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MIIA KONTTURI

INTERDIGITAL PHLEGMON IN FINNISH DAIRY HERDS

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DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, in lecture room 674, Porthania, on the 14th of August 2020 at 12 o´clock.

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Kallelle

Dedicated to my dear son Kalle, without you this thesis would not exist.

Abstract

Interdigital phlegmon (IP) is an infectious hoof disease of cattle. Typically, it causes severe clinical signs, such as lameness, and hence impacts cattle welfare. IP has been a well-known disease of both dairy and beef cattle all over the world for decades. In general, IP occurs as sporadic infections. Lately however, outbreaks of IP have been detected in dairy herds in Finland. Most of these outbreaks have occurred in recently built or renovated free stall barns. In these outbreaks morbidity in IP has been substantial, which has led to the extensive use of antimicrobials and heavy financial losses associated with affected herds.

The aim of this thesis was to investigate the characteristics of the IP outbreaks in Finnish dairy herds and to explore the morbidity, clinical manifestation and degree of inflammation of the affected animals in these outbreaks. Moreover, we aimed to investigate the bacteriology in IP, i.e. to investigate several bacteria detected earlier from hoof diseases. An additional objective was to identify herd level risk factors behind these outbreaks.

This thesis is based on three studies. The first two were observational, cross-sectional studies, which were performed on commercial free stall dairy herds. The majority of the herds suffered from an outbreak of IP and three herds were unaffected control herds. Altogether 100 cows with IP were clinically checked, diagnosed, and sampled for the bacteriological culture and PCR, and analysis of acute phase proteins; serum amyloid A, haptoglobin and albumin. Cows with other hoof diseases and control cows were sampled similarly for comparison. The third study was a survey of free stall dairy herds of ≥50 cows. We sought general herd data, barn characteristics, herd management details, and asked questions about leg and claw health of the herd. Based on the replies, the risk factors for an outbreak of IP to occur were investigated among farms that had experienced an outbreak and farms that had not.

Fusobacterium necrophorum ssp. *necrophorum* is the main IP pathogen. The most common finding in IP samples in the early, acute stage was a combination of *F. necrophorum* and *Dichelobacter nodosus. Trueperella pyogenes* was frequently associated with IP at the later, healing stage. However, various bacterial combinations existed in IP samples. In outbreak herds, the morbidity was either high (≥50%), or moderate (9 – 33%), and no herd had intermediate morbidity. Strong acute phase response was detected among IP cows in the early stage of the disease; the values for serum amyloid A and haptoglobin were clearly elevated, and albumin decreased in comparison with the case for other hoof diseases or control cows in our study. The acute phase response was even greater in herds of high morbidity and with a bacterial combination of *F. necrophorum* and *D. nodosus*. The possible herd level risk factors for an outbreak of IP to occur were animal movement between herds, i.e. animal purchase or contract heifer rearing, enlargement of the barn within three years, and fields under organic farming. Mechanical ventilation in the barn seemed to lower the

risk. Moreover, herds that had experienced an IP outbreak more often had other infectious hoof diseases.

Based on our study results, the same cow may have several hoof diseases and a thorough clinical inspection is essential in diagnosing IP, also during an IP outbreak. Furthermore, IP causes severe clinical signs and a very strong APR. Thus, an anti-inflammatory should be included in the treatment of affected animals. Even though *F. necrophorum* is the key pathogen in IP, in the disease process several other bacteria play a role, such as *D. nodosus*, which may affect the severity of IP. To lower the risk of an IP outbreak, new cattle should be purchased very cautiously, if at all, and enlargement of the barn should be constructed without undue restrictions being placed on time and labour inputs.

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Contents

List of original publications

This thesis is based on the following publications:

I Kontturi, M., Junni, R., Simojoki, H., Malinen, E., Seuna, E., Klitgaard, K., Kujala-Wirth, M., Soveri, T., Pelkonen, S., 2019. Bacterial species associated with interdigital phlegmon outbreaks in Finnish dairy herds. BMC Veterinary Research 15:44

II Kontturi, M., Junni, R., Kujala-Wirth, M., Malinen, E., Seuna, E., Pelkonen, S., Soveri, T., Simojoki, H., 2020. Acute phase response and clinical manifestation in outbreaks of interdigital phlegmon in dairy herds. Comparative Immunology, Microbiology and Infectious Diseases 68: 101375

III Kontturi, M., Kujala, M., Junni, R., Malinen, E., Seuna, E., Pelkonen, S., Soveri, T., Simojoki, H., 2017. Survey of interdigital phlegmon outbreaks and their risk factors in free stall dairy herds in Finland. Acta Veterinaria Scandinavica 59:46

The publications are referred to in the text by their Roman numerals (Study I-III). The original articles are reprinted by the kind permission of Springer Nature and Elsevier.

Abbreviations

Alb	albumin
AmpC	AmpC β-lactamase
AMR	antimicrobial resistance
APP	acute phase protein
APR	acute phase response
CIA	critically important antimicrobial
DD	digital dermatitis
DIM	days in milk
ESBL	extended spectrum β-lactamase
FAA	Fastidious Anaerobe Agar
HHE	heel horn erosion
Hp	haptoglobin
ID	interdigital dermatitis
IH	interdigital hyperplasia
IM	intramuscular administration
IP	interdigital phlegmon
*lkt*A	leukotoxin gene of *Fusobacterium necrophorum*
NV	Neomycin Vancomycin agar
PCR	polymerase chain reaction
RRR	relative risk ratio
OR	odds ratio
SAA	serum amyloid A
Se	selenium
UK	United Kingdom
US	United States
WHO	World Health Organization

1 INTRODUCTION

Interdigital phlegmon (IP) is an infectious hoof disease of cattle and has been a major cause of lameness for decades (Johnson et al., 1969; Alban et al., 1995; Hernandez et al., 2002; DeFrain et al., 2013; Davis-Unger et al., 2019). For the past fifteen years, outbreaks of IP have occurred in Finland. Most of these outbreaks have taken place in dairy herds, in recently built or renovated free stall barns, and where enlargement of the herd had taken place simultaneously. Before these outbreaks, IP occurred mainly as sporadic infections in Finland and only a few small outbreaks occurred on pasture.

In the IP outbreaks, the clinical signs of IP have been severe and morbidity substantial. Additionally, other infectious hoof diseases have been detected frequently in outbreak herds. The treatment of affected cows results in excessive use of antimicrobials and disinfectants, and therefore an outbreak or presence of infectious hoof diseases easily becomes an issue of human and environmental health as well. During an outbreak, the treatment of IP cases results in a substantial volume of discarded milk; e.g. in one herd with over 70 antimicrobial treatments, each treatment resulted in discarding milk from 11 days (Häggman et al. 2015). But if third generation cephalosporins are used, even though their use has been restricted, antimicrobial resistance (AMR) may worsen (Snow et al., 2012). AMR is considered a global threat and several operators, including the World Health Organization (WHO) and European Union (EU), have implemented an action plan on AMR (WHO publications, 2015; EU publications, 2017).

In any case, infectious hoof diseases can be regarded as representing an animal welfare issue, causing lameness, discomfort and pain (Whay and Shearer, 2017). Management of pain should be an essential part of the treatment of a lame dairy cow (Whay and Shearer, 2017; Federation of Veterinarians of Europe position paper, 2019). Though farmers consider IP to be a painful condition (Thomsen et al., 2012), lameness is often overlooked (Whay et al., 2003; Rutherford et al., 2009; Fabian et al., 2014) and a lame cow can occasionally wait for proper treatment for several days (Alefneh et al. 2012).

Currently in most of the countries with a modern dairy industry, the most troublesome infectious hoof disease is digital dermatitis (DD). Therefore, the research focus has been on DD and very few studies have recently dealt with IP. Most of the literature on IP is quite old; bacteriological and statistical methods, medicinal products, and the dairy industry have developed a great deal since most of the studies were done. In addition, the earliest reports are primarily clinical observations of a single person. Interpretation of the results is occasionally difficult also because of reclassification of bacterial species and changed nomenclature for IP.

This thesis concerns outbreaks of IP in dairy herds in Finland. We studied the outbreaks in general, the morbidity, clinical signs and degree of inflammation. The bacteriology of IP was investigated at various stages of the disease. Furthermore, we carried out a survey on risk factors for an outbreak of IP to occur, and to determine the current situation for infectious hoof diseases.

2 REVIEW OF THE LITERATURE

2.1 Interdigital phlegmon

The earliest referrals to IP date back to the 1800s. Flint and Jensen (1951) described that in 1889 Moore reported on a contagious disease, panaritium, locally known as foot rot. Since then many articles on IP have been published. In that literature, IP was referred to using several terms: foot rot, interdigital necrobacillosis, infectious pododermatitis, or foul-in-the-foot. Historical names also include mud fever, winter foot rot, stinky foot, panaritium, and clit-ill (Johnson, 1945; Flint and Jensen, 1951; Gupta et al., 1964). Due to confusion with nomenclature for several other claw diseases, the International Committee for Animal Recording (ICAR) established a working group of claw health experts, who harmonized the names of various foot and claw disorders, and subsequently published the ICAR claw health atlas (Egger-Danner et al., 2015). During this work, the name IP was agreed on.

Mainly based on clinical observations, a common consideration concerning the etiology of IP has been that the infection invades the body through traumatic injuries or macerated skin (Johnson, 1945; Gupta et al., 1964). Wet mud or faeces can soften and macerate the interdigital skin, facilitating entry of the pathogens into the tissue (Gupta et al., 1964; Johnson, et al., 1969). The same studies also reported that stones, or frozen or dried mud, could bruise the skin, making it easy for the bacteria to enter the interdigital tissues. In addition, dry faeces or straw around the coronet may create suitable anaerobic conditions for bacterial growth (Gupta et al., 1964). However, Johnson et al. (1969) already discussed that in IP, the host, microbiological agent and management and environmental factors must all interact before IP can develop. The pathology of an early IP lesion includes swelling and erythema of the soft tissues of the interdigital space and coronary band, and later necrosis and exudation may follow and can advance to cellulitis (Hargis and Myers, 2017). In advanced cases the deeper structures of the foot, including the distal phalanx, distal sesamoid bone, distal interphalangeal joint, and tendons may be affected. If necrosis is extensive, it can lead to sloughing of affected tissue (Mauldin and Peters-Kennedy, 2016; Hargis and Myers, 2017).

In general, IP is diagnosed based on clinical signs. Typical clinical signs of IP are lameness and swelling of the affected foot. Gupta et al. (1964) described that IP is first noticed by slight lameness, which becomes more evident as the infection progresses. Swelling is detected in the interdigital area and the bulbs of the heels. In severe cases, the swelling can be around and above the fetlock (Clark et al., 1985). A fissure with swollen protruding edges and necrotizing tissue soon appears along the interdigital cleft (Gupta et al., 1964). Also, a fetid odour is typical (Gupta et al., 1964; Prentice and Neal, 1972). In severe cases systemic signs occur: fever, recumbency, anorexia or decrease in milk production (Gupta et al., 1964; Clark et al., 1985). If the distal interphalangeal joint is infected, septic arthritis, severe prolonged lameness and rapid loss of bodyweight can occur (Clark et al., 1985). An

Iraqi study of 31 beef bulls suffering from IP reported clinical signs; fever (average 40.5°C), loss of appetite, increased respiratory and heart rates, and sudden onset of lameness. Often more than one foot was affected, commonly the front feet. The bulls stood only touching their toe to the ground (Saleh et al., 2019). Even though diagnosis of IP is usually made based on clinical signs, also a serodiagnostic enzyme-linked immunosorbent assay (ELISA) using leukotoxin protein of *Fusobacterium necrophorum* in diagnosis of cattle IP has been reported (Guo et al., 2010). The new investigative methods may become valuable in the future (Sun et al., 2013; Zheng et al., 2016).

IP reduces milk yield. A 10% reduction in mean milk production due to IP has been reported (Hernandez et al., 2002). At a milk price in the United States (US), at the time of the study, a case of IP was reported representing a loss of $301 per cow. Lower fertility in IP cows was reported in a Mexican study, where heifers with IP needed an extra service to become pregnant in comparison with healthy cows (Mellado et al., 2018). IP may lead to early culling of the affected animal. If IP was diagnosed in the second or third months of lactation, it decreased survival rate during the same period (Booth et al., 2004). The authors discussed IP at this stage of lactation as possibly having affected the cow´s health and production more. In a US study, 60% of the cows affected with IP were culled (Hernandez et al., 2001). A US survey investigated the treatment costs for various hoof diseases; average cost of treating IP was $8 for hoof trimmers (n=90) and $65 for veterinarians (n=12) (Dolecheck et al., 2018). In another US study, a cost of $121 per IP case included milk loss, decreased fertility and treatment costs, and was calculated using dynamic programming (Cha et al., 2010). In Finland, an average cost of IP per cow was €489, but the cost varied among farms from €246 to €652, including discarded milk, veterinary costs, labour, special hoof trimming, culling and other costs, such as increased use of copper sulphate ($CuSO_4$) in hoof baths (Häggman et al., 2015).

