Enteric Diseases in Pigs from Weaning to Slaughter

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Utan tvivel är man inte riktigt klok

Tage Danielsson

To my family

THE INDIVIDUALITY OF THE PIG

ITS BREEDING, FEEDING, AND MANAGEMENT

BY ROBERT MORRISON

SPECIALIST IN BREEDING, FEEDING, AND MANAGEMENT

Diarrhœa may be due to a number of causes. It principally attacks litters when being suckled by the mother—at the third, fourth, fifth, or sixth week.

It may be traced to cold, damp, unclean stys, unclean or damp bedding, or a change in the feed of the mother such as the introduction of an excessive quantity of green food when the pigs are too young, bringing on scouring, and where the other conditions mentioned are present developing into diarrhœa.

Treatment.—Cleanse the sty thoroughly and disinfect the place with a solution of Jeyes' fluid—apply with a hand spray. Supply warm dry bedding and give the mother a dose of castor oil, in the morning feed as already advised.

Prevention.—There is no reason whatever why litters should not escape this trouble entirely with ordinary attention to cleanliness in the sty, dry **bedding, and the avoiding of any sudden or excessive change in the mother's feed during the eight weeks she is suckling her young.**

*Figure 1. "*The individuality of the pig*" was written by Robert Morrison in 1926, and published by John Murray, London.*

Abstract

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The general aim of this thesis was to study enteric diseases in growing pigs, with special reference to diseases caused by *Brachyspira hyodysenteriae* and *Lawsonia intracellularis.* The occurrence of enteric diseases in "growers" is a problem of increasing importance in Sweden and an understanding of the mechanisms by which the microorganisms causes enteric diseases is essential to develop good prophylactic measures. The most important microorganisms involved in enteric diseases in grower pigs were identified as *Lawsonia intracellularis* and *Brachyspira pilosicoli*, as determined by necropsy, microbiological and histopathological examinations performed on representative growing pigs from good and poor performing herds.

Diagnostic methods based on polymerase chain reaction for *L. intracellularis* in tissue or faecal samples were established and the results related to those obtained by necropsy and serology. An internal control, a mimic, was constructed to demonstrate inhibition of the PCR reactions and to evaluate different preparation methods. The methods for the demonstration of *L. intracellularis* in tissue samples were sensitive and specific, and the bacteria were reliably identified in faeces from pigs with overt disease.

A number of factors interacting in the clinical expression of swine dysentery were evaluated. In this work, group-housing of pigs and the addition of 50% soybean meal in feed was shown to predispose for infection.

A model was developed that enabled the sequential monitoring of disease in single animals by repeated endoscopy and biopsy sampling through a caecal cannula. This reduced the number of experimental animals required and increased the accuracy of the study. The general condition of the animal was not affected. The model was used to study the development of experimentally induced swine dysentery and the sequential development of lesions was characterised by histopathology and immunohistochemistry. An increase in the acute phase proteins serum amyloid A and haptoglobin and in monocytes was seen when haemorrhagic dysentery occurred.

Keywords: Experimental animal model, cortisol, enteric pathogens, immune response, white blood cells, T lymphocytes, mucohaemorrhagic diarrhoea

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Sammanfattning

Tarmsjukdomar hos gris från avvänjning till slakt

Studiens syfte är att belysa diarrésjukdomar hos växande grisar, med särskild inriktning på de sjukdomar som orsakas av bakterierna *Brachyspira hyodysenteriae* och *Lawsonia intracellularis*. Diarréer hos s.k. tillväxtgrisar, dvs. djur som lämnat den kritiska avvänjningsperioden bakom sig men ännu inte förflyttats till slaktsvins-stallet, är ett ökande problem i Sverige. En fördjupad kunskap om de faktorer som medverkar vid uppkomst av sjukdom och om de bakomliggande mekanismerna är viktig för att finna adekvata förebyggande åtgärder.

I avhandlingen klarläggs vilka mikroorganismer som är vanligast förekommande i samband med diarré hos tillväxtgrisar. Detta samband studerades med hjälp av jämförelser av resultaten från obduktioner, mikrobiologiska och histopatologiska (mikroskopiska) undersökningar på grisar med och utan akut diarré. Grisarna var inremitterade från besättningar med sämre produktionsresultat och typiska problem, och från besättningar med goda produktionsresultat och friska grisar. Resultaten visade, att de två bakterierna *Brachyspira pilosicoli* och *Lawsonia intracellularis* är de vanligaste orsakerna till diarré hos växande grisar.

En molekylärbiologisk PCR-baserad diagnostik för bakterien *Lawsonia intracellularis* etablerades. För att påvisa falskt negativa resultat utvecklades en intern kontroll, en sk. mimic. Denna användes även för att utvärdera olika metoder för preparering av PCR-prover. Resultaten från PCR-diagnostiken jämfördes med resultat från undersökningar baserade på obduktion och serologi (påvisande av antikroppar i blodet). PCR-tekniken visade sig vara specifik och ha en hög känslighet vid påvisande av bakterien i vävnad och i faeces hos sjuka grisar.

Det är sedan tidigare känt att flera olika faktorer samverkar vid uppkomst av svindysenteri, den sjukdom som orsakas av *Brachyspira hyodysenteriae*. I en studie visades att en kraftig inblandning av sojamjöl i fodret hos grupphållna grisar bidrog till uppkomsten av sjukdom vid infektion.

Vidare utvecklades en *in vivo*-modell på gris för att kunna studera sjukdomsförloppet i tarmen. Tidigare har sådana studier baserats på obduktion av ett stort antal djur. Den nya modellen bygger på endoskopi och biopsitagning via en tarmfistel, och medför att sjukdomens förlopp kan följas hos ett och samma djur. Detta innebär att antalet djur som ingår i försöket kan minskas och att precisionen i försöken ökar. Metoden påverkade inte djuren negativt och de successiva förändringarna i tarmen vid svindysenteri kunde studeras i detalj. Det fastslogs att djurens immunsystem aktiverades i samband med blödande tarmskador, vilket avspeglades i att koncentrationerna av två s.k. akutfasproteiner, SAA och haptoglobin, ökade. De vita blodkroppar som benämns monocyter ökade också i samband med blödande skador i tarmen.

Contents

Abbreviations 5

Introduction 6

Is it important to study diarrhoea in pig? 6

Particularly hazardous periods in the pigs life 7
Are enteric diseases common in swine? 9
The causative relationships in enteric diseases 9
The host defence against an invading microbe 10
The physiological barriers in the gut 10
The innate immune system 11
The cellular adaptive immune system 11
The humoral immune system 11
The immune response to infection 12
The pathogenesis of enteric diseases 13
General aspects on diagnosis 17
Diagnosis of *Lawsonia intracellularis* 17
Experimental challenge studies 19

Aims of the present studies 20

Aspects on material and methods 21

Paper I 21
Paper II and III 22
Paper IV 23
Paper V 24
Paper VI 25

Results and Discussion 27

The diagnosis of *Lawsonia intracellularis* 27
Diarrhoea in growing pigs 29
Pathogenesis of *Brachyspira hyodysenteriae* 32
Experimental inoculation with *Brachyspira hyodysenteriae* 32
The possibility to study series of events in the intestine 33
Interactions between the host and the microbe 34
Strategies to prevent disease 35

Conclusions 36

Acknowledgements 37

References 40

Appendix

Papers I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals I-VI.

I. Jacobson, M., Hård af Segerstad, C., Gunnarsson, A., Fellström, C., de Verdier Klingenberg, K., Wallgren, P. & Jensen-Waern, M. 2003. Diarrhoea in the growing pig – a comparison of clinical, morphological and microbial findings between animals from good and poor performance herds. *Research in Veterinary Science,* 74:163-169

II. Jacobson, M., Englund, S. & Ballagi-Pordány, A. 2003. The use of a mimic to detect polymerase chain reaction-inhibitory factors in feces examined for the presence of *Lawsonia intracellularis*. *Journal of Veterinary Diagnostic Investigation,* 15:268-273

III. Jacobson, M., Aspan, A., Heldtander Königsson, M., Hård af Segerstad, C., Wallgren P., Fellström, C., Jensen-Waern, M. & Gunnarsson, A. Diagnosis of *Lawsonia intracellularis* performed by PCR, serological and post mortem examination, with special emphasis on sample preparation methods for PCR. Submitted for publication.

IV. Jacobson, M., Lindberg, J. E., Lindberg, R., Hård af Segerstad, C., Wallgren, P., Fellström, C., Hultén, C. & Jensen-Waern, M. 2001. Intestinal cannulation: Model for study of the midgut of the pig. *Comparative Medicine,* 51:163-170

V. Jacobson, M., Fellström, C., Lindberg, R., Wallgren, P. & Jensen-Waern, M. Experimental swine dysentery – comparison between infection models and studies of the acute phase protein response to infection. Submitted for publication.

VI. Jacobson, M., Lindberg, R., Jonasson, R., Fellström, C. & Jensen-Waern, M. Consecutive pathological and immunological alterations during experimentally induced swine dysentery – a study performed by repeated endoscopy and biopsy samplings through an intestinal cannula. In manuscript.

Abbreviations

APP	acute phase protein
B. hyodysenteriae	*Brachyspira hyodysenteriae*
B. pilosicoli	*Brachyspira pilosicoli*
B. vulgatus	*Bacteroides vulgatus*
b.w.	body weight
C.	*Campylobacter*
C. coli	*Campylobacter coli*
CD	cluster of differentiation
C. jejuni	*Campylobacter jejuni*
Cl. perfringens	*Clostridium perfringens*
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
IFN-γ	interferon-gamma
Ig G	immunoglobulin G
Ig M	immunglobulin M
Ig A	immunoglobulin A
IL	interleukin
I. suis	*Isospora suis*
L. intracellularis	*Lawsonia intracellularis*
M cells	microfold cells
MHC	major histocompatibility complex
NK cells	natural killer cells
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PED	Porcine epidemic diarrhoea
rRNA	ribosomal ribonucleic acid
SAA	serum amyloid A
S. choleraesuis	*Salmonella enterica* serovar *choleraesuis*
SDS	sodium dodecyl sulphate
sIgA	secretory immunoglobulin A
SFS	svensk författningssamling
SJVFS	statens jordbruksverks författningssamling
SPF	specific pathogen free
S. typhimurium	*Salmonella enterica* serovar *typhimurium*
T_C	cytotoxic T cell
TGE	transmissible gastroenteritis
T_H	T helper cell
TNF	tumour necrosis factor
T-RFLP	terminal restriction fragment lenght polymorphism
Y. enterocolitica	*Yersinia enterocolitica*

Introduction

Diarrhoea is the clinical manifestation of one of the most common disease complexes in pigs worldwide. One of the first enteric diseases described in swine was salmonellosis (Salmon & Smith, 1886), and the number of known agents and other non-infectious causes continously increases (Dunne, 1958; Straw *et al*., 1999). Diarrhoea can be defined as malabsorption of water and electrolytes (Jubb & Kennedy, 1970), the frequent passage of soft or watery faeces (Liebler-Tenorio *et al.,* 1999), or a condition with a water content in faeces exceeding 80% (Makinde *et al.,* 1996). Enteric diseases show a wide spectrum of clinical signs, ranging from a soft stool for a few days in a seemingly healthy animal, to profuse, watery faeces with dehydration and a rapid decrease in body condition (Svendsen *et al*., 1974; Morin *et al*., 1983; Thomson *et al*., 1998b; Johnston *et al*., 2001). The intestinal content may be mucous, haemorrhagic or necrotic but the disease may also appear so rapidly that death occurs without any preceding clinical sign (Alexander & Taylor, 1969; Svendsen *et al.,* 1974). Thus, the general condition of the pig may be unaltered or severely depressed, causing anything from no obvious signs to severe suffering in the individual animal.

