Clinicopathological and Electrophoretic Pattern of Serum Protein Alterations in Acute Pneumonic Sheep

Ali Arbaga1, Hany Hassan1*, Anis Anis2, Naemaa Othman2 and Ahmed Kamr1

1Department of Animal Medicine and Infectious Diseases (Animal Internal Medicine), Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Egypt
2Department of Pathology, Faculty of Veterinary Medicine, University of Sadat City 32897, Egypt
*Corresponding author: hany.youssef@vet.usc.edu.eg

ABSTRACT

The goals of this study were to determine serum protein fractions, acute phase proteins and inflammatory cytokine concentrations, as well as histological alterations, phagocytic activity, and index in sheep with pneumonia. An overall 50 adult sheep were assessed and categorized into control healthy (Group 1, n=20), and sheep with acute pneumonia (Group 2, n = 30). Pneumonia is medically diagnosed by physical examinations, ultrasonography, as well as histopathological investigation. Serum protein fraction concentrations were measured by protein electrophoresis, while inflammatory biomarkers were measured by immunoassays. Concentrations of serum total protein, globulins, 1α-globulins, β-globulins, γ-globulins, immunoglobulin G, immunoglobulin M, haptoglobin, serum amyloid A, and inflammatory cytokines (Interleukin-1β, interleukin-6, and tumor necrosis factor-α) were significantly increased while albumin and Interleukin-10 were decreased in acute pneumatic disease than healthy group. Phagocytic activity and index were significantly decreased in diseased sheep compared to healthy controls. Histopathological examination of lung tissues revealed thickening of interalveolar septa, congestion, alveolar hemorrhages, hemosiderophages, and lymphocytes and macrophages infiltration. In conclusion, acute pneumonia is associated with a severe pro-inflammatory condition that enhances the release of inflammatory cytokines, resulting in organ dysfunction and mortality in sheep. With strong sensitivity and specificity, serum haptoglobin and serum amyloid A concentrations act as diagnostic biomarkers for pneumonia in sheep.

INTRODUCTION

Sheep are valuable animals in Egypt for a variety of reasons, including the production of wool, meat and milk (Zeineldin et al., 2017). Respiratory diseases are a substantial and huge challenge due to the massive financial setbacks they generate and the high costs of the necessary prevention or treatment (Belkhiri et al., 2009).

Pneumonia is a common problem in sheep and it is usually associated with many predisposing factors such as inclement weather, weaning, transportation, poorly ventilated housing and dietary deficiency that, in turn, potentiate the pathogenic action of viruses and bacteria, leading to inflammatory reactions in the bronchioles and alveoli, which would lead to lung tissue consolidation (Lacasta et al., 2019).

Eighty-five percent of all circulating antibodies in ruminants are immunoglobulins (IgM and IgG), which are generated in the circulatory system and extravascular tissue fluid in reaction to antigenic activation of lymphocytes to stimulate host defenses (Youssef et al., 2015; Zhang et al., 2016). However, more research on serum immunoglobulins in pneumatic sheep is required.

Inflammatory cytokines were higher in pneumatic sheep due to pro-inflammatory circumstance (Smeed et al., 2007). Interleukin-10 is an anti-inflammatory cytokine that has immunomodulatory features that could be linked to the pathogenesis of pneumonia in sheep, but information on affected animals remains elusive.

In sheep, the acute phase reaction is characterized by a rise in some acute phase proteins (APPs), like haptoglobin, serum amyloid A and ceruloplasmin, as well
as a lowering in the concentration of other APPs. These proteins are all produced by hepatocytes (Iliev and Georgieva, 2018; Kushner and Mackiewicz, 2020). APPs are a helpful marker for detecting inflammation in sheep as they are considered to be more sensitive, specific, and efficient than hematological examination (Tothova et al., 2014; Donia et al., 2018).

Phagocytosis is a vital component of the cellular native immune response and integral to the defense of the hosts against microbial infections (Čobanová et al., 2017). Phagocytic macrophages and neutrophils, which have surface receptors for common bacterial surfaces chemicals, are responsible for the regulation of innate immunity. Phagocytosis and microbe apoptosis begin when these cells' receptors are activated (Lim et al., 2017). However, information on phagocytic activity and index needs further clarification in pneumonic sheep.

Histologically, there are serous exudates present in the lumen of the bronchioles and neutrophil infiltrates surrounding the bronchi and bronchioles in pneumonic sheep. In addition, the alveolar lumen exhibits inflammatory exudates, primarily neutrophils and a few mononuclear cells, together with increased interalveolar septal thickness (Dar et al., 2014).

