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IMMUNOLOGICAL STUDIES ON PASTEURELLA HAEMOLYTICA IN SHEEP

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SUMMARY

In the period from January-July 2001 respiratory manifestations appeared on 53 out of 239 sheep at Sakha station of reproduction Research center, causing death of 30 cases. Clinically history symptoms and necropsy findings were reported. Pasteurella haemolytica was incriminated as the causative agent of this problem. This organism was isolated from nasal swabs of clinically diseased sheep and from pharyngonasal swabs, lung tissue of six emergency slaughtered cases. The isolates were identified biochemically and serologically and using pathogenicity test. Two antigens leukotoxin and whole cell associated with Pasteurella haemolytica. SDS-PAGE of leukotoxin yield four protein bands ranged from 80 KDa to 98Kda while whole cell antigen yielded 10 protein bands ranged between 22 to 85 KDa. Antigens associated with P. haemolytica whole cell and leukotoxin were identified for their immunogenic activity by neutralization test and ELISA. The neutrilizing antibodies against leukotoxin in sheep were significantly higher than antibodies against the whole cell antigen. Also, antibodies titre to leukotoxin by ELISA were significantly higher than antibody titre to whole cell antigen. Pathological examination revealed that the sacrified sheep suffered from pneumonia with various lesions in lymph nodes, liver, kidney, heart and brain.

INTRODUCTION

Pasteurella haemolytica is the most important cause of bacterial respiratory mortality in cattle and sheep. It is also, identified as the major cause of systemic deaths in sheep Rowe et al. (2001). A variety of antigens that may serve as potential immunogens have been characterized as whole cell and leukotoxin antigen (Derek et al., 1989). Pathological changes were demonstrated in most of the internal organs especially lung. Pluritis and interstitial pneumonia were most dominant patho-

logical lesions detected in dead animals (Fadel, 2000). Antigens associated with whole cell P. haemolytica and leukotoxin were analysed by Sodium dedecyl sulfate ployacrilamide gel electrophoresis (SDS - PAGE) to reveal their molecular weight profile. Immunolgical response was detected serologically by netralization test and enzyme linked immmunosorbant assay (Tatum et al., 1998), high antibody levels were detected in the serum of immunized rabbits and naturally infected Sheep.

The aim of the present work was to characterize the whole cell and leukotoxin antigens of P. haemolytica isolated from clinical cases.

MATERIAL AND METHODS

A total of 239 nasapharyngeal swabs and lung samples were collected from Sakha station of Reproduction Research Center. Out of these samples 203 nasopharyngeal swabs were collected from 150 apparently normal and 53 from diseased animals). The remaining 30 samples were taken from dead animals and 6 samples from emergency slaughteredcases. All samples were; transported to the laboratory with the minimum delay.

Serum samples collected from 53 diseased animals. Nasal swabs and lung samples from emergency slaughtered and dead animals were inoculated onto blood and MacConkey agar. The isolation, purification and identification of the bacterial isolates were carried out according to Diker et al., (1999).

HISTOPATHOLOGICAL STUDIES

For histopathological examination ,paraffin blocks were prepared from different organs and tissue sections were stained by H&E (Cloyden, 1971).

ANTIGEN PREPARATION:

Whole cell antigen was prepared from fresh 18 hours cultures of P. haemolytica suspended to concentration of 109 c. f. u. in phosphate buffer saline pH 7.4 were utilized as whole cell antigen according to Derek et al. (1989). Leukotoxin was prepared by inoculation of brain heart infusion broth with 18h, culture of P. haemolytica following 4.5h incubation at 37°C on shaker rotator. Bacteria were pelted and suspended in RBMI 1640 medium for an additional one hour incubation. The supernatant was then precipitated by ammonium sulfate. The resulting pelt was suspended in 10 mM Tris hydrochloride buffer according to Jennifer et al. (1991).

