks after farrowing (Davies *et al.*, 1996). Internationally peer-reviewed scientific publications on shoulder ulcers are limited, and therefore several non-peer-reviewed sources are referred to in this chapter.

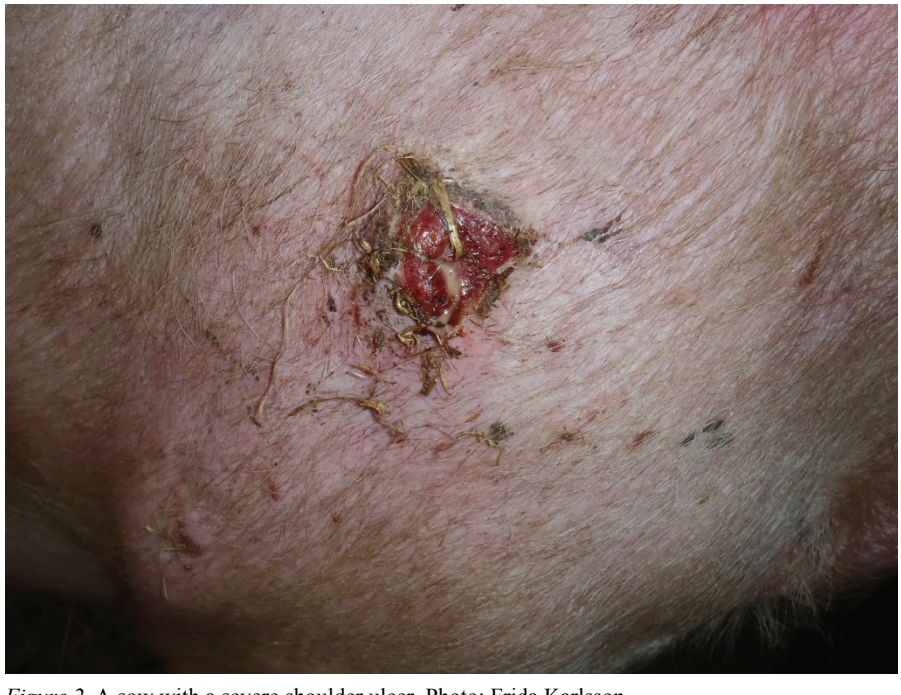

Figure 2. A sow with a severe shoulder ulcer. Photo: Frida Karlsson

Animal welfare and economic impact

Shoulder ulcers are considered as a welfare problem in pig production (Thorup, 2006; Zurbrigg, 2006; Rosendal & Nielsen, 2004; Davies *et al.*, 1997), and research has intensified during the last years. There is not much information available regarding pain or suffering associated with shoulder ulcers, and anecdotal reports from farmers and experienced veterinarians propose that sows with shoulder ulcers are not in pain (Reese *et al.*, 2005). Recent publications from Denmark suggest the opposite (Dahl-Pedersen *et al.*, 2013; Herskin *et al.*, 2011).

Another motive for studying shoulder ulcers is the economic impact the occurrence of ulcers may have on production. Some authors suggest that sows with shoulder ulcers are culled earlier, leading to inefficient production (Zurbrigg, 2006). Not all farms, however, use the presence of a shoulder ulcer as a criterion for slaughter of a sow (Davies *et al.*, 1996).

Prevalence

The prevalence of shoulder ulcers varies between herds and countries. The investigations are not always comparable due to variation in study design. It is also important to remember that housing systems vary over time and between countries.

In her Master's thesis, Billström examined 102 slaughtered sows at an abattoir in Sweden (Billström, 2007). Of these, 20.6% had shoulder ulcers or scars from healed ulcers. In a field study including 2578 sows in 60 Swedish pig herds, 34% of the sows had shoulder lesions (Ivarsson *et al.*, 2009). All registrations were performed during the third to fifth lactation week. In another study from Sweden, over 12000 sows in 148 herds were visited by veterinarians from Swedish Animal Health Service. Both lactating and pregnant animals were investigated. The occurrence of shoulder ulcers was 17.9% in lactating sows, and 2% in pregnant sows (Johansson, 2010).

In Denmark, a slaughter house study of 23794 culled sows from 207 herds reported a within-herd median prevalence of 7.2% (Cleveland-Nielsen *et al.*, 2004b). This was assumed to be an underestimation due to the fact that euthanized or exported animals not were included, and that the study was based on meat-inspection. In a Norwegian investigation at four abattoirs 10.2% of the in total 3048 investigated sows had shoulder ulcers (Baustad & Fredriksen, 2006).

An American study involving 1916 sows from one herd reported a prevalence of 8.3% (Davies *et al.*, 1996). That study included sows both from the farrowing and gestation houses. In a slaughter house study in United States the prevalence was 4.6% in 1751 sows (Ritter *et al.*, 1999). In another

American study conducted at a slaughter house, 12.5% of the 3158 culled sows had shoulder lesions (Knauer *et al.*, 2007). In England, KilBride *et al.* found a shoulder lesion prevalence of 10.4% in 288 sows from 86 farms (KilBride *et al.*, 2009). The prevalence in sows kept indoors was 12.1%, compared to an outdoor prevalence of 2.4%.

Risk factors

Many researchers agree that the cause of shoulder ulcers is multifactorial (Rosendal & Nielsen, 2004; Davies *et al.*, 1997; Davies *et al.*, 1996) and a number of risk factors have been identified. Davies *et al.* (1996) showed that ulcer prevalence was associated with a low body condition score. This has been confirmed in several studies (Ivarsson *et al.*, 2009; Knauer *et al.*, 2007; Zurbrigg, 2006; Rosendal & Nielsen, 2004; Davies *et al.*, 1997) and is usually explained by the fact that thin sows have less fat protecting the shoulder area from pressure. In the study by Davies *et al.*, an increased thickness of soft tissue covering the *tuber spina scapulae* reduced the risk of developing lesions (Davies *et al.*, 1997). A low back fat level at farrowing is associated with the development of shoulder ulcers (Thorup, 2006; Davies *et al.*, 1997). Zurbrigg (2006) performed flank-to-flank measurement and showed that sows with a low measure (97-104 cm) had an increased risk of developing shoulder ulcers compared to sows with a high measure (104.5-113.5 cm).

Prevalence of ulcers or scars from ulcers increases with parity (Rosendal & Nielsen, 2004; Davies *et al.*, 1997; Davies *et al.*, 1996). Davies *et al.* (1997) suggested this was due to an increasing body weight of older sows and that larger sows have less space in farrowing crates. It has also been shown that sows with signs of previous lesions have a higher risk of developing new lesions (Thorup, 2006). In a study by Zurbrigg (2006), sows in first parity and above fifth parity were at greater risk of developing shoulder ulcers. The increased risk for gilts was assumed to be attributed to longer lying periods.

Long periods of lateral recumbency have been mentioned as a potential risk factor in several studies (Zurbrigg, 2006; Davies *et al.*, 1997; Davies *et al.*, 1996). In one study from Sweden a correlation was found between the duration of the longest uninterrupted lying time for a sow, and the development of a shoulder ulcer (Rolandsdotter *et al.*, 2009). That was however a small study conducted in one herd, and the correlation was only observed for the right side. There are also studies indicating that lameness or MMA (metritis, mastitis and agalactiae syndrome) may increase the risk of developing shoulder ulcers (Ivarsson *et al.*, 2009; Rosendal & Nielsen, 2004; Nouws *et al.*, 1981). This has been explained by increased periods of recumbency for lame sows or animals with disease.

Another sow-related risk factor mentioned in the literature is the height of *tuber spina scapulae* (Penny & Muirhead, 1986b), however without any references. In her Master's thesis, Billström did not find a significant difference in the height of *tuber spina scapulae* between sows with or without shoulder ulcers (Billström, 2007).

Several environmental or herd related risk factors have also been investigated. In a Danish study Cleveland-Nielsen *et al.* found that having two instead of one person working in the farrowing stables increased the risk for shoulder ulcers, as well as using housing systems where the sows were tethered (Cleveland-Nielsen *et al.*, 2004a). Having one's own recruitment of gilts and using hospital pens decreased the risk. A higher level of welfare in the herd also decreased the risk of shoulder ulcers. This was defined as the use of straw, a 30% increase of space per pig than mandatory, solid floor in ≥ 60% of the pens for growers and finishers, four weeks as a minimum age for weaning, no use of antimicrobials as growth promoters and having loose-housed gestating animals.

Type of flooring is often discussed having an influence on the development of shoulder ulcers. It has been hypothesized that slatted floor increase the risk compared to solid floor and that floor material may have an importance (Zurbrigg, 2006; Davies *et al.*, 1996). In a study from England, slatted, wet or damaged floors increased the risk for shoulder ulcers (KilBride *et al.*, 2009). It was also showed that sows in crates with less space were at greater risk. In one Swedish study flooring and confinement of sows were studied (Ivarsson *et al.*, 2009). Confinement did not influence the frequency of shoulder ulcers, but there was an increased risk of shoulder ulcers for sows in pens where the slatted part of the floor consisted of plastic material compared to cast iron or concrete. No significant difference was observed between pens with a small part of solid floor (< 1.5m^2) compared to pens with a large part of solid floor (> 2.3m^2). Floor was also investigated in another study from Sweden (Holmgren & Lundeheim, 2010). The study was conducted in one herd, and the sows were all loose-housed in pens with partly slatted floor. The study compared size of solid floor (2.1m^2 or 1.3m^2) and structure of slatted floor (plastic with ridges or plastic without ridges). No significant differences were seen between the different types of floors. The position of the sows was also recorded. Sows often positioned on the slatted floor were at greater risk of developing ulcers than sows more seldom observed lying on the slatted floor.

Pathology and patogenesis

The actual progression from intact skin to ulcer has been a subject of discussion for many years. The big issue seems to be if the ulcers develop from

top-to-bottom, or bottom-to-top. As reviewed by Herskin *et al.,* parallels are often drawn between sow shoulder ulcers and human pressure ulcers (Herskin *et al.*, 2011). Due to similarities between human and porcine skin, pigs have been used as animal models for human pressure ulcers (Kokate *et al.*, 1995; Daniel *et al.*, 1981; Dinsdale, 1974; Dinsdale, 1973). Because research on shoulder ulcers is limited, knowledge about the pathology of pressure ulcers in pigs has been based on results from these studies.

