

Studies on the Acute Phase Reaction during Respiratory Infections in Calves

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To my family

*What is man without the beasts?
If all the beasts were gone,
man would die from great loneliness in spirit,
for whatever happens to the beasts
also happens to the man.
All things are connected.
Whatever befalls the earth
befalls the children of the earth.*

Chief Seathl of the Suquamish Tribe 1855

Abstract

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The overall aim of this thesis was to examine the acute phase response, as measured mainly by acute phase proteins (APP) and cellular response, in calves during different types of respiratory infection, and to identify potential markers useful in evaluation of their health status. Experimental infections were performed with virus, bovine virus diarrhoea virus (BVDV) or bacteria, *Mannheimia haemolytica* (*M. haemolytica*), or with a combination of the two micro-organisms. Special interest was focused on the differences between single BVDV or *M. haemolytica* inoculation and co-infection with both agents. In another study, calves were infected with the cattle lungworm, *Dictyocaulus viviparus* (*D. viviparus*) using different dose regimens. Finally, a study was performed to evaluate the usefulness of APP measurements as indicators of health in calf herds.

Co-infection with BVDV and *M. haemolytica* induced the most severe clinical signs, while single *M. haemolytica* inoculation induced none or very mild clinical signs, while BVDV inoculation induced mild to moderate signs. Increases in the APP haptoglobin, serum amyloid A (SAA) and fibrinogen were observed after inoculations with BVDV and/or *M. haemolytica*. The increases coincided in time with the onset of clinical signs. The duration of elevated serum concentrations of SAA and fibrinogen was larger in the group inoculated with both BVDV and *M. haemolytica*. A marked decrease in lymphocyte numbers was observed after BVDV inoculation. This was mainly explained by a decrease in the numbers of CD4+, CD8+ and WC1+ lymphocytes. In contrast, *M. haemolytica* inoculation induced an increase in total neutrophil numbers, while the numbers of CD8+ and WC1+ lymphocytes decreased somewhat. The results indicate that detection of supra-normal APP levels could be useful to identify animals that are, or have recently been, clinically or sub-clinically diseased.

Lungworm infection induced an increase in haptoglobin, SAA and fibrinogen, as well as in the numbers of eosinophils in blood. However, high numbers of eosinophils and low levels of APP do not exclude a diagnosis of lungworm. Thus lungworm infection may not be detected if measurements of APP are used to assess calf health.

In a herd with high disease incidence, a larger proportion of calves had elevated serum concentrations of APP, and a larger number of days per calf with elevated levels of APP compared to a herd with low disease incidence. The results indicate that measurements of serum concentrations of APP can be useful as indicators of herd health.

Keywords: serum amyloid A, haptoglobin, fibrinogen, calves, respiratory disease, acute phase proteins, BVDV, *Mannheimia haemolytica*, *Dictyocaulus viviparus*.

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Gånheim, C., Hultén, C., Carlsson, U., Kindahl, H., Niskanen, R. & Persson Waller, K. 2003. The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or *Mannheimia haemolytica*. *Journal of Veterinary Medicine B* 50, 183-190.
- II. Gånheim, C., Johannisson, A., Öhagen, P. & Persson Waller, K. Cellular response in calves infected with bovine virus diarrhoea virus and/or *Mannheimia haemolytica*. *Manuscript*.
- III. Gånheim, C., Höglund, J. & Persson Waller, K. 2004. Acute phase proteins in response to *Dicytocaclus viviparus* infection in calves. *Acta Veterinaria Scandinavica*, 45, 79-86.
- IV. Gånheim, C., Alenius, S. & Persson Waller, K. Acute phase proteins as indicators of calf herd health. *Manuscript*.

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Abbreviations

APC	antigen presenting cell
APP	acute phase proteins
APR	acute phase response
BAV	bovine adeno virus
BCV	bovine corona virus
BRSV	bovine respiratory syncytial virus
BVDV	bovine virus diarrhoea virus
CD	cluster of differentiation
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GLM	general linear model
IFN	interferon
IL	interleukin
Mh	<i>Mannheimia haemolytica</i>
MHC	major histocompatibility complex
pi	post inoculation
PGF _{2α}	prostaglandin F _{2α}
PIV-3	parainfluenza virus type 3
SAA	serum amyloid A
SD	standard deviation
TBL	tracheo-bronchial lavage
TNF-α	tumor necrosis factor α
WC	Workshop Cluster

Introduction

Background

An important contributor to beef production is the specialised rearing of dairy calves for slaughter. It is mostly bull calves, but also heifers that are not needed for recruitment of dairy cows, that are used for this type of production. Often, calves from many different dairy farms are collected and transported to specialised farms where they are raised to a suitable age for slaughter. This procedure, with transportation stress and mixing of animals from different farms, often causes disease in the calves. One important problem is respiratory disease due to infections with virus and/or bacteria, which can cause disease and suffering for the animals when host resistance is compromised (Dyer, 1982; Bengtsson & Viring, 2000).

The costs for disease outbreaks can be considerable because of veterinary treatment and medication, extra labour time, losses of animals and prolonged feeding period due to reduced growth rate. Moreover, the public concern about animal well-being and food security is increasing, emphasising the need for some kind of quality assurance of animal health in beef production.

There is an obvious need for objective parameters that are suitable as indicators of health, or disease, in herds. Several parameters, such as presence of potential pathogens or antibodies against potential pathogens, treatment incidence and growth have been discussed in this context (Fulton *et al.*, 2002). Lately, the acute phase proteins (APP) have gained increasing interest as objective parameters of animal health, and several reports have been published on this topic (reviewed e.g. by Niewold, Toussaint & Gruys, 2003; Petersen, Nielsen & Heegaard, 2004). APP are produced by the liver cells after stimulation by inflammatory mediators such as cytokines, and can be found in serum of animals during the acute phase response (APR) to different types of disturbances.

Calf diseases

The most important health problems in calves are infectious diseases, especially respiratory disease and enteritis (Olsson *et al.*, 1993). The most common respiratory disease affecting calves is enzootic pneumonia or bovine respiratory disease complex, which is of multi-factorial origin (Wikse & Baker, 1996; Ames, 1997). Among the causative agents are environmental factors, the animal's immunity and different infectious agents such as different virus and bacteria (Dyer, 1982). In Sweden, parainfluenza-3 virus (PIV-3), bovine virus diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine adeno virus-3 (BAV-3) and bovine corona virus (BCV) are associated with the disease (Bengtsson & Viring, 2000). Except for BRSV the viral infection is often sub-clinical or associated with only mild disease. However, it can induce damage to the respiratory organs, facilitating invasion of bacteria e.g. *Mannheimia haemolytica*, *Pasteurella multocida* or *Haemophilus somnus* (Wikse & Baker, 1996). All the major bacterial respiratory pathogens are commensal in clinically

normal cattle (Mosier, 1997). However, during co-infection with virus they can cause serious respiratory disease (Wikse & Baker, 1996).

Mannheimia haemolytica (*M. haemolytica*) (previously named *Pasteurella haemolytica*) is a common inhabitant in the respiratory tract of healthy cattle, as well as of cattle suffering from respiratory disease (Barbour *et al.*, 1997). It is a common isolate from lungs of calves with pneumonia, often in combination with various viruses (Allen *et al.*, 1992). Transportation, viral infections with agents such as PIV-3 or BRSV, overcrowding, housing of neonates and weaned animals together, and other stressful conditions predispose animals to *M. haemolytica* infection (Frank *et al.*, 1996; Brogden, Lehmkuhl & Cutlip, 1998).