S D S PAGE:

Antigen extracts were subjected to preparative sodium dedecyl sulfate ploy acrylamide gel electrophoresis. Analytical discontinuous method (Derek et al., 1989).

RABBIT IMMUNIZATION:

Two groups of New Zealand rabbits were inoculated with the two different prepared antigens (whole cell antigen and leukotoxin antigen) leukotoxin was inoculated in first group injecting each animal with 100µg leukotoxin mixed with Freund`s incomplete adjuvant (1ml) via subcutaneous route (Derek et al., 1986) and injecting 109 colony forming unit of whole cell antigens in the second group subcutaneous, third group were injected with physiological saline and kept as control serum (Young and Ryoung, 1987) .

NEUTRALIZATION ASSAYS:

10µl sample of partially purified toxin (100µ) was mixed with 10µl of serially diluted antiserum obtained from naturally infected sheep, after 1 hour incubation at 37°C.(10^6) in 1ml of growth medium was added, the cell showed morphological changes induced by leukotoxin young and Ryoung (1987).

ELISA ASSAYS:

P. haemolytica antigens (whole cell and leukotoxin antigen) were used as coating antigens in detecting antibody levels against these antigens in natrually infected sheep and experimentally infected rabbits. Horseradish peroxides (H R P) conjugated rabbit antisheep IgG was diluted in PBS in case of ovine serum. Labelled sheep anti-rabbit IgG was diluted in PBS according to Derek et al. (1989).

RESULT

Sakha station Reproductions Research Center is a collective breeding farm for different animal species. The lack of labour does not permits early detection and isolation of diseased cases. From January to June 2001 clinical manifestations characterized by emaciation, fever (40-41°C) loss of appetite, cough and sneezing were noticed. Through one month from the onset of this clinical problem, the morbidity rate reached 22.18%, mortality rate was 12.55% . (Table 1). Examination of 203 Nasopharengeal samples obtained from diseased and apparently healthy sheep suffering from pneumonia revealed that the prevalence rates were 75% and 50%, respectively. The rate of Pasteurella isolation among emergency slaughtered sheep and dead animals with symptom of shipping fever were 66% and 90% as in Table (2). SDS PAGE of leukotoxin and whole cell antigens extracted from the isolated strain indicated 4 bands of leukotoxin range between 85KD a to 98 KDa and 10 bands of whole cell antigens ranged between 22 KDa and 80 KDa. as in Table (3) and Fig. (1).

Neutralization antibodies against leukotoxin in sheep serum were significantly higher than whole

Table (1): Distribution of the fractured bones in relation to the methods of reconstruction.

Locality	Period of examiantion	Species	Total No. of animals	No. of died animal	Morbidity rate %	Morbidity rate %	Fatality rate %
Sakha Station	June 2001	Sheep	239	30/239	22.18%	12.55%	56.60%

Table (2): Prevalence rate of ***Pasteurella haemolytica*** in sheep.

Designation of animal	Total No. of samples	Total No. of animals	Lung smaples. * Positive *Pasturella*
Dead animal	30	-	27/30 (90%)
Emergengy slaughtered	6	5/6 (83%)	4/6 (66%)
Diseased animal	53	40/53 (75.5%)	
Apparently healthy	150	75/150 (50%)	
Total	236	120/239 (50.2%)	31/36 (86%)

* Number of positive *P. hamloytica/* total number of animal.

Table (3): Analysis of leukotoxin and whole cell antigens of *Pasteurella haemolytica* isolates by sDS-PAGE.

Marker		Leukotoxin antigen		Whole cell antigen	
M.W.	Amount	M.W.	Amount	M.W.	Amount
120	14.4	98.42	21.63	85	10.0
110	14.3	90.46	24	79.86	10
85	18.3	85	26	66.25	10.50
66	18.3	80.51	28	54.49	5.57
45	18.3			50.02	16.90
20	16.4			45.23	10.70
				40	10.57
				35	5.29
				30	11.20
				22	9.52

cell antibodies in the same sera as shown in Table (5) . In Table (4 and Fig.(2)) demonstrated that a high antileukotoxin antibody levels in serum of rabbits were significantly greater than antibody titre to whole cell antigen. Antibody titre against leukotoxin in sheep serum was significantly greater than antibody titre against whole cell antigens as shown in Table (6&7).