Is it important to study diarrhoea in pig?

Diarrhoea sometimes appears occasionally in single animals but more often, it occurs as a repeated problem in a herd involving many animals and on several occasions (Svendsen *et al.,* 1974; Jestin *et al*., 1985; Nabuurs *et al.,* 1993). The economic impact is substantial because of increased mortality rates, poor growth and additional medical costs (McOrist *et al*., 1997; Wills, 2000). Poor growth results in delayed marketing of some animals and an over-stocking in the resident herd. Subsequently, the failure to sustain segregated rearing systems leads to breaches in biosecurity and hygiene, thus, increasing the risk of further transmission of disease (Pearce, 1999; Wills, 2000; Morris *et al*., 2002).

Diarrhoea can cause large economical losses (Morris *et al.,* 2002). For example, in Australia in the late 1980s, production losses due to postweaning colibacillosis were estimated at approximately $80 per sow per year, and the corresponding figure for swine dysentery was ~$100 (Cutler & Gardner, 1988). In the United States, to produce a 100 kg pig cost $32 more in a conventional herd compared to a high health farm, which clearly indicates the economic losses caused by the various diseases (Batista & Pijoan, 2002). In 2001, the overall mortality rate in Swedish pigs from birth to 25-kg b.w. was ~15% (S. Anér, pers. comm.) with diarrhoea being considered one of the main causes. In Denmark, pork is a large export industry with a turnover of 25 billion Dkr. from a total production of 24 million pigs in 2002. The calculated losses due to the ban of antibacterial growth promoters in feed were estimated to ~10 Dkr. per pig and one of the main reasons for this were infectious enteric diseases (Prof. J. P. Nielsen, pers. comm.). Hence, diarrhoea in pigs causes substantial economic losses not only for the individual farmer but also for the country.

Diarrhoea in pigs might also have other implications for the society (Glossop, 2002; Hayes, 2002). Firstly, some of the porcine enteric diseases are zoonoses and transmitted to humans through direct contact, or through contamination of the environment or meat and meat products (Helms *et al*., 2001; Nielsen, 2002). Secondly, there is increasing concern about the development of antibiotic resistance and rest substances in the environment due to the use of antibiotics in both animals and humans (Wills, 2000; Glossop, 2002). Thirdly, animal welfare is one of the most pertinent questions in animal husbandry, and diseases must be regarded as an animal welfare issue (Fraser, 2002; Glossop, 2002). Lastly, concern about the environmental impact of the excessive release of nitrogen and phosphorous from the pig industry increases, and a good animal health with proper utilisation of nutrients reduces the amount of waste products (Hatfield, 2002).

Particularly hazardous periods in the pigs life

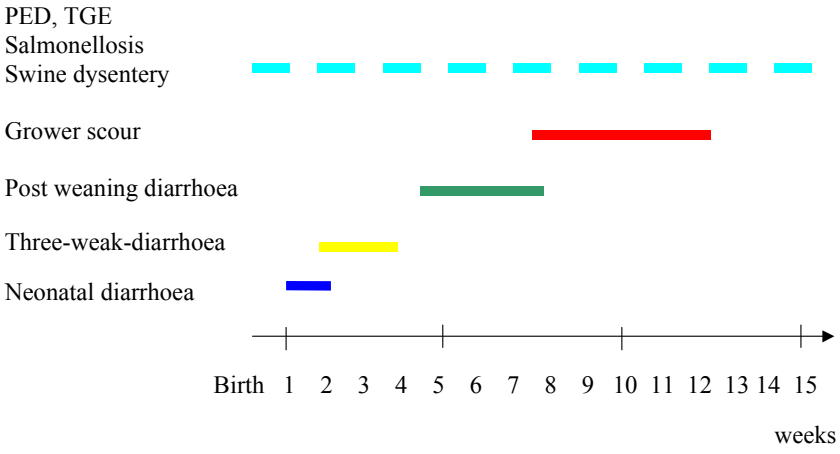

Fig. 2. Diarrhoea in pig is often related to certain ages or certain periods during rearing.

Neonatal diarrhoea caused by *Escherichia coli*, *Clostridium perfringens* type C (in Sweden this disease is referred to as "transmissible gut gangrene"), or coronavirus is seen during the first week of life (Haelterman & Hutchings, 1956; Bergeland, 1972; Morin *et al*., 1983). This period is particularly hazardous, since the epitheliochorial placenta of the sow makes the piglet dependent on the colostral transfer of maternal antibodies (Kohler, 1974; Tizard, 1987). From two weeks onwards the piglets own antibody production slowly increases (Bourne, 1976; Tizard, 1987). However, serological surveys indicate that Swedish swineherds are free from infection with the coronavirus-induced diseases transmissible gastroenteritis (TGE) and porcine epidemic diarrhoea (PED) (Elvander *et al*., 1997).

In the somewhat older pig, a steatorrhoea sometimes referred to as "white scour" is seen. The disease is presumably caused by the intestinal parasite *Isospora suis* or by rotavirus (Morin *et al*., 1983; Nilsson *et al*., 1984), and is in Sweden referred to as "three-week-diarrhoea", which indicates the average age of diseased piglets (Wills, 2000). At this age, the maternal immunity is vanishing whereas the piglets own immune functions has not yet fully developed (Bourne, 1973; Gaskins & Kelley, 1995; Tzipori *et al.,* 1980; Liebler-Tenorio *et al*., 1999).

The next critical period in the pig's life is weaning, which in Sweden by legislation is not allowed to take place before 28 days of age (SJVFS 2003:6). Post-weaning diarrhoea occurs during the first two weeks after weaning and is one of the most important diarrhoeal diseases worldwide (Moxley & Duhamel, 1999). The causative organism is enterotoxigenic *E. coli* (ETEC) (Richards & Fraser, 1961; Svendsen *et al.,* 1974) but post-weaning diarrhoea is still somewhat of an enigma as several predisposing factors such as heredity, feed, management, and environment interact to cause the disease (Bertschinger *et al*., 1978/1979; Svensmark *et al*., 1989; Wathes *et al.,* 1989; Nabuurs *et al*., 1993; Johansen *et al*., 2000; Löfstedt *et al*., 2000; Madec & Buddle, 2002).

In recent years, a disease referred to as grower scour or colitis has emerged (Thomson *et al.,* 1996; Thomson *et al*., 2002). The disease is assumedly caused by *Brachyspira pilosicoli* or by *Lawsonia intracellularis*. The growing pigs have passed the critical period of weaning but have not yet been transferred to the finishing herd. No alteration in feed or environmental factors usually takes place during this period and no obvious challenges to the immune system occur. It is purely speculative as to why this disease has not previously been recognised in Sweden. The Swedish ban of antibacterial growth promoting feed additives in 1986 was followed by an increased incidence of post-weaning diarrhoea (SFS 1985:295), (Robertsson & Lundeheim, 1994). During the following years, farmers began to cope with this problem and the mortality and morbidity rates due to diarrhoea post-weaning decreased. Hence, other diseases with less mortality and less obvious clinical signs became noted. In addition, herd structures changed dramatically in recent years and several alternative production systems have developed. This might in some way have promoted the incidence of grower scour (Duhamel, 1996; Wills, 2000; Morris *et al*., 2002). The infectious causes of this disease will be further discussed in Paper I.

During the finishing period, the importance of diarrhoeal diseases usually decreases. Within the first weeks after arrival to the finisher unit, the animals might be affected by diarrhoea induced by the stress during transport and the mixing of animals, or by environmental factors such as contaminated water remaining in the water system. Sometimes, mild diarrhoea that is considered osmotic and apparently not affects the pig's health or growth is seen (Jensen, 1995). None of this is considered as a major problem. However, outbreaks caused by certain pathogens can result in considerable production losses: swine dysentery caused by *B. hyodysenteriae* is an important disease in swine of all ages (Alexander & Taylor, 1969; Meyer, 1978) and salmonellosis is a very important global zoonotic disease (Nielsen, 2002). The latter is subjected to extensive

control programmes and is rarely seen in Swedish swineherds (Wahlström *et al*., 1998; Wahlström *et al*., 2000). Other enteric diseases affecting pigs of all ages includes, in immunologically naive herds, TGE and PDE, and in some cases, diseases caused by *L. intracellularis* (Haelterman & Hutchings, 1956; Pritchard, 1982; Rowland & Rowntree, 1972).

Are enteric diseases common in swine?

Enteric diseases are undoubtedly a large problem in swine production, but the prevalence of diarrhoeal diseases is difficult to interpret and available figures usually concern separate diseases. In a Danish study (Kjaersgaard *et al*., 2002) on the pre-slaughter cause of mortality performed on 12 481 pigs from three herds, 5.6% of the piglets, 25.5% of the weaners and 5.5% of the grower-to-finisher-pigs had a post-mortem diagnose of gastro-intestinal disease. In another Danish study (Petersen *et al*., 2002) performed on 98 finisher herds, clinical signs of diarrhoea were detected in 0.31% of pigs in the herds. At the last two International Pig Veterinary Society Congresses (2000 and 2002), the prevalence of some enteric pathogens in different countries was presented. *I. suis* were reported in 13.2% of the piglets in 28 of 40 herds in a Brazilian study (Rostagno *et al*., 2002). In Spain, 30.4% of the pigs in 15 of 24 farms were sero-positive for *L. intracellularis* and in Argentina, 19.8% of the pigs in 15 of 22 herds studied were positive (Corral & Valiente, 2002; Machuca *et al*., 2002). In Canada, 14.3% faecal samples in 47 of the 90 herds tested positive with faecal culture for *Salmonella* spp. and 12% of the samples in 75 herds were serologically positive (Rajic *et al*., 2002). In an English study published in 1999, 50.5% of the 105 herds questioned had experienced a scour problem in the previous three years. The cause had been identified as colitis in 34.3% of the cases, as swine dysentery in 10.5% and porcine enteropathy in 3.8% (Pearce, 1999). In a recent Swedish study by Löfstedt, (2003), 75% of 105 piglet-producing herds had experienced problems with “growing scour” during the previous year.

The causative relationships in enteric diseases

Diarrhoeal diseases are traditionally viewed as one microbe-one disease (Meyer, 1978; Stevenson *et al*., 1990), which may be true for some diseases such as transmissible gastroenteritis (TGE) in an immunologically naive herd (Morin *et al*., 1983; Pritchard, 1982). Further, diarrhoeal diseases can be regarded as a struggle between the infectious agent and the individual’s immune response, in which the pathogen is the winner (Bergeland, 1972; Stuart *et al*., 1982; Clarke & Gyles, 1987; Cano *et al*., 2000). However, this does not explain why some herds repeatedly suffer from diseases caused by microbes that do not seem to affect other herds, even if the microbe is present. For instance, certain herds in a sow-pool-system (Lundeheim *et al*., 2000) employing strict all in-all out with thorough cleaning and disinfecting between batches, repeatedly suffers from infection with *Cl. perfringens* type C, whereas other herds, utilising the same sows, never experience the disease (M. Lindblad, pers. comm.). Thus, a third way to consider enteric diseases is to regard them as an entirely multifactorial problem, where the

diarrhoea is the sum of a range of provocative and preventative factors (Morin *et al*., 1983; Madec & Buddle, 2002; Morris *et al*., 2002).

Monofactorial diseases tend to be easier to handle and have therefore become rare in modern pig husbandry. Instead, diseases of today are usually of complex, multifactorial origin (Madec & Buddle, 2002). Several interacting factors have been proposed. Environmental factors such as temperature, draught, humidity, and feed might negatively affect the hosts susceptibility to disease, hygiene level might interfere with pathogen load, and other diseases and stress might increase the host susceptibility (Morin *et al*., 1983).
For instance, swine dysentery was initially thought to be of unifactorial origin. Later, it was discovered that factors such as additional bacterial flora and feed interact to cause disease, but the mechanism of interaction is still unknown (Whipp *et al*., 1979; Pluske *et al*., 1996).

