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IMMUNOLOGICAL AND PATHOLOGICAL STUDIES ON THE PURIFIED TOXIN OF *PASTEURELLA MULTOCIDA* ISOLATED FROM CALVES

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SUMMARY

In the present study, a total of 235 buffalo calves (1-8 months of age) from different localities were investigated. They were grouped, 80 apparently healthy calves (group 1), 105 with respiratory manifestations (group 2) and 50 dead calves suffered from respiratory manifestations before death (group 3). Nasopharyngeal swabs were taken from group (1 and 2) and lung tissues, lymph nodes and liver from the groups (2 & 3) for detection of *P. multocida* and specimens from the lung, associated lymph node and liver were taken from each group for the histopathological studies.

P. multocida was isolated from 6.25%, 10.47% and 66% of each calf group respectively. The isolated *P. multocida* were pathogenic to mice and belonged to serotype B:2.

The histopathological studies revealed fibrinous broncho-pneumonia, severe pleuritis, hyperplasia in the lymphoid follicles of the lymph node and fibrinous hepatitis of group (2); hemorrhagic pneumonia with adhesive pleuritis, depletion of the lymphoid follicles of the lymph node and hepatitis were seen in calves of group (3).

Heat labile toxin from cell sonicate of the isolated *P. multocida* serotype was purified by ammonium sulphate precipitation followed by ion exchange of gel filtration chromatography and polyacrylamide gel electrophoresis. Toxic activity was assayed during toxin purification by intranasal insillation in rabbits. The most toxogenic band was the 150 KDa fraction as determined by SDS PAGE. Western blot analysis, rabbit antisera react with 150 KDa. Rabbits inoculated I/N with the purified toxin dead within 2 days, showing symptoms of septicaemia with hemorrhagic pneumonia and pleuritis. Non of the rabbits inoculated with the heated toxin at 70°C for 30 minutes dead or had clinical sings of toxicosis.

Pasteurella free rabbits were immunized I/N and I/conjunctival twice with 2 weeks interval and challenged I/N with a homologous *P. multocid.* for 2 weeks after the second immunization. The immunized challenged rabbits were shown to be protected against clinical sings of the disease and death with production of serum immunoglobulin against *P. multocida*. The lesions seen were slight emphsyma and few inflammatory cell infilteration of the lung, mild depletion of the lymphoid follicle of the lymph node with mild hepatitis. In contrast, the non-immunized challenged rabbits dead within 4 days with heamorrhagic pneumonia, pleurtitis and hepatitis.

Immunization with the purified toxin prevented all clinical sings of the disease and deaths.

INTRODUCTION

Respiratory diseases are the most frequently recorded causes of morbidity and mortality in calves (Healy et al., 1993). Pasteurella multocida is a common commensal organism found in the upper respiratory tract of cattle and buffaloes playing an important role in the induction of haemorrhagic septicaemia and bronchopneumonia (Donkersgoed et al. (1993).

Numerous techniques have been used in an attempt to control and eliminate pasterurellosis. Another approach that is promising the development of immunization procedures that will protect animals exposed to *P. multocida* (El-Eragi et al., 2001).

Immunization with killed *P. multocida* has produced poor protection against pasteurellosis in several animal species (Bapat and sawhney, 1977). In contrast, live mutant vaccines produced protection against *P. multocida* challenge (Lu and Pakes, 1981). However the protection was not complete, since focal lung lesions were noticed, i.e. the vaccination using *P. multocida* bacterins were moderetly successful in protection against pasteurellosis (Okerman and Spanoghe, 1981).

Due to the great economic losses from the fatal pulmonary infection produced by *P. multocida* and its effect on animal performance in the form of poor growth, reduced productivity, debility, lowered resistance, the aim of the present study was to investigate the prevalence of *P. mutocida* infection among calves and to evaluate its pathogenicity and protective efficacy of the purified toxin of the isolated serotypes.

MATERIAL AND METHODS

Field Samples:

A total of 235 buffalo calves (1-8 month old) from El-Giza, El-Kalubia and El-Sharkia were examined. They were grouped into 80 apparently healthy calves were (group 1), 105 calves showed respiratory manifestations (group 2) and 50 dead calves which were suffering from respiratory syndrome before death (group 3). Nasopharyngeal swabs were collected from calves of group 1 and 2, while heart blood, lung, associated lymph nodes and liver were collected from groups (2 and 3). Tissue specimens from the lung, associated lymph nodes and liver were collected from groups (2 and 3) and fixed in 10% buffered formalin for histopathological studies.

Isolation and identification:

The collected samples were inoculated directly onto 7% sheep blood agar (Oxoid) and incubated aerobically at 37°C for 24 hours. Identification of pasteurella organisms was carried out according to Makie and MacCarteny, 1996 and standard hyperimmune sera against *P. multocida* serotypes B, A, D and E (kindly obtained from Aerobic Bacterial Vaccine Research Department Vet. Serum and Vaccine Research Institute Abbasia, Cairo) were used for serological identification. Mice were employed for application of the pathogenicity test for *P. multocida* isolates (Bain et al., 1982).

Extraction of toxin:

Bacterial culture of *P. multocida* grown for 18 hours at 37°C on sheep blood agar plate was harvested with saline solution. Cells were centrifuged twice at 11.000 xg for 20 minutes. Nucleases (0.001% DNAase, 0.001% RNAase, protease

inhibitors) and 0.01% NaN_3 were added to cells suspended in saline solution and incubated for 1 hour at 37°C. After incubation, the cells were sonicated 3 times for 10 seconds at 4°C. The sonicate was centrifuged at 19.600 xg for 40 minutes and the supernatant was passed through 0.45 μm filter. Ammonium sulfate was added to the filtrate to 40% saturation at 4°C and kept in a refrigerator for overnight. After centrifugation at 19.600 xg for 30 minutes, the precipetate was suspended in phosphate buffered saline solution (PBSS) dialyzed against 0.05 Mtris Hcl buffer (pH = 6.0) at 4°C for 24 hours and filtrated twice through 0.45 μm and 0.20 μm filters respectively (Clarence et al., 1991).