In studies on normal and paraplegic pigs, Dinsdale applied pressure, or pressure combined with friction, to the skin above the posterior superior *spina iliaca*, and studied the alterations of the skin by microscopy (Dinsdale, 1974; Dinsdale, 1973). It was shown that animals were more prone to develop skin ulcers when exposed to both pressure and friction. From his results Dinsdale concluded that decubital ulcers were caused by a combination of at least two factors; ischemic pressure and friction.

Dinsdale was later criticized by Daniel *et al.* due to the fact that only superficial ulcers had been induced (Daniel *et al.*, 1981). Therefore only the skin and not the deep tissue had been studied. Daniel *et al.* reviewed previous animal models for human pressure sores and developed another porcine experimental model by applying pressure towards *trochanter major* of *femur* of normal and paraplegic pigs. It was shown that muscle damage occurred before damage to the skin. The authors stated that there were indications of a progression of the lesions from the musculature and outwards towards the skin. Daniel *et al.* emphasized that normal skin is very resistant to ischemia. It was hypothesized that this resistance may be lowered due to changes in the soft tissue caused by paraplegia, infection or repeated trauma/mechanical injury, and that pressure ulcers therefore may develop easier in such cases (Daniel *et al.*, 1981). Kokate *et al.* showed that pressure in combination with an elevation of temperature increased ulceration (Kokate *et al.*, 1995).

None of the above mentioned authors, as pointed out by Herskin *et al.* (2011), studied naturally occurring skin lesions in the shoulder area of pigs. There is a limited amount of studies regarding the pathology of shoulder ulcers in pigs. In his Master's thesis from 2003, Lund performed a thorough evaluation of shoulder ulcers, describing the gross pathology of ulcers from 3200 slaughtered sows and histopathology of 38 ulcers (Lund, 2003). A pathoanatomical scale (0-4) for the grading of shoulder ulcers was developed. The scale was further used in a study by Jensen, where 95 shoulders without ulcers and 421 shoulders with ulcers were cross-sectioned, examined by gross pathology and scored (Jensen, 2009). Histopathological evaluation was performed on approximately half of the shoulders, with an even distribution of non-ulcers and ulcers of all grades. The stage 1 ulcerations were characterized

by a missing or necrotic epidermis. Necrosis was at the deepest located in the superficial dermis. Subcutis or underlying bone was not affected. In stage 2 the larger part of dermis was affected by necrosis, but not subcutis. In stage 3 subcutis was necrotic, either only the superficial part of subcutis or a major part. The stage 4 ulcerations were characterized by an exposed and deformed *tuber spina scapulae*. Presence of fibrosis and granulation tissue and infiltration of inflammatory cells were increasing from stage 1 to 4. The findings from this study were interpreted as indications of a development of shoulder ulcers from top-to-bottom. The author suggests a pathogenesis with an initial thrombosis leading to ischemia, with a secondary infection. He also agrees with previous authors that infection, further pressure and friction would worsen the lesions (Daniel *et al.*, 1981; Dinsdale, 1973).

Interestingly, at cross-sectioning, lesions were revealed in 45 of the 95 normal shoulders (Jensen, 2009). The majority of these lesions were detected caudally of the *tuber spina scapulae*. The lesions were located in the subcutis, and were either acute, characterized by hemorrage and edema and fibrin deposits, or chronic, with fibrosis. At gross inspection, no alterations were noted in the musculature or bone. No thrombosis or necrosis was seen. In a three cases there were mild signs of muscle regeneration and new bone formation. The author hypothesized that the subcutaneous findings most likely were caused by blunt trauma during transport or contact with farrowing crates. It was pointed out that the lesions were located caudal of the *tuber spina scapulae* and they were not considered as evidence for a bottom-to top development. It was emphasized that the findings did not resemble deep tissue injuries in humans, which may start from the musculature or osseous tissue.

The pathoanatomical scale (Lund, 2003) has since then been used by the Danish Veterinary and Food Administration (Herskin *et al.*, 2011) for grading of shoulder ulcers. One disadvantage with this scale is however the difficulties in correctly identifying the tissue involved by visual examination (Herskin *et al.*, 2011; Lund, 2003). Often a histopathological evaluation of underlying bone tissue is required, which is difficult to perform on live animals. A clinical scale useful in the field and on live pigs has therefore been requested. Recently, such a scale was developed and evaluated (Jensen *et al.*, 2011). That clinical scale has been used in this work and is further explained in the material and methods section. An attempt to develop an official scale for clinical use in Sweden has been made in a student's report, but this scale has to the author's knowledge not yet been evaluated (Hedfors, 2011).

Bacteriology

Information on infections of shoulder ulcers is limited. It is often generally stated that shoulder ulcers may be infected (Dahl-Pedersen *et al.*, 2013) but without references to sources that confirm this statement. In the study by Davies *et al.* signs of infection were rare (Davies *et al.*, 1996). No sampling or culturing was however performed. Several pathological investigations also mention microcolonies of bacteria in the tissues, but without any further specification (Jensen, 2009).

In a study from Holland, bacteriological examination was performed on 315 slaughtered pigs with shoulder ulcers (Nouws *et al.*, 1981). Of these, 58.4% were positive for bacteria. Of ulcers with a diameter of > 5cm, 69.2% were positive compared to 39.8% of smaller ulcers. In more than 95% of the cases with shoulder ulcers positive for bacteria, *Trueperella pyogenes* was isolated from the spleen. In his Master's thesis, Lund investigated 33 shoulder ulcers by aerob culturing on blood agar plates (Lund, 2003). From 28 of the ulcers bacterial growth was confirmed. In 50% of the culture positive ulcers bacterial growth was also confirmed from the adjacent lymph nodes. The most commonly found bacterial species was *T. pyogenes*. Other bacteria found were β-hemolytic streptococci, *Staphylococcus aureus*, *Actinomyces hyovaginalis*, *Proteus* spp. and *Bacillus* spp.

1.4.2 Ear necrosis

Ear necrosis is a syndrome normally affecting young pigs a couple of weeks after weaning, or during the early grower period. The malady is described under a variety of names in the literature, such as porcine ulcerative spirochetosis (Harcourt, 1973; Blandford *et al.*, 1972), streptococcal auricular dermatitis (Maddox *et al.*, 1973), ear biting (Penny & Mullen, 1975), necrotic ear syndrome or ulcerative spirochetosis of the ear (Cameron, 2012). These names usually reflect the writers' hypotheses about the etiology of the disease. Richardson *et al*. (1984) suggested usage of the term "porcine necrotic ear syndrome" until the cause of the disease had been determined. Lately the shorter terms ear necrosis or ear necrosis syndrome have been commonly used (Park *et al.*, 2013; Busch *et al.*, 2010a; Lang, 2010b; Lang, 2010a; Lang, 2010c; Pringle *et al.*, 2009; Busch *et al.*, 2008a; Busch *et al.*, 2008b; Hansen & Busch, 2008).

Gross pathology

The lesions may be situated anywhere on the pinna, but the most common examples involve the margin, either the ventral margin (Park, 2011; Busch *et al.*, 2008a; Richardson *et al.*, 1984; Harcourt, 1973) or the tips of the ear

(Weissenbacher-Lang *et al.*, 2012; Park, 2011; Mirt, 1999; Richardson *et al.*, 1984). In severe cases the lesions are spread to involve a major part of the pinna (Blandford *et al.*, 1972), and affected pigs may lose the whole or a part of the ear (Park *et al.*, 2013; Richardson *et al.*, 1984).

In one study the progression of the macroscopic lesions of ear necrosis was followed (Richardson *et al.*, 1984). Twelve days after weaning, superficial scratches were seen on the tip, caudal or ventral part of the pigs' ears. These were regarded as caused by fighting among the pigs. In rare cases vesicles were seen, later progressing to erosions. The lesions were surrounded by mild edema and erythema. Some expanded locally and became covered with crusts.

On day twenty-six after weaning, the lesions could be divided into two groups; progressing severe or healing. The severe lesions were characterized by exudation, edema, hyperemia and ulceration. The tissue along the margin of the ear was necrotic. In some cases signs of bacteremia were seen. In other cases erosions were spread to a large part of the ear, or a cellulitis developed, the whole ear turned necrotic and was eventually lost.

A similar clinical picture, although less detailed, was given by Harcourt *et al.* (1973) from an outbreak of ear necrosis in one herd. The lesions were observed two to three weeks after weaning, started from the ventral part of the ears and were spread along the edge of the pinna. Harcourt noted that the lesions were bilateral. A ventral, bilateral occurrence of ear lesions was also observed in field cases (Penny & Mullen, 1975), in a slaughterhouse study (Penny & Hill, 1974), and in a cross-sectional study in Denmark, where 75% of the observed ear necroses were ventral, 25% were located to the ear tip, and 70% of the ventral lesions were bilateral (Busch *et al.*, 2008a). It should be noted, however, that even though ear necrosis may occur either on the tip or on the ventral part of the ear, it should be differed from damage due to circulation disturbances caused by systemic diseases (Park *et al.*, 2013; Cameron, 2012).

Histopathology

The histopathological picture naturally varies with the age, severity and progression of the lesions. To the author's knowledge, the most thorough histopathological investigation was performed by Richardson *et al.* (1984). This study revealed two histologic patterns in accordance to the stages of the gross lesions. In the early stage, where vesicles could be observed macroscopically, degeneration of basal cells in epidermis with forming of intraepidermal vesicles was detected microscopically. In dermis only mild changes as edema and infiltration of mononuclear cells could be observed. In the more progressive stage, hyperkeratosis, acanthosis and abscesses within epidermis were common findings. In many cases the epidermal-dermal

junction was damaged, resulting in focal ulcers. The reaction in dermis was characterized by edema, congestion and abundant neutrophils. In cases where the ulceration expanded the dermal reaction was more severe. Neutrophils were dominating closer to the surface and macrophages and fibroblasts were located deeper. In the even more severe cases, where extensive necrosis had developed, the changes in dermis were of a more chronic nature. This meant active granulation tissue and/or fibrosis, covered by necrotic tissue. In the ears with signs of cellulitis, both vasculitis and thrombosis leading to necrosis was common.