The host defence against an invading microbe

To handle potential threats, the animal has several defence mechanisms which can be referred to as the external barriers, the innate immune response and the adaptive immune response (Roitt *et al*., 1993; Galvin *et al*., 1997). Many species-specific differences exist.

The physiological barriers in the gut

Several barriers exist throughout the intestinal canal, such as acid secretion and low pH in the stomach (Bergeland, 1972; Savage, 1980). Further, invading pathogens can be trapped in mucus and removed by peristalsis (Savage, 1980; Galvin *et al*., 1997). Therefore, in order to colonise, a non-adherent bacterium must multiply faster than it is discharged (Savage, 1980). Shortly after birth, the commensal (indigenous or autochthonous) microflora are established by microbes with a high multiplication rate from the pigs' closest proximity, such as members of the *Enterobacteriaceae* (Adlerberth *et al*., 1991). As space and nutrients diminish, every location in the gut becomes occupied by the fittest microbe (Midtvedt, 1999). The commensals compete with pathogens for nutrients, or attachment sites in mucus or in the epithelium (Bibel *et al*., 1983). Further, the commensals can alter the pH or redox-potential in the intestine, resulting in a less suitable microclimate for the pathogen, or they can produce growth inhibitors such as hydrogen sulphide, bacteriocins, or short chain volatile fatty acids (Meynell, 1963; Savage, 1980; Freter *et al*., 1983; Galvin *et al*., 1997; Cebra, 1999). The epithelium provides an invasion barrier and the turnover rate of the epithelial cell is a mechanism by which infected cells can be excluded (Moon *et al*., 1975; Savage, 1980). In some cases, specific host receptors are needed to induce disease (Gibbons *et al*., 1977; Edfors-Lilja *et al*., 1995). In addition, sIgA molecules on the mucosal surface may inhibit adherence and prevent absorption of the antigen (Kraehenbuhl & Neutra, 1992; McGhee *et al*., 1992).

The innate immune system

The immune system is well described in textbooks such as Roitt *et al* (1993) and Mims *et al* (2001). The innate immune response non-specifically recognises foreign antigens and consists of cells such as polymorphonuclear leucocytes (*i.e.* neutrophils, eosinophils, and basophils), mononuclear phagocytes (i.e. monocytes and macrophages), dendritic cells, mast cells and platelets. Further, humoral inflammatory mediators such as complement components participate in the non-specific response.

The cellular adaptive immune system

The adaptive cellular immune response consists of B and T lymphocytes. The B cells and plasma cells constitute 20-40% of the lamina propria lymphocytes (McGhee *et al*., 1992). The lymphocytes are characterised by their different receptors and can thus be specifically recognised by the use of monoclonal antibodies. For instance, the CD 3 receptor is a general marker for T cells, $CD8^+$ is a cytotoxic T cell (T_C) marker and $CD79^+$ is a B cell marker. A subpopulation of T cells carrying the γ/δ receptor is seen at epithelial surfaces and is thought be important in early defence in pathogen-induced epithelial damage (Kraehenbuhl & Neutra, 1992 and Mims *et al*., 2001).

The humoral immune system

The humoral immune response consists of antibodies, complement factors and different mediators such as cytokines, leukotrienes and prostaglandins. The antibody response mainly takes place in the lymphoid organs and in the submucosa. The gastrointestinal tract could be considered as the largest immune organ in the body, containing 70-80% of the immunoglobulin producing cells. M-cells (microfold cells) overlying the Peyer's patches have a specialised mechanism for transporting and presenting antigens to the immune system (Kraehenbuhl & Neutra, 1992). Small amounts of a specific antibody are formed locally within a few days after stimuli, although antibodies are not usually detected in serum until a week later. A second exposure results in the formation of large quantities within two days. Immunoglobulin G (IgG) is mainly distributed into the circulation, but the levels in tissue increases during inflammation. Immunoglobulin M is confined to the vascular system, has a low affinity and short-lived memory, and its presence indicates a recent or a persistent infection. Secretory IgA is the main immunoglobulin (>80%) on mucosal surfaces (McGhee *et al*., 1992). Ig A has a limited ability to fix complement, which might be a way of preventing extensive tissue damage and maintaining the integrity of the mucosal barrier (McGhee *et al*., 1992; Galvin *et al*., 1997).

The complement factors consist of ~20 proteins which act as opsonins, promote chemotaxis, increase vascular permeability and are capable of damaging plasma membranes. The classic complement pathway is activated by the antigen-antibody binding, and the alternative pathway can be activated early in the inflammatory process by microbial polysaccharides and endotoxin. However, complement can cause considerable inflammation and tissue damage.

Cytokines act as soluble mediators and depending on the course of infection, upregulate or downregulate the immune response. At least 20 different cytokines are known. Some cytokines are released from damaged tissues and attract immune cells to the site of injury. Others are produced by lymphocytes (i.e. lymphokines) and induce inflammatory or immunological changes. Some cytokines, especially interleukin-1 (IL-1) and IL-6, are endogenous pyrogens, whereas others, such as TNF, tend to reduce elevated temperature.

Cytokines induce or modulate the acute phase response, which includes the production and systemic release of about 30 different proteins. The function of the acute phase proteins is not clear, but appears to be protective and to aid in restoring and maintaining homeostasis. For instance, the acute phase protein haptoglobin acts as an antioxidant and binds free hemoglobin, forming stable complexes that are rapidly eliminated through the liver (Wang *et al*., 2001). Several of the acute phase proteins are also part of the complement cascade. In humans, their presence is associated with headache, muscle pain, fever and anaemia. Further, they induce a decrease in iron and zinc, and an increase in copper and ceruloplasmin in the blood.
Other hormonal mediators including corticosteroids increase in more severe or widespread inflammations.

The immune response to infection

The immune response consists of two phases: the recognition of the antigen and the reaction to eradicate it. Once a microbe has penetrated the epithelial surface, the major host defences are NK cells, complement, phagocytic cells and interferon. Later, antibodies and T-cells occur.
The antigen is endocytosed by, or bound to, antigen presenting cells such as macrophages, dendritic cells, B cells or epithelial cells. In the cell, lysosomal enzymes degrade the antigen into short peptides that associate with MHC (major histocompatibility complex) molecules that are presented on the cell surface. Depending on the nature of the antigen, the antigen-presenting cell will express different MHC receptors. Intracellular organisms usually induce the expression of MHC I (the endogenous pathway), whereas organisms taken up by endocytosis will usually induce the expression of MHC II (the exogenous pathway). If the MHC I receptor is expressed on the cell surface, the antigen will be recognised by CD8 T_C cells, which expand, become activated, release antimicrobial cytokines and kill the infected cell by cytolysis. Cells that have a reduced expression of MHC class I, as well as some virus-infected cells and tumour cells, are recognised and killed by NK cells.

If the MHC II receptor is expressed on the cell surface, the antigen will be recognised by CD4 T_H cells: the predominating subgroup (T_{H1} or T_{H2}) will depend on the nature of the antigen. The triggered T_{H1} cells modulate the cell-mediated immune response (activation of phagocytes, proliferation of lymphocytes and delayed hypersensitivity reactions) by different cytokines (IFN-γ and IL-2). The triggered T_{H2} cells activate the polymorphonuclear cells, and induce proliferation

and maturation of the B cells to antibody-producing plasma cells by cytokines IL-4, IL-5, and IL-10. The antibody binds to the antigen, thereby exerting different antimicrobial activities, which include prevention of the antigen binding to the host cells and activation of macrophages, polymorphonuclear leucocytes and the classical pathway of complement, followed by destruction of the antigen.

Simultaneously, inflammatory mediators such as histamine, kinins, and the alternative complement pathway are activated by carbohydrates on the bacterial surface and by inflammatory materials released by the bacteria or by injured tissue. The mediators induce an inflammatory response consisting of dilatation of capillaries and increased permeability, resulting in an increased leakage of fluid, immunoglobulins, complement components and other proteins from blood to tissue. The inflammatory mediators attract leucocytes, especially neutrophils and monocytes, to migrate from the vessels to the site of injury. The monocytes are recruited from the blood by IFN-γ.

Bacterial diarrhoea is often considered as "hit and run" infections, with an incubation period of less than a week. The infection is mainly controlled by the early, innate immune system and has usually vanished before the T-cells and specific antibodies develop (Mims *et al*., 2001).

The pathogenesis of enteric diseases

Knowledge about the mechanisms for the induction of diseases, *i.e.* pathogenesis, is essential. In both human and veterinary medicine, most infectious diseases are treated with antibiotics. The current challenge to veterinarians is to develop better prophylactic measures to protect the animal from disease. To achieve this, understanding of how diseases emerge and evolve is necessary and comparisons of the mechanisms utilised by other microorganisms might be of great benefit. For instance, the mechanism behind *E. coli*-induced diarrhoea was elucidated by comparison to previous data on the pathogenesis of *Vibrio coli*. Still, more of these mechanisms are unknown than known (ter Huurne & Gaastra, 1995; McOrist & Gebhart, 2002).

A symbiotic relationship exists between the host and its indigenous gut flora (McFall-Ngai, 1998). The host benefits from the diverse metabolites produced by the bacteria, whereas the microorganism utilises the gut as a shelter provided with nutrients and other requirements that facilitate its survival and replication. The mechanisms for satisfying the different needs are specialised and vary between species. For example, viruses are devoid of systems for energy production and protein synthesis and instead they utilise their cellular hosts. For this purpose, they have to penetrate the host cell to gain access to the necessary machinery. Others, such as enterotoxic *E. coli*, attach to certain receptors on the small intestine epithelial cells by adhesins (fimbriae or pili) (Gibbons *et al*., 1997; Holland, 1990). Following binding, bacterial enterotoxins activate the cAMP and cGMP systems, causing secretory diarrhoea with excessive losses of fluid and electrolytes (Guerrant *et al*., 1974; Field *et al*., 1989; Gyles, 1994). However, the host cell remains intact. On the other hand, *Cl. perfringens* type C attach to the jejunal

epithelial cell and cause necrosis of the tissue by its α- and β-toxins (Bergeland, 1972; Arbuckle, 1972; Yoo *et al*., 1997). This disease normally occurs within the first 2-3 days of life (Figure 1), probably because the increased pH and low trypsin content in the stomach, as well as the content of trypsin inhibitors in sow colostrum, facilitates the infection (Arbuckle, 1972; Bergeland, 1972). Attachment of *Cl. perfringens* type A in swine has not been proved but the bacteria produces α-toxin and enterotoxin (Estrada Correa & Taylor, 1989; Johannsen *et al*., 1993) which binds to the colonic epithelial cells causing necrosis and fluid secretion (Taylor, 1999). Disease associated with enterotoxin is seen in 5-7 weeks old, weaned pigs. *Cl. perfringens* type A is ubiquitous in gut contents (Estrada Correa & Taylor, 1989) and colostrum usually contains antibodies to both toxins (Taylor, 1999).

