

On the Immune Response in Porcine Enteric Diseases

with Special Reference to Swine Dysentery, Proliferative
Enteropathy and PMWS

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Abstract

Enteric infectious diseases are a common problem worldwide among pigs. It is important to gain a better knowledge about the pathogenesis of the diseases and the local immune response, in which the intestine has a very important role. The aim of this thesis was to study the immune response in pigs infected with *Brachyspira hyodysenteriae*, *Lawsonia intracellularis* or Porcine circovirus type 2 (PCV2) that cause swine dysentery (SD), proliferative enteropathy (PE) and postweaning multisystemic wasting syndrome (PMWS), respectively. For SD and PMWS, material from both experimental infections and field cases were used, whereas material from pigs with PE was available from field cases.

The local immune response was studied by analysing the cytokine expression in intestines using the microarray and quantitative PCR techniques. For that purpose a porcine microarray with a limited number of cytokines representing different types of immune response was constructed and evaluated using *in vitro* stimulated porcine blood mononuclear cells, and porcine intestinal samples. A commercial available genome wide porcine cDNA microarray was applied for screening intestinal samples collected from experimental (PCV2) as well as field (*Lawsonia intracellularis*) studies.

The serum analyses showed that IL-1 β , IL-6 and TNF- α were increased during natural SD. In pigs with experimental PMWS, increased mRNA expressions in the intestine for IL-6, IL-10 and IFN- γ were found. The increased mRNA expression for IFN- γ was also observed in field cases. For pigs with PE no differences in cytokine levels in serum or intestine were found between control and case pigs.

cDNA microarray screening of intestinal samples from pigs with PE indicated that an uncomplicated infection with *L intracellularis* does not evoke an immune response and that the severe clinical signs of haemorrhagic diarrhoea can be regarded as a complication to the chronic form. In PCV2-infected pigs a marked up-regulation of interferon-stimulated genes was noticed. Comparison of two different isolates of PCV2 revealed differences in gene expression evoked by the two isolates. Expanded analyses using methods established in the present thesis will provide valuable insight into immune reactions elicited at porcine enteric diseases.

Keywords: pig, intestine, microarray, cytokine, *Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, PCV2, gene expression

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To my family

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List of Publications

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

- I Jonasson, R., Andersson, M., Råsbäck, T., Johannisson, A., Jensen-Waern. (2006). Immunological alterations during the clinical and recovery phases of experimental swine dysentery. *J Med Microbiol* 55, 845-55.
- II Andersson, M., Berg, M., Fossum, C., Jensen-Waern, M. (2007). Development of a microarray for studying porcine cytokine production in blood mononuclear cells and intestinal biopsies. *J Vet Med A Physiol Pathol Clin Med* 54, 161-8.
- III Jacobson, M., Andersson, M., Lindberg, R., Fossum, C., Jensen-Waern, M. Microarray and cytokine analyses in pigs with diarrhoea and in healthy pigs in herds confirmed to be infected with *Lawsonia intracellularis*. *Submitted*.
- IV Andersson, M., Ahlberg, V., Fossum, C. Intestinal gene expression in pigs experimentally infected with PCV2 (manuscript).

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Abbreviations

| | |
|----------------|--|
| CTL | Cytotoxic T lymphocytes |
| IFN | Interferon |
| IL | Interleukin |
| ISG | Interferon-stimulated gene |
| GO | Gene ontology |
| PBMC | Peripheral blood mononuclear cells |
| PCV2 | Porcine circovirus type 2 |
| PCVD | Porcine circovirus associated diseases |
| PE | Proliferative enteropathy |
| PMWS | Postweaning multisystemic wasting syndrome |
| qPCR | Quantitative polymerase chain reaction |
| RIN | RNA integrity number |
| SD | Swine dysentery |
| T _H | Helper T cells |
| Treg | Regulatory T cells |

Introduction

Enteric diseases in pigs

Enteric infectious diseases constitute a common problem worldwide among pigs. They are of significant economical importance for the producer due to increased mortality and unthrifty pigs, with a delay in growth. There are also indirect losses from increased use of antibiotics and changes in farm management practices trying to control the disease (Wood and Lysons, 1988; McOrist et al., 1997). Other aspects of infectious diarrhoea outbreak in pigs are compromised animal health, the risk of transmitting foodborne diseases and zoonoses to humans (Poppe et al., 1998) and antibiotic resistance (Aarestrup et al., 2000). Many of the pathogens that enter the intestine cause severe lesions, alterations in the transport of nutrients or alterations in enzyme activity (Holland, 1990). The clinical outcome varies widely, both between and within, the different enteric diseases. Some are self-limiting, some are associated with high morbidity and others are associated with high mortality. The impact of certain enteric diseases has been reduced with vaccines and advances in pig management, (Springer and Selbitz, 1999; Dean-Nystrom et al., 2002; Laine et al., 2008; Pejsak et al., 2009) such as the all in-all out management (Smith et al., 1998; Mirko and Bilkei, 2006).

Enteric infections are caused by a variety of bacteria, viruses or parasites, such as *Esherichia coli*, *Clostridium perfringens*, *Lawsonia intracellularis*, *Brachyspira* spp., *Salmonella* spp., rotavirus, PCV2 and *Isospora suis*, but the prevalences of these diseases vary worldwide (Stege et al., 2000; Thomson et al., 2001; Meriardi et al., 2003; Suh and Song, 2005; Katsuda et al., 2006). In Sweden, *Lawsonia intracellularis* and *Brachyspira* spp. are common causes of enteric infections in pigs (Jacobson et al., 2005), whereas *Salmonella* spp. rarely cause clinical disease in pigs (Boqvist et al., 2003). Diarrhoea is also

seen in pigs with PMWS, which is a relatively new disease in Sweden, with the first recorded outbreak in 2003 (Wallgren et al., 2004).

Pathogenesis

Diarrhoea is dependent on the secretion of fluids and absorption of water, electrolytes and nutrients in the intestine. The immature epithelial cells in the crypts are predominantly secretory, whereas the mature epithelial cells on the surface (the villi in the small intestine) are predominantly involved in absorption. This is important in various enteric diseases that cause lesions preferentially on the surface epithelium, which disrupts electrolyte and fluid absorption, while the secretion in the crypts are left normal or even increased. Fluid secretion flushes mucus into the lumen providing an environment required for digestion and the transit of food and waste products through the gastrointestinal tract. In addition increased intestinal permeability and increased osmotic load in the lumen also play a role in the process of diarrhoea (Moeser and Blikslager, 2007). Enterotoxigenic *E. coli* is an example of a pathogen that causes secretory diarrhoea. The bacteria produce enterotoxins, heat-stable toxin, heat-labile toxin and heat-stable enterotoxin-1 (Söderlind and Möllby, 1979; Savarino et al., 1993), which stimulate secretion in the intestine.

Villus atrophy and malabsorption are often seen in pigs with viral diarrhoea, such as rotavirus (Ward et al., 1996). Rotavirus attaches to the mature enterocytes on the villus. It then enters the cell, where it replicates and impairs glucose absorption, which contributes to the diarrhoea (Halaihel et al., 2000). Diarrhoea caused in part by malabsorption is also seen in infections caused by *Brachyspira* spp. The ability to absorb Na^+ and water is severely impaired resulting in diarrhoea (Argenzio et al., 1980). Although *B. hyodysenteriae* is the causative agent behind swine dysentery the presence of other bacteria (Whipp et al., 1979), or dietary changes (Durmic et al., 1998) seem to be required for the disease to occur. Most other bacteria are immobile in the viscous intestinal mucus, which forms a defence barrier over the epithelium. *B. hyodysenteriae* though, is highly motile in mucus and can easily reach the enterocytes through the mucus, and colonize the luminal surface (Kennedy et al., 1988). Two toxins, hemolysin and lipopolysaccharide (Hyatt et al., 1994; Nuessen et al., 1983), are produced but their importance in lesion induction is still not clear. The host's immune response has also been considered an important factor in disease development (Hontecillas et al., 2005). For instance, inflammatory bowel diseases result from a defective regulation of CD4^+ T-cell responses to

antigens from the normal intestinal flora by regulatory T cells (Cong et al., 2000).

Salmonella choleraesuis cause inflammatory diarrhoeal disease by invading enterocytes and thereby trigger activation of inflammatory signalling cascades (Galyov et al., 1997). This causes prostaglandin production which activates secretion by enterocytes (Bertelsen et al., 2003).

Lawsonia intracellularis invades the immature enterocytes in intestinal crypts, most commonly in the ileum, and multiplies within the cytoplasm. The infected cells start to proliferate progressively, thereby transmitting the organism to the daughter cells, but fail to develop into mature absorptive enterocytes (Kroll et al., 2005). The decreased absorptive capability is thought to contribute to the diarrhoea, but it is not clear whether *L. intracellularis* affects the secretory processes too. In infections with PCV2, the causative agent of PMWS, lymphoid depletion and granulomatous inflammation, with infiltrates of epithelioid cells and multinucleated giant cells in the Peyer's patches, are commonly seen. Intracytoplasmic inclusion bodies are often seen in the multinucleated giant cells as well as histiocytosis (Kim et al., 2004; Jensen et al., 2006).

However, intestinal pathogens solely are generally insufficient to cause diarrhoea but other factors, such as mixing, crowding, environmental factors, transportation and fluctuations of temperature affect the pig and make it more vulnerable to enteric infections (Laine et al., 2008).

It is important to gain a better knowledge about the pathogenesis and the host immune response to a certain pathogen in order to be able to prevent disease. Today, effective vaccines are available for some enteric infections, such as *E. coli*, *Clostridium perfringens* and PCV2. However, for the majority of enteric diseases this is not the case. A deeper knowledge about the immune response raised in the different enteric diseases is essential for producing a well functioning vaccine. Most vaccines are administered parenterally and give rise to a systemic immune response. However, pathogenic organisms infect the pig at the mucosal surface in the intestine and systemic immunisation has been shown to be ineffective for the generation of protective immunity in some enteric infections (Yuki and Kivono, 2003). Mucosal administration of vaccine can induce both systemic and mucosal immune responses. This could sometimes be more efficient than parenterally administered vaccines and prevent the infection in question at the site of initial invasion. However, protein antigens are poor immunogens when given mucosally, and can result, not in protective immunity, but in immunological tolerance (Czerkinsky et al., 1999).

The excessive use of antimicrobials causes increasing problems with resistance. Tiamulin has been used in *B. hyodysenteriae* infections all over the world and several studies have shown a reduced susceptibility to tiamulin (Lobová et al., 2004; Hidalgo et al., 2009).

Immune response

Most intestinal pathogens enter the body by the oral route and once the microbe is inside the gastrointestinal tract the immune system can differ between the pig's own tissue and intruding microbes, toxins or other foreign substances. The first set of response comprises the innate immune parameters, which are very quick in acting. The second line of response constitutes the adaptive immunity, which is composed of cells with specificity for the antigen - B and T lymphocytes. When the intruding microbe enters the body, cells with correct specificity for the antigen become activated. They then proliferate and differentiate, in order to generate a sufficient number of effector cells before an effective response is acquired against the microbe. The adaptive immune response also produces memory cells that persist for a long time. Thus, at a second encounter of a microbe the specific adaptive immune system responds much quicker. Both innate and specific immunity is mediated by and produce soluble factors, so called cytokines. The "key concepts in immunology" were recently reviewed (Moser and Leo, 2010) and are briefly summarized below.

