BS. VET. MED. J. 6TH SCI. CONF. VOL. 20, NO.1, P.374-378

Beni-Suef Veterinary Medical Journal

Biomolecular relationship of whole protein of Mannheimia haemolytica, Pasteurella trehalosi and Pasteurella multocida of sheep

Amal M. El-Sawah[1*], Eman M. El-Rawy[2]

[1]*Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo and*[2] *Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo*

P. multocida contains one or more antigenic determinant of different proportions responsible for partial protection offered by the heterologous serovars. SDS-PAGE analysis of the whole protein profile prepared from sheep local isolates of *P. multocida* types (A, D), *Mannheimia haemolytica* type (A), *P. trehalosi* type (T) as well as standard strain of *P. multocida* (B6) revealed that the protein profile exhibited some differences with variable molecular masses ranged between 14 to 116 kDa. There are sharing protein subunits of molecular masses of 66, 37 and 28 kDa as the unique cross-reactive antigens in all isolates. The protection percentage for the vaccinated mice with inactivated *M. haemolytica* against the challenge with virulent strain of *P. multocida* type A, D and B6 are 30%, 30% and 20% respectively and 25%, 25% and 20% respectively in the groups of mice vaccinated with inactivated *P. trehalosi* so, there is cross reaction but limited cross protection between pasteurella isolates. The suggested vaccine must contain local isolates of *P. multocida* serotypes A, D and B6 as well as *M. haemolytica* and *P. trehalosi*.

Pasteurellosis is one of the most important diseases of sheep and goats throughout the world (Zamri Saad *et al.*, 1987). Both *Pasteurella haemolytica* and *Pasteurella multocida* have been incriminated with septicaemic and pneumonic pasteurellosis (Gilmour, 1980). *Pasteurella multocida* is a heterogenous bacterial pathogen infecting domestic and wild animals. The heterogenicity of this bacterium is most evident from the fact that distinct capsular serotypes (A, B, D, E and F) occur within the species (Rimler and Rhoades, 1989). *Pasteurella haemolytica* was more frequently incriminated with pneumonic pasteurellosis than *P. multocida*. The isolated *P. multocida* showed considerable serological heterogenicity between the isolates of the types D, A and B (Shigidi and Mustafa, 1980). The agglutinagens of *P. multocida* contain one or more antigenic determinants of different proportions responsible for cross reaction (Rebecca *et al.*, 1995), there were partial cross protection afforded by the heterologous serovars. Alaa *et al.* (2004) stated that the antigenic extracts from a local isolate of *M. haemolytica* (A2) are effective potential candidates for serodiagnosis of wide serovars of *M. haemolytica* infection in livestock animals. A37 kDa antigenic determination was *M. haemolytica* (A2) specific immunoreactive protein subunit in its OMP extracts. The most unique cross-reacting antigens in OMP of P. trehalosi (T4) and *P. multocida* (B6) versus polyclonal anti-*M. haemolytica* A2 antiserum were at 66, 44, 43, 41 kDa that could be potential pasteurella related surface antigens for stimulating cross protecting immunity. These antigens are probably important resistance related antigens that should be considered a construction of subunit and/or adjuvant vaccine. Apparently, resistance to pasteurellosis is mediated by multiple antigenic determinants and therefore a combination of more than one serotype-specific antigen is needed for complete protection.

The present study was conducted to demonstrate biomolecular and cross reactivity relationship of the whole protein of *Mannheimia haemolytica*, *Pasteurella trehalosi* and *Pasteurella multocida* isolated from sheep.

Materials and methods

Bacterial isolates and culture. Sheep local isolate of *M. haemolytica* serotype A, P. trehalosi serotype (T) and *P. multocida* serotypes (A and D) were grown in brain heart infusion (BHI) or RPM1 media as needed and incubated aerobically overnight at 37°C. All bacterial isolates have been subjected to phenotypic, biomolecular and serological characterization.