The main histological findings by Richardson *et al.* (1984) have been described by others (Park *et al.*, 2013; Weissenbacher-Lang *et al.*, 2012; Mirt, 1999; Harcourt, 1973; Blandford *et al.*, 1972), with some exceptions. None of the other investigators have found the intraepidermal vesicles associated with the very early stage of the lesions. Harcourt (1973) noted that there was little inflammation in the investigated sections, and Blandford *et al.,* (1972), found the sections in their study less purulent than expected. Weissbacher-Lang *et al.* (2012) considered focal epidermal necrosis as the initial lesion in their samples. In addition, they noted re-epithelisation in the healing lesions. Park *et al.* (2013) found a minor part of the investigated sections to have signs of vasculitis or thrombosis. From these results they suggested the vascular alterations were secondary to inflammation in the epidermis.

Risk factors

Several risk factors for development of ear necrosis have been identified on herd level. In one study from Denmark, including over 30 000 pigs, high density of pigs, fully slatted floor, diffuse air intake and purchased feed showed an association with an increased risk of developing ear necrosis (Busch *et al.*, 2008b). It was suggested that diffuse air intake would influence the climate in the pig stalls, affecting both animal behavior and skin condition, thereby increasing the risk of infection. In a similar manner, high stocking rate would decrease the space and the availability to the feed dispensers, causing aggression among pigs. In another Danish study, risk factors on pen level and pig level were studied in one herd (Busch *et al.*, 2008a). In this study, increasing number of pigs in the pen increased the risk of ear necrosis. Pigs that were large at weaning or had scratches on the ears short after weaning had a higher risk of developing mild ear necrosis, but not severe ear necrosis. In a recent study from Canada, high humidity, ear biting and early weaning of pigs were associated with ear necrosis. The writers emphasized that the study was small and further studies were necessary.

Factors possibly contributing to disease, but not yet proved as risk factors, have also been discussed in the literature (Weissenbacher-Lang *et al.*, 2012; Richardson *et al.*, 1984; Penny & Mullen, 1975; Harcourt, 1973). These include e.g. suckling behavior, fighting, poor environment, dirt or feed on the ears, virus, bacteria, parasites and mycotoxins.

Bacteriology

Coccoid bacteria, cocco-bacillary bacteria and spirochetes have been microscopically confirmed in smears from ear lesions (Harcourt, 1973). Both staphylococci and streptococci have been isolated in several studies (Park *et al.*, 2013; Weissenbacher-Lang *et al.*, 2012; Hansen & Busch, 2008; Mirt, 1999; Richardson *et al.*, 1984). Due to the frequent findings of *Staphylococcus hyicus*, hypotheses have been formed suggesting ear necrosis as a localized form of exudative epidermitis (Park *et al.*, 2013; Mirt, 1999). Others find this sole explanation unlikely (Penny & Smith, 1999). One common hypothesis is that an initial trauma with a subsequent infection (possibly staphylococci and/or streptococci) is the cause of the syndrome (Park *et al.*, 2013; Richardson *et al.*, 1984; Maddox *et al.*, 1973). Another hypothesis, to be elaborated in this thesis, is that spirochetes are involved in the etiology of the disease (Pringle *et al.*, 2009; Harcourt, 1973; Blandford *et al.*, 1972). In a recent study from Canada the presence of both staphylococci and spirochetes of genus *Treponema* in ear necrosis was investigated (Park, 2011). Cultivation of *Treponema* spp. did not succeed, and only 8.6% of the samples were positive for spirochetes in Warthin-Starry silver staining.

In spite of the many investigations and the broad range of hypotheses about background factors and etiology, the cause of ear necrosis remains unknown.

1.4.3 Facial ulcers

Facial ulcers are most common in piglets less than one week of age (Cameron, 2012). Other names used to describe the condition are facial necrosis, facial pyemia, facial traumatic dermatitis or infectious necrosis of the cheeks (Hungerford, 1990; Cameron, 1984). Facial ulcers are caused by infection of traumatic injuries in the skin, many times caused by biting by other piglets (Cameron, 2012). The piglets fight about the teat order, and the problem is more often seen in large litters or in litters of a sow with agalactia. Weak piglets are more commonly affected. The initial skin lesions may spread to involve the whole face, eyes and lips and affected animals may starve to death. The ulcers are often bilateral and covered by a crust (Fig.3).

To prevent these problems, high hygienic standards, cross-fostering of pigs from large litters and prevention of agalactia are recommended (Cameron,

2012). In international literature tooth clipping with well disinfected instruments is recommended to prevent facial ulcers. This is forbidden in Sweden, and tooth grinding is not to be performed by routine. One Swedish study did not find any differences in occurrence of facial lesions in litters where the piglets' teeth had been grinded compared to control litters with ungrinded teeth (Hansson & Lundeheim, 2012). That was however a small study in only one herd. Some writers point out that tooth clipping may induce an entrance for bacteria through an exposed pulp, especially if not correctly performed (Penny & Muirhead, 1986a). In certain literature facial ulcers are described as one syndrome together with necrosis of the gum (oral necrobacillosis or necrotic stomatitis) (Straw, 1992; Penny & Muirhead, 1986a).

Figure 3. A facial ulcer of a piglet. Photo: Olov Svartström

The literature on microbiological findings in facial ulcers is sparse. One writer describes facial ulcers as part of the "porcine spirochete complex" (Hungerford, 1990). Bacteria mentioned as infecting the ulcers are *Fusobacterium necrophorum*, spirochetes or *Streptococcus* sp. (Straw, 1992; Cameron, 1984; Penny *et al.*, 1971) referring to reports which contain limited information on the subject (Hutyra, 1938; Hindmarsch, 1937) (Simon & Stovell, 1969).

2 Hypothesis

Bacteria of genus *Treponema* play the main role when shoulder ulcers and ear necrosis occur in an infectious or severe form, and perhaps also in other skin conditions in the pig. The treponemes reside in the mouth of the pigs, and are transferred to the skin through biting and licking behaviour.

3 Aims

- To determine the occurrence and diversity of *Treponema* spp. in skin lesions in pigs
- To isolate and characterize *Treponema* spp. from ear necrosis, shoulder ulcers and gingiva of pigs.
- To investigate a possible association between *Treponema* spp. from gingiva and porcine skin lesions.
- To study the localization and species-specific distribution of treponemes in shoulder ulcers and ear necrosis.
- To study if *Treponema pedis* can cause ear necrosis in a challenge model.
- To investigate the humoral response in pigs to recombinant *Treponema pedis* proteins.

4 Comments on materials and methods

4.1 Paper I, II and III

4.1.1 Animals and ulcers

From April 2010 until December 2011, eighteen Swedish pig herds with a history of shoulder ulcers or ear necrosis were visited. An overview of herds and sampled animals with skin ulcers is given in tables 2 and 3. The farms were selected based on contact with the herd veterinarian. It was not known in beforehand how many animals that would be affected at the time of the herd visit. Many of the "shoulder ulcer herds" were visited during the last weeks of lactation, and most "ear necrosis herds" were visited one to two weeks after the appearance of the first cases.

The aim was to sample 10 animals in each selected herd, but in practicality the number depended on the availability of animals with ulcers at the day of the visit. Furthermore, on some of the farms the available time to perform the sampling was limited. The animals to be sampled were selected at the time of visit, following a tour in the animal stables.

In addition to the animals with ulcers included in the tables 2 and 3, 60 piglets of 30 sows with shoulder ulcers were sampled from gingiva. Furthermore, one herd reporting problems with facial necrosis was visited and four animals sampled. In one "ear necrosis herd", in addition to the pigs with ear necrosis, five pigs with other types of skin ulcers were sampled.

In the visited herds no healthy pigs were sampled as controls, as we had no ethical permission to do this. Comparison material was collected from eight sows and six finisher pigs at slaughter. These slaughter house samples cannot be considered a substitution for outright controls, but provided information about differences in occurrence of *Treponema* spp. in ulcers compared to healthy skin tissue.

Table 2. *An overview of herds and sampled animals with shoulder ulcers (n=52)*

Shoulder ulcers						
Herd	No. of sampled animals	Date of sampling	Type of pig	Appr. age[1]	Breed[2]	Type of herd
A	7	100614	Sow	Unknown	LxY	Conventional
B	3	100901	Sow	Unknown	LxY	”
C	4	101026	Sow	Unknown	LxY	”
D	6	101119	Sow	1-2 y	LxY	”
E	3	110111	Sow	2-4 y	LxY	”
F	5	110113	Sow	2-3 y	L	Gilt producer
G	6	110223	Sow	2-3 y	LxY	Conventional
H	6	110614	Sow	Unknown	LxY	”
I	5	110825	Sow	Unknown	LxY	”
J	7	111017	Sow	1-2 y	LxY	”
Total	52					

[1.] y=years
[2.] Y=Yorkshire, L=Landrace

Table 3. *An overview of herds and sampled animals with ear necrosis (n=57)*

Ear necrosis						
Herd	No. of sampled animals	Date of sampling	Type of pig	Appr. age[1]	Breed[2]	Type of herd
K	5	100429	Grower	14 w	Y, H	Boar station
L	10	101025/101110	Grower	13-14 w	LxYxH	Conventional
M	4	101208	Weaner	12-13 w	LxY	”
N	10	110216	Weaner	9-13 w	LxY	Organic
O	5	111007	Weaner	12 w	LxYxH	Conventional
P	9	111109	Weaner	10 w	LxYxH	”
Q	9	111201	Weaner	12 w	LxYxH	”
R	5	111219	Weaner	8-9 w	LxYxH	”
Total	57					

[1.] w=weeks
[2.] Y=Yorkshire, H=Hampshire, L=Landrace
(Table 2 and Table 3 modified from Paper I)

4.1.2 Grading of ulcers

Before sampling the ulcers were graded. The sampled shoulder ulcers represented all variants of ulcers from mild to severe. The system used to grade shoulder ulcers in this study has recently been developed (Jensen *et al.*, 2011).

According to this system a severe shoulder ulcer is ≥ 5cm in diameter, with a thickened margin. A mild ulcer is ≥ 2cm, but without the characteristics of a severe shoulder ulcer. Ulcers < 2cm do not count as ulcers. As reviewed by Herskin *et al.* (2011) there are several scales for classifying shoulder ulcers. These scales differ only slightly between themselves, are often divided in multiple steps and some of them also require post-mortem investigations. The grading system by Jensen *et al*. (2011) was selected because of its simple and clinical approach and as it was possible to use ante-mortem.