In the gastrointestinal tract the acid pH in the stomach, the mucous membranes with a normal gut flora competing with intruding microbes and a mucus layer that acts as a trap constitute the first line of defence against invading organisms. Other systemic barriers include intact skin, increased body temperature, soluble factors, such as interferons, pro-inflammatory cytokines, acute phase proteins and the complement system. Cells of the innate immunity encompass mast cells, eosinophils, basophils, and natural killer cells as well as phagocytosing cells, such as macrophages, neutrophils, and dendritic cells (McWilliam et al., 1996; Yoshimura et al., 1999; Nadeau and Rivest 2001; Bannerman et al., 2004). Dendritic cells act as a link between the innate and adaptive immunity. They are abundant in the skin and the lining of the gastrointestinal tract (Summerfield and McCullough 2009) and are important in the intestine where they take up, process and present the antigen on MHC class II molecules together with co-stimulatory signals to T lymphocytes (Haverson et al., 2000; Inman et al., 2010; Rescigno, 2010). The activation of CD4⁺ T cells, also referred to as T helper (T_H) cells, requires and causes the release of cytokines that influence a variety of immune cells. The T cell response generated is essential for a

successful outcome from an infection. Currently three subsets of T_H cells are identified based on their cytokine secretion – T_H1 , T_H2 and T_H17 (Weaver et al., 1988, Park et al., 2005). In addition, T regulatory cells (Treg) constitute a subset of $CD4^+$ cells, of which some are believed to operate through cytokine production (Pandolfi et al., 2009). A Treg subset has also been identified in the pig (Käser et al., 2008, Germer et al., 2009) as well as various T helper subsets with similarities and differences to the human system as reviewed by Piriou-Guzylack and Salmon (2008). Endogenous antigens and intracellular organisms are presented by MHC class I molecules to $CD8^+$ T-cells that bind to this complex and become activated effector cells under the influence of cytokines. These activated cytotoxic T lymphocytes (CTL) travel throughout the body in search of their target cells and kill them. T cell killing of host cells is particularly important in preventing the replication of viruses. However, CTL has not been widely studied in the pig due to the need for autologous effector/target systems. Pigs also have a significant number of double-positive T cells, $CD4^+/CD8^+$, with probable memory functions (Zuckerman, 1999). A third type of T cell, besides the $CD4^+$ and $CD8^+$ T cells, are the $\gamma\delta$ T cells that recognize antigens that are not bound to typical MHC molecules. The $\gamma\delta$ T cells are more abundant in the young pig and decrease with age (Yang and Parkhouse, 1996) and the number of $\gamma\delta$ T cells in the intestinal epithelium is low (Rothkötter et al., 1999). Their function is still unclear but they are thought to act as sentinels against invading microbes and have immunoregulatory mechanisms. $\gamma\delta$ T cells are rapidly mobilized and act as a link between the innate and adaptive immunity (Meresse and Cerf-Bensussan, 2009). Intestinal $\gamma\delta$ T cells are also involved in regulation of inflammatory responses. Transfer of $\gamma\delta$ intraepithelial lymphocytes to mice, lacking their own $\gamma\delta$ T cells, improved chemically induced colitis (Inagaki-Ohara et al., 2004).

Activated B-cells that are specific for a certain antigen differentiate into antibody secreting plasmacells. The B lymphocytes retain their specificity but can switch to another immunoglobulin class (Butler et al., 2009). Non-activated B-cells express IgM and IgD on their surface, but as they mature, under the influence of cytokines from T-cells, they switch isotype. T-cell derived IL-10 and IFN- γ causes switching to IgG (Fujieda et al., 1996; Snapper et al., 1992), IL-4 and IL-13 cause switching to IgE (de Vries et al., 1993) and TGF- β to IgA (Briere et al., 1995). The antibodies circulate in the blood and lymph and bind to the pathogens, which marks them for phagocytosis or destruction by complement activation. In an enteric infection the majority of antibodies are of IgA-type, which are found in the

lymphoid tissue and the submucosa in the intestine. IgA efficiently binds to and neutralizes antigens but is regarded as non-inflammatory, which is important in the intestine.

When an immune response is successfully completed the majority of the antigen-responsive cells must be removed. This is easiest done by inducing the unwanted cells to undergo apoptosis. The cytokines belonging to the Tumor Necrosis Factor (TNF) family are strong signals for the apoptotic programmed cell death pathway (Hernández-Caselles and Stutman, 1993), but other signal mechanisms are also used to terminate an immune response.

In general, extracellular bacterial infections activate CD4⁺ T cells via MHC class II molecules on antigen-presenting cells, and induce an immune response that depends upon antibodies, whereas viral infections activate CD8⁺ cells via MHC class I molecules on virus-infected cells and trigger a cell-mediated immune response.

The immune response in the intestine

The intestine has a very special role in immune surveillance as it constantly must differ between the commensal microflora, harmless antigens in the food and pathogenic organisms or products. Intestinal dendritic cells play an important role in directing the immune system towards tolerance of harmless self- or food antigens or immunity to pathogens (Rescigno, 2010). Discriminating between pathogens and non-pathogenic antigens is especially important in the intestine, as it constitutes the main contact surface between the body and the outside environment. Enterocytes express pattern recognition receptors (PRR), both extracellular Toll-like receptors (TLR) and intracellular NOD-like receptors (NLR) that recognise conserved microbial products or structures (Wells et al., 2010). In a healthy intestine, TLRs and NLRs remain relatively unresponsive to the normal microflora, but invading pathogens start the induction of an inflammatory host response (Gribar et al., 2008). The intestinal microflora is also crucial for the development of both local and systemic immunity (Tlaskalová-Hogenová et al., 2004; Shirkey et al., 2006). The lymphoid tissue in the intestine is organized in mesenteric lymph nodes, isolated follicles and Peyer's patches, which are lymph follicles in the mucosa (Fig. 1). There are also lymphocytes scattered in the epithelium (mostly CD8⁺) and lamina propria (mainly plasma cells and CD4⁺) (Haverson et al., 1999; Wilson et al., 1996). The epithelium in the intestine is covered by a mucus layer that protects the mucosa. However, interspersed among the epithelial cells are M cells that do not secrete mucus or digestive enzymes and the brush border is much thinner. Thus they are more accessible for antigens in the intestinal lumen.

The M cells take up antigens and deliver them to dendritic cells in the underlying Peyer's patches. Certain subpopulations of dendritic cells can also take up the antigen directly from the lumen, mediating stimulatory and inhibitory signals. The dendritic cells present MHC/peptide complexes and deliver the costimulatory signals necessary for the activation of T cells (Inman et al., 2010; Rescigno, 2010). B-cells and memory cells are stimulated upon encountering antigen in Peyer's patches. B- and T cells then pass to the mesenteric lymph nodes where the immune response is amplified. Activated lymphocytes pass into the blood stream and home to the intestine where they carry out their effector functions. Under the influence of TGF- β the plasma cells secrete mainly IgA which is released into the intestinal lumen. The importance of Treg and T_H17 cells in the intestinal immune reactivity is clearly indicated in man (Pandolfi et al., 2009) and begins to unravel also in the pig (Bailey, 2009).

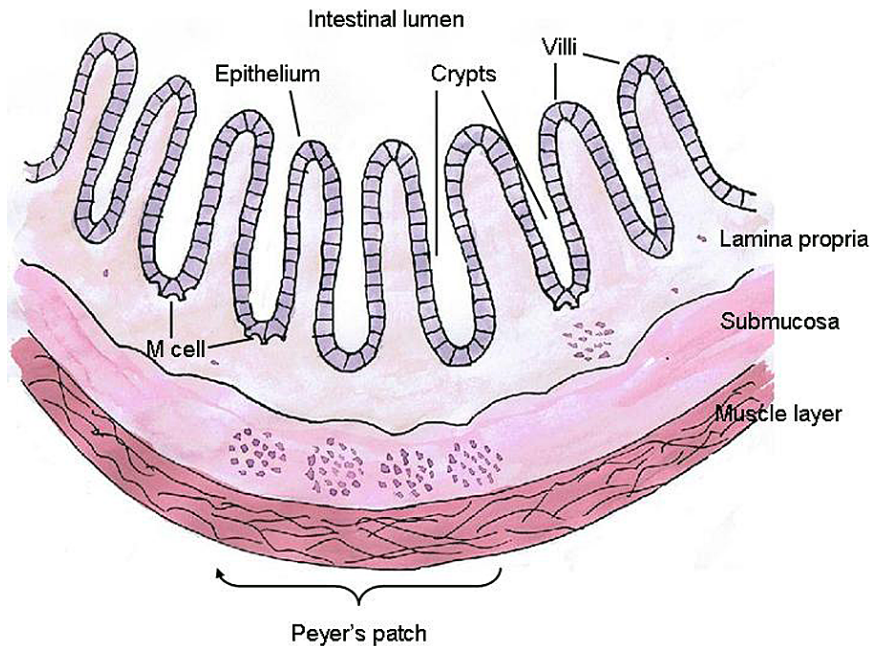


Figure 1. A cross-sectional view of the intestine, showing the different layers of the intestinal wall, the epithelium composed of enterocytes, M cells that take up antigen and deliver them to dendritic cells and a Peyer's patch (a nodule of lymph follicles in the submucosa).

The immune response in the intestine in certain enteric diseases

Naturally, the immune responses differ between the different enteric infections. Studies on the immune response in diarrhoea-associated diseases have mainly been focused on the systemic response, whereas not as much is known about the local response in the intestine.

In pigs infected with *B. hyodysenteriae* a loss of $\gamma\delta$ T cells in the epithelium and aggregates of $CD4^+$ T cells within the lamina propria and submucosa have been described (Hontecillas et al., 2005). In swine dysentery the loss of $\gamma\delta$ T cells might be due to the epithelial erosions, caused by the cytotoxic action of the hemolysins. Increased levels of monocytes and $CD4^+ CD8^+$ T systemically were seen in an experimental infection (Jonasson et al., 2004). IgG, IgM and IgA specific for *B. hyodysenteriae* are produced in serum during clinical disease together with local production of IgA in the intestinal mucosa. However, the production of antibodies does not seem to be related to protection, but rather indicative of recent or prolonged exposure to the pathogen (Rees et al., 1989).

In pigs experimentally infected with *Lawsonia intracellularis* reduced numbers of $CD8^+$ T cells and B cells were found in the intestine together with an increased number of macrophages (MacIntyre et al., 2003), but large amounts of IgA are present within enterocytes (Lawson et al., 1979; McOrist et al., 1992; Guedes and Gebhart, 2010). In the acute form of the disease the response in the intestine is more massive and IgM-producing B cells are also found (McOrist et al., 1992). In an experimental infection both serum IgG and IFN- γ , representing the cell-mediated immunity, were increased up to 13 weeks after infection (Guedes and Gebhart, 2003). However, as *L. intracellularis* is an obligate intracellular organism that infects epithelial intestinal cells, serum IgG is most probably not protective against infection, while secretory IgA and cell-mediated immune response might be more important. The inflammatory cell response in the intestine in proliferative enteropathy is limited (McOrist et al., 1992).

Not much is known about the immune response in the intestine during PCV2 infections. PCV2 seems to have a predilection for dendritic cells and macrophages. However, the virus does not replicate inside these cells, but they seem to play a major role in viral persistence and transmission throughout the body (Gilpin et al., 2003; Vincent et al., 2003). Lymphoid depletion and granulomatous inflammation in the Peyer's patches are a common find though (Kim et al., 2004; Jensen et al., 2006). PCV2 has an immunosuppressive effect on the host, with lymphoid depletion and leucopenia. Systemically reduced numbers of T-cells, B-cells, dendritic cells

and NK-cells are seen, whereas the number of monocytes and granulocytes are less affected (Nielsen et al., 2003).

The present thesis is aimed to further analyze the immune responses in the above mentioned diseases. New technology, such as qPCR and microarray, was used to gain a broader view of the processes taking place. A large part of the thesis is devoted to the studying of cytokines, as the induced cytokine profile can in hindsight help interpret what kind of immune response was raised.

Cytokines

Cytokines are small protein mediators capable of regulating various biological functions, including immune responses. The different T_H subsets differ in their cytokine production. T_H1 cells mainly secrete IL-18 and IFN- γ that activate a cell-mediated immune response with antibodies of certain subtypes, CD8⁺ T cells, macrophages and NK cells, whereas T_H2 produce IL-4, IL-5, IL-9, IL-10 and IL-13 that predominantly activate B-cells (Nagler-Anderson, 2001) and T_H17 characterized by their production of IL-17, IL-21 and IL-22 that is important for neutrophil recruitment, activation and migration in the host defence against bacteria and fungi (Cruz et al., 2006; Godinez et al., 2009).

Some cytokines (IL-1 β , IL-6, TNF- α) are produced early in a disease or injury, independent of antigen (Beck and Wallace, 1997). Macrophages and monocytes are the major producers of these pro-inflammatory cytokines. The inflammatory cytokines speed up inflammation to give the immune cells in the blood access to tissues. This is achieved by causing the blood vessels be more permeable or by helping white blood cells to leave the blood vessels. The pro-inflammatory cytokines can also induce fever and induce an acute-phase-response. If the inflammatory cytokines are over-expressed, they might exacerbate the severity of a disease condition (Dambrauskas et al., 2010).

Interferons are mainly referred to as type I (IFN- α , IFN- β) or type II (IFN- γ) but also type III interferons exist. Type I interferons are very important in combating viral infections where they bind to target cells and induce an antiviral state (Perry et al., 2005). Interferons do have other roles as well, such as increase the expression of MHC molecules on cells and activate NK cells which then kill infected cells (O'Mahony et al., 2008).

Some chemokines (IL-8, CXCR4) are induced during an immune response to promote cells of the immune system to a site of infection. They can thus regulate inflammatory responses by coordinating the movement of

immune cells. Other chemokines are involved in the control of the movement of cells during normal tissue maintenance (Zimmerman et al., 2008).

