Neonatal Porcine Diarrhoea

Aspects on Aetiology and Pathology

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Abstract

Diarrhoea in newborn piglets is an old but still relevant problem in pig production globally. During the last decades, reports from a number of countries describe problems with neonatal porcine diarrhoea (NPD) despite the use of previously effective preventive measures. The aim of this thesis was to investigate and characterise the problem with NPD in Swedish piglet-producing herds. The magnitude of the problem was estimated by a questionnaire study distributed to 170 randomly selected herds. A response rate of 58% was achieved. The total herd-level prevalence of NPD, including herds with sporadic cases, was 79.6%, indicating that NPD is a substantial problem. Ten herds affected by NPD were selected for in-depth studies on the potential causes of the diarrhoea. From each herd, five diarrhoeic and two healthy control piglets were selected. The piglets were blood-sampled for analysis of serum ɣ-globulin, and thereafter euthanized and necropsied. The intestines were sampled for histopathology, virology, and bacteriology. There was no difference in serum ɣ-globulin concentration between diarrhoeic and non-diarrhoeic piglets and pathological lesions in the intestines were generally mild. Porcine enterotoxigenic *Escherichia (E.) coli* was only found in two piglets. Further, extended virulence gene profiling did not suggest involvement of other diarrhoeagenic pathotypes of *E. coli*. *Clostridium (C.) perfringens* type C was not detected, and neither *C. perfringens* type A nor *C. difficile* could be related to the diarrhoea. Furthermore, no protozoa such as *Cystoisospora suis* were observed in the intestinal mucosa. By viral metagenomics analyses of intestinal samples, the only previously well-established porcine enteropathogen found was rotavirus that was present in two herds. Otherwise, the data did not suggest involvement of previously known viruses. The only consistent finding associated with diarrhoea was small intestinal colonisation by *Enterococcus (E.) hirae.* Enteroadherent *E. hirae* was detected in 60% of the diarrhoeic piglets from six of the herds and was associated with small intestinal mucosal lesions in more than 50% of the cases (10/18). Thus, our results show that diarrhoea in newborn piglets may have other causes than the well-established pathogens previously associated with NPD and a potential involvement of *E. hirae* is suggested.

Keywords: *Enterococcus hirae*, *Escherichia coli*, ETEC, *Clostridium perfringens*, *Clostridium difficile*, rotavirus, viral metagenomics, neonate, pig, swine, NNPD.

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Dedication

To my friends and family

Contents

List of Publications

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

I Larsson, J., Fall, N., Lindberg, M. & Jacobson, M. (2016). Farm characteristics and management routines related to neonatal porcine diarrhoea: a survey among Swedish piglet producers. *In manuscript.*

II Larsson, J., Aspán, A., Lindberg, R., Grandon, R., Båverud, V., Fall, N. & Jacobson, M. (2015). Pathological and bacteriological characterization of neonatal porcine diarrhoea of uncertain aetiology. *Journal of Medical Microbiology* 64(8), 916-926.

III Karlsson, O.*, Larsson, J.*, Hayer, J., Berg, M. & Jacobson, M. (2016). The intestinal eukaryotic virome in healthy and diarrhoeic neonatal piglets. Manuscript submitted to *PLoS One.*

IV Larsson, J., Linberg, R., Aspán, A., Grandon, R., Westergren, E. & Jacobson, M. (2014). Neonatal piglet diarrhoea associated with enteroadherent Enterococcus hirae. *Journal of Comparative Pathology* 151(2-3), 137-147.

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Abbreviations

AE	Attaching-effacing
ADG	Average daily gain
AIDA	Adhesin involved in diffuse adherence
cAMP	Cyclic adenosine monophosphate
CDAD	Clostridium difficile associated diarrhoea
cGMP	Cyclic guanosine monophosphate
CpA	Clostridium perfringens type A
CpC	Clostridium perfringens type C
DNA	Deoxyribonucleic acid
EAggEC	Enteroaggregative Escherichia coli
EAST1	Enteroaggregative heat-stable toxin 1
EPEC	Enteropathogenic Escherichia coli
ETEC	Enterotoxigenic Escherichia coli
FISH	Fluorescence in situ hybridization
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract
H&E	Haematoxylin and eosin
HDCD	Hysterectomy derived colostrum deprived
ICTV	International committee on taxonomy of viruses
Ig	Immunoglobulin
LEE	Locus of enterocyte effacement
LT	Heat-labile enterotoxin
MALDI-TOF MS	Matrix assisted laser desorption ionization – time of flight mass spectrometry
MIC	Minimum inhibitory concentration
NCBI	National centre for biotechnology information
NGI	National Genomics Infrastructure
NNPD	New neonatal porcine diarrhoea
NPD	Neonatal porcine diarrhoea

NVI	National Veterinary Institute
Paa	Porcine attaching and effacing associated protein
PAV	Porcine adenovirus
PCR	Polymerase chain reaction
PEC	Porcine enteric calicivirus
PEDV	Porcine epidemic diarrhoea virus
PPDS	Postpartum dysgalactia syndrome
PRCV	Porcine respiratory coronavirus
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SIP	Sows in production
SISPA	Sequence independent single primer amplification
SLU	Sveriges Lantbruksuniversitet (Swedish university of agricultural sciences)
SPE	Serum protein electrophoresis
ST	Heat-stable enterotoxin
SvDHV	Svenska djurhälsovården (Swedish animal health service)
TcdA	Clostridium difficile toxin A
TcdB	Clostridium difficile toxin B
TEM	Transmission electron microscopy
TGEV	Transmissible gastroenteritis virus
V/C	Villus/crypt

1 Introduction

This thesis concerns an old, but yet still common and relevant problem in pig production, namely diarrhoea in neonatal pigs. In the newborn piglet, diarrhoea is generally considered to be of infectious nature but the outcome also depends on factors related to the host and its environment (Martineau, 1995). Thus, to provide a background for the work presented in this thesis, the major enteropathogens associated with neonatal porcine diarrhoea (NPD) will be described together with some other aspects of importance for enteric disease in neonatal piglets.

The term 'neonatal' is sometime used to cover the entire suckling period but in this thesis, neonatal diarrhoea refers only to diarrhoea occurring during the first week of life.

1.1 Implications of neonatal porcine diarrhoea in pig production

Since the emergence of a more modern-like pig production during the late 1950s and 1960s, diarrhoea has been one of the most frequently encountered clinical signs of disease in neonatal piglets (Alexander, 1994). Enteric diseases in newborn piglets are often enzootic but may also occur as defined outbreaks with high morbidity and mortality (Wittum *et al.*, 1995; Svensmark *et al.*, 1988; Morin *et al.*, 1983; Svendsen *et al.*, 1975). As an example of the latter, *Porcine epidemic diarrhoea virus* (PEDV) has caused dramatic outbreaks in America and Asia during recent years with a mortality rate of 80-100% in suckling piglets (Authie *et al.*, 2014). The economic impact of such high death rates is of course huge. However, the economic consequences of enzootic NPD can also be substantial. In studies conducted in Sweden and Denmark, diarrhoea is estimated to account for 5-24% of the overall pre-weaning mortality (Westin *et al.*, 2015; Pedersen, 2010; Svendsen *et al.*, 1975) and to reduce average daily gain (ADG) by 8-14 g per day (Kongsted *et al.*, 2014a;

Johansen *et al.*, 2004).[1] Based on these effects, the cost of NPD was recently estimated to 134 € per sow and year (Sjölund, 2014).

Apart from the economic consequences, NPD has other important implications. The development and spread of antibiotic resistance is one of the greatest threats to both animal and human health (Wise *et al.*, 1998). A minimal and prudent use of antimicrobials is therefore of utmost importance. In a recent study of antimicrobial usage in Swedish farrow-to-finish herds, the highest incidence of antimicrobial treatment was shown to occur in suckling piglets (Sjölund *et al.*, 2015). Targeting improvement of health in this age group may therefore be the most efficient way to further decrease the use of antimicrobials in Swedish pig production. Furthermore, animal welfare is one of the most intensively debated questions regarding the pig industry and disease is an issue that should be addressed also from an animal welfare perspective (Mellor & Stafford, 2004).

1.2 Aspects of neonatal physiology in regard to intestinal health

After birth, the intestine undergoes a number of changes to fulfil the tasks of the postnatal life. The functional development of the porcine intestine is reflected by significant morphological changes. Already after 24 h the small intestine increases in weight by approximately 60-70%, and the large intestine, by 30-40% (Xu *et al.*, 1992; Widdowson, 1976). This dramatic increase is mainly confined to the mucosa, due to the accumulation of colostral proteins within the epithelium, and a high level of cell proliferation (Xu *et al.*, 1992; Widdowson, 1976). The small intestinal absorptive surface area will thus expand with approximately 50% during the first day of life, and by 100% during the following 10 days (Xu *et al.*, 1992; Smith & Jarvis, 1978). These early structural and functional changes of the intestine are however dependent on an early intake of colostrum as a source of both nutrients and growth-promoting peptides (Xu *et al.*, 2002; Zhang *et al.*, 1998; Widdowson, 1976). Furthermore, colostrum has the important function of providing the piglet with a maternally derived, passive immunity.

The newborn piglet is immunologically immature, depending both on an underdevelopment per se but also on the lack of antigen exposure which normally occurs after birth (Rooke & Bland, 2002; Curtis & Bourne, 1971). Thus, even though both innate immune cells and gastrointestinal associated lymphoid tissue (GALT) are present at birth, the intestinal immunity is not considered functionally developed until 4-7 weeks of age (Gaskins, 1995;

1. In 2014, the average pre-weaning mortality in Sweden was 17.8% (Farm and Animal Health, 2014).

Rothkötter & Pabst, 1989; Pabst *et al.*, 1988; Chu *et al.*, 1979). The porcine epitheliochorial placenta does not allow passage of maternal antibodies and the piglet is hence born without passive immune protection (Curtis & Bourne, 1971; Kim *et al.*, 1966). The passive immunity is instead dependent on colostral transfer and one of the key tasks of the neonatal pig intestine is hence uptake of colostrum. Sow colostrum contains high concentrations of mainly IgG but also IgA, IgM, immune cells, and various antimicrobial substances such as lactoferrin (Hurley, 2015; Wagstrom *et al.*, 2000). To facilitate the uptake of maternal immunoglobulins (antibodies), the small intestinal epithelial cells of the neonate is capable of bulk transport of macromolecules from the intestinal lumen and across the basolateral cell membrane (Clarke, 1971; Payne & Marsh, 1962). This ability is however transient, and by 24 h after birth the transfer of immunoglobulins to the circulation ceases, a process referred to as 'gut closure' (Rooke & Bland, 2002; Speer *et al.*, 1959). Immediate intake of colostrum is hence crucial for the newborn piglet's immune status.

The delivery of intact immunoglobulins to the small intestine is facilitated by a number of mechanisms. The secretion of gastric acid is lower in the newborn piglet compared to mature animals (Smith & Jones, 1963) and in combination with the buffering effect of colostrum the immunoglobulins are thus protected from denaturation (Wagstrom *et al.*, 2000). In addition, the dominant gastric protease after birth, chymosin, has a lower proteolytic ability as compared to pepsin (Moughan *et al.*, 1992). Colostrum also contains trypsin-inhibitors (Jensen, 1978; Laskowski *et al.*, 1957) that significantly increases the IgG-uptake (Weström *et al.*, 1985; Carlsson *et al.*, 1980). Altogether, these factors protect the immunoglobulins in colostrum from digestive degradation. However, they also promote survival of potentially harmful microbes and may prevent degradation of noxious substances (e.g. clostridial toxins).

Another key event in the functional development of the intestine is the acquisition of a commensal intestinal microbiota. Initial colonisation of the gastrointestinal tract (GIT) is generally believed to start during and shortly after birth by microbes in the immediate environment (Maxwell, 1995; Moughan *et al.*, 1992). Studies on the early colonisation of piglets show that the first colonizers are facultative anaerobic bacteria including *Escherichia coli*, *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp. but also anaerobic bacteria such as *Clostridium perfringens* (Petri *et al.*, 2010; Konstantinov *et al.*, 2006; Inoue *et al.*, 2005; Naito *et al.*, 1995; Swords *et al.*, 1993; Smith & Jones, 1963). Similar to infants, the major source of the microbiota is probably maternal faeces (Bäckhed *et al.*, 2015; Brown *et al.*, 2006). The composition of the intestinal microflora has been reported to show

a large inter-individual variability during the first 1-2 weeks of the piglet's life, followed by a cohabitation effect around 3-4 weeks when the microflora among piglets become more similar (Thompson *et al.*, 2008; Melin *et al.*, 1997; Katouli, 1995). However, compared to infants, information on the dynamics of the early post-natal microbiota is limited.

In summary, the barrier functions of the GIT in the neonatal piglet is not as developed as in mature animals due to the higher pH in the stomach, the lower proteolytic capacity, the lack of colonisation resistance by an established intestinal microbiota (Lawley & Walker, 2013; Van der Waaij *et al.*, 1971), and the dependence on passive immune protection due to immunological immaturity. Furthermore, the regeneration time of the small intestinal epithelium in day-old piglets is reported to be 7-10 days, as compared to 2-4 days in 3-week-old pigs (Moon, 1971). This difference is probably also contributing to the susceptibility of infectious enteritis in newborn piglets, as a rapid turnover of enterocytes is considered a defence mechanism by the expulsion of infected cells (Kim *et al.*, 2010; Moon *et al.*, 1973).

Another concern of the newborn piglet is the sensitivity to hypothermia due to the poor hair coverage, the large body surface-to-weight ratio, the lack of brown adipose tissue, and the paucity of body energy reserves at birth (Herpin *et al.*, 1994; Trayhurn *et al.*, 1989; Le Dividich, 1983). Hypothermia may not only impede colostrum intake (Blecha & Kelley, 1981; Le Dividich & Noblet, 1981) but has furthermore been shown to reduce the intestinal peristalsis and increase the severity of diarrhoea following enteric infections (Steel & Torres-Medina, 1984; Sarmiento, 1983; Kelley *et al.*, 1982).

Taken together, all of these factors contribute to the vulnerability of the neonatal piglet to enteric infections.

1.3 Basic pathophysiology of diarrhoea

The term diarrhoea is typically used to describe loose, watery stools that occur more frequently than usual. More specifically, diarrhoea can be described as faeces with an excess of water relative to faecal dry matter (Brown, 2007a) and a water content exceeding ~80% has been used to define diarrhoeic stool in pigs post weaning (Pedersen *et al.*, 2011; Kenworthy, 1970).

Mechanisms of diarrhoea are usually categorised as malabsorption, increased intestinal secretion, increased intestinal permeability, and motility disorders (Brown, 2007a; Moon, 1978). These mechanisms are however not mutually exclusive and thus, many diarrhoeic conditions are a result of the simultaneous action of a number of mechanisms.