Ion exchange chromatography:

Dialyzed and filtered crude toxin was applied to a 1x50 cm ionexchange column (DEAE-Sephacel, Sigma, USA) at 4°C after equiliberation with tris-HcL buffer. The column was eluted with 500 ml of tris buffer, 600 ml of tris buffer containing 0.2 M NacL and 900 ml of buffer containing 0.5 M NacL, respectively. Ten-milliliter fractions were collected at a rate of 20 ml/h (Wood and Cooper, 1970).

Gel filtration chromatography:

Fractions from ion exchange chromatography containing toxigenic activity were pooled and concentrated by ulterafiltration (Clearance et al., 1991). The concentrate was added to 100 x 1 cm gel filteration column (sephacel S-200 gel) equiliberated with tris-HcL buffer contining 0.1 M NacL (pH = 7.2). The column was eluted with 1000 ml of buffer and 3 ml fractions were collected at a rate of 12 ml/h. Fractions containing toxic activity were pooled and concentrated by ulterfilteration using Amecon pM-10, Danvers, Mass. USA (Himnelhoch, 1971).

Sodium dodecyl sulfate (SDS)-PAGE, (Laemmli, 1970):

This technique was used to determine the molecular weight of toxic protein. Briefly, vertical slab gels, 1.5 mm thick, were run with a discontinuous buffer system using 4% polyacrylamide 2.7%BIS staking gel and an 8% polyacrylamide, 2.7% BIS running gel. The tank buffer contained 0.025 M tris, 0.192 M glycine and 0.1%SDS at pH 8.3 samples (5 μg of protein) in 0.125 M Tris-HcL (pH 6.8), 4% SDS, 20% glycerol and 10% 2-mercaptoethanol were heated at 95°C for 5 minutes and layered under the tank buffer directly on the staking gel.

Electrophoresis was done in conjunction with marker proteins of known molecular weight (Biorad, USA). Gel were runs at 50 mA at 4°C for 3 hours and were silver stained. Molecular weights of unknown proteins were determined using image densitometer (G 700, Biorad). Protein concentration of the toxin was determined as described by Layne (1957).

Perparation of rabbit hyperimmun sera:

New Zealand white rabbits weighing about 2 Kg injecting with 10 mg protein per ml of formalized crude toxin subcutaneously (S/C) into rabbit twice per week (0.5 ml) given with 0.5 ml of freund complete adjuvant. Subsequent 1 ml injection of samples were given S/C 20 times at 3 day intervals. One week after the final injection, blood samples were withdrawn. The sera were pooled and stored at - 20°C. (Nakia et al., 1984).

Immunoblot analysis:

Polyacrylamide gel electrophoresis was done as described and the toxine was transferred to nitrocellulose membrane (0.45 μm) by use of a western blot transfere cell. The gel was placed on a sheet of nitrocellulose. Cut to the same size and subjected to electrophoresis overnight at 100 mA in mM Tris base, 192 mA glycine and 20% methanol in a transblot apparatus (Bio-Rad). After transfere, the nitrocellulose blots were shaken for 1.5 h at 37°C in a blocking buffer (PBS contaning 3% fraction V bovine serum albumin and 10% heat inactivated calf serum) to block non specific protein binding. The blots were then incubated for 2h at room temperature with rabbit hyperimmune serum previously prepared against crude toxin washed at room temperature by shaking with four changes of PBS containing 0.3% fraction V bovine serum albumin, 15 minutes per wash, then shaken for 1.5h at room temperature in peroxidase conjugated goat antirabbit IgG (Towbin et al., 1979). After incubation, the nitrocellulose was washed twice with PBS, transferred to a glass dish, washed and incubated with 4-chloronaphthol (0.1%) in 9.5% ethanol and 0.015% hydrogen peroxide for 30 minutes before rinsing with water.

Experimental animals:

New Zealeand rabbits (8-9 week old-2.5 Kg weight) obtained from Vacsera farm-Helwan, were used. Rabbits were housed in wire rabbitary. Before experiment, the nasal cavity of each rabbit was cultured for *P. multocida* and sera were tested in an ELISA for the IgG to *P. multocida* subsequently all rabbits were found to be free of *P. multocida* and specific antibody.

Toxicity of fractions from gel filtration chromatography:

Toxicity of fractions of the toxin were carried out according to Clarence et al. (1991). Each of six pasteurella free rabbits were inoculated I/N with the fraction from gel filtration chromatography (28 μg protein/ml).

Two additional groups of six rabbits were also inoculated I/N with toxin that has been heated at 50 °C and 70°C for 30 minutes. The rabbits were observed for 14 days to detect lethality of the extracted toxin.

Dead rabbits were necropised after death. Lungs, associated lymph nodes and liver were fixed in buffered formalin 10% for histopathological examination for the evidence of pneumonia, pleuritis and other lesions.

Immunological Studies of Toxin:

* **Experimental design:**

Rabbits were divided into four groups each group consisted of 6 rabbits.

Group I. (Challenged): Rabbits were challenged I/N (0.5 ml per rabbit) with $2x10^8$ organism/ml (Ringler et al., 1985). The survived animals were necropised after 2 weeks.

Group II. (Immunized-challenged): Rabbits were immunized I/N with 5 μg/ml (Sanckow, 2000) and then reimmunized 2 weeks later. Immunization consisted of one drop of purified toxin in each eye and the remaining dropped into nostrils. after the second immunization by 2 weeks, the animals were challenged. All animals were necropised after 2 weeks.

Group III. (Immunized): Rabbits were immunized as previously indicated in group II but they were not challenged. The animals were necropised after 2 weeks.

Group IV (neither immunized nor challenged): Rabbits were kept as control group. All animal were necropised after 2 weeks.

All of the rabbits that have died or necropised, were subjected to post mortem examination and their lungs, associated lymph nodes and livers were fixed in buffered formalin 10% for histopathological studies.

*** Collection of sera:**

Sera were collected from the immunized rabbits at 14, 21, 30 and 40 days from the beginning of the experiment. All sera were tested for antibodies to *P. multocida* by the Enzyme Linked Immunosorbed Assay (ELISA) as described by Marshall et al. (1981) and indirect haemagglutination test IH as described by Carter and Rappy (1962).

