Immunohistochemical detection of Serum Amyloid-A, Serum Amyloid-P, C-reactive protein, Tumour Necrosis Factor-α and TNF-α receptor in sheep and goat pneumonias

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SUMMARY

The study aims to investigate the lung expression of some positive acute phase proteins (APPs) such as CRP (C reactive Protein), SAA (Serum amyloid A) and SAP (Serum amyloid P) and of the cytokine TNF-α and its receptor according to the pneumonia severity in sheep and goats. After conventional histopathology, the lung tissue samples were classified according to the intensity of the inflammatory reaction during pneumonia into 3 groups (mild forms mainly characterized by hyperaemia and oedema in alveoli, moderate forms associating mild necrosis and inflammatory infiltrates in alveoli, bronchioles and bronchus and severe forms in which the inflammatory reaction associated intense necrosis, oedema and neutrophil infiltrates in all parts of lung), each group containing 10 cases (5 sheep and 5 goats) and then, the APP and cytokine expression in lung was evaluated by immunohistochemistry using specific primary antibodies in the all diseased animals and in 10 healthy ruminants (5 sheep and 5 goats). The immunoreactivity for all the investigated markers was greatly exacerbated in pneumonic lungs compared to the controls. Whereas the mean CRP expression was significantly higher and a strong immunolabelling was more frequently encountered in the mild forms than in the aggravated ones, the mean expression of the other selected markers was significantly increased in the moderate (except for the TNF-α) and severe forms compared to the mild forms and a strong immunoreactivity (except for the TNF-α and its receptor for which a moderate immunoreactivity was observed in 2 sheep and 2 goats) was evidenced in all animals from the 2 groups. This study demonstrated that acute phase proteins are produced in pneumonic lungs and are important markers for detecting the earliness (CRP) and the severity (SAA, SAP, TNF-α and TNF-α receptor) of pneumonias in sheep and goats.

Keywords: Pneumonia, severity, sheep, goat, immunohistochemistry, C-reactive protein, serum amyloid-A, serum amyloid-P, tumour necrosis factor-α, TNF-α receptor, inflammation.

Introduction

Respiratory system diseases can cause high morbidity and mortality and economic loses in ruminants [16]. Bacteria, viruses, toxic gases and particles may cause pneumonia in addition to parasites and haematogenic agents [13, 16]. Acute phase response (APR) is a defensive reaction of the organism to infections, tissue damage, trauma, neoplasia or immunological damage. APR restricts the spread of the affected area and clears the agent or at least isolates the agent from the organism [9, 20, 24]. Acute phase proteins (APPs) can be used to differentiate inflammatory reaction from non-inflammatory

RÉSUMÉ

Détection immunohistochimique des protéines SAA (serum amyloid A), SAP (serum amyloid P), PCR (Protéine C Réactive), du TNF-α (Tumour Necrosis Factor-α) et du récepteur au TNF-α lors de pneumonies chez le mouton et la chèvre.

L’objectif de cette étude a été de rechercher l’expression pulmonaire chez le mouton et la chèvre de différentes protéines positives de la phase aiguë (PCR (Protéine C Réactive), SAA (sérum amyloïde A) et SAP (sérum amyloïde P)) ainsi que de la cytokine TNF-α et de son récepteur en fonction de la sévérité de l’inflammation observée lors de pneumonia. Après étude histopathologique, les échantillons de tissus pulmonaires ont été classés en fonction de l’intensité de la réaction inflammatoire en 3 groupes (formes mineures caractérisées principalement par une hyperémie et un œdème des alvéoles - formes modérées associant des lésions nécrotiques peu étendues et des infiltrats inflammatoires importants dans les alvéoles, les bronchioles et les bronches - formes sévères présentant des lésions nécrotiques, des œdèmes et des infiltrats inflammatoires importants dans toutes les structures pulmonaires), chaque groupe contenant 10 cas (5 de moutons et 5 de chèvres). Par comparaison aux contrôles sains, tous les marqueurs étudiés ont présenté une immunoréactivité fortement amplifiée lors de pneumonia. Alors que l’expression moyenne de la PCR a été significativement plus élevée et qu’un marquage intense a plus souvent été rencontré dans les formes mineures que dans les formes aggravées, l’expression moyenne des autres marqueurs étudiés a été significativement plus importante dans les formes modérées (excepté pour le TNF-α) et sévères que dans les formes mineures et une forte immunoréactivité correspondante a été mise en évidence dans tous les cas de formes sévères et modérées sauf pour le TNF-α et son récepteur pour lesquels une réaction positive modérée a été observée chez 2 moutons et 2 chèvres. Cette étude démontre que les protéines de la phase aiguë sont produites dans les poumons atteints de pneumonia et qu’elles constituent des marqueurs fiables pour détecter la précocité (PRC) et la sévérité (SAA, SAP, TNF-α et son récepteur) de la pneumonia chez le mouton et la chèvre.