In general, enteropathogens cause diarrhoea either directly by affecting ion transports and barrier functions, or indirectly through inflammation or loss of absorptive surface (Hodges & Gill, 2010). For example, enterotoxigenic *E. coli* (ETEC) causes profuse secretory diarrhoea by the production of enterotoxins that by their effects on ion transports induce a hyper-secretory state in the intestine (Nataro & Kaper, 1998). *Clostridium (C.) perfringens* type C on the other hand induces severe damage to the intestinal mucosa via its potent beta-toxin, leading to obvious impairment of absorption as well as effusion of interstitial fluid and blood to the lumen (Sayeed *et al.*, 2008; Moon & Bergeland, 1965). Furthermore, other porcine enteropathogens, e.g. rotavirus and the coccidian *Cystoisospora suis,* multiply in the intestinal epithelium leading to an excessive loss of enterocytes and thereby diminished digestive and absorptive capacity (Graham *et al.*, 1984; Eustis & Nelson, 1981). As a consequence, the accumulation of undigested material will drive water into in the lumen, resulting in osmotic diarrhoea.

The clinical signs and outcome of enteric disease in the newborn piglet will vary depending on the infectious agent involved, as well as the piglet's susceptibility to infection (Thomson, 2012). However, the basic consequences of profuse, watery diarrhoea will, irrespective of its cause, be a rapid loss of water, electrolytes, and nutrients. Considering the limited body reserves of the newborn piglet, this will soon lead to a severe condition and the piglet may die within hours. Hence, acute diarrhoea in the neonatal pig should always receive immediate attention.

1.4 Causes of diarrhoea in neonatal piglets

Diarrhoea in newborn piglets are usually related to the presence of a single pathogen and mixed infections are considered less common (Katsuda *et al.*, 2006; Yaeger *et al.*, 2002; Johnson *et al.*, 1992; Morin *et al.*, 1983). The following section will address infectious agents regarded as important causes of NPD (Thomson, 2012), as well as some less common agents, and some of uncertain clinical importance.

1.4.1 Agents with well-established clinical importance

An overview of well-established causes of diarrhoea in suckling pigs, including typical age when pigs are affected and main pathological lesions, are given in Table 1.

Enterotoxigenic Escherichia coli

Escherichia (E.) coli is a gram-negative, facultative anaerobic bacterium that is part of the commensal intestinal microbiota in warm blooded animals (Gordon & Cowling, 2003). However, some *E. coli* strains have developed specific abilities which turn them into primary pathogens, responsible for a range of different diseases in both man and animals (Kaper *et al.*, 2004).

The principle pathotype[2] of *E. coli* responsible for intestinal disease in pigs is enterotoxigenic *E. coli* (ETEC) (Alexander, 1994). ETEC is defined by its capability to produce enterotoxins, exerting their effects locally in the intestine (Nataro & Kaper, 1998). Porcine ETEC is equipped with genes encoding one or both of the two enterotoxin classes heat-labile (LT) and heat-stable (ST) enterotoxin (Smith & Gyles, 1970). The ST toxins can subsequently be divided into two different types; STa and STb (Burgess *et al.*, 1978). LT and STa stimulates intestinal secretion by causing increased intracellular levels of cAMP and cGMP, respectively, leading to an increased Cl^- secretion from secretory crypt cells and decreased absorption of Na^+ and Cl^- by absorptive enterocytes on the villi tips (Gyles, 1994). Whereas the actions of LT and STa have been studied in detail (for an excellent review see Nataro & Kaper, 1998), much less is known about STb. One reason for this might be that STb primarily is associated with porcine ETEC (Nataro & Kaper, 1998; Gyles, 1994). However, the main effect of STb seems to be an increased secretion of HCO_3^- (Weikel et al., 1986). In addition, many porcine ETEC isolates also carry the gene for enteroaggregative heat stable enterotoxin (EAST1), an enterotoxin originally detected in enteroaggregative *E. coli* (EAggEC) in diarrhoeic humans (Choi *et al.*, 2001; Savarino *et al.*, 1991). The mechanism of action for EAST1 is proposed to be similar to that of STa (Savarino *et al.*, 1993). However, the role of EAST1 in the development of diarrhoea is questionable, since EAST1-positive *E. coli* frequently are isolated also from healthy piglets (Vu-Khac *et al.*, 2007; Ngeleka *et al.*, 2003). Moreover, experimental infections with *E. coli* expressing EAST1 as the only enterotoxin fail to induce diarrhoea in gnotobiotic pigs (Ruan *et al.*, 2012; Zhang *et al.*, 2006; Ngeleka *et al.*, 2003).

To cause diarrhoea, ETEC must also be able to persist and multiply in the small intestine (Alexander, 1994). This ability is acquired by the expression of adhesion factors that bind to specific mucosal receptors. Porcine ETEC associated with NPD usually possess one or a combination of the fimbrial adhesins F4, F5, F6 and F41 (Nagy & Fekete, 1999; Söderlind, 1988).

2. Pathotype refers to a group of strains of the same species that cause a specific disease using a common set of virulence factors (Kaper *et al.*, 2004)

The secretory diarrhoea induced by ETEC is unspecific and pathological lesions are mild (Table 1). Clinical signs of ETEC infection can be observed as early as a couple of hours after birth and may vary from mild diarrhoea in an otherwise unaffected animal, to profuse, watery diarrhoea that can result in severe dehydration and death within hours (Alexander, 1981).

The ultimate source of ETEC is believed to be sow faeces, as healthy animals may carry ETEC but at lower numbers (Schierack *et al.*, 2006; Alexander, 1981; Söderlind, 1979). Susceptible infected piglets will subsequently act as an enrichment vessel and excrete large amounts of bacteria, leading to a build-up of pathogenic strains in the environment (Fairbrother, 2012; Alexander, 1994). The contagious nature of ETEC is reflected by the fact that the same strain usually is found in several diarrhoeic piglets and often in consecutive farrowing batches (Fairbrother, 2012).

Diagnosis of colibacillosis in pigs has traditionally been based on serotyping, as certain serogroups of *E. coli* are associated with a particular disease in a certain area (Alexander, 1994; Söderlind, 1971; Sojka *et al.*, 1960). However, in routine diagnostic settings, typing of *E. coli* is now often performed by PCR-based screening of virulence genes (Nagy & Fekete, 2005).

ETEC is considered one of the most important causes of NPD globally, but can be controlled by vaccination of gilts and sows to induce a protective passive immunity in piglets via colostrum (Alexander, 1994; Moon & Bunn, 1993). Furthermore, the susceptibility to ETEC depends on the expression of specific mucosal receptors that varies both with age and genetics (Nagy & Fekete, 2005; Dean *et al.*, 1989; Runnels *et al.*, 1980). For example, some pigs are naturally resistant to colonization by F4-positive *E. coli* (Schroyen et al., 2012) and the genetics behind this trait are used for selective breeding for F4-resistance in Denmark (Pig Research Center, 2010).

Clostridium perfringens *type C*

Clostridium (C.) perfringens is an anaerobic but oxygen tolerant, gram-positive, spore-forming rod that is divided into five groups (toxinotypes A-E) based on the production of the major toxin types alpha, beta, epsilon, and iota (Songer, 2012). *C. perfringens* type C (CpC) produces alpha- and beta-toxin and can cause severe, necrohaemorrhagic enteritis in the newborn piglet (see Table 1). The extensive damage to the intestinal mucosa seen in CpC infections is largely attributed to the actions of the pore-forming, necrotizing beta-toxin (Sayeed *et al.*, 2008; Steinthorsdottir *et al.*, 2000). CpC is also described to adhere to the villous epithelium in early stages of disease (Walker *et al.*, 1980; Arbuckle, 1972) and subsequent, massive attachment to the damaged, necrotic

mucosa is a general histological finding (Niilo, 1988; Morin *et al.*, 1981). The mechanism of this adherence is however not known (Petit *et al.*, 1999).

The susceptibility of newborn animals to CpC-enteritis is considered to depend on the lack of an established intestinal flora combined with slower degradation of the beta-toxin, due to low secretion of trypsin and presence of trypsin-inhibitors in colostrum (Songer, 1996; Lawrence & Cooke, 1980; Jensen, 1978; Laskowski *et al.*, 1957).

In cases with low herd-immunity (such as nonvaccinated populations), CpC can cause dramatic outbreaks that may reach a litter-prevalence of 100% and very high mortality rates in newborn piglets (Morin *et al.*, 1981; Hogh, 1966; Moon & Bergeland, 1965). In this acute form, infected piglets develop haemorrhagic diarrhoea, cease nursing, and become moribund within 12-36 h (Hogh, 1966; Field & Gibson, 1955). However, as herd immunity rises, the disease will become milder and the clinical signs may be less typical (Songer, 2012).

Diagnosis of CpC infection is based on clinical presentation, typical pathological lesions, and demonstration of CpC in faeces or intestinal contents (Songer & Uzal, 2005). The latter is usually performed by bacteriological culture and toxinotyping of *C. perfringens* isolates by PCR (Songer & Uzal, 2005; Engström *et al.*, 2003).

CpC causes disease in piglets in many areas of the world but in a global perspective, it is considered less important than ETEC (Songer, 2012). In Sweden, CpC-enteritis is a notifiable disease in pigs. However, reported index cases during the last ten years have been few (n=3) and confined to the south-western parts of the country (Swedish Board of Agriculture, 2005-2014). NPD caused by CpC can be effectively prevented by administration of vaccines containing beta-toxoid to gilts and sows prior to farrowing (Niilo, 1988).

Table 1.*Overview of important infectious causes of neonatal porcine diarrhoea, typical age when pigs are affected, and main pathological lesions*

Cause	Age	Macroscopic lesions	Microscopic lesions	References
ETEC [1]	12 h-4 days, and/ or 2-3 weeks after weaning	Fluid, yellowish intestinal contents. Small intestinal dilation with some congestion of the intestinal wall	None or mild. Attachment of gram-negative rods to the small intestinal epithelium	Fairbrother (2012); Dean *et al*. (1989); Moon *et al.* (1970); Kohler & Bohl (1966)
Clostridium perfringens type C	12 h-7 days, may occur up to 2-3 weeks of age	Haemorrhagic intestinal contents. Mucosal necrosis in the small intestine. Intestinal and mesenteric hyperaemia	Pathognomonic segmental necrohaemorrhagic enteritis, gram-positive rods associated with lesions	Jäggi *et al.*(2009); Hogh (1966); Moon & Bergeland (1965); Field & Gibson (1955)
TGEV [2] and PEDV [3]	All ages	Fluid, yellowish intestinal contents, thin walled, transparent intestines	Severe villus atrophy, primarily in the distal small intestine	Stevenson *et al*. (2013); Pospischil *et al*. (1981); Debouck & Pensaert (1980)
Rotavirus	1 day-7 weeks, most frequent at 2-3 weeks of age	Fluid to creamy intestinal contents, pale intestines	Mild to severe villus atrophy, primarily in the distal small intestine	Pospischil *et al*.(1981); Tzipori & Williams (1978)
Cystoisospora suis	5-21 days	Fluid to creamy intestinal contents, fibrinous exudates and/or necrosis in the distal small intestine	Mild to severe villus atrophy, fibrinonecrotic enteritis. Intracellular stages of coccidia in the small intestinal epithelium	Eustis & Nelson (1981); Robinson & Turgeon (1980); Stuart *et al.* (1980)

1. ETEC, enterotoxigenic *Escherichia coli*
2. TGEV, *Transmissible gastroenteritis virus*
3. PEDV, *Porcine epidemic diarrhoea virus*

Transmissible gastroenteritis virus *and* Porcine epidemic diarrhoea virus

Transmissible gastroenteritis virus (TGEV) and *Porcine epidemic diarrhoea virus* (PEDV) are single stranded, positive sense RNA viruses belonging to the family *Coronaviridae* (ICTV, 2014). Both viruses are spread by the faecal-oral route and replicate in absorptive enterocytes in the small intestine, resulting in lysis of infected cells (Kim & Chae, 2000; Pospischil *et al.*, 1981; Pensaert *et al.*, 1970). The effect is an acute, malabsorptive diarrhoea associated with villus atrophy primarily in the distal small intestine (Table 1). Additional mechanisms described for TGEV are altered intestinal sodium transport and loss of extravascular protein (Butler *et al.*, 1974). Even if the viruses share a number of properties they are antigenically distinct, and pig TGEV antisera does not neutralize PEDV or vice versa (Lin *et al.*, 2015).

All age groups are susceptible to TGEV and PEDV infection, but the highest mortality rates (up to 100%) are seen in piglets less than two weeks of age (Stevenson *et al.*, 2013; Saif, 2012; Moon *et al.*, 1973; Haelterman & Hutchings, 1956). Typical clinical signs include inappetence, vomiting, and profuse, watery diarrhoea. A clinical history of severe clinical signs in pigs of all ages is indicative for the diagnosis of TGEV/PEDV but in enzootic situations, clinical signs may be less dramatic (Pritchard, 1987; Morin *et al.*, 1983). Laboratory diagnosis can be set either by direct detection of viral antigen or nucleic acid, or indirectly by serology (Saif, 2012; Song & Park, 2012).

TGEV is a cause of disease in most pig-producing areas of the world (Saif, 2012). However, the clinical impact of TGEV in Europe diminished during the 1980s and 1990s due to the extensive spread of a closely related porcine respiratory coronavirus (PRCV; a deletion mutant of TEGV) (Saif, 2012; Schwegmann-Wessels & Herrler, 2006; Pensaert *et al.*, 1986). The PRCV infection is usually subclinical but induces a cross-reactive immunity towards TGEV.

PEDV first appeared in the UK and Belgium during the 1970s and subsequently spread to a number of European countries (Jung & Saif, 2015; Chasey & Cartwright, 1978; Pensaert & De Bouck, 1978; Wood, 1977). After the 1980s, problems with PEDV in Europe declined and disease outbreaks have since only been occasional (Carvajal *et al.*, 2015; Martelli *et al.*, 2008). In 2013, PEDV was introduced for the first time on the American continent and resulted in severe outbreaks of disease in the naïve population (Carvajal *et al.*, 2015; Stevenson *et al.*, 2013). Recently, PEDV isolates similar to the variants described in America have also been detected in Europe (Boniotti *et al.*, 2016; Dastjerdi *et al.*, 2015; Grasland *et al.*, 2015; Hanke *et al.*, 2015; Stadler *et al.*,

2015). However, neither PEDV nor TGEV have ever been reported in Sweden (Wallgren, 2014).

Rotavirus

Rotaviruses are segmented double stranded RNA viruses that frequently cause diarrhoea in young animals and humans (Ramig, 2004). Porcine rotaviruses are divided in four serogroups (A-C, E) among which serogroup A accounts for the vast majority of rotavirus-associated diarrhoeas (Will *et al.*, 1994; Morilla *et al.*, 1991; Janke *et al.*, 1990). Rotavirus primarily targets mature enterocytes in the small intestine but has also been demonstrated in the epithelium of the large intestine (Gelberg, 1992; Pospischil *et al.*, 1981). The virus replicates in the cytoplasm resulting in lysis of enterocytes, subsequent villous atrophy (Table 1), and malabsorptive diarrhoea (Svensmark *et al.*, 1989a; Graham *et al.*, 1984). Additional mechanisms of rotavirus-induced diarrhoea have been suggested, including enterotoxin function of the NSP4-protein (Lundgren & Svensson, 2001; Ball *et al.*, 1996).