*** Isolation and identification :**

At necropsy, nasopharyngeal swab, heart blood, lung and the associated lymph nodes and livers were cultured for *P. multocida*. Each sample was inoculated onto tryptic soya agar with 5% sheep blood and incubated at 37°C for 24 hours. Colonies were identified and serotyped according to Makie and MacCarteny (1996).

Histopathological studies:

Tissue specimens (lung, associated lymph node and liver) collected from the slaughtered calves showing respiratory manifestation, from dead calves which had respiratory manifestations before death as well as from all of the rabbits used in the experiment, previously collected on neutral 10% buffered formalin, were processed to obtain five micron thick sections and stained with Haematoxylin and Eosin according to Drury and Wallington (1980) for histopathological examination.

RESULTS

P. multocida was isolated with a percent of 6.25%, 10.47% and 66% from apparently healthy, respiratory diseased and dead calves; respectively with a total incidence of 22.9% as shown in table (1). In respiratory diseased calves *P. multocida* was isolated with a percentage of 10.47 % from each of heart blood, lung and lymph node while from the liver the isolation percent was 9.5%. In the examined dead calves, *P. multocida* was isolated with a percent of 66% from each of the heart blood and lung, but from the lymph node and liver the isolation percent was 60% each.

Identification of *P. multocida* isolates was carried out on the bases of culture characters, staining reaction, biochemical properties in addition to serological identification which revealed that all *P. multocida* isolates were classified as B:2 and pathogenic to mice.

Concerning the calves which had respiratory manifestations (cough, nasal discharge, abdominal respiration, fever and grunting respiratory movement), the gross lesions showed severely congested and enlarged lungs and some parts were firm. The liver was pale in color, enlarged and some parts were firm in consistency.

The microscopic examination showed that the alveolar lumen was full of infiltrated inflammatory cells which include alveolar macrophages, lymphocytes, giant cells, plasma cells, polymorphnuclear cells and necrosed cells. Sometimes, complete necrosis of the cells lining the alveolar wall with infiltrated fibrinous threads in-between the inflammatory cells (Fig. 5). Severe hyperplastic proliferation of the cells lining the bronchioles (Fig 6). Hyperplasia of the cells lining the pleura. Thickening pleura may be due to the accumulation of fibrinous infiltration which lead to its adhesion over the lung surface or due to the accumulation of the infiltrated inflammatory cells (Fig 7). The associated lymph node showed proliferating lymphoid follicle with thickened capsular wall which infiltrated by mononuclear inflammatory cells and edema (Fig. 8). Moderate fibrosis was found in portal areas which infiltrated by mononuclear inflammatory cells and newly formed bile ductules, vacuolar degenerative changes were seen in the hepatocytes (Fig. 9).

The Gross lesion concerning the dead calves suffering from respiratory syndrome before death showed dark red enlarged lung.

The microscopic examination revealed that severe heamorrahges lead to obliteration of most of the alveoli associated with severe thickening of pleural membrane with severe heamorrhages which infiltrated by the inflammatory cells (Fig. 10). Severe depletion of the lymphoid follicles of the associated lymph node with infiltrated by polymorphnuclear cells (Fig. 11). The triad area of the liver showed moderate amount of fibrosis around the newly formed bile ductules, some hepatocytes suffered from vacuolar degenerative changes, others were necrosed (Fig. 12).

Crude *P. multocida* toxin prepared by sonication of isolated serotype was applied to DEAE-sephacel column and toxic fractions eluted from the column at concentration of 0.5 M NacL (Fig. 1). Toxic fractions were pooled and concentrated and prepared to gel filtration chromatography (Fig. 2). The toxicity of each fraction was assayed by instillation I/N in rabbit, the most toxogenic band eluted from gel filtration chromatography was that corresponded to molecular 150 KDa. Rabbits inoculated with this toxin fraction dead within 2 days post inoculation. Two of 6 rabbits inoculated with this purified toxin heated at 50°C for 30 min dead between 3 to 8 days post inoculation. Toxicity lost by heating at 70°C for 30 minutes (Table 3). The electrophoretic analysis of crude toxin revealed 8 protein bands ranging from 185 to 83 KDa (Fig 3 lane 2). Meanwhile, toxogenic fraction eluted at conc. of 0.5M NacL show 5 protein bands raning from 185 to 10^6 KDa (Fig 3 lane 3). The most separated toxic fraction eluted from gel filtration at 150 KDa recorded the same molecular weight at SDS. PAGE. The results of the western blot analysis is shown in fig. 4, the rabbit hyperimmune sera react with 185.26, 164.28, 149.41, 106.08 and 83.154 KDa protein in crude toxin . While, react with toxogenic fraction 185.26, 163.24, 150.35 and 106.08 KDa (lane 2). Also, the protein band 150 KDa immunologically react with hyperimmune sera (lane 3).

It can be clearly seen from the data illustrated in table (5), that there was a significant rise in the mean optical density in sera of immunized rabbits by day 21 after the initial immunization. Serum IgG activity remained elevated through day 40, when sampling was terminated.

Table (6) revealed that all the non-immunized challenged rabbits dead 3 to 4 days post challenge. *P. multocida* organisms of this group, was recovered at necropsy from the nasopharngeal swabs (2/6), heart blood, lung, associated lymph nodes and liver (6/6 each).

Whereas non of the six immunized challenged rabbit dead and *P. multocida* isolated only from nasopharengeal swabs 1/6.

No deaths occurred in the immunized non challenged rabbits or non immunized non challenged rabbits. *P. multocida* was not isolated at necropsy from these rabbits.

Table (1): Prevalence of *P. multocida* in apparently healthy, respiratory diseased and dead calves.

Condition of calves	No. of examined calves	Type of samples	+ ve culture		+ ve case	
			No.	%	No.	%
Apparently healthy	80	*Nasopharyngeal swabs (80)	5/80	6.25	5/80	6.25
Respiratory diseased	105	*Nasopharyngeal (105) *Heart blood (105) *lung (105) *Lymph nod (105) *Liver (105)	11/105 11/105 11/105 11/105 10/105	10.47% 10.47% 10.47% 10.47% 9.5%	1/105	10.47%
Dead	50	* Heart blood (50) * lung (50) * Lymph nod (50) * Liver (50)	33/50 33/50 30/50 30/50	66 66 60 60	33/50	66%
Total	235	805	185/805	22.9%	49/235	20.85%

( ) = No. of examined samples.
All isolated *P. mutocida* were belonged to serotype B:2.