The availability of reagents, such as antibodies to porcine cytokines, is still limited. It is therefore more convenient to study the expression of mRNA, since gene sequences for the various cytokines are more easily accessible. A restriction to be aware of, is that up-regulation of mRNA expression does not necessarily result in translation and production of the protein.

There are several different analysing techniques that can be used to gain a better insight in the immune response raised in the intestine in various infections. Examples are immunohistochemistry to determine what type of cells are present, *in situ* hybridization to detect specific mRNA sequences in tissues sections, histopathology with different staining of tissue sections to identify cellular components, and microarray and qPCR to study changes in gene expression.

Analysis of gene expression

Microarray

The microarray technology uses the same principle of base pairing as other traditional techniques such as Northern and Southern blotting (Southern, 2001). In the earliest arrays cDNA was spotted onto filter paper when studying the antiproliferative action of interferons (Kulesh et al., 1987) and the miniaturized microarray was first described by Schena et al. in 1995. The technique evolved quickly and the first commercial array was available in 1996 (Affymetrix, Santa Clara, CA). To start with, the number of genes on each array was limited but the first complete eukaryotic genome, from a yeast, was placed on microarray in 1997 (Lashkari et al., 1997). Porcine microarrays of various sizes have been in use since 2003.

A DNA microarray is a series of spots of DNA oligonucleotides on a solid surface (Fig. 2). Each spot represents a gene and the number of spots can vary from just a few to up to tens of thousands. The cDNA in the sample hybridizes to the DNA strands in the spots and are labelled with a fluorophore whose signal is measured after excitation with a laser beam of a defined wavelength. A high number of matching complementary base pairs in a nucleotide sequence makes the binding between the two strands stronger. The intensity of the fluorophore signal depends on the number of target bound to the spot. The signal intensity of the sample is compared to

that of a control in the same spot and hence a relative quantification of the sample can be made.

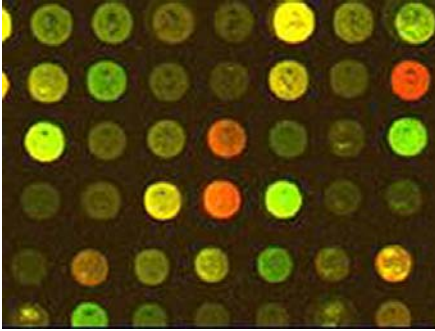


Figure 2. The scanned image of a two-channel microarray. Green spots represents up-regulated and red spots down-regulated genes, whilst yellow spots are equally expressed in both samples. Uncoloured spots represent genes that are not expressed in either sample.

The use of high density microarrays containing thousands of DNA fragments has the main advantage that the expression level of a large number of genes can be studied simultaneously. The major advantages with low-density microarrays are the lower cost and the short time taken for analysis and interpretation of the data. However, it is clear that with low-density microarrays, the effects on genes not selected will obviously be missed (de Longueville et al., 2003).

The microarray technology is used to study for instance the functional role of the various genes, gene regulation, influence on gene expression in disease, medication, mutations and diagnosis and classification of microbes. The efficiency and power of microarray have greatly improved with development of new surface substrates, more sensitive robots for printing, development of the dye labelling techniques and improved computer analyzing since the start.

quantitative PCR

In quantitative real time PCR (qPCR) the amount of PCR product is measured at each cycle (Fig. 3). This is what differentiates this method from ordinary PCR, which just amplifies a specific DNA sequence. The correlation between the starting template concentration and the amplification kinetics is exploited for the quantification of nucleic acids. Quantitative real time PCR is generally considered as the gold standard for nucleic acid quantification and it is commonly used for validation of microarray results (Ding and Cantor, 2004).

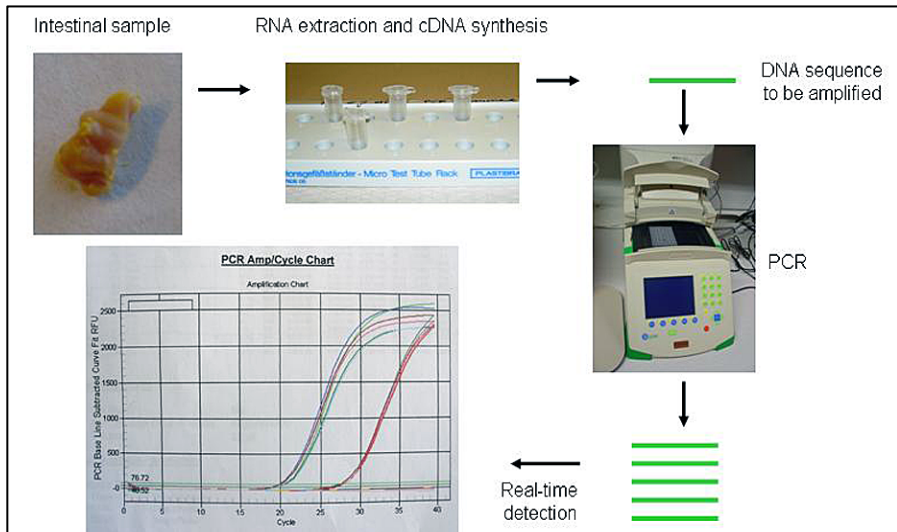


Figure 3. RNA is extracted from the intestinal samples and cDNA is synthesized. With the help of specific primers, the selected DNA sequence is amplified when the sample is run for a number of cycles in the PCR thermocycler. Each DNA copy has a fluorescent reporter molecule attached, and the fluorescence emitted manifolds as the DNA copy number accumulates with each cycle, thereby quantifying the PCR product.

The power of microarrays lies mostly in the simultaneous quantification of a very large amount of genes, which makes it possible for extensive comparisons to be made, either looking for differences in gene expression between a control group and a disease group, or among genes within the same sample (Ding and Cantor, 2004). Quantitative real time PCR has not the same level of throughput in terms of gene numbers and it is not possible, neither time-wise nor economically, to analyze the same number of genes by real-time PCR as by microarray. However, real-time PCR has a higher sensitivity than microarrays (Fink et al., 1998) and just a few cDNA copies are sufficient for detection and quantification (Palmer et al., 2003). The specificity is generally very high in both methods, but there is a risk of cross-hybridization in arrays whereby the specificity of low expression genes is likely to be lower since cross-hybridizations by homologous sequences can account for a larger percentage of the signal intensity.

The reproducibility is generally higher with qPCR than microarrays, and whereas absolute quantification is possible with qPCR, it is unlikely to perform with a microarray. The cost is also something to consider, as screening samples by microarray is much more costly than by qPCR (Ding and Cantor, 2004). The microarray is invaluable for getting an overall

picture of the genome wide expression when studying infectious diseases. Patterns in gene expression between the different experimental groups are detected and further analyses can then be performed with, for example, qPCR.

In the present thesis the immune response elicited by the infectious diseases swine dysentery, proliferative enteropathy and PMWS, was studied by qPCR and microarray techniques. Material from both experimental infections and field cases was studied in pigs infected with *Brachyspira hyodysenteriae* or PCV2, whereas material from field cases exclusively, were available from *Lawsonia intracellularis*-infected pigs.

Swine dysentery

Swine dysentery was first described in 1921 in USA (Whiting et al., 1937), but it was not until 1971 that the causative organism was confirmed to be an anaerobic spirochete (Taylor and Alexander, 1971), now named *Brachyspira hyodysenteriae*. The disease is found in most pig-producing countries.

It is characterized by muco-haemorrhagic diarrhoea (Fig. 4) and reduced general appearance with inflammation, excessive mucus production and necrosis of the large intestine (Alexander and Taylor, 1969). Transmission of the disease occurs mainly through the faecal-oral route and clinical signs usually appear 10-14 days after infection. If left untreated the mortality can reach more than 30% (Hamdy, 1974). The disease can affect pigs of all ages, but it primarily affects pigs between 15 and 70 kg. Pigs that have recovered can shed bacteria in the faeces for up to 70 days after recovery and thus infect new pigs for a long time after clinical signs have ceased (Harris et al., 1999). Wild rodents can also transmit the disease between pigs (Joens and Kinyon, 1982).

It has been suggested that the feed influences the development of the disease. However, the results concerning the effect of different types of carbohydrates on swine dysentery have so far been contradictory (Siba et al., 1996; Hansen et al., 2010).

At necropsy hyperaemia and oedema of the wall and mesentery of the large intestine are frequently seen. The mucosa is swollen, covered by mucus and fibrin and lesions are often visible. The contents in the large intestine are often watery and mixed with blood (Harris et al., 1999). Other spirochetes, most of them non-pathogenic, are present in the porcine intestine. They can be differentiated from *B. hyodysenteriae* either by biochemical tests or by PCR (Fellström et al., 1997). The control and treatment of clinical cases of swine dysentery usually involves antimicrobials.

There is, however, an increasing problem with reduced susceptibility in some strains of *B. hyodysenteriae* (Karlsson et al., 2001; Hidalgo et al., 2009). As a consequence, use of antimicrobials should be restricted (Karlsson et al., 2002).



Figure 4. Muco-hemorrhagic diarrhea is characteristic for pigs with swine dysentery. Photo: Märit Andersson.

Proliferative enteropathy

Proliferative enteropathy (PE) is caused by an obligate intracellular bacterium. The disease has been observed in pigs for over 80 years, but it was not until 1993 that an intracellular bacterium was isolated from the intestine obtained from pigs with proliferative enteropathy (Lawson et al., 1993) and it was later named *Lawsonia intracellularis* (McOrist et al., 1995). Cultivation of the microbe is difficult, as it is obligately intracellular and microaerophilic (Lawson et al., 1993).

The chronic form mainly affects young growing pigs in which diarrhoea, rough hair-coat and retarded growth are observed (Fig. 5), whereas the acute form is seen in older pigs and is characterized by hemorrhagic diarrhoea and sudden death (Bane et al., 2001). Mortality in the chronic form is low and is usually due to secondary infections (Winkelman and Dee, 1996). Poor growth is the only clinical sign in subclinically infected pigs. Clinical cases respond well to antibiotics (McOrist et al., 2000) and there is a vaccine available that is extensively used (Kroll et al., 2004).



Figure 5. Wasting, as in the left pig, is commonly seen in enteric infections. However, it is also a typical clinical sign in pigs with PMWS. Photo: Robert Kruse.

PE is most commonly found in pigs, but has also been observed in dogs, sheep, horses, rabbits and primates to name a few species (Duhamel et al., 1998; Lafortune et al., 2004; Frazer, 2008). It has, however, never been found in humans (Jacobson et al., 2007).

The prevalence of *L. intracellularis* in pigs is high all over the world. In a study in Korea, serum antibodies were detected in 100% of examined herds (Lee et al., 2001), and 93.7% of herds in Denmark (Stegé et al., 2000) and 48% of piglet-producing herds in Sweden were positive for *L. intracellularis* (Jacobson et al., 2005).

Transmission occurs via the faecal-oral route, and the incubation period is 1- 3 weeks (Smith and McOrist, 1997). Many pig producing farms have a sustained level of *Lawsonia intracellularis* and thus new groups of pigs get infected (Smith et al., 1998).

At necropsy the intestinal mucosa is thickened into deep folds and histologically enlarged, branching crypts lined with a multilayer of immature epithelial cells are seen in the chronic form. In the acute form, the affected intestine is thickened due to serosal oedema and the lumen usually contains formed blood clots. The mucosa shows little gross abnormalities, but histologically degeneration and haemorrhage within the proliferative epithelium are seen.

PCV2

Porcine circovirus (PCV) was discovered in 1974 as a contaminant of the porcine kidney cell line PK-15 (Tischer et al, 1982). The small virus contains a single-stranded, circular DNA genome and belongs the Circoviridae family.

So far, two genetically different strains of PCV have been found – PCV1 and PCV2 (Allan et al, 1999a). Only PCV2 is considered to be pathogenic. Three genotypes of PCV2 have been found. PCV2a is generally non-pathogenic under field conditions, but it can cause disease under experimental conditions (Allan et al., 2003; Hasslung et al., 2005). PCV2b, on the other hand, is usually considered to be the only isolate that is pathogenic under field conditions. PCV2c has so far only been found in Denmark in archived samples from pigs in which PMWS was not detected (Dupont et al., 2008).

PCV2 can be transmitted between pigs in several ways, but the main route for infection is by oro-nasal contact with infected faeces (Segalés et al., 2005a) or urine, or direct contact between pigs (Magar et al., 2000.; Bolin et al, 2001). The virus primarily infects the tonsils and lymph nodes of the head (Rosell et al., 1999) and from there it disseminates throughout the body via the lymphatic system.

