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Clinicopathological Alterations in Blood and Sera of Sheep due to Respiratory Affections

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Abstract

This study was conducted to investigate the effect of respiratory affections in sheep and their associated bacteriological, hematological, immune logical and antioxidant alterations. Nasal swabs, ocular swabs and blood samples were collected from 30 sheep of 2 farms of different ages (2-7 months); Farm A apparently healthy and diseased sheep before and after treatment by sulpha-trimethoprim, kanamycin 25%, ketoprofen and AD_3E, while, Farm B were treated with oxytetracycline 20% L.A, sulphadimidine 33.3%, declophen 5% and AD_3E for 5 days treatment. The result of isolated bacterial pathogens from farm A were *Enterobacter cloacae and Pasteurella multocida* (80% and 50%), while, *Klebsiella pneumonia*e and *Pasteurella haemolytica* in farm B (30% and 60%) respectively. The hematological findings of farm a revealed significant microcytic hypochromic anemia (iron deficiency anemia) and thrombocytopenia with significant increase in T.L.C coupled with lymphocytosis, monocytosis, eosinophilia and neutropenia. While, farm B showed significant decrease in RBCS count, Hb content, PCV% with significant increase in MCV with decrease in MCHC (regenerative stage of hemorrhagic anemia) as well as thrombocytopenia. Marked increase in T.L.C with neutrophilia, monocytosis and eosinophilia with lymphocytopenia. Immunological results in farm a showed marked increase in Interlukin 10 and insignificant change in Interleukin 1β. While, farm B showed marked increase in IL-1β and insignificant change in IL-10. Over and above, oxidative stress was noticed in both farms expressed by lipid peroxidation and diminished total antioxidant capacity. All alterations reported with pneumonia were improved after treatment. In conclusion, pneumonia can result in different types of anemia depending on the causative microorganism and affect the immunity adversely, also can lead to oxidative damage.

Keywords: Respiratory affections; Sheep; *Pasteurella spp*; *Klebsiella pneumonia*; *Klebsiella pneumonia*; Anemia; Oxidative stress; Interleukins

Introduction

Sheep play an important role in nutrition and yield of people around world. They act as a source of meat, and also provide milk, skin, and wool [1]. Respiratory affection is complex including stress factors, virus and bacterial infection. Bacterial pneumonia is consider the most common and serious causes of mortality and economic losses associated with respiratory diseases of sheep [2]. The selection of a suitable antibiotic and evaluation of its success in the treatment of respiratory diseases is an important consideration to the feedlot owner and his veterinarian [3]. Signs of acute phase of pneumonia in lambs are fever, bilateral nasal discharges, moist cough, congested mucous membrane, lacrimation, abnormal respiratory sounds, in appetence, weakness and recumbency, tachypnea and dyspnea, change in hematological and leukogram parameters [4,5].

Pasteurellosis considered as the most important respiratory disease affecting sheep caused mainly by *Pasteurella multocida* and *Pasteurella hemolytica*. *Pasteurella spp.* occur as a part of the normal flora of the nasopharynx but under initiation of complex geographic stresses and immunological factors will cause diseases [2,6].

So, this work aimed to study acute and chronic respiratory manifestations on sheep before and after treatment, by means of isolation and identification of bacterial pathogens, hematological examination, serum biochemical analysis, immunological assessment and determination of some serum oxidative stress indices.

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Materials and Methods

Animals

One hundred and fifty samples (nasal swabs, ocular swab and blood samples) were collected from sheep (at age 2-7 months) reared in 2 different farms (farm A and farm B) located in Ismailia Governorate; sheep were distributed in 3 groups; 5 as apparently healthy sheep and 10 as diseased sheep (pre and post treatment) in farm A and farm B (pre and post treatment).

Sampling

Blood samples: Blood samples were obtained from apparently healthy and diseased sheep before and after treatment for evaluation of erythrogram and leukogram, serum biochemical analysis, oxidative stress markers and immunological parameters analysis. Blood samples were obtained from jugular vein.

Laboratory investigation

Direct microscopic examination of blood smears: Blood smears were prepared by putting a drop of blood on a slide then spread it with another slide, fixed by heating, stained by Gram's stain and examined microscopically for the detection of *Pasteurella multocida* [7].

Isolation and identification of microrganisms: The nasal and ocular swabs were collected for isolation of causative microorganisms; these swabs were inoculated on peptone water and nutrient broth then incubated aerobically at 37°C for 12 hours. A loopful from incubated peptone water and nutrient broth were streaked on blood agar and MacConkey's agar plates then incubated for 24 hours at 37°C.