2.1.1 Occurrence of interdigital phlegmon

Generally, IP occurs as a sporadic infection of cattle. The occurrence of IP has been reported in several different ways, such as incidence, prevalence, based on lameness reports, and either reported by veterinarians or hoof trimmers. This explains some of the variation among studies. In Denmark an IP incidence of 1.5% per lactation was reported (Alban et al., 1995). In a US study, IP was diagnosed in 9% of the total lameness cases (Hernandez et al., 2002). Their study took place in one herd of 868 cows in Florida that calved during 1997-98. Based on data of 40 veterinary practitioners and six veterinary schools in the United Kingdom (UK) in 1997-2004, IP was the fourth most common cause of lameness in cattle; IP was diagnosed in 6.2% of the total lameness reports (Laven and Lawrence, 2006). A similar study from New Zealand reported IP in 8.3% of lameness-causing lesions in dairy cattle (Chesterton et al. 2008). In a Canadian study, a herd and cow-level prevalence of various foot lesions was determined. Recordings from 13 530 cows in 204 Ontario dairy herds were made by five hoof trimmers for 13 months; a cow level prevalence of IP was 0.2% in tie stalls and in free stalls, and mean herd level prevalence 0.3% and 0.0%, respectively.

Of tie stall herds 7.8% and free stalls 2.6% were affected with IP, i.e. had at least one animal affected with IP, and the cow-level prevalence of IP was 0.2% in both herd types (Cramer et al., 2008). A study from New Zealand detected an IP incidence risk as 6 cases per 100 cows (Alefneh et al., 2012). The study herd was a spring-calving and pasture-fed dairy herd with 452 cows. During the last decade in the US, the herd incidence per lactation is reported as 2–5% (DeFrain et al., 2013; Oberbauer et al., 2013). A study from Brazil reported 1% prevalence of IP in 48 all-year-round grazing dairy herds of 2267 lactating cows (Moreira et al., 2018b).

IP is a common cause of lameness in cattle also in feedlots. In an US study on six commercial feedlots during a 12-month period from 2012 to 2013, the overall lameness incidence rate was 1.04% and incidence of IP was 0.09%. Of all 222 IP cases, 29 (13.1%) died or were euthanized (Terrell et al., 2017). In a Canadian study of 28 feedlots and corresponding health recordings for ten years, from 2005 to 2015, the lameness prevalence estimation was 4.5% (Davis-Unger et al., 2019). The herd level lameness prevalence varied among the feedlots from 1.3% to 46% in different years. Of all lameness diagnoses in the study period, IP was the most common, with 74.5% of all recorded lameness cases. Additionally, IP was behind 3.9% of all deaths on feedlots (Davis-Unger et al., 2019). Another Canadian study estimated the economic impact of lameness on several Alberta feedlots over ten years; the net return for IP cattle was $568, IP in cattle near the market weight $695 and healthy cattle $690 (Davis-Unger et al., 2017).

2.1.2 Outbreaks of interdigital phlegmon

In the 1990s there were mentions of a more severe form of IP, a super foul. Some reports of IP outbreaks (David, 1993; Doherty et al., 1998) detailed clinical signs more severe and of a more rapid course of the disease in comparison with previously detected IP cases. Cook and Cutler (1995) described the disease as a more severe form of IP, peracute at onset and refractory to conventional therapy. They also reported that in chronic or severe cases, the infection could extend to the joint or tendon. Reports of earlier outbreaks of IP had incidences of 17–25% during outbreaks (David, 1993; Doherty et al., 1998).

In a report of three outbreaks of IP in the UK (David, 1993), in one outbreak a dirty hoof bath was presumed to spread the infection, and in another there was a sudden change in hoof bath product just before the outbreak. Two herds had a history of DD, but one did not. In Ireland (Doherty et al., 1998), 100 (25%) of feedlot cattle were affected in six weeks. In that outbreak, response to treatment was poor and they culled all the affected animals.

2.1.3 Treatment of interdigital phlegmon

In general, IP is treated with antimicrobials. The earliest reports of IP treatment are mainly clinical observations and include the use of intravenous sulphonamide therapy (Forman, 1946; Candlin, 1947; Case, 1948) and intramuscular procaine penicillin (Chambers, 1951), but also other treatment protocols (Levanthal and Easterbrooks, 1956). In a study of Berg and Loan (1975) they used either penicillin or oxytetracycline, and observed that if the treatment was delayed, more severe lesions developed, leading to a prolonged treatment and delayed recovery.

Some clinical trials have been performed to investigate IP treatment. Braun et al. (1987) performed a clinical trial of the use of 10 mg/kg amoxicillin trihydrate intramuscularly (IM), given within five days after the onset of IP for the treatment of experimentally induced IP in cattle. In their study, IP was induced with suspension of *F. necrophorum* and *Bacteroides melaninogenicus*. Amoxicillin trihydrate reduced the severity of lesions in comparison to non-treated controls. They also concluded that prompt diagnosis and rapid treatment with amoxicillin trihydrate could reduce the severity of the lesions and improve weight gain.

Later clinical trials deal with the use of ceftiofur. Morck et al. (1998) performed a clinical trial of IP treatment in feedlot cattle. They compared 1.0 mg/kg ceftiofur sodium with 6.6 mg/kg oxytetracycline; both antimicrobials were administered once a day IM for three days. The success rate for ceftiofur was 73.0% and for oxytetracycline 68.0% based on locomotion score being zero on day four and lack of re-treatment within ten days of initial treatment. No statistical difference existed between treatment groups, although the oxytetracycline dose used in that study was lower than the currently recommended 10 mg/kg for most cattle infections (del Castillo, 2013). A review of seven studies using either ceftiofur sodium or hydrochloride for treatment of bovine IP was written by Kausche and Robb (2003). The studies included dosage titration studies, clinical trials with ceftiofur versus placebo and ceftiofur versus oxytetracycline. The authors regard the injection site tolerance and short or no milk discard as being a benefit of ceftiofur. A US review of individual therapy for IP mentioned several antimicrobials, including ceftiofur, florfenicol, tulathromycin and oxytetracycline for which trial evidence is available (Apley, 2015). Most of this evidence is from approval trials of a new veterinary pharmaceutical, i.e. an antimicrobial treatment versus placebo.

Some studies exist on antibiotic usage for IP. A survey in dairy herds in Pennsylvania reported that the most commonly used antimicrobial for IP was sulfadimethoxine in 27.3% (9/33) of the study farms and ceftiofur was used in three farms (9.0%), being the second most common antimicrobial (Sawant et al., 2005). In their study herds, IP cases were reported for calves, heifers, lactating cows and dry cows, and the biggest treatment group was lactating cows; 16% (459/2783) of the cows treated because of IP (Sawant et al., 2005).

Nordic countries have a long history in prudent use of antimicrobials. The focus is on individual animal therapy instead of mass medication. Narrow spectrum antimicrobials are preferred, and the use of certain antimicrobials is restricted in animals, because they are considered critically important antimicrobials (CIA) in humans (Collignon et al., 2009). In a Swedish study of the use of antimicrobial drugs in dairy calves and replacement heifers (n=3081), all thirteen IP cases were treated with tetracycline or penicillin (Ortman and Svensson, 2004). In Finland there are national recommendations for the use of antimicrobials in the treatment of the most significant infectious and contagious diseases in animals (Finnish Food Authority publications, 2018). In those recommendations benzylpenicillin is considered the first-line treatment for IP and oxytetracycline the second one. In addition, Finnish legislation restricts the use of third and fourth generation cephalosporins (Decree on the use and distribution of medicines in veterinary practice, 2014). These medicines may only be used on the target animal species and the indication detailed in the marketing authorisation if no other antimicrobial is known to be effective.

Some reports exist on local treatment of IP without antimicrobials (Johnson, 1945; Woelffer, 1951; Chambers, 1951). Already Johnson (1945) observed that if affected animals were moved to drier ground or bedding, some animals recovered without treatment. He also reported that removal of necrotic tissue and application of copper sulphate as a powder to the affected area with a wrap was a beneficial treatment in some cases, and the use of 5% copper sulphate hoof bath prevented the disease in the herd. A few years later Williamson (1953) also wrote about hoof-bathing with copper sulphate in order to prevent IP. Chambers (1951) reported about his colleague´s use of salicylic acid and a bandage to treat IP. A recent Swedish study reported a successful cure of non-complicated IP with salicylic acid (Persson et al., 2019). In that study there was no control group and the diagnosis of IP was made by the farmer. The clinical cure was tested 3–5 days after initial treatment, when the bandage was taken off, with reduced lameness, lower body temperature, decreased swelling and improved general condition.

Surgical treatment of IP is mentioned in some articles, including removal of necrotic tissue of an IP lesion (Silva et al., 2004) or digit amputation in advanced cases with complications (Desrochers et al., 2008). Advanced cases with adjacent joint or tendon infection are not cured with antimicrobials and therefore digit amputation is suggested (Desrochers et al., 2008).

2.2 Bacteriology of interdigital phlegmon

In IP, *F. necrophorum* is considered the major infective agent (Flint and Jensen, 1951; Berg and Loan, 1975; Clark et al., 1985). However, various other bacteria are detected frequently in IP lesions (Gupta et al., 1964; Berg and Loan, 1975; Clark et al., 1985; Morck et al., 1998; Sweeney et al., 2009), but their role in the pathogenesis of IP is still obscure.

2.2.1 *Fusobacterium necrophorum*

F. necrophorum is a Gram-negative non-spore-forming anaerobe (Langworth, 1977; Nagaraja et al., 2005) and is currently classified into two subspecies, *necrophorum* and *funduliforme* (Shinjo et al., 1991). *F. necrophorum* produces several toxins able to damage tissue. Leukotoxin is regarded as a major virulence factor in cattle (Tan et al., 1996) and it is unique to *F. necrophorum* (Oelke et al., 2005). The gene encoding the leukotoxin is *lkt*A (Narayanan et al., 2001). Several other virulence factors are detected in *F. necrophorum*, including endotoxic lipopolysaccharide, haemolysin, haemagglutinin, capsule, adhesins or pili, platelet aggregation factor, dermonecrotic toxin and several extracellular enzymes (Tan et al., 1996; Nagaraja et al., 2005; Tadepalli et al., 2009).

As reviewed by Langworth (1977), *F. necrophorum* has been isolated from oral cavities, gastrointestinal and genitourinary tracts of animals and humans. In cattle, *F. necrophorum* is a normal inhabitant of the rumen (Robinson et al., 1951). It is found in the rumen contents and adherent to the rumen wall, and its role in fermentation is to metabolize lactic acid into volatile fatty acids, and digest feed or epithelial cell proteins (Tadepalli et al., 2009). Normally, *F. necrophorum* is not excreted in cattle faeces (Smith and Thornton, 1993).

F. necrophorum is an opportunistic pathogen; subspecies *necrophorum* is more commonly detected in animal infections and in pure culture, whereas *funduliforme* is found in mixed infections and is considered less pathogenic in animals (Lechtenberg et al., 1988; Nagaraja et al., 2005). In humans, *F. necrophorum* causes throat infections, localized abscesses and systemic lethal disease referred to as Lemierre´s syndrome (Brazier, 2006). In domestic and wild animals, *F. necrophorum* has been detected in various necrotic conditions (Jang and Hirsh, 1994; Tan et al., 1996; Nagaraja et al., 2005).

To date, little antimicrobial resistance is detected with cattle isolates of *F. necrophorum*. In a study of Cook and Cutler (1995), four *F. necrophorum* isolates were detected from six IP cases; three out of these four isolates were resistant to penicillin and potentiated sulphonamides, but all isolates were sensitive to tetracyclines, macrolides and ampicillin or amoxycillin. On the contrary, the isolates from bovine hepatic abscesses and rumen contents were susceptible to all tested antimicrobials, including penicillin G, oxytetracycline and cephaloridine (Berg and Scanlan, 1982; Lechtenberg et al., 1998). Swedish National Veterinary Institute´s reports regard *F. necrophorum* isolated from IP susceptible to treatment with penicillin or tetracyclines (SVARM, 2009; Swedres-Svarm, 2017). Also, all isolates of *F. necrophorum* (n=24) from IP cows were susceptible to penicillin in a study of Persson et al. (2019).

During recent years attempts to immunize cattle against *F. necrophorum* have been reported (Clark et al., 1986; Amachawadi and Nagaraja, 2016). At least one vaccine against IP and liver abscesses remains on the market; *F. necrophorum* bacterin in Canada (Fusogard™, Elanco, US). A clinical trial suggests that vaccination against *F. necrophorum* infection may decrease the prevalence of severe liver abscesses at slaughter and IP treatments in a certain forage based diet (Checkley et al., 2005). Earlier study on combination vaccine of leucotoxoid of *F. necrophorum* and *T. pyogenes* bacterin reduced the prevalence of liver abscesses in cattle (Jones et al., 2004), but the vaccine used in this study is no longer available.