Although PCV2 infection in a herd causes the majority of pigs to seroconvert, other factors are needed in order to trigger disease. Such factors include variations in virulence of PCV2 (Grau-Roma et al., 2010), host factors (López-Soria et al., 2004; Opriessnig et al., 2006; Meerts et al, 2005), co-infections (Allan et al., 1999b; Allan et al., 2000; Krakowka et al., 2000; Opriessnig et al., 2004a, 2004b; Ellis et al., 2008), immunomodulatory factors (Krakowka et al., 2001; Allan et al., 2007; Kyriakis et al., 2002; Opriessnig et al., 2003) and management-related factors (Rose et al., 2003; López-Soria et al., 2005; Woodbine et al., 2007). The amount of PCV2 found in tissues in the pig is directly correlated to severity of disease expression (Krakowka et al., 2005; Segalés et al., 2005b).

PCV2 is the infectious agent behind several porcine circovirus associated diseases (PCVDs). The most significant manifestation is postweaning multisystemic wasting syndrome (PMWS), which affects 5–12 week old piglets. Clinical signs are wasting, dyspnoea, lymphadenopathy, pallor, jaundice and diarrhoea (Harding and Clark, 1997). Necropsy findings include enlargement of the superficial inguinal, submandibular, mesenteric and mediastinal lymph nodes, white streaks or spots in the kidneys, granulomatous lesions in the lungs, liver, kidney, heart and intestines and failure of the lungs to collapse (Rosell et al., 1999; Opriessnig et al., 2007).

An enteric form of PCVD, with diarrhoea, retarded growth and unthriftiness is seen in pigs at 8 – 16 weeks old (Kim et al., 2004; Jensen et al., 2006). Another clinical manifestation of PCV2 is reproductive failure with abortions and still-births and pre-weaning mortalities (West et al., 1999). PCV2 also causes porcine dermatitis and nephropathy syndrome (PDNS), which usually affects grower pigs. The affected pigs experience purple skin lesions, fever, nephritis, vasculitis and deposition of immune complexes in the kidneys. The disease is often fatal (Rosell et al., 2000). Several vaccines against PCV2 are now commercially available. All of them have proved to be efficient in reducing the incidence of PMWS (Gillespie et al., 2009; Pejsak et al., 2009; Grau-Roma et al., 2010).

The aims of this thesis

The general aim of this thesis was to study the immune response elicited in pigs with various enteric infections in order to get a better understanding of what parts of the immune response that were activated in the different diseases.

To achieve this, the specific aims were:

- to study the changes in leucocyte population in blood during swine dysentery (paper I)
- to study the development of specific antibodies during the course swine dysentery (paper I)
- to evaluate different methods to isolate mRNA of good quality from intestinal samples (paper II)
- to develop a microarray for studying porcine cytokine expression in the intestine (paper II)
- to study the cytokine expression systemically and in the intestine in pigs with *Lawsonia intracellularis* infection (paper III)
- to further characterize the local immune reaction in the intestine in pigs affected by *Lawsonia intracellularis* or PCV2 (papers III, IV)
- to examine the gene expression in the intestine in pigs affected by *Lawsonia intracellularis* or PCV2 by microarray (papers III, IV)

Materials and methods

A brief presentation of the materials and methods used in the studies of this thesis is given here. A complete description is presented in the individual papers.

Animals and sampling

Paper I

Ten clinically healthy pigs from a commercial breeder were used. The pigs, aged 8-10 weeks old at arrival, were housed individually with free access to water and were fed twice daily. They were acclimatized for at least a week and had straw for bedding during this time. Clinical health examination was performed daily and the pigs were weighed weekly. Rectal swabs for detection of *Brachyspira* spp were collected weekly until clinical signs appeared, daily during the clinical signs of disease and then once a week until euthanization. Blood samples were obtained from the jugular vein before a provocative feeding with soya (Jacobson et al., 2004) started, for four consecutive days when clinical signs started and at day 1, 3, 7, 11, 15 during the recovery period. Pigs that did not show any clinical signs were sampled on days 4, 14, 21, 28 and 35 post-inoculation.

Paper II

Clinically healthy pigs from the University Research Station Funbo-Lövsta, Uppsala, Sweden or a conventional herd were used for blood and tissue sampling. Samples were taken from the vena jugularis. Intestinal biopsies from colon, were taken at euthanization or at slaughter. The biopsies were rinsed in saline, snap frozen in liquid nitrogen and stored in -70°C.

Paper III

The pigs originated from six piglet-producing herds with a history of proliferative enteropathy. In total, 15 pigs with clinical signs resembling chronic PE, i.e. non-haemorrhagic diarrhoea and rough haircoat, and nine matching control pigs were sampled. In addition, samples from four pigs with haemorrhagic diarrhoea from four finisher herds were collected. Blood samples were collected from the jugular vein. After euthanization samples from the ileum and other parts of the intestine with visible lesions were collected. Some intestinal samples were snap-frozen in liquid nitrogen within 15 min after death whereas others were frozen in -80°C .

Paper IV

Fortytwo snatch-farrowed piglets were divided into four experimental groups. They were housed group-wise with free access to water. They were fed a bovine colostrum-substitute for the first three days and thereafter pig milk-substitute. All pigs were treated with antibiotics throughout the experimental period. Clinical health examination was performed daily. Blood samples were collected on days 0, 8, 15, 22 and 28. On day 28 the pigs were euthanized and necropsied. Intestinal samples were snap-frozen within 15 min after death.

Design of the experimental infections

Paper I

Four days prior to inoculation the pigs were on a provocative feeding regime that included a pure soybean meal once daily for a week. The pigs were inoculated orally once daily with 30 ml brain heart infusion broth containing 10^7 - 10^9 bacteria per ml of *Brachyspira hyodysenteriae* strain B204^T. The straw bedding was replaced with a synthetic fur blanket.

Paper IV

The pigs were colostrum-deprived and snatch-farrowed, thus ensuring that the pigs were sero-negative for PCV2 prior to inoculation. At three days of age they were inoculated with mock (group 1), PPV only (group 2), PPV and PCV2-1010, originating from a Canadian outbreak of PMWS (group 3) or PPV and a Swedish isolate of PCV2 (group 4).

Analysis of peripheral blood leukocytes by flow cytometry (paper I)

Total and differential white blood cell counts were analysed with an electronic cell counter (Cell_Dyn 3500, Abbott, Wiesbaden, Germany). Erythrocytes were then lysed and the remaining leukocyte pellet was washed, resuspended in PBS with foetal calf serum. The leucocytes were doubly immunostained using porcine monoclonal antibodies (VMRD, Pullman, WA, USA) or isotype controls (DakoCytomation, Glostrup, Denmark) as primary antibodies and, after washing, phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. The cells were washed, resuspended and fixed in PBS with paraformaldehyde.

To examine the subpopulations of white blood cells in the individual pigs 30 000 cells per sample were analysed by flow cytometry. The cells were examined for forward light scatter, orthogonal light scatter and green and orange fluorescence in a BD LSR flow cytometer (BDBiosciences, San José, CA). Lymphocytes were gated for by light scatter parameters and evaluated for FITC and/or PE fluorescence. The results were assessed by region analysis, except for the CD4/CD8 α double staining where quadrant analysis was used. Assessment was performed using the Cellquest software (BDBiosciences, San José, CA).

Analysis of serum antibodies (papers I, III)

In paper I six pooled serum samples from pigs with clinical signs of swine dysentery and three pooled samples from clinically healthy pigs were analysed regarding antibodies against *B. hyodysenteriae*. The samples were from pre-inoculation, day 2 of clinical signs of disease, days 1, 7, 11, 15 of recovery and pre-inoculation, 14, 28 days after inoculation respectively. In addition, un-pooled sera from the pigs with clinical signs on days 7, 11 and 15 of recovery were analysed separately. General antibodies to *Brachyspira* spp. were removed from the samples using an absorption procedure, leaving only antibodies with the specificity for *B. hyodysenteriae* strain B204^T. Western blotting was used to identify and quantify antibodies against *B. hyodysenteriae*. Whole cell proteins from the type strain *B. hyodysenteriae* (B78^T) were separated by SDS-PAGE. Bacterial samples were applied onto an SDS-polyacrylamide gel together with a protein-size marker (New England Biolabs, Beverly, MA) and subjected to electrophoresis (Mini-protean II gel system, Bio-Rad, Hercules, CA). The separated proteins were transferred onto a PVDF membrane, which was blocked and incubated in sera from the pigs in the study. After applying secondary antibodies

(peroxidise-conjugated anti-swine IgG from DakoCytomation, Glostrup, Denmark) the membranes were washed and the bound antibodies were visualised with a chemiluminescent substrate system.

In study III a blocking ELISA was used for the demonstration of antibodies to *L. intracellularis* in sera (Bioscreen GmbH, Münster, Germany). Absorbance values with a calculated percent inhibition above 35 were considered as positive.

Lymphocyte activation (paper II)

Isolation of peripheral blood mononuclear cells (PBMC) was performed using gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). This was initiated within 20 minutes after sampling. The PBMC were suspended at a concentration of 5×10^6 cells per ml in supplemented RPMI medium (RPMI 1640; BioWhittaker). Cytokine production was induced by incubation of PBMC with either 5 µg per ml of the mitogen ConA (Amersham Pharmacia Biotech) for 10 or 24 hours, 2.5 µg/ml of LPS from *E. coli* serotype 0111:B4 (SIGMA Chemical Co, St.Louis, USA) for 6 h or 5 µg per ml of ODN 2216 (5'-ggGGGACGATCGTCgggggG-3', Cybergene AB, Huddinge, Sweden) for 6 or 18 hours. Parallel cultures with PBMC grown in plain growth medium served as controls. The PBMC were washed in PBS before RNA isolation.

RNA extraction (papers II, III, IV)

Total RNA was isolated from PBMC using TRIzol[®] following the manufacturer's manual (Invitrogen, Life Technologies, Carlsbad, CA).

When isolating RNA from the intestinal tissues in paper II, two different methods of RNA extraction were evaluated. TRIzol[®] extraction was performed following the manual from the manufacturer (Invitrogen, Life Technologies, Carlsbad, CA, USA), with slight modifications. In brief, the frozen biopsies were grinded with a mortar and pestle, with increasing amounts of TRIzol[®], until the final volume of 1 ml. The mixture was homogenised by passing it 10-15 times through a 1.2 mm needle fitted on a syringe. RNA was also extracted with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions including grinding the biopsies with a mortar and pestle and homogenising them with a needle and syringe. Extracted RNA was resuspended in 25 µl DEPC H₂O and the quantity and purity of RNA were determined by spectrophotometric analysis NanoDrop[®]ND-1000, NanoDrop

Technologies, Montchanin, DE, USA) at OD 260 nm and OD ratio 260/280 nm respectively. The quality of the RNA was tested by evaluation of the 18S and 28S band formation on 1% TAE-agarose gel before being used for cDNA production. As both methods yielded similar amounts of RNA of equal quality the less laborious TRIzol[®] method was used in papers III and IV. In these studies the RNA was also treated with DNase to eliminate contamination of genomic DNA.

cDNA synthesis (papers II, III, IV)

cDNA was synthesized using 1 µg of sample RNA as template and Superscript II reverse transcriptase following the protocol from Invitrogen (Stockholm, Sweden) with the modification of a specially designed oligo (dT) primer (530 µg/ml) which was included in the mixture to incorporate 21 new bases at the end of the synthesized cDNA (Wattrang et al., 2005). A mixture without the reverse transcriptase enzyme was run in parallel to those with the enzyme included to ensure that there was no disturbance from genomic DNA.

Detection and quantification of cytokine mRNA in the intestine (papers II, III, IV)

In paper II the expression of cytokine mRNA in the induced PBMC and in the intestinal samples was analysed by ordinary PCR. PCR amplification of cDNAs was performed in one step for the reference genes and for the cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-18, IFN-γ and TNF-α and in two steps for the cytokines IL-4, IFN-α, IFN-β, MIF and GM-CSF. The two step amplification was carried out using a specific forward primer and a common back primer (p23A) (Berg et al., 1999). This was not performed in the one step amplifications. In the second step, specific primers for each cytokine were used. The samples were first heated at 95 °C followed by a cycle consisting of denaturation for 30 s at 95 °C, annealing for 30 s and extension for 30 s at 72 °C with a final extension for 5 min at 72 °C. The PCR products were analysed by agarose gel electrophoresis.