In Denmark, Busch *et al*. have used two different numerical scales to score ear necrosis, ranging from 0-4 and 0-3 (Busch *et al.*, 2010a; Busch *et al.*, 2010b; Busch *et al.*, 2008a; Hansen & Busch, 2008; Busch *et al.*, 2007). We found these scoring systems too detailed and therefore decided to divide the ear necroses in two groups, mild or severe, similar to the shoulder ulcers. Severe ear necroses were defined as lesions involving 1/3 or more of the ventral margin of the ear (Fig. 4). Less spread ear lesions were classified as mild. According to the pictures in the reports by Busch *et al.*, a score of 1-2 would correspond to a mild ulcer and a score of 3-4 to a severe ulcer in our study, but this is only a rough estimation (Busch *et al.*, 2010b; Busch *et al.*, 2007).

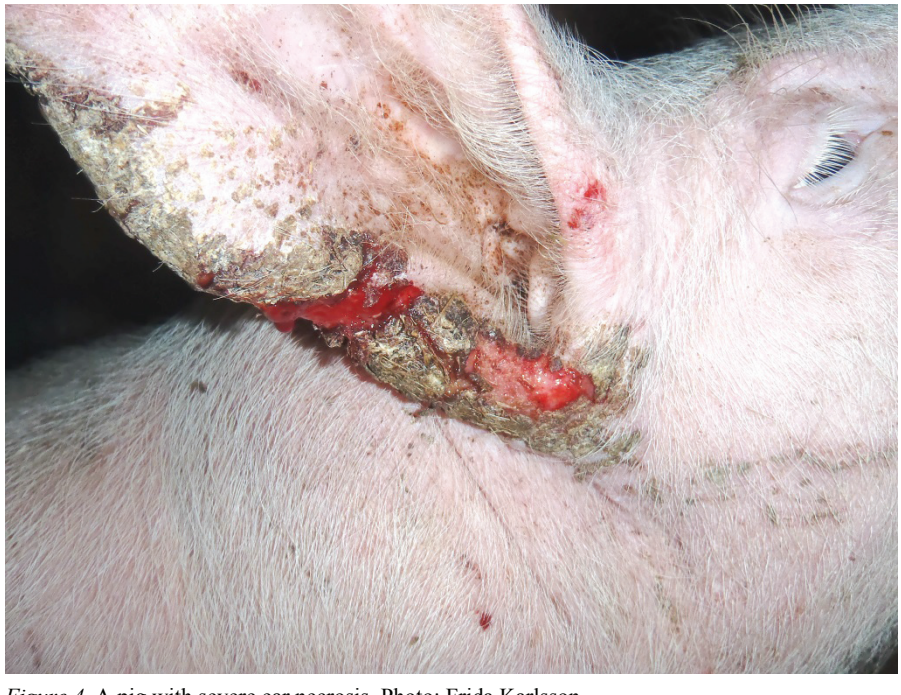

Figure 4. A pig with severe ear necrosis. Photo: Frida Karlsson

4.1.3 Sampling

Assessment of the ulcers was followed by sampling. In order to get enough material both for culturing and PCR, material was collected from the ulcer using a sterile scalpel blade. For sampling of the mouth both cotton swabs or cotton rope used when straining the pigs were tested. When analyzing the PCR results from the first herds (unpublished material), the samples from the cotton ropes were all negative. It was suspected that extra material stuck to the cotton rope (such as feed and straw material) inhibited the PCR. Therefore this method was abandoned in the continued studies and cotton swabs were used instead. When sampling the mouth with cotton swabs, the intention was to collect the sample close to the dental pockets, as the treponemes reside in these pockets, providing an anaerobic environment. However, sampling the dental pockets of live, non-sedated animals proved difficult in practice. In many cases the PCR on the gingival samples was negative, even though it can be expected that treponemes are present in the mouth of pigs, as in other animal species (Dewhirst *et al.*, 2012; Dewhirst *et al.*, 2010; Valdez *et al.*, 2000). A reason for these negative results could have been that we did not get enough material or did not sample deep enough in the tooth pockets.

One or two biopsies were taken from each ulcer for histopathological evaluation, Warthin-Starry silver staining and FISH. Attempts were made to collect the biopsy at the margin of the ulcer so that both damaged and healthy tissue would be included in the biopsy. Due to the degree of necrosis in especially some shoulder ulcers, sometimes this was not possible in practice.

From animals with ulcers, serum samples were collected during straining, from *vena jugularis externa.* An overview of the samples collected from the field is given in table 4. An example of a type of skin lesion sampled is given in figure 5.

Table 4. *Overview of samples collected from the field (Material in paper I, II, III)*

	Piglet[1]	Shoulder[2]	Ear[3]	Facial[4]	Other[5]
Scraping	-	52	57	4	5
Biopsy	-	51	54	2	5
Gingiva	30[6]	52	52	4	-
Serum	-	43	56	-	-

[1.] Piglet from sow with shoulder ulcer. [2.] Sow with shoulder ulcer. [3.] Pig with ear necrosis. [4.] Piglet with facial ulcer. [5.] Pig with other type of skin ulcer. [6.] 60 piglets were sampled from gingiva, the samples were pooled in pairs and analysed as 30 samples.

Figure 5. Example of other type of skin ulcers sampled. Photo: Frida Karlsson

4.1.4 ISR2-based PCR

With the intention to detect a possible transmission from mouth to skin ulcers, both sites were sampled and PCR performed on lysates from ulcer material and gingiva material. The region we chose to amplify is located between the genes for 16S rRNA and $tRNA^{Ile}$, a part of one of the two 16S-23S rRNA intergenic spacer regions (ISR). The 16S-23S rRNA intergenic spacer region is more variable than the 16S rRNA gene, and analyses of this region have a higher discriminatory resolution than analyses of the 16S rRNA gene (Stamm *et al.*, 2002). Bacterial species differ in the number of rRNA operons (Gürtler & Stanisich, 1996), the length of the interspacer regions and which tRNA genes they contain. Stamm *et al*. (2002) showed that *T. denticola*, *T. medium* and *T. phagedenis* as well as six BDD-associated *Treponema* isolates had two rRNA operons, the operon with ISR1 containing $tRNA^{Ala}$ and the operon with ISR2 containing $tRNA^{Ile}$. This had previously also been shown for *T. pallidum* subsp. (Centurion-Lara *et al.*, 1996). In the study by Stamm *et al.* (2002) it was only possible to detect one rRNA operon for *T. vincentii* (containing ISR1). A PCR protocol for the 16S rRNA-$tRNA^{Ile}$ intergenic spacer region of *Treponema* spp. was developed and it was suggested that interpretation of the length of the PCR product on a gel could be used as a rapid method to

discriminate between treponemal species. In our study, however, we could not discriminate between different phylotypes only by comparing fragment lengths, as some phylotypes showed similar lengths of their ISR2 fragments.

The amplicons were run on a gel, cloned, purified and sequenced as described in paper I. A large number of sequences were obtained. After sorting out identical sequences originating from the same sample site (an ulcer or gingiva from one animal), 132 unique sequences were used to construct a phylogenetic tree. This tree revealed a broad diversity of treponemes in both ulcers and mouths of pigs. Some of the phylotypes could not be species identified due to the lack of ISR2 sequences of described species deposited in GenBank. This was one disadvantage of using the ISR2 region for PCR. Should we have used the 16S rRNA region, identification on species level would probably have been possible for a larger number of clones. On the other hand a possible transmission pattern would most likely not have been detected because of less variability in the 16S rRNA gene. We chose the ISR2 region because it is highly variable with primer sites in conserved regions, and with the intention to use it as a fingerprinting method.

4.1.5 Culturing and isolation

Samples for culture of *Treponema* were taken from ulcers and mouths and transported anaerobically to the laboratory. Culture media were prepared as described in paper II. Before inoculating the media, ulcer material was examined for detection of spirochetes using a phase contrast microscope. In many cases *Treponema*-like spirochetes were observed in the microscope in samples which later did not result in growth of treponemes. It frequently occurred that treponemes were successfully cultured for a period, but later showed weak growth, died, remained as mixed phylotypes or proved impossible to isolate with our methods. This must be considered as normal when working with treponemes, as they are known to be extremely difficult to culture.

To obtain pure isolates 100 µl of broth containing motile *Treponema* bacteria were put on a membrane filter with pore size 0.22 µm on an FAA plate. This method was described by Smibert (1991). The treponemes moved through the filter and started growing in the FAA. A piece of agar could then be moved into a flask with fresh medium. If the new culture was assessed pure by phase contrast microscopy it was prepared for PCR and sequencing of the 16S rRNA gene and/or ISR2 (Paper II). This method of isolation was used as the growth on agar was very slow, resulting in diffuse and confluent colonies. The term “isolate” was used to clarify that we consider them as isolates, and not as pure strains.

4.1.6 Fingerprinting methods

To further characterize the isolates, two established fingerprinting methods were tested. For random amplified polymorphic DNA (RAPD) a commercial kit with six primers was used, of which the included Primer 4 resulted in the most informative patterns. Repeated trials with PFGE, using different cell and enzyme concentrations, were also performed but resulted in bands too weak to be correctly interpreted. The PFGE protocol used was modified from an original study on *Campylobacter* spp. (Höök *et al.*, 2005), and has been used in a previous study on *T. phagedenis*- like isolates (Pringle *et al.*, 2008).

4.1.7 Probe design for FISH

FISH (Paper III) was performed to complement study one. The aim was to get a clear picture of the occurrence and spatial distribution of treponemes in the tissue samples. As we had to restrict the number of phylotypes for investigation, we focused on the main phylotypes detected in study I and study II. These were *T. pedis*, *T. parvum* and one unknown phylotype. All were represented by large clusters (A-C) in the phylogenetic tree (Paper I). One cluster (C) remained unidentified due to fact that we had not obtained an isolate from this cluster. Hence the sequence of the 16S rRNA gene was not known. Therefore it was not possible to design a probe for this group, and we restricted our investigations in study III to *T. pedis* and *T. parvum*. For *T. pedis* a probe targeting 16S rRNA was already available as it had been designed and used in previous studies on BDD in cattle (Rasmussen *et al.*, 2012). For *T. parvum* a new probe was designed (5'ccactggcttcgggtatcct 3'). This was performed using methods described by Klitgaard *et al.* (2008).

The probes were tested on small tissue samples from porcine lung prepared with one isolate each of *T. pedis* (T A4) (Pringle *et al.*, 2009), *T. phagedenis*-like (V1) (Pringle *et al.*, 2008) and *T. parvum* (B1119) (Paper II). The results are shown in table 5.

Table 5. *Test of probes for FISH*

Isolate (Species)	T A4 (*T. pedis*)	V1 (*T. phagedenis*-like)	B1119 (*T. parvum*)
T. pedis probe	+	-	-
T. parvum probe	-	-	?[1]

[1.] The *T. parvum* probe generated a signal too weak to be interpretable.