Group A rotavirus is primarily associated with mild diarrhoea at 2-3 weeks of age, a diarrhoea often referred to as 'white scours' (Svensmark *et al.*, 1989b; Bohl *et al.*, 1978). As the virus is widespread, piglets are usually protected against disease during the first weeks of life via maternal immunity (Ward *et al.*, 1996; Fu *et al.*, 1990; Shaw *et al.*, 1989). Many piglets may hence be subclinically infected and disease occurs only if colostrum intake is insufficient or if the infectious dose exceeds the protective immunity (Ward *et al.*, 1996; Debouck & Pensaert, 1983; Tzipori & Williams, 1978). Outbreaks of group C rotavirus have previously only been described occasionally (Chasey *et al.*, 1986) but recent studies indicate that rotavirus group C has become more common in piglets less than a week of age (Lorenzetti *et al.*, 2014; Marthaler *et al.*, 2013). Diagnosis of rotavirus infection is usually set by detection of viral antigens or nucleic acid in faeces, intestinal contents, or in tissue (Chang, 2012).

Porcine rotaviruses are widespread among pigs throughout the world including Sweden (Nilsson *et al.*, 1984), and in some countries up to 100% of the adult pig population is seropositive (Chang, 2012).

Cystoisospora suis

Cystoisospora suis is an intracellular protozoan parasite with a direct life cycle that can be divided into three stages: sporulation of oocysts in faeces, excystation and release of infectious sporozoites, and the endogenous stage when the parasites multiply within the small intestinal epithelium (Lindsay *et al.*, 1997; Lindsay *et al.*, 1980). The endogenous stage leads to necrosis of

enterocytes, followed by villus atrophy and malabsorptive diarrhoea (Lindsay, 2012; Eustis & Nelson, 1981). Pathological lesions (Table 1) and clinical signs develop around 3-5 days post infection and excretion of oocysts in faeces can be seen after five days (Stuart *et al.*, 1982; Lindsay *et al.*, 1980; Stuart *et al.*, 1980). Clinical signs are commonly seen in piglets between five and 21 days of age and include fluid to creamy diarrhoea, rough hair coat, and reduced weight gain (Lindsay, 2012; Roberts & Walker, 1982; Eustis & Nelson, 1981; Robinson & Turgeon, 1980). The severity of the disease depends on the infectious dose (Stuart *et al.*, 1982) as well as concurrent infections with other enteropathogens (Eustis & Nelson, 1981).

Diagnosis of *Cystoisospora suis* can be made by demonstration of oocysts in faeces, or the endogenous life stage in the intestinal mucosa by microscopy of tissue sections or mucosal smears (Lindsay, 2012). *Cystoispospora suis* is present in commercial pig herds worldwide including Sweden and is, similar to rotavirus, primarily associated with 'white scours' in 2-3-week-old piglets (Nilsson *et al.*, 1984; Stuart *et al.*, 1980).

1.4.2 Clostridium perfringens type A and Clostridium difficile

During the last decades, there has been an increased focus on clostridia in association with NPD. Both *C. perfringens* type A and *C. difficile* have been pointed out as important causes of NPD (Songer, 2012) but the clinical importance of these agents has also been questioned as both the organisms and their toxins can be equally demonstrated in healthy newborn piglets (Farzan, 2013; Hopman *et al.*, 2011; Yaeger *et al.*, 2007).

Clostridium perfringens *type A*

C. perfringens type A (CpA) produces alpha-toxin as its sole major toxin and is considered a part of the normal intestinal microbiota in piglets (Petri et al., 2010; Smith & Jones, 1963). The pathogenesis of CpA enteritis is poorly understood but due to the negligible pathological lesions seen in the intestine, a secretory mechanism has been suggested (Songer & Uzal, 2005). In contrast to CpC infection, adhesion or destruction of the epithelium does not seem to be a feature of CpA enteritis (Johannsen *et al.*, 1993b). Moreover, the role of toxins produced by CpA is unclear. Inoculation of colostrum-deprived piglets (n=23) with alpha-toxin alone resulted in diarrhoea in only 11% of the cases and pathological changes in the intestines were minor (Johannsen *et al.*, 1993a; Johannsen *et al.*, 1993b). Further, a large proportion of CpA isolates from diarrhoeic piglets carry the beta2-toxin gene (Bueschel *et al.*, 2003; Garmory *et al.*, 2000; Klaasen *et al.*, 1999). However, more recent studies demonstrate that beta2-toxin-positive CpA is highly prevalent also in healthy animals (Farzan,

2013; Chan *et al.*, 2012; Jäggi *et al.*, 2009) and that the toxin itself can be detected at comparable levels in intestinal contents from healthy and diarrhoeic piglets (Farzan, 2013; Jonach, 2013). In humans, CpA is associated with food poisoning due to the production of an enterotoxin. This toxin has also has been suggested to be involved in CpA enteritis in pigs (Collins *et al.*, 1989; Estrada & Taylor, 1989) but presence of the enterotoxin gene in porcine CpA is uncommon (Czanderlova *et al.*, 2006; Garmory *et al.*, 2000; Klaasen *et al.*, 1999; van Damme-Jongsten *et al.*, 1990).

Experimental infection of colostrum-deprived-piglets with CpA isolated from clinical NPD-cases have shown inconsistent results, ranging from necrotic haemorrhagic enteritis to absence of diarrhoea and intestinal lesions (Johannsen *et al.*, 1993a; Olubunmi & Taylor, 1985; Nabuurs, 1983).

Clinical signs of naturally occuring CpA enteritis in newborn pigs is reported to include creamy, whitish diarrhoea that commence around one to two days after birth (Songer, 2012; Collins *et al.*, 1989). Pathological changes range from no apparent lesions to focal, superficial necrosis of villi tips. A common observation is large amounts of gram-positive rods, consistent with *C. perfringens,* in the intestinal lumen (Silva *et al.*, 2013; Collins *et al.*, 1989).

Since pathological lesions are mild and CpA isolates allegedly associated with disease currently cannot be differentiated from CpA in healthy piglets the diagnosis of CpA-enteritis is equivocal.

Clostridium difficile

C. difficile is an important cause of hospital- and antibiotic-associated diarrhoea and pseudomembranous colitis in humans (Mylonakis *et al.*, 2001), and aspects of the disease have been studied in hysterectomy-derived colostrum-deprived (HDCD) piglets (Steele *et al.*, 2014; Steele *et al.*, 2010).

The pathogenesis of *C. difficile*-associated diarrhoea (CDAD) is mainly mediated through the actions of *C. difficile* toxin A (TcdA) and toxin B (TcdB) (Steele *et al.*, 2014; Kuehne *et al.*, 2010). Both TcdA and TcdB disrupt the actin cytoskeleton in intoxicated cells, leading to impairment of important cellular functions and ultimately disorganisation and cell death (Janoir, 2015). However, the toxins may also add to the development of intestinal inflammation and effect the enteric nervous system (Keel & Songer, 2006). Further, some porcine strains carry the gene for binary toxin (Silva *et al.*, 2011) but the role of this toxin in the pathogenesis of CDAD is unclear (Janoir, 2015).

C. difficile-associated diarrhoea in newborn piglets seems unrelated to antibiotic treatment. Clinical signs include soft to semifluid faeces, loss of body condition, and sometimes dyspnoea (Songer *et al.*, 2000; Waters *et al.*,

1998). Affected piglets are usually 1-7 days old and present at necropsy with mesocolonic oedema and, occasionally, hydrothorax and ascites (Yaeger *et al.*, 2002; Songer *et al.*, 2000; Waters *et al.*, 1998). Intestinal lesions are generally mild and confined to the cecum and colon. Characteristic microscopic findings are 'volcano lesions' consisting of focal mucosal damage with effusion of fibrin and neutrophils to the lumen (Yaeger *et al.*, 2002; Songer *et al.*, 2000; Waters *et al.*, 1998).

The organism is prevalent also among healthy neonatal piglets (Hopman *et al.*, 2011; Weese *et al.*, 2010; Yaeger *et al.*, 2007) and the confirmation of CDAD is based on detection of TcdA and TcdB in faeces or colonic contents (Songer *et al.*, 2000). However, *C. difficile* toxins were recently shown to be more common in non-diarrhoeic neonatal piglets (23/29) compared to diarrhoeic piglets (42/100) (Yaeger *et al.*, 2007). The study by Yaeger *et al.* (2007) is however the only large-scale study comparing the prevalence of *C. difficile* toxins in healthy and diarrhoeic animals. Hence more studies are needed to assess the diagnostic value of TcdA/TcdB-detection in newborn piglets.

Prior to the current study, *C. difficile* had not been investigated with regard to NPD in Sweden.

1.4.3 Additional agents associated with neonatal porcine diarrhoea

Several other agents have been associated with NPD. Some are regarded to be of lesser or unknown clinical importance, whereas others are restricted to certain geographical areas. Below follows a brief description of selected additional bacterial, viral, and parasitic infections that have been described in relation to diarrhoea in suckling pigs.

Variants of E. coli *other than 'classical' porcine ETEC*

Except 'classical' ETEC, some other variants of *E. coli* may also cause diarrhoea in newborn pigs but are considered much less common (Alexander, 1994). ETEC isolates of the STb or STb:EAST1 virotype[3] may produce a non-fimbrial adhesion factor called adhesin involved in diffuse adherence (AIDA-I). AIDA-I was originally described in *E. coli* isolates from diarrhoeic humans (Benz & Schmidt, 1989) and later found in porcine ETEC isolates associated with post-weaning diarrhoea (Niewerth *et al.*, 2001). Subsequently, this virotype was also isolated from cases of NPD and diarrhoea was reproduced experimentally in colostrum-deprived newborn piglets (Ravi *et al.*, 2007; Pritchard *et al.*, 2004; Ngeleka *et al.*, 2003). *E. coli* positive for STb:EAST1:AIDA-I has also been associated with NPD in Sweden (Eriksson,

3. Virotype refers to a set combination of virulence factors in a specific isolate

2012). Since commercially available vaccines against ETEC only contain fimbrial antigens (and in some cases LT toxoid), STb:EAST1:AIDA-I positive *E. coli* has been suggested as a cause of NPD in herds that experience problems in spite of adequate vaccination routines (Eriksson, 2012).

Another pathotype of *E. coli* related to porcine diarrhoea is enteropathogenic *E. coli* (EPEC) (Higgins *et al.*, 1997; Helie *et al.*, 1991; Janke *et al.*, 1989). EPEC is characterised by its ability to induce characteristic attaching and effacing (AE) lesions in the intestine (Nataro & Kaper, 1998), an ability regulated by a number of genes located in a pathogenicity island called locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). Typical for AE lesions is extensive or multifocal attachment of bacteria to the intestinal mucosa, accompanied by erosions and detachment of enterocytes to the lumen (Helie *et al.*, 1991; Janke *et al.*, 1989). Distinctive features noted by transmission electron microscopy (TEM) are effacement of microvilli and protrusions of the cell membrane underlying the bacteria, a phenomenon called pedestal formation (Helie & Higgins, 1999; Tzipori *et al.*, 1985; Moon *et al.*, 1983). Gnotobiotic pigs have been used to study the pathogenesis of EPEC (Tzipori *et al.*, 1985; Moon *et al.*, 1983) but reports on naturally occurring AE lesions in newborn piglets are few (Higgins *et al.*, 1997; Janke *et al.*, 1989). In post-weaning pigs however, porcine EPEC of serotype O45 has been associated with occasional outbreaks of diarrhoea (Zhu *et al.*, 1994; Helie *et al.*, 1991). Although investigations of porcine EPEC O45 show that presence of LEE correlates with the ability to induce AE lesions (An *et al.*, 2000), genes within LEE, such as the gene for the adhesin intimin, are frequently detected also in healthy pigs (Vu-Khac *et al.*, 2007; Malik *et al.*, 2006; Krause *et al.*, 2005; Ngeleka *et al.*, 2003). Likewise, porcine attaching and effacing-associated protein (paa) is important for the formation of AE lesions (Batisson *et al.*, 2003; An *et al.*, 1999) but has also been demonstrated to be present in porcine ETEC (Leclerc *et al.*, 2007; Zhang *et al.*, 2007; Boerlin *et al.*, 2005; An *et al.*, 1999). Hence, the mere detection of these genes cannot be used to diagnose porcine EPEC.

Furthermore, due to the detection of *E. coli* isolates from diarrhoeic pigs with EAST1 as the only known virulence factor, it has been suggested that enteroaggregative *E. coli* could be involved in porcine diarrhoea (Choi *et al.*, 2001; Penteado *et al.*, 2001; Tzipori *et al.*, 1992). However, typical EAggEC similar to isolates associated with diarrhoea in humans have so far not been demonstrated in animals (Uber *et al.*, 2006; Kaper *et al.*, 2004).

Enterococcus *spp.*

Although many *Enterococcus (E.)* spp. are regarded as commensals of the intestinal tract, some species belonging to the *E. faecium*-species group have also been associated with diarrhoea in suckling animals (Nicklas *et al.*, 2010; Vela *et al.*, 2010; Collins *et al.*, 1988; Etheridge *et al.*, 1988; Tzipori *et al.*, 1984). In pigs, a few case reports of NPD describe dense, small intestinal colonization with enterococci identified as either *E. durans* or *E. villorum* (Vancanneyt *et al.*, 2001; Cheon & Chae, 1996; Drolet R., 1990; Johnson, 1984). The condition has also been reproduced by experimental infection of gnotobiotic piglets with *E. durans* but very little is known about the pathogenesis (Johnson, 1984; Tzipori *et al.*, 1984). Prior to the present study, enterococci had not been investigated in relation to NPD in Sweden.

Salmonella

Diseases in swine caused by salmonella are mainly caused by *Salmonella (S.) enterica* serotype *cholerasuis* or *S. enterica* serotype *typhimurium* (Carlson, 2012). *S. cholerasuis* is primarily associated with septicaemia, whereas *S. typhimurium* causes enterocolitis. Disease is however rarely seen in suckling pigs. In Sweden, the prevalence of salmonella in the pig population is low, thanks to an extensive national control program (National Board of Health and Welfare, 2013).

Chlamydia suis

Chlamydiae are gram-negative bacteria that only multiply within their host cells. In pigs, chlamydial infections may have various manifestations including enteritis (Schautteet & Vanrompay, 2011; Nietfeld et al., 1993). Most intestinal infections are believed to be subclinical (Nietfeld et al., 1997; Szeredi et al., 1996) but diarrhoea is reproducible by experimental infection of gnotobiotic pigs with *Chlamydia suis* (Guscetti et al., 2009). Intestinal chlamydia infections are common in Swedish growing pigs but do not correlate with the presence of diarrhoea (Englund et al., 2012).

