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IMMUNOLOGICAL STUDIES ON PORIN PROTEINS OF PASTEURELLA HEAMOLYTICA IN GOAT

SAHAR R. MOHAMED AND HODA ABD EL-MONEM
Bacteriology Department. Biotechnology Department
Animal Health Research Institute, Doki, Giza, Egypt

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SUMMARY

This study was carried out on 120 goats, 45 of them suffering from pneumonia with its clinical symptoms, 55 of them were died and 20 were apparently healthy. The morbidity and mortality rates reached 22.5% and 27.5%, respectively.

A total of 25 nasal swabs were collected from pneumonic goat for isolation and identification of the causative agent. *P. haemolytica* was isolated pure in 44%. Other bacteria isolated coupled with *Staphylococcus aureus* in 24% and 32% Escherichia coli. The protein profiles of the purified bacteria was carried out using SDS polyacrylamide gel electrophoresis by comparing with molecular weight marker changed from 10 - 250 KDa. This study was conducted to characterize and I dentify the purity of major porin proteins in outer membrane with molecular weight of 38 - 42 kDa . Of pasteurella heamolytica using SDS-PAGE. In the active immunization assay, porin proteins 50µg conferred 100% protection to challenged mice. In addition crud extract give 70% protection when challenged mice. Immunization with porin proteins of *P.heamolytica* developed asignificant increase in serum antibody titre, as shown by enzyme linked immunasorbent assay., Stability assay , and opsonophagocytic - killing assay.

INTRODUCTION

Goats and sheep are very important source of meat and milk production in Egypt. Respiratory affection is a common problem of goat through economic losses and mortalities rate (Yehia, 2000). Pneumonia represents one of most important disease of goat and sheep. The main causes are bacteria and *Mycoplasma* (Soroor, 1999). Pasteurella (P) spp., Klebsiella, *Psuedomonas* spp. and *Staphylococcus aureus* are the most bacteria cause goat pneumonia (Martin, 1996 and Sayed,

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1996). *Pasteurella* spp. and *Staphylococcus* were described by Soroor (1999). Haematological and biochemical changes associated with pneumonia in goat were described by Yehia (2000). Sheikh et al. (1995) studied that pneumonia in sheep can be either chronic (*Mycoplasma* spp.) fibrinous (*Pasteurella* spp.)

Mannheima haemolytica is the most common bacterial isolate in pneumonic sheep and goat currently used vaccines against *M.haemolytica*. Do not provide complete protection against the disease. Research with *M.haemolytica* outer membrane proteins has shown that (Ayalew et al 2004). Outer membrane proteins in Gram negative bacteria possess known B.barrel three dimensional structures. These proteins including channel - forming trans membrane porins are diverse in sequence but exhibit common structural features. (Hari et al 2005). Porin proteins function serve as receptors for bacteriophages, and also appear to be targets of the immunological system.

The tightly bound nature of porin proteins in the presence of sodium dodocyl sulphate (SDS) has been described by (Choi et al 1989).

The aim of the present study was directed to:

a- Study isolation, identification of bacteria from diseased and apparently healthy goats.

b- Determine of protein profile of bacteria using SDS-PAGE by electrophoretic analysis and identified potential outer membrane porin proteins antigen of *P.haemolytica*.

c- Immunological studies on porin proteins.

MATERIALS AND METHODS

Bacterial isolation:

Nasal swabs were incubated into nutrient broth and incubated at 37°C for 24 h and then subcultered into 7% sheep blood agar and MacConkey's agar at 37°C for 24 - 48 h.

The surface of lung specimens were sterilized with a hot spatula, then with sterile platinum loop to be inoculated in previously mentioned media. The colonies were identified by their morphology, biochemical and pathogenicity to laboratory animal according to Baily and Scott (1974).

P. haemolytica isolated and serotyping by rapid plate agglutination test by Buchman and Gibon (1974). The colonies were cultured in brain heart infusion broth. Mortality were observed and LD50 was calculated according to Baily and Scott (1998).

Blood and sera samples:

About 10ml of blood aseptically collected from each diseased animal and apparently healthy , in sterile tubes. Sera samples were kept in sterile clean screw bottle at -20 until used.