NECROPSY FINDING :

Six sheep were emergency slaughtered and subjected to post-mortern examination. The pleura was thickened in 2 cases. The lungs showed hepatization with white different sizes nodules and multiple abscess formation (Fig. 4) the bronchial lymph node appeared larger than normal (Fig.3) in four cases. No lesion in other organs was detected.

Table (4): and Fig (2): Antibody response of immunized rabbits to leukotoxin and whole cell antigens using ELISA.

Weak post immunization	Leukotoxin	Whole cell
1st	0.514 ± 0.0193	0.4168 ± 0.0166
2nd	0.663 ± 0.0264	0.5532 ± 0.0183
3rd	0.7514 ± 0.0408	0.5814 ± 0.0221
4th	0.6928 ± 0.0325	0.5492 ± 0.0257

Table (5): Neutralization of leukotoxin and whole cell antigens of *Pasteurella haemolytica* from sheep (using sera from naturally infected sheep).

Type of antigens	1/25	1/64	1/128	1/256	1/512	1/102	1/2048
Leukotoxin	+	+	+	+	-	-	-
Whole cell	+	+	-	-	-	-	-

Table (6): Antibody response of sheep to *P. haemolytica* leukotoxin using ELISA

Antibody titre	Diseased animals	Apparently healthy
0.95> Strong positive	8/40 (20%)	
0.95-0.85 = Positive	22/40 (55%)	11/40 (27.5%)
0.85-0.75 = Weak positive	10/40 (25%)	19/40)47.5%)
0.70 -0.60 = Control positive		10/40 (25%)
0.40 < Control negative		

Table (7): Antibody response of sheep to *P. haemolytica* whole cell antigen using ELISA

Antibody titre	Diseased animals	Apparently healthy
0.80> Strong positive	3/40 (7.5%)	
0.80-0.70 = Positive	15/40 (37.5%)	8/40 (20%)
0.70-0.60 = Weak positive	16/40 (40%)	13/40 (32.5%)
Control positive	6/40 (15%)	19/40 (47.5%)
Control negative		

HISTOLOGICAL EXAMINATION :

The pleura was thickened and showed oedema. It was infiltrated by inflammatory cells mainly neutrophils the visceral layer of pleura was thickened showing infiltration with fibrinous exude (Fig. 6). Many alveolar lumens were filled with massive masses of ploymorphonuclear cells and macrophages with homogenous eosinphilic sub stance (Fig. 7&8). The bronchioles showed hyperplastic proliferation of it epithelium with inflammatory reaction in its lumen.

In the heart, the cardiac muscle fibers showed server infiltration with mononuclear cells (Fig 10). The liver showed dilatation of sinusoids, which were filled with R.B.Cs. (Fig 10). The spleen showed depletion in the number of lymphocytic cell. The kidney showed glomerulonephritis. The bronchial lymph node showed depletion of the lymphoid follicles with sever infiltration with neutrophils (Fig. 11) while in other cases the lymph nodes showed lymphoid hyperplasia with homogenous eosinophilic fibrin threads in between the lymph follicles intangled in it leucocytic inflammatory cells.