Hemolysis colony and non-hemolysis colony from blood agar and lactose and non-lactose fermenter were picked up and streaked on brilliant agar and XLD agar and kept in slope agar for biochemical identification. The biochemical reactions were carried out according to [8].

Bacteriological identification by API 20E (Analytical Profile Index): Analytical Profile Index (API) and is a commercial system to identify different bacteria. One of the API systems is specific for differentiating between members of the Gram-negative bacterial Family *Enterobacteriaceae* and is called API-20E. API test strips consists of wells containing dehydrated substrates to detect enzymatic activity, usually related to fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms. A bacterial suspension is used to rehydrate each of the wells and the strips are incubated. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.

Hematological assessment: Including erythrocyte count, Hb concentration, PCV value, MCV, MCH, MCHC, total and differential leukocytic count were performed by manual method.

Estimation of interleukin-10 (IL-10) (Pg/ml): Using purified sheep IL-10 and IL-10 antibody that has been labeled with HRP towels, then reactants becomes antibody-antigen-antibody complex (My BioSource, USA).

Estimation of Interleukin-1beta (IL-1β) (Pg/ml): Anti-Interleukin 1β antibody was pre-coated onto 96-wells plate. And the biotin conjugated anti-IL-1β antibody was used as detection antibodies (My BioSource, USA).

Determination of serum malondialdehyde level (MDA) (nmol/ml): MDA serum level was determined by using Bio diagnostic kit, which is based on the spectrophotometric method of [9].

Determination of serum Total Antioxidant Capacity level (TAC) (nmol/ml): The determination of the antioxidant capacity is performed by using Bio diagnostic kit. The reaction of antioxidants in the sample with a defined amount of (H_2O_2). The residual of reaction is determined by enzymatic reaction according to [10].

Field treatment

a) Sulpha-trimethoprim (BIOTUR) (1 ml/10 kg b.w, twice daily I/M) for 5 days

b) Kanamycin 25% (NOVA) (2 ml/50 kg b.w, daily I/M) for 5 days

c) Ketoprofen (Pharma Swid company) (1 ml/33 kg b.w, daily I/M) for 5 days

d) Oxytetracycline 20% L.A (Arab Company for Medical Products) (1 ml/10 kg b.w, 3 times weekly)

e) Sulphadimidine 33.3 % (ADWIA) (Initial dose: 6 ml/10 kg b.w at first day, maintenance dose: 3 ml/10 kg b.w) for 5 days.

f) Declophen 5% (ARABCOMED) (1 ml/50 kg b.w daily I/M) for 5 days.

g) AD_3E (Arab Company for Medical Products) (0.5-1 ml/10 kg b.w daily S/C) for 5 days.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS for widows (Version 16.0:SPSS Inc., Chicago, III).

One way analysis of variance (ANOVA), evaluated the significant of differences between groups. If one way ANOVA indicated a significant difference then differences between individual groups were estimated using Fishers Least Significant Difference (LSD) test, results were expressed as the mean (±) Standard Error of Mean (SEM). A P-value of less than 0.05 was considered significant [11].

Results

Farm (A)

Clinical signs: The main clinical signs observed among diseased sheep in farm A were fever up to 40°C, bilateral nasal discharges, cough, dyspnea, tachycardia, lacrimation, abnormal respiratory sounds, consolidation, off food, weakness and recumbency.

Bacteriological results: The bacteriological examination of nasal samples collected from farm (A) revealed isolation of *Klebsiella pneumonia* (8/10) and *Pasteurella multocida* (5/10) in percentage of 80% and 50%. But, the bacteriological examination of ocular samples obtained from farm (A) showed negative results for isolation of bacteria.

Hematological results: Erythrogram results of farm (A) were expressed in table 1, there were significant decreases in Hb content, PCV%, MCH, MCV and MCHC leading to microcytic hypochromic anemia (iron deficiency anemia) together with thrombocytopenia. While, leukogram showed significant increase in T.L.C with marked lymphocytosis coupled with monocytosis, eosinophilia and neutropenia in lambs suffering from pneumonia table 2. Hematological parameters were improved post treatment.