BVDV is a viral agent that is often isolated from pneumonic lungs of cattle (Reggiardo, 1979). However, during the last decades, the Swedish eradication program for BVDV has reduced the incidence of this infection. It is known that experimental BVDV infection alone can cause respiratory disease (Potgieter, McCracken & Hopkins, 1984a), and it can also make calves more susceptible to infections with other micro-organisms (e.g. Potgieter, 1995).

Lung worm (*Dictyocaulus viviparus*) is another agent that can cause respiratory disease in cattle. It is a pathogenic parasitic nematode that causes verminous bronchitis, which typically affects young cattle during their first grazing season in temperate areas. Outbreaks vary in severity from sporadic coughing to acute cases with a rapidly fatal outcome, depending on the number of larvae ingested and the immunity of the animal (Radostits *et al.*, 2002).

General aspects on the bovine immune response

The acute phase response (APR)

The APR is a non-specific reaction of the body to various forms of tissue damage. The response is a part of the innate, non-specific immune defence and is essentially the same regardless of the type of disturbance to the body (e.g. infection, trauma, surgery, neoplasia, or immunological disorders) (e.g. Gruys, Obwolo & Toussaint, 1994). The APR consists of several reactions including behavioural, haematological, metabolic, biochemical and immunological changes, and is initiated at the site of the tissue damage (e.g. Baumann & Gauldie, 1994; Gruys, Obwolo & Toussaint, 1994; Petersen, Nielsen & Heegaard, 2004).

Macrophages are the cell type that recognise products from micro-organisms, and thereby become stimulated to release pro-inflammatory cytokines, like interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF- α) (Van Miert, 1995). Virus-infected cells produce interferons (e.g. IFN- α) that act as antiviral cytokines in order to protect other cells against infection and stimulate the immunological response (Tizard, 2004a). The production of cytokines stimulates cells to produce more cytokines and other inflammatory mediators, which circulate in blood (Niewold, Toussaint & Gruys, 2003). The pro-inflammatory cytokines activate different target cells and stimulate the hepatocytes to alter their production of proteins (Gruys, Obwolo & Toussaint, 1994; Van Miert 1995). The proteins with altered production are called APP and can be divided into positive and negative

APP, depending on if their concentrations increase or decrease in plasma during an APR. The cytokines also induce systemic changes such as fever, anorexia, leukocytosis, production of other inflammatory modulators (e.g. prostaglandins) and cortisol (Baumann & Gauldie, 1994; Gruys, Obwolo & Toussaint, 1994; Suffredini *et al.*, 1999). The half lives of the pro-inflammatory cytokines are very short, making them unsuitable for diagnostic purposes, while increases in APP concentrations are persistent for several days (Gruys, Obwolo & Toussaint, 1994).

Acute phase proteins (APP)

Extrahepatic synthesis of APP have been demonstrated in several species (Kalmovarin *et al.*, 1991; Rygg, Husby & Marhaug, 1993; McDonald *et al.*, 2001; Hiss *et al.*, 2003). However, the main source of APP production is the liver, and within hours after tissue damage the protein synthesis by the hepatocytes is drastically altered. This results in decreased production of negative APP such as albumin (Eckersall & Conner, 1988) and transferrin (Kaneko, 1989), and increased production of positive APP such as C-reactive protein, serum amyloid A (SAA), haptoglobin and fibrinogen (Baumann & Gauldie, 1994; Conner *et al.*, 1988; Petersen, Nielsen & Heegaard, 2004).

The positive APP consists of a heterogenous group of proteins, which are species specific. As an example, C-reactive protein is frequently used in human medicine to distinguish between viral and bacterial infections, but is not an APP in cattle (Petersen, Nielsen & Heegaard, 2004; Tizard, 2004b). There are several positive APP in cattle, but SAA and haptoglobin are the only major APP. Major APP are absent, or present in very low levels, in plasma of healthy animals and their concentrations can increase over 100 times after stimulation (Conner *et al.*, 1986, Eckersall & Conner, 1988; Conner *et al.*, 1988; Gruys *et al.*, 1993; Alsemgeest *et al.*, 1994). In protein electrophoresis these APP migrate in the α -globulin fraction (Thomas, 2000). Recently, two new proteins have been proposed as APP in cattle, namely lipo-polysaccharide binding protein (Schroedl *et al.*, 2001) and inter-alpha-trypsin inhibitor heavy chain 4 (Pineiro *et al.*, 2004). Fibrinogen is another APP that has been extensively used to monitor inflammation in cattle. It is a moderate APP, i.e. the levels increase two to three-fold during an APR (Conner *et al.*, 1988 and it migrates in the β -globulin fraction during protein electrophoresis (Thomas, 2000).

The functions of the APP are not fully understood, but they are considered as mediators, inhibitors and scavengers in the inflammatory process (Whicher & Westacott, 1992). Their main functions are opsonization and trapping of microorganisms and their products, binding of cellular remnants (Whicher & Westacott, 1992), complement activation, neutralisation of enzymes, and scavenging of free radicals and haemoglobin (Niewold, Toussaint & Gruys, 2003). SAA is involved in high density cholesterol transport, attracts inflammatory cells (Xu *et al.*, 1995), inhibits the respiratory burst of leukocytes (Linke *et al.*, 1991) and modulates the immune response (Gruys, Obwolo & Toussaint, 1994). Haptoglobin binds free haemoglobin, thereby eliminating it from circulation and conserving the haemoglobin iron. Thus, the toxic and pro-inflammatory effects of free haemoglobin are eliminated (Wagener *et al.*, 2001). Haptoglobin also has anti-

inflammatory effects through inhibition of chemotaxis and leukocyte phagocytosis (Rossbacher, Wagner & Pasternack, 1999). Fibrinogen is involved in blood coagulation as a precursor to fibrin. It binds to red cells and reduces their charge leading to cell aggregation, and is also involved in tissue repair, providing a matrix for migration of inflammatory cells, fibroblasts and endothelial cells (Eckersall & Conner, 1988; Thomas, 2000).

Cellular immune responses in peripheral blood

Total white blood cell counts and differential cell counts is widely used, e.g. to differentiate between inflammatory diseases and disease of other types. Circulating leukocyte numbers vary considerably between calves and adult animals. At birth, calves have a high number of white blood cells and the neutrophils dominate over lymphocytes, while their ratio is reversed in adults. For calves in the age of 3-16 weeks, the neutrophil:lymphocyte ratio is approximately 0.5, which is about the same as in adults (Taylor, 2000). In cattle, the white blood cell count in the early stages of inflammation generally does not reflect the seriousness of the disease. This is due to the fact that in healthy animals, the lymphocytes exceed the neutrophils in number, and that numbers decrease in response to corticosteroids released due to stress. Neutrophils and monocytes also decrease initially as they leave the circulation to participate in the inflammatory reaction. This is compensated by an influx of immature neutrophils from the bone marrow. Thus, transient leukopenia with an increased proportion of immature neutrophils is the common initial response to severe inflammatory disease in cattle. Later, leukocytosis occurs due to neutrophilia, and monocytosis (Jain 1986).

The blood leukocytes play important roles in both the innate and the adapted immune system. Monocytes/macrophages and neutrophils belong to the innate immune system, while lymphocytes are a part of the adaptive immune system. The monocytes constitute 2 to 7% of the bovine blood leukocytes (Jain, 1986), and mature into macrophages when they leave the circulation and enter the tissues. Macrophages can recognise bacterial compounds, and engulf and kill invading micro-organisms (Suffredini *et al.*, 1999). They are also important as antigen presenting cells (APC). After processing the engulfed micro-organism they present the antigen in association with the MHCII molecule on the cell surface. The antigen is recognised by T-lymphocytes, resulting in activation of both T-cells and macrophages and cytokine production is induced. Neutrophils are phagocytic cells and constitute 15 to 45 % of the circulating bovine leukocytes (Jain 1986). They are the first cells to migrate into tissues when inflammation is induced. They engulf and kill micro-organisms, and thereafter undergo apoptosis and die (Tizard, 2004c).