Standard strain. A vaccinal strain of P. multocida serotype B6 was grown in similar culture conditions that used for growing of the local isolates. The patterns of these isolates were

* Corresponding author. Tel.: +202 3422505;
Fax: +202 3449204
E-mail address: clevb@tedata.net.eg
(Amal M. El-Sawah)

analyzed in SDS-PAGE for the whole membrane protein.

Sodium Dodecyl sulphate polyacrylamide gel eletrophoresis analysis (SDS-PAGE). It was used according to the method of (Johnson *et al.*, 1991). Protein marker mixtures of different molecular weight of concentration of 1 mg/ml were used in polyacrylamide gel electrophoresis SDS. Its molecular weight ranging from 14-116 kDa Cat. No. P.S.101 (Gibco BRL).

Rabbits. Six Boscat rabbit weighing 2-3 kg were used for passage of *P. multocida* strains.

Mice. A total of 180 Swiss Albino mice weighing about 18-20 g were used for the cross protection test. They were obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Bacterial Culture. Two local isolates of *P. multocida* serotypes (A and D) and one standard strain of *P. multocida* serotype B6 were inoculated S/C in Boscat rabbit. Reisolation of these strains from dead rabbits was done using sheep blood agar medium.

Biochemical identification. The suspected colonies of all serotypes were confirmed morphologically, culturally and biochemically by using API 20E according to (Quinn *et al.*, 2002).

Serotyping of the isolates. Pure colonies of *P. multocida, M. haemolytica* and *P. trehalosi* were serotyped by IHA using standard specific antisera according to (Davies *et al.*, 1997).

Preparation of whole protein membrane (Bacterial lysate). The whole bacterial cells of *P. multocida* serotypes A, D and B6, *M. haemolytica* type A and *P. trehalosi* type (T) were harvested from cultures grown overnight at 37°C onto 7% sheep blood agar. The harvested cultures were washed twice in sterile normal saline. The bacterial cell pellets were treated with lysis buffer producing cell lysate according to (Broden and Rimler, 1982). Equal volume of Pasteurella isolates and 2x sample buffer (10% glycerol, 4% SDS, 0.02 % bromophenol blue + 10% B. mercapto, 0.125 M Tris HCl, pH 6.8) were mixed and placed in boiling water bath for 5 minutes according to (Davies *et al.*, 1997).

Electrophoresis. The bacterial antigens were separated by discontinuous SDS-PAGE to obtain electrophoretic profiles of *P. multocida* and *M. haemolytica* isolates as described by (Johnson *et al.*, 1991).

Vaccination of mice. A total of 180 albino mice about 18-22 g/weight were divided into 3 groups (60 mice for each): The first group was vaccinated with inactivated oil adjuvant *M. haemolytica* type (A). The second group was vaccinated with *P. trehalosi* type (T). The third group was kept as a control non-vaccinated. The vaccinated mice were inoculated with 0.2 ml S/C in 2 doses with an interval of 15 days. The vaccinated and control groups were challenged with 0.1 ml S/C of virulent strains of *P. multocida* after 21 days post the booster vaccination.

Each vaccinated group was subdivided into 3 subgroups and challenged 21 days post the second dose with 0.1 ml S/C of virulent inoculum of P. multocida type A, D and B6 separately according to Ose and Muenster, (1986). The control groups received the same dilution of each challenged strains. Readings were recorded for 7 days.

Results and Discussion

Mannheimia (Pasteurella) haemolytica and other pasteurella species (*P. trehalosi* and *P. multocida*) are Gram -ve bacteria responsible for economically important respiratory tract infections of cattle and sheep known as pneumonic pasteurellosis (Davies *et al.*, 2002). Although the overall pathogens of bovine and ovine pneumonic pasteurellosis are very similar (Gilmour and Gilmour, 1989). *M. haemolytica* consists of genetically distinct subpopulation that are differentially adapted to elicit disease in either cattle or sheep (Davies *et al.*, 1997).