Rotavirus and *Isospora suis* (Figure 1) replicate in the cytoplasm of differentiated villous epithelial cells in the small intestine (Lindsay *et al*., 1980). The replication results in lysis and desquamation of infected cells with villous atrophy and fusion together with crypt hyperplasia, resulting in decreased digestion and absorption (Stuart *et al*., 1982; Graham *et al*., 1984). The degree and distribution of the lesions are generally related to age and infectious dose (Stuart *et al*., 1982; Stevenson *et al*., 1990), although a low ambient temperature resulting in increased energy demands might contribute to increased mortality (Steel & Torres-Medina, 1984). However, sporulation of *Isospora suis* is favoured by the supplemented heat provided to newborn piglets (Lindsay *et al*., 1982) and oocysts may be seen in faeces five days later (Stuart *et al*., 1982). Colostral antibodies are not protective but previous infection renders the piglet resistant to subsequent challenge (Lindsay *et al*., 1999). In addition, age related differences in susceptibility to the infection occur (Stuart *et al*., 1982).

The main *Salmonella* species responsible for disease in pigs are *S. choleraesuis* and *S. typhimurium* (Levine *et al*., 1945; Reed *et al*., 1986). *S. choleraesuis* generally invades through the tonsils or intestine, causing septicaemia followed by enterocolitis preferentially in ileum and colon (Reed *et al*., 1986; Pospischil *et al*., 1990). *S. typhimurium* has a low tendency to invade (Pospischil *et al*., 1990) and is endocytosed by the M cells and localised in the mesenteric lymph nodes and lamina propria where it causes an acute enterocolitis (Takeuchi & Sprinz, 1967). The spread is probably executed by macrophages and infection results in microvascular thrombosis, inflammation and necrosis, leading to malabsorption, fluid leakage and diarrhoea (Reed *et al*., 1986; Clarke & Gyles, 1987; Gröndahl *et al*., 1998; Moxley & Duhamel, 1999; Schwartz, 1999). Locally, neutrophil infiltration is prominent (Reed *et al*., 1986) and cytokine signals are important in regulating the intestinal response (Trebichavský *et al*., 1997). Over 200 virulence factors have been described, such as fimbriae, flagella and lipopolysackarides (Schwartz, 1999) and several predisposing factors exist (Hentges, 1970; Clarke & Gyles, 1987; Jörgensen *et al*., 2001).

The obligate intracellular bacterium *L. intracellularis* enters the crypt enterocytes in the distal jejunum, ileum, caecum and proximal colon within a membrane-bound endocytic vacuole (McOrist *et al*., 1995b). The bacteria divide in the

cytoplasm and appear to be dependent on host cell proliferation to be able to spread (Lawson *et al*., 1995). Further, an increased mitosis and cell division, and proliferation of immature enterocytes with depletion of goblet cells are induced (Jensen *et al*., 2000; Lawson & Gebhart, 2000). The immature enterocytes do not express MHC II molecules on the surface, which might be a bacterial strategy to escape the immune system. In addition, a marked accumulation of IgA at the apical cytoplasm of the enterocytes is seen. In the chronic form of the disease, i.e. intestinal adenomatosis, a mild infiltration of $CD8^+$ and $CD25^+$ T cells in lamina propria is noted. In the acute form, *i.e.* hemorrhagic enteropathy, a moderate infiltration of $CD8^+$, $CD25^+$ T cells and IgM^+ B cells in lamina propria is seen (McOrist *et al*., 1992). The mechanism for diarrhoea has not been described, but a proliferation of the secretory crypt cells and a lack of absorptive mature enterocytes could explain some symptoms. The disease cannot be reproduced in gnotobiotic pigs, and a synergistic action of other bacteria is suspected (McOrist *et al*., 1993; McOrist *et al*., 1994b).

Little information is available on the pathogenesis of the potentially zoonotic pathogen *B. pilosicoli* (Trott *et al*., 1996b), although blockage of the absorption by spirochaete "end-on" attachment to the mature enterocytes might be a mechanism of diarrhoea (Trott *et al*., 1996a). The infection induces an increased crypt cell mitotic rate and bacteria have been described in lamina propria and within goblet cells (Trott *et al*., 1996a). *In vitro* uptake by coiling phagocytosis by the monocytes have been reported (Cheng *et al*., 1999). The disease is characterised by a mild colitis and a mixed population of neutrophils and lymphocytes are seen in the mucosa in response to infection (Thomson *et al*., 1996; Trott *et al*., 1996a).

The pathogenesis of *B. hyodysenteriae* is still not fully understood. The significance of the acid secretion in the stomach has yet to be elucidated (Doyle, 1948; Blaha *et al.,* 1984). A concomitant infection with commensal gut bacteria has been shown to enhance the infection but the mechanism is unclear (Meyer *et al*., 1975; Harris et al 1978). A feed-induced alteration of the intestinal microflora might alter the oxygen tension (Hughes *et al*., 1975) or change the rate of fermentation in the large intestine (Durmic *et al*., 1998) resulting in a low pH (Prohászka & Lukács, 1984). Further, the microflora may provide growth factors, essential nutrients (Meyer, 1978) or produce other favourable conditions (Whipp *et al*., 1979). In addition, the microbes might be secondary invaders that exacerbate the lesions (Hughes *et al*., 1975; Meyer, 1978). *B. hyodysenteriae* is strongly chemotactic to mucus, and it has been suggested that chemotaxis and motility are important factors for association with the mucosa, by penetration or trapping in the mucus gel (Kennedy *et al*., 1988; Milner & Sellwood, 1994). The bacterium is suggested to primarily invade the goblet cells, thereby causing an excessive mucus-secretion, multiply and spread to adjacent enterocytes (Pohlenz *et al*., 1983). Hughes *et al*. (1975) suggested that the goblet cell hyperplasia and increased mucus production was caused by a toxin. The importance of attachment as a pathogenicity mechanism is uncertain and it is not clear whether invasion of the tissue is necessary to induce disease (Taylor & Blakemore, 1971; Wilcock & Olander, 1979b; Jensen *et al*., 1998). Other factors possibly involved in the pathogenesis are haemolysin, endotoxin or other toxins (Albassam *et al*., 1985;

Wilcock & Olander, 1979b; Nibbelink *et al*., 1997) and strains mutant in the haemolysin gene had reduced pathogenicity (Hyatt *et al*., 1994). Further, the enzyme NADH oxidase protects the bacteria against oxygen toxicity (Stanton *et al*., 1999). Several authors report an increased crypt cell proliferation, but it is not clear whether this is part of the defence against invading microorganisms or whether it is part of a repair process (Nuessen *et al*., 1983; Hughes *et al*., 1975; Wilcock & Olander, 1979a; Pohlenz *et al*., 1983). Diarrhoea occurs due to colonic absorptive failure (Argenzio *et al*., 1980).

Further, the immune mechanisms elicited are poorly understood. Attempts to suppress the immune response by induction of stress achieved by withdrawal of feed (Kinyon *et al*., 1977; Moreng *et al*., 1980), or by intramuscular injections of dexamethasone (Eriksen & Andersen, 1970), have been performed. The effect of feed withdrawal has not been separately evaluated but injections with dexamethasone worsen the condition (Eriksen & Andersen, 1970). However, in experimental inoculation with *L. intracellularis*, dexamethasone did not change the course of disease (Joens *et al*., 1997; Knittel *et al*., 1998). Altogether, reports concerning the cellular immune response are few (Galvin *et al*., 1997; Waters *et al*., 1999; Waters *et al*., 2000a; Waters *et al*., 2000b; Jonasson *et al*., 2003). Several authors report neutrophil infiltration, and some authors also report an increase in lymphocytes or macrophages in lamina propria during disease (Hamdy & Glenn, 1974; Hughes *et al*., 1975; Albassam *et al*., 1985). Systemic leucocytosis has been reported (Meyer et al 1975), but others report inconsistent results or no increase (Eriksen & Andersen, 1970; Kinyon *et al*., 1977). Galvin *et al*., (1997) claimed that spirochaetes are non-invasive organisms and that phagocytic activity would be of little benefit, but that release of inflammatory mediators might contribute to the inflammatory process. Mast cells appear to play a limited role, as concluded by experimental inoculations in mice (Nibbelink & Wannemuehler, 1990). Data indicate that a specific proliferative T cell response is induced in the mucosa following infection. An increase in the percentage of $CD8^+$ T cells in peripheral blood and in the mucosa in response to vaccination and experimental infection has been demonstrated (Waters *et al*., 1999; Waters *et al*., 2000a; Waters *et al*., 2000b; Jonasson *et al*., 2003). In contrast, an increase in the percentage of CD 4^+ and a decrease in $CD8^+$ cells were observed in peripheral blood, colonic lymph node, epithelia and lamina propria in experimental challenge studies (Galvin *et al*., 1997). Little is known about the cytokine and APP response to infection. Experimental intravenous injections with *B. hyodysenteriae* endotoxin resulted in increased levels of IL-6 but no TNF activity was recorded (Nibbelink *et al*., 1997). A TNF-like activity has been identified in serum from swine infected with *B. hyodysenteriae*, and the authors speculated that TNF might contribute to necrosis and vascular thrombi. Further, an increase in IL-1, experimentally induced in cell cultures, would contribute to mucus secretion (Greer & Wannemuehler, 1989). The humoral response has been more extensively studied and several studies focus on the specific antibody response. An increase in circulatory IgG, IgA and IgM and in local IgA is seen in response to infection (Rees *et al*., 1989). However, opinions differ regarding whether specific serum antibodies are protective or not (Eriksen & Andersen, 1970; Joens *et al*., 1979; Rees *et al*., 1989). Sera from convalescent pigs provided local protection against

subsequent challenge in colonic loops, possibly by complement components and serum IgG secreted through microscopic lesions in the intestine (Joens *et al*., 1985). Colonic washings containing specific IgA inhibited growth of *B. hyodysenteriae in vitro* (Joens *et al*., 1984). Additional evidence is needed to demonstrate the sIgA-mediated protection from swine dysentery (Galvin *et al*., 1997). The increased amount of total circulatory antibodies following infection suggests that B cells specific for other antigens are also activated (Galvin *et al*., 1997).

General aspects on diagnoses

For the study of infectious diseases, a reliable demonstration of the causative organism is crucial. Hence, analytical methods should preferentially be well established, have good specificity and sensitivity, and good reproducibility. The diagnosis of bacterial diseases is usually based on direct demonstration of the microbe by techniques such as cultivation and PCR, or by indirect methods such as necropsy and serology. Each of these techniques has different advantages and limitations. Thus, to be able to choose the most adequate diagnostic method in a given situation, basic knowledge about the techniques as well as the particular microorganism is necessary. However, the demonstration of a certain microbe and simultaneous occurence of a certain disease do not necessarily imply a causal relationship (Evans, 1976). Thus, diagnosis also includes the interpretation of the results from the diagnostic investigation in relation to clinical signs and current information about the disease.

Diagnosis of *Lawsonia intracellularis*

L. intracellularis is a member of the *Proteobacteria*, family *Desulfovibrionaceae*, genus *Lawsonia* and up to now the only known species of the genus (Gebhart *et al*., 1993; McOrist *et al*., 1995a). It is most closely related to *Desulfovibrio desulfuricans*, a non-pathogenic organism that is found in freshwater, soil, and intestines of animals (Holt *et al*., 1994). For several years, the causative organism of porcine proliferative enteropathy was an enigma. A 1.5 x 0.35 μm intracellular organism was observed in silver stained sections, and culture consistently yielded profuse growth of *Campylobacter*. Several *Campylobacter* species have been proposed as the causative organism, but experimental inoculations were not successful and Koch's postulate was not fulfilled (Lawson & Gebhart, 2000). A monoclonal antibody that specifically bound to the intracellular organism was produced (McOrist *et al*., 1987). Part of the chromosomal DNA and 16S rRNA were sequenced and a novel organism was proposed (Gebhart *et al*., 1993; McOrist *et al*., 1995a). Subsequently, specific primers for single and nested PCR were constructed (Jones *et al*., 1993b; McOrist *et al*., 1994a). *L. intracellularis* grows in a commercial rat enterocyte cell line under micro-areofil conditions (Lawson *et al*., 1993b). The successful culture has only been reported by a few laboratories (Stills, 1991; McOrist *et al*., 1993; Joens *et al*., 1997) and diagnosis is based on necropsy, PCR or serology. Although PCR has a good sensitivity when it is performed on purified DNA, a decreased sensitivity is seen in complex biological samples because of the presence of inhibitory factors. Amplification

might be inhibited by interference with the cell lysis step, binding to the template or nucleotides, or by interaction with the enzyme. Certain specimens, such as blood, soil, cheese and faeces, contain more inhibitors but only a few of those have been identified (Wilson, 1997; Lantz *et al*., 2000). The inhibitors vary between different kinds of samples and probably also between animal species, and the degree of inhibition appears to vary between different microorganisms (Lantz *et al*., 2000). In faeces, several different inhibitors seem to be present (Lantz *et al*., 1997).