Preparation of porin proteins and sonicated bacte-

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ria according to Hansan et al. (1989) and Choi (1991). *P. haemolytica* strain were grown at 37°C in medium containing per liter {20 gm of tryptose, 1gm of glucose, 5gm of Nacl and 2.5gm of yeast extract (ph. 7.4)}. The cells were harvested after 12h under gentle agitation. Then culture was centrifuged at 12000 xg for 15min at 4°C. The pellets were dispersed in sodium phosohat buffer (ph 7.4) and centrifugation for 15min at 265.000 xg at 15°C. Pellet dispersed in 2ml of 1% SDS tris puffer.

Sonicated bacteria: bacteria harvested in PBS after 2 washing in the same buffer. The cells were suspended in 10 mM Heps buffer pH 7.4 (Sigma) and sonicated at 8 u for 2 min, unbroken cells and debris were removed by centrifugation at 2000 rpm for 20 min.

Determination of proteins: Proteins was determined by method of Lowry et al (1951), serum albumin as a standard. Specific titration of porin with complex protein mixtures was performed by scanning in electrophoregrams.

SDS polyacrylamide gel electrophoresis: According to Choi et al (1989) and Pati et al. (1996). *P. haemolytica* outer membrane porin proteins and sonicated extract were electrophoresed using 10% SDS-PAGE under reducing conditions. The fractionate antigen were determined by staining with 5% Coomassi blue.

Mice for immunization: Chevalier et al 1993 (Three groups of mice 20-25 gm were used each group contain 20 mice). Porin proteins and sonicated antigen were used to immunize mice intraperitoneally along with Freunds complete adjuvant. On day 14 the same antigen with freunds incomplete adjuvant were injected mice I/p. On day 28 the same antigen were injected mice intravenously, blood serum were collected from immunizing mice on day 32.

Determination of serum antibody titres by ELISA: According to Ayalew et al. (2004). ELISA plates were coated with 10 µg porin proteins in 100 µ of coating buffer per ml remaining unbound sites in the well were saturated with 5% skim milk powder in phosphate buffered saline. Tween 20 incubated at 37°C for 1 hour. Serial two fold dilutions from 1 : 2 to 1 : 256, positive and negative control sera were included in each plate. 100 µl of peroxidase conjugated goat anti mouse IgG diluted 1 : 1000 was added to each well. Colour development was observed after adding a substrate solution containing orthophenylene diamine. Optical density (OD) reading were taken at 490 nm in Wellscan ELISA reader.

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Stability of porin proteins IgG.

Porin proteins was dminstered I/V to three rabbits (2-2.5 kg)Via a marginal ear vein atr a dose 10mg in 10ml of 0.9% seline. Blood specimens were collected from vain of the other ear at 1.2.3.5.8.24.48.96 h, After completion of the infusion, allowed to clot at room temperature for 1h and centrifuged. The rabbit sera were analyzed by ELISA. For mice 50µg of porin proteins was administered I/P in 0.5ml of saline. At 1.2.4.7 and 24 h after injection, 8 mice randomly selected and bled. The mice sera analyzed by ELISA.

Opsonophagocytic - killing assay :

Opsonic activity of anti porin IgG was determined by , three week old white rabbits were bled and prepared sera were pooled and stored at -70°C whole blood collected from healthy goats mixed with an equal volume of 3% dextran in 0.9% saline and allowed to stand at room temperature for 20 min . The upper lyer was collected and centrifuged at 250xg at 4 °C . The cell pellet was sus pended in ice cold 0.2% Nacl solution to lyse red blood cells , followed by addition an equal volume of 1.6% Nacl solution . Lysed red blood cell were , removed . The recovered cell were washed twice with Hank balanced salt solution. The assay mixture contained (anti body , bacterial cells , rabbit sera , PMN preparation) the reaction mixture was incubated with agitation at 37°C for 90 min . After the reaction an aliquot of the reaction mixture was removed , and spread on TSB agar plates and incubated at 37°C overnight , and the number of colonies grown was counted next day . Method described by (Pandher et al. 1998).

RESULTS

Clinical finding:

The pneumonic goat showed dullness, nasal discharge, congested mucous membrane, cough and anorexia.