The aims of this study were to measure the serum protein fractions, acute phase proteins, inflammatory cytokine concentrations, as well as histopathological changes, phagocytic activity, and index in pneumonic sheep. We hypothesized that acute phase proteins would be sensitive and specific diagnostic indicators of pneumonic sheep and that higher levels of inflammatory cytokines would be associated with pro-inflammatory circumstances in acute sheep pneumonia.

MATERIALS AND METHODS

Animals' criteria: From December 20th, 2020 to May 30th, 2021, fifty sheep of various ages and sexes were examined in various farms and locations in Menuofia Governorate, Egypt. Sheep were reared on private farms and fed on 1.5 kg of concentrates in addition to hay and green berseem. Sheep were given broad-spectrum anti-parasitic medications at regular intervals and vaccinated against clostridial diseases. Sheep were categorized into control healthy (n=20) and pneumonic (n=30) sheep. Physical, ultrasonography, and blood biochemical examinations were used to determine the healthy sheep control group. In contrast, pneumonic sheep were confirmed based on clinical signs including nasal discharge, cyanosed mucosa, labored breathing, and an exaggerated lung sound with cough, in addition to an ultrasonic examination and histopathological findings.

Clinical examination: Case histories as well as physical examination, including rectal temperature, heart and respiration rates and ruminal motility, were completed for all included animals (Radostits et al., 2007).

Ultrasound examination: In a vertical plane, a 5-cm-wide strip of skin was shaved from below the elbow's point to the scapula's caudal border on both sides of the thorax. The thorax was examined in a vertical plane at the 6th and 7th intercostal spaces (Scott and Gessert, 1998; Scott et al., 2008), using a 5.0–6.5 MHz micro convex transducer attached to real-time B-mode ultrasound equipment (Soundscape E1V portable abdominal veterinary ultrasonography).

Sampling: Blood was collected in EDTA and heparin containing tubes for the hemogram, phagocytic activity, and index analyses. In serum clot tubes, serum samples were collected, centrifuged for 10 minutes at 2000 x g, divided into smaller quantities and preserved at -80°C prior to bio-chemical examinations. At the abattoir, approximately forty grams from the lung were taken then dissected from both control healthy sheep and diseased animals exhibiting signs of acute pneumonia for histological examination. Lung tissues were preserved on formalin (40%) until examination.

Serum protein concentrations and fractions: Spectrophotometry was used to determine serum total protein and albumin concentrations using commercial kits (Bio-Diagnostics Ltd., Egypt). A polyacrylamide gel electrophoresis was used to determine serum protein fractions (Bain and Lewis, 2006). Serum globulins were calculated by subtracting serum albumin from total protein (TP) concentrations.

Measuring immunoglobulins, cytokines, and acute phase proteins: Serum immunoglobulins (IgG and IgM), interleukins (IL-1β, IL-6, IL-10, and TNF-α), haptoglobin, serum amyloid A, and ceruloplasmin concentrations were measured using ELISA kits (Shanghai Coon Koon Biotech, Ltd.; China). For ELISA test validation, inter- and intra-assay coefficients of variation (CV) were studied using sheep samples in duplicate (low and high concentrations) using the following equation: (CV = standard deviation/mean). Inter- and intra-assay coefficients of variation were <15% with appropriate linearity.

Determination of phagocytic activity and phagocytic index: Phagocytosis of polymorphonuclear cells using candida albicans (C. albicans) was performed (Wilkinson, 1981).

Phagocytic activity % (PA) = number of neutrophils ingested C. albicans / 100 neutrophil cells

Phagocytic index (PI) = number of phagocytosed C. albicans / phagocytic activity

Histopathological examination: The healthy and pneumonic lung tissues collected were settled in 10% buffered formalin for 72 hours. Lung tissues were trimmed, washed, dehydrated, soaked in wax paraffin, and cut off into 5μm thick sections, then streaked with hematoxylin and eosin stains and examined under microscope with digital camera (Leica, Wetzlar, Germany).

Statistical analyses: The data were regularly handed out according to Shapiro-Wilk statistics and expressed as means with standard deviations (M±SD). T- test was conducted to compare between both two groups. A Pearson correlation (r) was executed to locate the association among variables. The sensitivity and
RESULTS

Clinical judgments: Sheep with acute pneumonia (Group 2) showed nasal discharge, cyanosed mucosa, labored breathing, and an exaggerated vesicular sound with cough. When compared to healthy sheep, pneumonic animals had significantly lower ruminal motility and significantly higher body temperature, heart and respiratory rates (P<0.05; Table 1). The ultrasonic examination revealed the reverberation artifacts that represent normal lung tissue (Fig. 1A), while the pneumonic sheep showed hyperechoic dots that indicate fibrinous pneumonia (Fig. 1B).