In the diagnosis of *L. intracellularis* by PCR, inhibition is poorly defined. Some authors propose that diluting and boiling of the sample circumvents inhibition (McOrist *et al*., 1994a; Möller *et al*., 1998). Most studies claim that PCR has good sensitivity, without any further specifications (McOrist *et al*., 1994a; Cooper *et al*., 1997; Jordan *et al*., 1999). Instead, variations in the outcome of analyses on faecal samples from experimentally inoculated animals is usually ascribed to an intermittent shedding of the organism (McCormick *et al*., 1995; Knittel *et al*., 1998). In PCR for other microorganisms, several methods for diminishing the effect of inhibitors or to remove them from the sample have been described. Optimising the PCR system will increase sensitivity and specificity and the use of ”Hot start” will prevent the formation of unspecific PCR products, but these measures will not usually overcome the inhibition (Williams, 1989; Lantz *et al*., 2000). Some enzymes are less sensitive to inhibition and *Pwo* DNA polymerase and r*Tth* DNA polymerase is capable of amplifying DNA in the presence of 0.4% faeces without reduced sensitivity (Lantz *et al*., 2000). Dilution increases the distance between the inhibitory factors and the target, thereby decreasing possible interactions. Centrifugation could remove soluble inhibitors, but some might instead be co-concentrated with the target. Lytic methods such as boiling and/or incubation with proteinase K and sodium dodecyl sulphate (SDS) increase the accessibility of DNA and inactivate some heat labile inhibitors, proteinases and polypeptides. Methods based on filtration or immunomagnetic capture concentrate or specifically bind DNA. The remaining sample containing the inhibitors is removed and DNA is subsequently released and subjected to PCR (Lantz *et al*., 2000). For instance, a method based on the binding of DNA to a silica membrane was reported to have a sensitivity of 10 to 100 *Helicobacter pylori* per tube (Lantz *et al*., 2000). DNA can further be purified by phenol/chloroform extraction followed by ethanol precipitation. However, the target might bind to substances in the sample with a subsequent reduction in sensitivity. The preparation of large amounts of samples to concentrate a low amount of target might also concentrate inhibitors. A large amount of unspecific DNA might also interfere with PCR by random binding of the primers (Rossen *et al*., 1992; Wilson, 1997).

PCR products can be detected by determining the size or sequence of the fragment. The size can be determined by ethidium bromide staining of an agarose gel or by polyacrylamide gel electrophoresis (PAGE) with a sensitivity of 1-10 ng DNA. Agarose gel electrophoresis is suitable for products with a size from 200 base pairs (bp) to 50 kbp and PAGE from 5 bp to 500 bp. Hybridisation with a digoxigenin-marked probe increases the sensitivity 20 to 100 times (Lantz *et al*., 2000).

Under controlled conditions, competitive DNA fragments (MIMICs) have been used to correlate the yield of amplified DNA to the original number of target molecules. However, several factors can affect the result and the ratio of the mimic to the target DNA must be relatively close (0.66 to 1.5) to achieve an accurate estimate. For instance, the technique has been used to quantify nonculturable bacteria in soil (Lantz *et al*., 2000).

A serological test based on the binding of IgG to wells coated with antigen-containing cells, followed by the detection of antibodies by staining with fluorescein isothiocyanate conjugate, is commercially available. The test was concluded to be more sensitive than PCR ante mortem (Knittel *et al*., 1998; Ohlinger *et al*., 2000). Detectable levels of antibodies to *L. intracellularis* usually develop 14 days after stimuli and re-exposure is usually essential for maintaining a high level of antibodies (Knittel *et al*., 1998; Guedes *et al*., 2000). However, positive serology indicates that the animal has been exposed to the microorganism but does not indicate whether this exposure has resulted in disease (Knittel *et al*., 1998).

Experimental challenge studies

In the studies of pathogenesis of infectious diseases, experimental challenge studies are necessary. Certain aspects of a disease can be studied on material submitted from the field, but factors such as feed, management, other infections *etc.* vary substantially between herds and will most probably interfere with the study (Madec & Buddle, 2002). Thus, it is difficult to obtain repeatable results from which conclusions can be drawn. However, experimental challenges are time consuming, expensive and difficult to perform. As discussed above, most diseases of today are of multifactorial origin and the interacting factors are often poorly defined (Hentges, 1970). Therefore, experimental reproduction of disease might be hampered by lack of certain essential interacting factors. Conversely, experimental inoculations enable the identification of those factors by the exclusion or addition of single factors.

Aims of the present studies

The general aim of this project was to study enteric diseases in growing pigs, with special reference to diseases caused by *Brachyspira hyodysenteriae* and *Lawsonia intracellularis*. This objective was further outlined in the following specific aims:

- To identify the most important microbiological agents causing diarrhoea in Swedish grower pigs (I).

- To develop a fast and reliable method for the diagnosis of *L. intracellularis*. To construct an internal control to demonstrate inhibition of the PCR reactions and evaluate different preparation methods (II, III).

- To develop a pig model enabling sequential *in vivo* examinations of the intestine during disease. The demand for using a limited number of experimental animals without reduction of the methodological accuracy should be fulfilled (IV, VI).

- To establish a procedure for experimentally induced swine dysentery (V).

- To use the novel animal model in studies concerning the pathogenesis of swine dysentery (VI).

Aspects on materials and methods

The materials and methods used are detailed in each paper but are based on altogether 20 surgical operations; 89 endoscopies and ~800 biopsies; 242 necropsies; 369 serum sample analyses for cortisol, haptoglobin and SAA each; 201 analyses of blood samples for white blood cell count; 1816 cultures for *Brachyspira* sp; 430 cultures for other bacteria; 72 parasitological examinations; 206 investigations for diversity of the coliform flora; 87 examinations for rotaviruses; 220 analyses for microflora-associated characteristics, 1498 PCR analyses on tissue samples; and 1300 single and/or nested PCR analyses on faecal samples. A summary of specific aspects are presented below.

Diarrhoea in the growing pig – a comparison of clinical, morphological and microbial findings between animals from good and poor performance herds. (I).

The herds and animals in this study were selected as representative of the particular problem, *i.e.* they should suffer from poor performance and grower scour. In contrast, the control herds and animals should not experience these problems. The figures were obtained from the Swedish Animal Health Service database that covers approximately 95% of the Swedish piglet-producing herds. The herds were situated in the mid-east and mid-west parts of Sweden, where 16.5% of the Swedish swineherds are located. It was important that the regions had access to quality assessed laboratories within a short distance from the herds. Further, the laboratories should have well-established collaboration routines with the reference laboratory (National Veterinary Institute, Uppsala, Sweden) performing the histological and microbiological investigations. To exclude post-weaning diarrhoea, the pigs selected should have been weaned at least two weeks prior to submission and to ensure that the pigs were in the acute phase of the disease, diarrhoea should have commenced within two days. Other diseases that might obscure the findings at necropsy should not be apparent, therefore, pigs that had not reached market weight at an age of 13 weeks or had been treated with antibiotics were excluded. It is possible that the ability of the farmer to immediately detect sick animals varied, as indicated by a difference in weight recorded between the selected experimental and control animals. Another explanation for the difference in weight could be that overt diarrhoea was preceded by a period of subclinical disease. Some owners may also have been more prone to submit animals of low weight. However, the mean age in the herd is calculated per three-month period in the official control and these values would not be expected to match the values for individual pigs on a single occasion. Some bias could still be present in the selection of the animals, but as indicated by the necropsy results, this did not appear to interfere with the results.

In post mortem studies of the intestines, necropsy must be performed immediately because of the rapidly occurring autolysis due to different enzymes (Kumar et al 1997). Therefore, the animals were submitted alive and euthanised by stunning with electricity and exsanguination immediately prior to necropsy.

A limited amount of sample can be a bottle-neck for further analyses (Lantz *et al*., 2000). Cotton swabs were used to obtain bacteriological samples, whereas stool samples were collected for analysis of parasites and viruses. A swab often contains a very limited amount of faeces and at least 100 particular organisms/g faeces should be present for reliable results (Wilson, 1997). Most bacteriological analysis begins with a pre-enrichment from which suspected colonies are selected for further examination. If a bacterium is present in low numbers or grows slowly, it might be overlooked. For instance, if a culture contains small numbers of *Campylobacter jejuni* and large numbers of *C. coli*, the colony picked for further identification will probably be *C. coli*. In addition, several techniques such as blotting or PCR could be combined with culture to increase sensitivity (Nesbakken *et al*., 1991). The standard methods applied at the National Veterinary Institute were chosen as they are standardised, cheap and easy to perform. In addition, the number of organisms excreted during overt enteric disease are probably sufficient to be detectable.

The use of a mimic to detect polymerase chain reaction-inhibitory factors in feces examined for the presence of *Lawsonia intracellularis* (II).

Diagnosis of *Lawsonia intracellularis* performed by PCR, serological and post mortem examination, with special emphasis on sample preparation methods for PCR. (III).

Detection by PCR is based on four steps (Lantz *et al*., 2000): sample collection; sample preparation; amplification of the nucleic acid; and detection of the product. As discussed above, sample size and the number and distribution of the microorganism in a sample are factors influencing sensitivity. Some preparation methods are time consuming and difficult to apply on large amounts of samples, which renders them inappropriate for routine diagnosis. Furthermore, several controls need to be included: – negative, to show possible contamination; positive, to control PCR conditions and reagents; and internal, to demonstrate the presence of inhibition and reaction conditions in single tubes. If reaction conditions are not optimal, unspecific reactions might occur and structures such as primer dimers can develop (Williams, 1989).

For the identification of *L. intracellularis* by PCR, the specificity of the primers is crucial. Known sequences collected in a database are compared to the target sequence and should differ in at least two nucleotides. The specificity is tested by hybridisation techniques, nested PCR and against DNA from related microorganisms (Jones *et al*., 1993b). The primers and reaction conditions used in the present study have previously been tested against: porcine intestinal DNA; *B. hyodysenteriae*; *Brachyspira* sp.; *C. hyointestinalis*; *C. mucosalis*; *C. coli*; *C. jejuni*; *C. fetus*; *C. concisus*; *C. laridis*; *C. cinaedi*; *C. fennelliae*; *C. cryaerophila*; *C. sputorum*; *Cl. perfringens* types A, B, C; *S. typhimurium*; and *E. coli*. No cross reactivity was reported (Gebhart *et al*., 1991; Jones *et al*., 1993a; McOrist *et al*., 1994a; Cooper *et al*., 1997; Möller *et al*., 1998). However, the primers have not

been tested against the most closely related organisms *Desulfovibrio desulfuricans* and *Myxococcus xanthus* (Gebhart *et al*., 1993). Further, the possibility still exists that some unknown organism carries a sequence that could cause false positive reactions. In addition, primers directed to 16S rRNA have been constructed, but these have not been as extensively used as the primers directed against the chromosomal DNA (McOrist *et al*., 1994a).