Local isolates of *M. haemolytica* serotype (A), *P. trehalosi* (T), *P. multocida* serotypes (A, D) and standard strain of *P. multocida* (B6) in the present study were characterized morphologically, culturally, biochemically, serologically and molecularly as in Tables (1 ,2).

Electrophoresis of the whole protein extracts, prepared from local isolates and standard strain of *M. haemolytica*, pasteurella species were exhibited some differences in their electrophoretic patterns as in Fig. 1, 2 and Table (3). The whole protein profile of local isolates of *P. multocida* type A and D, standard strain of *P. multocida* type (B6) as well as *M. haemolytica* type A and P. trehalosi (namely 1, 2, 3, 4, 5, 6) showed protein subunits of variable molecular masses ranged between 14 and 116 kDa. These results agreed with (Punchalski *et al.*, 2002) who found that the presence of about 11 to 20 protein subunits with molecular masses ranged between 16 and 109 kDa in OMP (outer membrane protein) of all serotypes of *M. haemolytica* and P. trehalosi. The data in Fig. (1 and 2) revealed that there are sharing protein subunits of

molecular masses of 66, 37.6 and 28.5 kDa in all isolates of Pasteurellae. These findings are supported by (Alaa *et al.,* 2004) who found a considerable similarity of approximately (69%) between *M. haemolytica* and *P. trehalosi.* Comparatively OMP profile of *P. multocida* (B6) showed slight similarity with that of *M. haemolytica* and *P. trehalosi* confined to protein subunits at molecular masses of about 66, 44, 41 and 35 kDa. The data illustrated in (Table 2, Fig. 1, 2) for the protein profile of local isolates of *P. multocida* type A, D and standard strain of serotype B6 revealed that all isolates of *P. multocida* have similar protein profile with little additional protein as 56, 33 kDa in *P. multocida* type D, 51, 16 kDa in P. multocida type A and the unique protein extra antigens as 53, 35, 33 kDa in *P. multocida* type B6. These findings were in agreement with (Eman *et al.,* 1998) who stated that the electrophoretic pattern of the overall protein pattern of *P. multocida* strains of cattle origin were visually similar, but there were some substantial difference between individual strains. The most majority of protein bands were located between 13-99 kDa molecular weights range. The minor protein of low amount of apparent molecular masses 17, 18, 31, 34 kDa appeared to be shared by all *P. multocida* field strains as well as standard type B6 strain and the minor protein band ranging from 20-35 kDa. The whole protein extracted from local isolate of *M. haemolytica* type (A) and *P. trehalosi* type (T) exhibited similar profiles with limited variation. The prominent proteins are 66, 37, 28 kDa and a faint protein at 87 kDa. *M. haemolytica* showed additional protein at molecular weight of 116 kDa. *P. trehalosi* showed some protein variation in the protein bands of molecular weights 47, 41, 14 kDa and these data agree with Puchalski *et al.,* 2002) who stated that the sharing protein at 44 kDa was responsible for cross protection of *P. trehalosi* (the intensity of reaction of anti-haemolytic serotype A1 with heterologic serum was stronger compared to the haemologic reaction for protein obtained from the non-typical strain (N2) localized in 16-31, 32, 44, 45, 72 and 100 kDa. Bosch *et al.,* (2002) stated that the correlation between electrophoretic pattern and serotypic properties of isolates were established but no single protein band could be identified as unique to all strains that caused haemorrhagic septicaemia. OMP (outer membrane protein) bands at (27, 34, 36 kDa) were common to all isolates regardless to serotype. One of the major virulence protein in OMP was haemoglobin binding protein which was considered as specific receptor for hemoglobin. The findings in Table (4) revealed that there was a weak cross protection for the group of mice vaccinated with inactivated *M. haemolytica* type (A) against the challenge with virulent strains of *P. multocida,* the protection percentage were 30%, 30% and 20% against *P. multocida* type A, D and B6 respectively with the overall of protection percentage of 30%. In case of group of mice vaccinated with inactivated *P. trehalosi* (T) and challenged with the virulent strains of *P. multocida* serotypes A, D and B6, the protection percentage was 25% against the challenge with the virulent strains of *P. multocida* type (A, D) and 20% against the challenge with *P. multocida* type B6 with overall of protection. The control group of non-vaccinated mice showed a 100%, 86, and 90% mortality due to inoculation with virulent strains of *P. multocida* type A, D and B6 respectively with an overall mortality of 95%. These results agreed with those obtained by (Eman *et al.,* 2001) who stated that there is a cross reaction but limited cross protection between the isolates of *P. multocida, M. haemolytica* and *P. trehalosi.* In conclusion, the evolutionary relationship among *M. haemolytica* and *P. trehalosi* isolates indicates that the ancestral host is the sheep and several distinct colonal lineages have crossed the species barrier into cattle heterologous serovars and the agglutinogens of *P. multocida* contain one or more antigenic determinant of different properties are responsible for cross reaction between *P. multocida* isolates.