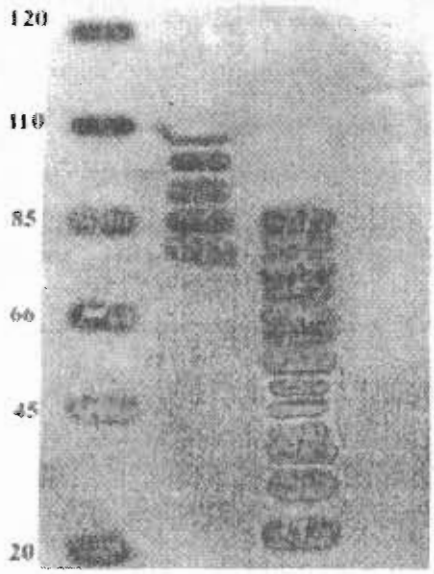

Fig. (1): SDS-PAGE (leukotoxin and whole cell antigen) of *P. haemolytica*

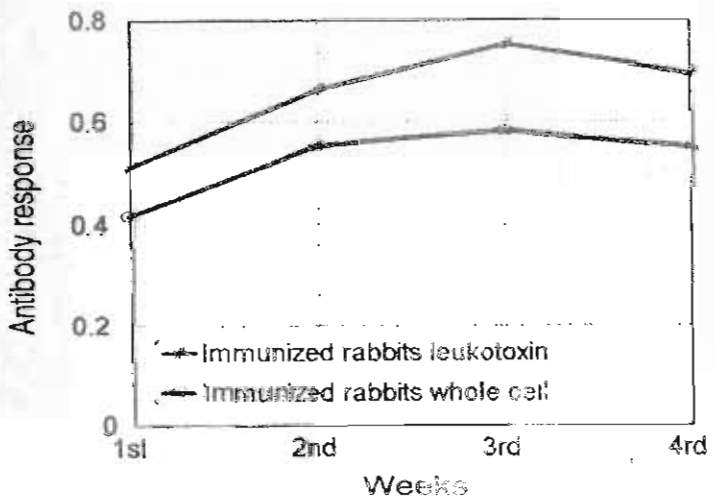

Fig. (2): Higher antibody titres were shown in rabbits immunized by leukotoxin.

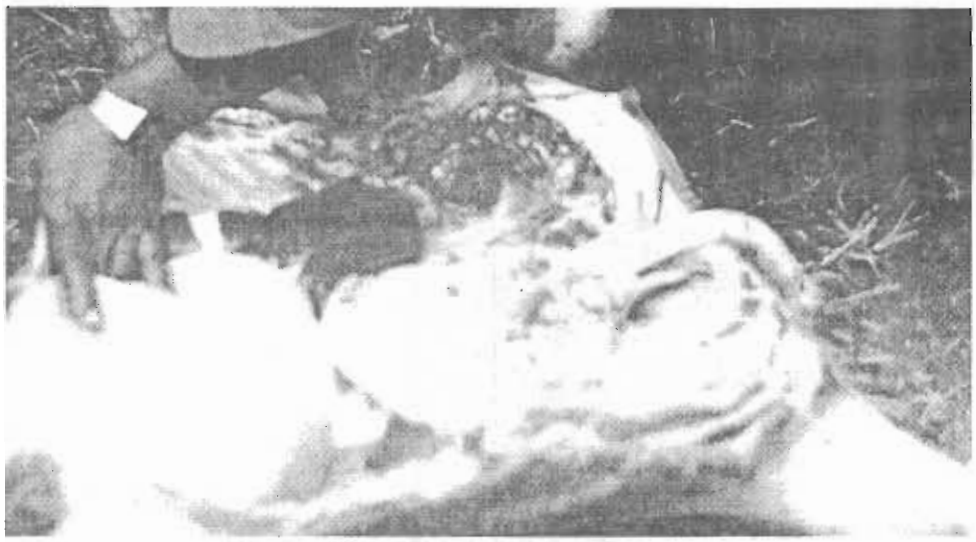

Fig. (3): Lung showing multiple nodules scattered all over the lung.