Miscellaneous viral infections

Aichivirus C (previously known as porcine kobuvirus) belongs to the *Picornaviridae* family (ICTV, 2014) and has been found at a high prevalence in faecal samples from diarrhoeic piglets (Park et al., 2010; Khamrin et al., 2009). However, *Aichivirus* C is frequently detected also in healthy, young piglets and hence an association with diarrhoea is uncertain (Di Bartolo et al., 2015; Verma et al., 2013; An et al., 2011). No experimental infections have yet been published.

Porcine adenoviruses (PAV) includes three species (A-C) within the *Adenoviridae* family (ICTV, 2014). PAVs are mainly associated with intestinal disease in swine but are regarded to have low to moderate pathogenicity (Benfield, 2012). Outbreaks of NPD where PAV was identified as the only known enteropathogen have however been reported from the US (Abid *et al.*, 1984) and diarrhoea is reproducible in HDCD pigs (Ducatelle *et al.*, 1982; Coussement *et al.*, 1981). Typical for intestinal PAV infection is the presence of intranuclear inclusion bodies in enterocytes of the distal small intestine (Ducatelle *et al.*, 1982; Coussement *et al.*, 1981).

Astroviruses (family *Astroviridae*) have also been implicated as a cause of NPD (Wallgren, 2012). Mild diarrhoea can be induced experimentally in HDCD pigs (Indik *et al.*, 2006; Shimizu *et al.*, 1990) but in most natural outbreaks, other well-known enteropathogens have been detected concurrently (Mor *et al.*, 2012; Shirai, 1985; Bridger, 1980). Porcine astroviruses have also been found to be common in healthy pigs at slaughter (Luo *et al.*, 2011) and hence the clinical importance of astrovirus in porcine enteric disease remains to be determined.

Porcine enteric caliciviruses (PEC), including porcine norovirus and porcine sapovirus (family *Caliciviridae*) have also been discussed in relation to NPD (Knowles, 2012). Porcine enteric caliciviruses are widespread in the pig population globally and have mostly been investigated in healthy pigs (Knowles, 2012). However, one European prevalence study, including both diarrhoeic and healthy pigs from Denmark and Spain, found no association between the presence of sapovirus and diarrhoea (Reuter *et al.*, 2010). Experimental infections of gnotobiotic pigs with PEC (both noro- and sapovirus) result in mild diarrhoea and viral shedding in faeces (Wang *et al.*, 2005; Guo *et al.*, 2001; Flynn *et al.*, 1988) but little is known about the association of PEC with naturally occurring disease (Knowles, 2012).

Parasitic infections other than coccidiosis

Cryptosporidia are obligate intracellular parasites that have been detected in pigs worldwide (Lindsay, 2012). The endogenous stages are extracytoplasmatic, and hence the parasites appear to be located on the luminal surface of the intestinal epithelium. In suckling pigs, *Cryptospordium (C.) suis* is the most frequently detected species but the association between faecal shedding of oocysts and diarrhoea is vague (Němejc *et al.*, 2013; Hamnes *et al.*, 2007; Vítovec *et al.*, 2006). Experimental infection of 2-day-old conventional pigs resulted in mild diarrhoea in only a proportion of pigs (4/9) (Enemark *et al.*, 2003). In Denmark and Norway, the prevalence of faecal shedding of cryptosporidial oocysts in suckling pigs is low (6% of 488 piglets

and 8% of 684 litters, respectively) (Hamnes *et al.*, 2007; Maddox-Hyttel *et al.*, 2006).

Finally, the nematode *Strongyloides ransomi* can cause diarrhoea in suckling pigs, especially in tropical areas (Greve, 2012). The small hairlike nematode (3-5 mm) resides embedded in the mucosa of the proximal small intestine where it can cause villus atrophy and inflammation if present in sufficient numbers (Brown, 2007c). The eggs that are excreted with faeces hatch into larvae that either develop into an infective L3 stage or turn into free-living adults (Viney & Lok, 2015). The infective L3 larvae can be transmitted to new hosts by percutaneous, oral, transcolostral, or prenatal spread (Greve, 2012). The principal route of infection for neonatal animals is believed to be transcolostral. The prevalence of *Strongyloides* in Scandinavian pigs has been investigated in a number of studies and has invariably been reported as very low or non-existent (Haugegaard, 2010; Roepstorff *et al.*, 1998; Roepstorff & Jorsal, 1989).

1.4.4 Non-infectious causes of neonatal porcine diarrhoea

Descriptions of non-infectious causes of NPD are limited and generally attributed to nutritional causes. 'Indigestive scours' is described as mild diarrhoea in otherwise healthy-looking piglets and has been attributed to overfeeding. This type of diarrhoea is therefore most common at approximately 10 days after farrowing, concurrent with the peak in lactation (Alexander, 1981). The condition has however also been described in neonatal piglets and is believed to be associated with feeding the sows an energy-rich, high-protein diet before and after farrowing (Kongsted *et al.*, 2013; Alexander, 1981). The association seem however to be based mainly on practical experience. It has furthermore been proposed that the presence of mycotoxins in sow feed could contribute to diarrhoea in suckling piglets (Alexander, 1981). The most relevant mycotoxins for Swedish pig production (deoxynivalenol and zearalenon) are however primarily associated with reproductive problems, feed refusal, and reduced weight gain (Nordkvist & Häggblom, 2014; D'mello *et al.*, 1999).

Starvation or 'deprivation scours' may be another non-infectious cause of NPD (Alexander, 1981). In such cases, diarrhoea is described not only to depend on a deficient passive immunity but also to have a malabsorptive component, as colostrum is vital for the early development and maturation of the intestine (Kongsted *et al.*, 2013; Lippke *et al.*, 2011; Jensen *et al.*, 2001). Deprivation scours is usually seen during the first week of life and may affect single piglets in a litter. Piglets become thin, rough-coated and present with empty or nearly empty stomach and intestines at necropsy (Alexander, 1981).

1.5 Aspects on the diagnosis of enteral disease in neonatal piglets

For investigations of infectious diseases, identification of the causative agent is crucial. Microbiological diagnosis of enteric infections is however complicated by the fact that the gastrointestinal tract normally contains a complex mix of microorganisms. The mere detection of a specific organism in the intestine of a diseased animal does therefore not automatically indicate that disease resulted from its presence. In infectious medicine, Koch's postulates have a central role in the establishment of causation and can be described in four steps: 1) establishment of an association between the presence of an organism and the disease, 2) isolation of the organism, 3) reproduction of the disease in a susceptible host, and 4) re-isolation of the organism from the experimentally infected animal. Since the original postulates were proposed in the 1880s, they have been shown to have some limitations and have hence been revised repeatedly (Mokili *et al.*, 2012; Falkow, 1988; Evans, 1976). However, although different biomedical disciplines of today use slightly different but overlapping criteria, most are derived from the basic concepts stated above.

Most of the previously established causes of NPD have been shown to fulfil the general guidelines for defining a pathogen as denoted by Koch's postulates. The diagnosis of NPD has thus largely focused on the detection of these agents, traditionally by direct methods such as culturing, microscopy, and antigen-based tests. However, with the introduction of highly sensitive molecular diagnostics, also low-level infections of uncertain clinical significance may be detected. Microbiological findings must therefore be interpreted with care and in relation to the clinical signs and pathological lesions in the diseased animal, as well as current knowledge of the associated disease.

Apart from determining the infectious cause, investigations of NPD in a herd should also include examination of other, potentially contributing factors. Major determinants for the manifestation of NPD are depicted in Figure 1 and include the passive immunity transferred by colostrum, the environmental temperature, and the infection pressure of the specific pathogens present in the herd (Martineau, 1995; Drew & Owen, 1988; Sarmiento, 1983; Kelley *et al.*, 1982; Blecha & Kelley, 1981). An inadequate passive protection can result from a weak colostral immunity (lack of protective antibodies towards the infectious agent present) or an insufficient colostrum intake (Martineau, 1995). Prophylactic measures against NPD often focus on the former by implementing vaccination schemes, but inadequate colostrum intake is also a frequent problem (Martineau *et al.*, 1992). Hypo-consumption of colostrum can both be related to maternal factors such as postpartum dysgalactia syndrome (PPDS)

and to factors related to weak piglets, such as low birth-weight and hypothermia (Svensmark *et al.*, 1988; Le Dividich & Noblet, 1981). Due to the piglet's sensitivity to cold-stress, the environmental temperature is an important factor in determining the susceptibility to disease (Pedersen *et al.*, 2013; Malmkvist *et al.*, 2006; Herpin *et al.*, 2002). In addition, a low ambient temperature together with high humidity can contribute to the prolonged survival of many infectious agents (Fairbrother, 2012). However, an all in-all out batch farrowing system is the most important factor to avoid a build-up of pathogens in the environment, as it allows for thorough sanitation of the farrowing unit between batches (Bowman *et al.*, 1996; Dewey *et al.*, 1995; Martineau, 1995).

Thus, presence of NPD in a herd should be viewed as the result from the interaction of a multitude of factors that need to be examined in order to find rational means for intervention.

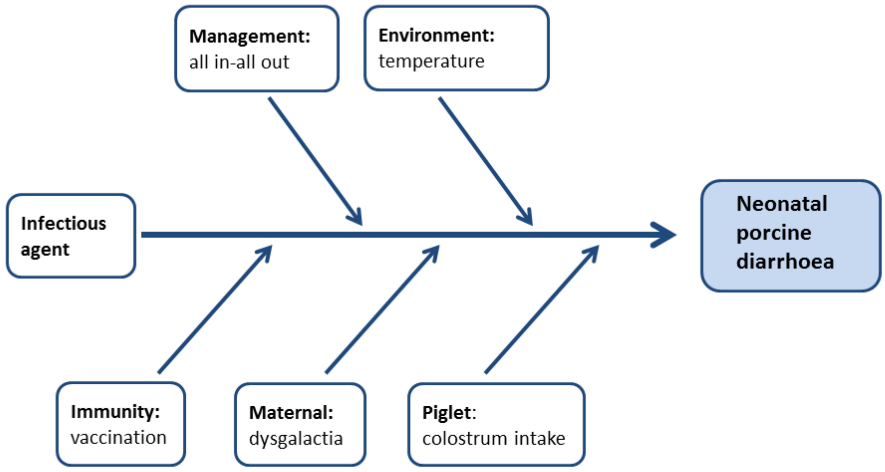

Figure 1. Fishbone diagram of factors important for the occurrence of neonatal porcine diarrhoea. Modified from (Ramirez, 2012)

1.6 New neonatal porcine diarrhoea

During the last decades, some studies from North America have indicated a relative increase in NPD not caused by ETEC, *C. perfringens* type C, or TGEV (Farzan, 2013; Yaeger *et al.*, 2002). In 2006 to 2010, a few reports from European countries including Sweden, Denmark and France described a similar situation with NPD in well-managed herds, despite the use of

previously effective preventive measures (Gin, 2010; Melin, 2010; Svensmark, 2009; Goillandeau, 2006). The condition was described to be unrelated to previously well-known enteropathogens and was by some referred to as ‘new neonatal porcine diarrhoea’ (NNPD). However, at the start of this project in 2011, studies characterising microbiological findings in relation to pathological changes and passive immunity were lacking.

Photo by Tim Meier

2 Hypothesis and aims

The hypothesis of the present study was that diarrhoea in newborn piglets in Sweden has other causes than the well-established enteropathogens previously associated with NPD.

The overall aim was to characterise the problem with NPD present in Swedish piglet-producing herds despite the use of previously well-functioning preventive measures such as maternal vaccination against ETEC. The specific objectives were:

- To assess the occurrence and manifestation of NPD in Swedish piglet-producing herds
- To characterise the pathology associated with neonatal diarrhoea in piglets from herds affected by NPD
- To investigate the presence of bacterial enteropathogens previously associated with piglet diarrhoea, the piglets' passive immune status, and important environmental factors in herds affected by NPD
- To investigate the presence of mammalian viruses in the intestine of diarrhoeic and healthy piglets from herds affected by NPD

3 Comments on materials and methods

The material and methods used are detailed in each paper. The studies can be divided into two separate parts; a questionnaire study on herd characteristics and management routines related to NPD in piglet-producing herds (paper I), and a case control study performed in ten selected herds (paper II-IV) including investigations on pathology, microbiology, passive immune status, and some environmental factors. Considerations regarding the methods used are presented below.

3.1 Occurrence and characteristics of neonatal porcine diarrhoea in Swedish piglet-producing herds (I)

Reports from the field indicated that NPD had become a common clinical problem in Swedish piglet-producing herds, despite the continuous use of previously effective preventive measures. This seemingly altered manifestation of NPD warranted investigations on the magnitude and characteristics of the problem. The herd-level prevalence of NPD had previously not been estimated in Sweden.

To include as many piglet-producing herds as possible in the sample frame, contact was established with the two main organisations carrying out organised porcine health management; the Swedish Animal Health Service (SvDHV; presently known as Farm and Animal Health) and Lunden's Animal Health Service. Together these organisations were estimated to cover the majority (~86%) of all piglet-producing herds in Sweden[4] (Statistics Sweden, 2012). A total of 170 herds were selected at random from the sample frame. However, herds with less than 10 sows were substituted, as these herds were considered not to be representative for Swedish pig production.

4. Personal communication Erik Lindahl (Lunden's Animal Health Service) and Sten-Olof Dimander (Swedish Animal Health Service)

We hypothesised that a higher response rate could be achieved if the questionnaires were distributed and completed at the routine herd visits performed by the veterinarians, compared to being distributed by mail. However, the approach could also introduce some bias as the veterinarians may be more motivated to ensure that the questionnaire was carried out in herds affected by NPD. Moreover, some veterinarians (4 out of 17) were not able to complete the study and thus, the corresponding questionnaires were distributed directly to the herds by mail, after an initial telephone contact. Potential differences between veterinarians and distribution method was however not included in the analysis due to the small number of questionnaires per veterinarian and hence these potential biases must be taken into consideration when interpreting the results.

To increase the comprehensiveness of the questions, the questionnaire was pilot-tested in six herds but despite this, 12 out of the total 59 questions had to be excluded in the data-editing process because of inadequately designed questions.

The outcome variable, presence of neonatal diarrhoea, was specified in the questionnaire as diarrhoea in piglets younger than seven days with the possible responses “Yes”, “No”, and “Occasional cases”. The third option “Occasional cases” was included to separate herds with only sporadic cases of NPD, from herds having a recurrent problem. In the statistical analyses of potential risk factors associated with NPD, the outcome was treated as a binomial variable where “No” and “Occasional cases” were merged.