In studies III and IV the mRNA expression for the cytokines IL-1β, IL-6, IL-10, IL-12p40, IFN-α, IFN-γ, TNF-α, TGF-β, CXCR4, RGS16 and the two reference genes, CyclophilinA and HPRT in the intestine were analysed using quantitative TaqMan real-time PCR (Timmusk et al., 2009; Hasslung Wikström et al., 2010). The samples were amplified in triplicate. A

negative control, with no template added and an internal laboratory standard, were included in each run. When analysing IFN- α , a control that had been run in the cDNA synthetization, but without the reverse transcriptase enzyme, was included to ensure that there was no disturbance from genomic DNA. The expression of cytokine mRNA was calculated relative to the mean expression of the cytokine mRNA in the control pigs using the geometric mean method (Livak and Schmittgen, 2001).

Immunoassay and bioassay for IFN- α and bioassay for IL-6 (paper II)

IFN- α activity in culture supernatants was measured using a cytopathic effect inhibition bioassay and a laboratory standard IFN- α as previously described (Artursson et al., 1989). The samples were also analysed by dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) using pairs of monoclonal antibodies against porcine IFN- α (Artursson et al., 1995), one for coating and one Europium-labelled for detection. The fluorescence emitted (counts per second, cps) was measured in a time-resolved fluorometer (Wallace, Oy, Turku, Finland). The lower limit for detection was 3 and 0.3 U IFN- α per ml in the bioassay and immunoassay, respectively.

Interleukin-6 activity was detected by bioassay using B9 cells as previously described (Fossum et al., 1998). The cell viability after 72 h of incubation was determined as the ability of the B9 cells to reduce tetrazolium salts into formazan (EZ4U; Biomedica, Wien, Austria), measured as the absorbance value at 450 nm (A_{450}) and related to a preparation of recombinant murine IL-6 (BioSource, Int.). The lower limit for detection was 0.3 U IL-6 activity per ml.

Light microscopy (paper III)

Two to three, $\sim 4 \mu\text{m}$ thick, slices were cut transversely of the intestine, dehydrated and embedded in paraffin. All samples were stained with haematoxylin and eosin. Selected sections were also stained for mucosubstances with Alcian blue-periodic acid Schiff, pH 2.5. In selected pigs, slides were stained with Ziehl-Neelsen and Gram-Twort for bacteria.

Quantification of porcine circovirus type 2 in sera and intestine (papers III, IV)

Quantitative real-time PCR was performed on sera (paper III) and the intestines (paper IV) to analyse the presence of PCV2 DNA. The following primer pairs were used: S-PCV2 Forward: TGG CTG GAA GTA ATC AAT AGT TCT; Reverse: CTT CGG ATA TAC TGT CAA GGC T and PCV2-1010 Forward: GTC AAG GCT ACC ACA GTC AC; Reverse: AGG GTA TAG AGA TTT TGT TGG TC. All reactions were performed in triplicate. The fluorescence was measured at the last step of each cycle. After 40 cycles, a melting curve analysis was performed. The quantification was made from a standard curve generated by titration of a plasmid containing a full-length genomic insert of PCV2 (S-PCV2) or a PCR product (PCV2-1010), using an in-house assay. In brief, PCV2-DNA was amplified from lymph nodes obtained from two of the PCV2-1010 infected pigs using the following primers: GAT CCG GCT GGC TGA ACT TTT GAA AGT and GGT CCG CTT CTT CCA TTC TT. The restriction pattern of the PCR product was verified by gel electrophoresis before purified on the Qiagen PCR purification kit. The copy number was determined to 6.8222×10^8 copies per ng DNA and the lower detection limit for both assays was set to 50 copies/100 ng DNA.

Analysis of Serum amyloid A and cytokines in sera (papers I and III)

Analysis for the acute phase protein SAA was performed using a commercially available ELISA kit (Tridelta Phase range SAA kit, Tridelta Development Limited, Greystones, Wicklow, Ireland). Commercially available ELISA kits (Quantikine Porcine Immunoassays, R&D Systems Europe Limited, Abingdon, UK) were also used to determine the serum concentrations of IL-1 β , IL-6, IL-10, IFN- γ , TNF- α and TGF- β . The minimum limits of detection were; IL-1 β , 10 pg/mL; IL-6, 10 pg/mL; IL-10, 1.8 pg/mL; IFN- γ , 2.7 pg/mL; TNF- α , 2.8 pg/mL and; TGF- β , 1.7 pg/mL. Analyses of serum concentration of IFN- γ was analysed in 18 pigs, *i.e.* two pigs with haemorrhagic diarrhoea, ten case pigs and six control pigs, using a dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA). The detection limit for IFN- γ was 0.3 U/mL.

Development of a microarray for studying cytokine expression in the intestine (paper II)

PCR products, representing 14 cytokines and four reference genes, were spotted in quadruplicate on glass slides (UltraGAPS from Corning Life Sciences, Acton, MA, USA). External control genes (Lucidea score card clones, Amersham Biosciences, Uppsala, Sweden) were included on the slide in order to normalize data. 5 mg RNA from PBMC induced with either ConA, ODN2216 or medium or RNA from intestinal material were used on the arrays. cDNA was produced from the RNA and were hybridized to the spots on the array and labelled with either the fluorophore Cy3 or Cy5. The hybridizations were repeated with exchanged dyes for labelling, a so-called dye-swap, to avoid bias due to variations in signal intensity. After hybridization the arrays were scanned (GenePix 4000B, Axon Instruments, Union City, California, USA) and analyzed with the software GenePix Pro 5.0 (Axon instruments). Spotting of the slides and data analyses were carried out at the Microarray platform, Wallenberg Laboratory, Uppsala University. The gene expression for each spot was compared between duplicate slides and within each slide between the sub-grids, thereby evaluating the sensitivity and reproducibility of the array. The gene expressions were also compared between the two dye-swaps. Spots with intensities of at least 2.5 times the background intensity were further analyzed. When comparing the level of gene expression in various samples a two-fold change was used as cut-off value.

However, the microarray technology has developed fast and a few years ago a microarray with 20 201 porcine genes represented on it was commercially available (Affymetrix GeneChip® Porcine Genome expression arrays). This array was used in studies III and IV.

Analysis of gene expression in the intestine by microarray (papers III, IV)

The gene expression in the intestine was analysed with the Affymetrix GeneChip® Porcine Genome Array at the Uppsala Array Platform, Uppsala, Sweden. In paper III, intestine from six pigs were analysed - two pigs with haemorrhagic diarrhoea, two with non-hemorrhagic diarrhoea and two control pigs ("control 6" and "control 10"). Of the two pigs with hemorrhagic diarrhoea one suffered from swine dysentery ("haem 1") and one from PHE ("haem 3"). One of the pigs with non-hemorrhagic diarrhoea was judged to be in the early stages of PE ("case 3") whereas the other one was considered to be in a more progressed stage of PE ("case

12”). In paper IV, intestine from three pigs in each experimental group were subjected to analyses by microarray: group 1 (control): nos. A5, B2, D11; group 2 (PPV): nos. B10, D6, E6; group 3 (PCV2-1010 and PPV): nos. B1, B11, E5 and group 4 (S-PCV2 and PPV): nos. A3, B8, C3.

RNA quality was evaluated with the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA). 250 nanograms of total RNA from each sample were used for array analyses. In brief, the arrays were hybridized for 16 hours in a 45°C incubator, and thereafter washed, stained and scanned.

The gene expression data was analysed and a principal component analysis (PCA) was performed in order to identify correlations between samples. The PCA showed that the overall gene expression in one pig in each infection group diverged from the other pigs in the same group in paper IV. These pigs were excluded from further microarray analysis and thus the pigs analysed by (data not shown) the microarray were in group 1 (control): A5, B2, D11; group 2 (PPV): D6, E6; group 3 (PCV2-1010 and PPV): B1, B11, and group 4 (S-PCV2 and PPV): A3, C3.

Genes that were differentially expressed (DE) in the infected pigs when compared to the control pigs, were subjected to further analyses. Only genes with a fold change greater than 2 and $p < 0.01$, were further analyzed. The exception was the pigs with non-hemorrhagic diarrhoea in paper III, where no consideration was given to the p-value due to few DE genes.

The selected DE genes were analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov>). In paper III, the genes were assigned specific Gene Ontology (GO) terms, based on biological processes. The fold enrichment (FE) for each term was calculated as the ratio between the percentage of the DE genes in the sample (“haem 1” and “haem 3” or “case 3” and “case 12”) and the percentage of background population genes, *i.e.* all genes represented on the Affymetrix Porcine Array annotated to that GO term. The number of genes contributing to each GO term varied between 30 and 990. In paper IV, cluster analysis of differentially expressed genes was performed to detect similarities and differences between the two PCV-2 isolates and a heatmap was constructed using the Gene Cluster 3.0 and Java Treeview software (Stanford University, 2002).

In order to find the most active genes in each experimental group, genes with the greatest significant fold changes were selected and studied individually.

Results and discussion

Animals and experimental infections

The diseases studied in the present thesis are all found in pigs of the same age group, i.e. from weaning until slaughter. The gut microbiota in the young piglet shows low homogeneity initially, but the homogeneity increases after weaning (Melin et al., 1997). Thus, young pigs are more susceptible to enteric infections as the balance between the development of commensal microbiota and the establishment of a bacterial intestinal disease can easily tip towards disease. Some pigs are transported and/or mixed with new pigs in large grower-finisher units, thereby being exposed to new infections at a time when the passive maternal immunity is declining. Several of the enteric diseases, such as PE, PMWS, porcine intestinal spirochetosis, swine dysentery, Salmonellosis and *E. coli* infection have similar symptoms in the form of diarrhoea, wasting and retarded growth, and in some cases it is only with histopathology combined with antigen demonstration that a final diagnosis can be made (Jensen et al., 2006). As the aetiology and pathogenesis differs between these microbes, variations in the immune response raised are expected.

In the present thesis both swine dysentery and PMWS were induced experimentally (papers I, IV), but both diseases were also diagnosed in the field material (paper III). One study of the cytokine production in serum obtained from pigs at various stages of experimentally induced swine dysentery showed that IL-1 β was increased at the end of the incubation period and at the beginning of the dysentery period, TNF- α was increased post-inoculation and stayed high during the dysentery period and the levels of IL-10 were increased during the recovery period. Furthermore, low levels of IL-6 were found in some pigs during the dysentery period (Kruse et al., 2008). These results were confirmed in the pig diagnosed with swine

dysentery in the field study (paper III), where increased levels of IL-1 β , IL-6 and TNF- α were found in serum collected when the pig displayed clinical signs of disease. In pigs experimentally infected with PCV2 and PPV (paper IV) the mRNA expression for various cytokines in the intestine was studied at the time for development of PMWS showing an increased expression for mRNA for IL-6, IL-10 and IFN- γ . In accordance, the mRNA expression for IFN- γ was also found to be increased in field cases infected with PCV2 (paper III). Taken together, these results indicate that the experimental infections used in the present thesis correspond fairly well to the diseases observed in the field. The advantages of controlled experimentally induced infections, compared to field studies, are that selection of the pigs ensure that they are of the same age and weight, free from certain pathogens, all pigs are infected at the same time, the infectious dose is known and the same for all pigs, the control pigs are treated and handled in the same way as the infected pigs, feed intake can be measured, fewer pigs can often be used in experimental infections and the pigs are monitored more closely. The drawbacks are that the infectious load in most conventional herds is higher than in experimental studies. Also, the experimental conditions might not always correspond to the pigs' normal environment, and the influence of other factors, mostly unknown, that might influence or trigger the infection under field conditions are lacking. It is therefore vital to study enteric infections in pigs both under experimental conditions and in the field.

Leukocyte subpopulations in experimental swine dysentery (paper I)

Alterations in leukocyte subpopulations were studied in blood collected during the course of experimentally induced swine dysentery (paper I). The total numbers of leukocytes increased after inoculation. There was also an increase in the levels of monocytes, neutrophils, CD8 α^+ lymphocytes and CD45RA $^-$ lymphocytes (activated lymphocytes) in the blood during clinical signs of swine dysentery, whereas the CD21 $^+$ cells decreased at that time. During the recovery period increased numbers of neutrophils, $\gamma\delta$ T cells and antibodies to *Brachyspira hyodysenteriae* were seen.

In comparison, studies of the intestine showed that *B. hyodysenteriae* induced a mucosal CD4 $^+$ T cell response (Hontecillas et al., 2005). Among lymphocyte subpopulations in the intestine in pigs with experimental swine dysentery the amounts of CD21 $^+$ cells as well as lymphocytes expressing T cell receptor γ , were decreased in Peyer's patches during the dysentery period (Kaleczyc et al., 2010), whereas the CD8 $^+$ cells tended to be increased, in

pigs with swine dysentery. Other lymphocyte subpopulations did not differ from the control group. Thus, the alterations observed in the blood samples mimicked to some extent those reported by others to occur in the intestine. It should be kept in mind however, that different techniques when sampling the intestine, as well as age and differences in the diet influences the outcome of lymphocyte phenotype analyses (Solano-Aguilar et al., 2001).