The lymphocytes can be divided into B- and T-lymphocytes. The activated B-cell matures into an antibody producing plasma cell. Antibodies are important in the defence against extra-cellular invaders, such as many bacteria. B-cells can also act as APC, and activate T-cells (Tizard, 2004d). T-cells can be divided into T-helper cells, T-cytotoxic cells, and $\gamma\delta$ Tcells. T-helper cells (CD4+) are important for regulation of the immune response as neither B-cells nor cytotoxic T-cells can

respond optimally to antigens unless they are stimulated by T-helper cells (Tizard, 2004e). Cytotoxic T-cells (CD8+) are important in the defence against intracellular invaders, e.g. virus. The cytotoxic T-cell kill infected cells after it has been activated by antigen presented by APC. Thus, the spreading of the micro-organisms is prevented (Tizard, 2004f). $\gamma\delta$ T-cells cells (WC1+) are a minor lymphocyte subset in humans and mice, but they are rather common in cattle, and in young cattle they can constitute up to 60% of blood lymphocytes (Wyatt *et al.*, 1994; Wilson *et al.*, 1996). Their functions are not fully understood, but they are considered to be important in the early stages of inflammation and to have cytotoxic activity (Pollock & Welsh, 2002; Skinner *et al.*, 2003). According to McBride *et al.* (1998), the proportions of CD4+, CD8+ and WC1+ cells were 29%, 14.4% and 9.6% respectively in healthy one year old cattle.

Local immune response in the respiratory organs

The local immune response in the respiratory tract consists of both innate and acquired defence mechanisms. Intact epithelium, mucus, ciliated epithelial cells, complement, IFN, natural killer cells and phagocytes are parts of the innate immune system, while antibodies and cytotoxic T-lymphocytes form part of the acquired immune system (Perino, 1996). There are considerable differences in the micro-flora between the upper and lower respiratory tract. The upper tract normally harbours a variety of micro-organisms, while the lower tract is sterile. There are also great differences in the immune defence between the upper and lower parts of the respiratory tract. In the upper parts, the defence is non-inflammatory to a great extent, and directed towards prevention of adherence of pathogens. Mucus and ciliated epithelium effectively clear the mucus membranes of micro-organisms. Large particles are trapped in the upper airways and only the smallest particles ($<5\mu$) reach the alveoli (Perino, 1996; Tizard, 2004g). IFN produced by virus-infected cells is an important contributor to the defence, helping to protect neighbouring cells from virus infection (Biron, 1998). Acquired immunity also plays a role, contributing to the defence by antibody activity, of which secretory IgA is the most important isotype (Tizard, 2004g).

In the lower respiratory tract the defence is mostly inflammatory and directed towards killing invading organisms. There are innate components such as alveolar macrophages that form the first line of defence followed by complement and neutrophil activity, once infection is present (Perino, 1996). The acquired immunity contributes with cytotoxic T-lymphocytes and antibodies of which IgG forms a large proportion (Tizard, 2004g). Cattle, and most other domestic animals, differ from humans, rodents and dogs in that their lungs contain large numbers of intravascular macrophages. This leads to a greater capacity to clear bacteria from blood in the lungs compared to the liver and spleen in these species (Tizard, 2004g).

The macrophages are the pre-dominant leukocyte population in the lungs of healthy animals. These cells may constitute approximately 90% of the cells present in lavage fluid from the respiratory tract (Pringle *et al.*, 1988). The second most

common cell type is the neutrophil, while lymphocytes and epithelial cells are less common (Allen *et al.*, 1992). During inflammation in the lung, the cell populations typically change and the neutrophil proportion is increased to become the predominant cell type (Allen *et al.*, 1992).

Immune response to M. haemolytica infection

Through mechanisms that are poorly understood, *M. haemolytica* breaches the innate mucosal defense, including the mucociliary apparatus and antimicrobial factors, to establish infection in the lung. *M. haemolytica* pneumonia is characterized by an acute fibrinosuppurative and necrotizing inflammation with infiltrates of neutrophils, fibrin, seroproteinaceous fluid and blood (Ackermann & Brogden, 2000). The bacterium produces leukotoxin, lipopolysaccharides and polysaccharides. In addition, inflammatory products released during the inflammatory process by neutrophils, and other cells, are present and contribute to the parenchymal damage (Slocombe *et al.*, 1985; Weiss *et al.*, 1991). Macrophages and neutrophils in the alveoli and in the alveolar capillaries phagocytose micro-organisms, and release pro-inflammatory cytokines e.g. IL-1 and TNF- α , which in turn stimulate hepatocytes to produce APP. A rapid increase in haptoglobin and fibrinogen (Cheryk, Hooper-McGrevor & Gentry, 1998) and SAA (Horadagoda *et al.*, 1994) has been reported after intratracheal inoculation of *M. haemolytica*.

Immune response to BVDV infection

The immunosuppressive effect of BVDV infection has been extensively reported (Reggiardo, 1979; Potgieter, McCracken & Hopkins, 1984a; Potgieter, McCracken & Hopkins 1984b; Chase, Elmowalid & Yousif, 2004), and is due to decreased numbers and impaired functions of immune cells. Transient immunosuppression occurs in animals acutely infected with BVDV as cells pivotal in control of both the innate and the acquired immune systems are infected (Chase, Elmowalid & Yousif, 2004). Archambault *et al.* (2000) found a significant drop in the numbers of circulating leukocytes (neutrophils, lymphocytes and monocytes) in calves experimentally inoculated with non-cytopathogen BVDV type 2. BVDV infection has an important impact on circulating T-lymphocytes. Their numbers decrease (Ellis *et al.*, 1988; Brodersen & Kelling, 1999), and the relative proportions of T-lymphocyte subpopulations are affected. The largest decrease is observed in cytotoxic T-lymphocytes (CD8+) followed by helper T-lymphocytes (CD4+), while the circulating $\gamma\delta$ -cells seem to be unaffected (Ellis *et al.*, 1988; Brodersen & Kelling, 1999). BVDV infection also has an effect on B-cells, mainly the follicular B-cells. Varying effects of BVDV infection on circulating B-lymphocytes have been reported. Ellis *et al.* (1988) found a decrease in numbers of B-cells, while Brodersen & Kelling (1999) found a transient increase, and Archambault *et al.* (2000) no effect on the B-cells.

Immune response to lungworm (Dictyocaulus viviparus)

Parasites are totally dependent on the survival of their host for their own existence. Therefore they generally induce only mild or sub-clinical disease (Tizard, 2004h).

However, if the parasite burden is large, severe disease can occur (Radostits *et al.*, 2000). Infective *D. viviparus* larvae are ingested with herbage (Eysker, 1994). After penetration of the intestinal mucosa, the larvae follow blood and lymph to the lungs where they penetrate the alveoli (Urquhart *et al.*, 1996). Migrating *D. viviparus* larvae cause little tissue damage until they reach the lungs. Thereafter, passage of larvae into the bronchioles causes inflammation with recruitment of eosinophils and other inflammatory cells.

Health assessment in calves

Today, assessment of calf health is mostly based on observation of clinical signs, such as depression and body temperature, in combination with specific disease signs, such as nasal discharge, coughing and/or dyspnea in cases of respiratory disease. In the longer perspective, low weight gain may also be a sign of a compromised health. Except for weight gain and body temperature, these parameters are all depending on the observer's capacity to notice clinical signs. In individual animals, blood sampling for evaluation e.g. of the total leukocyte count may help to indicate infectious disease. However, using total leukocyte counts to detect infection are not as informative in cattle as in many other species (Taylor, 2000). On herd level, recordings of treatment frequencies may be used as a measurement of animal health. However, this parameter is also subject to arbitrary decisions of different persons handling the animals. Therefore, objective parameters of animal health could be useful for identifying unhealthy animals both on individual level and on herd level.