Serum protein and immunoglobulin concentrations: Concentrations of serum total protein and globulin were significantly higher, while albumin and A/G ratios were significantly lower in sheep with acute pneumonia than control animals (P<0.05; Table 2). In pneumonic sheep, serum 1α-globulins, β-globulins, γ-globulins, IgG and IgM were significantly elevated than control ones (P<0.05; Table 2), while 2α-globulin concentrations were not different (P>0.05).

Acute phase proteins, cytokine concentrations as well as their ratios, phagocytic activity and index in healthy control and pneumonic sheep (M±SD): Table 1:

<table>
<thead>
<tr>
<th>Items</th>
<th>Group 1 (n = 20)</th>
<th>Group 2 (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>38.4±0.5</td>
<td>40.2±0.49*</td>
</tr>
<tr>
<td>Pulse (Beat/min)</td>
<td>80.7±6.18</td>
<td>105.7±9.76*</td>
</tr>
<tr>
<td>Respiration (Cycle/ min)</td>
<td>25.7±2.9</td>
<td>45.7±13.9*</td>
</tr>
<tr>
<td>Ruminal motility</td>
<td>4.7±1.1</td>
<td>2.7±0.9*</td>
</tr>
</tbody>
</table>

In pneumonic sheep, the IL-1β concentrations (P>0.05) were not different compared with healthy sheep (P>0.05; Table 3). In pneumonic sheep, the IL-6 concentrations (P>0.05) was significantly associated with the severity of acute pneumonia among sheep with 92% sensitivity and 85% specificity (AUC=0.90; Fig. 2A). Serum Hp concentrations >98.2 μg/mL had 100% sensitivity and 100% specificity (AUC = 0.99; P<0.01; Fig. 2B) to predict acute pneumonia in sheep. Serum Cp concentrations were not significantly sensitive and specific for predicting pneumonia in sheep (P>0.05). When compared to healthy sheep, pneumonic sheep had significantly greater IL-1β, IL-6, and TNF-α value, while having lesser IL-10 value (P<0.05; Table 3). In pneumonic sheep, the IL-1β /IL-10, IL-6/IL-10, and TNF-α /IL-10 ratios were significantly higher than in healthy sheep (P<0.05; Table 3). Phagocytic activity and phagocytic index were significantly lesser in ill sheep compared with control sheep (P<0.05) (Table 3; Fig. 3 A&B).

Correlation between serum proteins and cytokine concentrations: In sheep with acute pneumonia, values of serum Hp were positively correlated with IL-1β and TNF-α (r = 0.41; P<0.05; r = 0.53; P<0.05, respectively), but not with IL-6 (P>0.05). SAA concentrations in pneumonic sheep were positively associated with IL-1β values (r = 0.50; P<0.05), but not with IL-6 and TNF-α values (P>0.05). Serum Cp values were not correlated with inflammatory cytokines in pneumonic sheep (P>0.05). Serum IL-10 value in pneumonic sheep was inversely related to IL-1β concentrations (r = -0.42; P<0.05), but not to IL-6 and TNF-α concentrations (P>0.05).

Histopathological finding: Lung tissue samples were collected from sheep suffering from acute pneumonia; some samples showed that interalveolar septa were thickened with capillaries congestion (Fig. 4A). Other samples exhibited remarkable interalveolar septa thickening with lymphocytes and macrophages infiltrates with congestion of interalveolar capillaries and hemosiderophages (Fig. 4B). Some lung samples showed acute hemorrhagic pneumonia with widespread alveolar hemorrhages, activation of alveolar macrophages, and pneucmoniosis in lung tissues (Figs. 4C & D).

DISCUSSION

In the existing study, pneumonic sheep exhibited respiratory distress, fever, and decreased ruminal motility in addition to an increased pro-inflammatory state, increased serum globulins, acute phase proteins, and inflammatory cytokines. Besides decreased anti-inflammatory cytokines (IL-10), phagocytic index and
Fig. 1: Ultrasonography examination of healthy and pneumonic sheep. Fig. 1A: shows the reverberation artifacts that represent the normal lung tissue. Fig. 1B: Hyperechoic dots representing fibrinous pneumonia.

Fig. 2: Sensitivity and specificity of SAA and Hp concentrations in pneumonic sheep. Fig. 2A: SAA > 0.88 g/mL had 92% sensitivity and 85% specificity (AUC = 0.90; P<0.02). Fig. 2B: Serum Hp concentrations > 98.2 μg/mL had 100% sensitivity and 100% specificity (AUC = 0.99; P<0.01).

Fig. 3: Photomicrographs of blood films showing phagocytic activity and index in healthy and pneumonic sheep. Fig. 3A: shows a strong phagocytic activity of active neutrophils in healthy sheep that engulfed large numbers of *candida albicans* (arrow). Fig. 3B: shows a weak phagocytic activity of neutrophils in pneumonic sheep (arrow) or non-degraded yeast particles (arrowhead). Giemsa stain; bar 100 µm.

