Haptoglobin and SAA concentrations and enzyme activities in bronchoalveolar lavage fluids from calves with bronchopneumonia

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SUMMARY

The aim of this study was to evidence acute phase response in bronchoalveolar lavage fluids (BALF) from calves with bronchopneumonia by measuring Haptoglobin and Serum Amyloid A (SAA) concentrations and some enzyme activities. For that, 30 calves with bronchopneumonia and 8 clinically healthy calves were selected on the basis of the clinical signs and examination and laboratory analysis. Haematological analysis (White Blood Cell counts) was performed using an automated haematology cell counter. In blood and BALF samples, gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities and the total protein concentrations were measured using an automatic analyzer, whereas Haptoglobin and SAA concentrations were measured with commercially available ELISA kits. Proteinemia, GGT activity, haptoglobin and SAA concentrations in sera from diseased calves were significantly and dramatically increased compared to the healthy controls, while increases in other parameters (leukocyte count, ALP and LDH activities) were not statistically significant. Significant increases in LDH and GGT activities and in concentrations of the 2 acute phase proteins were also evidenced in BALF samples from calves with bronchopneumonia compared to the controls. In addition, except in 2 diseased animals, haptoglobin and SAA concentrations in BALF samples were above 250 and 25 µg/L, respectively, whereas they have remained below the threshold values in all clinically healthy calves. These results show that BALF haptoglobin and SAA concentrations are useful acute phase proteins (APPs) for the determination of pulmonary inflammation in calves and future studies are needed to determine the importance of local acute phase response in the respiratory system.

Keywords: Calves, acute phase response, haptoglobin, serum amyloid A, bronchoalveolar lavage fluid, bronchopneumonia.

RÉSUMÉ

Concentrations en haptoglobine et en SAA et activités enzymatiques dans le liquide de lavage bronchoalvéolaire chez les veaux atteints de bronchopneumonie

Le but de cette étude a été de mettre en évidence une réponse inflammatoire en phase aiguë dans le liquide de lavage broncho-alvéolaire (LLBA) obtenus chez des veaux atteints de bronchopneumonie en mesurant les concentrations de 2 protéines inflammatoires, l’haptoglobine et la protéine sérique amyloïde A (SAA) ainsi que plusieurs activités enzymatiques. Pour cela, 30 veaux atteints de bronchopneumonie et 8 veaux cliniquement en bonne santé ont été inclus dans cette étude au vue des signes et examens cliniques et des analyses de laboratoire. L’analyse hématologique (numération leucocytaire) a été effectuée par comptage automatique. Dans les sérums et les liquides de lavages bronchoalvéolaires, les concentrations totales en protéines et les activités enzymatiques de la GGT (gamma-glutamyl transférase), de la LDH (Lactate déshydrogénase) et des phosphatases alcalines (PAL) ont été déterminées par un analyseur automate et les concentrations en haptoglobine et en SAA ont été mesurées par des trousses de dosages ELISA disponibles dans le commerce. La protéinémie, l’activité de la GGT, les concentrations en haptoglobine et en SAA ont été significativement et considérablement augmentées dans le sérum des veaux malades par rapport aux témoins tandis que les augmentations des autres paramètres (numération leucocytaire, activités de la LDH et des PAL) n’ont pas été statistiquement significatives. Des élévations significatives des activités de la LDH et de la GGT ainsi que des concentrations de 2 protéines inflammatoires ont aussi été mises en évidence dans les LLBA recueillis chez les veaux atteints de bronchopneumonie. De plus, les concentrations de l’haptoglobine et de la SAA dans les LLBA étaient respectivement supérieures à 250 et 25 µg/L chez tous les animaux malades sauf chez 2 alors qu’elles sont restées au-dessous de ces valeurs seuils chez tous les veaux sains. Ces résultats montrent que l’haptoglobine et la SAA du LLBA peuvent constituer des marqueurs pertinents d’une réponse inflammatoire en phase aiguë dans le poumon mais des études supplémentaires sont nécessaires afin de determiner l’importance d’une réaction inflammatoire locale dans le système respiratoire.

Mots clés : Veau, phase aiguë de l’inflammation, haptoglobine, protéine sérique amyloïde A, liquide de lavage bronchoalvéolaire, bronchopneumonie.