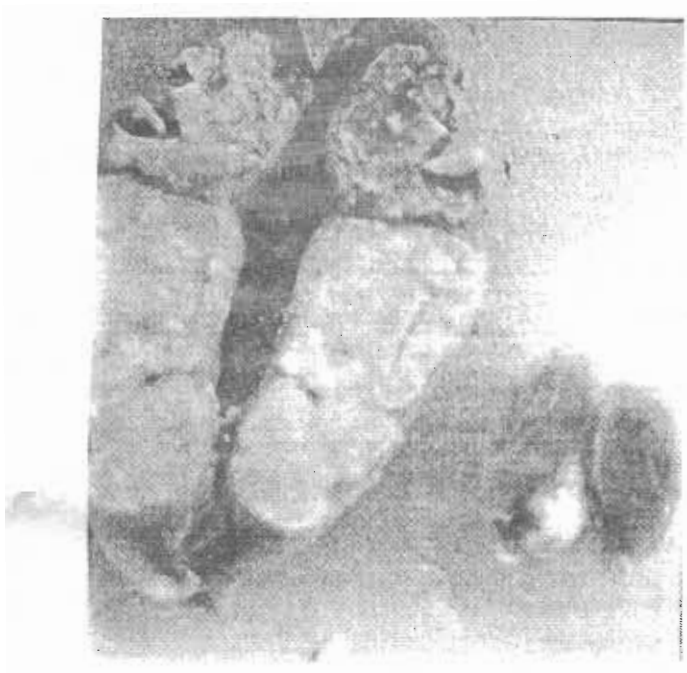

Fig. (4): Enlargement of bronchial lymph node in comparing with normal one

Fig. (5): Lung showing multiple nodules in cut section.

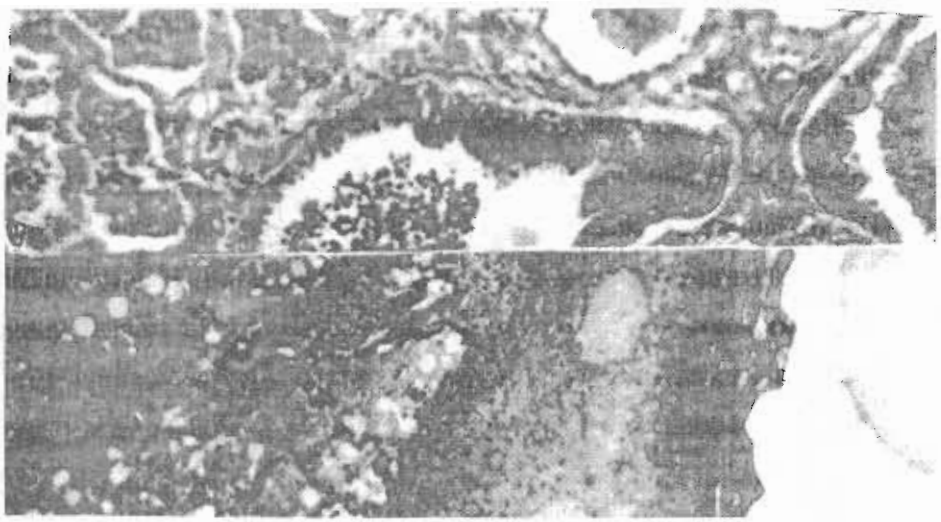

Fig. (6): Lung showing pleural thickening, with fibrinous exudate and inflammatory cells H & E X 100.

Fig. (7): Lung showing alveoli filled with polymorph nuclear cells and fibrin deposition between the alveoli H & E x 100.