Fig. 4: Histopathological examination of the sheep lung tissue. 4A: Acute pneumonia with thickening of the interalveolar septa (arrow) and congestion of the interalveolar capillaries (arrowhead). HE stains, Bar 100 µm. Fig. 4B: Acute pneumonia with marked thickening of the interalveolar septa, infiltration of lymphocytes (thin arrow), macrophages (bent arrow), and congestion of interalveolar capillaries (arrowhead), and hemosiderophages (thick arrow). H&E stains Bar 50 µm. Fig. 4C: Acute hemorrhagic pneumonia showing widespread alveolar hemorrhages (arrow). H&E stains, Bar 100 µm. Fig. 4D: Acute hemorrhagic pneumonia showing alveolar hemorrhages (arrowhead) with activation of alveolar macrophages (thin arrow) and the presence of pneumoconiosis (thick arrow). H&E stains Bar 50 µm.
activity indicate that sheep were affected systemically by pneumonia. These findings were in line with previous studies in sheep with pneumonia (Radostitis et al., 2007; Rad et al., 2011; Donia et al., 2018).

The current study found that pneumatic sheep had lower serum albumin concentrations as well as higher serum total protein and serum globulins (1a-globulins, β-globulins, and γ-globulins). The increase in serum globulin concentrations could be a compensatory mechanism against bacterial infection as a part of immunomodulation to generate immunoglobulins IgG and IgM, which was the case in our study (Gaber et al., 2000; Ceron et al., 2011; Omidi et al., 2016; Donia et al., 2018). At the same time, a decrease in serum albumin concentrations might be due to increased capillary permeability of albumin due to bacterial toxins in the interstitial spaces (Omran et al., 2005); Furthermore, the A/G ratio was reduced in sick sheep compared to healthy control ones due to the convey in albumin and globulin concentrations that could be a diagnostic biomarkers for animal immune status (Stockham and Scott, 2013).

In pneumatic sheep, serum Hp and SAA concentrations were increased, while Cp concentrations were not significantly different from healthy ones. Similar results were observed in former studies on sheep (Saleh and Allam, 2014; El-Deeb and Tharwat, 2015). It is likely that the hepatic response to acute inflammation, amplified by inflammatory cytokines, resulted in higher Hp and SAA concentrations in pneumatic sheep (Godson et al., 1996; Urieli-Shoval et al., 2000; Orro et al., 2011). SAA and Hp have been reported as diagnostic biomarkers for pneumonia in sheep with good sensitivity and specificity, which is the case in the current study (Godson et al., 1996; Orro et al., 2011).

Increased levels of IL-1β, IL-6 and TNF-α were found in pneumatic sheep, which could be attributed to alveolar macrophage activation in response to bacterial infection of lung tissues, resulting in an acute phase response (Saleh and Allam, 2014). Serum IL-10 concentrations were decreased among pneumatic sheep in this study, which could indicate a severe inflammatory reaction and predominant inflammatory cytokines that suppress IL-10.

Phagocytic activity and the phagocytic index of neutrophils were lowered in pneumatic sheep than healthy ones. Phagocytic activity is a non-specific innate immune response that contributes to the clearance of germs by producing cytotoxic and microbiocide free radicals. Furthermore, neutrophils have a definitive role in the origin and direction of adaptive T-cell immunity by bacterial opsonization (Mehrzad et al., 2014). The mechanism of decreased phagocytic activity in pneumatic sheep has not been well established; however, one could speculate immunosuppression through altered phagocytic function by bacterial infection.

Pneumonia is a disease complex characterized by interactions among multiple agents, including the host, etiological agents, and environmental factors (Dar et al., 2014). A previous study by (Mekibib et al., 2019) reported that 25.7% of the examined cases showed acute suppurative bronchopneumonia and 24.3% had acute fibrinous broncho pneumonitis. The histopathological changes in lung tissues were agreed with previous records that others have reported (Rajya and Singh, 1964; Lindström et al., 2018).

Conclusions: Pneumonia in sheep is associated with pro-inflammatory conditions. Elevated SAA and Hp concentrations could predict the acute inflammatory response in pneumatic sheep with good sensitivity and specificity. Elevated serum globulins with decreased albumin concentrations could reflect altered immune status in pneumatic sheep.

Acknowledgments: We are grateful to the veterinarians and technicians at the Faculty of Veterinary Medicine, University of Sadat City, Egypt, for their assistance and support during this work.

Authors contribution: HH, AA, and AK designed the study; AA, NO, and AK shared in the experiments and wrote the manuscript; AnA finalized the histopathological examination beside his sharing in it the script; HH revised the final version of the manuscript for publication. All authors agreed the final manuscript.

Conflict of interest: None of the authors disclosed any conflicts of interest.

REFERENCES