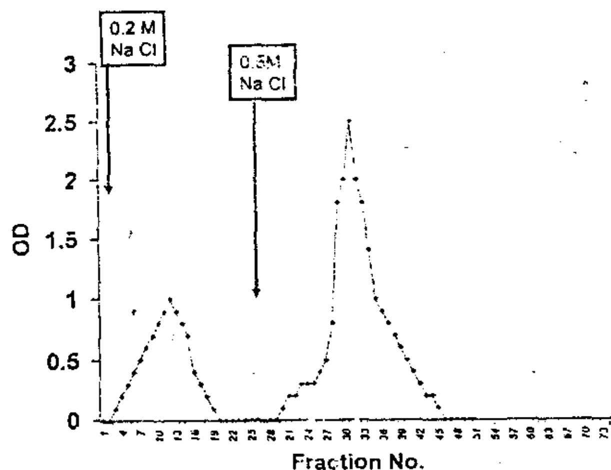

Fig. (1): Ion exchange column chromatography of dissolved NH_4So_4 peripitalte of crude sonicate of *P. multocida*.

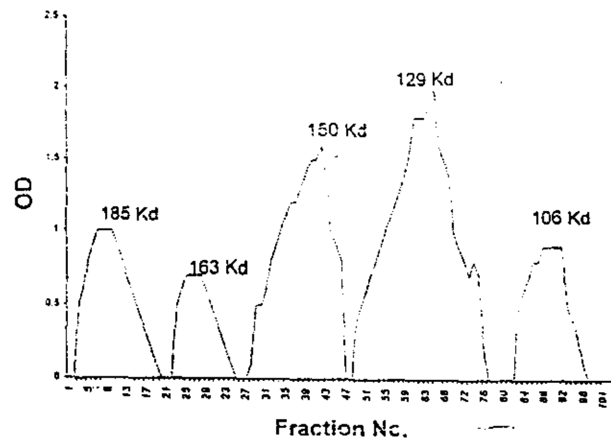

Fig. (2): Chromatogram represent the elution pattern of *P. multocida* toxin fractionated on sephacryl S-200 gel filtration 100 x 1 cm.

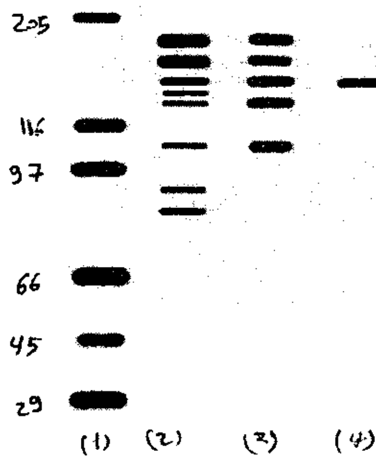

Fig. (3): Pattern of SDS-PAGE of fractions during purification of *P.multocida* toxin. Lane 1 molecular weight marker, Lane 2 ion exchange column, Lane 3 gel filtration column elute, Lane 4 purifiied toxin.

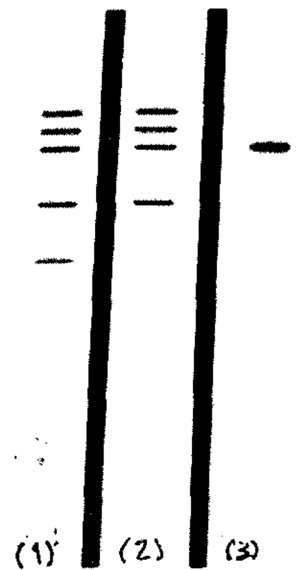

Fig. (4): Western blot analysis of *P. multocida* toxin with rabbit antiserum. Lane 1 crude toxin, Lane 2toxogenic fractions, Lane 3 purified toxin.

Table (2): Pattern of SDS PAGE of *P. multocida* toxin.

Lanes Bands	Lane 1 (marker)		Lane 2 *		Lane 3 *		Lane 4**	
	M.W (KDa)	Amount (%)	M.W (KDa)	Amount (%)	M.W (KDa)	Amount (%)	M.W (KDa)	Amount (%)
1	205	11.114	185.26	17.450	185.26	18.867	150.35	100
2	116	15.728	164.28	17.758	163.24	16.944		
3	97	17.762	149.41	10.333	150.35	20.839		
4	66	24.720	136.74	4.589	129.99	18.035		
5	45	16.961	129.99	7.637	106.08	25.315		
6	29	13.741	106.08	13.820				
7			89.810	13,819				
8			83.154	14.594				
Aum		100		100		100		100
In lane		100		100		100		100

Lane 1, molecular weight markers. Lne 2 ion exchange column
Lane 3 gel filtration column elute. Lane 4 purified toxin.

Table (3): Toxicity of fractions eluted from gel filtration chromatography in rabbits 14 days post inoculation.

Fractions (Mo. Wt. KD)	Treatment of toxin		
	Non heated toxin	Heated at 50°C for 30 minutes	Heated at 70°C for 30 minutes
185.26 KDa	2/6	1/6	0/6
163.24 KDa	1/6	0/6	N.D
150.35 KDa	6/6*	2/6**	0/6
129.99 KDa	0/6	N.D	N.D
106.08 KDa	0/6	N.D	N.D

Dead/Total.
N.D = Not Done.

* Survival time 2 days.
** Survival time 3 -8 days.

Table (4): Survival of immunized and non immunized rabbits after challenge with a lethal dose of *P. multocida* B:2.

Treatment	Dead/total	Total No. of survivals	Survival %	Survival time (days)
Non immunized challenged	6/6	0	0	2-4
Immunized challenged	0/6	6	100	More than 14 days
Immunized non challenged	0/6	6	100	More than 14 days
Non immunized non challenged (control group)	0/6	6	100	More than 14 days

Table (5): Antibody titre against *P. multocida* detected by ELISA and IH test in immunized and nonn immunized rabbits.