To include as many responses as possible in the regression analyses, missing data was managed by multiple imputation. Only variables applicable for all respondents were imputed. Considering the large number of variables registered for the in total 98 responses used, selection of variables for analyses by logistic regression models were based on univariable associations with NPD of $P<0.2$, which limited the number of explanatory variables to 12. However, as the association of NPD and herd size (measured as number of sows in production; SIP) can be assumed to be partially mediated by differences in management between larger and smaller herds, management factors can be defined as intervening variables in the causal pathway between herd size and NPD. Since variables identified as “intervening” should not be included in a response model (Martin, 2014), the effect of SIP was estimated in a univariable logistic regression model, whereas the other variables were analysed in a multivariable logistic regression model.

Data from non-responders were unfortunately not available and it was therefore not possible to investigate if responder and non-responder herds differed in terms of size, production results etc. Hence, the external validity of

the study is difficult to estimate and generalization of the results to the entire target population must therefore be made with care.

3.2 Pathological and bacteriological characterisation of neonatal porcine diarrhoea of uncertain aetiology (II)

3.2.1 Selection of herds and animals

The included herds (n=10, A-J) were selected as representative of the particular problem, i.e. NPD should be present as a clinical problem despite maternal vaccination against ETEC. Moreover, the herds had to be located in the middle of Sweden (Figure 2) for two reasons: 1) to enable submission of live animals for necropsy to obtain intestinal samples of good quality for histopathology (Cross & Kohler, 1969), and 2) to ensure standardized sampling and rapid access to laboratory processing at the Section of Pathology, Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden) and the National Veterinary Institute (NVI, Uppsala, Sweden), respectively.

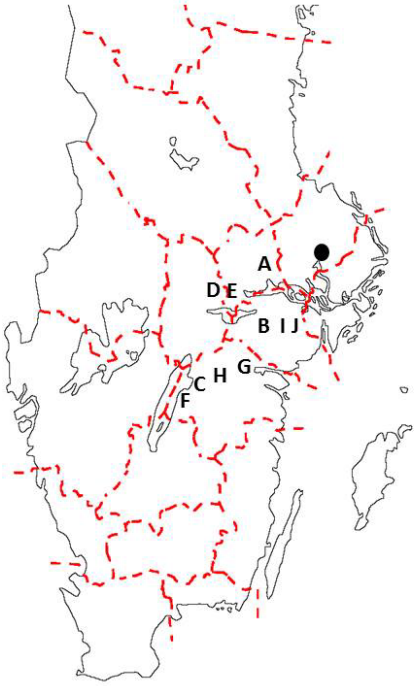

Figure 2. Approximate location of herds included in paper II-IV. Herd A and E were satellite herds in the same sow pool. Herd I and J were in proximity to each other and partially employed the same personnel. ● = Uppsala. (Source: Swedish Land Survey).

Each herd was visited during two consecutive days, at time-points when diarrhoea typically occurred in the individual herd. At each farm, five diarrhoeic piglets and two non-diarrhoeic piglets younger than one week of age were selected. The presence of diarrhoea was assessed based on apparent faecal consistency. Animals with loose to watery faeces were considered diarrhoeic and animals with shaped, firm to soft faeces were considered non-diarrhoeic (Figure 3). Piglets were selected from as many different litters as possible. None of the selected piglets were treated with antibiotics since that would have affected the bacteriological investigations. To avoid interference from secondary infections all piglets had been ill for less than 24 h. The control piglets should preferably be of the same age as the diarrhoeic piglets and selected from litters with no signs of diarrhoea. However, the options for selection were limited to the litters present at the time of the visits. Finding suitable controls was in general more difficult and therefore the control piglets ended up to be slightly older, a difference that was considered in the analyses when necessary. In one herd (J), all litters fulfilling the criteria (less than a week of age and untreated) where affected by diarrhoea at the visit and hence the healthy control piglets were selected from the least affected litter.

Investigation of environmental risk factors was not the main focus of this study but intended to reveal obvious shortcomings, possibly contributing to the problem in the individual herd. Thus, the temperature of the creep area was measured and the cleanliness of the farrowing pens estimated for all pens in use at the visit. The cleanliness estimates were based on the percentage of the non-slatted floor in the pens that was wet and/or covered with faeces (0-20%, >20-60% or >60-100%). It should however be pointed out that since these estimates were made before noon, the pens had been recently cleaned. An overview of characteristics of selected herds is given in Table 2.

Table 2. *Descriptive data on the ten herds (A-J) selected for investigation. (TS= trimethoprim/sulphonamide combinations, mv= missing value)*

Herd	A	B	C	D	E	F	G	H	I	J
Type of production	Satellite	Farrow-to-finish	Piglet production	Piglet production	Satellite	Piglet production	Piglet production	Farrow-to-finish	Satellite	Farrow-to-finish
Sows in production	[1]	800	600	1100	[2]	160	500	120	[3]	250
Recruitment of gilts	Internal	mv	Internal	Internal	Internal	Internal	Purchase	Internal	Purchase	Purchase
Days empty between batches	5	<1	4	5	2-5	7-14	4	<1	7	mv
Sanitation between batches	Washing + disinfection	Washing	Washing + disinfection	Washing	Washing + disinfection	Washing	Washing	Washing	Washing	Washing + disinfection
Antimicrobial used to treat NPD	Neomycin	TS	Penicillin	Penicillin	TS	TS	TS	Enro-floxacin	TS	TS
Proportion of diarrhoeic litters at the visit	30% (7/23)	36% (12/33)	43% (16/37)	47% (8/17)	63% (12/19)	17% (4/24)	33% (9/27)	19% (6/31)	67% (14/21)	50% (9/18)
Median temperature in creep area (°C)	23	32.7	28.3	26	22.4	30.7	28.5	33.6	31.5	31.5
No of litters sampled	7	5	5	7	7	6	7	7	6	3
Time of visit	June and September 2011	October 2011	November 2011	January 2012	February 2012	March 2012	March 2012	April 2012	April 2012	May 2012

1. Farrowing on a weekly basis, 50 or 10 sows alternating every other week
2. Farrowing every two weeks, 44 sows per batch
3. Farrowing every seven or eight weeks, 72 sows per batch

Figure 3. Photo of a healthy piglet with normal faecal consistency (left) and a diarrhoeic piglet with perineal staining (right). (As seen in the left photo, defecation is often prompted when the rectal temperature is measured; photo by Tim Meier and Jenny Larsson)

Upon arrival at the Section of pathology, SLU, one of the 20 control piglets was diarrhoeic and therefore excluded from the study. It is unclear if the animal had fallen ill during the transport (a maximum of three hours) or if it was misclassified as non-diarrhoeic during the clinical examination performed in the herd.

All animals were cross-breeds (Yorkshire × Landrace × Hampshire/Duroc). Clinical data on the diarrhoeic and non-diarrhoeic piglets used in study II-IV is given in Table 3.

Table 3. *Median age, weight, rectal temperature and the proportion female/males among 50 diarrhoeic and 19 non-diarrhoeic piglets from ten herds selected for pathological and microbiological investigations.*

	Diarrhoeic	Non-diarrhoeic
Age [1]	1 day	2 days
Female	66%	63%
Male	34%	37%
Weight	1.6 kg	1.8 kg
Rectal temperature	38.8°C	39.0°C

1. Age was registered as <1 day (born the night before the visit), 1 day old (born the day before the visit), etc.

3.2.2 Analysis of the piglets' passive immune status

Blood was collected prior to euthanasia and serum analysed by serum protein electrophoresis (SPE) that separates serum proteins into six different fractions (albumin, α_1, α_2, β_1, β_2 and γ). Immunoglobulins consitute the major part of the ɣ-fraction (especially IgG) but may also be found at lower levels in the other fractions (mainly in the β-fractions) (Vavricka *et al.*, 2009). In this study we focused only on the ɣ-fraction and hence this has to be taken into account when comparing the results with other studies.

Moreover, the serum concentration of immunoglobulins in newborn piglets is dynamic. The highest concentrations are usually observed at 24 h after birth but a decline can be seen already within a couple of days (Kruse, 1983; Frenyo *et al.*, 1980). To adjust for the slight difference in age between diarrhoeic and non-diarrhoeic piglets, the association between immunoglobulin concentration and diarrhoea was analysed with a linear regression model including age as an explanatory factor.

All serum samples were analysed simultaneously (Nov 2012) to avoid inter-analysis variation, hence the storage time (at -70°C) prior to analysis varied between the samples. However, no substantial effect on the concentration of ɣ-globulins was evident as in two animals, whose sera had been stored the longest, the concentrations were 10g/L higher than the average.

3.2.3 Pathology

Piglets were euthanized by initial anaesthesia with an intramuscular injection of tiletamine and zolazepam, followed by intracardiac injection of pentobarbital sodium. The method was chosen since it is quick and easy to perform. Moreover, intracardiac injection of the barbiturate was preferred over intra-peritoneal injection (which often is used under field conditions) to minimize any effects on the intestinal tissues.

All intestinal tissue samples were put in 10% neutral-buffered formalin within 30 min *post mortem*. This procedure proved to be rapid enough as very few autolytic changes were observed on histopathology. For an overview of samples collected during the necropsies see Table 4.

Table 4. *Overview of samples collected from each piglet at necropsy.*

Type of sample	Sampling site	Number of samples per pig
Intestinal swabs for bacteriology	Distal jejunum, proximal colon, rectum	6
Intestinal contents for bacteriology	Proximal colon, rectum	2
Intestinal tissue samples for histopathology	Duodenum, proximal jejunum, distal jejunum, ileum, cecum, proximal colon, distal colon	7
Intestinal tissue samples frozen in liquid nitrogen	Duodenum, proximal jejunum, distal jejunum, ileum, cecum, proximal colon, distal colon	7
Intestinal contents	Small intestine, large intestine	2-4
Mucosal scrapings for parasitology	Duodenum, proximal, mid and distal jejunum, ileum, proximal colon	6
Additional tissue samples	Stomach, liver, pancreas, spleen, kidney, thymus, lung, heart, abdominal wall, bone marrow	10

To avoid overfixation that may impede subsequent analyses (e.g. immunohistochemistry), tissue samples were transferred to 70% ethanol after 24 h, and cut and embedded in paraffin within 48 h.

As descriptions of pathological changes are subjective, it is important that the investigators are blinded to the health status of the animals. This was however difficult to achieve in the macroscopic examinations, as the presence of diarrhoea in most cases was obvious. Presence of macroscopic changes was

recorded using a standardized protocol. The parameters included are given in Table 5. For consistency, all piglets were examined by the same two persons (the author and Rodrigo Grandon, pathologist at SLU).

Table 5. *Gross changes recorded during necropsy. Unless stated otherwise, changes were graded as mild, moderate, or severe.*

Parameter	Comments on grading
Body condition	Graded as 'poor', 'poor to normal' or 'normal' based on the protrusion of ribs and backbone
Stomach contents	Presence of stomach content was recorded as absent, sparse, or normal amounts of clotted milk
Changes in mesenteric lymph nodes	Enlargement of mesenteric lymph nodes was difficult to assess due to the young age of the animals. However, no animal had clearly enlarged or hyperaemic mesenteric lymph nodes
Dilation of the intestine	Grading of increased diameter of the intestinal lumen. Assessed individually for the small and large intestine
Hyperaemia of the intestine	Grading of presence of diffuse redness and congested blood vessels. Assessed individually for the small and large intestine
Intestinal contents	The content of the distal colon/rectum was assessed using the same criteria as described for clinical categorisation of animals as diarrhoeic or non-diarrhoeic
	Since the consistency of the contents in the small intestine normally is fluid, categorising the contents as abnormal or normal is difficult in the absence of blood or fibronecrotic debris. Neither was present in any animal
Mesocolonic oedema	Graded based on the separation of colonic loops as described by (Yaeger *et al.*, 2007)
Macroscopic lesions in other organs	Lesions outside of the GI tract were described briefly

Histopathological investigations included descriptions of villus:crypt (V/C) ratio in the small intestine, epithelial morphology, and cellularity in the lamina propria. The V/C ratio was estimated with aid from the scale bar in the ocular. As this rather crude measure was used, only cases with clear alterations of the villous morphology were recorded as atrophy. The reported V/C ratio in

healthy newborn piglets is approximately 6:1 or more in the jejunum, but can be lower in the duodenum and ileum (Jonach, 2014; Gancarčíková, 2012; Hooper & Haelterman, 1969). Based on these considerations, villous atrophy was registered if the V/C ratios were ≤ 3:1 in any of the small intestinal segments. V/C ratios were not possible to determine in all segments (proximal small intestine in 12 piglets and distal small intestine in 4 animals), as reliable assessments require that tissue sections are well oriented and cut trans-sectionally. To achieve this, intestinal samples can be nailed onto pieces of corkboard before fixation. This was tried on samples from five piglets (from herd A) but since the results were not substantially improved, this approach was abandoned. If a more sensitive method had been used for the determination of V/C ratio, such as quantification by morphometric measurements, a proportion of diarrhoeic piglets had probably been regarded to have mild, small intestinal atrophy. However, morphometry is very time-consuming and since investigation of villus atrophy was not the main purpose, this investigation was excluded.

Special attention was given to the potential presence of microbes within or attaching to the intestinal epithelium, e.g. intracellular protozoa or bacterial colonisation. As intracellular bacteria may be difficult to detect by light microscopy, immunohistochemistry was performed for *Chlamydia* spp. that is commonly detected in Swedish pig herds (Englund *et al.*, 2012).

Mucosal scrapings from the small and large intestine, as well as samples of intestinal contents, were collected for the purpose of parasitological investigations. However, due the low age of the piglets and the prepatent period for *Cystoisospora suis* and *Cryptosporidium* spp. (5 and 2-9 days, respectively) it was considered more likely to detect developmental stages of protozoa on histological sections (Lindsay, 2012) and as no protozoa were observed in the intestinal mucosa in any animal, further parasitological investigations were not considered motivated.

3.2.4 Bacteriology

Sampling and culturing procedures

Initial bacteriological investigations focused on bacterial agents previously associated with NPD, i.e. *E. coli*, *C. perfringens,* and *C. difficile*. The sites chosen for sampling were based on previous descriptions on the typical site of infection. In addition, samples were collected from the rectum, to mimic the field situation where samples usually are collected from live animals.

To avoid environmental contamination and *post mortem* overgrowth, bacterial samples were collected immediately after death by incising the

intestinal wall with a sterile scalpel blade and rubbing a cotton swab against the mucosa. All samples were cultured at the NVI immediately after the completion of the necropsies. A delay between sampling and culture can have adverse effects on bacterial viability, especially for sensitive organisms such as *C. difficile*. Thus, bacterial swabs were kept in Amies transport medium with charcoal until cultured. The maximum time-interval between sampling and culture was approximately 8 h. The effect of this delay on the viability of *C. difficile* should be minor as storage in commercial anaerobic transport medium has been shown to preserve viable *C. difficile* in faecal samples for 72 h (Weese *et al.*, 2000).