Introduction

Acute-phase response (APR) occurs during infection, inflammation, tissue injury, trauma, burns, and neoplastic formations, and is the leading systemic reaction seen during disease. One of the main features of APR is hepatic production of acute-phase proteins (APP) [17, 20, 38-40, 43]. The secretion of APPs is regulated by proinflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and IL-1β [52]. Increase in circulating haptoglobin (Hp) concentrations during acute inflammation is the major APP event seen in cattle [15, 16, 38, 42]. In healthy animals, serum Hp concen-
trations are very low, often below detection [12, 16]. Serum amyloid A (SAA) is an acute-phase apolipoprotein found in the high-density lipoprotein fraction of plasma and is an indicator of infection, especially at the early stage, in companion animals [34, 38, 40]. Many studies have indicated the significance of Hp and SAA as clinically useful parameters for measuring the occurrence and severity of inflammatory responses in cattle with mastitis, pneumonia, enteritis, peritonitis, endocarditis, abscesses, endometritis, and other natural or experimental infectious conditions [12, 15, 16, 22, 26, 38, 40]. Therefore, using assays to determine concentrations of APPs, especially Hp and SAA, may be better able to differentiate chronic and acute inflammation in cattle than the currently used haematological tests [30].

Respiratory diseases in calves cause great economic losses for the dairy and beef industry worldwide and is one of the most important causes of morbidity and mortality in beef and dairy calves [45]. Respiratory diseases are multifactorial, caused by a variety of aetiological agents. Studies on experimental infections have shown that some APPs have good properties, functioning as markers of respiratory infections in calves after viral, bacterial, or combined challenges [43]. Cellular enzymes such as gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) in bronchoalveolar lavage fluid (BALF) can be used as sensitive markers of cellular damage in organisms with pulmonary diseases [10]. Additionally, although SAA and lipopolysaccharide binding protein (LBP) are known to be more sensitive APPs than Hp, some studies have concluded that SAA is not a useful marker of respiratory diseases in field conditions [6, 9]. Finally, although many studies have indicated the significance of serum Hp and SAA as clinically useful parameters, acute-phase response in BALF had not been evaluated in calves with bronchopneumonia.

The aim of this paper was to determine some APPs (Hp and SAA) concentrations and significant enzyme activities in the BALF samples from calves with bronchopneumonia.

**Materials and Methods**

**ANIMALS**

The study was approved by the Ethics Committee of the Faculty of Veterinary Science. For the study, 30 calves with bronchopneumonia and 8 clinically healthy calves were selected on the basis of clinical signs, clinical examination, and laboratory analyses. The mean age of calves was 38 (range 15-65) days, and the mean body weight was 60 kg (range 45-80). Selective criteria for healthy calves were rectal temperature below 39.5°C, no nasal discharge, no coughing, and respiration frequency lower than 40 breaths/minute. Selective criteria for calves with bronchopneumonia were coughing, abnormal auscultation findings over the thoracic cage (crackles and harsh breath sounds), respiration frequency higher than 40 breaths/minute, nasal discharge, anorexia, depression and high rectal temperature (> 39.5°C).

Blood and BALF samples were taken from clinically healthy and diseased calves. Blood samples were collected from external jugular vein aseptically. An aliquot of blood was placed into EDTA-containing plastic tubes for routine haematological examination, and another aliquot was placed into glass tubes without anticoagulant for serum biochemical analyses. The tubes were centrifuged (2000g, 10 minutes, room temperature) after clotting (for 1 hour at room temperature) and the serum was carefully harvested and stored at -20°C until analyzed. Bronchoalveolar lavage samples were taken from the right side of the calves without sedation. An area of 5×5 cm², located 10 cm distal to the larynx, was shaved and sterilized with 70% alcohol and polyvidone-iodine. The calf was restrained by assistants while the trachea was perforated with an infusion needle between 2 cartilage rings. A feeding catheter (Feeding tube, Bicakcilar, Turkey) was inserted into the infusion needle (10 G) and pushed down into the airway until there was slight resistance. Then, 30 mL of sterile 0.9% NaCl was injected into the catheter by syringe, and the BALF was aspirated immediately. The volume of BALF retrieved was nearly 8 mL. The lavage samples were collected into EDTA-containing plastic tubes and gently mixed. The BALF was divided equally into 2 portions. One portion was used for bacterial examination, while the other portion was centrifuged (1100g, 5 minutes, room temperature) and supernatant stored at -20°C until APP and enzyme analyses.

**MICROBIOLOGICAL AND HAEMATOLOGICAL ANALYSES**

**Identification of bacteria:** BALF samples were examined for bacterial growth within our laboratory. Bacteria were identified using standard procedures [4].