The *T. parvum* probe generated a signal too weak to be interpretable. The most likely reason for this weak signal was the location of the region for which the probe had been designed. Inspection of the higher order structure of the

ribosome showed that the region was located in an area from where the fluorescent signal may be very low. Attempts were made to find alternative regions for a new probe, but no regions specific enough were found, and *T. parvum* was therefore excluded from the study.

The *T. pedis* probe gave a signal only in the specimen prepared with *T. pedis* and remained in the study. The signal was intense and easy to read. With only one species-specific probe left we had to reform our initial idea. As planned, we investigated the presence and localisation of *Treponema* spp. in the sections, and we were also able to compare these with serial sections hybridized with *T. pedis* probe. But instead of continuing by detecting other phylotypes by FISH we moved forward to high-throughput sequencing (HTS) on material from a selection of ulcers (Paper III).

4.2 Paper IV

4.2.1 Animals

The animals used in the challenge study were of crossbreed Yorkshire/ Hampshire. The piglets were born in the animal facilities at the department of Clinical Sciences, SLU, by two Yorkshire gilts and were during the first four weeks of life included in another study (Chalkias *et al.*, 2014), where clinical examination of piglets was carried out daily. Therefore, the animal material in the present study was well defined and we could exclude piglets that had previously been ill and/or medicated. We also knew that the mothers of the piglets had not had any signs of shoulder ulcers during the lactation period.

4.2.2 Selecting an isolate

At the time of the challenge study, the research group had access to 15 treponemal isolates originating from shoulder ulcers, ear necrosis or gingiva of pigs (Paper II). Two isolates of *T. pedis*, isolated from ear necrosis, were available (T A4 and E1186). To perform the inoculation using an isolate as well defined as possible, T A4 was selected to be used in the challenge study. T A4 has been well characterized phenotypically, and the whole genome was recently sequenced, *de novo* assembled and annotated (Paper II; Svartström *et al.*, 2013; Pringle *et al.*, 2009)

The decision to use one single isolate, or one single *Treponema* species, was not obvious. Findings from the previous studies (Paper I, Paper II) indicated presence of various treponemal phylotypes in porcine skin ulcers. Therefore, an alternative study design could have included several treponemal species. With the intention to work with a well-defined material, and with the

objective to re-isolate the agent if lesions were induced, the decision to use one single isolate was made.

4.2.3 Experimental design

No challenge studies on pure isolates of *Treponema* spp. in pigs have previously been reported. Therefore, adequate infection dose or inoculation procedure was not known. Based on information available from studies in mice (Elliott *et al.*, 2007; Kesavalu *et al.*, 1997) and cattle (Gomez *et al.*, 2012), an inoculation dose of 10^9 spirochetes per inoculum was injected to the ears of the pigs.

During the acclimatization period as well as during the first week of the study, a wet bandage was applied to one ear of each animal to keep the skin moist (Fig.6). This was an attempt to mimic methods applied in a BDD challenge trial, where lesions had been induced in cattle of which the skin first had been kept moist and under anaerobic conditions (Gomez *et al.*, 2012; Read & Walker, 1998a; Read & Walker, 1996). To keep the bandages on the ears in the present study proved difficult, and the bandages were therefore removed after one week.

Figure 6. During the first part of the challenge study one ear of each pig was bandaged.

As our initial studies had shown *Treponema* spp. to be commonly present in the mouth of pigs (Paper I), the animals were initially kept in single pens to avoid contamination of the inoculated area. We also intended to investigate if treponemal species present in the mouth differed from any treponemes isolated from possible skin ulcers. Therefore, cotton swab samples were continuously collected from the gingivae of the pigs and regularly investigated for presence of treponemes by phase contrast microscopy.

As no lesions were induced following the first inoculation, the pigs were kept in pairs after the second inoculation. This was to study if the outcome would be different when biting or licking occurred, similar to field conditions.

4.2.4 The *T. pedis* protein TPE0673

One of the aims of this thesis was to investigate the IgG response in pigs to recombinant *T. pedis* proteins. Several putative virulence factors had previously been identified in the genome of *T. pedis* strain T A4 (Svartström *et al.*, 2014; Svartström *et al.*, 2013). Two proteins were successfully expressed in *Escherichia coli* and purified (Svartström *et al.*, 2014; unpublished). One of these proteins, TPE0673, a homologue to *T. denticola* virulence factor IdeT (Ishihara *et al.*, 2010), was further characterized (Svartström *et al.*, 2014). Investigation of a subset of serum samples by western blot suggested a humoral immune response towards TPE0673 was elicited in pigs with shoulder ulcers but not in pigs with ear necrosis. When the challenge study was performed (Paper IV) we wanted to investigate the humoral response in serum samples from challenged pigs as well as in a larger set of sera from previously sampled field cases of ear necrosis and shoulder ulcers (Paper I) by using ELISA. The other protein, TPE1291, that had been purified was tested in pilot runs by the use of ELISA, but was excluded from further analysis due to inconsistent results.

5 Results and discussion

5.1 Summary of results

- Spirochetes of genus *Treponema* were frequently occurring in shoulder ulcers and ear necrosis of pigs.
- The number of treponemal phylotypes was higher than previously reported, revealing a broad diversity of treponemes in shoulder ulcers and ear necrosis.
- In spite of the high variability of treponemes, three main phylotypes were found; *Treponema pedis*, *Treponema parvum* and one phylotype without designation.
- The three main phylotypes could be found both in ulcers and gingiva.
- By use of ISR2-based PCR and sequencing, identical sequences from ulcers and gingiva were detected, which indicate transmission between mouth and ulcer.
- Culturing resulted in 12 isolates of species *Treponema pedis* (n=5), *Treponema parvum* (n=1) and one phylotype most similar to *Treponema* sp. OMZ 840 (n=6).
- Except for two, all isolates showed unique RAPD patterns.
- The isolates could not be differentiated by use of biochemical tests.
- *Treponema pedis* and *Treponema* sp. OMZ 840 were hemolytic, while *Treponema parvum* was not.
- The MICs of the tested antimicrobials were in general low.
- Not all phylotypes detected by molecular techniques could be cultured or species identified.
- By histopathological evaluation the majority of the sampled ulcers were classified as chronic.
- Studies by FISH confirmed a frequent occurrence of *Treponema* spp. in the investigated ulcers, and an abundance of treponemes was visualized in a majority of the tissue samples.

- A predominance of *Treponema pedis* in the ulcers was confirmed by HTS in a subset of samples.
- *Treponema* spp. were also detected in cases of facial necrosis and other skin lesions on the body.
- The *Treponema pedis* strain T A4 did not induce skin ulcers or clinical signs of infection when injected in the ears of healthy pigs in a challenge study.
- A strong IgG response towards the *Treponema pedis* protein TPE0673 was detected in sows with shoulder ulcers, but only a weak response was detected in pigs with ear necrosis, and no response was seen in pigs challenged with *Treponema pedis* T A4.

5.2 Occurrence

In 2010, when this work began, the knowledge of *Treponema* spp. in porcine skin ulcers was based on two pilot studies (Pringle & Fellström, 2010; Pringle *et al.*, 2009). In those studies, spirochetes of genus *Treponema* had been identified and isolated from single cases of ear necrosis and shoulder ulcers in three Swedish pig herds, as well as from porcine gingiva. These pilot studies were, to the author's knowledge, the first studies in which molecular techniques were used to identify spirochetes occurring in porcine skin lesions. The studies were carried out based on results from previous studies identifying spirochetes in various types of skin disorders of pigs (Harcourt, 1973; Blandford *et al.*, 1972; Osborne & Ensor, 1955; Neitz & Canham, 1930; Gill, 1929; Schmid, 1925; Nomi & Matsuo, 1922; Gilruth, 1910; Cleland, 1908). In addition, previous experience from *Treponema* research on BDD in cattle and comparisons to treponemal disease in cattle, but also in other animal species, contributed to the hypothesis that was formed and elaborated in this thesis (Sayers *et al.*, 2009; Stamm *et al.*, 2009; Pringle *et al.*, 2008; Holt & Ebersole, 2005).

The work performed in this thesis confirmed the results from the pilot studies. Spirochetes of genus *Treponema* were indeed present in shoulder ulcers and ear necroses, and also in gingiva of pigs, and not an occasional finding. The occurrence of spirochetes in ulcers was detected by three different culture independent methods; phase contrast microscopy (PCM), Warthin-Starry silver staining (W-S) and ISR2-based PCR (Paper I). The treponemal occurrence in gingival samples was investigated only by PCR. The percentages of positive samples by each method are shown in table 6.

Table 6. *Percentage of samples found positive for spirochetes by three different methods.*

Method	W-S[1]	PCM[2]	PCR[3]	All[4]
Origin of sample				
Shoulder ulcers	67%	46%	52%	73%
Ear necroses	22%	21%	46%	53%
Gingivae	Nt[5]	Nt	9.7%	9.7%

[1.] Warthin-Starry silver staining. [2.] Phase contrast microscopy. [3.] Intergenic spacer region 2-based PCR. [4.] Combining the results from all three methods, a positive sample= positive in at least one of three methods. [5.] Not tested.

The aim of using three methods in parallel was not to compare the different methods, but rather to increase the possibility to detect spirochetes. The W-S investigation was carried out on sections from full thickness skin biopsies, the material for PCR consisted of scrapings, and PCM was only performed on part of the scraping material. This should be taken into account when recording differences in the results of the three methods. When the results from all three methods were combined, the total occurrence of spirochetes in shoulder ulcers was 73% and in ear necrosis 53%. This approach is however somewhat problematic, as neither W-S nor PCM, though considered as well established methods to detect spirochetes, can be regarded as genus specific. The PCR system used was specific for *Treponema* (Stamm *et al.*, 2002), and sequencing further confirmed treponemal presence in these samples.

In addition to the findings from ear necrosis and shoulder ulcers, spirochetal occurrence was investigated in four cases of facial necrosis (Karlsson *et al.*, 2012). W-S staining revealed spirochetes in two out of two biopsies, and in two out of four samples investigated by PCM. Spirochetes of genus *Treponema* were confirmed in three out of four cases of facial necrosis by ISR2-based PCR. Five cases of other skin ulcers were also sampled. Spirochetes were identified by PCM in one of these samples, but could not be confirmed by W-S or ISR2-based PCR (Karlsson *et al.*, 2014, to be published). No spirochetes were found in samples from intact skin by W-S, ISR2-based PCR or FISH (unpublished; Paper III).