Studies of the sensitivity are usually tested by a known amount of organism that is serial diluted, prepared and subjected to PCR. The highest dilution (*i.e.* the lowest amount of organisms) that results in a visible amplicon is determined as the detection limit. If the organism cannot be cultured, indirect measures must be utilised. In this study, a mimic containing a piece of DNA consistent with the primer sequence from *L. intracellularis* was constructed and a known quantity was used. This gave good apprehension of the sensitivity in the final solution prepared for PCR. However, when samples were spiked prior to preparation consistent results was difficult to achieve. Probably, the mimic plasmid behaves very different from the microorganism in the unprepared faecal sample.

The specificity of the commercially available, serological test used in this study has previously been tested against *B. hyodysenteriae*; *B. pilosicoli*; *B. innocens*; *S. typhimurium*; *S. choleraesuis*; *C. mucosalis*; and *C. hyointestinalis:* no reactions were observed. However, non-specific reactions have been observed in sera from gnotobiotic pigs (Knittel *et al*., 1998).

Intestinal cannulation: Model for study of the midgut of the pig. (IV).

Experimentally inoculated, cannulated pigs have not previously been used in the study of infectious diseases. Thus, it was necessary to ascertain that the cannulation *per se* does not interfere with the study. The surgical procedure, as well as possible secondary infections, cause an immunological response that must have vanished before the experimental inoculation can take place. The use of antimicrobials was avoided by strict aseptic surgical procedures and the inflammatory response was monitored by measurements of SAA, haptoglobin, white blood cell counts and serum cortisol. Cortisol is used as a stress parameter and was included to assess the stress the animals were subjected to during surgery and endoscopy. However, cortisol quickly increases in response to all kinds of stress, such as restraint during blood sampling. This could have been circumvented by the use of an indwelling catheter. On the other hand, the adverse effects of a second surgical procedure, an increased risk of bacterial infections, and interference with the inflammatory response were considerable. As the measurement of cortisol was not the main purpose of the study, the use of an indwelling catheter was dismissed and when possible, samples were collected during anaesthesia. However, the results regarding cortisol must be interpreted with caution. The general anaesthetic used during endoscopy might also influence the animal. Halothane was chosen as inhalation anaesthetic as it is commonly used and cheap. Because repeated anaesthesia can induce liver necrosis, glutamate dehydrogenase and γ-glutamyltransferase were analysed. In addition, the intestinal

cannula or the partial resection of caecum might alter the gut motility, or the intestinal microenvironment and indigenous gut flora. Therefore, the transit time of the digesta and the diversity of the coliform flora were examined.

To enable endoscopy through the cannula, it is necessary to empty the gut. In humans, this is achieved by a 24-hour starvation period combined with administration of laxative. In this study, the repeated endoscopy combined with the drowsiness after anaesthesia would have meant a prolonged starvation for the animals. Therefore, endoscopy was restricted to every two days and the amount of anaesthetic drug reduced. The starvation period was shortened to approximately 18 hours prior to endoscopy and the adjacent meals were given earlier and postponed for some hours, respectively. Although the gut was not completely emptied, inspection of the mucosa and biopsy sampling were still possible. Endoscopy of the large intestine is difficult to perform in the pig since the anterior part of the colon is coiled in a double helix. Consequently, the entire spiral colon is easily pushed forward when the endoscope is introduced into the intestinal lumen. However, this problem decreased with increased experience. As with every species, it is essential to always view the next part of the gut during insertion. The quality of the 2 x 4 mm-sized biopsy specimen for morphological examination varied, but when the specimen was placed in formalin without previous mounting, the quality improved substantially. Possibly, differing resistance in the paper and in the tissue made them difficult to cut simultaneously.

Experimental swine dysentery - comparison between infection models and studies of the acute phase protein response to infection. (V).

The outcome of an experimental bacterial challenge depends on a number of factors. The importance of the commensal microflora has been convincingly shown, and therefore pigs from conventional herds were used. However, conventional apparently healthy pigs might be subclinical carriers of potential pathogens whose impact might be difficult to assess (Raynaud *et al*., 1980; Fisher & Olander, 1981; Lawson & McOrist, 1993a). The use of specific pathogen-free pigs could circumvent this problem to some extent. In Sweden, SPF pigs are declared free from the diseases listed in the A-list of International Office of Epizootics, and from Aujeszky's disease, atrophic rhinitis, transmissible gastro-enteritis, porcine epidemic diarrhoea, porcine reproductive and respiratory syndrome, *Brachyspira hyodysenteriae* and salmonellosis (Melin & Wallgren, 2003). The status regarding other microorganisms are unknown. The pigs in the present study originated from herds with a well-known health status that had been supervised and inspected by the University swine practising veterinarians once a month for at least ten years.

The role, if any, for other *Brachyspira* species in swine dysentery is not known. On the University farm, no *Brachyspira* species at all have been detected during the last ten years and the animals should be fully susceptible to infection. On the other hand, this herd has been the subject of extensive breeding programmes, and

it cannot be excluded that freedom from infection with *Brachyspira* sp. may depend on a hereditary resistance.

To test the detrimental effect of a low pH in the stomach, two pigs were inoculated through a caecal cannula and eight pigs were given antacids prior to inoculation. As different practices are utilised (Kinyon *et al*., 1977; Raynaud *et al*., 1980), different routes for administration of the inoculum were tested. However, no congruity seems to exist.

Dexamethasone has been extensively used as a suppressor of the immune system prior to experimental inoculation. The dosage used is recommended for terapeutical purposes in pigs and consistent with other studies (Knittel *et al*., 1998). However, no study has convincingly proved that the dosage and duration of treatment used actually achieves this effect.

Straw is a common bedding material, as the heat-insulating and water-absorbing properties are good and it activates the animal (Fellström, 2001). However, straw also has nutritional properties and contains a large amount of cellulose and hemicellulose, which might influence the fermentation in the large intestine (Pluske *et al*., 1996). Straw was therefore replaced with a fibre-fur blanket before inoculation.

Dysentery is defined as an inflammation of the intestine, characterised by pain, rectal tenesmus, intense diarrhoea with the frequent passage of small amounts of mucus and blood, and symptoms of toxaemia (Blakiston's New Gould Medical Dictionary, 1956). The term swine dysentery has been used for all different clinical manifestations of disease caused by *B. hyodysenteriae*, although not all signs described above may be present in all, or even in most animals (Lee *et al*., 1976; Meyer, 1978; Raynaud *et al*., 1980). This terminology was also applied in the present study.

One hypothesis was that an alteration of the gut flora would enhance the establishment of *B. hyodysenteriae*. As it would be useful to be able to measure such an alteration, measurements of the diversity of the coliform flora and of certain microflora-associated characteristics were included.

Consecutive pathological and immunological alterations during experimentally induced swine dysentery – a study performed by repeated endoscopy and biopsy samplings through an intestinal cannula. (VI).

In this study, a new anaesthetic protocol was applied and the halothane used in previous studies was replaced with isofluran, an inert drug that is not metabolised in the body. As no systemic effects were suspected, the measurement of liver parameters was omitted. This anaesthetic gave a smooth and fast recovery: after endoscopy the pigs usually tried to raise immediately on return to the pen. Further, a new protocol for pain relief during and after surgery was applied with very

promising results. These studies are reported elsewhere (Malavasi *et al*., 2003). The lesions during the experimentally reproduced swine dysentery in the present study did not differ from previous descriptions (Wilcock & Olander, 1979a). Hence, it was concluded that the use of endoscopy through a caecal cannula did not influence the development of the intestinal lesions. Endoscopy was performed essentially as previously described, but a new protocol for emptying the gut before endoscopy was applied. This protocol is based on an osmotically active laxative and was given before the first endoscopy. In some pigs, this produced sufficient emptying of the gut, but in some cases, an additional enema was given. However, the intestines were not completely emptied during endoscopy and the mucosa was usually visualised in snap-shots. This was sufficient to allow inspection of the proximal part of the spiral colon and biopsy sampling. During overt dysentery, the emptying of the gut was not a problem. Further, at three occasions, the ileum was accidentally penetrated instead of the colon. As discussed in paper IV, this is usually difficult to achieve and it is possible, that the tension of the ileal papilla was reduced during overt disease.

Morphometrical studies of the intestine are difficult to perform as the size of the biopsies do not provide any surplus material, thus biopsies must be well oriented and cut trans-sectionally. A good orientation is usually achieved by mounting the biopsy on a paper, but based on our previous experience this was excluded. As a result, some parts of the biopsies were not adequate for this purpose and only five well-oriented crypts were included. However, four sections covering the base and the surface were measured in each area between two adjacent crypts, which allowed thorough study of the different compartments in lamina propria without inclusion of the epithelium. As only a few animals were examined, further studies are needed to draw proper conclusions.

Results and Discussion

The diagnosis of *Lawsonia intracellularis*

In the diagnosis of *L. intracellularis*, necropsy has previously been the only method available and the "gold standard" (Cooper & Gebhart, 1998; Lawson & Gebhart, 2000). Post-mortem examinations enable the linkage between clinical signs and the lesions found, which is important in showing causative relationships: the main disadvantage being that the animal must be euthanised. This represents an economic loss that together with the labour- and time-consuming necropsy makes the method expensive (Zhang *et al*., 2000). Thus, necropsy is usually performed on a limited number of animals that must be carefully selected. Further, in the absence of macroscopic lesions, microscopic lesions can be over-looked and discrepancies between the results occur (Zhang *et al*., 2000). Among the 66 pigs in Paper I, 48% tested positive for *L. intracellularis* based on necropsy and the presence of the bacteria in silver stained sections, whereas 67% tested positive with nested PCR performed on faeces. In a study in UK by Thomson *et al*. (1998a), diagnosis was based on the presence of intracellular bacteria in silver stained sections and histological lesions, and proliferative enteropathy was confirmed in 15% of the 85 units investigated.

PCR is a fast technique and the results can be obtained within one day (Jordan *et al*., 1999; Lantz *et al*., 2000). PCR is also a sensitive and specific method and in theory, it is possible to detect a single DNA molecule (Saiki *et al*., 1988). However, the method does not differentiate between dead or living microorganisms. Depending on the aim of the investigation, this could be either an advantage or a disadvantage. PCR is superior if the microorganism can be expected to succumb during non-optimal transport conditions or if the animal has recently been treated with antibiotics. On the other hand, the evaluation of a treatment might be difficult (Altwegg, 1995). Usually, faecal samples are subjected to primary culture from which a colony is selected for PCR (Altwegg, 1995). L. intracellularis is difficult to cultivate, and therefore PCR is performed directly on prepared faecal samples (McOrist *et al*., 1994a; Holyoake *et al*., 1996; Jordan *et al*., 1999). Besides the obvious necessity for the presence of at least one microorganism in the sample, sensitivity varies depending on the amount of inhibitory factors, as demonstrated in Papers II and III. The presence of inhibitory factors can be monitored by the inclusion of internal controls in each tube (Siebert & Larrick, 1993; Ballagi-Pordány & Belák, 1996). In the absence of these controls, a sample in which the reaction has been inhibited will be judged as negative (Wilson, 1997; Englund *et al*., 1999). The inhibitors are suggested to act by one of three mechanisms: inactivation of the DNA polymerase by degradation, denaturation or reduction of the enzymatic activity; by degradation or capture of the nucleic acids; or by interference with the cell lysis step (Wilson, 1997; Lantz *et al*., 2000). The sensitivity for inhibitors varies between the DNA polymerases. In Paper III, the ability to sustain inhibition was tested on five polymerases and *Pwo* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) was

found to be superior to the others. However, the usefulness of this enzyme is limited by its 3'-5' exonuclease activity.