2.2.2 Other bacteria in interdigital phlegmon

In the 1960s in the US, Gupta et al. (1964) took smears from IP feet and detected a *Spirochete* and a Gram-negative bacillus that resembled bacteria currently known as *Dichelobacter nodosus.* They also detected other bacteria from the lesions, like haemolytic and non-haemolytic staphylococci and streptococci, bacteria currently known as *Trueperella pyogenes,* and Gram-positive rods. After a decade, Berg and Loan (1975) took biopsies from IP lesions of cattle and isolated large numbers *F. necrophorum* and *B. melaninogenicus;* currently reclassified as several *Porphyromonas* and *Prevotella* species (Jousimies-Somer and Summanen, 2002). In that study, they also isolated from some of the IP lesions species of *Bacteroides, Peptostreptococcus, Propionibacterium*, and *Staphylococcus*. Additionally, they experimentally inoculated a mixture of *F. necrophorum* and *B. melaninogenicus* to cattle feet and were able to induce IP and reisolate both bacteria from the lesions (Berg and Loan, 1975). Ten years later, Clark et al. (1985) inoculated subcutaneously various isolates of *F. necrophorum*, either alone or with *B. melaninogenicus*, in calves. They were able to induce IP with *F. necrophorum* alone and with both bacteria. However, they reisolated *B. melaninogenicus* only from two feet out of five inoculated, while *F. necrophorum* was reisolated from all inoculated feet (n=11). The isolates used in their study originated from cattle and sheep.

On two farms in UK, young stock suffered from severe IP affecting all four feet. The bacteriological swabs revealed *F. necrophorum* and *Bacteroides fragilis* and *B. melaninogenicus* from one animal. These animals tested positive for bovine viral diarrhoea (BVD) (Daniel et al., 1995). Doherty et al. (1998) demonstrated *Spirochetes* from direct smears (14/20; 70%) and isolated *B. melaninogenicus* from culture of IP samples during an outbreak in Ireland. *B. melaninogenicus* was cultured from 15/20 (75%) lesions and *F. necrophorum* from 4/20 (20%). In a clinical trial, Morck et al. (1998) took biopsy specimens from IP cattle and isolated several bacteria from the specimens; *Prevotella intermedia, Porphyromonas levii, Peptostreptococcus indolicus, Bifidobacterium* sp., *B. fragilis, Lactobacillus* sp., *T. pyogenes* and an unidentified Gram-positive coccus. Sweeney et al. (2009) investigated needle biopsy and swab specimens from cattle IP lesions, which were collected in a clinical trial of ceftiofur and tulathromycin. Of the isolates that were originally classified as *Prevotella* or *Porphyromonas* spp. using conventional methods, 241/264 (91.3%) and 156/275 (56.7%) were identified as *P. levii* by 16S rRNA sequencing.

A study conducted in ten dairy farms in UK investigated the microbiome of several lameness causing foot lesions; complicated claw horn disruptions, i.e. complicated sole ulcers, toe ulcers and white line lesions, interdigital hyperplasia (IH), and IP (Bay et al., 2018). They obtained three IP samples, all from the same farm, and found at the genus level *Fusobacterium* spp., *Porphyromonas* spp., *Helococcus* spp., *Parvimonas* spp. and *Peptostreptococcus* spp. to be more prevalent in IP lesions compared with samples from healthy control cows. Additionally, they reported that in healthy skin samples the diversity was increased over samples obtained from various lesions. Furthermore, they noticed that complicated claw horn disruption lesions and IP had similar bacterial profiles, suggesting many bacteria in these lesions may act opportunistically.

Apart from Bay et al. (2018), most of this research is quite old; the taxonomical changes in bacteriology and changes in the nomenclature of various infectious hoof diseases complicate interpretation of the results. In addition, improvement in molecular diagnostics markedly increases the capacity of bacterial detection. Also, a recent review explains that the role of various bacterial species in the pathogenesis of IP is still unresolved (Van Metre, 2017).

2.2.3 *Fusobacterium necrophorum* in other cattle infections and bacterial synergism

F. necrophorum is observed in many cattle diseases, repeatedly in mixed infections with bacteria also commonly detected in IP. In liver abscesses, *F. necrophorum* is frequently detected with *T. pyogenes*, the latter being the predominant facultative anaerobic bacterium isolated (Berg and Scanlan, 1982; Lechtenberg et al., 1988). However, several other bacterial species were detected from the same abscesses in those studies. A possible synergy between *F. necrophorum* and *T. pyogenes* has been suggested in liver abscesses (Takeuchi et al., 1983; Nagaraja et al., 1999). It is hypothesized that *T. pyogenes* helps *F. necrophorum* in the establishment in the rumen wall or in the liver by utilizing oxygen and providing anaerobic conditions (Tadepalli et al., 2009). Furthermore, the leukotoxin of *F. necrophorum* could offer protection to *T. pyogenes* (Narayanan et al., 2002). Additionally, a nutritional interaction between the two bacteria exists; lactic acid, being an end product produced by *T. pyogenes,* serves as an energy substrate for *F. necrophorum*, as reviewed by Tadepalli et al. (2009).

F. necrophorum is also detected in summer mastitis with *Peptostreptococcus indolicus*, a microaerophilic coccus, *T. pyogenes, B. melaninogenicus* and *Streptococcus dysgalactiae* (Madsen et al., 1990; Madsen et al., 1992), and regarded as a key pathogen also in calf diphtheria, i.e. necrotic laryngitis, where necrotic lesions occur in the larynx, oral cavity, or pharynx (Panciera et al., 1989; Tan et al., 1996; Nagaraja et al., 2005). Calf diphtheria can affect animals up to three years of age and is characterized by laryngeal dyspnea causing a roaring noise on inhalation and in severe cases both when inhaling and exhaling (Mackey, 1968). In two case reports of Panciera et al. (1989), in addition to *F. necrophorum,* they also isolated *T. pyogenes, Mycoplasma* spp., a pigmented *Bacteroides*, which they presumed to be *B. melaninogenicus,* and several other bacterial species in some of the specimens.

Additionally, *F. necrophorum* has an important role in metritis in dairy cattle (Bicalho et al., 2012; Cunha et al., 2018). In metagenomics studies an increased abundance of *Fusobacteria* and *Bacteroidetes* were detected in metritic cows (Bicalho et al., 2017b) and the increased abundance of *Fusobacteria* and *Trueperella* in the cows with purulent vaginal discharge, which emphasizes their role in the pathogenesis of this disorder (Bicalho et al., 2017a). Also, evidence of interactions or possible synergism is detected with *F. necrophorum* and other bacteria – with *T. pyogenes* in uterine infections in cows (Ruder et al., 1981) and with *P. levii* in cattle pyometra (Karstrup et al., 2017).

2.3 Risk factors for interdigital phlegmon

Several studies have investigated the risk factors for IP. Already Johnson (1945) observed that the increased movement of cows, like cattle auctions, enhance the incidence of IP. Monrad et al. (1983) studied occurrence of IP and possible risk factors on pasture in several feedlots in Denmark. They concluded that weather conditions affect the occurrence, discussed how soil pH might be one risk factor, and that heavier breeds than Jersey were affected more often. In another Danish study, first parity and free stall cows had a higher risk for IP. In addition, breed was a significant factor; Danish Jersey cows having the lowest risk (Alban et al., 1995). Another study of Alban et al. (1996) in 12238 tie-stall dairy cows in 1366 Danish dairy herds reported that first parity, summer calving combined with outdoor grazing, use of yoke tie and high average herd milk production (>7500kg energy corrected milk) were associated with increased risk of IP.

The data on the seasonality of IP are inconsistent and partly difficult to compare. The seasons and e.g. pasture conditions vary among countries and different years. In addition, housing conditions have changed considerably since the first studies on seasonality. In a Danish study of approximately 9500 dairy cows in 170 herds during a two-year follow-up, half of the treatments were done during June-September (Alban et al., 1995). A German study on heat stress indicators of dairy cattle detected increasing incidences for IP with increasing temperature and relative humidity indexes of the previous week (Gernand et al., 2019). In contrast, in an UK study more cases of IP occurred during winter (Murray et al., 1996). Also, in a study of seasonality of lameness in the UK Laven and Lawrence (2006) observed that IP had low seasonality but there was a peak in January. Nevertheless, they reported that if compared with DD, IP was more common during June-August. In a Mexican study, the seasonal incidence of IP was significantly higher ($P<0.05$) in cows calving in spring than cows with parturition in all other seasons (Mellado et al., 2018). Otherwise in Canadian feedlots, most IP cases were treated during spring or summer (Davis-Unger et al., 2019).

IP often occurs during early lactation (Alban et al., 1995; Hernandez et al., 2002; Booth et al., 2004; DeFrain et al., 2013; Oberbauer et al., 2013). In a study of Alban (1995) the incidence risk of IP was 0.6% during the first month after calving. In a study of Hernandez et al. (2002) 80% of the IP cases were during early lactation. Similarly, 59% (Booth et al., 2004) and 42% of cases of IP (DeFrain et al., 2013) occurred in first 60 days in milk (DIM). In addition, DeFrain et al. (2013) said that IP was most troublesome in the first 120 DIM especially in first lactation. Their study was based on about 50,000 foot lesion recordings from 17 dairy herds in the US.

Recently, the probable deficiency of trace minerals and hoof health has been investigated, e.g. (Formigoni et al., 2011; Osorio et al., 2016; Faulkner et al., 2017). A US study investigated if a feed supplement with either copper sulphate, zinc sulphate, and zinc methionine complex or basic copper chloride and zinc hydroxychloride affected IP incidence and carcass weight on feedlot bulls, but no differences were established (Hilscher et al., 2019).

2.3.1 Other infectious hoof diseases

In addition to IP, other infectious hoof diseases include heel horn erosion (HHE), interdigital dermatitis (ID) and DD. These diseases are known to predispose to each other, and almost the same bacteria are detected in the diseases.

HHE is a progressive destruction of heel horn in the axial surfaces of the bulbs of the heels. In general, the lesion forms a series of ridges, gets darker in colour and ends up as a dark V-shaped erosion (Greenough, 2007). In severe cases, the posterior third of the sole is absent (Prentice and Neal, 1972). Normally HHE does not cause lameness, except in advanced cases that disrupt the longitudinal balance of the claw (Greenough, 2007). HHE has been associated with hooves contacting manure (Bergsten and Pettersson, 1992). HHE is quite common among dairy cows. Already in 2002 it was reported that 41% of cows and heifers had HHE in a cross-sectional study on Swedish dairy farms (Manske et al., 2002). In a longitudinal study in Denmark (Capion et al., 2009), HHE was detected in almost all the cows (93–100%).

ID is described as a mild, superficial dermatitis between the claws with no swelling or lameness, nor any systemic signs (Greenough, 2007). More chronic cases are hyperkeratotic and create a roughened appearance to the interdigital skin and dorsal and palmar commissural skin folds. An exudate may be present, and mild sensitivity to pressure detected (Berry, 2001). These more chronic conditions are frequently accompanied with HHE (Berry, 2001). An association of ID and HHE was detected also in other studies (Enevoldsen et al., 1991; Manske et al., 2002; Capion et al., 2009; Knappe-Poindecker et al., 2013). *D. nodosus* has been detected in ID lesions (Laing and Egerton, 1978; Knappe-Poindecker et al., 2013). A Swedish study considered that improved foot health regarding HHE and ID was an indirect effect of the improved hygienic conditions (Hultgren and

Bergsten, 2001). A Norwegian study reported that in addition to *D. nodosus*, *Treponema* spp. and hygiene are involved in the pathogenesis of ID (Knappe-Poindecker et al., 2013).

DD was first described in Italy by Cheli and Mortellaro (1974) as a disease that causes painful ulcerative lesions in the coronary band. Currently, DD lesions are classified into M-stages (Döpfer et al., 1997; Berry et al., 2012) or scored using the Iowa DD scoring system (Krull et al., 2014). The clinical signs of DD include lameness, lifting of the affected foot, and in severe cases shaking of a leg in partial flexion and reluctance to move (Read and Walker, 1998). Little or no diffuse digital swelling was observed in the US, and most of the DD lesions occurred in the hind feet (Read and Walker, 1998). DD reduces milk yield (Gomez et al., 2015a). The etiology of DD is still partly unknown, but several *Treponema* spp. play a key role in its etiopathogenesis (Klitgaard et al., 2008; Evans et al., 2009; Zinicola et al., 2015). However, other bacteria are suggested to play a role in DD, including *D. nodosus, F. necrophorum*, and *P. levii* (Sullivan et al., 2015; Nielsen et al., 2016; Moreira et al., 2018a). As reviewed by Evans et al. (2016), DD is a contagious disease and responds poorly to treatments.

DD has spread all over the world and is considered endemic in many countries (Holzhauer et al., 2006; Capion et al., 2009; Relun et al., 2013; Solano et al., 2016; Plummer and Krull, 2017). In a study in the Netherlands, based on 20 hoof trimmers' recordings in 383 herds and 22454 cows, DD was present in 21.2% of the cows (Holzhauer et al., 2006). In a Canadian study of 156 farms and 28607 cows, as recorded by seven hoof trimmers, the herd level prevalence of DD was 93.6% and cow-level prevalence 21.8% (Solano et al., 2016). In Brazil, among the 2267 lactating cows evaluated on 48 farms, the herd level prevalence of DD was 96% and cow-level 33% (Moreira et al., 2018b). It has been estimated that the economic impact on milk production losses due to DD every year in the US is $190 million (Losinger, 2006). Another US study calculated a cost per DD case of $133, including milk loss, decreased fertility and treatment costs (Cha et al., 2010).