5.3 Diversity

The differences in results between culture (Paper II) and culture independent methods (Paper I) clearly illustrate the difficulties in isolating *Treponema* spp., but also emphasizes that not only cultivable phylotypes were present in the skin ulcers. By culture, 12 treponemal isolates were obtained. These were of species *T. pedis*, *T. parvum* and *Treponema* sp. OMZ 840. By ISR2-based PCR, 52% of shoulder ulcers, 46% of ear necroses and 9.7% of

gingivae were positive for treponemes. Cloning and sequencing yielded a large number of ISR2 sequences, of which 132 were used to construct a phylogenetic tree, together with ISR2 sequences acquired from GenBank (Paper I). This tree revealed a great diversity of treponemal phylotypes in porcine skin ulcers.

Three main clusters were apparent, designated A, B and C. Cluster B was identified as *T. pedis*. This cluster contained sequences from UMD and BDD in cattle (Stamm *et al.*, 2009; Stamm *et al.*, 2002), as well as from shoulder ulcers, ear necrosis and gingiva of pigs (Pringle & Fellström, 2010; Pringle *et al.*, 2009). Both group A and group C clustered with different sequences derived from UMD (Stamm *et al.*, 2009). Group A was later identified as *T. parvum* (Paper II). Cluster C remained unidentified by species. Several small clusters or single sequences were also present in the phylogenetic tree. One small cluster was later identified as *Treponema* OMZ 840-like (Paper II) and two single sequences were found to be identical to other sequences derived from UMD in cattle (Paper I; Stamm *et al.*, 2002)

The great variability in the phylogenetic tree resembles the findings in BDD in cattle, where increasing evidence points towards a broad treponemal diversity in such skin lesions (Klitgaard *et al.*, 2013; Rasmussen *et al.*, 2012; Klitgaard *et al.*, 2008; Choi *et al.*, 1997). In the present studies, however, no evidence of *T. phagedenis*-like or *T. denticola*-like treponemes (except for *T. pedis*) was found, which is a difference from studies on BDD (Klitgaard *et al.*, 2013; Yano *et al.*, 2010b; Evans *et al.*, 2008; Klitgaard *et al.*, 2008; Nordhoff *et al.*, 2008; Moter *et al.*, 1998; Choi *et al.*, 1997). In addition, the phylotype *T. medium/T. vincentii*-like is often identified and isolated from BDD lesions, but only few *T. medium*-like sequences were identified by ISR2-based PCR and sequencing in these studies (Paper I).

When discussing which phylotypes are the most frequent it should be emphasized that three main phylotypes were demonstrated, but that the result from the ISR2-PCR and sequencing may not reflect the whole truth. The primers used were designed to be general for genus *Treponema*, but there is always a possibility that not all phylotypes are detected, or that the primer affinity is higher to some species. In addition, the number of clones analysed varied from three to six and this may also have affected the outcome.

One of the aims with this work was to investigate a possible association between treponemes from gingiva and skin ulcers. The phylogenetic tree clearly showed that ISR2 sequences from ear necrosis, shoulder ulcers and gingiva were represented in all the three main clusters A-C (Paper I), but also visualized the great variability of ISR2 sequences. Despite this variability, identical sequences of different origin (ear necrosis, shoulder ulcers and gingiva) were detected both within and between herds. In two cases, identical

sequences were observed from sows with shoulder ulcers and from the mouth of their piglets. These findings indicate a transmission from mouth to ulcer, but to prove this, established fingerprinting methods or genome analyses of isolates obtained from both sites would be required. A transmission from ulcer to mouth is of course also possible. Isolates both from ulcers and gingiva were obtained, and at one occasion from pigs in the same pen (Paper II). Two of these isolates were both of species *T. pedis*. However, analysis of ISR2 sequences and RAPD patterns indicated differences between these two isolates.

Identical ISR2 sequences were detected from pigs with ear necrosis, residing in the same pen (Paper I). This indicates a transmission of treponemes between pigs. As only one isolate was obtained from ear necrosis, further investigations using fingerprinting with RAPD could not be performed.

The substantial variability of ISR2 sequences, showed in the phylogenetic tree, supports the option to use this method as a fingerprinting method. One advantage of the method is that it can be used not only on cultured isolates, but also directly on sample material from for example an ulcer. To evaluate the degree of resolution compared to fingerprinting methods established for other bacteria, a large number of isolates need to be tested.

5.4 Characterization of isolates

Twelve isolates were obtained and characterized in this work (Paper II). These isolates were all related to oral treponemes from dogs or humans. *Treponema pedis* was first isolated from BDD in cattle, and was initially designated as *T. denticola*-like, but proposed as a new species in 2009 (Evans *et al.*, 2009b; Walker *et al.*, 1995). Whole genome sequence analysis of *T. pedis* has confirmed a high similarity of this species to *T. denticola* (Svartström *et al.*, 2013). Furthermore, *T. parvum* was originally isolated from periodontitis in humans (Wyss *et al.*, 2001), and *Treponema* sp. OMZ 840 from dental plaque in a dog (Correia *et al.*, 2003).

The results from the enzyme production tests showed they could not discriminate between isolates or be used for species identification. In addition, the enzyme production patterns of *T. pedis* isolates and the *T. parvum* isolate in this work differed from the type strains (Evans *et al.*, 2009b; Wyss *et al.*, 2001). *Treponema pedis* isolates were hemolytic, which is in agreement with the description of the type strain for *T. pedis*. Isolates of *Treponema* sp. OMZ 840 were also hemolytic, but *T. parvum* was not. No previous reports on hemolytic activity for *T. parvum* or *Treponema* sp. OMZ 840 were found.

RAPD was carried out to test if this technique could work as a fingerprinting method. Unique patterns were seen for all isolates except two

gingival isolates (T M1 and isoM1220). In addition, these isolates showed identical ISR2 sequences. Identical ISR2 sequences were also recorded for two more sets of isolates (isoM11886, isoM1188, isoB1175) and (isoB1173 and isoB1177). These isolates did not show identical RAPD patterns. Hence RAPD showed a higher discriminatory power as a fingerprinting method in these cases. RAPD may be more discriminative than ISR2 because this method investigates differences in the whole genome of isolates, instead of just a short region.

In general, the susceptibility to the antimicrobials tested (tiamulin, valnemulin, tylosin, tylvalosin, lincomycin, doxycycline) was high. Antimicrobial susceptibility tests for *T. pedis* isolates from BDD and CODD have been performed in two studies from UK (Evans *et al.*, 2012; Evans *et al.*, 2009a). Those studies differ from our studies in methodology and in antimicrobials tested, but similar MICs of lincomycin were seen; 0.75-6 µg/ml (Evans *et al.*, 2009a) compared to 1-4 µg/ml (Paper II), as well as of oxytetracycline; 0.375-0.75 µg/ml (Evans *et al.*, 2009a) compared to doxycycline 0.125-0.25 µg/ml (Paper II). For macrolides the MICs were in general low in all studies; 0.017-0.0235 µg/ml of erythromycin (Evans *et al.*, 2009a) and 0.0234-0.0469 µg/ml of azithromycin (Evans *et al.*, 2012) in UK, and ≤ 0.5 µg/ml of tylosin and ≤ 0.25 µg/ml of tylvalosin for most isolates in Sweden (Paper II). It should be noted that a low MIC of an antimicrobial does not automatically indicate that the drug is the most appropriate choice for treatment.

For a subset of isolates from one herd included in this investigation, elevated MICs of tylosin and tylvalosin were observed. In Sweden, macrolides are allowed to use for treatment of pigs. It is therefore possible that the use of macrolides in this herd might have selected for these clones. This was however not further investigated. A lower MIC of lincomycin and a higher MIC of doxycycline was recorded for the *T. parvum* isolate in this study. This could reflect a normal susceptibility pattern for this species. To the author's knowledge, no previous antimicrobial susceptibility tests for *T. parvum* or *Treponema* sp. OMZ 840 have been reported.

It should be noted that the results from the antimicrobial susceptibility tests in this work only reflect the tested isolates susceptibility *in vitro*. If *Treponema* spp. would be proven as a causative agent of skin disorders of the pig, or shown to be crucial for development of severe ulcers, antimicrobial therapy might be necessary for treatment of diseased animals to prevent animal suffering or transmission of the agent. In that case, clinical treatment studies to study selected antimicrobials effectiveness *in vivo* would be necessary to establish any general treatment recommendations.

5.5 Localization and species specific distribution

The studies of occurrence had demonstrated several phylotypes present in the ulcers (Paper I). Furthermore, the density of spirochetes in the tissue samples had been assessed by investigation of W-S stained sections. The localization of treponemes in the tissue and the distribution of certain treponemal phylotypes was not known. The idea of performing FISH was to elucidate these matters, as has already been described under the materials and methods section in this thesis.

The results from FISH confirmed the presence and abundance of *Treponema* spp. in shoulder ulcers, and to a lesser extent also in ear necrosis (Paper III). Of the shoulder ulcers, 69% were positive for treponemes. The corresponding percentage for ear necrosis was 59%. In addition, *Treponema* spp. were confirmed in sections from two out of two facial ulcers and two out of five other skin ulcers.

The occurrence of treponemes was scored from 0-3, according to a scale described previously (Rasmussen *et al.*, 2012; Paper III). The most frequent score of *Treponema* positive sections was score 3, which indicated that treponemes constituted > 10% of bacteria observed in that section. As a general observation, in sections where *Treponema* spp. were abundant, presence of bacteria other than treponemes was sparse.

Specific investigations on *T. pedis* were performed on serial sections. The results were strikingly similar to the results achieved with the general probe for genus *Treponema*. Of all samples positive for *Treponema* spp. (n=71), 69 samples were positive for *T. pedis*. Furthermore, the scoring results (0-3) only differed in six cases. The results suggested *T. pedis* as a predominating species in the investigated samples from porcine skin ulcers.

The results from the histopathological examination of HE stained sections were basically in agreement with previous descriptions of shoulder ulcers and ear necrosis (Jensen, 2009; Richardson *et al.*, 1984) (Paper I, Paper III). A majority of the investigated skin lesions were of chronic nature, showing varying degrees of ulceration (Paper III). In general, the degree of severity appeared uniform in sections from shoulder ulcers, whereas in the sections from ear necrosis the age and severity of the lesions seemed to vary within each lesion. Even though treponemes were identified also in lesions assessed as acute, the findings of treponemal occurrence in shoulder ulcers and ear necrosis mainly concern chronic lesions.