Immunological results: Immunological results of sheep in farm (A) were represented in table 3 showing marked increase in

Table 1: Erythrogram of apparently healthy, diseased and treated groups with sulphatrimethoprim, kanamycin, ketoprufen and AD_3E in farm A.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
RBCs(×10⁶/µl)	5.65 ± 0.39ᵃ	5.01 ± 0.35ᵃ	5.20 ± 0.26ᵃ
Hb(g/dl)	12.13 ± 0.58ᵃ	8.04 ± 0.57ᶜ	10.28 ± 0.53ᵇ
PCV (%)	27.06 ± 0.74ᵃ	22.89 ± 0.96ᶜ	23.97 ± 0.93ᵇ
MCV(Fl)	47.89 ± 0.18ᵃ	45.69 ± 0.11ᶜ	46.09 ± 0.22ᵇ
MCH(pg)	21.46 ± 1.33ᵃ	16.05 ± 1.01ᶜ	19.77± 1.15ᵇ
MCHC (%)	44.83 ± 3.06ᵃ	35.12 ± 3.25ᶜ	42.89 ± 2.90ᵇ
Platelets(x10³/µl)	326.6 ± 38.5ᵃ	300.6 ± 29.3ᵇ	322.0 ± 35.3ᵃ

Means in the same row with the different superscripts differ significantly ($p \leq 0.05$).

Table 2: Leukogram of apparently healthy, diseased and treated groups in farm A.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
WBCs(x10x³/µl)	11.29 ± 1.57ᶜ	20.35 ± 0.91ᵃ	15.85 ± 0.63ᵇ
Neutrophil(x10³/µl)	4.99 ± 0.42ᵃ	3.09 ± 0.43ᶜ	3.66± 0.31ᵇ
Lymphocyte(x10³/ µl)	5.56 ± 0.65ᶜ	16.33 ± 0.59ᵃ	11.34 ± 0.26ᵇ
Monocyte(x10³/µl)	0.10 ± 0.01ᶜ	0.18 ± 0.01ᵃ	0.15± 0.01ᵇ
Eosinophil(x10³/µl)	0.64 ± 0.07ᶜ	0.75 ± 0.09ᵃ	0.70 ± 0.05ᵇ

Means in the same row with the different superscripts differ significantly (p≤ 0.05).

Table 3: Immunological studies in apparently healthy, diseased and treated groups in farm A.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
IL-10(Pg/ml)	2.89 ± 0.09ᵇ	4.06 ± 0.05ᵃ	3.09 ± 0.08ᵇ
IL-1β(Pg/ml)	23.33 ± 0.28ᵃ	22.32 ± 0.25ᵃ	22.59 ± 0.30ᵃ

Means in the same row with the different superscripts differ significantly ($p \leq 0.05$).

Table 4: Oxidative stress/antioxidant markers studies in apparently healthy, diseased and treated groups in farm A.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
MDA(nmol/L)	15.03±1.31ᶜ	19.10±0.11ᵃ	16.49±0.87ᵇ
TAC(mM/L)	1.55±0.05ᵃ	0.95±0.07ᶜ	1.32±0.16ᵇ

Means in the same row with the different superscripts differ significantly (p≤ 0.05).

Table 5: Erythrogram in apparently healthy and diseased and treated groups in farm B.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
RBCS(×10⁶/µl)	8.08 ± 0.42ᵃ	5.39 ± 0.37ᶜ	7.40 ± 0.54ᵇ
Hb(g/dl)	11.71 ± 0.57ᵃ	7.63 ± 0.3 7ᶜ	10.78 ± 0.50ᵇ
PCV(%)	32.84 ± 1.13ᵃ	24.56 ± 2.21ᵇ	32.60 ± 1.42ᵃ
MCV(Fl)	40.64 ± 2.31ᵇ	45.56 ± 3.81ᵃ	44.05 ± 2.02ᵃ
MCH(pg)	14.49 ± 0.92ᵃ	14.16 ± 1.26ᵃ	14.56 ± 1.16ᵃ
MCHC (%)	35.66 ± 1.69ᵃ	31.07 ± 2.34ᶜ	33.08 ± 1.48ᵇ
Platelets(x10³/µl)	390.3 ± 53.6ᵃ	328.2 ± 58.4ᶜ	336.8 ± 52.87ᵇ

Means in the same row with the different superscripts differ significantly (p≤ 0.05).

Interlukin10 (IL-10) and insignificant change in Interleukin 1β (IL-1β) which were improved after treatment.

Oxidative/Antioxidant markers: Oxidant/Antioxidant results as shown in table 4 in farm (A) showed marked increase in MDA and decrease in Total antioxidant capacity (TAC) that were improved after treatment.

Table 6: Leukogram studies in apparently healthy and diseased and treated groups in farm B.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
WBCs(x10³/µl)	23.23 ± 2.23ᶜ	30.25 ± 1.21ᵃ	25.46 ± 1.02ᵇ
Neutrophil(x10³/µl)	10.55 ± 1.37ᶜ	16.66 ± 0.82ᵃ	13.35 ± 0.52ᶜ
Lymphocyte(x10³/ µl)	9.78 ± 0.94ᵃ	8.69 ± 0.71ᶜ	8.85 ± 0.56ᵇ
Monocyte(x10³/µl)	1.73 ± 0.21ᵇ	2.75 ± 0.27ᵃ	1.91 ± 0.17ᵇ
Eosinophil(x10³/µl)	1.17 ± 0.17ᵇ	2.15 ± 0.25ᵃ	1.35 ± 0.18ᵇ

Means in the same row with the different superscripts differ significantly (p≤ 0.05).