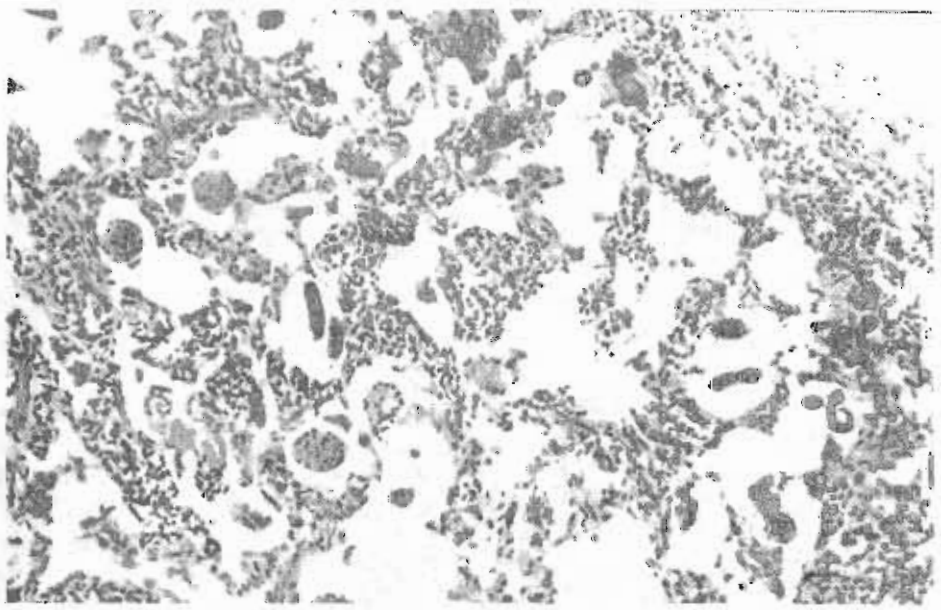

Fig. (8): Heart sowing sever infiltration with mononuclear cells in between the cardiac muscles H & E x 400.

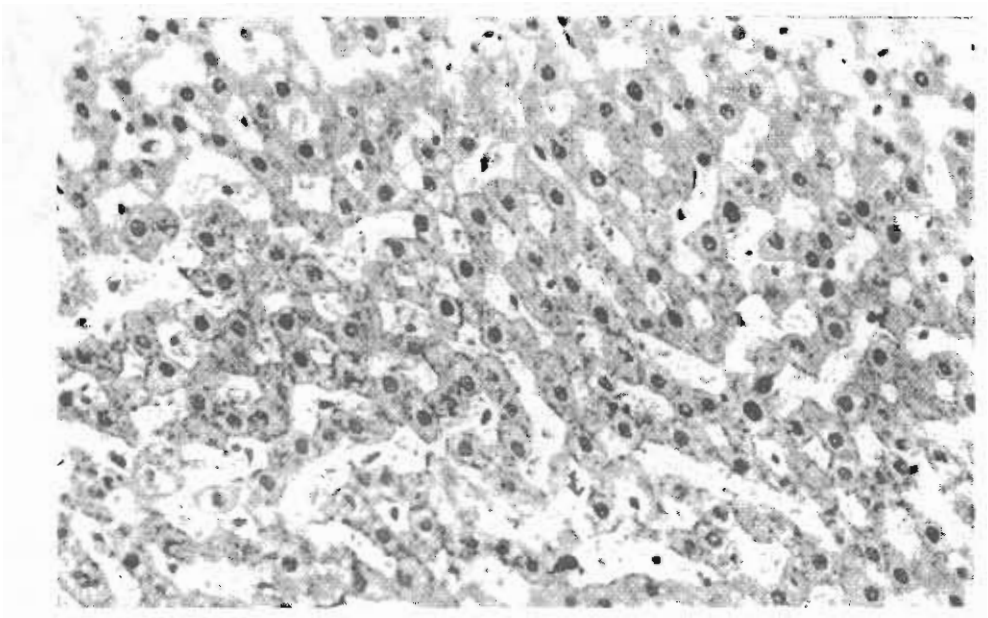

Fig. (9): Heart sowing sever infiltration with mononuclear cells in between the cardiac muscles H & E x 400.