The severity of the investigated lesions and the fact that epidermis was missing or completely necrotized in many of the sections made it difficult to determine the location of *Treponema* spp. in epidermis/dermis. As a general observation, treponemes were mainly located on the border between the

necrotic tissue and vital granulation tissue. Other bacteria, if present, were located on the surface of the ulcers, while treponemes were found deeper. In investigations on BDD, a spatial distribution of various treponemal phylotypes in epidermis has been shown, and some phylotypes found deeper in the epidermis have been implicated as more important in the etiology of BDD (Moter *et al.*, 1998). The involvement of *Treponema* spp. in the etiology of shoulder ulcers and ear necrosis is not clear. It is possible that samples collected in an acute stage would tell us more about the specific distribution of treponemes in epidermis/dermis. It is also possible that bacteria of genus *Treponema* are of less importance in the acute stage of the lesions, but arrive later, as secondary invaders.

In sows, multiple risk factors predispose for the development of shoulder ulcers (Zurbrigg, 2006; Davies *et al.*, 1997; Davies *et al.*, 1996). The progression is believed to start with a pressure induced alteration of circulation to the skin, leading to thrombosis and ischemia, causing damage to epidermis, with subsequent ulceration and secondary infection (Herskin *et al.*, 2011; Jensen, 2009). The literature on previous microbial findings in shoulder ulcers is sparse, and does not include culturing of anaerobes (Lund, 2003; Nouws *et al.*, 1981). The frequent and abundant occurrence of *Treponema* spp. shown in this work (Paper I, Paper III) suggests that treponemes play an important role in severe and chronic shoulder ulcers.

The cause of ear necrosis is not known, but a multifactorial background has been proposed (Park *et al.*, 2013; Weissenbacher-Lang *et al.*, 2012). Trauma caused by biting, with subsequent infection involving staphylococci or streptococci is one hypothesis (Park *et al.*, 2013; Richardson *et al.*, 1984). In this work, specific investigation of bacteria other than *Treponema* spp. was not performed, but other bacteria, mostly cocci, were sometimes observed superficially in the ulcers (Paper I, Paper III). In one study from Canada, *S. hyicus* was isolated in 68% and *S. aureus* in 88% of the investigated cases of ear necrosis, while spirochetes were only confirmed in 8.6% of the samples, investigated by W-S (Park, 2011). In our study 22% of the sections from ear necrosis were positive for spirochetes by W-S (Paper I). The difference in results may either reflect a true difference in occurrence between the two countries or a difference in methodology. Another aspect to consider is that in the Canadian study a majority of sampled lesions were ear tip necroses whereas in our study most cases were ventral ear necroses. If these variations in clinical signs characterize two different syndromes, a difference in spirochetal occurrence is possible. Another possibility to take into account is the time aspect. If initial infection is caused by staphylococci and spirochetes are secondary invaders, the difference in the results between the Canadian

study and this work may be due to a difference in sampling time during the course of infection. Interestingly, in the present work investigation of the same material (serial sections of the same biopsies) by FISH (paper III) compared to by W-S (paper I) resulted in a higher percentage positive samples, which indicate an underestimation of treponemal presence may occur using W-S staining for detection.

In this work, spirochetes of genus *Treponema* were confirmed in 59% of the ear necrosis cases by the use of FISH (Paper III). The distribution of treponemes in tissue was not as abundant and uniform as in the shoulder ulcers, but the frequent findings suggest *Treponema* spp. may be of importance in ear necrosis lesions, especially chronic and severe ones.

The predominance of *T. pedis* was confirmed by partial 16S rRNA PCR and high-throughput sequencing in 36 samples (26 from shoulder ulcers and 10 from ear necrosis). In addition, *T. medium*/*T. vincentii*-like spirochetes were identified as the second most prevalent phylotype in investigated samples. These results differed from the first study (Paper I), where only few *T. medium*-like sequences were identified, however even though *T. medium/T. vincentii*-like treponemes were second most prevalent in the high-throughput sequencing study, they were still only present to a limited extent compared to *T. pedis*.

It should be pointed out that even though the primers used targeted a broad spectrum of treponemal phylotypes, they were specifically designed to amplify members of the *T. pallidum*, *T. phagedenis* and *T. denticola*- subgroups of genus *Treponema* (Klitgaard *et al.*, 2013). These subgroups include most phylotypes hitherto identified in BDD, but not *T. parvum*. Therefore, the findings from the first study (Paper I), indicating *T. parvum* as a species frequently occurring in porcine skin ulcers, was not further investigated in paper III. It is also possible that other treponemes were present in the ulcers but failed to be detected with the primers used.

5.6 A challenge study

A challenge study was performed to study if *T. pedis* strain T A4 would induce ear necrosis in healthy pigs (Paper IV). The pigs in the challenge study did not develop any signs of ear necrosis or other clinical signs of disease. An initial erythema was observed at the injection site, but no difference was noted between challenged pigs or control pigs. After the second inoculation six of the pigs were kept in pairs. Fighting resulted in superficial scratches in the skin on the ears and body, but these lesions healed without any complications. No difference was seen between bandaged or non-bandaged ears.

To the author's knowledge, this is the first reported challenge study aiming to induce ear necrosis in pigs using a pure isolate of *Treponema* spp. In old reports, other skin lesions were reproduced in healthy pigs by inoculation of scraping material from ulcers of affected pigs (Osborne & Ensor, 1955; Dodd, 1906). It was noted that only scrapings containing motile spirochetes had the ability to cause new skin ulcers. It was not known to which species or genus the spirochetes belonged.

Recently it was reported that in a challenge study in cattle, using a pure *Treponema* culture, a BDD-like lesion was induced in one heifer (Gomez *et al.*, 2012). The lesion was classified as incipient, as it did not have all the features of an acute field case of BDD. It was stated that the isolate used was of species *T. vincentii*. Treponemal occurrence in the induced lesion was confirmed by dark-field microscopy, silver staining, immunohistochemistry, re-isolation and PCR, but the treponemes were not species determined by sequencing. Contrary to those studies, one pilot study from Sweden described unsuccessful results using a *T. phagedenis*-like isolate to induce BDD (Pringle *et al.*, 2008). Previously, successful results in inducing BDD lesions in calves by using scraping material from diseased animals were reported (Read & Walker, 1998a; Read & Walker, 1996).

Interestingly, comparing the studies on pigs and cattle, it appears as if the use of ulcer scraping material in general has a higher success rate than the use of pure *Treponema* isolates. The reason for this is unknown, but the results suggest something is missing in the studies with pure isolates or that the bacteria loose virulence when cultured *in vitro*. In some of the old studies on skin ulcers in pigs, other microorganisms were observed in the ulcers, together with spirochetes (Neitz & Canham, 1930; Nomi & Matsuo, 1922; Gilruth, 1910; Cleland, 1908). Both the ideas that 1) spirochetes alone, or spirochetes together with other agents were causing the ulcers (Dodd, 1906) or that 2) other microorganisms were the causative factor of the skin ulcers and spirochetes were merely commensals (Gill, 1929), were expressed. More recent investigations, specifically on ear necrosis, have suggested the syndrome has a multifactorial background (Park *et al.*, 2013; Weissenbacher-Lang *et al.*, 2012). Except for spirochetes, other bacteria commonly found in ear necrosis, mainly staphylococci or streptococci, have been implicated as possible etiological agents (Park *et al.*, 2013; Mirt, 1999; Richardson *et al.*, 1984). To the author's knowledge no challenge trials to induce ear necrosis using other agents, for example staphylococci, have been performed, but the need for these types of studies was recently pointed out by Park *et al.* (2013).

In BDD, other etiological agents than *Treponema* spp. have been suggested and different bacteria have been isolated or detected from the lesions

(Rasmussen *et al.*, 2012; Schlafer *et al.*, 2008; Schroeder *et al.*, 2003; Dopfer *et al.*, 1997). The findings of *Treponema* spp. in BDD lesions are however consistent, and results from several research groups point toward a treponemal involvement in the etiology of disease (Klitgaard *et al.*, 2013; Yano *et al.*, 2010b; Evans *et al.*, 2008; Moter *et al.*, 1998). Recent studies show that one single BDD lesion may contain several treponemal phylotypes (Klitgaard *et al.*, 2013; Nordhoff *et al.*, 2008). A high variability of treponemes has been found, although some phylotypes seem to be more common than others. From this aspect, the results in this thesis (Paper I) have many similarities to results from studies on BDD. As samples from field cases indicate a polytreponemal occurrence both in porcine ear necrosis and BDD, a possible approach in further challenge studies could be to include experiments with mixed treponemal phylotypes. Another approach could be to consider including other putative pathogens.

5.7 IgG response towards a *T. pedis* protein

The last aim of this thesis was to investigate the IgG response in pigs to recombinant *T. pedis* proteins. One protein, TPE0673, was successfully expressed, purified and characterized in another study (Svartström *et al.*, 2014; unpublished), and the IgG response towards this protein was further analyzed by ELISA (Paper IV).

The results from the ELISA showed that the challenged pigs did not have an IgG response towards TPE0673. Of the sera from the field, the majority of the sera from sows with shoulder ulcers showed a strong IgG response compared to negative controls. On the contrary, most cases of ear necrosis showed a weak IgG response. The results from the sera from animals with shoulder ulcers indicate that the protein is immunogenic, and is expressed *in vivo*.

The difference in IgG response in field cases of ear necrosis compared to shoulder ulcers is interesting, and not easily explained. *Treponema pedis* was detected both in ear necrosis and shoulder ulcers (Paper I, II, III), but of the *Treponema* positive sections, a higher percentage (71%) of sections from shoulder ulcers had a score of 3 (indicating a high treponemal occurrence) compared to sections from ear necrosis (38%) (Paper III). In shoulder ulcers, the treponemes were often visualized in the tissue organised as a broad band or brim. This observation was also seen in sections from ear necrosis, but was not as common. A general impression was that in sections from ear necrosis, the treponemes were more scattered than in sections from shoulder ulcers. The absorbance values detected by ELISA may reflect a difference in expression of

TPE0673 in ear necrosis lesions compared to shoulder ulcers, which in turn may be dependent on an abundance, or a certain organisation pattern of treponemes in the tissue. This is only a hypothesis, and remains to be tested.