Mots clés : Pneumonie, intensité, mouton, chèvre, immunohistochimie, Protéine C-réactive, sérum amyloïde A, sérum amyloïde P, TNF-α, récepteur du TNF-α, inflammation.
reaction or are used in treatment and as prognosis markers [28]. The major positive APPs that are secreted after hepatocyte stimulation are C Reactive Protein (CRP), Serum Amyloid A (SAA) and haptoglobin (Hp) [11]. CRP is an APP that exhibits both pro-inflammatory and anti-inflammatory effects [14]. SAA is an apolipoprotein, chiefly synthesized by hepatocytes [8, 30] and the Serum Amyloid P (SAP) is a pentraxin serum protein [7, 17]. The most commonly used APP is the C Reactive Protein (CRP) [4, 18, 25]. Although the Serum Amyloid A (SAA) is thought to be the more susceptible, it is not used extensively [2, 5, 26, 31]. Some cytokines such as the interleukin-6 (IL-6), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ), transformed growth factor-β (TGF-β) and probably also the interleukin-8 (IL-8) can be considered as APPs [5, 8, 15, 20]. The most important sources of cytokines are monocytes and macrophages in the inflammatory areas [10, 15, 20]. TNF-α, an important pro-inflammatory cytokine that is generally synthesized by activated macrophages has been the focus of several studies on pneumonias [5, 19].

Studies on APPs and cytokines are generally focused on their blood concentrations and their variations during disease. Therefore in this study, the secretion of APPs and cytokines and their specific concentrations in inflammatory areas during pneumonias in sheep and goats were evaluated. This is the first study of immunohistochemical detection of APPs and cytokines in sheep and goat pneumonias.

Materials and Methods

LUNG SAMPLES AND HISTOPATHOLOGICAL ANALYSIS

Pneumonias were macroscopically and microscopically diagnosed in 15 sheep and 15 goats. For both animals species, 5 mild, 5 moderate and 5 severe cases were selected from different slaughterhouses near the Burdur city. Only the severity and the distribution of the lesions were taken into consideration for classifying the pneumonia degree and aetiological factors were ignored. The gender, the age and the race of animals have not been taken in consideration.

Lung samples were fixed in 10% buffered formalin, routinely processed and then blocked in paraffin and sectioned 5-μm.

<table>
<thead>
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<th>Primary antibody</th>
<th>Type</th>
<th>Laboratory reference</th>
<th>Dilution</th>
</tr>
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<tr>
<td>Anti-SAP antibody</td>
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<tr>
<td>Anti-TNF-α antibody</td>
<td>Polyclonal Mouse IgG</td>
<td>PA1079 Boster Bio-Technology Co. Ltd, Wuhan, China</td>
<td>1:50</td>
</tr>
<tr>
<td>Anti-TNF-α R antibody</td>
<td>Monoclonal Rabbit IgG</td>
<td>MT0003 Boster Bio-Technology Co. Ltd, Wuhan, China</td>
<td>1:50</td>
</tr>
</tbody>
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SAA: Serum Amyloid A; SAP: Serum Amyloid P; CRP: C Reactive Protein; TNF-α: Tumour Necrosis Factor α; TNF-α R: Receptor of the Tumour Necrosis Factor α.

TABLE I: Primary antibodies used in the present study for detecting APPs and cytokines in lung samples from sheep and goats.

For immunohistochemical examination, paraffin blocks were sectioned at 5-μm and sections were attached to glass slides coated with poly-L-lysine. The slides were dried overnight at 37°C to optimize adhesion. Sections were deparaffinised through xylene, and tissues were rehydrated in sequentially graduated ethyl alcohol. To reduce nonspecific background staining due to endogenous peroxidase, slides were incubated in 3% hydrogen peroxide in methanol for 10 minutes. The sections were washed in phosphate buffer solution (PBS) twice. Then tissues were boiled with 1:100 citrate buffer for 10 minutes and cooled for 20 minutes. The cooled tissues were washed 4 times in PBS prior to application of blocking serum for 5 minutes and then primary antibody (Table I) was applied. Tissues were incubated for 30 minutes at room temperature. Novostain Universal Detection Kit (ready to use) [Novocastra Laboratories Ltd., Newcastle, UK (NCL-RTU-D)] was used as the revelation system. The biotinylated anti-polyvalent antibody, which recognizes rabbit IgG and mouse IgG, was then added and incubated for 10 minutes at room temperature. After washing in PBS 3 times, the streptavidin peroxidase conjugate was added. After incubation for 10 minutes at room temperature and 4 washes in PBS, slides were further incubated for 20 minutes at room temperature in a solution of DAB (3,3’ diaminobenzidine) chromogene. After washing in PBS, tissues were counterstained with Mayer’s haematoxylin, washed in water, and cover slips were applied with mounting media.