Escherichia coli

The investigation of *E. coli* was based on the routine method used at the NVI for detection of ETEC in clinical samples. Two representative *E. coli* colonies per sample (i.e. four per pig) were subcultured and subsequently analysed for the presence of selected virulence genes. Studies performed by Söderlind and Möllby (1979) show that at least one out of three colonies per pig (from faecal cultures) will be enterotoxin-positive in samples from diarrhoeic piglets harbouring ETEC. Although that study was performed on a limited number of piglets (n=20) it clearly demonstrates that ETEC predominates the intestinal *E. coli* flora in piglets suffering from ETEC diarrhoea.

Detection of virulence genes were performed by PCR systems previously validated at the NVI. The investigations focused on the presence of virulence genes associated with ETEC, as well as other types of diarrhoeagenic *E. coli* that have been associated with NPD.

Attempts were made to set up a PCR for paa (porcine attaching effacing associated protein). However, our preliminary results indicated that previously described PCR-systems gave ambiguous results and due to the limited time frame, the presence of paa was not investigated further.

Based on results from the PCR-screening, interesting isolates were selected for further analysis of additional virulence genes associated with pathogenic *E. coli* by a microarray, and for O-typing by seroagglutination. The test panel used for O-typing was developed at the NVI and cover the O-groups that previously have been associated with piglet diarrhoea in Sweden. Since a recent study of NNPDS in Denmark indicated that O111-isolates could be associated with the diarrhoea, this O-group was screened for by PCR (Hermann-Bank, 2014).

Clostridium perfringens *type A and C (CpA, CpC), and* Clostridium difficile

As the role of CpA and *C. difficile* in NPD is unclear, the sampling were designed to allow for possible extended investigations of these agents. Thus, in addition to direct culturing, spore selection and enrichment was performed to increase the isolation rate. Further, attempts were made to subculture up to five colonies from the direct cultures of one randomly selected diarrhoeic and one non-diarrhoeic animal per herd.

Step one in the investigations of clostridia was to examine the potential presence of CpC and thus toxinotyping was performed on isolates from the distal jejunum, being the typical site of CpC infection. As it is recommended to investigate more than one isolate per animal (Buogo *et al.*, 1995), toxinotyping was performed on all isolates from positive jejunal samples. From 20 animals only one isolate was available, and even if this decreases the sensitivity, presence of CpC was considered highly unlikely combining the negative PCR-results for the beta-toxin gene and the lack of typical lesions in the intestine. In addition to the PCR screening for major toxin genes, isolates of *C. perfringens* were also examined for the presence of enterotoxin and beta2-toxin genes, as these toxins have been suggested to be involved in the pathogenesis of CpA-enteritis.

Detection of toxins in intestinal contents has been suggested as diagnostic for NPD caused by both CpA (beta2-toxin) and *C. difficile* (toxin A and B) but the value of these tests is questionable since these toxins also are found in healthy pigs (Farzan, 2013; Yaeger *et al.*, 2007). Isolation of CpA and *C. difficile* could not be related to diarrhoea or intestinal lesions in the present study. Thus, examination of toxins in intestinal contents was regarded as unmotivated.

3.3 The intestinal eukaryotic virome in healthy and diarrhoeic piglets (III)

A potential viral contribution to the investigated diarrhoea was examined by viral metagenomics. In contrast to other diagnostic methods that are based on the detection of viral nucleic acid or viral antigens of a certain virus (or virus group), viral metagenomics does not require targeting of a specific pathogen. In that sense, viral metagenomics is often described as an unbiased screening of the viruses present in a sample (Mokili *et al.*, 2012; Delwart, 2007). It thus allows the detection of viruses not previously associated with the condition under investigation, or variants of viruses that may be overlooked using specific sequence-based detection methods.

The herds and animals used in this study were the same as in paper II. The sample material consisted of transverse sections from the distal jejunum collected at the necropsies and snap-frozen in liquid nitrogen. This sample site was chosen as most enteric viral infections primarily affect the distal small intestine.

The steps in viral metagenomics studies can be divided into three main steps: sample preparation, high-throughput sequencing, and bioinformatics analyses. However, since viral metagenomics is a relatively new method, the protocols used are continuously being developed and improved. Some important considerations of the different steps will thus be briefly mentioned below.

In most samples, viral nucleic acids only constitute a fraction of the total DNA and RNA. The purpose of the sample preparation is therefore to preserve the viral component while other contaminating nucleic acids (primarily from the host and bacteria) are depleted (Delwart, 2007). As viral nucleic acids are protected by the viral capsid, a frequently used method is to treat samples with nucleases to degrade non-capsid protected DNA and RNA (Allander *et al.*, 2001). However, samples will still contain residual non-viral nucleic acids, especially rRNA, due to its abundance and stability (Blomström, 2011). This must hence be considered in subsequent analyses. An overview of the sample preparation methods used is depicted in Figure 4.

Due to the low concentration of viral nucleic acids (nanograms or even pikograms), amplification is usually necessary prior to sequencing. This pre-amplification should ideally be completely sequence-independent to preserve the genetic composition in the sample. Unfortunately this is very difficult to achieve and hence, different amplification methods will induce different types of bias. Sequence-independent single-primer amplification (SISPA) is one of the most widely used approaches for pre-sequencing template amplification in virology (Reyes & Kim, 1991). In the present study, we used a modified SISPA protocol that has been shown to decrease the bias introduced, by using longer random primers (12 instead of 6 nucleotides) and two different amplification-tags (Rosseel *et al.*, 2013). However, some bias will unavoidably still be present due to annealing bias combined with the non-linear amplification and thus, this prevents the results from being interpreted quantitatively (Karlsson *et al.*, 2013; Rosseel *et al.*, 2013).

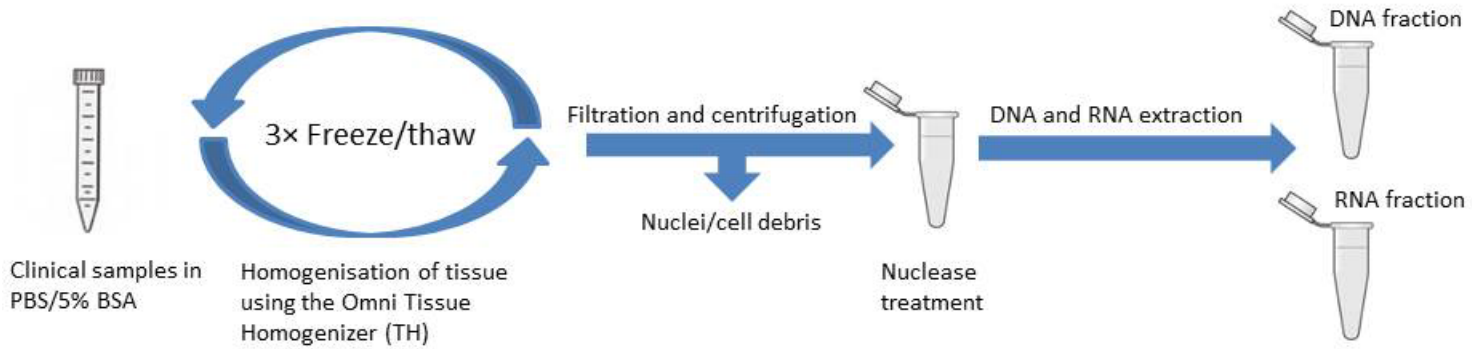

Figure 4. Overview of sample preparation before sequence-independent amplification. Transverse sections of the jejunum was put in 1 ml of PBS 5% BSA solution and subjected to three consecutive cycles of freeze-thawing and homogenisation. After homogenisation, samples were centrifuged and the supernatants were collected and filtered through a 0.45 µm sterile filter. The filtered supernatant was subsequently treated with a nuclease cocktail containing both DNase and RNase. Each sample was thereafter split into two fractions, one for DNA and one for RNA extraction.

Sequencing was performed using the IonTorrent-platform at the National Genomics Infrastructure (NGI) in Uppsala to ensure that library preparation and sequencing was performed in a standardized way. Prior to taxonomic classification, the datasets were quality-assessed using PrinSeq (Schmieder & Edwards, 2011) and host-sequences removed by mapping the datasets towards the pig reference genome using Bowtie2 (Langmead & Salzberg, 2012). Taxonomic classification on nucleotide level was performed by Kraken (Wood & Salzberg, 2014) using an in-house database covering all sequences from the viral (VRL) and phage (PHG) divisions of GenBank (NCBI; available on the 2015-07-03, release 208), as well as the bacterial and archaeal sequences from RefSeq. In addition, reads were classified on protein-level by Diamond (Buchfink *et al.*, 2015) using the NCBI non-redundant protein sequences database and by scanning the predicted protein sequences with a database of hidden Markov model profiles, vFam, specifically built using viral sequences (Skewes-Cox *et al.*, 2014). As the primary focus of the study was to find a potential cause of the diarrhoea, we chose to focus on mammalian viruses.

Pooling of samples prior to sequencing had to be performed to limit the costs. We chose to pool samples from diarrhoeic animals for each herd whereas the samples from healthy piglets were analysed separately due to the relatively low number of healthy pigs. Thus, detected viral sequences could not be related to individual piglets and since the data from diarrhoeic and healthy samples were not directly comparable they are presented descriptively.

The sensitivity of viral detection by viral metagenomics has previously been reported to be comparable to PCR (Li *et al.*, 2015; Greninger *et al.*, 2010) and it is hence unlikely that a previously described virus responsible for causing diarrhoea should be overlooked. However, more studies are needed to determine the levels of detection in different types of complex clinical samples.

3.4 Neonatal piglet diarrhoea associated with enteroadherent Enterococcus hirae (IV)

The selection of animals was based on the observation of enteroadherent coccoid bacteria in the small intestine by light microscopy (paper II). This finding was made in piglets from six herds, denoted as A-F, which corresponds to herd B, C, F, H, I and J in paper II. Non-diarrhoeic animals from the same herds were included for comparison. The aim of the study was to identify the colonising agent and to characterise the associated intestinal pathology in detail.

16S rRNA analysis of colonised tissue from one animal identified the enteroadherent bacterium as *E. hirae*. As indicated by light microscopy, only

one type of bacterium (as judged by morphology) was observed adhering to the epithelium in large numbers, and thus it was possible to obtain a "clean" sequence by amplifying the 16S rRNA gene from intestinal tissue by PCR, followed by traditional Sanger sequencing.

As a next step we wanted to analyse the presence of *E. hirae* in all colonised animals. However, since enterococci normally can be found in the intestinal flora, the mere detection of *E. hirae* in intestinal contents would not be sufficient to indicate that this species was the colonising agent. We therefore decided to use two different approaches: identification of the colonising agent by fluorescence *in situ* hybridization (FISH) and investigation of the composition of the enterococcal flora by culturing. The latter was based on the hypothesis that if *E. hirae* was the agent responsible for this massive colonisation it would also be the predominating enterococcal species on culture.

As species in the *E. faecium* group are very closely related, it was difficult to design a FISH probe targeting 16S or 23S rRNA that were specific for *E. hirae* (Vancanneyt *et al.*, 2001). Instead, a 16S rRNA probe targeting the genus *Enterococcus* (Enc221) previously used to investigate intestinal colonisation in kittens, was employed (Nicklas *et al.*, 2010; Wellinghausen *et al.*, 2007). The Cy3-labelled Enc221 probe yielded a strong signal when tested on a positive control consisting of lung tissue prepared with a reference strain of *E. hirae*. In addition, an eubacterial probe (Eub338) labelled with 6-FAM was used simultaneously on all samples as a positive hybridization control. Some unspecific hybridization with rod-shaped bacteria in the lumen was observed with the Enc221 probe in a few animals. However, these bacteria were easily distinguished from the coccoid bacteria outlining the villi.

To investigate the enterococcal flora, bacterial swabs were collected from the spiral colon that had been stored at -70°C. Slanetz and Bartely agar was used for selective culturing displaying enterococci as pink colonies. However, *Streptococcus (S.) gallolyticus* can also exhibit pink colonies on this medium (Devriese *et al.*, 1998), which explains why a large number of colonies (predominantly from healthy piglets) were identified as *S. gallolyticus*. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was used for species identification as the method is cheap and fast, thereby allowing for examination of a larger number of colonies per sample. Species identification of *E. hirae* by MALDI-TOF MS was confirmed by PCR.

Transmission electron microscopy (TEM) was performed to examine the interaction between the bacteria and the microvillous border. Although the samples were not optimally collected for TEM, the samples had been similarly treated and were hence comparable.

Apoptosis of enterocytes at the tips of villi is a physiological process but seldom evident by routine microscopic investigation of H&E-stained small intestinal sections from healthy animals. However, in segments with enteroadherent colonisation, cells with a rounded shape and eosinophilic cytoplasm were observed. As a number of pathogenic bacteria are able to induce apoptosis (Grassme *et al.*, 2001) we wanted to further validate these observations. Activated caspase-3 has been demonstrated to be a good marker for apoptosis (Duan *et al.*, 2003) and immunohistochemistry for activated caspase-3 confirmed our findings.

For antimicrobial susceptibility testing of *E. hirae* (one *E. hirae* isolate per herd) we chose to use the VetMIC panels GN-mo and GP-mo instead of the enterococcal panel (VetMIC E-cocci) as the former two together cover a larger number of clinically relevant antimicrobials.

4 Results and discussion

In 2011 when this work began, single European reports had described the occurrence of a seemingly new form of NPD, present despite the continuous use of previously effective preventive measures (Gin, 2010; Melin, 2010; Svensmark, 2009; Goillandeau, 2006). Previous investigations in Sweden was limited to one microbiological investigation on diarrhoeic piglets (n=17) from six herds (Melin, 2010). However, the magnitude of the problem among Swedish piglet-producing herds was unknown and studies characterising microbiological findings in relation to pathological changes and passive immunity were lacking.

4.1 Occurrence and characteristics of neonatal porcine diarrhoea in Swedish piglet-producing herds

The high herd-level prevalence described in paper I (79.6%) suggests that NPD is common among Swedish piglet-producing herds. However, although a relatively good response rate of 58% was achieved (Cummings *et al.*, 2001; Asch *et al.*, 1997), selection bias must be considered since farmers from herds with NPD might have been more motivated to complete the questionnaire. Nonetheless, even if all of the 72 non-responders were herds not experiencing NPD, the prevalence would still be considerable (45.9%).

Notably, many herds had a recurrent problem with NPD despite maternal vaccination against ETEC and, in some herds, also *C. perfringens* (39 of the 42 herds with recurrent problems). Eight of these herds estimated that the average proportion of piglets affected per batch was >25%, which indicates a substantial problem. The use of manure backfeeding [5] in six herds further underlines the dissatisfactory effect of commercial vaccines.

5. Feeding sows and gilts manure from diarrhoeic piglets prior to farrowing with the intention of preventing disease

The most frequently used antimicrobial treatment of NPD was trimethoprim/sulphonamide (sulfadiazine or sulfadoxine) which is the recommended treatment for colibacillosis (Medical Products Agency, 2012). However, 43% of the herds used other antimicrobials such as amoxicillin, penicillin, fluoroquinolones, neomycin or tylosin.