APP as indicators of animal health

APP as indicators of individual health

Over the last two decades measurement of APP, especially haptoglobin and SAA, as indicators of health in cattle has gained increasing interest among researchers (for review see Murata, Shismada & Yoshioka, 2004). Fibrinogen has also been used for many years as an indicator of inflammatory disease in cattle (McSherry, Horney & deGroot, 1970; Eckersall & Conner, 1988). Haptoglobin and SAA have been found to increase in serum of cattle with many different diseases (e.g. Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1994; Godson *et al.*, 1996; Hirvonen, Pyörälä & Jousimies-Somer, 1996; Heegard *et al.*, 2000). Measurement of SAA and haptoglobin has also been used to discriminate between acute and chronic inflammation (Alsemgeest *et al.*, 1994; Horadagoda *et al.*, 1999). Some researchers have studied the usefulness of APP as indicators of effect of treatment. Berry *et al.* (2004) found that fibrinogen and haptoglobin concentrations were higher in calves treated multiple times for respiratory disease compared to in those never treated, or treated on a single occasion.

APP as indicators of herd health

The usefulness of APP measurements as a tool for herd health evaluation has mostly been examined for pigs, as reviewed by Petersen, Nielsen & Heegard (2004). The authors emphasized that sub-clinical infections that do not lead to overt disease, but may cause suboptimal growth and concerns for animal welfare,

is of considerable practical importance. They conclude, from several reports, that haptoglobin seems to be a promising marker of health status in pig herds by reflecting a broad spectrum of ongoing clinical, as well as sub-clinical, diseases. In veal calves, Gray *et al.* (1996) found that the predictive value of a negative haptoglobin test for the absence of gross lesions was 90%, making haptoglobin a useful tool for separating healthy animals from those that may need further examination at slaughter.

Cole, Roussel & Whitney (1997) stated that APP profiles show promise in multiple animal investigations to screen groups of cattle for sub-clinical and clinical disease. In addition, Saini *et al.* (1998) suggest that haptoglobin determination can be an important tool for application at the farm and the slaughterhouse to improve food safety, as it is effective in identifying diseased and healthy cattle. Toussaint, van Enderen & Gruys (1995) found that an Acute Phase Index (API) calculated from both positive (haptoglobin and SAA) and negative (albumin and α -2-macroglobulin) APP was a much more reliable parameter for monitoring health in cattle than the individual parameters.

Aims

The overall aim of the present study was to examine the APR, as measured mainly by APP and cellular response, in calves during different types of respiratory infections, and to identify inflammatory markers useful in evaluation of their health status. The specific aims were to:

- examine the APR during respiratory infections with BVDV and/or *M. haemolytica*, and to investigate the differences in APR between single and dual infections.
- characterize the APR during respiratory infection with the lung worm *D. viviparus*.
- investigate whether the measurement of one, or several, APP can be useful for objective evaluation of calf herd health.

Material and methods

Material and methods used in the present study are described in detail in papers I-IV. Here, only general comments are made.

Animals

All experimental animals (n=92 calves) used in these studies were of the dairy breeds, namely Swedish Red and White or Swedish Holstein, or crosses between the two breeds. They were male calves, which came from commercial dairy units located in the middle of Sweden. The calves used in papers I, II and III, and in group B in Paper IV, came from herds that were declared free from BVDV and enzootic bovine leucosis, according to the specifications for the Swedish eradication programmes. At the start of the experiments, the age of the animals was 2-3 months. All calves, except group A in Paper IV, were kept at the Department of Clinical Sciences, Division of Ruminant Medicine and Epidemiology, Faculty of Veterinary Science Medicine and Animal Science, Swedish University of Agricultural Sciences. Group A in Paper IV was kept in a commercial farm close to Uppsala, Sweden.

Experimental designs

Animal ethics approval of the experimental designs for all studies was sanctioned by the Swedish National Board for Laboratory Animals, Uppsala, Sweden.

Papers I and II

Before the start of the study, which lasted for 23 days, the calves were allowed a three weeks adjustment period in pens. Twenty-four calves were divided in four equal groups. The groups were denominated as control, BVDV, Mh and BVDV/Mh respectively, depending on the type of infectious agent inoculated in the respiratory tract. The BVDV and BVDV/Mh groups were inoculated with BVDV on day 4 of the study, and the Mh and BVDV/Mh groups were inoculated with *M. haemolytica* on day 9 of the study. Thus, the BVDV/Mh group received both types of infection, five days apart. No inoculations were given to the calves in the control group.

Rectal temperatures and general appearance were assessed daily during the adjustment period. If signs of depression were observed in an individual, a more detailed examination was performed. After inoculations, thorough daily clinical examinations were undertaken. Blood samples were obtained at two to three occasions before inoculations, and thereafter daily, or twice daily. Tracheo-bronchial lavage (TBL) was performed once before, and at three occasions after, the inoculations.

The BVDV strain used was a non-cytopathogenic field strain of BVDV type 1 obtained from serum of a persistently infected calf, while the *M. haemolytica*

strain was obtained from tracheo-bronchial lavage in a calf with clinical signs of pneumonia.

Paper III

Three groups of calves were studied, with 11, 5 and 6 calves in each group, and the animals were allowed an adjustment period of four weeks before the experiments started. The three groups of calves were inoculated orally with third stage infective larvae of the lungworm, *D. viviparus*, using different dose regimens. The larvae were obtained from infected donor calves. The animals in the three groups were studied for 35, 30 and 28 days, respectively. Rectal temperatures were recorded daily throughout the adaptation period and throughout the study. Clinical signs, such as coughing and depression, were also recorded daily. Blood samples were taken before inoculations and at six to eight occasions after inoculation. Faeces samples were collected at the start of the experiments and then once weekly.

Paper IV

Two groups of calves (35 calves in group A and 11 calves in group B) in two different herds were studied during six weeks after arrival at the farms. The animals were blood sampled and observed clinically twice weekly for four weeks, followed by once weekly for another two weeks. All diseases were naturally acquired. However, the calves in group B were originally part of another study in which they were inoculated with larvae of *D. viviparus*. However, the infection never reached patency, and none of the calves shed parasites in faeces. Brief clinical observations and blood samplings were done twice weekly for four weeks after arrival, and then once weekly for another two weeks. Coughing, diarrhoea, depression and other clinical signs of disease were recorded.

Analyses

Acute phase proteins, interferon- α , prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and cortisol (Papers I-IV)

In Papers I-IV, the serum haptoglobin concentrations were analysed using a commercial kit based on the haemoglobin-binding capacity of haptoglobin. The serum SAA concentrations were analysed using a commercial ELISA kit, with extra standard points added to the standard curve. In Papers I-IV, fibrinogen concentrations were determined in EDTA-plasma using a kinetic method, which measures increasing turbidity of the sample following activation of the clotting with a snake venom (batroxin).

In Paper I, IFN- α concentrations were measured as antiviral effect in serum, plasma concentrations of the major $PGF_{2\alpha}$ -metabolite, 15-ketodihydro- $PGF_{2\alpha}$, were measured by a radio immuno assay, and the Immulite Cortisol Assay was used to determine cortisol levels in serum.

Cell counts in blood and TBL (Papers II-IV)

Total and differential blood cell counts were analysed by an automated blood analyser (Paper II-IV). Flow cytometry was used to examine lymphocyte subpopulations that were labelled with antibodies to WC1, CD4, CD8, B-cells and IL-2R antigens (Paper II). The differential cell counts in TBL fluids were evaluated microscopically (Paper II). The TBL fluid was also examined for bacterial growth.