Antibody response during experimental swine dysentery and proliferative enteropathy in field cases (papers I, III)

Specific antibodies to *Brachyspira hyodysenteriae* were detected in sera from the first day of the recovery period in pigs with experimentally induced swine dysentery (paper I). It has previously been shown that pigs that have recovered from experimental infection with swine dysentery are immune to reinfection with the same serotype of *B. hyodysenteriae* (Joens et al., 1983). However, antibodies to *B. hyodysenteriae* are generally not related to protection, but mainly an indication of recent or prolonged exposure to the pathogen (Rees et al., 1989).

Antibodies to *Lawsonia intracellularis* were detected in sera from two pigs with haemorrhagic diarrhoea and from three of the 15 pigs with non-haemorrhagic diarrhoea, whereas all control pigs in the field study were negative (paper III). However, as *L. intracellularis* is an obligate intracellular organism infecting epithelial cells in the intestine, it is not likely that serum IgG is protective against infection, but rather secretory IgA and cell-mediated immune response may be of more importance (Guedes and Gebhart, 2003). It is difficult to study the cytotoxic cell-mediated immune response in pigs directly, as there is a need for MHC compatibility between effector cell and target cell. Instead, the production of IFN- γ at re-exposure to the antigen is commonly used as a measure of cell-mediated immunity in the pig. Thus, this is another reason to monitor the local cytokine production in pigs.

RNA extraction (papers II, III, IV)

As the two methods to isolate RNA that were compared (paper II) yielded similar amounts of RNA of equal quality the less laborious TRIzol[®] method was used in papers III and IV.

It is well known that RNA degrades quickly by post-mortem processes and inadequate handling or storage of samples (Holland et al., 2003). The difference in gene expression in samples with intact or degraded RNA was

assessed in a study by analyzing the expression of various reference genes in various tissues (Pérez-Novo et al., 2005). It was shown that it is of utmost importance that only RNA of good quality is used for gene expression profiling, both on microarrays and by qPCR.

The RNA quality can be assessed by different methods, such as measuring the optic density by Nano-drop or otherwise, assessment of formation of 18S and 28S bands by denaturing gel-electrophoresis or with lab-on-chip technologies like the Bioanalyzer (Fleige and Pfaffl, 2006). With lab-on-chip technologies the RNA samples are separated by an automated capillary electrophoresis system on a micro-fabricated chip and subsequently detected via laser induced fluorescence emission. Only a very small amount of RNA sample is needed and this method has become widely used, particularly in the gene expression profiling platforms (Mueller et al., 2000; Fleige et al., 2006). The Bioanalyzer determines the RNA Integrity Number (RIN) on basis of the shape of the curve in the electropherogram (Fig. 6). A RIN value of 1 is the most degraded and 10 the most intact. One study, using calf cells and tissue samples, showed that RNA isolated from single cells like white blood cells had a higher RIN value (around 9) than RNA isolated from tissues and organs (6-8) (Fleige and Pfaffl, 2006). The reason for this was that cells were easier to access and thus sampling and RNA extraction was quicker compared to tissue sampling and handling. Tissues contain connective and fatty tissue that further complicates the RNA extraction.

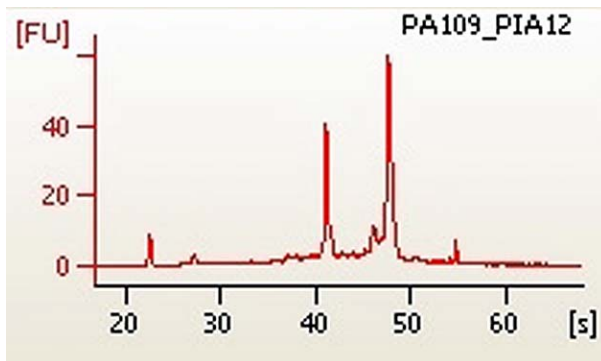


Figure 6. Assessment of RNA quality by determining the RIN value. RNA samples are separated by electrophoresis on a micro-fabricated chip and subsequently detected via laser induced fluorescence emission. Degradation of the RNA sample results in a larger proportion of small size RNA fragments and a decrease in fluorescence signal, since the sites where the fluorophores are situated are destroyed. The sample integrity is determined by the entire electrophoretic trace of the RNA sample. The figure shows a RIN value of 8.7. s = seconds, FU = Fluorescence units.

Data from studies on samples from human colon cancer suggest that the sample should be snapfrozen within 20 minutes of tissue extirpation, in order to provide relatively stable gene expression profiles as detected through microarray analysis (Huang et al., 2001). The RNA quality can also be affected by storage temperature and length of storage, both before and after RNA extraction (Holland et al., 2003). This became obvious when analyzing intestinal biopsies from pigs with experimental swine dysentery (paper I). The biopsies were taken via fistulae in the large intestine during the course of the disease and were stored in -80°C for three years before RNA extraction was performed. None of the 35 samples yielded RNA of reasonable quality for further analyses (data not shown). In comparison, the quality was acceptable of RNA extracted from intestinal samples that were stored in liquid nitrogen for the same length of time. How long time the samples have been stored does also affect the quality of the RNA. When comparing the RIN numbers from intestinal samples that had been snapfrozen in liquid nitrogen and then stored in liquid nitrogen for six years or in -80°C for one year, we found that the samples with the shorter storage period had RIN numbers between 6.2 – 8.7, whereas the RIN values for the samples that had been stored longer varied between 2.4 – 7.3. In another comparison RNA was also extracted from the same intestinal samples twice. cDNA was prepared from the first extraction and stored in -20°C . Four years later new RNA was extracted from the samples and the expression for various cytokines in the two cDNA preparations were analyzed in parallel by qPCR. The results were similar for the two preparations except that in the samples that had been stored as cDNA the threshold cycle values (Ct values) were consequently reached earlier than in the sample that had been stored as biopsies.

Also, in acute intestinal infections the intestinal lesions might be so severe that degradation of RNA has started, and perhaps it is difficult to obtain RNA of reasonable quality from a sample taken from such a lesion. It was shown, from studies on *Actinobacillus pleuropneumoniae* infection in lungs, that damaged areas in the lungs yielded RNA with lower RIN values than visually unaffected areas (Mortensen et al., 2009).

With correct tissue sampling, handling and storage technique, it is thus possible to obtain RNA of good quality. These improvements combined with established techniques for taking biopsies via a cannulae into the intestine (Jacobson et al., 2004) generate great opportunities to follow what happens in the intestine during the course of an enteric disease. When using repeated samplings via cannulae instead of consecutively sacrificed animals,

the number of pigs used in an experiment can be reduced by up to 70 % (Emanuelson et al., 2007)

Thus, in order to obtain the best results, the intestine should be sampled within a short time after the pig has been euthanized. The samples should be no bigger than approximately 0.1 cm³ (Medeiros et al., 2007), to enable quick freezing of the whole piece. The sample should be immediately snapfrozen in liquid nitrogen, or stored in RNAlater[®] solution (Applied Biosystems/Ambion, Austin, TX, USA), which permeates the tissue and stabilizes and protects the RNA. If snapfrozen, the samples should be stored in liquid nitrogen, but RNA should be extracted as soon as possible. In order to avoid thawing and refreezing of the RNA, the extracted RNA should be aliquoted into several tubes. Synthesize cDNA from the RNA as soon as possible, if cDNA will be used in later analyses, as cDNA is more stable and will not be degraded like the RNA.

Different cytokine responses in enteric diseases (papers III, IV)

Several cytokines, such as IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF- α and IFN- γ (Oswald, 2006; Devriendt et al., 2010; Inman et al., 2010) are constitutively expressed in the porcine intestine where they are involved in many processes, for instance epithelial cell growth, homeostasis and transport of immune cells into the mucosa (Oswald, 2006). However, the cytokines, both systemically in the blood and in the intestine, are up- or down-regulated in response to microbial infection.

In pigs experimentally infected with *Brachyspira hyodysenteriae* increased levels of IL-1 β and TNF- α in serum were detected during incubation and when symptoms occurred, whereas low levels of IL-6 were found in some pigs during the dysentery period and IL-10 was increased during recovery (Kruse et al., 2008). In accordance, the pig that was diagnosed with swine dysentery (paper III) showed elevated levels of IL-1 β , IL-6 and TNF- α in serum as well as increased expression of mRNA for IL-1 β in the intestine. Dysenteric pigs have previously been shown to express increased levels of mRNA for IFN- γ in colonic lymph nodes (Hontecillas et al., 2002) and it might be that IFN- γ is produced locally in the intestine during the infection. These findings indicate that the above cytokines are produced to a greater extent during acute swine dysentery.

Knockout mice, in which a specific gene is inactivated, are used for studying that gene during a certain condition. It is established that IFN- γ plays a significant role in protective immunity to intracellular pathogens (Reljic, 2007). Infection with *L. intracellularis* in IFN- γ R⁻ mice was both

longer and more severe than in normal mice, indicating that IFN- γ is important in limiting intracellular infection and restricting the cellular proliferation associated with *L. intracellularis* (Smith et al., 2000). In pigs with experimental *L. intracellularis* infection IFN- γ production was increased two weeks after infection (Guedes and Gebhart, 2003), but no increase of IFN- γ in serum or mRNA expression of IFN- γ in the intestine could be solely related to the *L. intracellularis* infection in the clinical cases sampled in paper III. In fact, the increased mRNA expression for IFN- γ appeared to be more influenced by the PCV2 infection in this field study and no differences in cytokine (IL-1 β , IL-6, IL-10, IL-12p40, IFN- α , IFN- γ , TNF- α and TGF- β) expression in the intestine could be documented when comparing *L. intracellularis*-positive pigs with *L. intracellularis*-negative pigs. This supports the hypothesis that the immune system is weakly activated during chronic infection with *L. intracellularis* (McOrist et al., 1992; MacIntyre et al., 2003), but also illustrates the complicated interpretation of data from field studies.

In pigs experimentally infected with PCV2, increased levels of IL-10 in serum has been correlated with the viraemic phase of infection (Darwich et al., 2008), and thus the expression of mRNA for IL-10 or the levels of IL-10 can be increased or lowered depending if the pigs are sampled early or late in infection (Sipos et al., 2004 and 2005, Stevenson et al., 2006). IFN- γ production increased in PBMC from clinically affected pigs in response to recall antigen (Darwich et al., 2003b) and in porcine blood mononuclear cells (PBMC) from experimentally PCV2-infected pigs without overt symptoms (Steiner et al., 2009). Both under experimental conditions and in the field, PCV2 has been found to induce the expression of IFN- γ in the intestine (papers III, IV) and in tonsils (Darwich et al., 2003a). IFN- α could be detected in intestines from all PCV2-infected pigs but the expression was comparatively low (paper IV). However, microarray analyses of the intestine revealed an up-regulation of several ISGs (interferon-stimulated genes), indicating that IFN- α was expressed in higher quantities at an earlier stage of infection. It has been shown that PCV2 modulates the immune response of the host, leading to suppressed production of IFN- α (Vincent et al., 2007).

Development of a cDNA microarray with a limited number of Cytokines (paper II)

A porcine microarray with a limited number of cytokines was constructed and evaluated in paper II (Fig. 7). Cytokines reflecting early inflammatory response of bacterial (IL-1 β , IL-6, TNF- α) or viral (IFN- α , IFN- β) infections and the subsequent Th1-type (IL-12 p35, IL-12 p40, IL-18, IFN-

γ) or Th2-type (IL-4, IL-10) of immune response were included on the array as well as the chemokine IL-8, the colony-stimulating factor GM-CSF and the pro-inflammatory cytokine MIF. The main evaluation was performed using porcine PBMC stimulated *in vitro* with ConA or ODN 2216, but samples obtained from intestinal biopsies from healthy pigs were also screened on the array.

While large arrays with up to tens of thousands of genes are invaluable for genome-wide expression analyses, low-density microarrays with a limited number of genes are a common tool for diagnostic use (Townsend et al., 2006). Most diagnostic microarrays can achieve optimal classification with only 5-50 discriminative genes (Jaeger and Spang, 2006) and an array with a limited number of genes can e.g. indicate a certain cytokine profile. On the other hand, genomewide arrays can give valuable new, non-predicted information, and as the porcine Affymetrix genomewide array became available during the course of these studies it was applied for screening intestines collected in the field (paper III) and an experimental study (paper IV). One problem with large arrays is how to make the huge amount of data, obtained from screening tens of thousands of genes at the same time, comprehensible and give it biological meaning. There are now web-based analysis sites, such as DAVID (<http://www.david.niaid.nih.gov>) available that facilitates the transition from data collection to biological relevance.