STATISTICAL ANALYSIS

For statistical analysis, 10 randomly chosen microscopic fields (X40) were examined in pneumonic areas. Immunopositive cell numbers from 10 cells in each area were counted (total of 100 cells) and statistically analyzed. In the statistical evaluations, a one-way analysis of variance test was used to
determine differences between groups relating to APPs and the severity of lung lesions. Duncan's multiple comparison method was used for the determination of different groups. SPSS 10.0 program pack (SPSS, Inc., Chicago, USA) was used for all calculations. The differences were considered as significant when $P$ value was less than 0.05.

Results

During the histopathological examination, mild, moderate and severely affected areas were all observed in the same lung, but evaluations were based on the most severe lesions. Mild form pneumonia was evaluated as hyperaemia, with oedema in the alveoli lumens, slight haemorrhage and only a few neutrophil leukocytes in the alveoli lumens (figure 1A). Severe neutrophil infiltrations together with lymphocytes and plasma cells in the alveoli lumens, bronchiolo and bronchus with mild necrosis were the considered criteria for a moderate pneumonia (figure 1B). Severe neutrophil leukocyte infiltrations in alveoli, bronchiolo and bronchus, in the interlobular and interalveolar septal tissues and a severe infiltration with inflammatory cells in pleura coupled to oedema and necrosis have characterized a severe pneumonia (figure 1C).

In the control lungs, all APPs were commonly slightly and more rarely moderately expressed in a small number of cells. In lungs with pneumonia areas, immunopositive reactions for CRP, SAA, SAP, TNF-$\alpha$ and TNF-$\alpha$ receptor were observed in the parenchyma and the stroma. The APPs and cytokines were found in parallel with the inflammatory reaction and the number of positive cells between control and all forms of pneumonia were markedly increased in pneumonic lungs. There was no difference in the positive immunohistochemical staining for CRP, SAA, SAP, TNF-$\alpha$ and TNF-$\alpha$ receptor between sheep and goats, but these markers were differentially expressed according to the pneumonia severity.

In the mild form of pneumonia, CRP was strongly expressed, especially if notable alveolar oedema and alveoli epithelial cells were present. Strong CRP expression was observed in 4 sheep and 4 goats (figure 2A) whereas 1 sheep and 1 goat slightly expressed CRP in this form of pneumonia. In moderate pneumonias, a severe CRP immunopositive reaction was observed in areas where oedema and neutrophil leukocytes were abundant. A markedly increased CRP expression was found particularly from neutrophil leukocytes in oedema fluid and from inflammatory cells in inflammatory areas (figure 2B) whereas the protein expression decreased when no or only slight oedema was present. However, the mean CRP expression in the group of moderate pneumonias was significantly decreased compared to the group of mild cases ($P < 0.001$) and CRP was slightly expressed in 2 sheep and 2 goats, moderately expressed in 2 sheep and 2 goats and markedly expressed in both 1 sheep and 1 goat. In the cases of severe pneumonias, the CRP expression has remained moderate in 8 animals (4 sheep and 4 goats) and was markedly increased in one case in which oedema and neutrophils were abundant. In this group, the mean CRP immunoreactivity was strongly depressed compared to the 2 other groups (mild and moderate cases) (Table II).