**Haematological analyses:** White blood cells (WBC) in blood were measured using an automated haematology cell counter (MS4e, Melet Schloesing Lab., Osny, France).

**BIOCHEMICAL ANALYSES**

The GGT, LDH and ALP activities and the total protein (TP) concentrations were measured using an analyzer (BT 3000plus, Biotechnica Instruments SpA, Italy) using test kits [7, 19, 36] in serum and BALF samples.

Hp concentrations in serum and BAL fluids were determined using a sandwich ELISA (Life Diagnostics Inc., West Chester, PA) previously used for the analysis of Hp concentrations in cattle [14, 21, 27]. Serum samples were diluted according to the manufacturer's instructions (Life Diagnostics Inc., West Chester, PA). The manufacturer of this assay reported a limit of detection in bovine serum of 0.25 mg/L. Cut points for serum haptoglobin concentrations of > 150mg/L or > 500mg/L have been recommended to identify an acute phase response in postparturient dairy cows, and > 670 mg/L was recommended to identify cattle with traumatic reticulo-peritonitis [21, 27, 46]. Estimated values for sensitivity and specificity (versus clinical examination as the gold standard) for a cut point of serum haptoglobin concentrations of > 150mg/L or > 500mg/L are 0.83 and 0.58, respectively [21, 32]. For determination of Hp concentrations in BAL fluids, a standard curve (0.0, 7.81, 15.63, 31.25, 62.50, 125 and 250 µg/L) was used, and optical density of ELISA plates was measured at 450 nm using a spectrophotometer (MWGt Lambda Scan 200, Biotek Instrument Inc., USA).

SAA concentrations in serum and BALF samples were measured with a commercially available ELISA kit (Tridelta De-
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Development Ltd., Maynooth, Co. Kildare, Ireland). Serum samples were diluted according to the manufacturer’s instructions. The manufacturer of this assay reported a limit of detection in bovine serum of 0.3 mg/L and a reference range of 9-150 mg/L. Cut points for SAA concentrations of > 9 mg/L or > 600 mg/L have been recommended to identify an acute phase response in cattle [30, 32]. Estimated values for sensitivity and specificity (versus clinical examination as the gold standard) for a cut point of > 600 mg/L are 0.79 and 0.60, respectively [21, 32]. For determination of SAA concentrations in BAL fluids, a standard curve (0.0, 9.4, 18.7, 37.5, 75 and 150 µg/L) was used, and optical density of ELISA plates was measured at 450 nm using a spectrophotometer (MWGt Lambda Scan 200, Biotek Instrument Inc., USA).

STATISTICAL ANALYSIS

Data was expressed as means ± standard errors of the means (SEM). The level of statistical significance was at P < 0.05. Comparisons of values between the two groups were analyzed with the independent sample t test. A statistical software program (SPSS 10.0) was used for statistical analysis.

Results

CLINICAL SYMPTOMS AND HAEMATOLOGICAL FINDINGS

All sick calves showed clinical symptoms of bronchopneumonia, such as high rectal temperature (39.5°C–41.0°C), anorexia, nasal discharge, coughing, abnormal auscultation findings, and high respiration frequency (> 40 breaths/minutes).

Total leukocyte count in the blood of diseased calves has increased and was higher than in healthy control calves, but no statistically significant difference between the 2 groups was evidenced (Table I).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy calves (n = 8)</th>
<th>Calves with BP (n = 30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^9/L)</td>
<td>8 975 ± 838</td>
<td>13 328 ± 849</td>
<td>0.093</td>
</tr>
<tr>
<td>Total Protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum (g/L)</td>
<td>61.2 ± 2.3</td>
<td>71.6 ± 3.5</td>
<td>0.031</td>
</tr>
<tr>
<td>BALF (g/L)</td>
<td>0.69 ± 0.27</td>
<td>2.10 ± 0.20</td>
<td>0.117</td>
</tr>
<tr>
<td>ALP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum (U/L)</td>
<td>102.75 ± 14.64</td>
<td>151.04 ± 16.69</td>
<td>0.200</td>
</tr>
<tr>
<td>BALF (U/L)</td>
<td>6.62 ± 1.72</td>
<td>18.87 ± 1.61</td>
<td>0.129</td>
</tr>
<tr>
<td>LDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum (U/L)</td>
<td>766.87 ± 73.93</td>
<td>812.31 ± 60.77</td>
<td>0.108</td>
</tr>
<tr>
<td>BALF (U/L)</td>
<td>6.88 ± 1.26</td>
<td>20.40 ± 7.56</td>
<td>0.046</td>
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<tr>
<td>GGT</td>
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<tr>
<td>Serum (U/L)</td>
<td>19.50 ± 2.12</td>
<td>30.35 ± 5.63</td>
<td>0.024</td>
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<tr>
<td>BALF (U/L)</td>
<td>11.82 ± 2.94</td>
<td>28.86 ± 5.53</td>
<td>0.043</td>
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<tr>
<td>Hp</td>
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<tr>
<td>Serum (mg/L)</td>
<td>9.67 ± 2.99</td>
<td>413.46 ± 54.28</td>
<td>0.000</td>
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<tr>
<td>BALF (µg/L)</td>
<td>30 ± 7</td>
<td>2 408 ± 202</td>
<td>0.001</td>
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<tr>
<td>SAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum (mg/L)</td>
<td>10.57 ± 1.04</td>
<td>111.29 ± 7.78</td>
<td>0.000</td>
</tr>
<tr>
<td>BALF (µg/L)</td>
<td>31 ± 10</td>
<td>737 ± 96</td>
<td>0.002</td>
</tr>
</tbody>
</table>