Several studies have associated various infectious hoof diseases with each other. A connection with dermatitis and HHE has been detected (Manske et al., 2002). In that study, the authors did not make a distinction between ID and DD, because the early stages of DD are very similar to ID lesions. In a Dutch study, when grade 2 or 3 (scale 1-5) HHE was present, a cow was significantly more likely to be affected by DD (Holzhauer et al., 2008). In a Danish study (Capion et al., 2009) HHE was strongly correlated with DD, and ID significantly correlated with both HHE and DD. A Swiss study detected a strong association between HHE and DD (Becker et al., 2014). In a study on the effect of DD on hoof conformation, feet with DD lesions experienced a 46%-point increase in severe HHE (Gomez et al., 2015b).

There are also studies that have associated IP with other infectious hoof diseases. ID in a previous lactation was found to be a risk factor for IP in the next lactation (Alban et al., 1996). In a Dutch study, IP was strongly associated with DD and other infectious hoof diseases, and a combination of ID and HHE, IH, and IP appeared to predispose to DD (Holzhauer et al., 2006).

IH is recognized as a proliferative growth in the interdigital space (Berry, 2001), and large IH, or if infected, can cause lameness (Prentice and Neal, 1972; Berry, 2001). The etiology of IH is not quite clear and several backgrounds are speculated. Peterse (1985) tried to place infectious hoof diseases into a causal context and suggested that either IP or ID leads to IH. A review of Berry (2001) mentions poor hygiene, claw overgrowth or poor conformation, or uneven or slippery surfaces, that may lead to formation of IH, or that IH could be a result of ID or IP. A Swedish study suggested that chronic irritation caused by dermatitis is more likely to be of importance in the development of IH than other causes such as abnormal claw shape (Manske et al., 2002). Another theory is that outwards spreading of the claws and poor ligamentous structure results in stretching of the interdigital skin and leads to hyperplasia (Desrochers et al., 2008). In a Dutch study, it was suggested that DD is an important factor in the development of IH or vice versa, but in their study design it was impossible to confirm the causality (Holzhauer et al., 2008).

2.4 Acute phase response

Acute phase proteins (APP) are used in clinical veterinary medicine as an option to monitor animal health. Because acute phase response (APR) is non-specific, an exact diagnosis cannot be made, but APPs can add information on extent of ongoing lesions or serve as a prognostic tool (Petersen et al., 2004).

APR is described as an early systemic and non-specific defence mechanism of the host to infection, inflammation or trauma and it aims to restore normal body functions (Gabay and Kushner, 1999; Murata et al., 2004; Petersen et al., 2004; Eckersall and Bell, 2010; Ceciliani et al., 2012). During APR, metabolic, endocrinal, haematological and behavioural changes occur and clinical signs – fever, anorexia, apathy and pain can be identified (Gabay and Kushner, 1999). APR is started when cytokines function as messengers between the local injury sites and induce the synthesis of APP in the liver (Murata et al., 2004; Petersen et al., 2004; Eckersall and Bell, 2010). APPs have multiple functions and play a direct role in the protection of the host (Petersen et al., 2004). In general, the maximum serum concentration of APPs is reached within 24–48 hours and the feedback mechanisms will limit the response normally within 4–7 days (Petersen et al., 2004).

APPs are categorized as positive when they increase, or negative when they decrease, and based on the magnitude of their increase as minor, moderate or major (Murata et al., 2004; Petersen et al., 2004; Eckersall and Bell, 2010). In ruminants, Hp and SAA are major, alpha 1-acid glycoprotein, C-reactive protein, and fibrinogen moderate, and ceruloplasmin minor APPs (Murata et al., 2004; Eckersall and Bell, 2010).

Several studies have investigated APR and APPs in various diseases of cattle, such as mastitis, as in Eckersall et al. (2001) and Grönlund et al. (2003), metritis (Schneider et al., 2013), and bovine respiratory disease (Abdallah et al., 2016). Also, there are studies on APPs and lameness or non-infectious hoof diseases (Kujala et al., 2010; Tóthová et al., 2011; Tadich et al., 2013; O'Driscoll et al., 2015; Zhang et al., 2015). However, studies that focus on infectious hoof diseases are few (Smith et al., 2010; Nazifi et al., 2012; Tóthová et al., 2017; Ilievska et al., 2019).

3 AIMS OF THE STUDY

The overall aim of the study was to explore the characteristics of outbreaks of IP in Finnish dairy herds. Specific aims were to investigate:

1) the morbidity, clinical manifestation, and degree of inflammation in the outbreaks of IP (Study I-II)

2) the bacteriology of IP; to investigate bacteria earlier detected from hoof diseases (Study I-II)

3) the herd level risk factors for an outbreak of IP (Study III)

4 MATERIALS AND METHODS

This study was part of a research project on infectious hoof diseases in Finnish dairy herds in 2012–2015. The project was funded by the Ministry of Agriculture and Forestry (project number 2066/312/2011), a dairy company, Valio Ltd, the Finnish Food Authority and the University of Helsinki. The overall study designs, laboratory analyses and main statistical analyses are summarized in this paragraph. All details concerning materials and methods are described in the original articles reprinted at the end of this thesis, which are also available as open access publications.

4.1 Study design and sampling

4.1.1 Study design

Studies I and II were observational, cross-sectional studies and Study III was a survey.

4.1.2 Study herds and sampling (I, II)

During 2012–2014 we performed one-day farm visits to 19 commercial dairy herds affected by outbreaks of IP (IP herd). A requirement for a herd was a free stall barn less than eight years old. The IP criteria to visit a herd were; at least three cows affected by IP in one week, and no previous history of IP in the herd for ten years. Furthermore, we collected convenience samples from three non-outbreak herds (IP free herds).

The herd visits were restricted to one day only and therefore not all cows were inspected in the herds visited. In each outbreak herd, the focus was on IP and all detected, non-treated IP cows were selected for sampling. If few non-treated IP cases were detected, IP cows recently treated with antimicrobials were also sampled. Some of the cows diagnosed as IP had other minor infectious lesions, including ID, HHE or mild DD lesions (M1, M3). In these cows however, IP was regarded as the main reason for a cow being lame. While examining the feet and looking for IP and healthy control cows, we detected several cows with hoof diseases other than IP. Of these cows, those with a possible bacterial involvement in the detected lesions were chosen for sampling for comparison. The cows with other infectious disease, in addition to infectious hoof disease, were excluded from the sampling. At sampling time, 148 (73%) of all sampled cows were not treated with antimicrobials, 35 (17%) had current, and 20 (10%) previous antimicrobial treatments. However, none of the control cows were treated with antimicrobials.

The cows were selected for sampling based on lameness, long lying times, or a “trouble report” from an automatic milking system. The lameness was recorded according to Sprecher et al. (1997); non-lame cows were scored 1–2 and lame 3–5 on this scale. A clear odour was detected from a distance of 25–30 cm of sampled feet and categorized as present or absent. The cows were clinically checked and sampled in a trimming chute:

bacteriological samples for culture and PCR analysis, and blood samples for the analysis of APPs. In IP herds, we sampled mainly cows that had IP (n=100). These IP lesions we further classified as acute stage of IP (Acute IP) or healing stage of IP (Healing IP). If detected, we also sampled cows with various other hoof lesions, such as DD, ID, white line abscesses and sole ulcers (n=53). Moreover, we sampled control cows: 1–5 control cows per IP herd (n=45) and 4–8 control cows in IP free herd (n=19). The control cows were non-lame and otherwise clinically healthy cows with no sign of IP, DD, ID, sole ulcer, white line disease, or HHE, as categorized by Manske et al. (2002). **Figure 1 A-D** shows photographs of some of the sampled cows. Bacteriological samples were taken either from the affected region (IP and other hoof diseases) or from the interdigital cleft (control cows).

All diagnoses were based on clinical examination made by one of two study veterinarians. IP was diagnosed based on Gupta et al. (1964). If symmetrical swelling was detected in a distal foot and a possible ulceration in the interdigital cleft, acute IP was diagnosed. Contrarily, healing IP was defined with proliferation tissue or scar formation in the affected region. Diagnosis of DD was based on Döpfer et al. (1997). All sampled DD lesions were either stage M1, M2 or M3 and less than 3 cm in diameter. We photographed the sampled feet and by analysing the photographs, standardized the diagnoses between the two study veterinarians.

In Study I, two legs were sampled from 11 cows, thus overall number of hoof samples was 228. In Study II, one herd was excluded because of a different barn construction and missing data. One sample per cow and only cows with APP results were included and therefore the overall number of cows was 203.

4.1.3 Study herds in survey (III)

A questionnaire was sent via mail to all Finnish dairy farms in a dairy herd recording database of ProAgria that had ≥50 dairy cows in 2012 (n=1134). Only free stall herds were included in the study. The questionnaire contained altogether 35 questions; the questions of general herd data, barn characteristics, and herd management details are presented in **Table 1.**

Figure 1 A-D Most of the study cows were either A) cows suffering from interdigital phlegmon in an acute stage (Acute IP) either with or without a fissure in the interdigital cleft, B) cows in a later, healing stage of IP (Healing IP), C) digital dermatitis or D) healthy control cows. Mild heel horn erosion was detected on some of the control cows. Photographs by DVM Reijo Junni.


B)


C)


D)

Table 1 Study questions concerning general herd data, barn characteristics, and herd management details.

	Survey questions
Herd data	Herd size, mean milk yield, region in Finland
Barn characteristics	Construction year, stall type (freestall, tiestall), free stall type (insulated, partly insulated, non-insulated) number of compartments for milking cows, flooring (slatted or solid concrete and with or without rubber), milking system (parlour, automatic milking system), ventilation system (natural, mechanical)
Herd management	Enlargement or renovation of the barn within 3 years (yes, no), total or partial mixed ration feeding (yes, no), outdoor access during summer (yes, no), outdoor access during winter (yes, no), hoof trimming frequency per annum, stocking density (less cows than stalls, equal number of cows and stalls, more cows than stalls), animal purchase (yes, no), contract heifer rearing (yes, no), fields in organic farming (yes, no)

Some questions on the leg and claw health of the herds were included in the questionnaire. Based on the replies, we categorized the respondent herds to outbreak herds, herds with few cases of IP, and no IP herds. An outbreak of IP was defined if the morbidity in IP was ≥5% within the first month of the outbreak. We investigated the risk factors for an outbreak of IP to occur. The herds with few cases of IP were excluded from the risk analysis.

We surveyed the current occurrence of some infectious hoof diseases and disorders, including IH, verrucose dermatitis, i.e. wart growth, ID, DD, and IP-associated putative disorders, i.e. buccal abscesses in suckling calves. The signs of ID and DD were listed and included lesions between the hooves or in the heel, and reddish, painful lesions in the hoof region. Based on the replies, it was not possible to make a correct diagnosis of which hoof disease, ID or DD, was in question and therefore they were united and termed "other infectious hoof disease".

4.1.4 Ethical considerations

The study protocol was reviewed and approved by the Viikki Campus Research Ethics Committee of Helsinki University in 2012.

A written informed consent to use the cows in our research was obtained from the herd owners before sampling. The sampling took place in a normal trimming chute with minimal stress to sampled cows. If needed, the affected cows were treated after sampling by the farm veterinarian. At the time of sampling, 66 (30.4%) of 217 cows sampled were currently being or had previously been treated with antimicrobials. It would have been unethical to leave the affected cows untreated until our sampling visit took place.

4.2 Analytical methods

4.2.1 *Bacteriology (I)*

4.2.1.1 Bacterial culture

A field laboratory was set up during the farm visits. We used swabs for sampling and Fastidious Anaerobe Agar (FAA) and Fusobacterium Neomycin Vancomycin (NV) agars for primary culture. Agar plates were prereduced in Genbox containers and within two hours after culture the containers were sealed to maintain anaerobic conditions. The plates were incubated anaerobically for two days at 37 °C.

From cultures we picked greyish, umbonate colonies of various shapes and sizes typical of spp. *necrophorum,* and smaller, yellowish, and waxy colonies typical of spp. *funduliforme*. Both expressed strong beta-haemolysis on FAA and NV agars. We identified the colonies by conventional methods to species and subspecies level and verified them using PCR assays for *lktA* and *haemagglutinin* genes. From culture, other bacteria were not analysed further.

4.2.1.2 PCR

Total DNA was extracted from cytobrush samples with a commercial kit following the manufacturer's instructions. The samples were stored at –20 °C until the PCR analysis. The PCR analysis of *D. nodosus, F. necrophorum and T. pyogenes* took place in the Food Safety Authority, Evira in Helsinki, Finland, the analysis of *P. levii* and *P. melaninogenica* at ThermoFisher Scientific in Vantaa, Finland, and the analysis of *Treponema* group 1 (*T. medium/ T. vincentii*-like), 2 (*T. phagedenis*-like) and 3 (*T. putidum/ T. denticola*-like) in the Danish Technical University in Copenhagen, Denmark.