*Parasitological examination for *D. viviparus* (Paper III)*

Confirmation of infection, and determination and enumeration of excreted *D. viviparus* larvae was performed in fresh faeces. The specific antibody response reflecting patent *D. viviparus* infection was measured in serum using a diagnostic ELISA-kit. Infections were also confirmed by demonstration of lungworms at slaughter of the calves.

Statistics

In Paper I, mean values and standard deviations (SD) for haptoglobin, SAA and fibrinogen were calculated from all pre-inoculation values and control group values. These mean values plus two SD were considered as the basal level, and values above were considered supra-normal. The two-sided Student's t-test was used to test for significant differences between groups in days with supra-normal values for each parameter.

In Paper II, a general linear model (GLM) in the SAS system, with Dunnett's adjustment for multiple comparisons was used for analyses of maximum and minimum values for total leukocyte, neutrophil, lymphocyte and monocyte counts in BVDV, Mh and BVDV/Mh group compared to control group. The numbers of each lymphocyte subpopulation were calculated using the total lymphocyte number and the proportion of the subpopulation given at the flow cytometric analysis at each time point. For flow cytometry results, a mixed procedure in the SAS system was used for analyzing over all effect of time and group. A GLM in the SAS system with Dunnett's adjustment for multiple comparisons was used for analyzing days with significant change from pre-inoculation values within groups.

In Paper III, a GLM for repeated measures was used in SAS for making statistical inferences of the dependent variables, eosinophils, haptoglobin, SAA and fibrinogen. The values of the different days were also tested pairwise with the values day 0 using Dunnett adjustment to avoid mass significances.

In Paper IV, APR scores were calculated for each group of animals. The occurrence of supra-normal values of SAA, haptoglobin, fibrinogen or total leukocyte count, was given one point each. A sum of points was calculated for each individual and sampling occasion, and the mean (SD) value for each group was established. Leukocyte counts, APP concentrations, numbers of days with supra-normal APP values and APR scores were compared using one-sided Student's t-tests. The Bonferroni correction was used to avoid mass significances.

In all studies, a p-value <0.05 was considered significant, and all results presented are significant, if nothing else is stated.

Results

Experimental infections with BVDV and/or *M. haemolytica* (Papers I and II)

Clinical examinations

All calves in the control group remained healthy throughout the study. In all inoculated groups, some or all animals had elevated body temperatures and affected general appearance in varying degrees after inoculation. In the BVDV-inoculated group, all individuals had fever and affected general appearance starting day 7 post inoculation (pi), while in the Mh group, two calves were mildly depressed and three had a rise in body temperature the day after the inoculation. The most severe clinical signs were observed in the BVDV/Mh group, where all calves had fever and depression for several days.

APP concentrations (Paper I)

The concentrations of APP in the animals in the control group were generally low or below detectable levels. After BVDV-inoculation, all animals had increased concentrations of all three APP, with maximum values around days 8-9 pi (day 12-13 of the study). Inoculation with *M. haemolytica* induced an increase in concentrations of all three APP in all inoculated animals within 24 hours. The concentrations decreased quickly, starting with SAA, followed by haptoglobin, and a few days later by fibrinogen.

In the group inoculated with both BVDV and *M. haemolytica*, the concentrations of all three APP had a more complicated pattern with larger individual variations. In general, the APP concentrations did not increase until after the *M. haemolytica* inoculation, and for SAA, a biphasic pattern was observed. In most cases, the concentrations for all three APP decreased somewhat slower than in the groups inoculated with a single infectious agent.

The number of days with supra-normal levels of all three APP was greater in the inoculated groups compared to the control group. However, there were no differences in haptoglobin between the inoculated groups. For SAA and fibrinogen, the number of days with supra-normal levels was higher in the BVDV/Mh group compared to the BVDV group.

IFN- α , PGF_{2 α} -metabolite and cortisol (Paper I)

All BVDV inoculated animals were positive for IFN- α on days 4 and 6 pi. Thereafter, five of six calves in the BVDV/Mh group were positive for IFN- α at least once, but only two calves in the BVDV group. There were no differences in PGF_{2 α} -metabolite values between the calves in the different groups, or between pre- and post-inoculation values within groups. Cortisol values, analysed at four occasions from two animals did not differ between animals, or between pre- and post-inoculation time points.

Cellular responses in blood (Paper II)

Inoculation with BVDV induced decreases in total leukocyte counts and lymphocyte counts compared to the control group. The decrease was observed 3 days pi and lasted for five days. Non-significant decreases in neutrophil and monocyte numbers were also observed. Inoculation with *M. haemolytica* in animals not inoculated with virus, induced increases in total leukocyte counts and neutrophil counts, which appeared the day after inoculation and lasted only for one day. A non-significant increase in monocyte numbers was also seen, while the lymphocyte count decreased for one day starting the day after inoculation. In BVDV-inoculated animals, *M. haemolytica* inoculation only induced a small increase in total leukocyte counts, which did not reach pre-inoculation values and was not significant compared to the control group. The same was seen for monocytes. The decrease in lymphocyte counts observed in the Mh group also appeared in the BVDV/Mh group. The same pattern of decrease was observed on the day post *M. haemolytica* inoculation and made the decrease induced by the BVDV inoculation even more accentuated. In this group, the lowest leukocyte and lymphocyte counts were observed.

The most common lymphocyte sub-population at the beginning of the study was WC1+ cells, followed by B-cells. Effects of groups and time was observed in the numbers of CD4+, CD8+ and WC1+ lymphocytes. No effects were seen on the B-cells and IL2R+-cells. The numbers of CD4+, CD8+ and WC1+ cells were lower after BVDV inoculation than in the control group, and the most pronounced decrease was found in the BVDV/Mh group. *M. haemolytica* inoculation induced decreased numbers of CD8+ and WC1+ cells.

Cellular responses in TBL (Paper II)

The results from differential counts of TBL cells did not show any significant differences between groups. There were large individual differences, both between and within groups. No clear trend towards a more pronounced neutrophil influx in inoculated animals, or animals with signs of respiratory disease was observed. In TBL with growth of *M. haemolytica*, neutrophils were the dominant cell type in four out of six cases.

Experimental infections with *D. viviparus* (Paper III)

Infection with *D. viviparus* induced increases in numbers of eosinophils and in serum concentrations of haptoglobin, SAA and fibrinogen. These changes occurred at the same time as the calves began to show clinical signs, such as respiratory distress and coughing, coinciding with the time when the migrating parasites were expected to reach the lungs.

APP as indicators of calf herd health (Paper IV)

The APR score was numerically higher in the group with the higher disease incidence (group B) during the whole study except at the last sampling occasion. However, the difference was only significant at days 4 and 8 of the study. The proportion of calves with supra-normal levels of APP were generally numerically higher in group B compared to group A throughout the study. The largest difference in the proportion of calves with supra-normal levels appeared for haptoglobin. In group B, the mean maximum concentrations of haptoglobin and fibrinogen were higher than in group A. The maximum concentration of SAA was numerically higher in group B, but not significantly different, from in group A.

General discussion

Methodological considerations (Papers I-IV)

Experimental designs

In paper I, the calves were allowed a three week adjustment period before the experiment started. As a comparison, the animals in group B in Paper IV did not have any adjustment period before sampling started, and in that experiment many calves had elevated levels of APP when the study started. This was probably due to stress and maybe also to some small traumatic damages from transportation and co-mingling. Elevation of APP due to infections acquired during the mixing of animals at their arrival in the new environment is not likely to occur as early as after 2-3 days, as the incubation time for the infectious agents are longer.