Table (1) shows the prevalence of the morbidity and mortality rates in goats in which the morbidity rate reached 22.5%, mortality rate 27.5% and apparently healthy 20%.Table (2) shows that 45 nasopharyngeal swabs were positive for bacteriological examination with incidence 20% and 66.6% in apparently healthy and diseased goat respectively, while from 55 lung of dead animal 63.6% were positive for bacteriological examination.Table (3) shows 25 isolated of P. haemolytica were found with percentage 44% from nasal swabs, mixed with *E. coli* with percentage 32% and mixed with Staphylococcus at percentage 24% from nasal and pneumonic lungs. To evaluate the protective effect of the porin proteins and sonicated extract (anti porin IgG and anti sonicat-

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ed Antigeu IgG) against P.heamolytica infection in goat . We Carried out protection assay , Groups of mice were infected with porin and sonicated antigen , followed by lethal challenges with *P. heanolytice* . The result are shown in table (4) . Administration of 50 ug porin protins conferred protection all mice from *P.heamolytica* . Protection rate decreased to 70% at sonicated extract antigen administrated . anti body response of immunized mice to porin proteins reached (2.633 ± 0.970) and reached to (0.575 ± 0.202) when mice immunized with sonicated antigen. In table (5) antibody response p.heamolytica porin proteins and sonicated antigen in diseased and apparently healthy animal were ( ). From table (6) the stability of porin proteins antibody titer of rabbit and mice sera determined by ELISA found the cut of value reached to (90.5 ± 10.1) in immunized rabbit and reached to (19.9 ± 2.1) in immunized mice. To determine whether the IgG reains biological functions , assessed the ability of ant proin IgG to promote phagocytic killing of *P.heamolytica* as in table (7). Characterization of *P. haemolytica* porin proteins and sonicated extract by SDS-PAGE technique: SDS-PAGE analysis of porin proteins of *P. haemolytica* revealed three protein bands. The molecular weight of polypeptide bands estimated by comparison with standard MW markers (Rainbow TM) run in parallel were of 32 - 70 KDa in the sonicated extract (SE).

Table (1): Percentage of apparently healthy, morbidity and mortality rate in goat examined.

Total goat	Apparently healthy		Diseased animal		Dead animal	
	Number	Percentage	Number	Percentage	Number	Percentage
120	20	20%	452	2.5%	552	7.5%

Table (2): pneumonic cases detected by bacteriological examination of nasal and lung of goat.

Positive cases of nasal swabs				Positive causes	
Apparently healthy animal (20)		Diseased animal (45)		Dead goat (55)	
Positive case	Percentage	Positive case	Percentage	Positive case	Percentage
4	20%	30	66.6%	35	63.6%

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Table (3): Serotype of microorganism isolated from nasal swab and lung of living and diseased animals.

Type of MO isolated	Number of isolate bacteria	Number of isolate bacteria	Percentage
P. haemolytica	25	11	44%
P. haemolytica + E. coli	25	83	2%
P. haemolytica + Staphylococcus	25	62	4%

Table (4): Antibody response of immunized mice to porin proteins and sonicated antigen using ELISA:

Serum	Animal	Anti porin proteins response	Anti sonicated antigen respons
Preimmune	Mice	0.2 12 ± 0.156	0169 ± 0.57
Immune	Mice	2.633 ± 0.970	0.575 ± 0.202

Table (5): Antibody response of goat to P.heamolytica porin proteins and sonicated antigen using ELISA:

Antigen	Diseased animal (30)	Apparenty healthy (30)
Porin proteins	20/30 66 %	12/30 40 %
Sonicated antigen	11/30 37 %	7/30 23 %

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38- 42 KDa molecular mass region and 32 - 70 KDa, respectively.

Porin proteins Elisa detected the presence of Pasteurella antibodies in the serum of immunizing mice and in the serum of pneumonic goat.

The cut off value was (2.633 ± 0.970) in case anti porin protein respons and (0.575±0.202) in anti sonicated antigen response as in table (4), while porin proteins and sonicated antigen ELISA results detected the presence of *P.heamolytica* antibodies in the serum of pneumonic goat 66 % of animals (20 out of 30), 37% (11 out of 30) in sonicated antigen. In apparently healthy goat, ELISA detected the presence of P.heamolytica antibodies in their serum 40% of animals (12 out of 30), 23 % (7 out of 30) as in table (5). The present data indicate the immunogeicity of the protein should be evaluated and considered that porin proteins of *P.heamolytica* have been demonstrated to form water filled channels which allow the diffusion of low molecular mass solutes and to serve as receptor sites for the binding of phages and bactriocins (Davies and Lee 2004, Paupit et al 1991). The prophylactic efficacy of porin protein IgG against *P.heamolytica* infiction evaluated in the mice , indicate that prepation is far mor effective thran the sonicated extract . Antigan preparation , considering that porin IgG antibody level was 50 times higher than the sonicated extract antigen, this difference could be explained by the observation that the titer of anti porin IgG against *P.heamolytica* , which is in good agreement with the result . (Lubke, et al 1994).