References

Alaa, A. E.; Eman, M. E.; Manal S. M. and Abd El-Salam, Z. H. (2004): Molecular characterization of *Mannheimia haemolytica* extracts and utility in serodiagnosis of pasteurellosis in Egypt. J. Egypt. Vet. Med. Assoc., 64 (3): 321-343.

Bosch, M.; Garrido, M. E.; Perez DeRozas, A. M.; Badiola, I. and Barbe, J. (2002): Characterization of *P. multocida* hgba gene encoding haemoglobin binding protein. Infect. Immun., 70 (11): 5955-5964.

Brogden, K. A. and Rimler, R. B. (1982): Lysate of turkey-grown *P. multocida* and examination of vaccine preparations by electron microscopy. Am. J. Vet. Res., 43 (2): 304-309.

Davies, L. R.; Susan, C. and Thomas S. W. (2002): Mosaic structure and molecular evolution of the leukotoxin operan in *Mannheimia (Pasteurella) haemolytica, Mannheimia glucosidal* and *Pasteurella trehalosi.* J. Bacteriol., 184 (1): 266-277.

Davies, R. L.; Arkinsaw, S. and Selander, R. K. (1997): Evolutionary genetics of *Pasteurella haemolytica* isolates recovered from cattle and sheep. Infect. Immun., 65: 3583-3593.

Table (1): Biochemical properties of ovine pasteurella according to Quinn *et al.*, (2002).

Fermentable substrates	*P. multocida*			*M. haemolytica*	*P. trehalosi*
	Serotype D	Serotype A	Serotype B	(A)	(T)
Haemolysis onto blood agar	-	-	-	+	+
Growth on MacConkey	-	-	-	+	+
Indole test	+	+	+	-	-
Vogus Proskaur	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-
Citrate utilization	-	-	-	-	-
Catalase test	+	+	+	+	+
Oxidase test	+	+	+	+	+
Sugar fermentation					
Salicin	-	-	-	-	+
Inulin	-	-	-	-	-
Lactose	-	-	-	+	-
Mannose	-	-	-	-	+
Sucrose	+	+	+	+	-
Trehalose	-	+	-	-	+
Arabinose	-	-	-	+	-
Xylose	-	+	-	+	-

Table (2): Serotyping of ovine pasteurellae.

	Pasteurella species		
	P. multocida	*P. haemolytica*	
Serotypes	A D B6	*M. haemolytica* type A	*P. trehalosi* type T

Table (3): SDS-PAGE analysis of the major subunits of whole protein extracted from type D, A, B6, *M. haemolytica* type A and *P. trehalosi*.