Out of 228 samples of *D. nodosus, F. necrophorum* and *T. pyogenes*, 205 were successfully amplified in PCR. Only control and IP samples (n=149) were analysed with real time PCR of *P. levii* and *P. melaninogenica,* and for *Treponema* spp. 168 samples were analysed.

4.2.2 *Acute phase response (II)*

The whole blood samples were centrifuged at 3500 rpm for 15 minutes, and serum was placed in a 2 ml tube, and frozen to –20 °C in 24 hours from sampling. Of the APPs, we analysed Alb, Hp and SAA in a laboratory at the University of Helsinki, Finland. The determination of Alb was performed with an automatic chemistry analyser (the intra-assay coefficient of variation, CV was 2.7% at the serum level 43.1 g/l and inter-assay CV 3.6% at the serum level 42.6 g/l) with spectrophotometric methods (Doumas et al., 1971). Serum Hp concentration was determined with a haemoglobin-haptoglobin binding assay (the intra-assay CV was 2.4% at the serum level 0.42 g/l and 1.1% at the serum level 1.14 g/l, and inter-assay CV 2.6% at the serum level 0.42 g/l and 1.8% at the serum level of 1.15 g/l) and SAA with a commercially available solid phase sandwich ELISA kit (the intra-assay CV was 7.8% at the serum level 13.4 μg/ml and 5.1% at the serum level 84.7 μg/ml, and inter-assay CV 17.2% at the serum level 15.3 μg/ml and 8.6% at the serum level of 88.5 μg/ml) according to the manufacturer's instructions for cows.

4.3 Statistical methods

All statistical analyses were carried out using Stata IC version 14 or 15 (Stata Corporation, Texas, USA).

APPs were analysed separately. The values of Alb, Hp and SAA were described using medians, means, standard errors and 95% confidence intervals in various disease categories (Study II). Pearson chi square tests were used to explore differences between various control cow groups (IP herd and IP free herd), culture results for fusobacteria and IP (Study I), and the possible outbreak of IP-associated putative disorders (Study III). For this thesis, additional tests for measuring the effect size were performed, e.g. a Cramer´s V test was used to measure the effect size of these putative disorders. The interpretation of the results was considered none or very weak if >0, weak if >0.05, moderate if >0.10, strong if >0.15 and very strong if >0.25. The possible association of bacteria in IP samples and high or moderate morbidity outbreak of IP were tested using Fisher exact tests; only cows not receiving antimicrobial treatment were included in the analysis (Study I). A Student´s t-test was used in Study II to compare whether two control cow groups (IP herd and IP free herd) differed from each other. A t-test with unequal variances was performed within various animal groups when comparing APP values of cows in high and moderate morbidity and control herds, and when comparing acute IP cows with *F. necrophorum* and with or without *D. nodosus* (Study II). Cohen´s d was used to measure the effect size of these APP results. The interpretation of Cohen´s d was considered as a small effect, if d=0.2; medium effect, if d=0.5; and large effect, if d=0.8.

In all studies, statistical models were also used. The effect of antimicrobial treatment on each bacterium was tested separately with a logistic regression model. The dependent variable was each bacterium separately and independent variables were various disease categories (1–4) and antimicrobial treatment categories (1–3). Herd was included as a random factor in these models (Study I). We studied the association of disease categories and various bacteria in a multinomial logistic regression model (Study I). The herd had no effect on the results and was not included in the final model. The outcome of the model was disease categories (control, acute IP, healing IP) and variables were *F. necrophorum, D. nodosus, T. pyogenes, Treponema* group 2 and 3, *P. levii* and *P. melaninogenica* (all dichotomous, not present/present). In Study II we investigated the association of APPs and various disease categories. The values for Hp and SAA were not normally distributed and were transformed into a 0/1 variable and a logistic regression model was used. The 90% level of the values of both groups of control cows (IP free herd and IP herd) was used as a reference value for a healthy animal. We used a regression mixed model with Alb, because the values were normally distributed. In these models, a possible antimicrobial treatment, lactation stage, parity and breed were considered as confounding variables and were kept in all models, and herd was included as a random factor (Study II). A simple logistic regression model was used in Study III to establish the risk factors for an outbreak of IP to occur. The outcome was an outbreak of IP and variables were barn flooring, ventilation system, stocking density, possible outdoor access, fields under organic farming, enlargement of the barn in three years and open or closed herd. Region, herd size and milk yield were considered confounding variables and were kept in the model.

In every model, all biologically plausible interactions were tested but no significant associations detected. The models were evaluated by sensitivity and specificity test and ROC curve of the model. The assumptions of the models were controlled by normality and scatter plots of the residuals.

5 RESULTS

5.1 Characteristics of outbreaks of interdigital phlegmon (Study II)

5.1 Morbidity and clinical signs

In outbreak herds of Study II (n=18), in the first two months of IP outbreak the morbidity was high (≥50%) in seven herds, moderate (9–33%) in eleven herds, and no herd had an intermediate morbidity (Study II). The most typical clinical signs of the IP cows were lameness and swelling. **Figure 2** shows the clinical signs detected in IP cows.

In the visited outbreak herds, in addition to milking cows, also cattle less than two years old, i.e. calves and heifers, had cases of IP in six herds (33%). Most farmers (15/83%) felt that cows cured well of IP with parenteral antimicrobial treatment; the most common antimicrobial used in study herds was benzylpenicillin (16 herds/89%), but six herds (33%), four high morbidity and two moderate morbidity herds, had treated at least one cow with third generation cephalosporins. Those three (17%) farmers that felt cows did not cure well were all farmers of a high morbidity herd. In one of those herds 22/55 (40%) of the affected cows needed a re-treatment with antimicrobials. In 11/18 herds (61%), 1–3 cows were culled as a result of IP: the highest culling rate being 12% (11/90) of the current herd size. After one year from the outbreak, in eight herds (44%) new sporadic cases of IP appeared regularly. Farmers replied that these sporadic cases appeared typically after first calving or first oestrus after calving.

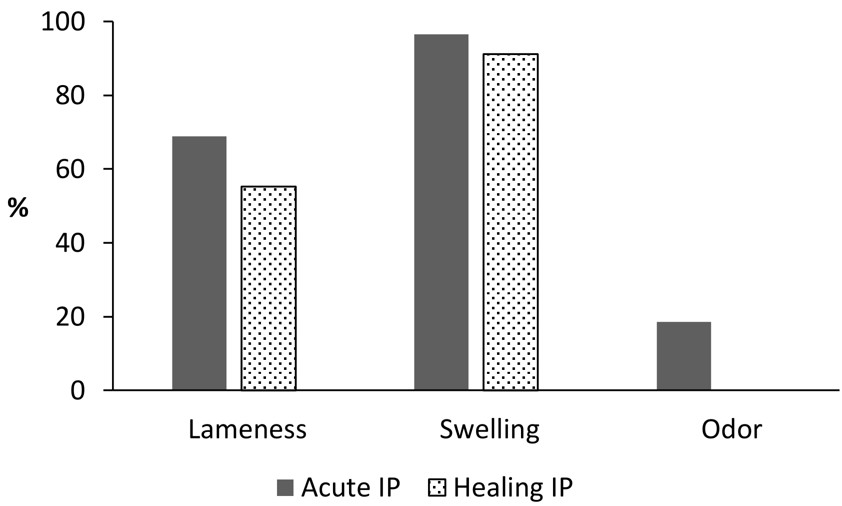

Figure 2 The clinical signs detected in study cows with interdigital phlegmon (IP) in an acute stage (Acute IP, n=60) or in a healing stage (Healing IP, n=34). Lameness was observed in 36/53 (69%) of Acute IP cows and in 16/29 (55%) Healing IP cows, swelling 57/59 (97%) and 31/34 (91%), and odour 8/43 (19%) and 0/26 (0%) respectively (Study II).

The farmers reported several incidents that occurred before the IP outbreaks. All 18 outbreak herds included newly purchased cattle during one year before the outbreak; 16 (89%) herds included more than five animals and two herds (11%) fewer. An enlargement of the barn or a new barn was constructed on 12 farms (67%) in the two years before the outbreak. The feeding changed one month before the outbreak on 12 farms (67%), and either too watery faeces or undigested feed particles in faeces of milking cows before the outbreak were reported by eleven farmers (61%). Either one or both of these feeding related problems had occurred in 16 herds (89%). In the milking cow compartment, two herds (11%) had overstocking and six farmers (33%) problems with the cow flow. Moreover, seven farmers (39%) had problems with ventilation systems. Thirteen farmers (72%) were not satisfied with the cleanliness of the alleys, and ten (44%) with the cleanliness of the cows.

5.1.2 Severity of inflammation

IP causes a strong APR. The values of SAA and Hp were clearly elevated ($p<0.01$), and Alb decreased ($p<0.01$) when compared to other hoof diseases or control cows in our study. **Figure 3 A-C** shows the concentrations of SAA, Hp and Alb in various disease categories. The median values, mean values, standard errors and 95% confidence intervals are presented in **Table 2**.

Odds ratio (OR) for SAA to exceed 80 µg/ml was 38.16 ($p<0.01$) and Hp to exceed 0.27 g/l was 37.93 ($p<0.01$) in acute IP cows. In DD cows OR for SAA to exceed 80 µg/ml was 6.42 ($p=0.05$). The Alb values were lower in acute IP cows when compared with control cows (coefficient -1.85, $p<0.01$). Lactation stage ($p<0.01$) and parity ($p=0.04$) affected the Alb values. The herd had an effect on values of SAA ($p=0.06$, a trend), Hp ($p=0.05$) and Alb ($p=0.02$).

The mean values for SAA and Hp in acute IP cows (n=60) differed between high and moderate morbidity herds. The mean value of SAA was 421.51 µg/ml ± 58.56 (95% CI: 304.33-538.71) in high morbidity and 169.37 µg/ml ± 32.10 (105.14-233.59) in moderate morbidity herds ($p<0.01$, Cohen´s d= 0.92) and the mean values of Hp 2.71 g/l ± 0.35 (1.99-3.43) and 1.47 g/l ± 0.29 (0.89-2.05) respectively ($p<0.01$, Cohen´s d 0.67). The Alb values did not differ between acute IP cows from herds of various morbidities.

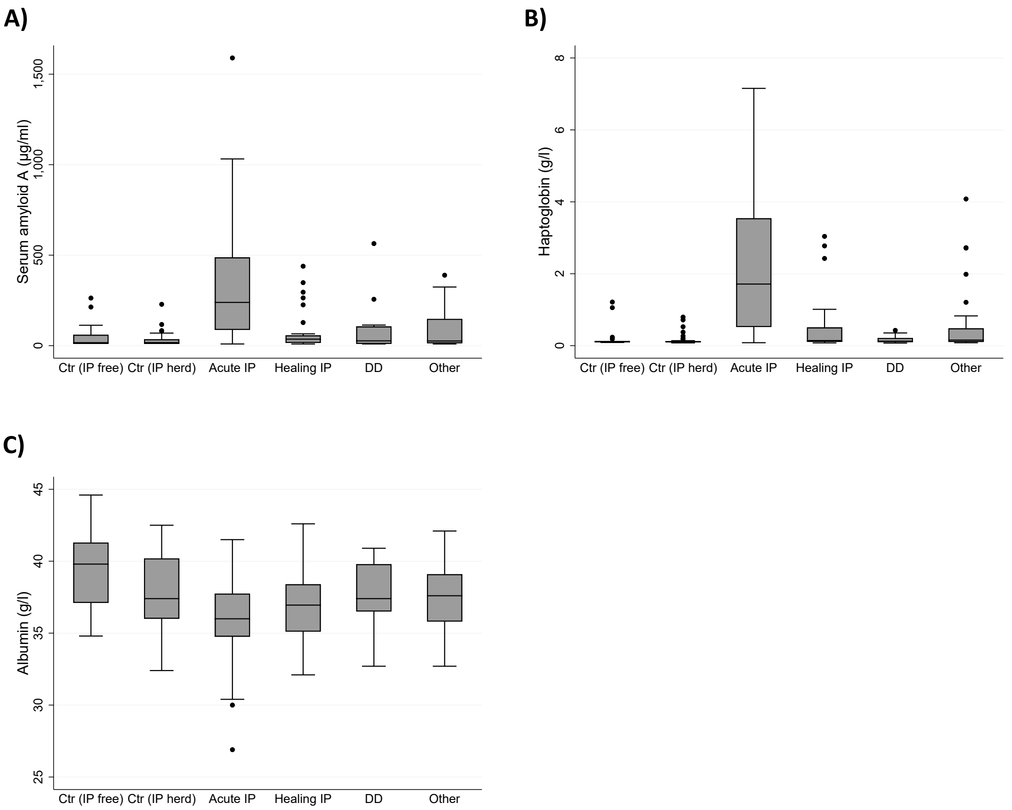

Figure 3 A-C The concentrations of A) serum amyloid A, B) haptoglobin and C) albumin in control cows in herds with no outbreak of interdigital phlegmon, IP (Ctr IP free, n=19), control cows of herds with an outbreak of IP (Ctr IP herd, n=40), group of animals suffering from acute IP (acute IP, 61), animals in a healing process for IP (healing IP, 36), animals with digital dermatitis (DD, 13) and other hoof diseases (other, 38). In the box plots, the boxes are the first and third quartiles and the band inside the box is the median. Ends of the whiskers represent the 2nd percentile and the 98th percentile of sampled animals. IP = interdigital phlegmon (Study II). In acute IP cows, the values for SAA and Hp were clearly elevated ($p<0.01$), and Alb decreased ($p<0.01$) when compared to DD, other hoof diseases or control cows in our study in our study.