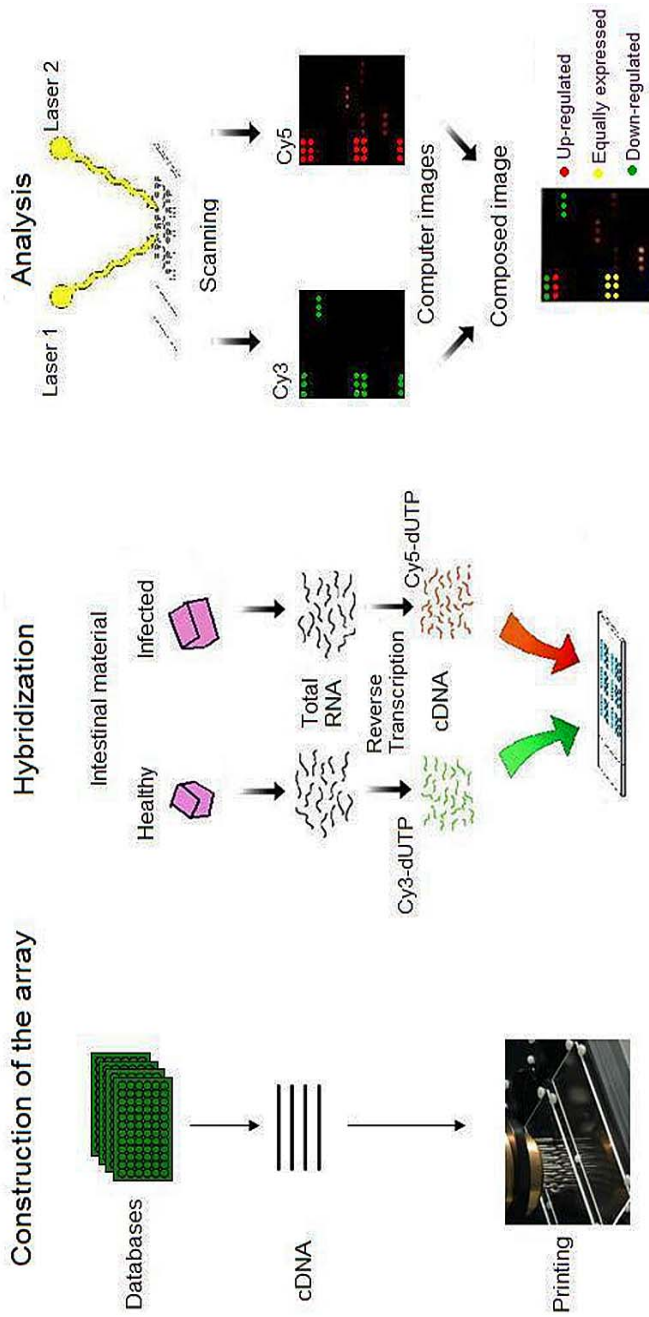


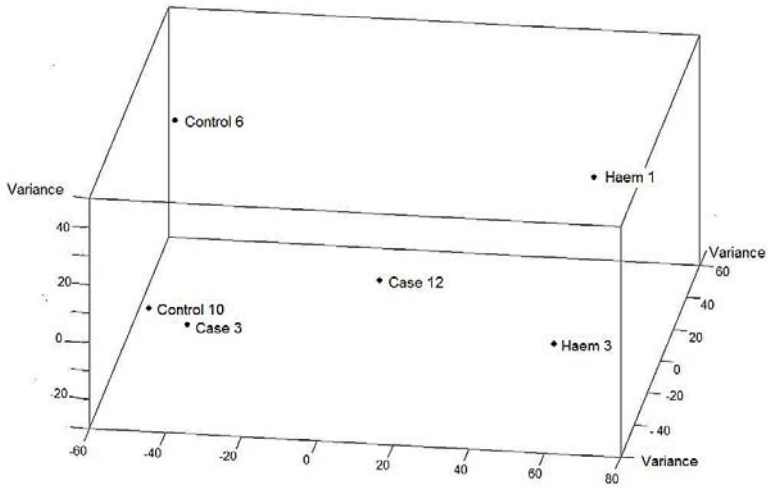
Figure 7. The microarray technique involves three steps: construction of the array, preparing the samples and hybridizing to the array and analysis. The first step, construction of the array, starts with selecting gene sequences that represents the different genes. cDNA of the selected sequences is produced and printed on to the slide. Step two involves isolating RNA from the samples, produce cDNA that are labelled with different fluorophores and let the cDNA hybridize to the cDNA on the slides. In the last step, the slides are scanned, and the signal intensity in each spot is measured after excitation with a laser beam of a defined wave length. The intensity of the signal depends on the number of target bound to the spot.

Analyses of the gene expression in intestine by cDNA microarray (papers III, IV)

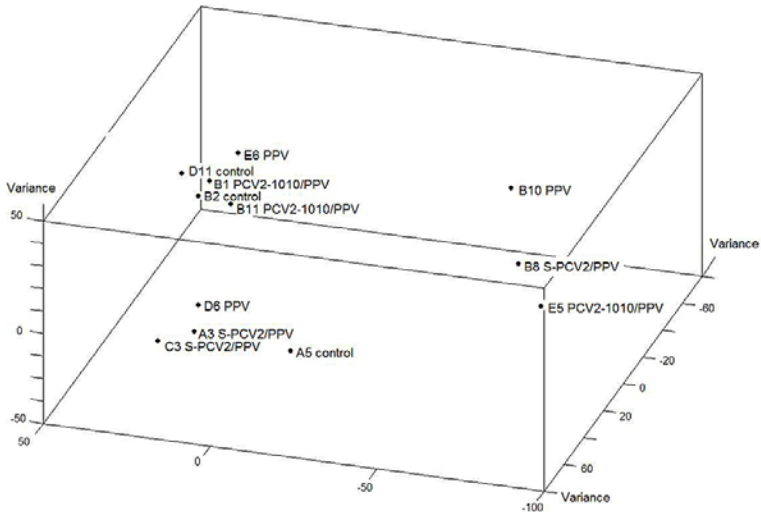
Principal component analysis (PCA), which is a mathematical procedure that simplifies complex data sets, was applied on the data from the microarray in papers III and IV (Fig. 8). This method reduces a number of variables, which might be correlated, into a smaller number of uncorrelated variables called principal components (Raychaudhuri et al., 2004).

In paper III, the enrichment of selected GO terms was studied (Fig. 9). This revealed that no GO term was significantly enriched ($FE > 3$) with up-regulated genes in pigs with the acute haemorrhagic form of proliferative enteropathy, whereas the down-regulated genes were significantly over-represented in several GO terms. In pigs with the chronic form, with non-haemorrhagic diarrhoea, only one GO term was significantly over-represented with down-regulated genes, but several were significantly enriched with up-regulated genes.

Pigs with haemorrhagic diarrhoea (“haem 1” and “haem 3”), displayed more than six times as many genes (2467 and 385 genes respectively) that were differentially expressed when compared to the control pigs, as the pigs with non-haemorrhagic diarrhoea (“case 3” and “case 12”). However, as the PCA indicated a low correlation between the gene expressions in the two pigs with non-haemorrhagic diarrhoea individual analyses of the pigs were performed. This revealed that the enrichments noted above in these pigs were mainly related to “case 12”. No significant enrichments of DE genes were found in “case 3”, which was considered to be in the early stage of the infection. The gene expression in “case 3” was similar to that of the control pigs, indicating that the immune response might not yet be evoked. “case 12” was considered to be in a later stage of the disease, based on serology and morphology, as did “haem 3”. This could suggest that an uncomplicated infection with *L. intracellularis*, affecting immature enterocytes, does not evoke any immune response. The pigs “case 12” and “haem 3” displayed greater enrichments with up-regulated genes in GO terms related to the immune response, suggesting that PE with haemorrhagic diarrhoea may not be the acute stage of the disease, rather these severe symptoms can be regarded as a complication to chronic proliferative enteropathy. These findings indicate that further microarray analyses of intestinal material during a controlled experimental infection with *L. intracellularis* would be very interesting.



a.



b.

Figure 8. Principal component analysis (PCA) of the data from the Affymetrix GeneChip[®] Porcine Genome expression array of intestinal samples from pigs in study III (a) and study IV (b). PCA is a way to identify predominant gene expression patterns, and visualize correlations between samples. Variance in gene expression are shown on the x-, y- and z-axes, with ~80% of the variance accounted for on the x-axis, ~80% of the remaining variance on the y-axis and ~80% of the residual variance on the z-axis. To the right in fig. b are the three pigs, which were excluded from further analyses as their overall gene expression diverged from the other pigs in the same group.

In “haem 1”, the pig diagnosed with swine dysentery, several GO terms, i.e. “complement activation”, “humoral immune response” and “antigen processing and presentation” were significantly enriched with down-regulated genes. The GO terms “digestion” and “complement activation” were at least twofold significantly enriched among the up-regulated genes. However, as these results are based on one pig, they should be viewed with caution. Further microarray screenings on a larger number of pigs with swine dysentery would be very interesting though.

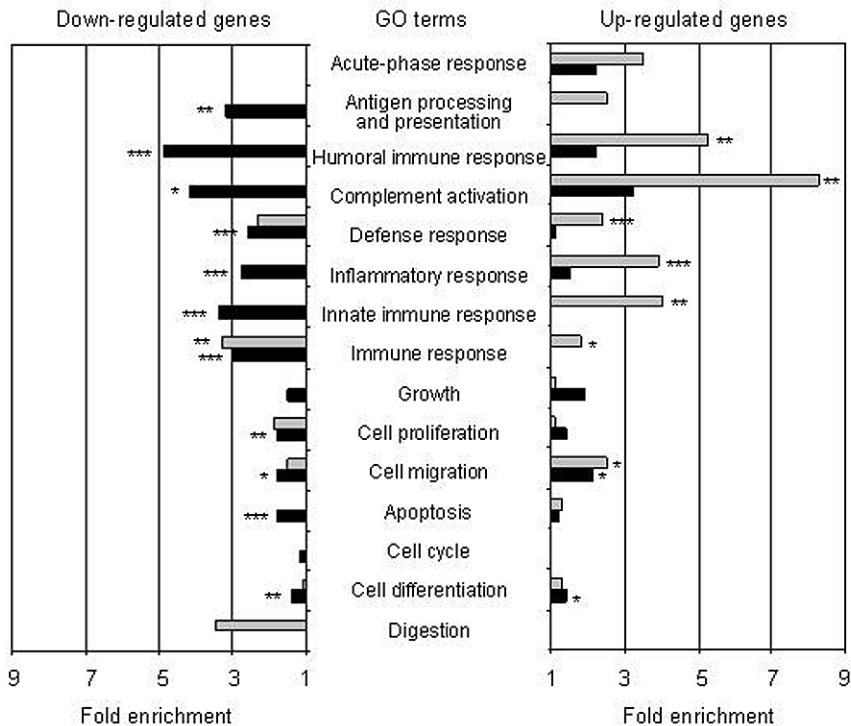


Figure 9. Fold enrichment (the ratio between the percentage of differentially expressed (DE) genes and the percentage of background population genes in each category) of specific gene ontology (GO) biological processes terms. Functional annotation was performed on DE genes in intestinal samples obtained from pigs with haemorrhagic diarrhea (black bars) and pigs with non-haemorrhagic diarrhea (grey bars) when compared to the control pigs (“control 6 and 10”). Up-regulated and down-regulated DE gene lists were analysed with the Database for Annotation, Visualization and Integrated Discovery (DAVID), based on GO terms. Statistical significance is shown with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

In paper IV, the gene expression in intestine obtained from three control pigs and two pigs from each infection group were analyzed by cDNA microarray. The main finding in this study was the up-regulation of several interferon-stimulated genes (ISGs). In both PCV2-infected groups the most pronounced up-regulated gene (FC 23.3 and 36.7 respectively) was *IFITM3*, an ISG. *IFITM3* was not up-regulated in the intestine in the pig (“haem 3”) analysed on microarray in paper III that was positive for both PCV2 and *L. intracellularis*.