Table 7: Immunological studies in apparently healthy and diseased and treated groups in farm B.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
IL-10(pg/ml)	4.04± 0.41ᵃ	3.98±0.04ᵃ	4.01±0.09ᵃ
IL-1β(pg/ml)	21.60±0.34ᵇ	54.55±3.15ᵃ	22.14±0.29ᵇ

Means in the same row with the different superscripts differ significantly ($p \leq 0.05$).

Table 8: Oxidative stress/antioxidant markers studies in apparently healthy and diseased and treated groups in farm B.

Species/ Parameters	Apparently healthy sheep	Diseased sheep	
		Before treatment	After treatment
MDA(nmol/L)	10.90±0.73ᶜ	17.74±0.43ᵃ	12.45±0.25ᵇ
TAC(mM/L)	1.60±0.04ᵃ	0.81±0.07ᶜ	1.52± 0.10ᵇ

Means in the same row with the different superscripts differ significantly ($p \leq 0.05$).

Farm (B)

Clinical signs: Signs of diseased sheep in farm (B) were fever up to 41°C, moist cough, abnormal respiratory sound, bilateral nasal discharges, congested mucous membranes, in appetence, and lacrimation.

The bacteriological results: The bacteria isolated from nasal samples obtained from sheep in farm (B) were *Klebsiella pneumonia*e (3/10) and *Pasteurella haemolytica* (6/10) in percentage of 30% and 60%. Moreover, the bacteriological examination of ocular samples collected from farm B isolated *Klebsiella pneumonia*e and *Pasteurella haemolytica* in percentage of 30% and 20%, respectively.

Hematological results: The obtained data in table 5 of farm (B) showed a significant decrease in RBCS count, Hb content, PCV% with significant increase in MCV and decrease in MCHC (regenerative stage of hemorrhagic anemia) with thrombocytopenia. Marked leukocytosis, neutrophilia, monocytosis and eosinophilia with lymphocytopenia were recorded. These parameters showed improvement post treatment as shown in table 6.

Immunological results: Farm B showed marked increase in serum IL-1β and insignificant change in IL-10 as shown in table 7.

Oxidative/Anti-oxidant markers: Antioxidant results of farm (B) as shown in table 8 showed marked increase in lipid peroxidation and decrease in TAC, which were improved after treatment.

Discussion

Respiratory diseases of sheep particularly pneumonia continues

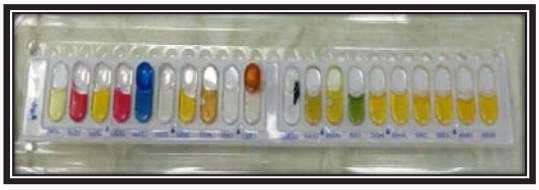

Figure 1: Biochemical identification of *Enterobacter cloacae* by API 20.

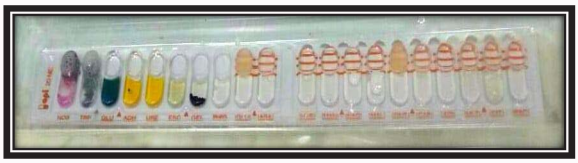

Figure 2: Biochemical identification of *Pasteurella haemolytica* by API 20.

being a major problem commonly encountered in sheep flocks, affecting all ages and types [12].

Age, geographic location, nutrition and climate are specific factors on the type of microorganism causing pneumonia. Plus, rearing systems, stress factors, climatic changes, unhygienic conditions, sudden changes in feed [13].

Concerning hematological results of farm (A); diseased sheep with respiratory affections showed a significant decrease in Hb, PCV, MCV, MCH and MCHC prompting microcytic hypochromic anemia with leukocytosis. Similar findings were acquired by [14,15] who reported significant decrease in Hb, PCV, MCV, MCH and MCHC values in the diseased group indicating the presence of microcytic hypochromic anemia which is characterized by significant reduction in RBCs size with increased central pallor of the cells. These changes could be due to the mononuclear phagocytic system under the effect of inflammatory conditions becomes trapping free iron and increases iron storage in phagocytic cells. In addition, decreases iron transfer to developing erythroid cells in bone marrow leading to reduction of Hb synthesis and production of microcytic hypochromic RBCs [16].