Treatment	ELISA *				IH**			
	Days at which sera were taken***				Days at which sera were taken***			
	14	21	30	40	14	21	30	40
Non immunized challenged	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
Immunized challenged	1.323	1.641	1.993	1.997	80	2500	5000	5000
Immunized non challenged	1.243	1.521	1.742	1.821	80	2500	1500	5000
Non immunized non challenged (control group)	0.122	0.174	0.134	0.341	20	40	20	20

* The result is expressed as mean of optical density.
** The results is expressed as inverse of dilution.
*** These days were calculated from the beginning of the experiment.

Table (6): Isolation of P ... m rabbits a ... v.

Treatment	Nasopharyng swabs	eart ood	Lung	Lymph node	Liver
Non immunized challen	2/6	6/6	6/6	6/6	6/6
Immunized challenged	1/6	0/6	0/6	0/6	0/6
Immunized non challen	0/6	0/6	0/6	0/6	0/6
Non immunized non ch (control group)	0/6	0/6	0/6	0/6	0/6

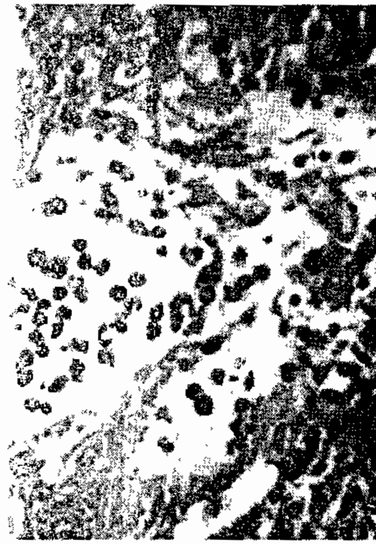

Fig. (5): Lung of ... diseased calv ... g the alveolar lumen full with infiltrated inflammat ... d the alveola ... ages, lymphocytes, giant cells, plasma cells, poly ... ar cells and ... ells (a). Sometimes, completely necrosis of the cel ... alveolar wal ... ltrated fibrinous threads in-between the inflammat ... (H & E. X

Fig. (6): lung of re ... seased calves ... severe hyperplastic proliferation of the cells lining the br ... n fibrous con ... ssues surrounded it. (H & E. X: 200).

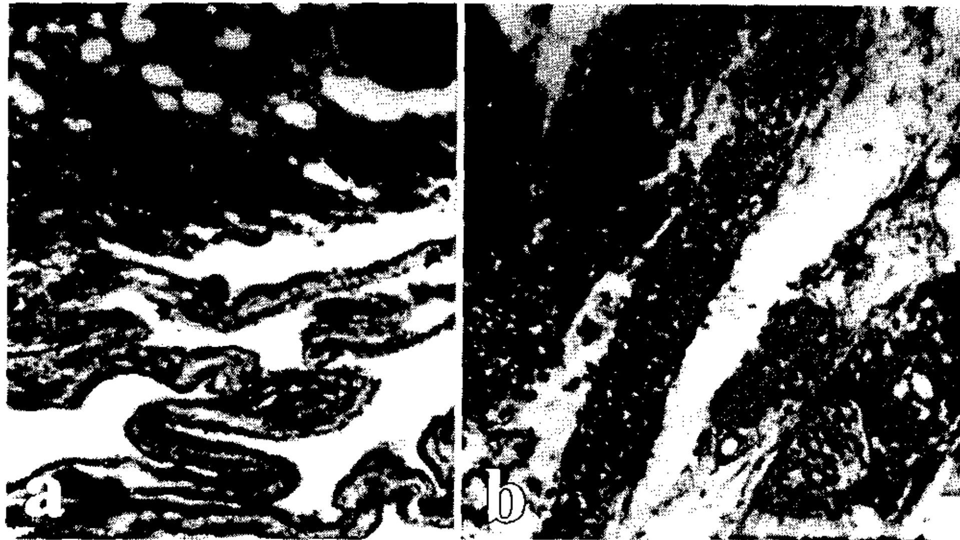

Fig. (7): Pleura of respiratory manifested calves showing hyperplasia of the cells lining the pleura and thickening pleura due to the accumulation of fibrinous exudate (a) thickening of pleura due to accumulation of infiltrated inflammatory cells (b). (H & E. X: 200).

Fig. (8): Lymph node of respiratory diseased calves showing proliferating lymphoid follicle with thickened capsular wall infiltrated by mononuclear inflammatory cells and sub-capcullar edema. (H & E. X: 400).

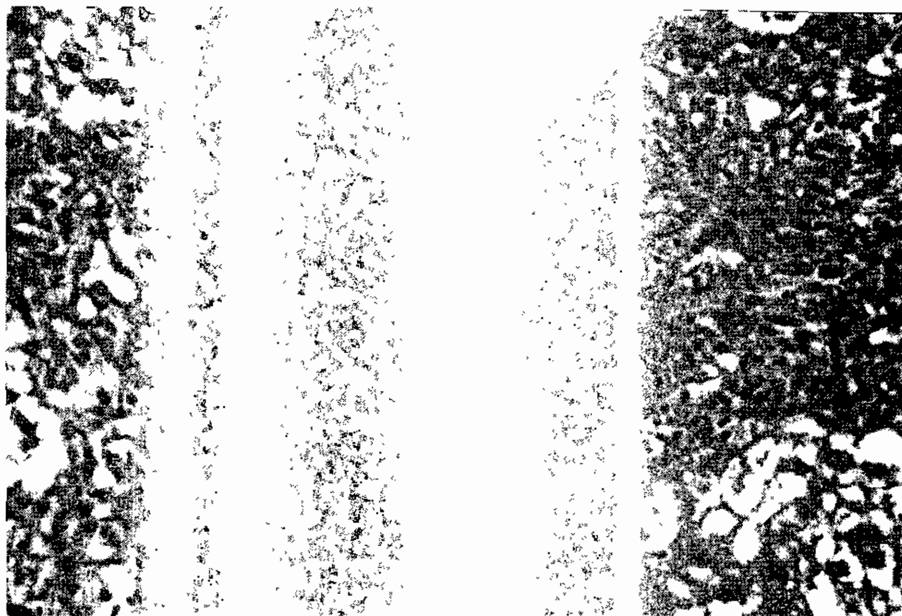

Fig. (9) Liver ... formed bile ductues with fibrous ... nerative changes hepatocytes. (H ...