Further, different methods to separate the inhibitors from the DNA in the sample were evaluated. These methods included extraction by phenol/chloroform; buoyant density gradient centrifugation; binding of DNA to a silica membrane in combination with the inactivation and removal of the inhibitors; filtration; and binding of DNA to magnetic beads followed by removal of the inhibitors. In addition, different methods for cell lysis, such as boiling and incubation with proteinase K, were tested. To diminish the effect of the inhibitors, dilution of the sample or nested PCR can be used (Lantz *et al*., 2000). In addition, inhibitors can be neutralised by the boiling or by a component in the Inhibex-tablet (QIAamp® DNA Stool mini kit, Qiagen Inc., Valencia, California, USA). Extraction with phenol/chloroform, followed by dilution, resulted in good sensitivity for PCR diagnosis on tissue samples. An excessive amount of DNA seemed to account for some of the inhibition present in the undiluted sample (Rossen *et al*., 1992; Altwegg, 1995). However, none of the methods tested on faecal samples were sufficiently sensitive to detect low amounts of bacterial DNA. Some methods (*i.e.* boiled lysate combined with nested PCR and QIAamp® DNA Stool mini kit combined with single PCR) were sufficiently sensitive to detect clinical cases of proliferative enteropathy, as judged by the demonstration of the microorganism by at least two methods (*e.g.* necropsy) in each case. However, compared with the boiled lysate and nested PCR, the QIAamp® kit failed to detect the microorganism in 40% of the subclinically infected animals. The method did remove most of the inhibitors present in the sample, but it seems like some DNA was also lost in the sample preparation. However, as it was not possible to spike the samples prior to preparation, this could not be accurately demonstrated. To avoid false positives due to cross-contamination, precautions such as dedicated equipment and separation in time and room between the preparation of samples and analysis of PCR products were undertaken (Kwok & Higuchi, 1989; Belák & Ballagi-Pordány, 1993). Further, a negative control was included every fifth sample. Although the possibility of occasional false positives can not be excluded, they were probably not common, as none of the altogether 90 negative controls in the nested PCR showed positive.

The nature of the inhibitory factors is unclear. Some inhibitors have been studied and substances such as bile salts, large amounts of other bacteria, urea, hemoglobin degradation products, bilirubin and bile acids are suggested to cause inhibition in faecal samples (Lantz *et al*., 2000). Faeces from wild boar (*sus scrofa*) seem to contain more inhibitory factors than faeces from domestic pigs kept indoors, as illustrated in Paper II. Inhibited wild-boar samples contained more urobilinogen and less fibre, compared to samples with little inhibition from domestic pigs (data not shown). The inhibition in faeces has been suggested to be caused by binding of inhibitors to the DNA polymerase (Lantz *et al*., 2000). Since urobilinogen is the final breakdown product of hemoglobin, a known inhibitor that forms a stable complex with the polymerase (Lantz *et al*., 2000), this might be a candidate for further investigation. However, at present no method exists to remove urobilinogen from faeces. Bovine serum albumin, which is known to

reverse the inhibitory effect of hemin, does not relieve the inhibition from the degradation product bilirubin, a precursor of urobilinogen (Kreader, 1996).

The diagnosis of *Brachyspira* spp. based on culture has a good sensitivity and specificity (Fellström *et al*., 2001) and the isolation of the microbe in faeces and its presence in tissue correlates well (Jacobson *et al*., 2002). The method used in the present study for diagnosis of *Y. enterocolitica* was evaluated by Nesbakken *et al.* (1991) who determined that 5% of 50 samples were positive. However, with colony hybridisation techniques, 45.8% of 24 samples were positive. Hence, in the present studies pigs with low-degree infections could have falsely been judged as negative.

The detection of subclinical carriers is a clinical and diagnostic problem. Usually, only a few animals harbour the microorganism, without any clinical signs (Fisher & Olander, 1981; Hampson *et al*., 1992). Thus, a large number of animals must be sampled to ascertain that at least one subclinical carrier is included. These carriers may also shed the microorganism intermittently, which means that in addition to sampling the right animal, sampling must be performed at the right time. As previously discussed, a sufficient number of organisms must have been shed if they are to be readily detected. A similar problem arises in the case of histological examinations, a 4-6 μm thick slice exhibiting microscopical lesions should be picked from a 30 m long, macroscopically normal intestine from an apparently healthy animal (Jordan *et al*., 1999).

Diarrhoea in growing pigs

The main microorganisms involved in enteric diseases in Swedish grower pigs are *Lawsonia intracellularis* and *Brachyspira pilosicoli*, as shown in Paper I. This is supported by a recent epidemiological study (Löfstedt, 2003), where 64% of growing pigs with diarrhoea were positive for *L. intracellularis* and 27% for *B. pilosicoli* ($p<0.001$). Further, the results are in consistency with other epidemiological investigations in Europe (Möller *et al*., 1998; Thomson *et al*., 1998a). In the present study, the demonstration of *L. intracellularis* was significantly ($p<0.001$) related to herds with poor performance and to animals with diarrhoea ($p\leq0.05$). In a previous study on animals submitted for necropsy from different herds and for different purposes (Jacobson *et al*., 2000), a relationship ($p<0.001$) between the occurence of diarrhoea and the demonstration of *L. intracellularis* by PCR performed on tissue samples was observed. However, of 25 pigs with a case history of poor growth only, 12 were negative for *L. intracellularis* (data not shown). This indicates that diarrhoea in growing pigs is probably a more reliable indicator of proliferative enteropathy, than the commonly held belief that poor growth is the main clinical sign. In the study described in Paper I, it was further concluded that the clinical symptoms appeared closely related to the extent of intestinal damage. Whether this implies that infection with *L. intracellularis* often causes more severe lesions in the intestine, or that the bacterium is commonly involved in mixed infections, is not known. In 10 cases, *L. intracellularis* was the only pathogen found, and in 19 cases, other presumptive pathogens were also demonstrated.

Table 4. The correlation between the frequency of isolation of entero-pathogenic bacteria and the occurence of gross intestinal lesions in pigs from the ”poor performance herds”. n.s. = not significant. The significance was calculated by the chi square test.

	Gross lesions		
	Yes (n=26)	No (n=28)	significance
Lawsonia intracellularis	18	11	$p<0.05$
Brachyspira pilosicoli	11	9	n.s.
Escherichia coli	10	4	$p<0.05$
Campylobacter jejuni	4	10	n.s.
Campylobacter coli	16	10	n.s.

The interpretation of the findings of other potentially pathogenic microorganisms is obscure. An attempt was made to correlate the macroscopic lesions and the microorganisms detected (Table 1). The lesions in the small intestine were significantly correlated to *L. intracellularis* and *E. coli*, whereas no correlation was determined between *B. pilosicoli* and the lesions in the large intestine. However, these correlations must be carefully interpreted. Enterotoxic *E. coli* is not known to induce any lesions in the intestine (Holland, 1990) and the present result is probably related to the concomitant demonstration of *L. intracellularis* in six of the eight cases. In addition, the macroscopic lesions induced by *B. pilosicoli* are less prominent and consist of a slightly enlarged, thin walled and flaccid colon (Trott *et al*., 1996a).

The interpretation of the findings of *Campylobacter jejuni* and *Yersinia enterocolitica* in enteric diseases in pigs is difficult. Previous experimental reproductions of disease with clinical isolates of *C. jejuni* have been unsuccessful (Lawson & McOrist, 1993a). However, these pigs were probably suffering from infection with *L. intracellularis*. The occurence of strain-specific differences in pathogenicity is not known, although variation in strains between species has been reported (Broman, 2003). *Y. enterocolitica* exhibits a strain-specific pathogenicity but the possible involvement of this microbe in enteric disease in pigs has not been extensively studied. In a study by Zheng & Xie (1996), *Y. enterocolitica* was more frequently isolated in diarrhoeic pigs, whereas, Schiemann (1988) claimed that it is very unlikely that piglets develop serious disease following infection with *Y. enterocolitica*. In one herd in the present study, *Y. enterocolitica* was isolated in four pigs, three of which showed an profuse growth of the bacteria. In one pig, it was the only pathogen found. Two pigs also had crypt abscesses in the small and large intestine, one of which had a concomitant infection with *L. intracellularis*. Hence, the results from this study cannot exclude the possibility that *Y. enterocolitica* and *C. jejuni* can occasionally induce overt disease. However, they are probably not common causes of diarrhoea in growing pigs. Of the other microorganisms included, rotavirus and parasites were only shed to a lesser extent.

Further, a correlation between the presence of the commensal *C. coli* (Kinyon *et al*., 1977; Lawson & McOrist, 1993a) and good performance was demonstrated.

As discussed above, several presumptive pathogens were found in the poor performance herds but they were not considered as the primary cause of disease. Possibly, these microbes are opportunists that are normally present in low numbers but increases if the gut flora is disturbed. Thus, detectable levels of these microbes would indicate poor intestinal health. Alternatively, an increased amount of various pathogens might arise because of poor environmental hygiene (Löfstedt *et al*., 2000). If disturbances in the intestinal eco-system increase the risk for enteric diseases, it would be important to monitor these alterations. This would enable identification of perturbing factors and the establishment of proper prophylactic measures for avoiding them. As discussed in Paper V, several methods are available. Attempts have been made to monitor alterations in the intestinal flora by non-selective aerobic and anaerobic culture during experimental inoculation with *B. hyodysenteriae* (Durmic *et al*., 1998). However, the results are difficult to interpret. The colon might contain 10^{10} to 10^{11} bacteria/ g ingesta (Durmic *et al*., 1998; Leser *et al*., 2000), of which >99% are anaerobes (Rolfe, 1984) and only 20-40% can be cultivated at present (McCracken *et al*., 2001). Thus, if the alteration does not have a profound effect on the flora, it will probably not be detected. Another approach is the monitoring of selected indicator bacteria, such as the coliform flora, that might be affected by alterations in other bacterial populations. This method is established and much experience has been gained in studies of post-weaning diarrhoea (Melin *et al*., 2000a, Melin *et al*., 2000b). However, in the present studies no changes in the diversity were apparent. Other studies also indicate that the coliform flora remains stable in grower pigs (Kühn *et al*., 1995). In addition, the microbial conversion of certain substances in the intestine, *i. e.* the microflora associated characteristics, can be monitored. Some of these reactions are probably performed by a limited number of bacterial species and a change in these populations would result in altered conversion of the substrate (Midtvedt, 1999). In Paper V, some alterations in metabolism occurred in response to feeding with soybean meal and to swine dysentery. However, the number of samples analysed was to few to enable any extensive conclusions to be drawn. Further, different techniques for molecular fingerprinting of the intestinal flora, such as terminal restriction fragment length polymorphism (T-RFLP) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE), might be used (Leser *et al*., 2000; Simpson *et al*., 2000). These techniques overcome the difficulties of a non-cultivable flora. A fluctuating T-RFLP pattern has been reported after experimental inoculation with *B. hyodysenteriae* and T-RFLP was considered more sensitive than DGGE (Leser *et al*., 2000). However, as these techniques are based on PCR performed on faecal samples, the problems with inhibition must be considered.

No comparison between the different methods has apparently been performed regarding the possibility to use them as markers for a healthy gut flora and monitoring of changes during disease.