The SAA and SAP immunopositive reactions were demonstrated in alveolar epithelial cells and neutrophils and few macrophages. In the cases of mild pneumonias, the SAA and SAP were markedly expressed in inflammatory areas (figure 3A) in 4 sheep and 4 goats whereas a slight immunoreactivity of the 2 markers was observed only in 2 animals. The mean
SAA and SAP immunoreactivity was significantly higher in inflammatory zones of lungs with moderate (figures 3B and 4A) or severe pneumonia (figure 4B) forms ($P < 0.05$ for SAA and $P < 0.01$ for SAP) (Table II). Nevertheless, the mean SAA expression was slightly reduced in severe pneumonias compared to moderate cases ($P < 0.05$) and whereas all ruminants

![Image](https://example.com/image1.png)

**Figure 2**: CRP immunolabelling during mild, moderate and severe forms of pneumonia in sheep and goats. Immunohistochemistry with anti-CRP polyclonal mouse IgG and streptavidine-biotin-peroxidase revelation system, Haematoxylin-Eosin counterstain, bar: 100 µm. A.: Strong expression of CRP in alveolar epithelial (arrows) and interstitial cells (arrowheads) in mild pneumonia. B.: Strong expression of CRP in the alveolar (arrows), bronchiolar (●) and interstitial cells (arrowheads) in moderate pneumonia.

<table>
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<tr>
<th>Markers</th>
<th>Mild cases</th>
<th>Moderate cases</th>
<th>Severe cases</th>
<th>$P$ value</th>
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<tr>
<td>CRP</td>
<td>70.50 ± 6.34$^a$</td>
<td>39.00 ± 9.80$^b$</td>
<td>9.50 ± 3.11$^c$</td>
<td>$&lt;0.001$</td>
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<td>SAA</td>
<td>75.00 ± 8.94$^a$</td>
<td>90.00 ± 0.00$^c$</td>
<td>86.50 ± 1.50$^b$</td>
<td>$&lt;0.05$</td>
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<tr>
<td>SAP</td>
<td>65.41 ± 10.55$^a$</td>
<td>90.00 ± 0.00$^b$</td>
<td>90.00 ± 0.00$^b$</td>
<td>$&lt;0.01$</td>
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<tr>
<td>TNF-α</td>
<td>65.51 ± 9.13$^a$</td>
<td>62.50 ± 7.82$^a$</td>
<td>82.00 ± 2.80$^b$</td>
<td>$&lt;0.05$</td>
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<tr>
<td>TNF-α R</td>
<td>75.00 ± 8.94$^a$</td>
<td>87.00 ± 1.52$^b$</td>
<td>89.00 ± 0.66$^b$</td>
<td>$&lt;0.05$</td>
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</tbody>
</table>

**Table II**: Immunoreactivity (expressed as the mean number of positive cells / microscopic field at X 40 ± standard deviation) for APPs and cytokines (CRP, SAA, SAP, TNF-α and TNF-α R) in lung tissues with mild (n = 10), moderate (n = 10) and severe (n = 10) pneumonias from sheep and goats.

SAA: Serum Amyloid A; SAP: Serum Amyloid P; CRP: C Reactive Protein; TNF-α: Tumour Necrosis Factor α; TNF-α R: Receptor of the Tumour Necrosis Factor α. Different superscripts $a,b,c$ in the same row indicate significant difference between the 3 forms of pneumonias ($P < 0.05$ or more).

![Image](https://example.com/image2.png)

**Figure 3**: SAA immunolabelling during mild, moderate and severe forms of pneumonia in sheep and goats. Immunohistochemistry with anti-SAA monoclonal rabbit IgG and streptavidine-biotin-peroxidase revelation system, Haematoxylin-Eosin counterstain, bar: 100 µm. A.: Strong SAA expression in cells (arrows) and oedema (arrowheads) in mild pneumonia. B.: Strong SAA expression in the alveolar (arrows) and inflammatory cells (arrowheads) in moderate pneumonia.
with the moderate form exhibited strong SAA and SAP immunolabelling, slight SAA and SAP positivity was observed in 3 (2 sheep and 1 goat) and 4 (2 sheep and 2 goats) cases with severe pneumonia, respectively. Moreover, the SAA positive cells were more abundant than the SAP positive cells in mild and severe cases while the density of the 2 cell types was similar in the moderate forms (Table II).

As far as the TNF-α and TNF-α receptor immunolabelling was concerned, the expression of this cytokine and of its receptor was encountered in epithelial, interstitial and inflammatory cells whatever the pneumonia intensity. However, the TNF-α and TNF-α receptor positivity has remained moderate in all cases of mild pneumonia (figure 5A), whereas 5 animals (3 sheep and 2 goats) with moderate pneumonia and in all cases with the severe form exhibited a strong immunoreactivity for
the cytokine (figure 5B) and its receptor (figure 5C). The mean TNF-α immunolabelling was significantly higher in the group with the severe form compared to the 2 other groups (P < 0.05) and the mean TNF-α receptor immunolabelling was significantly more extended in the groups of moderate and severe forms than in the group of the mild form (P < 0.05) (Table II).