BP: bronchopneumonia; WBC: White blood cells; BALF: bronchoalveolar lavage fluid; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; GGT: gamma-glutamyl transferase; Hp: haptoglobin; SAA: serum amyloid A.

Table I: Haematological and biochemical findings in serum and BALF samples from calves with bronchopneumonia (n = 30) and healthy calves (n = 8). Results are expressed as means ± standard errors (SE).

BIOCHEMICAL FINDINGS

As shown in Table I, proteinemia was significantly increased in diseased calves compared to the healthy ones (P < 0.05) but the increase in total protein concentrations observed in BALF samples from calves with bronchopneumonia was not statistically significant. Serum ALP, LDH and GGT enzyme activities were increased in diseased calves: differences with healthy calves were not significant for the ALP and LDH activities whereas difference in serum GGT activity between the 2 groups was significant at P < 0.05. In addition, the enzyme activities determined in BALF samples from calves with bronchopneumonia were markedly higher than in healthy calves (P < 0.05 for LDH and GGT activities; not significant for ALP activity). In the same way, the Hp and SAA concentrations determined in serum and in BALF samples dramatically increased in sick calves (P < 0.01 or more) compared to healthy animals. The APP concentrations in BALF samples in 3 of 8 healthy calves were undetectable in this study and they were below 70 µg/L in all other samples and serum Hp and SAA concentrations remained below 22 mg/L and 14 mg/L, respectively in all healthy animals (figure 1). In the group of calves with bronchopneumonia, serum Hp concentrations above 150

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mg/L and SAA concentrations above 9 mg/L were found in 21 (70.0%) and 30 (100%) cases, respectively. In BALF samples, the Hp and SAA concentrations were systematically higher than 250 µg/L and 25 µg/L, respectively except in 2 animals in which they were not detected (figure 1).

IDENTIFICATION OF BACTERIA

BALF samples from calves with bronchopneumonia and clinically healthy calves were cultivated. *Pasteurella* spp., *Mycoplasma* spp., *Corynebacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., and *Escherichia coli* were identified in BALF samples from 25 calves with bronchopneumonia. The most common bacteria were the *Pasteurella* spp. (44.0%) among positive samples. At least 2 bacterial species were detected in 7 (28.0%) BALF samples. No bacteria were identified in the BALF samples from all healthy calves and from 5 calves with bronchopneumonia.

Discussion

A total of 30 sick calves were diagnosed as suffering from bronchopneumonia. In this group, calves exhibited typical clinical symptoms such as coughing, elevated body temperature, abnormal auscultation findings, and nasal discharge. Clinical findings showed similarities with the study of HUMBLET et al. [31]. Leukocytes are sensitive indicators of acute inflammatory responses and total leukocyte count was increased in most of the sick calves. In general, a positive relationship between the severity of disease and total leukocyte count was observed.

Biochemical changes in BALF have been implicated as useful tools for the detection of pulmonary injury [7, 19, 24, 36]. Increases in the activities of LDH, ALP, or certain other intracellular enzymes found in the recovered BALF samples reflect lung structural cell damage or cell death [11, 25]. In the current study, LDH and GGT activities in the BALF samples from calves with bronchopneumonia were significantly increased compared to the controls, suggesting directly lung structural cell damage. ALP is a membrane-bound enzyme either present in neutrophils or secreted by pulmonary type II cells along with surfactants. In BALF samples, ALP activity has been associated with type II cell damage or stimulation [8, 13, 25, 33]. However, in the present study, the mean ALP activity measured in BALF samples from diseased calves has not significantly differed from the control values although it tended to increase.