Taken together, these results suggest that NPD in Swedish herds may not always respond to prophylaxis and treatment targeting ETEC or *C. perfringens*. The results also conform to the bacteriological investigations in paper II, demonstrating the presence of NPD unrelated to ETEC and *C. perfringens*.

Further, herds with >200 sows in production had a higher risk (OR=4) of having NPD, compared with herds <200 SIP ($P<0.01$). The underlying biological explanation for this difference is unknown, and detailed studies on management practices in larger vs smaller herds are needed to untangle these relationships. Management routines indicated to be important in study I included washing routines in the farrowing unit ($P=0.05$) and efforts made to save week-born piglets ($P=0.02$). Interestingly, less rigorous washing routines were associated with a lower risk for diarrhoea. One explanation for this may be that a problem with NPD could be an incentive to increase the hygienic measures. The estimated ORs for the different efforts made to save weak-born piglets were uncertain and the association with NPD hence requires further investigation.

4.2 Pathological and microbiological investigations

An overview of pathological and microbiological findings in diarrhoeic piglets (paper II-IV) is presented in Table 5.

Approximately 40% of the diarrhoeic piglets (20/49, data missing for one piglet) had a poor body condition compared to 5% of the non-diarrhoeic piglets (1/19). As described in paper II, the intestinal pathological gross lesions found in diarrhoeic piglets were unspecific and consistent with the changes commonly seen in neonatal diarrhoea irrespective of the cause (Brown, 2007b). In agreement with previous studies, mesocolonic oedema was equally present in diarrhoeic and healthy animals (Farzan, 2013; Yaeger *et al.*, 2007).

On histopathology, the only consistent finding was small intestinal colonisation by gram-positive, enteroadherent cocci, subsequently identified as *E. hirae*. This colonisation was present in 18 of 30 diarrhoeic piglets (60%) in six herds. Further, villous epithelial damage and villous atrophy in the small intestine were only observed in diarrhoeic piglets and mainly in animals colonised by *E. hirae* (10/13 piglets with small intestinal epithelial damage and 4/5 piglets with villous atrophy were simultaneously colonised).

In the large intestine, mild miscellaneous epithelial lesions were only noted in a few animals. Further, no association was seen between large intestinal epithelial damage and the presence of mesocolonic oedema (P=0.46, Fisher's exact test). Thus, similar to most intestinal infections in newborn piglets, the small intestine seemed to be the primary site involved (Table 1).

Investigations of previously well-known causes of NPD (paper II) demonstrated that ETEC was rare (2/50 diarrhoeic piglets from herd A and E, respectively) and *C. perfringens* type C absent. This is consistent with the absence of these agents in the study by Melin *et al.* 2010 and data from the Swedish board of agriculture, reporting that *C. perfringens* type C is confined to the south-western parts of Sweden (Swedish Board of Agriculture, 2005-2014). The low presence of ETEC may thus suggest an adequate effect of the routinely performed vaccination against ETEC.

Moreover, no difference in the serum ɣ-globulin concentrations were detected between diarrhoeic and healthy piglets (21.8±7.7 g/L versus 19.8±9.1 g/L, P=0.74) suggesting that the colostrum intake was sufficient (Svendsen *et al.*, 1972). Further, a large proportion of the diarrhoeic piglets originated from first parity sows (23/45 diarrhoeic piglets, compared to 3/17 of the healthy piglets; information missing for piglets from herd A). These results could indicate that the quality of colostrum may be more important. Interestingly, serum ɣ-globulin concentrations also seemed adequate in the two ETEC-positive pigs from herd A and E (30 g/L and 33 g/L, respectively). However, in these herds the temperature in the creep area was low (median temperature of 23°C, and 22.4°C respectively) which potentially could contribute to an increased susceptibility to disease (Steel & Torres-Medina, 1984; Sarmiento, 1983). Otherwise, no obvious shortcomings were identified in the farrowing environment (paper II).

It would have been interesting to investigate the presence of NPD in relation to maternal factors influencing early lactation. It is e.g. well-known that the presence of post-partum dysgalactia syndrome (PPDS) in sows results in an increased risk of pre-weaning diarrhoea (Wittum *et al.*, 1995; Svensmark *et al.*, 1988; Halgaard, 1980; Svendsen *et al.*, 1975). However, due to the practice of cross-fostering piglets after colostral intake, it was not possible to link individual piglets to their original sows.

C. perfringens type A (CpA) and *C. difficile* have been put forward as important causes of NPD during the last decades (Songer & Uzal, 2005; Yaeger *et al.*, 2002). CpA has previously been suggested as a cause of NPD in Sweden (Holmgren, 2004), but *C. difficile* has previously not been investigated in relation to NPD. In the present study (paper II), CpA and *C. difficile* was

commonly found both in diarrhoeic and healthy piglets, however moderate to profuse growth on direct culture was not related to diarrhoea.

In agreement with previous studies, the gene for beta2-toxin was highly prevalent among CpA isolates (151 positive isolates out of 152 tested) whereas the gene for enterotoxin was absent, implying that these genes are of little value for distinguishing commensal *C. perfringens* type A from strains allegedly associated with disease (Farzan, 2013; van Damme-Jongsten *et al.*, 1990). Furthermore, moderate to profuse growth of *C. perfringens* was not associated with small intestinal epithelial lesions in diarrhoeic piglets (54% of the diarrhoeic piglets with small intestinal lesions had moderate to profuse growth of *C. perfringens* compared to 83% of the piglets with no lesions, P=0.06, Fisher's exact test).

The isolation of *C. difficile* from all investigated piglets corresponds to previously reported, high detection rates of *C. difficile* in neonatal piglets (Hopman *et al.*, 2011; Weese *et al.*, 2010; Yaeger *et al.*, 2007). Moreover, lesions described as characteristic of *C. difficile*-induced NPD, including mesocolonic oedema and microscopic typhlocolitis (Songer & Uzal, 2005), were not related to diarrhoea and typical volcano lesions were not observed.

Thus, in the present material, a pathogenic role of CpA or *C. difficile* seems unlikely. However, the diagnosis of NPD associated with CpA and *C. difficile* is equivocal, and further work is needed to elucidate the pathogenic potential of these organisms in enteric disease of newborn piglets.

The presence of non-ETEC diarrhoeagenic *E. coli* not included in the vaccines has previously been discussed as a cause of NPD (Eriksson, 2012). The results from study II do however not support this. Isolates positive for STb:EAST1:AIDA-I were only occasionally found (6/276 isolates from two diarrhoeic and one healthy piglet) and neither did porcine EPEC seem to be involved. Moreover, extended investigations of EAST1-positive isolates did not demonstrate virulence gene profiles previously associated with enteropathogenicity. The results conform to previous studies demonstrating that pathotypes other than ETEC are uncommon in newborn pigs (Alexander, 1994). Thus, in agreement with the results from Melin *et al*. 2010, *E. coli* does not seem to play a major part in the investigated cases of diarrhoea.

Enteric virus infections in newborn piglets are generally associated with lysis of infected enterocytes, resulting in small intestinal villous atrophy and malabsorptive diarrhoea (Pospischil *et al.*, 1981; Moon, 1978). Thus, to cause diarrhoea, the virus must multiply and cause cell lysis in amounts sufficient to impair the absorption. Hence, viral diarrhoea is seldom seen in piglets younger than 24 h of age (Martineau, 1995). The very early onset of diarrhoea in herd A, D, E, G and J (the majority of the diarrhoeic piglets were ≤ 1 day old) may

thus speak against a primary viral cause. Moreover, a clearly altered V/C-ratio was only observed in five piglets (herd B, F, and H). Accordingly, few mammalian viruses were found by viral metagenomics analyses of the distal jejunum from diarrhoeic piglets (paper III). Among the viruses found in diarrhoeic pigs (Table 5), only rotavirus is considered an established enteropathogen in piglets (Thomson, 2012; Will *et al.*, 1994; Morin *et al.*, 1990). Rotavirus was detected in the pooled diarrhoeic sample from herd A (*Rotavirus* A) and herd B (*Rotavirus A* and *C*) and also in the healthy piglets from herd B. Since samples from the diarrhoeic animals were pooled, findings could not be related to individual animals but it is possible that rotavirus contributed to the small intestinal atrophy observed in two diarrhoeic piglets from herd B. Otherwise, the most common finding was sequences classified as belonging to the genus *Kobuvirus* with specific matches on nucleotide level to *Aichivirus C*. However, in agreement with previous studies (Di Bartolo *et al.*, 2015; Verma *et al.*, 2013; An *et al.*, 2011), the presence seemed unassociated with diarrhoea (detected in 10/19 healthy piglets and in 4/10 pooled diarrhoeic). Further, a larger number of different virus families were detected in the healthy piglets (≥ 2 were found in 8/19 healthy piglets and in 2/10 pooled diarrhoeic samples). This could be related to age, as healthy piglets were slightly older. Alternatively, the diarrhoea could have led to a decreased complexity of the intestinal virome. However, only one previous study has investigated the neonatal virome in healthy mammals (Lim *et al.*, 2015) and further studies including individual analyses of diarrhoeic piglets are needed to clarify this.

The current work (paper II-III) thus demonstrate the occurrence of neonatal porcine diarrhoea where well-known enteropathogens found in the Swedish pig population were of minor importance (*E. coli* and rotavirus), absent (*C. perfringens* type C) or apparently unrelated to the diarrhoea (*C. perfringens* type A and *C. difficile*). Neither were protozoa such as *Cystoisospora suis* observed on histopathology. Overall, the only consistent finding was an association between enteroadherent *E. hirae* and NPD, since intestinal colonisation associated with mucosal lesions only was observed in diarrhoeic animals (paper II and IV).

Table 5. *Overview of major pathological and microbiological findings in diarrhoeic piglets from ten herds (A-J) affected by NPD despite maternal vaccination against ETEC. Five diarrhoeic animals were examined per herd. (Figures within brackets indicate the number of pigs per finding).*

Herd	Necropsy findings	Histopathology	Bacteriology[1]	Virology[2]
A	Moderate mesocolonic oedema and hydrothorax (1) Mild dilation of small and large intestine (1) Only mild hyperaemia of the small intestine (1)	Unspecific, apart from superficial epithelial damage in the small and large intestine in one piglet.	*E. coli:* STa:F5:F41 (1), STb:EAST1:AIDA-I (1) *C. perfringens* type A (5) *C. difficle* (2)	*Rotavirus*
B	Mild mesocolonic oedema (2) Mild to moderate dilation of small and/or large intestine (4) with mild hyperaemia of the small intestine (1/4)	Small intestinal colonisation by enteroadherent enterococci accompanied by superficial epithelial damage (3), villous atrophy (2/3), and large intestinal superficial epithelial damage (1/3).	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (3)	*Kobuvirus* *Rotavirus*
C	Moderate mesocolonic oedema (2) Mild to moderate dilation of small and/or large intestine (5) with moderate hyperaemia of the small intestine (2/5)	Small intestinal colonisation by enteroadherent enterococci (4), accompanied by superficial epithelial damage in the small intestine (1/4). Superficial large intestinal lesions (2/4). Extensive infiltration of neutrophils in the ileum despite intact epithelium with no bacterial colonisation (1).	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (3) *C. difficle* (3)	No mammalian viruses detected

Herd	Necropsy findings	Histopathology	Bacteriology	Virology
D	Mild mesocolonic oedema (4) Mild dilation of small and large intestine (2) Only mild hyperaemia of the small intestine (1)	Unspecific, apart from one piglet with superficial epithelial damage in the large intestine	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (5) *C. difficle* (2)	No mammalian viruses detected
E	Mild mesocolonic oedema (1) Mild to moderate dilation of small and large intestine (3) with mild hyperaemia of the small intestine (1/3) Only mild hyperaemia of the small intestine (1)	Colonisation of distal small intestine by rod-shaped bacteria and extensive infiltration of neutrophils in the distal small intestine (1) Extensive infiltration of neutrophils in the ileum despite intact epithelium with no bacterial colonisation (1)	*E. coli:* STa:F5:F41 (1) *C. perfringens* type A (5) *C. difficle* (1)	*Iotatorque-virus* *Unclassified circoviridae*
F	Mild mesocolonic oedema (2) Mild to moderate dilation of small and large intestine (5) with mild to moderate hyperaemia of the small intestine (4/5)	Small intestinal colonisation by enteroadherent enterococci (4), accompanied by superficial epithelial damage (1/4).	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (1) *C. difficle* (1)	*Kobuvirus*
G	Mild or severe mesocolonic oedema (1 piglet respectively) Mild dilation of small and/or large intestine (4) with mild hyperaemia of the small intestine (1/4) Only mild hyperaemia of the small intestine (1)	Unspecific, apart from extensive infiltration of neutrophils in the ileum despite intact epithelium with no bacterial colonisation (2)	*E. coli:* STb:EAST1:AIDA-I (1) *C. perfringens* type A (5) *C. difficle* (3)	*Kobuvirus*

Herd	Necropsy findings	Histopathology	Bacteriology	Virology
H	Mild mesocolonic oedema (1) Mild dilation of small and/or large intestine (3) with mild hyperaemia of the small intestine (1/3) Only mild hyperaemia of the small intestine (1)	Small intestinal colonisation by enteroadherent enterococci (4), accompanied by superficial epithelial damage (3/4) and villous atrophy (1/4). Superficial epithelial damage, villous atrophy, and extensive infiltration of neutrophils in the distal small intestine without colonisation (1).	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (3) *C. difficile* (3)	No mammalian viruses detected
I	Mild to moderate mesocolonic oedema (4) Mild dilation of small and/or large intestine (3) with mild hyperaemia of the small intestine (2/3)	Small intestinal colonisation by enteroadherent enterococci accompanied by superficial epithelial damage (1) Extensive infiltration of neutrophils in the ileum despite intact epithelium with no bacterial colonisation (1) Superficial epithelial damage in the large intestine (2).	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (4) *C. difficile* (1)	*Kobuvirus*
J	Mild mesocolonic oedema (3) Mild to moderate dilation of small and/or large intestine (4) with moderate hyperaemia of the small intestine (1/4) Only mild hyperaemia of the small intestine (1)	Small intestinal colonisation by enteroadherent cocci accompanied by superficial epithelial damage (1) or severe damage and extensive infiltration of neutrophils (1) Extensive infiltration of neutrophils in the ileum despite intact epithelium (2)	*E. coli:* No diarrhoeagenic virotypes detected *C. perfringens* type A (4) *C. difficile* (2)	No mammalian viruses detected

1. Results for *E. coli* correspond to detection of virotypes previously associated with diarrhoea in newborn piglets. Results for *C. perfringens* or *C. difficile* correspond to diarrhoeic piglets with moderate to profuse growth on direct culture from either of the sample sites (jejunum/rectum and colon/rectum, respectively). All diarrhoeic piglets from herd B were negative for *C. difficile* on direct culture.