Bacterial isolate	Approximate subunit molecular masses in kilodalton kDa SDS-PAGE	
	Prominent	Faint
P. multocida type D	66, 56, 37.6, 35	87, 33, 28.5, 18.4
P multocida type A	66, 51, 37.6, 42, 28.5, 18.4	116, 87, 16
P. multocida type B6	66, 42, 37, 35, 28.5, 18.4, 14.6	116, 87, 53, 33
M. haemolytica type A	116, 66, 37.6, 33, 28.5, 18.4	87, 31
P. trehalosi	66, 47, 41, 37, 28, 25	116, 87, 18, 14

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

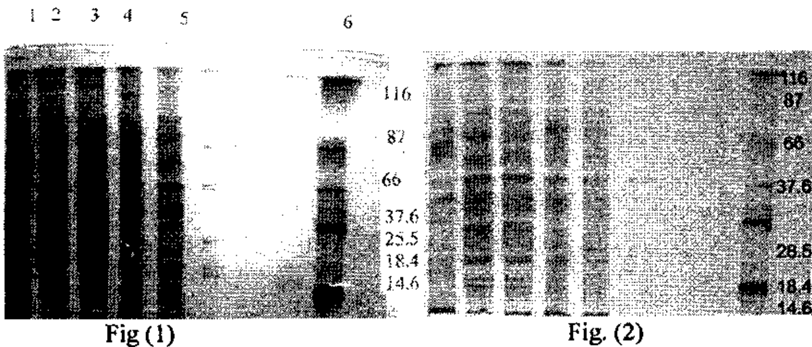

Fig (1) Fig. (2)

Fig. (1 and 2): SDS-PAGE analysis of whole protein extracts received from local isolates of ovine pasteurella separated through 2% denaturating polyacrylamide gel and stained with 0.1 % bromophenol blue, 10 % glycerol. Lane (1): *P. multocida* type (D), Lane (2): *P. multocida* type (A), Lane (3): *P. multocida* type (B), Lane (4): *M. haemolytica* type (A), Lane (5): *P. trehalosi*, Lane (6): Marker with molecular mass standard 14-116 kDa.

Table (4): Determination of the cross protection for mice vaccinated with oil adjuvanted *M. haemolytica* and *P. trehalosi* against challenge with virulent strains of *P. multocida*.

Vaccinated Groups	No. of mice	Types of challenging serotypes						Total No. of survival	Overall protection %
		P. multocida type A	P1 %	*P. multocida* type D	P2 %	*P. multocida* type B6	P3 %		
Group (1)	60	6/20	30 %	6/20	30 %	4/20	20 %	16/60	30 %
Group (2)	60	5/20	25 %	5/20	25 %	4/20	20 %	16/60	23 %
Control Group	60	0/20	0 %	1/20	2 %	2/20	10 %	3/60	5 %

Group (1): Vaccinated with oil adjuvanted *M. haemolytica* in 2 doses of 0.2 ml S/C
Group (2): Vaccinated with oil adjuvanted *P. trehalosi* in 2 doses of 0.2 ml S/C
P1 %: The No. of survived vaccinated against the challenge with virulent strain of P. multocida type A / total No. of vaccinated mice within the same group.
P2 %: The No. of survived vaccinated mice against the challenge with virulent strain of P. multocida type D / total No. of vaccinated mice within the same group
P3 %: The No. of survived vaccinated mice against the challenge with virulent strain of P. multocida type B6 / total No. of vaccinated mice within the same group

Eman M. E.; Susan F. G.; Gergism S. M. and Hussein, A. Z. (2001): Evaluation of the local prepared vaccine for protection against caprine pasteurellosis. J. Egypt. Vet. Med. Assoc., 61 (6C): 89-102.

Eman, S.A. Zaki; Abd Ekl-Ghani, M. and Gergis, S.M. (1998): Immunological studies on P. multocida in cattle. A Thesis of MD, Cairo Univ., Egypt.