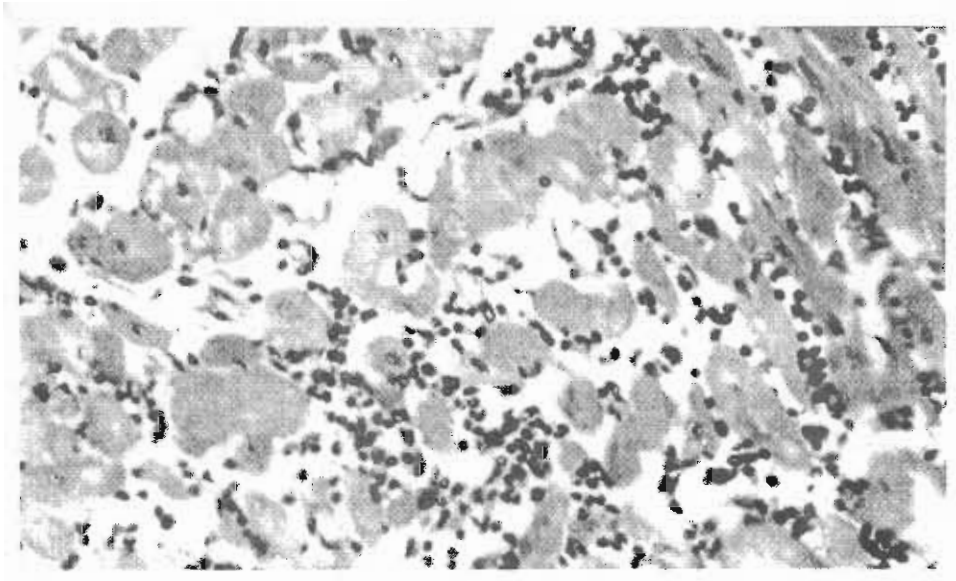

Fig. (10): Liver showing sinuses congestion and dilatation H & E x 650.

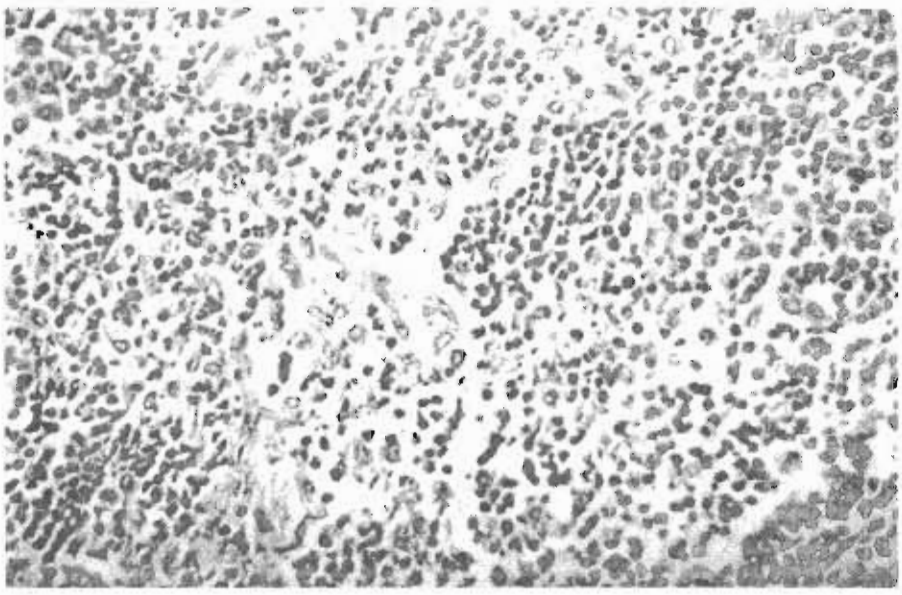

Fig. (11): Lymphonode showing sever infiltration with inflammatory cells

DISCUSSION

P. haemolytica is more recently associated with epizootic of shipping fever and are commonly normal inhabitant in nasopharyngeal mucosa and associated with pneumonia or septicaemia in lambs Rowe et al. (2001).

The clinical signs of respiratory infection were dullness, elevated temperature, anorexia, dispnea coughing, sneezing and nasal discharge, these finding were nearly the same recorded by Diker et al. (1999) and Mishears et al (2000).