Table 2 The number of cows, medians, means, standard errors and 95% confidence intervals for serum amyloid A, haptoglobin and albumin in blood samples from cows in the various disease groups[1].

	n	Median	Mean	SE	95% CI
Serum amyloid A (µg/ml)					
Control (IP-free herd)	19	16.68	52.59	16.49	20.08–85.10
Control (IP herd)	42	16.96	33.00	6.13	20.83–45.02
Acute IP	60	239.10	308.05	38.65	231.83–384.27
Healing IP	34	36.02	74.12	18.50	37.65–110.60
DD	14	26.77	90.91	40.70	10.66–171.15
Other	34	26.07	89.33	19.40	51.07–127.59
Haptoglobin (g/l)					
Control (IP-free herd)	19	0.11	0.23	0.07	0.08–0.37
Control (IP herd)	42	0.11	0.16	0.03	0.11–0.21
Acute IP	60	1.71	2.15	0.25	1.66–2.64
Healing IP	34	0.14	0.50	0.13	0.25–0.76
DD	14	0.12	0.17	0.29	0.11–0.23
Other	34	0.16	0.55	0.15	0.24–0.87
Albumin (g/l)					
Control (IP-free herd)	19	39.80	39.54	0.58	38.39–40.69
Control (IP herd)	42	37.40	37.79	0.38	37.04–38.55
Acute IP	60	36.00	35.91	0.36	35.20–36.62
Healing IP	34	36.95	36.76	0.44	35.90–37.63
DD	14	37.40	37.70	0.59	36.53–38.86
Other	34	37.60	37.50	0.39	36.72–38.28

[1] Control cows with no outbreak of interdigital phlegmon (IP-free herd), control cows in herds with an outbreak of IP (IP herd), IP in acute stage (acute IP) and healing stage (healing IP), digital dermatitis (DD) and other hoof diseases (other).

n = number of cows, SE = standard error; 95% CI = 95% confidence interval; IP = interdigital phlegmon

Moreover, we compared APP values for acute IP cows with *F. necrophorum* and with or without *D. nodosus* (n=44) in high and moderate morbidity herds. In cows with *F. necrophorum* and *D. nodosus* (n=31) the mean SAA was 450.73 µg/ml ± 59.29 (95% CI 331.15-570.31), and for cows with *F. necrophorum* but without *D. nodosus* (n=13), 140.22 µg/ml ± 41.13 (57.27-223.16) ($p<0.01$). The mean values for Hp were 2.85 g/l ± 0.38 (2.08-3.61) and 1.54 g/l ± 0.39 (0.73-2.34), respectively ($p=0.02$). Furthermore, statistical difference existed between acute IP cows with *F. necrophorum* and *D. nodosus* in high (n=21) and moderate morbidity (n=10) herds in SAA ($p<0.01$, Cohen´s d=0.90) and in haptoglobin ($p<0.05$, Cohen´s d=0.63) values.

5.2 Bacteriology of interdigital phlegmon (I, II)

F. necrophorum is the main pathogen in IP. Subspecies *necrophorum* was detected by culture in 48/65 (74%) of the samples from acute IP and in 26/41 (63%) from healing IP and was clearly associated with IP (p<0.01) when all IP samples (n=106) were compared with control samples (n=64). Moreover, we compared control, acute IP, and healing IP samples with various bacteria detected by PCR in a multinomial logistic regression model; *F. necrophorum* was strongly associated with IP at both stages; acute IP (relative risk ratio, RRR 74.9; p<0.01) and healing IP (RRR 58.4; p<0.01). *T. pyogenes* was detected frequently with the healing IP (RRR 22.4; p=0.01), and a trend existed with acute IP samples (RRR 10.8; p=0.06). Antimicrobial treatment affected detection of *D. nodosus* (current treatment OR 0.2;p=0.01, previous treatment OR 0.1; p<0.01) and *Treponema* group 2 and 3 (current treatment OR 0.1; p<0.01, previous treatment OR 0.1; p=0.03), but not the detection of other bacteria (Study I).

The most common finding in acute IP samples was a combination of *F. necrophorum* and *D. nodosus* (24/36; 67%). However, various combinations existed, and all bacteria studied were detected in IP. **Figure 4** shows the bacteria observed in various disease groups. Because control cows (IP herd) and control cows (IP free herd) did not differ bacteriologically, the control groups were united for the analysis. *D. nodosus* was more often detected in IP in high than moderate morbidity herds (p=0.05, n=35, Study I).

Furthermore, *F. necrophorum* ssp. *necrophorum* was also detected frequently in hoof diseases other than IP: in 7/20 (35%) DD samples, and in other hoof diseases, including ID, white line abscesses and sole ulcers, in 11/38 (29%) of the samples.

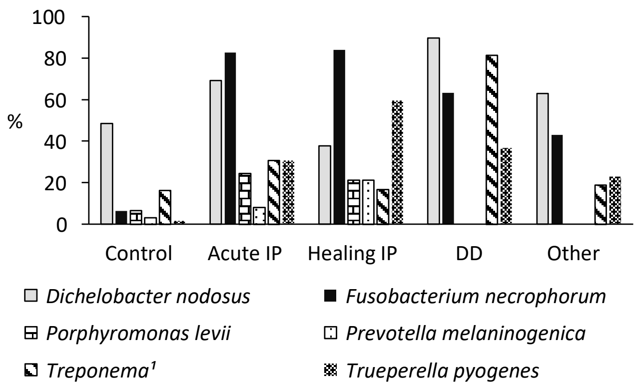

Figure 4 PCR detected bacteria in hoof samples from various disease categories; control cows (n = 62), acute interdigital phlegmon (Acute IP, n = 52), IP in a healing stage (Healing IP, n = 37), digital dermatitis (DD, n = 19) and other hoof diseases (Other, n = 35). The group other hoof diseases included hoof samples from interdigital dermatitis, white line abscess and sole ulcer. Total number of hoof samples is 205, except with *P. levii* and *P. melaninogenica* (142) and *Treponema* group 2 and 3 (168). *P. levii* and *P. melaninogenica* were investigated only among control and IP samples. *Treponema*[1] = *Treponema* group 2 and 3.

5.3 Risk factors for an outbreak of interdigital phlegmon (III)

From farmers of all 1134 dairy herds who received the survey questionnaire, the response rate was 34%. An outbreak of IP with morbidity ≥5% within the first month of the outbreak had occurred in 64/355 (18%) herds. Furthermore, 32 (9%) respondents reported few cases of IP in the herd, i.e. incidence of IP was less than 5% in one month.

Of the outbreak herds, 14 (22%) reported cases of IP in heifers and 11 (17%) in calves. A year after the outbreak, in 21 of the 47 outbreak herds (45%) still had sporadic cases of IP in cows, in four herds (9%) in heifers, and in one herd (2%) in calves. All reported outbreaks had occurred in 2004–2013.

There were several clinical signs of IP cases in outbreak herds: in 33 herds (52%) fever, 62 (97%) lameness, 62 (97%) swelling above a hoof, 36 (56%) shaking of a leg, 34 (53%) bad odour in the hoof region, 50 (78%) lesions in between the hooves and 25 (39%) lesions in the heel.

IH ($p<0.01$, Cramer´s V=0.28), verrucose dermatitis ($p<0.01$, Cramer´s V=0.11) and signs of other infectious hoof diseases (ID or DD) ($p<0.01$, Cramer´s V=0.36) were detected more frequently in outbreak herds. Buccal abscesses in suckling calves were also observed more often ($p<0.01$, Cramer´s V=0.43).

In Study III, we also investigated the possible herd level risk factors for an outbreak of IP to occur; animal purchase or heifer contract rearing (OR 4.9; $p<0.01$), enlargement of the barn within three years (OR 3.3; $p<0.01$), and fields under organic farming (OR 3.8; $p=0.02$) were detected as risk factors. Any kind of mechanical ventilation in the barn tended to lower the risk (OR 0.2; $p<0.01$).

6 DISCUSSION

6.1 Characteristics of outbreaks of interdigital phlegmon (II)

Our aim was to investigate the characteristics of IP outbreaks in Finnish dairy herds – the morbidity, clinical manifestation and degree of inflammation.

6.1.1 Morbidity and clinical signs

IP outbreak herds of our study included only herds of high morbidity (≥50%), or herds of moderate morbidity (9–33%), and no herd had intermediate morbidity. This was an interesting finding since we had heard a view of several clinical practitioners that in the field there seemed to be two kinds of outbreaks; severe with almost all the cows in the herd suffering from IP and those where an outbreak quite easily subsided. The number of study herds was limited; hence the analysis of differences between herds of various morbidities was not feasible and would need further investigation. However, one very simple reason for this could be that in some farms the actions to eliminate the outbreak were intense and in other farms the actions taken were minimal. In either case, the outbreak in all herds represented an exhausting situation; even the medication of affected animals took time, and in addition, possible new IP cases had to be detected and the normal farm duties had to be taken care of. Moreover, an outbreak represented a financial burden. Häggman et al. (2015) reported herd-level costs of €4560 to €28386 on four Finnish dairy farms with an outbreak of IP. The lowest cost was on a farm where 18% of the cows were affected, while the highest was for a farm where 77% of the cows were affected with IP. The costs per affected cow ranged from €246 to €652, the average cost being €489. The major cost was associated with discarded milk, hence the farms that used ceftiofur as a treatment incurred the lowest costs (Häggman et al., 2015). However, the authors pointed out that ceftiofur was used despite the recommendations, and suggested that rapid treatment and control measures to limit the outbreak would greatly reduce the total cost (Häggman et al., 2015).

Even though several cows were affected on all outbreak farms, most farmers (83.3%) felt that cows cured well from IP with parenteral antimicrobial treatment, the most common of which was benzylpenicillin, as recommended by the Finnish Food Authority (Finnish Food Authority publications, 2018). However, third generation cephalosporins were used on six farms to treat a minimum of one cow.

IP cows in our study had severe clinical signs; lameness and swelling were detected most commonly. Although bad odour is described as a typical clinical sign of IP (Gupta et al., 1964), we detected a clear bad odour only in 18.6% (8/43) of cases of acute IP and in none of the healing IP cows. This could be because we were on a farm during an outbreak and therefore we might have observed some of the IP cases earlier than normally detected. It might be that very early in the acute stage of IP, no odour exists, and odour relates to more advanced IP cases with necrotizing tissue, or one could speculate involvement of certain bacterial species. This speculation could be supported by the fact that we detected no bad odour in any of the cows in the healing stage of IP either.

We observed several cases of acute IP, with extensive swelling but no visible injury to the skin in the early stage of infection, though in previous studies an interdigital trauma was regarded as a predisposing factor for IP (Johnson, 1945; Gupta et al., 1964; Johnson et al., 1969). Some of the inspected feet were quite dirty and occasionally covered with dried faeces around the claws. It was suggested earlier that maceration of the skin facilitates entry for the pathogens into the deeper tissues (Johnson, 1945; Gupta et al., 1964; Johnson et al., 1969), or dry faeces around the coronary band may create an effective anaerobic condition for the pathogens (Gupta et al., 1964). However, some of the sampled feet were clean and dry. Hence there may also be some new mechanisms of pathogenesis in these cases.

Even though *F. necrophorum* is a normal inhabitant of the rumen (Robinson et al., 1951), it is not normally excreted in the faeces (Smith and Thornton, 1993). Similarly, a recent microbiota analysis of environmental slurry detected relative abundances below 1% of families *Spirochaetaceae* and *Fusobacteriaceae* (Klitgaard et al., 2017). That study comprised 135 slurry samples from 22 dairy farms with and without DD cows. Interestingly, they also established that *Actinomycetaceae* were more abundant in environmental slurry in DD herds, *Trueperella* being the most prevalent. However, Smith and Thornton (1993) suggested that disturbance of the gastrointestinal microflora may lead to intestinal multiplication and faecal excretion of *F. necrophorum*, which may then give rise to IP. As in our study, several study farms had experienced some feeding related problems just before the outbreak. A possibility exists that these problems might have led to excretion of *F. necrophorum* in the faeces.

It remains unknown whether *F. necrophorum* invades the body through the interdigital skin. For example, the liver abscesses in feedlot cattle result from heavy grain-feeding programmes as reviewed by Nagaraja and Lechtenberg (2007). Liver abscesses occur commonly with rumenitis; the ruminal lesions are regarded as the primary foci of infection and liver abscesses the secondary one (Scanlan and Hathcock, 1983). In the formation of liver abscesses, the ingestion and rapid fermentation of dietary carbohydrates result in rumen acidosis and induce the ruminal lesions. Rumen bacteria, mainly *F. necrophorum*, invade these lesions and form abscesses in the deeper layers of the rumen wall. From here bacterial emboli penetrate the hepatic venous system, ending up in the liver parenchyma where they form abscesses (Scanlan and Hathcock, 1983).