The most obvious difference between the pigs with ear necrosis and pigs with shoulder ulcers is age. The sows were adults and the pigs with ear necrosis were young (8-14 weeks of age). One possibility is that the differences in IgG serum titers between the two groups reflect age related differences in the reactions of the immune system. Another factor to consider is that sows may have been exposed to *Treponema* spp. previously, if they have been afflicted with shoulder ulcers during previous lactations. The high absorbance values may therefore reflect a boost of the immune response at re-infection.

Interestingly, the ELISA results from serum samples from one shoulder ulcer herd differed from the other herds (Paper IV). Where the majority of sera from shoulder ulcers showed high absorbance values, the sera from this herd showed very low titers. When looking into our records, it was found that *T. pedis* had not been identified in any samples from this herd using *Treponema* specific detection techniques, nor had any other treponemal phylotypes. The phase contrast microscopy on ulcer samples from the same herd was also negative, but two of the biopsies from this herd were positive on W-S. It should be kept in mind though, that W-S is not a method specific for detection of *Treponema* spp.

6 Concluding remarks and future perspectives

The frequency and abundance of *Treponema* spp. in chronic shoulder ulcers and ear necrosis found in this work suggest that treponemes have an important role in these types of ulcers, possibly as secondary invaders. The occurrence of *Treponema* spp. in initial stages of the lesions is however still unknown and a causative role of treponemes in the etiology of these ulcers was not shown. A transmission between mouth and ulcer was indicated but not finally proven. Although most of the results from this work support the original hypothesis, additional studies are still needed for a final verification or rejection. In addition, new questions and ideas have been raised.

A longitudinal study on the progression of ulcers coupled to the presence of *Treponema* spp. in various development stages of the ulcers is one way to verify or exclude *Treponema* spp. as a secondary invader. Other putative pathogens could be included in the study. It would also be interesting to study if the presence of *Treponema* spp. has any influence on wound healing. And if so, what can be done in terms of treatment or prevention?

To identify the source of infection, or to follow a transmission pattern, fingerprinting methods with sufficient resolution to differ between clones are desirable. The results from the ISR2 sequence analyses are promising, but this type of analysis needs to be evaluated by comparison of fingerprinting methods established for other bacteria.

A working challenge model should be developed. On the basis of the findings in this work, and in previous studies on BDD and periodontitis, a polytreponemal/polymicrobial etiology should be considered. A new experimental study design could therefore include several treponemal phylotypes, and other putative pathogens. Porcine challenge models could prove valuable in comparative studies on BDD in cattle and possibly also in diseases associated with *Treponema* spp. in other species. A working challenge

model would be useful for studying the local immune response during the progression of ulcers as well as the response to treatment.

7 Populärvetenskaplig sammanfattning

Den här avhandlingen fokuserar på ett nygammalt forskningsområde, nämligen bakterier av släktet *Treponema* i hudsår hos grisar. Ända sedan i början av förra seklet finns vetenskapliga notiser om spiralformade bakterier, spiroketer, i sår hos grisar. Exempel på hudlidanden som beskrivs i dessa rapporter är klövröta och sår på bål, ben och huvud hos grisarna. I några av artiklarna berättas att sårmaterial från sjuka grisar kunde orsaka nya sår på friska grisar. Spiroketerna identifierades med hjälp av mikroskop, men inga försök gjordes att odla dem. Därför kunde det heller inte utföras vidare undersökningar för att bestämma vilket släkte eller art dessa bakterier tillhörde.

Fram tills alldeles nyligen fanns inga odlingsförsök av spiroketer från sår på grisar beskrivna. Det är kanske inte så underligt med tanke på att denna typ av bakterie är känd för att vara svårodlad. För några år sedan lyckades dock vår forskargrupp isolera spiroketer från öronsår, bogsår och munslemhinna på grisar. Bakterierna identifierades och visade sig tillhöra släktet *Treponema*. Upptäckten lade grunden till vidare studier som har mynnat ut i två avhandlingar, varav den här är den ena.

Inför studierna i avhandlingen utformades hypotesen att bakterier av släktet *Treponema* har en viktig roll när bogsår och öronsår förekommer i smittsam och/ eller allvarlig form, och kanske även vid förekomsten av andra hudåkommor hos grisar. Våra mål med studierna var att undersöka förekomsten och diversiteten av *Treponema*-arter i hudsår hos grisar, att isolera och karaktärisera bakterierna och att undersöka ett möjligt samband mellan treponemabakterier från mun och i hudsår. Ytterligare mål var att studera hur hudlesionerna såg ut, både makroskopiskt och mikroskopiskt, samt var bakterierna var lokaliserade i såren. Vi ville också utveckla en modell för att testa om bakterier av arten *T. pedis* kunde framkalla hudsår.

Prover samlades in från grisar i 19 svenska besättningar under perioden från april 2010 till december 2011. Proverna bestod av skalpellskrap och biopsier från sår och svabbar från munslemhinna. Dessutom togs blodprover från varje

gris. De hudförändringar som provtogs bestod av 52 bogsår, 57 öronsår, 4 ansiktssår och 5 andra sår på bål eller huvud. För att undersöka förekomsten av spiroketer användes ett antal olika tekniker som ej är beroende av att man lyckas odla bakterien. Metoderna som användes var PCR (där man detekterar bakteriens DNA), silverfärgning av vävnadsprov (där man med mikroskop kan identifiera spiroketerna i fixerad vävnad), faskontrastmikroskopi (där man kan se bakterien direkt i prover från såren) och Fluorescerande *In Situ* Hybridisering (FISH) (där man märker in bakterierna i ett vävnadsprov med en prob som sedan fluorescerar när man lyser med ljus av en viss frekvens). För att undersöka artförekomsten av treponemabakterierna använde vi oss av två olika typer av sekvenseringstekniker. Dessa tekniker fastställer sekvensen för delar av treponemornas DNA.

Försök gjordes att odla treponemabakterier från varje enskilt sår och munprov. När isolat erhölls karaktäriserades de med hjälp av biokemiska analyser, test för känslighet av antimikrobiella substanser och RAPD (en metod för att jämföra genetiska fingeravtryck mellan bakterieisolat).

Ett infektionsförsök utfördes också, för att testa om treponemabakterier av arten *T. pedis* kunde orsaka hudsår på grisar. Fem grisar av blandras Yorkshire/Hampshire, sex veckor gamla, injicerades med *T. pedis*-isolatet T A4 under huden vid kanten av öronlappen. Tre grisar fungerade som kontroller och injicerades istället med steril koksaltlösning. Blodprov togs även på dessa grisar. För att testa om grisarna i infektionsförsöket eller från fältstudierna utvecklat ett antikroppssvar mot ett specifikt *T. pedis*-protein utfördes ELISA på blodproverna.

Spiroketer fanns i alla typer av hudsår och i alla besättningar. Förekomsten av *Treponema*, detekterad med PCR, var 52 % i bogsår, 46 % i öronsår och 9,7 % i munslemhinna (artikel I). Med hjälp av tekniken FISH identifierades treponemabakterier i 69 % av bogsåren och i 59 % av öronsåren (artikel III). Förekomst av *Treponema* kunde också bekräftas i ansiktssår hos spädgrisar och i andra hudsår på kropp och huvud hos grisar (artikel III). Sekvensering av delar av DNA: t hos treponemorna och konstruktion av ett släktträd visade att variationen av bakterierna i de undersökta såren och munnarna var stor (artikel I). Tre huvudgrupper i släktträdet kunde dock ses; *T. pedis*, *T. parvum* och så ytterligare en grupp som också var framträdande men som förblev okänd. Med hjälp av trädet kunde man se att identiska DNA-sekvenser från sår och munslemhinna förekom.

Totalt ledde odlingsförsöken fram till tolv isolat av släktet *Treponema* (artikel II). Isolaten visade sig tillhöra arterna *T. pedis* och *T. parvum* och en art som är mest lik *Treponema* sp. OMZ 840. Med undantag av två isolat hade alla unika RAPD-fingeravtryck. Man kunde inte skilja mellan isolaten enbart

baserat på resultaten från biokemiska tester. Känsligheten för de testade antimikrobiella substanserna var i allmänhet hög.

Mikroskopiska studier av vävnad från såren visade att utseendet på såren stämde överens med tidigare beskrivningar av liknande sår (artikel I & artikel III). En majoritet av de undersökta skadorna var av kronisk karaktär (artikel III). Med hjälp av FISH kunde man se att treponemorna var lokaliserade djupt ned i såren. Andra typer av bakterier kunde ses på ytan av såren. Treponemabakterierna utgjorde huvuddelen av de bakterier som fanns i såren. Vidare undersökningar med FISH och även sekvensering visade tydliga indikationer på att *T. pedis* var en dominerande *Treponema*-art i de undersökta proverna.

Grisarna i infektionsförsöket utvecklade inte några hudförändringar eller kliniska tecken på infektion (artikel IV). Vi kunde heller inte påvisa något antikroppssvar mot det testade *T. pedis*-proteinet hos dessa grisar. Bland grisarna från fältstudierna uppvisade suggor med bogsår generellt ett tydligt antikroppssvar, medan grisar med öronsår bara uppvisade ett svagt, eller inget antikroppssvar. Jämförelser gjordes med negativa kontroller från infektionsförsöket.

Resultaten från studierna i avhandlingen visar att bakterier av släktet *Treponema* är vanligt och rikligt förekommande i öronsår och bogsår hos grisar. De kan också finnas i ansiktssår och i andra sår på kroppen. Släktskap mellan treponemabakterier förekommande i sår och munslemhinna kunde konstateras, och identiska DNA-sekvenser från båda ställen kan tyda på en spridning av bakterier från mun till hudsår. En stor diversitet av treponemabakterier i sår och mun kunde påvisas, även om det fanns indikationer på att vissa arter var vanligare än andra. Vi upptäckte också att det förekom arter som vi inte lyckades isolera. Skillnaden mellan resultaten från detektionsmetoder oberoende av odling och resultaten från odlingsförsöken pekar på svårigheten att odla bakterier av släktet *Treponema*. Resultat både från FISH och från sekvensering tyder på en dominerande förekomst av *T. pedis* i de undersökta såren. Treponemabakteriernas roll som en del i etiologin av öronsår och bogsår behöver fortfarande klargöras, men våra resultat pekar på att dessa bakterier kan ha en viktig betydelse i kroniska och i allvarliga hudsår hos grisar.

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