2. Virus genera detected on nucleotide level and confirmed on protein level

4.2.1 Enteroadherent Enterococcus hirae

By FISH analysis, the enteroadherent Gram-positive cocci were identified as *Enterococcus* spp. in all 18 colonised animals (paper IV). Moreover, selective culture of intestinal samples for enterococci demonstrated a higher proportion of *E. hirae* in diarrhoeic piglets than in the controls (P=0.01). The species identification by MALDI-TOF mass spectrometry was confirmed by PCR and the identification of *E. hirae* also agreed with the 16S rRNA gene analysis of colonised intestinal tissue. Taken together these results suggested *E. hirae* as the colonising agent in the investigated animals. This finding was rather unexpected as *E. hirae* is not a previously well-established cause of NPD. Prior to the present study, enteroadherent enterococci associated with NPD had only been described in single case reports. In these cases, the colonising agent was identified as *E. durans* (Gin, 2010; Cheon & Chae, 1996; Drolet R., 1990; Johnson, 1984). More recently, a number of *E. durans* isolates from pigs have further been re-classified to the species *E. villorum* (De Graef *et al.*, 2003; Vancanneyt *et al.*, 2001) and it is hence possible that NPD can be associated with any of the closely related species *E. durans/hirae/villorum.* However, these species are difficult to distinguish biochemically and thus, an association between *E. durans* and NPD remains to be confirmed by genotypic methods.

The pathogenesis of diarrhoea associated with enteroadherent enterococci is unknown. In the 1980s, the condition was experimentally reproduced in gnotobiotic piglets (Johnson, 1984; Tzipori *et al.*, 1984). No enterotoxin production could be demonstrated and a malabsorptive mechanism was suggested. Although epithelial lesions in the small intestine has been previously reported in association with enteroadherent enterococci (Nicklas *et al.*, 2010; Johnson, 1984), further investigations on the ability of *E. hirae* to induce cellular damage have, to the best of the author's knowledge, not been performed. The results from study IV suggest that apoptosis could be involved in the increased loss of epithelial cells. However, apoptosis of jejunal cells may also be related to rotavirus infection (Boshuizen *et al.*, 2003) and one of the animals displaying apoptotic enterocytes confirmed by immunolabelling of activated caspase-3 was indeed from herd B. However, similar epithelial lesions were also present in diarrhoeic animals from other herds (paper III). Thus, further studies on the association of *E. hirae* and apoptosis are warranted, preferably by comparing the rate of apoptosis in infected and non-infected animals in an experimental setting, to definitely rule out the presence of other potential pathogens.

The investigation by TEM demonstrated hair-like filaments radiating from the surface of the bacteria towards the microvillus brush border. These protruding filaments, consistent with fimbriae, have been proposed to mediate

the epithelial adhesion (Nicklas *et al.*, 2010; Rogers *et al.*, 1992; Collins *et al.*, 1988; Johnson, 1984; Tzipori *et al.*, 1984). Interestingly, *E. faecium* and *E. faecalis* that are considered important nosocomial pathogens in human medicine, display a number of virulence properties associated with the ability to adhere and colonize different surfaces (Arias & Murray, 2012). However, the presence of virulence genes similar to those described for *E. faecalis* and *E. faecium* have hitherto not been investigated in enteroadherent *E. hirae* isolated from neonatal animals with diarrhoea.

Antimicrobial susceptibility testing has previously not been performed for *E. hirae* associated with NPD. Decreased susceptibility to ciprofloxacin was indicated for *E. hirae* from herd H (designated herd D in paper III) which notably was the only herd routinely using fluoroquinolones for treatment of piglet diarrhoea. None of the other isolates showed decreased susceptibility to the antimicrobials tested.

A similar diarrhoeic condition in newborn piglets has recently been investigated in four Danish herds (Kongsted *et al.*, 2013). Interestingly, enteroadherent enterococci were demonstrated in 27% of the diarrhoeic piglets (n=51) compared with 2% of the healthy controls (n=50) (Jonach *et al.*, 2014). The species was not identified but subsequent characterisation of the intestinal microbiota in the same piglets showed an abundance of the genus *Enterococcus,* and *E. hirae* in particular (Hermann-Bank *et al.*, 2015). Similar to the present work, colonisation was found to be associated with small intestinal mucosal lesions including villous atrophy and epithelial damage (Jonach *et al.*, 2014). However, in these studies, enterococcal colonisation was observed simultaneously with adherent non-ETEC *E. coli* and it was suggested that the lesions may be primarily associated with the latter. This was however not supported in the present study (paper II).

Further, an abstract by Gin *et al*. (2010) describe NPD in eight French high performance herds that was not related to classical enteropathogens. Instead, *E. durans* was reported as the aetiology in three of the herds (in one herd together with *C. difficile*) and suggested as a 'new' pathogen associated with NPD.

The similarities of the present work with studies on piglet diarrhoea in Denmark and France are intriguing and may reflect a shift in the relative importance of the enteropathogens involved in NPD (Hermann-Bank *et al.*, 2015; Jonach *et al.*, 2014; Kongsted *et al.*, 2013; Gin, 2010). However, the ten herds investigated in the present study may not be representative for all Swedish herds experiencing problems with NPD. Thus, extended studies on the prevalence of enteropathogens related to NPD including enteroadherent enterococci are warranted.

4.2.2 Other causes?

In four herds (A, D, E and G) no definite aetiological cause was determined, although ETEC was detected in two single cases. Incomplete diagnosis of NPD is however not uncommon (Lippke *et al.*, 2011; Yaeger *et al.*, 2002; Wieler *et al.*, 2001; Johnson *et al.*, 1992) and was noted in Scandinavian studies already in the 1970s (Söderlind, 1978; Svendsen *et al.*, 1975).

Possible explanations for the failure to detect an infectious cause could be that pathogenic organisms were present but not detected, or that the diarrhoea was non-infectious. Suggested non-infectious causes of diarrhoea in suckling newborn piglets includes overfeeding, starvation, antibiotic-associated diarrhoea (Goillandeau, 2006), and speculations on the presence of mycotoxins in sow feed (see section 1.4.4). None of these seem plausible considering the large number of animals affected, the presence of milk filled stomachs in all diarrhoeic piglets at necropsy, and the absence of piglet antimicrobial treatments and overt signs of disease in the sows. Moreover, NNPD in Denmark was demonstrated to be infectious by the reproduction of disease in healthy piglets inoculated with intestinal content and tissue from NNPD-piglets (Jonach, 2014).

It has also been suggested that diarrhoea in piglets <1 day old could be considered normal (Kongsted *et al.*, 2014a). This assumption was based on the observation that piglets only diarrhoeic on the first day of life did not show a decreased ADG compared to non-diarrhoeic piglets. However, the biological reason for this distinction was not discussed and further examinations subsequently showed that diarrhoea on the day of birth was a risk factor for having diarrhoea on the second to fifth day of life (Kongsted *et al.*, 2014b). An interpretation of these results may be that some piglets that are diarrhoeic already at the day of birth are mildly affected and recover quickly.

Although all piglets were sampled in the acute phase of the disease and immediately after death to ensure a high quality of the samples, the possibility that a potential infectious cause of the diarrhoea was overlooked cannot be excluded:

Bacteriological investigations focused on agents previously associated with NPD and subsequently, on the results from histopathology. The rationale for this strategy was that adherence and colonisation of the intestinal mucosa is considered a key step in the pathogenesis of bacterial enteric infections (Lu & Walker, 2001). Although samples for histopathology was collected from multiple sites of the intestine (n=7) and approximately three sections (of 4 µm each) were examined per site, this does not exclude the presence of localised lesions or bacterial colonisation in parts of the intestine that was not examined. One may speculate that pathological changes and/or bacterial adherence to the

intestinal epithelium may be less widespread in early stages of disease. This might explain the lack of specific findings among the youngest piglets and it would hence have been interesting to examine a larger number of two-day-old diarrhoeic piglets from some of the herds. However, that approach would also have increased the risk for secondary infections that may have concealed the primary cause. Furthermore, some intracellular bacteria may not be visible by light microscopy. However, all piglets (n=30) investigated for *Chlamydia* spp. by immunostaining were negative and no other intracellular bacteria have, to the best of the authors' knowledge, previously been associated with NPD.

In addition, the viral metagenomics analyses are capable of detecting any previously described mammalian viruses present in a sample. However, although unlikely, the presence of novel viruses, genetically highly dissimilar to previously described viruses, cannot be ruled out.

5 Concluding remarks and future perspectives

Taken together, the results presented in this thesis demonstrate that diarrhoea in newborn piglets in Sweden may have other causes than the well-established enteropathogens previously associated with NPD. Thus, the initial hypothesis of the project could not be rejected.
Our results show that:

- NPD is common in Swedish piglet-producing herds and many herds experience recurrent problems despite maternal vaccination against ETEC.
- The gross pathological lesions associated with diarrhoea were mild and unspecific in all of the investigated herds. On histopathology, small intestinal colonisation by enterodherent cocci accompanied by mild mucosal lesions was the only consistent finding among diarrhoeic piglets.
- *Enterococcus hirae* was implicated as the colonising agent. Colonisation by enteroadherent *E. hirae* was only detected in diarrhoeic piglets and was present in piglets originating from six of the ten investigated herds, suggesting that this bacterium is involved in the disease.
- Bacteriological enteropathogens previously associated with piglet diarrhoea, a deficient passive immune status of the piglets, or obvious short-comings in important environmental factors were generally not related to diarrhoea in the investigated herds.
- A number of viral families were present in both healthy and diarrhoeic piglets but the only previously well-established porcine enteropathogen found was rotavirus, which was detected in two herds only. Overall, a major contribution of previously known viruses was not indicated.

As so often is the case, the new knowledge obtained has also generated new questions:

To establish a potential, causative role of *E. hirae* in the aetiology of NPD, Koch's postulates needs to be fulfilled by experimental challenge studies with *E. hirae* strains isolated from diarrhoeic piglets. This would not only shed light on the role of *E. hirae* as a potential pathogen but an appropriate challenge model would also allow for investigations of host-pathogen interactions and evaluation of treatment strategies.

It would also be interesting to investigate the presence of *E. hirae* among healthy neonatal piglets from herds without NPD. *E. hirae* has been described as a normal part of the intestinal microbiota in older pigs (Leser *et al.*, 2002) but information on the composition of the enterococcal flora in healthy newborn pigs is lacking.

Furthermore, potential virulence properties of enteroadherent *E. hirae* associated with NPD need to be examined and compared with *E. hirae* isolated from healthy, non-diarrhoeic animals. As a first step, virulence determinants previously described for *E. faecium* and *E. faecalis* could be examined. This would add information on the pathogenic potential of this organism and in addition reveal if it is possible to distinguish *E. hirae* strains associated with diarrhoea from those present in the normal intestinal microbiota.

A method to diagnose the condition in live piglets should also be developed.

In four herds, the aetiology of NPD was not established. This underlines the complexity of this problem and warrants further studies on potential additional factors or agents contributing to the disease.

Finally, to investigate the relative importance of different enteropathogens associated with NPD, prevalence studies on randomly selected herds are needed.

6 Populärvetenskaplig sammanfattning

Diarré hos nyfödda grisar är ett vanligt problem i grisuppfödning världen över och kan orsakas av en rad olika bakterier, virus och parasiter. Många av dessa smittor går att minska genom att planera produktionen så att grisningsmiljön kan rengöras och desinficeras mellan grisningarna. Dessutom kan vissa infektioner förebyggas genom vaccination av suggan innan grisning, så att spädgrisarna får ett skydd via råmjölken. Trots dessa rutinmässiga åtgärder har man på senare år upplevt en ökning av spädgrisdiarré i många besättningar. Studierna i den här avhandlingen syftar till att beskriva problemet och undersöka möjliga bakomliggande orsaker.

För att uppskatta hur vanligt spädgrisdiarré är i svenska besättningar genomfördes en enkätstudie som innefattade 170 slumpmässigt utvalda besättningar. Av de 58 % som svarade, angav ~80 % att de hade haft spädgrisdiarré i sin besättning under föregående år. Undersökningen visade även att diarré var vanligare i större besättningar. Majoriteten av besättningarna vaccinerade mot kända, sjukdomsframkallande kolibakterier, som anses vara den vanligaste orsaken till spädgrisdiarré. Trots vaccinering så uppgav 40 % att man hade återkommande fall av spädgrisdiarré.

För att utreda orsaken till diarrén gjordes omfattande provtagningar i tio utvalda besättningar. Från varje besättning undersöktes fem grisar med diarré och två friska kontrollgrisar. Grisarna obducerades och en rad prover togs för att undersöka sjukliga förändringar i tarmen samt förekomst av olika infektionsämnen. Dessutom analyserades koncentrationen antikroppar i blodet för att undersöka om grisarna fått i sig tillräckligt mycket råmjölk.

Det var ingen skillnad i antikroppskoncentration mellan sjuka och friska grisar. Sjukliga förändringar i tarmarna var generellt milda och inga parasiter kunde ses i tarmslemhinnan. De bakteriologiska undersökningarna visade på en låg förekomst av sjukdomsframkallande kolibakterier. Den mer ovanliga bakterien *Clostridium perfringens* typ C, som orsakar smittsam tarmbrand,

kunde inte påvisas. Två andra bakterier som diskuterats som orsak till spädgrisdiarré (*Clostridium perfringens* typ A och *Clostridium difficile*) hittades hos både sjuka och friska grisar och det fanns ingen koppling mellan förekomst av dessa bakterier och diarré.

Närvaro av virus i tarmen undersöktes med en ny metodik, så kallad viral metagenomik, som tillåter en bred screening av olika virus i ett prov. Med denna metod påvisades rotavirus i två av besättningarna. Rotavirus är vanligt förekommande hos svenska grisar men orsakar sällan diarré hos nyfödda spädgrisar eftersom de är skyddade via antikroppar i råmjölken. I övrigt kunde inget annat virus kopplas till diarrén.

Det enda gemensamma fyndet hos grisar med diarré var att tunntarmsslemhinnan hos 60 % av de sjuka spädgrisarna i sex av besättningarna var koloniserad med en bakterie som kallas *Enterococcus (E.) hirae*. I drygt hälften av fallen sågs även lindriga skador i tarmslemhinnan. Fyndet var överraskande då *E. hirae* inte tillhör de bakterier som vanligen förknippas med spädgrisdiarré.

Sammantaget visar det här projektet att spädgrisdiarré är ett vanligt problem och att det inte alltid orsakas av de smittämnen som tidigare förknippats med sjukdomen. Däremot hittades ett samband mellan diarré och tunntarmskolonisation av bakterien *E. hirae* i merparten av besättningarna. Fyndet är intressant och betydelsen av *E. hirae* som potentiell orsak till diarré hos nyfödda grisar bör undersökas vidare.

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