In this study, farm (A) showed leukocytosis coupled with lymphocytosis, monocytosis, eosinophilia with neutropenia which agreed with [17] who discussed that Hb of the lambs with pneumonia pretreatment were found to be lower compared to the control animals, while, T.L.C and lymphocyte is higher than the control group.

Who discussed that *Klebsiella pneumonia* cause microcytic anemia, mild thrombocytopenia and lymphopenia reactive protein had slightly raised white cell count and neutrophil count [18].

Whereas for, the hematological assessment of the pneumonic sheep in farm (B), revealed significant decrease in RBCs count, Hb, PCV, MCHC with increase in MCV causing macrocytic hypochromic anemia. In addition, there was significant increase in T.L.C count with neutrophilia, monocytosis, eosinophilia and lymphopenia. These outcomes were concur with [19,20] who reported that T.L.C had increased in acute inflammatory diseases particularly due to bacterial infections. This could be attributed to that infectious agents and products of tissue injury which stimulate a variety of cells to release cytokines, growth factors and other mediators of inflammation that act as prompt stimuli and causing the increase in total WBCs and more production, proliferation, maturation and bone marrow release of mature and immature neutrophils.

Furthermore, the results obtained from farm B agreed with [21] who found upon hematological assessment of the pneumonic sheep; significant decrease in RBCS, Hb, PCV and increase in T.L.C and neutrophil together with decreased lymphocytic count and no progressions in both monocyte and eosinophil. The lymphopenia might be due to stress response and endogenous corticosteroids that may play a secondary role in redistribution of recirculating lymphocytes leading to the lymphoid tissues rather than entering efferent lymph and blood to participate in the developing inflammation [22,15].

Another opinion for this leukocytosis represented by neutrophilia; is the stress to which the animal exposed during the respiratory illness that results in releasing of endogenous corticosteroids which have a role in regulating circulating number of leukocytes in moderate and severe pneumonia [14].

The production of cytokines plays an important role in inflammatory reaction and development of infection. Cytokines may also be synthesized by several other immune or non-immune cells including lymphocytes, neutrophils and fibroblasts [23]. However, if cytokines levels become too much, tissue damage and even death of the host can occur. Therefore, the presence and the quantity of pro-inflammatory cytokines can lead to an increased understanding of the pathogenesis of disease and the corresponding host's immune response.

Interleukin 10 (IL-10) is considered an immunoregulatory cytokine [24,25]. Despite the previous fact, IL-10 is a pleiotropic cytokine that can exert either immune-stimulatory or immune-suppressive effects on many cells [25].

In the present study, the IL-10 in affected sheep in farm (A) showed significant increase which was similar to [6] who reported a significant interaction between the microorganisms and cytokine markers that leads to significant increase in IL-10 in diseased lambs.

In our study, IL-1β was elevated in farm (B) that agreed with [26] who said that IL1-β was elevated in the respiratory airways and lung lesions of diseased calves with pneumonic pasteurellosis.

Discussed that P. *multocida* presented the highest mean of immunolabelled cells, showing statistically significant differences in IL-6 and IL-10 [6]. On the other hand, M. *haemolytica* presented the IL-1 marker in highest mean value, presenting statistically significant differences when compared with IL-10. High level of IL-10 and a lesser expression in the rest of cytokines markers were observed in respiratory portions.

IL-1 promotes neutrophil mediated tissue injury by stimulating neutrophil degranulation and the extracellular release of arachidonic acid metabolites, toxic oxygen radicals, and proteolytic enzymes [27,28].

Oxidative stress results from an imbalance between reactive oxygen species production and the antioxidant enzymatic and non-enzymatic systems, leading to cell damage [29] Malondialdehyde (MDA) is a major and stable end formed of peroxidation is regarded as a marker of lipid peroxidation [30].

Antioxidants protect the cell from oxidative stress by preventing the initiation of peroxidation process and production of end products that are capable of causing sever cellular damage [31].

In present investigation, non-treated sheep in farm (A and B)

showed increased MDA level and deceased TAC compared with apparently healthy sheep which concurred with [32]. As it is known that inflammatory diseases are associated with enhanced oxidative reactions and limit antioxidant defense capabilities [33].

These results may be due to the excessive lipid peroxidation in plasma and cells due to many factors or diseases lead to excessive formation of NADPH which in turn promote lipid peroxidation in presence of cytochrome P450 system [34,35,17] discussed that TAC of pneumonic lambs before treatment were found to be lower than those of control group and the level of TAC in pneumonic lambs due to mixed infection were found to be lower than the single causative agent and the control group.

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