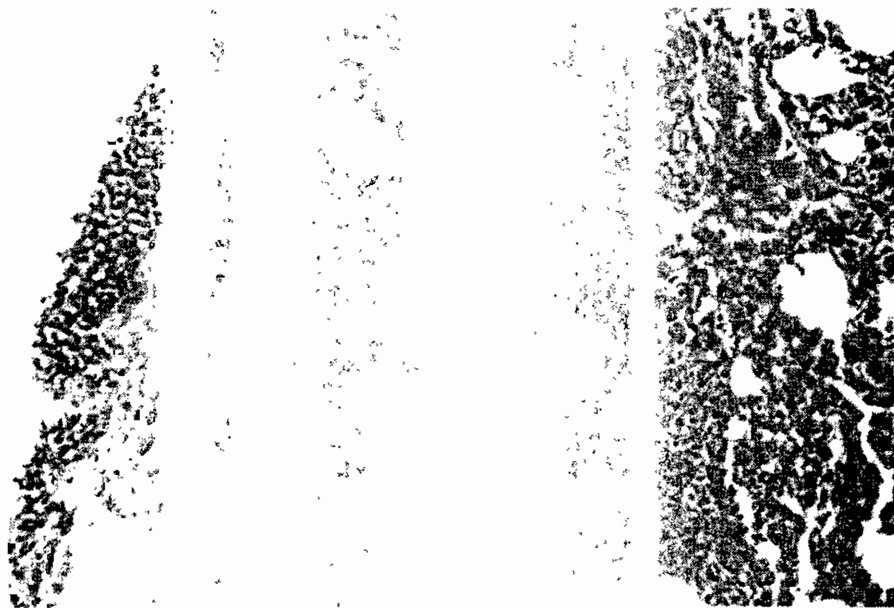

Fig. (10): Lung ... a) with pleuritis (b). (H. & E. X:

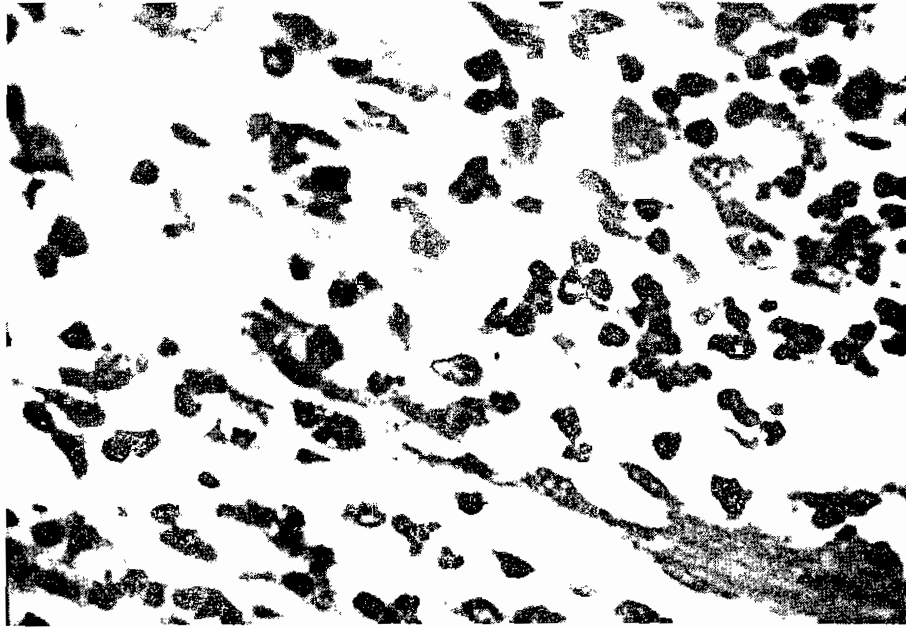

Fig. (11): Medulla of lymph node of dead calves showing infiltration with by polymorph-nuclear inflammatory cells (H. & E X: 650).

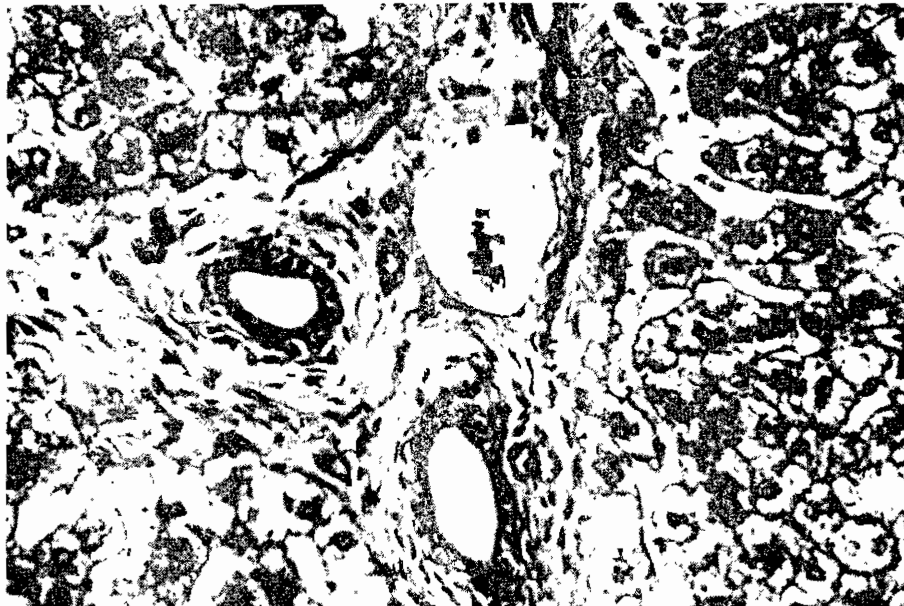

Fig. (12): Liver of dead calves showing slight amount of fibrosis around the newly formed bile ductules, some hepatocytes suffered from vacuolar degenerative changes, others were necrosed. (H. & E X: 400).

Fig. (13): Gross lesions of the dead rabbits received *P. multocida* toxin showing dark red, edematous lung with enlarged liver.