Pathogenesis of *Brachyspira hyodysenteriae*

The external barriers in the intestine clearly play a prominent role in the infection with *B. hyodysenteriae*. The results presented in Paper V indicated that gastric pH does not have a major influence on the onset of infection. Mucus plays a prominent role in the disease, but it is unclear whether excessive mucus production is an advantage or disadvantage for the bacteria. A low pH (6.0 ± 0.3) in the large intestine has been suggested to have an antibacterial effect with respect to *B. hyodysenteriae* and *E. coli*, whereas a pH of 7.0 ± 0.3 is considered to predispose for dysentery (Prohászka & Lukács, 1984). Of note is that pigs from the healthy herds had a mean pH in the large intestine of 6.5 and the corresponding value in pigs with diarrhoea was 7.1 (Paper I). However, the measurements were performed with litmus paper that only renders approximate values.

Substantial evidence has gathered that both feed and the commensal gut flora plays an important role in swine dysentery. This was further elucidated in the present work, since soybean meal enhanced the experimental reproduction of swine dysentery. Soybean meal and diets based on soybean and corn have previously been shown to predispose for post-weaning diarrhoea (Armstrong & Cline, 1977; Newport, 1980; Dewey, 1993). The suggested mechanisms include hypersensitivity reactions, interference with the mineral bioavailability and digestibility of proteins and carbohydrates, binding to receptors and fluid accumulation caused by prostanoids (Jager *et al*., 1986; Huisman & Jansman, 1991; Reddy & Pierson, 1994; Dréau *et al*., 1994). Thus, feed may influence intestinal flora and alter the fermentation pattern, pH, dry matter or nitrogen content in the large intestine (Newport, 1980; Makinde *et al*., 1996). In addition, some studies report that feed containing a high level of soluble non-starch polysaccharides results in an increased viscosity, an increased amount of fluid, a low pH and an increased amount of coliforms in the intestines (McDonald *et al*., 2000a; McDonald *et al*., 2000b). This might in some way create favourable conditions for *B. hyodysenteriae*. Neef *et al* (1994) suggested that feed including 38% soybean meal and tapioca stimulated the growth of attaching and effacing *E. coli* present in the normal intestinal flora, thus causing histopathological lesions in the large intestine that predisposed for other infections. Further, lectins are reported to damage the small intestinal epithelium in the presence of *E. coli* (Hillman *et al*., 1996).

Experimental inoculation with *Brachyspira hyodysenteriae*

Clinical disease is often difficult to reproduce under experimental conditions. In infections such as salmonellosis, inoculation of gnotobiotic pigs with a mutant strain of *S. typhimurium* prohibited subsequent infection by a virulent strain (Trebichavský *et al*., 1997). In *B. hyodysenteriae* infection, the commensal flora seems to play a more complex role. In the study reported in Paper V, the inclusion of 50% soybean meal in group-housed pigs induced dysentery in 9 out of 9 animals following challenge. When the same protocol was applied to pigs of the same origin but kept in single pens none developed dysentery. Subsequently, the

pigs were moved between the pens four times daily, and then 50% of the pigs developed disease. This might indicate that besides a provocative feeding regimen, an additional factor that is transmissible through the faeces is needed; however, it was not possible to relate the development of disease to the shedding of *B. hyodysenteriae* (data not shown). Several bacteria are known to colonise the gut by binding to the mucus and degrading its glycoproteins (Sharma *et al*., 1995). In experimental inoculation of gnotobiotic pigs, commensal bacterial species such as *Prevotella* (formerly *Bacteroides*) produce enzymes that desulphate mucus (Robertson *et al*., 1993) and some species of *Bacteroides* are also known to predispose for dysentery (Meyer *et al*., 1975; Whipp *et al*., 1979). Sulphated gastric mucin has previously been suggested to inhibit the colonisation of *Helicobacter pylori* (Piotrowski *et al*., 1991). However, the subsequent inoculation of *B. vulgatus* only induced dysentery in one out of four pigs and the same result was seen when glycosulphatase was administered through the caecal cannula prior to inoculation with *B. hyodysenteriae* (data not shown).

The non-infectious causes of diarrhoea were not addressed in the present study. These have been thoroughly studied and are generally related to feed (Thomson *et al*., 2002). In rats, different diets have been shown to alter the villi and crypt morphology, as well as the composition and amount of intestinal mucus (Sharma *et al*., 1995). Some studies discuss the importance of processing the feed, such as pellets versus meal feed (Smith *et al*., 1988; Jörgensen *et al*., 2001; Thomson *et al*., 2002). Liquid feeding was beneficial in increasing digestibility by activating phytases and other enzymes in feed, and by reducing the viscosity, pH and rate of passage in the intestine (Brooks *et al*., 1996; Lindecrona *et al*., 2000). Further, an increased viscosity in feed predisposed for diarrhoea (McDonald *et al*., 2000b). A wheat-based diet with a high content of arabinoxylan seems to predispose for intestinal disorders (Thomson *et al*., 2002; Strachan *et al*., 2002) and an increased inclusion of barley was protective against *Salmonella*, since less separation of the digesta, increased production of lactic acid and increased retention time was achieved (Jörgensen *et al*., 2001).

The possibility to study a series of events in the intestine

The study of the pathogenesis of enteric diseases is challenging since the interactive events take place inside the body. Usually, to enable the study of this series of interactions, several animals are experimentally inoculated and euthanised at scheduled times, the intervals being decided based on existing knowledge about the disease. Because of the complexity of these interactions, individual variations will be seen at all levels (Takeuchi & Sprinz, 1967). To relate the findings for one individual taken at a certain time, to findings from other animals taken at other times, account for the individual variation, and to draw conclusive results from the studies, requires a large number of animals. In Paper IV, a method to overcome this problem is presented. Although some difficulties were experienced, the technique proved to be very useful in the study of *B. hyodysenteriae* pathogenesis. As evidenced by the results in Paper IV, no serious adverse effects that might affect the animal or interfere with the results occurred. However, the method was hampered by the failure to induce overt swine dysentery

by experimental inoculations in animals kept in single pens. Therefore, it would be advantageous to use the method for studying very early events in the course of disease. As there is a lack of knowledge regarding the interactions between the spirochaete and the external barriers of the gut, the possibility that the precautions taken to empty the gut or that alteration of the gut motility during anaesthesia can interfere with the establishment of the infection, could not be excluded. Hence, no endoscopy was performed until the first clinical signs of dysentery were evident.

Interactions between the host and the microbe

The pathogenesis of enteric diseases involves the entire series of interactions between the microorganism and the defence mechanisms of the body. In subclinical carrier pigs, it is uncertain as to where *B. hyodysenteriae* resides (Hampson *et al*., 1992). Theoretically, they could reside inside macrophages, free in the mucosa, in goblet or epithelial cells, in the crypt lumen, in association with mucus, or free in the intestinal lumen. However, most reports state that the spirochaete does not penetrate beneath the basal membrane, which would exclude localisation in the lamina propria, and it is rarely reported inside macrophages. A local invasion in the tissue would result in an inflammatory response, and penetration of the goblet cells is reported to result in excess mucus production, which is not a feature of the carrier pigs. However, if the microorganism is not associated with the mucosa, it has to multiply faster than it is expelled (Savage, 1980). If the microorganism is retained in the intestine without interacting with the mucosa, the immune system will not be challenged, which might explain the discrepancies seen in results regarding the local and systemic immunity following disease (Joens *et al*., 1985; Smith *et al*., 1991). The absence of protection reported in some studies on re-challenge in subclinical carrier pigs, or in pigs suffering only mild lesions, might suggest that the adaptive immune response has not been evoked (Lee *et al*., 1976; Hampson *et al*., 1992). Regardless of the clinical symptoms, an increased number of antibody secreting cells in inoculated but healthy animals, has been reported (Galvin *et al*., 1997).

An increase of the acute phase proteins during overt dysentery indicates that cells releasing IL-1 and IL-6 are activated early in the course of disease (V, VI). These cells might be locally situated macrophages, since IL-1 is mainly released from macrophages and IL-6 from macrophages and lymphocytes (Mims *et al*., 2001). Further, the increased number of monocytes (V, VI) might indicate that IFN-γ has been released from NK cells or T lymphocytes (Roitt *et al*., 1993; Mims *et al*., 2001). T-cells appear to play an important role in the defence against *B. hyodysenteriae* (Waters *et al*., 2000b). However, it is not known whether NK cells and the activation of the complement system are prominent features of the early response to *B. hyodysenteriae* infection. The explanation for the decreased number of intraepithelial lymphocytes noted (study VI) is unknown. Intraepithelial lymphocytes are considered to consist of mainly γδ T cells (Mims *et al*., 2001) but this subpopulation was not further investigated in the present study. Further, the mechanisms by which erosions and haemorrhage occur are not known. Hypothetically, the lesions could be caused mechanically by invading spirochaetes, by the action of different toxins or haemolysins, or by an aberrant

immune response to luminal antigen (Galvin *et al*., 1997). In the Warthin-Starry stained sections presented in Paper VI, invading spirochaetes were not detected near the lesions and it is still not elucidated whether invasion is a necessary feature of the infection (Wilcock & Olander, 1979b; Galvin *et al*., 1997).

The number of neutrophils in the intestines did not increase during the course of infection (Paper VI). Infiltration of neutrophils and macrophages has previously been reported to occur three days after the onset of diarrhoea (Albassam *et al*., 1985), but it is not clear if the infiltration is elicited by invading *B. hyodysenteriae*, by the lesions induced by the disease, or by secondary invading microorganisms. As discussed in Paper VI, a local infiltration of neutrophils might be related to tissue necrosis, and the number of circulatory neutrophils and monocytes increased at the same time as the onset of haemorrhagic dysentery (paper V). Only a minor increase was observed in animals with non-haemorrhagic diarrhoea, which could imply that only more severe lesions demand increased recruitment of phagocytes. Previous reports differ regarding the recruitment of neutrophils, as demonstrated by a systemic neutrophilia. In addition, the haemorrhagic diarrhoea elicits a metabolic response, seen as increased levels of glucose and lactate (Somchit *et al*., 2003).

Strategies to prevent disease

As evident from the discussion above, overt disease is the sum of a series of events, starting with the ingestion of the microorganism. To be able to prevent disease, one of two possible strategies can be chosen. The first is to eliminate the causative microorganism, which is a very efficient way to deal with some diseases (Batista & Pijoan, 2002). The second strategy is an indirect intervention, aimed at reducing the risk for disease by the removal of one or several predisposing factors (Madec & Buddle, 2002). For some diseases, such as salmonellosis, elimination of the causative agent is desirable. However, for diseases such as proliferative enteropathy and porcine intestinal spirochaetosis this is probably not an achievable goal within the near future. To achieve efficient intervention, the predisposing factors must first be identified. The only way to identify these factors is to understand the mechanisms by which they act and interact. To understand the underlying mechanism by which predisposing factors intervene in the development of the disease, it is necessary to understand how the disease arise, *i. e.* to understand the pathogenesis.

Conclusions

- The most important microorganisms involved in enteric diseases in Swedish grower pigs are *Lawsonia intracellularis* and *Brachyspira pilosicoli*.

- PCR technique is superior to necropsy and serology for detecting *L. intracellularis* in pigs with acute enteritis. The internal control developed is necessary to interpret the PCR-results since inhibitory factors are common in faeces.

- The animal model enables repeated endoscopy and biopsy samplings through a caecal cannula. The experimental procedure does not affect the general condition of the animal. The course of the enteric disease can thus be studied by macroscopic inspections and histological and immunological examinations of biopsy specimens.

- Among a number of factors, soybean meal and group housing of pigs facilitates the experimental induction of swine dysentery.

- A better understanding of the pathogenesis of swine dysentery is obtained since the morphological and immunological properties can be related to the sequential development of lesions in an individual. An increase of SAA, haptoglobin and the number of monocytes is related to intestinal haemorrhages. Histopathological alteration such as crypt hyperplasia, mucin production and erosions, as well as immunohistological characteristics, can be studied.

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