Occasionally, it was difficult to make the exact diagnosis, and it was common to detect other infectious hoof diseases, like ID and HHE in a same foot as IP. Some of the IP cases had also claw lesions or heavily overgrown claws due to neglected regular hoof trimming of the herd. This might have predisposed cows to IP, as absence of hoof trimming increased the risk of developing DD (Relun et al., 2013). Some of the cows had both IP and DD – occasionally also in the same foot. In most of these cases, DD lesions were quite small (M1) and IP clearly the reason for the cow being lame.

6.1.2 Severity of inflammation

We investigated the association of clinical signs, bacteriological findings and APPs to evaluate the degree of infection in naturally occurring IP. APR in IP cows was evident; values for SAA and Hp were elevated and Alb decreased in cows suffering from IP at an acute stage. This APR was clearly stronger than in other study cows suffering from DD, and other hoof diseases or control cows. Interestingly the APR was even more apparent in acute IP cows in high morbidity herds and with a bacterial combination of *F. necrophorum* and *D. nodosus*. A reason for this might be a weaker immunity, a more virulent agent or a higher infection pressure in high morbidity herds.

In the acute stage of IP, the values for SAA and Hp were clearly increased. This was predictable because SAA and Hp are major APPs in cattle (Murata et al., 2004; Eckersall and Bell, 2010). Currently SAA is regarded as the first line and Hp the second line APP; first line APPs are primarily induced by IL-1 type cytokines whereas second line APPs are induced by IL-6 type (Gabay and Kushner, 1999; Petersen et al., 2004). Because we do not know exactly when the IP infection appeared in our study cows, our IP cases, especially the healing IP group, could be quite diverse. This might be the reason for observing only a trend ($p=0.06$) among Hp values in the group of healing IP cows, i.e. at the later stage of IP.

It has been known for a long time that Alb is a major plasma protein, maintaining the plasma colloidal osmotic pressure and being associated with the balance of body fluids, as reviewed by Eckersall in a textbook of clinical biochemistry (2008). Several pathological processes or physiological conditions result in hypoalbuminemia (Eckersall, 2008). In cattle, also feeding influences blood albumin, e.g. as shown in recent studies (Amanlou et al., 2017; Zhang et al., 2017). During APR, synthesis of Alb is probably downregulated and amino acids are used for the synthesis of the positive APPs (Gabay and Kushner, 1999). In our study cows, the values for Alb were decreased in acute IP, but they were also affected by DIM and parity. Thus, albumin alone is not a useful marker of infection in a single cow. However, it can add information on the severity of the disease in question, if several APPs are analysed.

Previous research on APR and infectious hoof diseases is limited. Additionally, in earlier studies, either only few IP cows were included, or they did not form a study group of their own but were investigated among other hoof diseases. Smith et al. (2010) studied Hp in various claw disorders. They detected elevated Hp concentrations, and a reduction in the values after antimicrobial treatment in seven IP cows. Nazifi et al. (2012) investigated APR in lame cattle with ID. They had a lameness group ($n=11$) and *F. necrophorum* positive lameness group ($n=4$) in their study and detected elevated concentrations of SAA and Hp when both groups were compared to control cows (n=10). Because ID normally does not cause lameness, a possibility exists that these cows might have had IP instead. Occasionally the nomenclature of various infectious hoof diseases complicates interpretation of the results. Tóthová et al. (2017) studied the electrophoretic pattern of serum proteins in dairy cows with various inflammatory diseases. They detected significantly lower values of Alb in 23 dairy cows with hoof diseases than in controls. Among the hoof diseases were DD, pododermatitis, laminitis and sole ulcer. The exact number of pododermatitis cows is unexplained. Ilievska et al. (2019) measured SAA and Hp in various claw diseases; DD cows (n=15) had elevated concentrations of both measured APPs in comparison with healthy control heifers, but HHE cows (n=7) had not.

6.2 Bacteriology (I, II)

We investigated the bacteriology in IP outbreaks, the presence of various hoof-associated bacteria in IP, and compared the findings with other hoof diseases, and control cows. Based on our results, *F. necrophorum* is the main pathogen in IP, which is in accordance with previous studies (Flint and Jensen, 1951; Berg and Loan, 1975; Clark et al., 1985). Furthermore, our culture results identified *F. necrophorum* ssp. *necrophorum.*

Contrarily, *F. necrophorum* was almost non-existent in samples from control cows. Likewise, a microbiome study of bovine DD identified very low abundance of bacteria from the *Fusobacteria* phyla in healthy digital skin, instead it was dominated by *Firmicutes* and *Actinobacteria* (Zinicola et al., 2015). In a US study of development of DD lesions, the microbiome of normal skin of the bovine foot was more diverse than the microbiome in DD lesions consisting several families, including *Staphylococcaceae, Streptococcaceae, Bacteroidaceae, Corynebactericeae* and *Pasteurellaceae* (Krull et al., 2014). In an UK study however, *F. necrophorum* detected by PCR was present in 44% of DD lesions, but also in 33% of healthy foot tissues (Sullivan et al., 2015). Similarly, we detected *F. necrophorum* also in DD lesions and other hoof diseases, but not as often as in IP. Also, other studies detected *F. necrophorum* in DD lesions (Nielsen et al., 2016).

In most of the acute IP samples (66.7%), we detected the combination of *F. necrophorum* and *D. nodosus*. Moreover, the acute IP cows with this bacterial combination had more elevated concentrations of SAA than acute IP cows with *F. necrophorum* but without *D. nodosus* (Study II). These values were even more increased in cows of high morbidity herds, indicating that *D. nodosus* might affect the severity of IP. The same bacteria, *D. nodosus* and *F. necrophorum,* are also detected in ovine foot rot, but *D. nodosus* is considered the primary etiological agent (Beveridge, 1941; Egerton et al., 1969; Raadsma and Egerton, 2013; Witcomb et al., 2015). The clinical picture of ovine foot rot differs from bovine IP; ovine foot rot is initiated by infection of the interdigital skin, which may lead to separation of the sole, soft, and hard horn from the underlying hoof matrix (Beveridge, 1941; Raadsma and Egerton, 2013). In a Norwegian study, an experimental infection with several benign and virulent strains of *D. nodosus* led to developments of ID lesions in heifers (Knappe-Poindecker et al., 2015).

Additionally, *D. nodosus* was observed in several samples of all disease categories; controls, IP in acute and healing stages, DD and other hoof diseases. However, our investigation was qualitative not quantitative, so the number of bacteria might differ among various lesions and healthy hooves. Other studies have also detected *D. nodosus* in healthy hooves and ID (Knappe-Poindecker et al., 2013), and DD (Capion et al., 2012; Rasmussen et al., 2012; Knappe-Poindecker et al., 2013; Sullivan et al., 2015; Moreira et al., 2018a). In their study on DD, Rasmussen et al. (2012) speculated that *D. nodosus* might weaken the epidermal barrier and thus provide a better environment for other bacteria. A recent study suggests *D. nodosus* as another potentially important pathogen in DD (Moreira et al., 2018a). In our study, we detected ID in some of the cows, also in some of the IP cows. However, some of the IP feet had a massive fissure and there was no interdigital skin left for us to detect any possible ID on the same foot. Additionally, we only visited the farms once. No information on possible previous hoof lesions on study cows was available, neither was follow-up done.

In addition, other bacteria might also be involved in the IP pathogenesis and explain the differences in clinical manifestation and epidemiology. In our study, *T. pyogenes* was associated with IP at the healing stage ($p=0.01$) and a trend ($p=0.06$) existed at the acute stage of IP. Also other studies detected *T. pyogenes* in IP lesions (Gupta et al., 1964; Morck et al., 1998). It is hypothesized that *T. pyogenes* helps *F. necrophorum* by utilizing oxygen and providing anaerobic conditions (Tadepalli et al., 2009), and that the leukotoxin of *F. necrophorum* protects *T. pyogenes* (Narayanan et al., 2002). In addition, a nutritional interaction exists between these two bacteria; lactic acid, produced by *T. pyogenes*, serves as an energy substrate for *F. necrophorum*, as reviewed by Tadepalli et al. (2009).

In addition, all other bacteria studied were detected frequently in cases of IP, either in the acute stage or during the healing process. Earlier studies on the bacteriology of IP detected *B. melaninogenicus* as an etiological agent in IP together with *F. necrophorum* (Berg and Loan, 1975; Clark et al., 1985). Later, *B. melaninogenicus* was reclassified as several *Porphyromonas* and *Prevotella* species (Jousimies-Somer and Summanen, 2002). In a study of Morck et al. (1998), the anaerobes most frequently isolated from IP biopsy specimens were *P. levii* and *P. intermedia.* A study of Sweeney et al. (2009) identified anaerobes isolated from IP cases by 16S rRNA sequencing. Based on their studies they consider *P. levii* to be an associative agent in cattle IP.

Some earlier studies mention the detection of *Spirochetes* in IP lesions (Gupta et al., 1964; Doherty et al., 1998), but whether these organisms were *Treponemes* or not, is unknown. *Treponema* spp. are the key bacteria in DD (Klitgaard et al., 2008; Evans et al., 2009; Zinicola et al., 2015), but various studies have discussed also a role for *P. levii, F. necrophorum* and *D. nodosus* as etiological agents in DD (Sullivan et al., 2015; Nielsen et al., 2016; Moreira et al., 2018a). Apparently almost the same bacterial flora is behind various infectious hoof diseases, but the main pathogens vary among diseases. The studied bacteria are also frequently detected with *F. necrophorum* in several infectious cattle diseases other than hoof diseases, like calf diphtheria (Panciera et al., 1989), liver abscesses (Berg and Scanlan, 1982; Lechtenberg et al., 1988), summer mastitis (Madsen et al., 1990; Madsen et al., 1992) and uterine infections (Bicalho et al., 2012; Cunha et al., 2018). Also indication of interactions and even possible synergism between *F. necrophorum, T. pyogenes* and *P. levii* is described (Ruder et al., 1981; Takeuchi et al., 1983; Nagaraja et al., 1999; Kan, 2009; Karstrup et al., 2017).

It is extremely interesting that mostly the same bacterial populations, at family and genus level, that are associated with infectious hoof diseases play a role in human periodontal disease, as reviewed by Takahashi (2015). Plummer and Krull (2017) evaluated the similarities between human periodontal disease and DD in their review in the following way; in periodontal disease the first colonizers, including the Gram-positive cocci, are followed by Gram-positive and Gram-negative rods and later the anaerobic Gram-negative rods. In this process, the microenvironment changes from totally aerobic to more anaerobic. These early and mid stage colonizers include *Campylobacter* spp., *Bacteroides* spp., and *Fusobacterium* spp. The later colonizers, like *Treponema* spp. and *Porphyromonas* spp., are largely microaerophilic or anaerobic and do not grow in the initial aerobic environment. At the same time, the overall metabolic profile is changed from mainly saccharolytic, i.e. use of glucose and sugars for energy, to more proteolytic metabolism (Takahashi, 2015; Plummer and Krull, 2017).

To summarize the bacteriological findings of IP: in our study, as in previous studies, several bacterial species were detected in IP. However, it remains unclear what the role of individual bacterial species in IP is.

6.3 Risk factors for an outbreak of interdigital phlegmon (III)

We investigated the herd level risk factors for IP outbreak to occur by carrying out a survey. In a cross-sectional survey however, one cannot confirm the causality. Therefore, the results should always be interpreted with caution.

In our survey, we identified recent enlargement or renovation of the barn as a risk factor for an IP outbreak. There may be several factors behind this. In the middle of the enlargement process, both the cattle and the dairy farmers may be stressed in adapting to a new building and different management (Brouzek et al., 2017). During the process, some overstocking may occur in the old barn. Overstocking may increase the infection pressure, but overstocking is regarded as a stress factor e.g. by altering feeding and lying behaviour (Krawczel and Lee, 2019). At the time the cattle move into the new free stall, it can still resemble a construction site and pose an increased risk for injuries. Moreover, as part of the enlargement process the tradition of the family-managed farm may end, and the cattle will be taken care of by hired labour. If the management is inadequate or there are too few stockmen to look after even more cows than earlier, the risk of mistakes being made and overlooked signs of diseases may increase. Additionally, if the farm´s finances are not stable, it may lead to low-grade feeding, and as a consequence to increased disease susceptibility, or even reduced labour on the farm. To conclude, an enlargement of a barn or construction of a new barn is a big investment and greatly increases the workload. Since the amount of time is limited, extra labour is needed either with management of the construction site or with management of the cattle.

Additionally, we found that animal transport between herds, i.e. animal purchase or contract heifer rearing, to be a risk factor for an IP outbreak. This was quite expected because IP is an infectious disease (Gupta et al., 1964; Clark et al., 1985). In addition, a new animal in a herd experiences a different environment and management, possibly affecting its immunology and predisposing it to disease. In our survey, we were unable to investigate the possible causality of the animal transport between herds and the IP outbreak. Therefore, animal transport between herds may not be a causal factor in the IP outbreaks, but just a simultaneous action in the study farms. In other studies, purchase of new heifers has been reported to represent a risk for DD (Rodriguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Wells et al., 1999), and purchase of cattle associated with higher DD prevalence within a herd (Oliveira et al., 2017). Nonetheless, if purchase of cows is mandatory, new animals should be bought with extreme caution; purchase only from herds with the best possible hoof health and have an experienced veterinarian clinically examine the herd and purchased cattle with a special focus on detection of infectious hoof diseases.