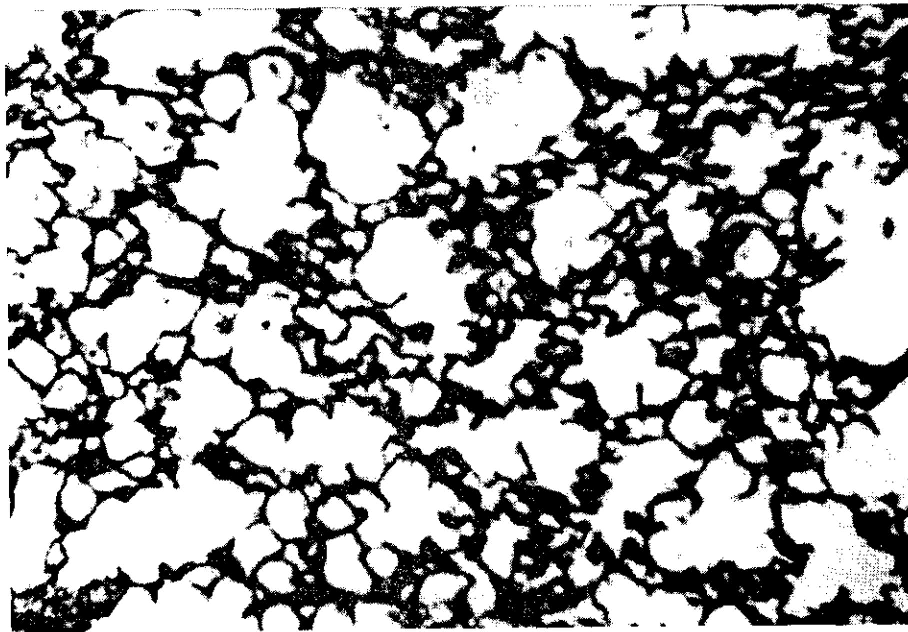

Fig. (14): Lung of immunized challenged rabbits showing slight emphsyma and few inflammatory cell infiltration. (H. & E. X: 200).

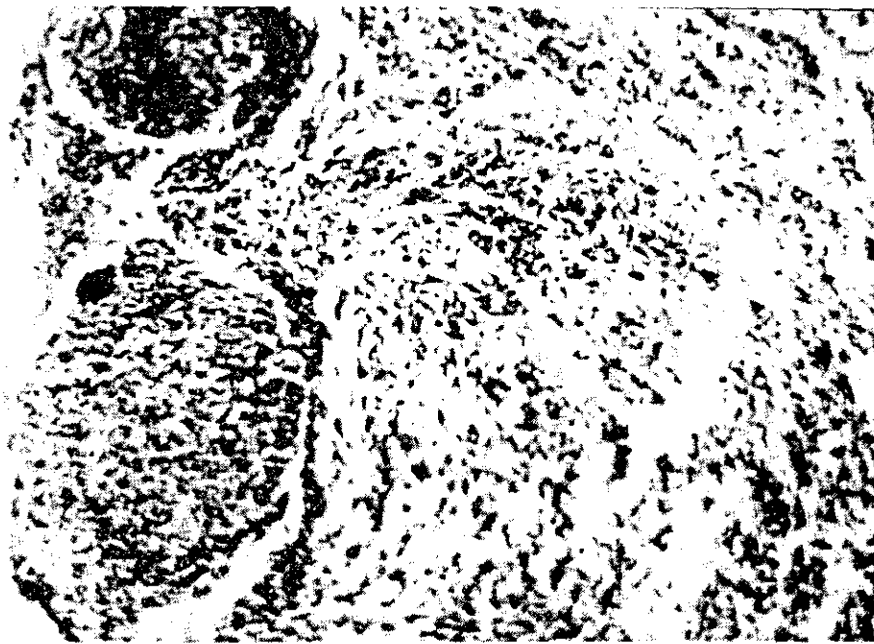

Fig. (15): Lymph node of Immunized challenged rabbits showing slight depletion of the lymphoid follicles with mild edema. (H. & E. X: 200).

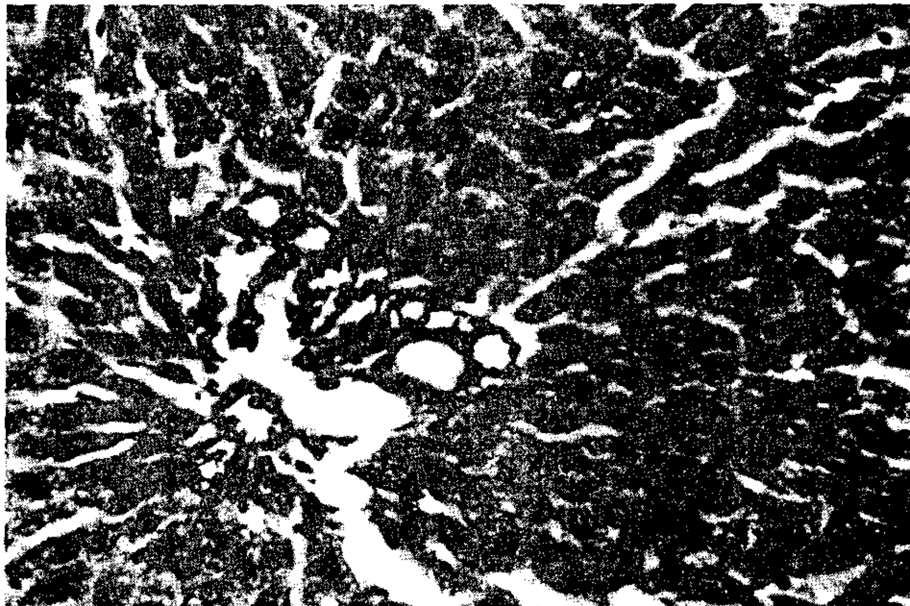

Fig. (16): Liver of immunized challenged rabbits showing few lymphocytic infiltration at the triad portal area with few necrosed hepatocytes. (H. & E. X:400).

The gross lesions of dead rabbits from the (extracted toxin or the heated toxin at 50°C) showed dark red, enlarged lung, with enlarged liver (Fig. 13). The microscopic examination revealed that there was severe heamorrhagic pneumonia with adhesive pluritis and vacuolar degenerative changes affected hepatocytesas as previously seen in respiratory manifested calve's lung, lymph node and liver. While the rabbits given I/N heated toxin at 70°C didn't showed any gross lesions even after 14 days after inoculation. There were not any pathological lesions neither in lung, associated lymph nodes or in liver.