In paper IV, the two groups studied were somewhat different in age and group size, and they were kept in two different stables managed by different personnel. The two latter factors may have influenced the recordings of disease as the ability of the keeper to identify diseased animals may differ considerably. The larger group size in group A may have resulted in a poorer surveillance of the animals. However, no calf died in this group, which indicates that diseased calves received the necessary treatment.

Concerning the age of the calves, the younger age in group A than in group B (paper IV) is likely to give a higher proportion of diseased animals, as younger animals are considered to be more susceptible to infections. However, this was not the case, since the incidence of disease was higher in group B. It is possible that the difference in disease incidence would have been even greater if the two groups had been of the same age.

In paper IV, the calves in group B were also part of another study in which they were infected with larvae of the cattle lung worm, *D. viviparus*. The infection was considered a failure as none of the calves developed a patent infection demonstrated by shedding of larvae in faeces. However, the increased peripheral blood eosinophil numbers that were observed in the group indicated that the immune system had responded to the parasite infection. This may have affected the clinical picture and the levels of APP. Anyhow, the inclusion of this group in the study was considered appropriate, as that study focused on the general health status in the herd and not on specific infectious agents.

Tracheo-bronchial lavage (Paper II)

The TBL method used was originally developed for field use to do bacteriological examinations of calves with respiratory disease (Bengtsson *et al.*, 1998). It was slightly modified by increasing the amount of fluid used. The method is easy to perform and has a small impact on the animals, compared to methods where sedation or general anesthesia is required. However, its main deficiency was that it was not always possible to obtain a sample, or to obtain sufficient amounts of

lavage fluid and cells for subsequent examinations, e.g. cytoplots. Despite this limitation, we chose to use this method as we had the necessary equipment and were experienced in using it. In retrospect, it may have been better to try to find a more reliable method, however, methods requiring sedation or general anaesthesia were not considered good alternatives.

The differential cell counts in TBL were evaluated microscopically with cytoplots. However, these evaluations were sometimes difficult due to destruction of cells. Therefore, the initial intention to do detailed differential counts was abandoned. Instead, the samples were categorized into those where macrophages were the dominating cell type, those where neutrophils dominated, and those where both cell types were approximately equal. In calves inoculated with *M. haemolytica*, the severe destruction of cells in the cytoplots may have been an effect of leukotoxin produced by these bacteria (Ackermann & Brogden, 2000).

The APR during experimental infection with BVDV and/or *M. haemolytica* and during infection with *D. viviparus* (Papers I, II and III)

The experimental model used in papers I and II was very successful. The calves in the control group remained healthy throughout the study and maintained very low levels of APP, while the animals in the infected groups showed reactions at clinical examination and in APP levels. As expected, the results showed that animals that were infected with both virus and bacteria developed more serious disease compared to those infected with monospecific infections. Clinical signs were most severe and long-lasting in the BVDV/Mh group. Mild clinical signs were also observed in the BVDV group, but in the Mh group only three out of six calves were mildly affected the day after inoculation. During the rest of the study they were essentially unaffected. Thus, the *M. haemolytica* strain used, which was isolated from a calf with pneumonia, seemed to have low pathogenicity when it was used as a single infection despite the high inoculum doses. On the contrary, calves that had been infected with BVDV five days before the *M. haemolytica* infection became more seriously affected and one calf did not recover during the study. This indicates that the bacterium needs a predisposing factor to cause disease which is consistent with previous reports (Dyer, 1982; Ackermann & Brogden, 2000). Co-infection with BVDV and *M. haemolytica* also induced a longer duration of elevated APP concentrations compared to single BVDV or *M. haemolytica* inoculation, reflecting the duration of the clinical symptoms. However, only the difference between the BVDV and the BVDV/Mh groups was significant.

No differences in PGF_{2α}-metabolite or cortisol concentrations were seen between the animals in the different groups. An increase in these parameters might have been expected after *M. haemolytica* inoculation, since intravenous injections of endotoxin in cattle have been reported to induce significant increases in both PGF_{2α} and cortisol (Fredriksson, 1984; Werling *et al.* 1996). However, it is possible that the endotoxin content of the bacteria used in our study was too low, or that the time of blood sampling was unsuitable to discover changes in PGF_{2α}

and cortisol. Moreover, the route of inoculation differed which may have been of importance.

In our study, the total leukocyte, neutrophil and lymphocyte counts decreased after BVDV inoculation. A decrease, although not significant, was also seen in monocyte numbers. This is consistent with Archambault *et al.* (2000) who observed significant decreases in total leukocytes, neutrophils, lymphocytes and monocytes after inoculation with BVDV type 2, and Tråvén *et al.* (1991) who reported decreases in total leukocyte and lymphocyte numbers. We observed that the kinetics of total leukocyte counts and neutrophil and lymphocyte numbers in the BVDV/Mh group were, in general, a combination of the kinetics for each infection alone. The decreases that were seen after BVDV inoculation also appeared in the BVDV/Mh group and the peaks following Mh inoculation appeared both in the Mh and in the BVDV/Mh group. The decreases were generally greater in the dual infected group compared to single BVDV infection, and the peaks were smaller than for the single Mh infected group.

Flow cytometry analysis showed that BVDV inoculation induced decreases in total numbers of CD4+, CD8+ and WC1+ lymphocytes. The numbers of CD8+ and WC1+ cells also decreased after *M. haemolytica* inoculation. As mentioned earlier, these cells have important functions in the immune response and depletion of these cells seriously compromises the animals ability to defend it self against the infections (Chase *et al.*, 2004). According to our findings, the numerically largest decreases of CD4+, CD8+ and WC1+ cells were seen in the BVDV/Mh group, causing the most serious damage to the immune response which was also reflected in the severity of the disease. While we studied total numbers, other authors have studied proportions of cell types (Brodersen & Kelling, 1999; McBride *et al.*, 1999). This makes comparisons between studies difficult. However, at least for BVDV infection, the trends seem to be similar. Brodersen & Kelling (1999) found decreased proportions of peripheral CD8+ lymphocytes after BVDV inoculation. However, McBride *et al.* (1999) found no significant changes in peripheral blood lymphocyte subsets after infection with *Pasteurella haemolytica* in one-year old calves. In our study, no effects on B-cell numbers were observed after inoculation. This is maybe not surprising, as BVDV infection is reported to have its major effect on follicular B-cells (Chase *et al.*, 2004) whereas the effect on circulating B-cells varies in different studies as mentioned earlier.

Lung worm infection induced an APR that could be measured as increased concentrations of haptoglobin, SAA and fibrinogen. The time of onset of clinical symptoms coincided with the APP reaction, which is consistent with results from experimental viral and/or bacterial infections (Gånheim *et al.*, 2003). It also coincided with the time when the lung worm larvae reached the lung. This indicates that migration of larvae from the gut to the lungs do not induce enough tissue damage to start an APR with APP production. Significant increases in eosinophil numbers were observed after lung worm infection and have been well documented earlier (Taylor, 2000; Höglund, Gånheim & Alenius, 2003).

APP as indicators of individual calf health (Paper I-III)

From our results, and from other studies (Murata, Shimada & Yoshioka, 2004), we can conclude that healthy animals have very low levels of APP in serum. If any APP reaction occurs, this is likely to be due to a stimulation of the immune system indicating some kind of damage to the animal. In many cases this damage may be mild without clinically observed symptoms. Despite this, it may have caused discomfort for the animal. As have been reported earlier, bacterial and viral infections cause APP elevations. It has generally been considered that viral infections give a weaker APR, or no reaction at all (Spooner & Miller, 1971; van Leeuwen & van Rijswijk, 1994). However, recently there have been several reports supporting our results that viral infections can induce an APR which is comparable to that induced by bacteria (Höfner *et al.*, 1994; Heegard *et al.*, 2000). In these studies, the APP increase occurred at the same time as clinical signs appeared, i.e. several days pi. Our observations are consistent with this, and indicate that analysis of APP as a tool for the prediction of disease, e.g. during incubation time, is not possible.