Porin proteins administered to rabbit by I/V infusion , was detected in the rabbit sera for 7 days after the infusion, and antibody was detected in the mice sera for 24h after the infusion. Which is similar to the result reported (Li et al 2004), also normal serum IgG showed some opsonic activity and reduced the number of surviving bacteria in the reaction mixture by 57% . Addition of anti porin proteins IgG Further enhanced the phagocytosis of *P.heamolytica* and the number of surviving organism in the reaction mixture decreased by 95%. This result demonstrated that the anti porin IgG possesses opsonphagocytic killing capacity . yet out of this result we could conclude that *P.heamolytica* porin proteins can play an essential role in the induction of immune response in the animal and can be employed as an effective vaccine which can confer solid and active immunity.

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Table (6):Stability of porin proteins antibody titers of rabbits and mice sera were determined by ELISA:

Animal	Porin proteins	Antibody level	
		Minimum Cut off value	Maximum Cut off value
Rabbits	I/V 1mg/ml	30.4 ± 4.6	90.5 ± 10.1
Mice	I/P 50μg/ml	10.8 ± 5.1	19.9 ± 2.1

Table (7) : Opsonophagocytic-killing Capacity of goat anti porin protens IgG antibody.

Antybody	No. of colonies (mean + S.D)	Phagocytosis (%)
Normal serum IgG	146 ± 17	57
Anti - porin proteins	18 ± 7	95

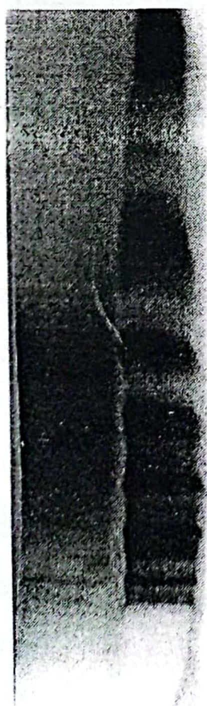

Fig (1): Comassic blue stained SDS - PAGE (10% gel electrophoresis) profile of porin proteins and sonicated antigen of *P.heamolytica*. The MW marker low moloculer weight. The row head indicated-orin proteins (38-42 KDa).

DISCUSSION

Respiratory diseases constitute one of the major causes of morbidity and mortality in goats and sheep (Sayed, 1996).

Bacteriological examination of pneumonia revealed that the predominant isolated, the results were in agreement with Hyziroglu et al. (1997).

The results recorded in table 1 revealed that morbidity and mortality rates were 22.5 and 27.5%, respectively. Similar results were obtained by Talab (2002). In the present study, it was evident that *P. haemolytica* with different serotypes in goats associated with *Staphylococcus aureus*, *E. coli* were responsible for severe bronchopneumonia. This observation was in accordance with that mentioned by Foldar et al. (1984) who found that *Pasteurella* spp. occurred in either single or mixed infection in sheep.

The microbiological examination in table (2&3) revealed that *P. haemolytica* was isolated alone in 44%. *P. haemolytica* was mixed with Staphylococcus aureus with an incidence of 24% by nasal swabs and similar results were obtained by Niang et al. (1997), while it was coupled with *E. coli* 32%. These results agreed with those mentioned by Zaitoun (2001) who noticed that *E. coli* was associated with respiratory disorder.

Porin proteins are major proteins found in the outer membrane of gram negative bacteria. Where they found channels for the nonspecific permeation of small solutes (Wesis et al 1991). *P.heamolytica* gram negative opportunistic pathogen contains a major specied of porin, under most growth condition it forms substantially large pores, approximately $2X10^5$ porin molecules in the outer membrane begin apparently function at any given time (Lubk et al 1994).

Electrophoretic separation of *P. haemolytica* porin proteins in polyacrylamide gel in the presence of SDS was used for qualitative analysis of protein as a method for molecular weight determination. The protein was judged by visual evaluation by Coomassie blue stain (R-250) and by comparing its electrophoretic motilities with standard rainbow molecular weight marker of fig. 1, which ranged from the top to the bottom 250 to 10 KDa. The proteins were fractionated into different bands according to their molecular weights.

This current study was undertaken to evaluate the immunogenic properties of porin proteins of *P. haemolytica* by using SDS-PAGE.

SDS-PAGE analysis of OM Porin proteins sonceted and extrat E of *P. haemolytica* have permitted the identification of protein components in

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