The analysis of the lavage fluid is a useful tool for determining the level of pulmonary response during respiratory diseases [23]. APPs such as Hp, LBP, and CRP are produced by the liver in response to cytokine activity and play a role in the pathogenesis of respiratory diseases [37]. Studies on the use of serum APPs as markers of naturally occurring respiratory diseases, however, are somewhat controversial [41]. For instance, Hp has been reported to be useful for identifying calves with respiratory diseases that require treatment and monitoring [6, 9, 31], while other studies have found Hp to have only limited capacity as a clinical tool in the diagnosis of respiratory diseases in feedlot cattle [41, 48, 53]. In the current study, both serum and BALF Hp concentrations in calves with bronchopneumonia were significantly increased compared to the control calves, and increased APP concentrations in serum and in BALF samples were observed in nearly all sick calves. In one study, APP concentrations in BALF samples were described to be lower than serum concentrations in pigs with acute swine influenza infection [5].

ANGEN et al. [3] reported that serum Hp may be the best choice for detecting respiratory diseases under field conditions. In the present study, Hp concentration in BALF was very low or undetectable in clinically healthy calves whereas in 93.3% calves with bronchopneumonia this concentration was superior to 250 µg/L, leading to a variation percentage of 7.927%. Elevated Hp concentrations in BALF samples are more indicative of pulmonary cell injury than serum Hp concentrations, and Hp is considered as a distinguishing marker between viral and bacterial diseases, because it significantly rises during bacterial infections [18]. In this study, BALF samples contained different bacteria in 25 out of 30 calves. Although other studies have reported similar high Hp values during the viremic stage of foot-and-mouth disease [22, 29], viral agents were not identified in the BALF samples here. In the present study, serum Hp concentrations remained lower than 150 mg/L in 9 cases of calves with bronchopneumonia and the variation percentage of serum Hp concentrations between diseased and healthy calves was 4.176%.

Although SAA [30] and LBP [44] have been shown to be more sensitive APPs than Hp, some studies have concluded that SAA is not a useful marker of respiratory diseases under field conditions [6, 9]. Serum SAA concentrations are reported to be influenced by physical stress [2], which may partially explain its inconsistent behaviour [41]. The SAA concentrations in serum and BALF from calves with bronchopneumonia were significantly increased compared to the control calves. Serum SAA concentrations were above 9 mg/L in all calves with bronchopneumonia however, the variation percentage of SAA concentrations in diseased animals was higher in BALF samples (2.277%) than in sera (9.3%). The SAA concentrations...
in BALF samples were very low in the clinically healthy calves, whereas they considerably increased in diseased calves by a factor of 23.77 and 93.3% of affected animals exhibited a SAA concentration above 25 µg/L, showing that the SAA concentration in BALF is a good indicator for pulmonary cell injury.

Our primary observation was that APP concentrations in both serum and BALF in calves with bronchopneumonia were increased compared to the clinically healthy calves. In addition, the intensity of the increases in APP concentrations was higher in BALF samples than in sera. According to our knowledge, this is the first study evaluation of APR in both BALF and serum of calves with bronchopneumonia. There was only a study conducted on only 3 calves by KATOH et al. [35] which reported detectable Hp concentrations in BALF samples from some calves but not in all calves experimentally inoculated with Pasteurella haemolytica, coupled to changes in serum proteins such as albumin. In the present study conducted on 30 calves, BALF SAA and Hp concentrations in BALF samples were detected in 28 out of 30 cases and were generally greater than the control values. Some studies showed that APPs is part of the local defence system of the lung rodents, humans and pigs [28, 47, 49]. KATOH et al. [35] reported that Hp in the lavage fluid resulted from leakage from the circulation across the blood-lung barrier. However, in humans, mice and pigs, the synthesis of Hp is also demonstrated in non-hepatic tissues amongst others in lung epithelial cells and in alveolar macrophages [1, 28, 49-51]. It could also state that APPs in calves with bronchopneumonia are produced locally in the lung epithelial cells.

As a conclusion, Hp and SAA concentrations in BALF samples are useful markers for the determination of pulmonary inflammation in calves. In addition, elevated LDH and GGT activities in BALF could be indicative of lung tissue injury. APP can play an important role in defence mechanisms of inflamed lungs in calves as reported for humans, pigs and rodents. Future studies are needed to determine the importance of local APR in the respiratory system.

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References