There are contradictory reports on the usefulness of APP concentrations as a measurement of disease severity. Heegaard *et al.* (2000) found that the magnitude and the duration of the haptoglobin response in general correlated well, and better than SAA, with the severity of clinical signs and with lung consolidation at necropsy. Gray *et al.* (1996) found no correlation between haptoglobin levels and the relative severity of inflammatory or degenerative processes found at necropsy. In Paper I, we found that the three calves in the BVDV/Mh group that had the most severe clinical symptoms also had the highest concentrations of haptoglobin, and the longest duration of the haptoglobin response. The same animals were also among those that had the highest concentration of fibrinogen and duration of elevated fibrinogen levels. However, disease severity and duration did not correlate with magnitude or duration of SAA response. From the studies presented in Papers III and IV, we cannot conclude that more severe clinical signs always correlate with higher levels of APP. However, in those studies the clinical signs of the calves were, in general, less severe than in the experimentally infected animals in Paper I. Heegard *et al.* (2000) observed that haptoglobin did not increase in some cases with mild clinical signs, while SAA increased slightly without correlation with fever or pathology score.

In our study of experimental infections with BVDV and *M. haemolytica*, we observed that an animal with a very high concentration of one or two APP did not always have a high concentration of the third APP. This was most obvious in the BVDV/Mh group in Paper I. The only calf (number 21) that did not recover during the study had very high concentrations of haptoglobin and fibrinogen, but the SAA concentration remained low. Interestingly, this calf had the lowest total leukocyte and lymphocyte counts of all animals in the group during the whole study, and it also had low total numbers of CD8⁺ and WC1⁺ lymphocytes in blood.

There are few reports in the literature about APP response during bovine parasitic infections. Conner *et al.* (1989) found that an APR was not a consistent feature of ostertagiosis, although some animals showed a rise in haptoglobin. In a study of cows persistently infected with *Neospora caninum*, Guy *et al.* (2001) did not find any change in haptoglobin during recrudescence of parasitosis during pregnancy. However, Glass *et al.* (2003) recorded an APP response in cattle infected with *Theileria annulata*. An increase in SAA concentrations was observed in all animals, whereas haptoglobin only appeared at low levels in some of the animals. Also in this type of infection, the APP elevation coincided with onset of clinical signs. In our studies, experimental infection with BVDV or *M. haemolytica* generally gave a higher concentration of haptoglobin, SAA and fibrinogen compared to infection with lung worm. However, there were significant increases in all three APP following lung worm infection. Consistent with previous reports, these elevations coincided in general with the onset of clinical signs. The above mentioned reported variations in APP reactions during parasitic infections probably reflect the different extent of tissue damage and inflammatory reaction that the parasite induces.

APP as indicators of calf herd health (Paper IV)

A large proportion of calves in both groups with different health status had elevated concentrations of APP at one or several occasions during the first six weeks after introduction into a new environment. This was not surprising, as we had expected a number of calves to experience infection after transportation and mixing with individuals from other farms. However, in our study we found that a larger proportion of calves in the herd with a high disease incidence had supra-normal concentrations of APP. The number of days per calf with supra-normal concentrations was also larger in the more unhealthy group, with the biggest difference for haptoglobin, indicating that this may be the most useful APP to measure. This is consistent with Carter *et al.* (2002) who concluded that analysis of serum haptoglobin was a better tool for discrimination between calves that became ill and those that did not, compared to other APP. SAA is reported to be more sensitive to stimulation (Horadagoda *et al.*, 1999; Heegaard *et al.*, 2000), and as an increase can be induced also by other factors than disease, e.g. stress (Alsemgeest *et al.*, 1995), it may be less reliable as an indicator of herd health. The APR score that was created to get an overview of the APR in the different groups was higher in the high disease incidence group. Other researchers have reported that measurement of a single APP is not reliable for health evaluation, and recommend combined analysis of several parameters (Toussaint, van Ederen & Gruys, 1995; Young *et al.*, 1996). In our study, the differences in health status between the groups were consistent with the differences in APR score. In our study on experimental infections (Paper I), we observed that the healthy animals in the control group generally had low, or undetectable, concentrations of APP. Our results indicate that if there is an increase in APP, there has been some kind of activation of the animals defence, e. g. fever. In veal calves, Gray *et al.* (1996) found that the predictive value of a negative haptoglobin test for the absence of gross lesions was 90%, making haptoglobin a useful tool for distinguishing healthy animals from those that may need further examination at slaughter. This is

in accordance with my opinion, that if there is no APP reaction, the animal is likely to be healthy. In conclusion, my opinion is that measurement of APP can be a useful tool for evaluation of herd health. However, many more herds need to be examined to establish thresholds for acceptable health. Moreover, today the high cost for the analyses is a problem. If cheap and simple methods would be available, sampling at appropriate occasions during the rearing period would probably give a good impression of the animal health in a farm, and constitute an objective parameter for quality assurance in beef production, which could be beneficial both for animal health and food safety.

Conclusions

- Serum concentrations of haptoglobin, SAA and fibrinogen in healthy animals were low, or below detectable limits.
- Inoculation of calves with BVDV or *M. haemolytica* in the respiratory tract, or co-infection with both micro-organisms, induced an APR that could be observed as an increase in serum concentrations of haptoglobin, SAA and fibrinogen. Co-infection with BVDV and *M. haemolytica* induced a longer duration of elevated SAA and fibrinogen concentrations compared to single BVDV inoculation.
- The increase in APP concentrations generally appeared simultaneously with the onset of clinical symptoms, about one week after BVDV inoculation, and within 24 hours after single inoculation with *M. haemolytica*.
- BVDV inoculation induced decreases in peripheral total leukocyte and lymphocyte numbers. *M. haemolytica* inoculation induced increases in peripheral total leukocyte and neutrophil numbers and a decrease in lymphocyte numbers. In calves inoculated with BVDV, *M. haemolytica* inoculation induced only minor increases in peripheral total leukocyte and neutrophil numbers.
- BVDV inoculation induced decreases in peripheral numbers of CD4+, CD8+ and WC1+ lymphocytes. *M. haemolytica* inoculation induced decreases in peripheral lymphocyte subsets CD8+ and WC1+.
- Lungworm (*D. viviparus*) infection can induce an APR as measured by an increase in SAA, haptoglobin, fibrinogen and eosinophils. The increase in APP concentrations after lungworm infection coincided, in general, with the onset of clinical symptoms, when the migrating larvae reached the lung.
- The APR score was higher in a group of calves with high incidence of disease compared to a group with low incidence of disease. In a group with high disease incidence, a larger proportion of calves had supra-normal concentrations of APP, compared to a group with low disease incidence. The largest difference appeared for haptoglobin, indicating that it may have the best potential as indicator of herd health. The number of days per calf with supra-normal concentrations of haptoglobin, SAA and fibrinogen was higher in the group with higher disease incidence compared to the healthier group.
- Measurement of serum concentrations of haptoglobin, SAA and fibrinogen as indicators of health status in calves can be useful both on individual level and on herd level.