Otherwise, the gross examination of challenged non immunized rabbits revealed enlarged lung had dark red color. The histopathological examination revealed the same picture like the dead calves and the rabbits which inoculated by the toxin as shown in figures (10, 11 & 12).

There was neither clinical signs of the disease nor gross lesions appeared in any organs of the immunized challenged rabbits group.

Meanwhile, the microscopic examination of the lung showed slight emphsyma and few inflammatory cells infiltration. (Fig. 14). As well as slight depletion of the lymphoid follicles lymphocytes of the associated lymph node with mild edema (Fig. 15). Moreover, few lymphocytic infiltration at the triad portal area of the liver associated with few necrosed hepatocytes (Fig. 16) was also seen.

Neither clinical sings, gross lesions nor pathological lesions appear among the organs of the immunized non challenged rabbits.

DISSCUSION

Bovine pneumonic pasteurelosis (shipping fever) is estimated to be the economically most important disease of feedlot cattle (Panciera and Corstvet, 1984).

P. multocida was isolated from nasopharyngeal swabs of both apparently healthy calves and respiratory diseased calves (6.25% and 10.47% respectively) as well as from heart blood, lung, associated lymph nodes and liver of the respiratory diseased and dead calves (10.47% and 66% respectively). These results come in agreement with Fayed et al., (2000) who isolated *P. multocida* from respiratory diseased and apparently normal calves with a percent of 8% and 6% respectively, and with Nakaya et al. (1998) recovered *P. multocida* from calves which had dead from respiratory disorder with an incidence of 64.3%.

The high prevalence of *P. multocida* in calves may be attributed to the lack of immunity. such animals and the degree of susceptibility of these calves as a result of concurrent infections by pathogens especially viruses and exposure to stress condition especially cold, overcrowding and malnutrition (Ajamal et al,. 1992 and Donkersgoed et al., 1993).

Meanwhile, the respiratory manifested calves showed depression, reduced appetite, nasal discharge, fever, cough and signs of dyspnea. These results coincided with Panciera and Corstvet (1984). The gross lesions showed highly congested, enlarged lungs with consolidation of some parts of it and enlarged pale liver, These lesions

come in accordance as previously shown Clarence et al. (1991).

Whereas the microscopic lesions revealed the chronic phase of the disease represented by fibrinous bronchopneumonia in lung with fibrosis adhesive pleuritis (Laila et al., 1996), edema and hyperplasia of the lymphocytic lymphoid follicles of the associated lymph node (Fadel, 2000) and liver fibrosis with vacuolar degenerative changes of the hepatocytes (Chevill and Rimler, 1989; Gourlary et al., 1989 and Clarence et al. 1991).

Otherwise, the dead calve suffered from the acute septicemic form of the disease which occurred in the outbreaks during the environmental stresses and to the immunosuppresed calves as it appeared in the microscopic lesions represented by severe heamorrhagic pneumonia with severe heamorrhagic pleuritis which may be the main cause for their death (Panciera and Corstvet, 1984). Moreover, the associated lymph node lesions represented by edema, depletion of lymphocytic lymphoid follicles accompanied with infiltration of few neutrophils were concided by Fadel (2000). While, the few inflammatory cells infiltration at the triad portal area of the liver with necrotic hepatocytes were partially agreement with (Glavits and Magyra, 1990) who found acute hepatitis with necrotic foci in liver.

The molecular weight (150 KDa) of purified toxin extracted from *P. multocida* isolated from calves was similar to that previously reported for rabbit isolates (Clearance et al., 1991). A number of biologic activities have been reported for *P. multocida* toxin including cytopathic effect in embryonic bovine lung cells (Chunter et al., 1986), dermonecrosis in guinea pigs (Nakai et al., 1984), lethality for mice after intrapertoneal injection (Foged et al., 1988).

When rabbits were exposed I/N to lethal dose of unheated toxin, resulted in the death of the rabbits within 2 days and the pathological changes observed in lung and their associated lymph nodes and liver were similar that appeared among the dead calves, these results proved that the purified toxin of *P. multocida* is responsible cause of the pathological alteration showed (Clearance et al., 1991).

Rabbit hyperimmune sera immunologically reacted with purified toxin. The previous results castin the light on the possibility of Western blot to be used as a diagnostic tool in pastrella infection. This result may be supported by conclusion of Sankow (2000) who stated that the purified toxin is an important virulence factor in isolates of *P. multocida* and immunization with this toxin stimulated the protective immunity against *P. multocida* challenge.

From the data illustrated in table 4, it can be observed that immunization with the toxin from the isolated *P. multocida* serotype B:2 is effective in preventing the clinical sings and deaths in rabbits. These observations were similar to that noted by Sanckow (2000) who suggested that the toxin induced the production of antitoxin which prevented induction of any respiratory manifestation but not colonization of in the nasopharynx

While, the mild lesions appeared in lung and as-

sociated lymph node of immunized challenged rabbits indicated that the used toxin dose stimulated the lung defense mechanism, these observations are in accordance with that reported by Fahmy et al. (1985) and Amina et al. (1996). Otherwise the lesions appeared in liver my be due to the effect of the adsorbed pasteurella endotoxin (Amina et al., 1996).

Moreover, the results obtained by using ELISA and IH tests for testing sera of rabbits at different time intervals after immunizations with toxin of *P. multocida* indicated that serum IgG activity against *P. multocida* purified toxin antigen was significantly elevated by day 21 after initial I/N immunization. This was 7 days after the second immunization on day 14, serum IgG activity remained elevated up to 40 day. These was the Meanwhile, the used dose of the toxin has improved the immunity status of the rabbits (Mukkar et al., 1982). Otherwise *P. multocida* was cultured only from nasopharnx of one of the six immunized and challenged rabbits, these finding indicate that immunization did not provide complete protection against colonization of nasopharynx (Ringler et al., 1985).

Also, the immunized rabbits and the non immunized non challenged (control) rabbits did not show any clinical sings, no pasteurell isolation or even pathological lesion of internal organs of them.

Our results clearly indicates that the purified toxin of *P. multocida* is an effective immunogen protecting rabbits against pasteurellosis.

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