In order to find similarities and differences in the immune response induced by the two PCV2-isolates, a cluster analysis was performed. In total 439 genes were differentially expressed (FC > 2) in group 3 (140 DE genes) and/or group 4 (351 DE genes) when compared to pigs inoculated with PPV only. A heatmap was constructed and four clusters of genes (A-D) were selected (Fig. 10). Genes in cluster A were up-regulated in both groups. The majority of these genes were immune response related. Several genes involved in cytotoxic T-cell response, such as genes related to MHC class I were found here. This might indicate that CD8+ T-cells are important in the defense against PCV2. Ten IFN-stimulated genes (ISGs) were found in this cluster. Thus, PCV2 is most probably responsible for the induction of these ISGs, even though IFN- α was not expressed in these experimental groups at this stage of infection. The most marked induced ISG (more than 23 times up-regulated in both groups 3 and 4) was *IFITM3*, that can be induced by IFN- γ as well as IFN- α . ISGs are genes, which expression is induced by interferons. The products from these genes mediate the many effects interferons have in the body (Sen and Sarkar, 2007). Infection by virus triggers different signalling pathways that subsequently lead to the induction of ISGs. Naturally, different viruses might trigger different pathways, but a single virus can also trigger separate pathways, depending on the cell type. The various pathways are probably dominant in different cell types, suggesting that the type of pathway triggered might play a role in determining pathogenesis, or the lack of it, when a pig is infected by a virus (Sen and Sarkar, 2007)

The ISG *IFITM3* has been studied in infection with influenza A H1N1 virus in humans, where it was found to restrict an early step in influenza A viral replication (Brass et al., 2009). It is also involved in the pathogenesis of inflammatory bowel disease and polymorphism of the *IFITM3* gene was associated with susceptibility to ulcerative colitis (Seo et al., 2010). Thus, further studies on the expression of *IFITM3* in other viral enteric infections in the pig, such as those caused by coronavirus, transmissible gastroenteritis

virus and rotavirus, might enlighten our knowledge about the immune reactions in these diseases.

Cluster B incorporated genes up-regulated in group 3 and down-regulated in group 4. Several of these genes were involved in metabolic processes, which is interesting as wasting is an important and distinct clinical finding in pigs with PMWS. Cluster C included genes that were down-regulated in group 3 and up-regulated in group 4, and these genes all had different functions. Genes in cluster D were down-regulated in both PCV2-infected groups. Genes involved in cell drug response, inflammatory response and homeostasis were found in cluster D.

In paper III, seven of the cytokines that were analysed by qPCR were represented on the microarray. These were IL-6, IL-10, IL-12p40, IFN- α , IFN- γ , TNF- α and TGF- β . In paper IV, six of the cytokines were represented on the on the microarray, namely IL-1 β , IFN- α , TNF- α , TGF- β , CXCR4 and RGS16. The qPCR results were in accordance with the results from the array in both studies. Cytokines that were expressed in high relative levels on the qPCR showed strong signal intensities on the microarray for each pig and vice versa (data not shown).

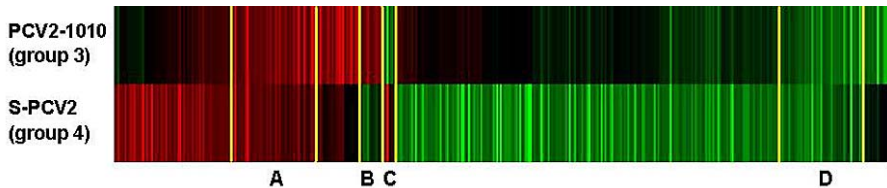


Figure 10. Cluster analysis of 439 DE genes in pigs experimentally infected with either PCV2-1010/PPV or S-PCV2/PPV. The heatmap was constructed using the Gene Cluster 3.0 and Java Treeview software. Green represents down-regulated genes and red represents up-regulated genes, when compared to pigs infected with PPV only. Four clusters showing similarities (A, D) and differences (B, C) between the isolates were defined. The number of genes in each cluster was: $n_A = 46$, $n_B = 11$, $n_C = 7$, $n_D = 46$

Microarray analyses have been used to study different immunological processes in the intestine. Such studies have suggested that pigs have evolved specialized immunological processes in the small intestine that differs from mouse and human, species which most immune function models are based on (Dvorak et al., 2006). Thus, results from these species should be applied with caution in the pig. However, many genes in the intestine still have unknown functions which remain to be discovered and the local immune response in enteric infections, others than those studied here, still needs to

be elucidated and the results in the present thesis clearly show the potential for microarray analyses in screening the immune response to porcine enteric diseases.

Conclusions

- Experimentally induced swine dysentery induced changes in leucocyte sub-populations with increasing levels of monocytes, neutrophils and CD8 α^+ lymphocytes during clinical disease. Specific antibodies to *B. hyodysenteriae* were detected in sera from the first day of recovery.
- The two different methods for isolating RNA, yielded RNA of similar quality, but the TRIzol[®] method was considered less laborious.
- The sampled material should be snapfrozen, and stored for as short time as possible, in order to obtain RNA of acceptable quality.
- A microarray for studying porcine cytokine expression in the intestine was developed, but not used in further studies, as porcine microarrays are now commercially available.
- Analyses of cytokines in sera and intestine in pigs with natural *Lawsonia intracellularis* infection showed no differences between infected and control pigs.
- Analyses of cytokines in the intestine in pigs experimentally infected with PCV2 and PPV showed an up-regulation of the mRNA expression for IL-6, IL-10 and IFN- γ in pigs infected with PCV2/PPV compared to control pigs.
- Microarray analyses of intestine from pigs with natural *Lawsonia intracellularis* infection indicated that an uncomplicated infection with *L. intracellularis* does not evoke an immune response and that PE with haemorrhagic diarrhoea may not be the acute stage of the disease, but can be regarded as a complication to chronic proliferative enteropathy.
- Microarray analyses of intestine from pigs experimentally infected with PCV2 and PPV showed that PCV2 induced the expression of several interferon-stimulated genes, underscoring that these genes are important in the defence against viral infection.

Aspects on future work

- The local immune response in the intestine in pigs with swine dysentery is still largely unknown. Further studies, both with microarray and other techniques, are needed to shed light on the immunological processes that take place in the gut.
- The field study on *Lawsonia intracellularis* infected pigs, warrants further screenings on intestinal material by microarray, to pursue the indications that the immune response is weak in pigs with PE and that the haemorrhagic form might be a complication to chronic PE. It would be interesting to follow a larger number of animals experimentally infected with *L. intracellularis*.
- As a common route for infection of PCV2 is orally, the virus encounters the intestinal environment within shortly. Microarray studies on intestines from a larger number of animals would be of value to learn more about the interaction between PCV2 and the immune system in the intestine.
- Further microarray analyses on intestines from pigs infected with PCV2 are needed in order to gain deeper insight in the difference in pathogenicity between different isolates of PCV2.
- The microarray technique could enrich the knowledge on the local immune response in a large number of other enteric infections, caused by bacteria, virus or parasites.

Populärvetenskaplig sammanfattning

Diarré sjukdomar är ett vanligt problem hos grisar världen över, och medför stora ekonomiska förluster för grisproducenterna genom ökad mortalitet, försämrad tillväxt, ökade läkemedelskostnader och merarbete i svinstallarna. Tarminfektioner medför också, förutom ökat lidande för grisarna, även en viss zoonosrisk. Behandling med antibiotika kan också innebära risk för resistensutveckling. Sjukdomarna kan orsakas av bakterier, virus eller parasiter, till exempel *Esherichia coli*, *Brachyspira* spp, *Lawsonia intracellularis*, *Clostridium perfringens*, *Salmonella* spp, rotavirus, PCV2 och *Isospora suis*.

Det är viktigt att immunförsvaret kan skilja de farliga mikroorganismerna från vanliga harmlösa bakterier som finns normalt i tarmen och från näringsämnen som ska tas upp av tarmen. För att kunna bekämpa de olika tarmsjukdomarna är det viktigt att veta vad bakterien eller viruset gör när den kommer in i kroppen och hur grisens immunförsvaret reagerar. Immunförsvaret kan studeras på många olika sätt. Bland annat kan man undersöka vilken sorts vita blodkroppar som dominerar, eller titta på vilka cytokiner som produceras. Cytokiner är små proteiner som fungerar som signaler mellan cellerna i immunförsvaret. Olika cytokiner utsöndras av olika slags celler och om man vet vilka cytokiner som bildas kan man skapa sig en bild av immunsvaret. Cytokinproduktionen kan mätas med hjälp av t ex ELISA. Man kan även titta på genuttrycket, dvs om en viss gen är på- eller avslagen, för cytokiner, med PCR eller microarraytekniker. De ovan nämnda metoderna är de som använts i denna studie.

Immunsvaret, både systemiskt i blodet och lokalt i tarmen, undersöktes hos grisar med svindysenteri, som orsakas av bakterien *Brachyspira hyodysenteriae*, samt proliferativ enteropati, som orsakas av den intracellulära bakterien *Lawsonia intracellularis*. Dessutom studerades det lokala immunförsvaret i tarmen hos grisar infekterade med Porcint circovirus typ 2 (PCV2), som ger sjukdomen PMWS.

Tio grisar infekterades experimentellt med *Brachyspira hyodysenteriae*, och av dessa blev åtta kliniskt sjuka i svindysenteri, med blodig diarré. Blodprov togs före infektionen och upprepade gånger under inkubationstiden, sjukdomstiden och återhämtningsfasen. Andelen olika sorters vita blodkroppar under infektionsperioden undersöktes med flödescytometri. Under sjukdomstiden sågs en ökning av monocyter och neutrofiler i blodet. Båda dessa vita blodkroppstyper ”äter upp” och förgör främmande mikrober

och bidrar på så sätt till att bekämpa infektionen. Andelen aktiverade (CD45RA⁺) lymfocyter ökade också. Majoriteten av dessa var CD8α⁺ lymfocyter, en sorts vita blodkroppar som inte använder sig av antikroppar, utan har andra sätt att döda invaderande organsimer. Under återhämtningsfasen kunde man se så kallade γδ T celler och antikroppar mot *B. hyodysenteriae* i blodet. I en senare studie i avhandlingen återfanns en gris som insjuknat naturligt i svindysenteri. Denna gris undersöktes med avseende på förekomst av olika cytokiner i blodet samt genuttryck för cytokiner i tarmen. Cytokinerna IL-1β, IL-6 and TNF-α fanns alla i förhöjd mängd i blodet, och genuttrycket för IL-1β var även ökat i tarmen. Alla de här cytokinerna är inflammationsframkallande och ger bla feber och startar akutfasreaktionen som motverkar infektionen.

Eftersom många tarmsjukdomar inte har så stor systemisk påverkan koncentrerades fortsatta studier mer på tarmen. Som ett led i detta utvecklades en microarray med ett begränsat antal cytokiner. En microarray kan ge svar på om en gen är upp- eller nedreglerad, d.v.s. i detta fall om genen för ett visst cytokin uttrycks mer eller mindre än hos normala grisar. Vanligtvis innebär en uppreglerad gen att mer cytokin produceras. Under de senaste åren har dock den biomedicinska teknikutvecklingen gått fort framåt och det finns nu ett antal kommersiellt tillgängliga microarrayer. Dessa innehåller tiotusentals gener, och finns även för djurslaget gris. I det fortsatta avhandlingsarbetet användes därför dessa microarrayer.

En fältstudie på grisar med kliniska symptom på proliferativ enteropati, orsakad av bakterien *Lawsonia intracellularis*, genomfördes. Denna sjukdom finns i två former: en kronisk med diarré och nedsatt tillväxt, samt en akut med blodig diarré och plötslig död. Inga skillnader i cytokinnivåerna hittades mellan sjuka grisar och kontrollgrisar, varken i blodet eller i tarmen. Resultaten från microarrayanalyser av tarmar pekar på att en okomplicerad infektion med *L. intracellularis* inte ger upphov till något markant immunsvaret och att de allvarliga symptomen med blodig diarré snarare är en komplikation till den kroniska sjukdomen än en akut sjukdomstyp.

Hos grisar med experimentell PMWS sågs ökat genuttryck för IL-6, IL-10 and IFN-γ i tarmen. En ökning av genuttrycket för IFN-γ i tarmen återfanns även hos naturligt sjuka grisar. Dessutom sågs ett markant ökat genuttryck för olika s.k. interferon-stimulerade gener (ISGs) hos grisar med PCV2-infektion. ISGs är gener som triggas igång av interferoner, vilka är en speciell sorts cytokiner. Stimulering av ISGs ses framför allt vid vissa virusinfektioner, där de är viktiga för att bekämpa infektionen.

Utökade analyserar med de metoder som använts i denna avhandling kan ge fördjupad kunskap om immunsförsvaret vid olika tarminfektioner, orsakade av såväl bakterier, virus och parasiter, hos gris.

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