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Populärvetenskaplig sammanfattning

En viktig del av nötköttproduktionen i Sverige utgörs av den specialiserade uppfödningen av kalvar av mjölkkras till slakt. Det är oftast tjurkalvar som utnyttjas i denna produktionsform, men även kvigkalvar som inte behövs för nyrekrytering av mjölkkor används. Uppfödningens form innebär att kalvar köps in från flera olika ursprungsbesättningar och transporteras till gårdar som specialiserat sig på uppfödning av ungdjur till slakt. Kalvarna är ofta i åldern en till två månader när de byter miljö. Att blanda djur från olika miljöer och att utsätta dem för stress i form av transport och konfrontation med nya kamrater och ny miljö innebär stora risker för sjukdomsutbrott bland djuren.

Under uppfödningstiden i den specialiserade produktionsformen kan djurhälsan variera betydligt mellan olika besättningar. De vanligaste sjukdomarna är luftvägsinfektioner och diarréer. Hur hälsoläget blir beror dels på ovan nämnda faktorer, men också på hur miljön och skötseln ser ut i besättningen. Om sjukligheten är hög kommer antibiotikabehandlingar att behöva sättas in i större utsträckning än om hälsoläget är gott. Den sjuklighet som kräver behandling kan lätt beräknas, men i många besättningar kan man misstänka att ett antal djur är utsatta för sjukdom utan att visa tydliga symtom, s.k. subklinisk sjukdom. Denna subkliniska sjukdom kan störa djurens välbefinnande. I takt med att konsumenterna blir mer medvetna och efterfrågar god kvalitet på köttet samtidigt som det ska vara producerat på ett djur- och miljövänligt sätt, ökar behovet av kvalitetssäkring av produktionen. Någon form av objektiva parametrar på hälsoläget i en besättning skulle därför kunna vara värdefulla som en del i en sådan kvalitetssäkring.

Alla individer, människor och djur, försvarar sig ständigt mot angrepp av yttre faror. Skador, som infektion med bakterier, virus eller parasiter, sårskador, kirurgiska ingrepp, UV-strålning, tumörer mm utlöser ett försvar hos individen. Försvaret består av många delar, varav en del kallas det medfödda eller icke-specifika immunförsvaret. Akutfasreaktionen (APR) utgör en del av detta medfödda försvar, och är i stort sett den samma oavsett vilken typ av skada som kroppen utsätts för. APR består av ett flertal reaktioner som inkluderar förändringar i beteende, blodbild och ämnesomsättning. Även biokemiska och immunologiska processer sätts igång vid platsen för skada. Skadad vävnad frisätter ämnen som attraherar celler med uppgift att ta hand om invaderande organismer eller aktivera fler celler. Dessa ämnen kallas cytokiner och de förekommer i ett flertal former med olika uppgifter i immunförsvaret. Cytokinerna har ett kortvarigt liv och försvinner snabbt ur blodet.

En av effekterna av cytokinerna är att aktivera celler i levern så att dessa börjar producera s.k. akutfasproteiner (APP) som har ett flertal olika uppgifter för att styra immunförsvaret. Positiva APP är proteiner som normalt finns i mycket låga nivåer, eller saknas helt, i blodet hos friska individer, men som stiger i

koncentration vid skada. Vilka proteiner som räknas som APP varierar mellan olika djurslag. C-reactive protein (CRP), som används mycket inom sjukvården för att skilja mellan virus- och bakterieinfektion hos människor, fungerar t ex inte alls som APP hos nötkreatur. De mest reaktiva APP hos nötkreatur är haptoglobin och serum amyloid A (SAA). Även fibrinogen, som stiger i måttligare nivåer, är betydelsefullt. I letandet efter objektiva parametrar för att mäta hälsoläget hos djur har man kommit att intressera sig för dessa APP. Man vet sedan tidigare studier att proteinerna stiger vid sjukdom och har även sett att subklinisk sjukdom ger utslag i form av förhöjda nivåer i blodet av APP. Då APP har en längre "livstid" i blodet än cytokinerna utgör de en mer lämplig faktor att undersöka.

Vi har undersökt haptoglobin, SAA och fibrinogen i blodet hos kalvar som infekterats med bakterier och/eller virus i luftvägarna. En del kalvar hade bara infektion med virus eller med bakterier, medan en del infekterades samtidigt med både virus och bakterier. Detta är en vanlig kombination som orsakar luftvägsinflammationer bland kalvar i besättningar. Vanligen slår virusinfektionen till först och försvagar kalvens motståndskraft mot bakterien. Som väntat var de kalvar som hade en dubbelinfektion sjukast och de fick också förhöjda APP under en längre tid än de som var infekterade med antingen virus eller bakterier. Höjningen av APP-nivåerna efter virusinfektionen kom först efter flera dagar och nådde sitt maxvärde efter en dryg vecka, vilket sammanföll i tiden med när kalvarna visade kliniska symtom. Efter bakteriell infektion steg nivåerna av APP mycket snabbt. Inom 24 timmar efter infektion hade de allra flesta kalvarna kraftigt förhöjda nivåer av alla APP. De dubbelinfekterade djuren fick först virus och 5 dagar senare bakterier i luftvägarna. Dessa djur fick i allmänhet sin APP-stegring först efter att de även infekterats med bakterier. Stegringen hos dessa djur var inte alltid lika snabb som hos de som enbart fått bakterier, men höjningen kvarstod längre.

Vi utförde även studier av hur de vita blodkropparnas antal och inbördes förhållanden ändrades vid dessa olika typer av infektioner. Man vet sedan tidigare att infektion med bovint virus diarré virus (BVDV) ger en sänkning av antalet vita blodkroppar. Det är bl a denna sänkning som orsakar att djurens immunförsvar blir försämrat vid denna infektion. Vi observerade att flera undergrupper av lymfocyter, en typ av vita blodkroppar, minskade kraftigt i antal ffa efter BVDV-infektion. Dessa celler har betydelse för immunförsvaret genom att hjälpa till att aktivera fler celler (CD4+T-helper cells) eller genom att döda celler som är angripna av virus och därigenom hindra vidare spridning (CD8+ cytotoxiska T-celler). En tredje grupp som också minskade är de sk WC1+ cellerna som är viktiga i den tidiga fasen av försvaret mot infektioner.

Uppgifterna i litteraturen om hur APP reagerar vid parasitinfektion är fåtaliga. Inflammation i luftvägarna kan orsakas även av parasiter. Vi gjorde därför en studie där vi infekterade kalvar med lungmasklarver. Vi kunde då påvisa att lungmaskinfektion kan ge stegringar av APP. Vi mätte även eosinofiler, ett slags vita blodkroppar i blod. Dessa stiger bl. a. vid parasitinfektioner och som väntat fick även kalvarna i studien förhöjda nivåer av eosinofiler i blodet. Vår slutsats är

att om blodprov visar både förhöjda eosinofiler och APP kan det tyda på lungmaskinfektion.

För att få en uppfattning om huruvida mätning av APP kan användas som ett mått på hälsoläget hos kalvar genomförde vi en studie i två besättningar med olika sjuklighet. Vi tog blodprov och analyserade APP, samt gjorde noteringar om sjukdomsfall och djur som behövde behandling. Vi kunde konstatera att antalet djur med förhöjda nivåer av APP var högre i den besättning som uppvisade den större sjukligheten. Med utgångspunkt från våra resultat anser vi att mätning av APP skulle kunna vara till nytta för att bedöma hälsoläget i en besättning och kunna användas som en del i ett kvalitetssäkringsprogram för specialiserad uppfödning av kalvar till slakt. För att fastställa vilka nivåer som ska bedömas som dålig respektive god djurhälsa behöver flera besättningar undersökas. Sammanfattningsvis kan vi konstatera att friska kalvar har mycket låga nivåer av APP i blodet. Har man en stegring av APP tyder det på att immunförsvaret har stimulerats på något sätt, av en infektion eller annan störning av djurets immunförvar.