In the present study (Table 2) *P. haemolytica* could be isolated from apparently healthy (56%) and diseased animals (75%). While 66% and 90% from emergency slaughtered and dead animals, respectively. These findings supported by Black and Duganzich (1995) and Fodrol et al. (1999).

P. haemolytica produces leukotoxin that is important in the pathogenesis of disease associated with this micro-organism, LKT produce number of biological effects include cytotoxin effect upon leukocytes, death of leukocytes by apoptosis, and the down regulation of proteins on the surface of macrophage affecting their ability to present antigen. Activation of macrophage results in the relase of cytokines and stimulation of polymorphonuclear leukocytes leading to relase of H2O2 which in turn is converted by alveoler endothelial cell in the presence of Fe to hydroxyl radicals. Hydroxyl radicals kill the cell, result accumulation of edema fluid and fibrin San Jeer et al., (2002)

Haemolysis of sheep blood by *P. haemolytica* organisms indicate that the greater potential of these organisms to produce disease is attributable to their ability to produce leukotoxin, which reported by Clinkenteard and Upton (1991) and Morphy et al. (1995) and Peters et al. (1995).

SDS-PAGE analysis of leukotoxin revealed four protein bands of molecular weight ranged from 80 to 98 KDa as in Table (3) and Fig. (1). This result nearly similar to that recorded by Derek et al. (1989), while SDS-PAGE analysis of whole cell Ag revealed ten protein bands of molecular weight ranged from 25 to 80 KDa. This result might be similar as Yung and RyYoung (1987).

Leukotoxin produced by *P. kaemolytica* was considered to be the primary virulence factor in the pathogenesis of shipping fever pneumonia in sheep Sweeney et al. (1994). The leukotoxin in serum antibody titre have been associated with increase resistance to pneumonic pasterulosis in sheep Krable et al. (1998), Menal et al. (2000) and Aqiular et al. (1991), suggested that whole cell antigen stimulate somatic antibody response which doesn't consistently provide protection to evaluate the humoral immune response of rabbits immunized with leukotoxin and whole cell antigen using ELISA.

Data presented in Table (4), (5) and Fig. (2) illustrated the significant increase in antibody titre observed during different intervals post immunization in rabbits immunized by leukotoxin, that the rabbit immunzied by whole cell antigen according to (YungFu Chang et al., 1987).

In this concern, nuetralizing test and ELISA were used to detect antibody titre of sera from naturally infected sheep using leukotoxin and whole cell antigens. The result of nuetralizing test and ELISA as presented in Table (6) and (7) showed a significant increase in antibody titre to leukotoxin than antibody titre to whole cell antigen.

The results indicate that the protective immunity could be manifested by leukotoxin antigen than the whole cell antigens, this could be attributed to the higher immunogenic feature of leukotoxin as concluded by Derek et al. (1986) and Black and Duganzech (1995).

The results of this study suggest that exposure to leukotoxin antigens may be necessary to produce an antileukotoxic immune response and that this response is a better predictor of resistance to pneumonia than the immune response to whole cell antigen , ELISA leukotoxin antibody response significantly higher than those for the whole cell antigen recorded by Jennifer and Reggie (1991) and Tantum et al. (1998) and Sanjeev et al. (2002) found that laktoxin activation of peripheral blood monocytes, macrophages, and when incubation of neutrophilo with leukotoxin resulted neutrophil activation..

In the present investigation, it was evident that the *P. haemolytica* was responsible for the severe pathological changes developed in the lung and other organs of sheep. The observed pathological lesions in the lungs of sheep which were characterized by pleuritis, interstitial pneumonia could be attributed to *P. haemolytica*. These results coincided with finding of Fadel (2000). The occurrence of pathological changes in liver, kidney, spleen, lymph node could be attributed to the superimposed infection with *P. haemolytica* and their circulating toxins (Hugo, 1987).

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