Another risk factor was represented by a farm having organically cultivated fields. In our statistical model, there were few herds from organically cultivated fields (n=24, 8.1%), but among outbreak herds there were 3.75 times more organic herds than among control herds. Finnish soil is mostly deficient in selenium (Se), which results in low Se content in plants (Oksanen and Sandholm, 1970). Therefore, the use of fertilizers with Se supplement is routine in conventional farming. Organic farming instructions limit the use of fertilizers, and if no Se is added in the feed, a deficiency might occur. One could speculate that low blood Se level might affect the immunological status of the cows and thus increase disease susceptibility. As investigated in other studies, an association was detected with low Se and vitamin E deficiency and udder health (Smith et al., 1984; Jukola et al., 1996). Moreover, another issue that needs further investigation is the abundant use of clover in silage in organic farming. Already Johnson (1945) thought that clover might be a reason for an increased incidence of IP. The European Union (EU) regulations for organic farming guide the purchase of new animals to occur among other organic herds (Council regulation 2007/834/EC). This further increases the risk of IP spreading from one organic farm to another, if IP is more common in organic farms than conventional ones. However, based on our survey data, it is uncertain which aspect, if any, is behind organically cultivated fields being a risk factor. More information on cattle nutrition as a whole and trace element status would be needed to establish possible risks associated with organic farming.

In contrast, any kind of mechanical ventilation decreased the risk of an outbreak of IP. The reason for this could be that better ventilation dried the indoor air and reduced the levels of ammonia in the barn. Other studies have reported that barns with low air humidity encouraged cleaner animals (Hauge et al., 2012) and if relative air humidity increased, the risk of cattle dirtiness increased (Ruud et al., 2010). Previously, moisture beneath the hooves was associated with IP (Gupta et al., 1964; Johnson et al., 1969). Other infectious hoof diseases and dirty claws or legs, or dirty alleys, were positively associated in several studies (Rodriguez-Lainz et al., 1996; Somers et al., 2005; Knappe-Poindecker et al., 2013; Relun et al., 2013). In our survey, we were not able to establish the effect of cattle cleanliness or cleanliness of the alleys for the respondent farms.

The role of other infectious hoof diseases is interesting; study farms that had experienced an outbreak of IP often supported other infectious hoof diseases. However, based on our study data we cannot say which disease appeared first in the herds. Similarly, other studies detected an association between various infectious hoof diseases (Manske et al., 2002; Capion et al., 2009; Knappe-Poindecker et al., 2013). A study from the Netherlands reported that the presence of ID and HHE together or IP increased the risk for DD, and IP was strongly associated with DD (Holzhauer et al., 2006).

Our survey replies included herds (32/9%) that had had few cases of IP, i.e. incidence of IP was less than 5% in one month. It would have been interesting to investigate these herds further. However, we think that some of those herds might have experienced IP outbreak previously. Therefore, they were more likely to resemble the herds visited during this research (8/44%), where new sporadic cases of IP appeared regularly after the actual outbreak. Another problem with these herds with sporadic cases of IP arises if some other hoof problem is misdiagnosed as IP. In Finland, we do not have good data on hoof health of dairy cattle. For example, the veterinary treatment codes for infectious hoof diseases were modified concurrently with our research project.

6.4 Observations regarding the treatment of interdigital phlegmon

In our research, we investigated IP outbreaks - a phenomenon not detected in Finland earlier. Because the treatment is an essential part of the phenomenon, some points of the treatment are discussed here. Currently, outbreaks of IP still occur in Finland, but seem to be fewer in number, probably because so many farms have already experienced an outbreak. In addition, farmers and veterinary practitioners are more aware of the IP problem and prompt actions are usually taken to put an end to an outbreak.

Most IP cows in our study expressed severe clinical signs, including lameness. Also 55% of our study cows in the later healing stage remained lame. Whay and Shearer (2017) reviewed lameness on a welfare perspective based on the five freedoms; freedom from hunger and thirst, from discomfort, from pain, injury and disease, to express normal behaviour, and from fear and distress (Farm Animal Welfare council, 1993). They point out the altered feeding behaviour and pain associated with lameness and how lameness interferes with a cow's ability to exhibit natural behaviour (Whay and Shearer, 2017). At the acute stage of IP, in addition to severe clinical signs, we detected an apparent APR – a defence reaction of the host to infection. Hence, IP cows must be treated without delay and a proper analgesic and anti-inflammatory should be included in IP treatment. Also previous studies report that if IP treatment is delayed more severe condition is detected (Berg and Loan, 1975; Braun et al., 1987; Cook and Cutler, 1995). Moreover, a recent Danish study observed that lame cows recovered better if, in addition to the proper treatment, they were kept in a hospital pen with deep-litter straw (Thomsen et al., 2019). Furthermore, all lame cows should be properly clinically examined, even during an IP outbreak. In an outbreak, one easily presumes that every new lameness case is IP even though also other causes for lameness can occur.

An IP outbreak easily leads to abundant use, and probable misuse, of antimicrobials and disinfectants. Based on the recommendations by the Finnish Food Authority benzylpenicillin should be the first-line antimicrobial in IP treatment, and based on Finnish legislation, third and fourth generation cephalosporins should only be used on the target animal species and the indication detailed in the marketing authorisation if no other antimicrobial is known to be effective (Decree on the use and distribution of medicines in veterinary practice, 2014). WHO has categorized the third and fourth generation cephalosporins as CIAs for humans (Collignon et al., 2009) and based on the new WHO ranking of May 2019, the highest priority CIAs (WHO, 2019). The use of third and fourth generation cephalosporins correlates with the appearance of extended-spectrum β-lactamase (ESBL) or AmpC β-lactamase producing bacteria in animal populations, such as ESBL producing *E. coli* on dairy farms (Snow et al., 2012). ESBL and AmpC are enzymes that provide bacteria with resistance to a variety of β-lactam antimicrobials (Jacoby and Munoz-Price, 2005). Infections with ESBL and AmpC producing bacteria cause high mortality in humans and therefore, they are considered to be a global threat, as reviewed in a meta-analysis by Rottier et al. (2012).

Without doubt the affected animals must be treated in the best possible way. But to lower antimicrobial use the focus should be on early detection of IP cows and on disease prevention. Fortunately, also the veterinary sector has become aware of AMR. For example, to reduce antimicrobial use, a non-antimicrobial salicylic acid was studied in treatment of early detected, non-complicated IP (Persson et al., 2019). This is a good start but needs further investigation.

In addition to antimicrobial use, another problem is walk-through hoof bathing for dairy herds; many disinfectants with various regimens are used with the intention of preventing spread of infectious hoof diseases. However, as informed by several veterinary practitioners and noticed in our study herds, quite often hoof bath products are used improperly, either against instructions or extended periods without change. To date there is no evidence that hoof bathing prevents IP even though it was suggested by our research group as being one mean during a prolonged IP outbreak to prevent healthy cows from getting IP. In contrast, a dirty hoof bath has been suggested to be a cause of an IP outbreak (David, 1993; Cook and Cutler, 1995). Also, the evidence for hoof bathing with copper sulphate or other products, in the treatment and prevention of infectious hoof diseases, has been questioned (Thomsen, 2015; Jacobs et al., 2019). Because some hoof bath products, like copper sulphate, are hazardous to the environment (Flemming and Trevors, 1989; Ippolito et al., 2010) and AMR and metal resistance are co-selected (Baker-Austin et al., 2006), these products should not be used carelessly. Instead one should focus on better on-farm biosecurity (Oliveira et al., 2017).

6.5 Limitations and strength of the studies

Studies I and II depended on spontaneous IP outbreaks. At times there were no outbreaks, but occasionally several occurred at the same time in various parts of Finland, making it impossible for our small study group to attend all the simultaneous outbreaks. Because our farm visits lasted only one day, we were not able to inspect all the cows in the herd, but our IP selection criteria were mainly based on lameness. Therefore, our IP cows could have been the most severe cases and the mildest ones were not detected and selected for sampling. This might have had some effect on our results. Nevertheless, the overall number of IP cows in each herd during an outbreak was inquired a year after, and even though spontaneous recoveries occur (Johnson, 1945), the groups of various morbidity herds are very likely to be correct. On our farm visits we were able to collect numerous samples; altogether 100 IP cows were sampled in our research project. We were able to divide the IP samples into two groups, acute and healing stage of IP, and thus better investigate the APR, but also IP bacteriology in various stages of the disease.

In our research however, we did not study the IP microbiome, we only investigated various bacteria, previously detected in hoof diseases, using PCR methods. With new methods we would have obtained a more comprehensive result on the bacteriology of IP in its various stages. Also, sampling on several days would have been more reliable than taking a single sample, to ensure that the specific bacteria of the study truly colonized the lesions and were not just contaminants from the environment. Mainly due to long distances in Finland, we were not able to visit the farms several times. In addition, for ethical reasons, the animals were treated with antimicrobials as soon as possible. Antimicrobial treatment alters the microbiome and thus affects the bacteriological studies.

Our study III was a survey. In this survey, the regional differences in Finnish dairy farming were well represented in the replies. The survey replies were gathered all over Finland and we gathered more replies from regions with more dairy cows, including the provinces of Pohjanmaa and Savo. Also, the average milk yield of the study herds at the time of sampling was associated with free stalls of similar size. In our survey, the clinical signs were interpreted by the farmer. Luckily IP can be quite easily identified by a farmer but diagnosing other infectious hoof diseases is more problematic. That is why we had to settle for a very simple description of clinical signs without exact diagnosis of a certain hoof disease – ID or DD. In addition, it is likely the farmers that had problems with infectious hoof diseases answered our survey more eagerly and therefore some bias may exist in our results.

In our research, we could not establish the reason behind so many IP outbreaks in Finland. On the other hand, some countries might have experienced similar outbreaks earlier (David, 1993; Cook and Cutler, 1995; Doherty et al., 1998) and due to Finland's relative isolation, Finnish dairy herds are still immunologically quite naïve and therefore more susceptible to IP. Furthermore, structural change in agriculture has occurred in Finland relatively recently. Based on the herd data recording system of ProAgria, the average herd size has more than doubled since 2000 (National Resources Institute, 2019). With the structural change and increasing herd size new technology has just entered Finnish dairy farms; automatic milking system, total mixed ration, and new machinery to feed cattle.

6.6 Future studies

IP studies would benefit from the new investigative methods in bacteriology. Studies on the IP microbiome would be of great importance; the comparison between healthy digital tissue and IP at its various stages. The detection of bacteria needed for the initiation of IP is important, as are all bacteria involved in the IP lesions. Comparison with DD lesions would be of interest. Additionally, based on bacteriological findings of our study, further investigations on *F. necrophorum* and *D. nodosus* are required. We would improve our understanding about the infective nature of IP with a genetic analysis of *F. necrophorum* isolates collected in this study. This might also give insight into *F. necrophorum* virulence factors associated with IP.

Also, transmission route of *F. necrophorum* and accompanied bacteria needs further research. Currently we are unaware of how *F. necrophorum* invades the interdigital tissue – through faecal contamination or is for example haematogenous infection possible?

Another research focus should be the significance of other infectious hoof diseases and their association with IP – whether mild ID with *D. nodosus* is enough for *F. necrophorum* to enter deeper tissues.

More research is required on risk factors for an outbreak of IP to occur. Firstly, herd level risk factors, like importance of proper feeding, or whether problems in feeding can predispose cows to IP requires further research. Also, the factors such as fields in organic farming being a risk factor for an outbreak of IP, need more investigation. Organic farming is becoming more common and farmers are encouraged to change from conventional farming to organic. Secondly, cow level risk factors should be studied; why a single animal gets sick. Although some risk factors, like recent calving, are well documented, there are still several factors we know little about. For example, in the outbreaks is it the animals that are introduced to a new farm that become ill first or the other way around? Studying the genetics would provide information that might help answer these questions.

7 CONCLUSIONS

Based on our research, it can be concluded that:

- A proper clinical inspection is key to diagnosing IP. Lameness and symmetrical swelling of the affected foot were the most observed clinical signs of IP. All lame cows should be clinically inspected – also during an IP outbreak.
- Outbreak herds were either high or moderate morbidity herds and no herd was of intermediate morbidity. Special attention should be paid to high morbidity herds while dealing with their IP outbreak. Further research could be focused on these cows, farms and the bacteriology.
- IP causes a very strong APR, which should be remembered when treating affected animals. Anti-inflammatories should be included in the treatment of IP cows.
- *F. necrophorum* ssp. *necrophorum* is the main pathogen in IP. However, in the disease process several other bacteria play a role, such as *D. nodosus*, which may increase the severity of IP.
- Because animal transport between herds was identified as a risk factor, new cows should be purchased very cautiously, and alternative methods to expand a herd should be considered.

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