Discussion

Pneumonia is an important issue for the sheep and goat industry, particularly in goats where contagious pneumonias cause a major health problem. In addition, treatment of pneumonias is generally difficult and disease can result in a high mortality [13]. The increased knowledge in the pathogenesis of pneumonias may lead to new and effective treatments. In this study, the expression of some APPs and cytokines focused in lung inflammatory areas were investigated in sheep and goat pneumonias. Indeed the detection of APPs and cytokines may help in evaluating the severity of pneumonia lesions and in understanding the pathogenesis of pneumonias in sheep and goats.

The acute phase response in inflammation is characterized by the increase in the circulating concentrations of some APPs, called positive APPs, and by the decrease in the circulating concentrations of others, called negative APPs. Plasma concentration of APPs may change because of inflammation, infection, trauma, operation, burn, infarct, some immunological reactions and cancer [8]. During bacterial pneumonia, APPs correlate with the severity of disease, serve as biomarkers and are functionally significant [8, 21]. However, the kinetics and regulatory mechanisms of the liver APP induction during a lung infection have yet to be defined [21].

In this study, CRP, SAA, SAP, TNF-α or TNF-α receptor expression was examined in lungs from sheep and goats with pneumonias and analysed using both negative control slides (no primary antibody applied) and slides obtained from apparently healthy animals (control group). Only slight positive reactions were encountered in healthy ruminants whereas the expression of all investigated proteins was greatly enhanced in lungs from diseased animals.

In numerous studies [1, 22, 23], the plasma CRP concentration was known to increase in acute inflammatory reaction. In the present study using immunohistochemistry, CRP immunopositivity was evidenced in inflammatory areas, specifically in oedema fluid, alveoli epithelium, neutrophils and macrophages and this finding confirms that extra-hepatic cells may secrete CRP [3, 8, 14, 29]. The basic function of CRP is to recognize, connect and detoxify or remove from blood toxic substances that arise from injured tissues [1, 3, 22, 29]. As the CRP is metabolized during opsonisation, its anti-inflammatory effects can be related to the neutrophil adhesion to the endothelial cells and to the inhibition of the superoxide formation in neutrophils that stimulates mononuclear cells to synthesize an IL-1 receptor antagonist [32]. The marked positive CRP reaction in pneumonic areas, particularly in the early stage of inflammation observed in the present study, supports that CRP is an important APPs and may be considered as a precocious indicator of the acute inflammatory reaction.

During inflammatory reactions, the circulating SAA concentrations can increase up to 1000-fold [12, 23, 26]. In addition, SAA can influence the cell adhesion, migration, proliferation and aggregation [27]. In this study, an immunopositive SAA and SAP reactions was observed in alveolar epithelial cells, neutrophils and in some macrophages. Furthermore, a strong immunoreactivity was observed whatever the pneumonia intensity, but particularly in moderate and severe cases, suggesting that the serum amyloid proteins may locally accumulate according to the amplification of the inflammatory process and therefore can be probably play some important roles in the aggravation of the sheep and goat pneumonias.

Measurement of the APP concentrations in serum or plasma can provide useful quantitative diagnostic information on the presence of disease and can be used to monitor responses to therapy [6]. In addition, cytokines are important components of the inflammatory reaction and cytokine concentrations increase with inflammation [8]. Their effects on the target cells may be stimulated or inhibited by other cytokines, hormones, cytokine receptor antagonists and circulatory receptors [5, 6, 8]. In the present study, the TNF-α and its receptor were expressed in almost all cell types in the inflammatory areas, particularly when the inflammation reaction was well established and intense (severe form of pneumonia).

The study showed that CRP, SAA, SAP, TNF-α and TNF-α receptor were markedly expressed in inflammatory areas from pneumonic lungs in sheep and goats, since the start of inflammation, i.e. in the mild form. Except for the CRP expression which decreased with the severity of the inflammation, the immunoreactivity for the other selected markers was strengthened in moderate and severe forms. However, while the SAA expression was strongly elevated in moderate pneumonias, it has slightly decreased in the severe forms of pneumonia. Additionally, the counts of the SAA and SAP immunopositive cells were higher than those of TNF-α positive cells in aggravated forms, particularly in the moderate forms, leading to consider the SAA and SAP as mainly reliable markers for sheep and goat intense pneumonias. These results emphasize the importance of these markers in pneumonic lungs in sheep and goats.

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References