Gilmour, N. J. L. (1980): Veterinary Annual (20): 234-235 Cited in Zamri M. S.; Sharif, H. and Basri, K. (1989): Microbiological and pathological evaluation of vaccination against naturally occurring caprine pasteurellosis. Vet. Rec., 124: 171-172.

Gilmour, N. J. L. and Gilmour, J. S. (1989): Pasteurellosis of sheep. P. 223-261. In C.F. Adlam and J.M. Rutter (ed). Pasteurella and Pasteurellosis. Academic Press, London, England.

Johnson, R. B.; Dawkins, H. J. S. and Spencer, T. L. (1991): Electrophoretic profiles of pasteurella isolates from animals with haemorrhagic septicaemia A. Am. Vet. Res., 52 (10): 1644-1648.

Ose, M. S. and Muenster, B. S. (1986): A method for evaluation of vaccines containing *Pasteurella multocida*. Am. J. Vet. Res., 29 (9): 1863-1866.

Puchalski, A.; Wernicki, A.; Mikucki, P. and Urban-Chemiel, R. (2002): Profiles of outer membrane proteins from *M. haemolytica, M. glucosida, P. trehalosi* and non-typical strains. J. Bacteriol., 184 (1): 266-277.

Quinn, P. J.; Markey, B. K.; Carter, M. E.; Donnelly, N. J. C. and Leonard, F. C. (2002): Veterinary Microbiology and Microbial Disease. Ch. 22. Pasteurella species and Mannheimia haemolytica, pp. 137-143 Blackwell Science, MPG Books LTD, Bodmin, Carnwall, England.

Rebecca, J. M.; Paniciera, J.; Rober, W.; Fulton, Glynu, H. F.; Sidney A. E.; John T. H. and Anthony W. C. (1995): Vaccination of cattle with outer membrane protein enriched fraction of *P. haemolytica* and resistance against experimental challenge exposure. Am. J. Vet. Res., 56 (1): 875-879.

Rimiler, R. B. and Rhoades, K. R. (1989): In Pasteurella and Pasteurellosis, New York, Academic Press, pp. 37.

Shigidi, M. T. A. and Mustafa, A. A. (1980): Serological relationship between strains of P. multocida. Cornell Vet., 70: 27-36.

Zamri, S. M.; Sheikh Omar, A. R.; Chaoi, K. F. and Chulan, U. (1987): Isolation of *P. haemolytica* from the nasal cavity of goats perianika, 10: 246-247.

المقارنة الجزينية للبروتين الكلى لعترات مانيهماهيموليتكا والباستريلا تريهالوزى والباستريلا مالتوسيدا للأغنام

يحتوى ميكروب الباستريلا مالتوسيدا على العديد من المحددات الأنتيجينة بنسب مختلفة وهى المحددات هى المسئولة عن الحماية الجزئية ضد الأنواع السيرولوجية متعددة التجانس وذلك عن طريق اختبار الفصل البلمرى الكهربائى للبروتينات الحقلية الباستريلا مالتوسيدا أنواع (A) ، (D) والمانهيماهيموليتكا النوع (A) والباستريلا تريهلوزى النوع (T) والعترة القياسية لميكروب الباستريلا مالتوسيدا (B_6) فى الأغنام وقد نتج عن هذا الإختبار أن بروتين البروفين لهذه العترات جميعها تتراوح الكتل الجزيئية له ما بين ١٤ : ١١٦ كيلو دالتون. وقد وجدت وحدات بروتينية مشتركة لجميع العترات التى لها الوزن الجزيئى التالى ٦٦ ، ٣٧ ، ٢٨ كيلو دالتون وتعتبر هى المسئولة عن التفاعلات المتبادلة بين هذه العترات ، أما عن نسبة الحماية للفئران المحصنة باللقاح المثبط لميكروب المانهيماهيموليتكا ضد أختبار التحدى المناعى بالعترات الضارية لميكروب الباستريلا مالتوسيدا أنوع (A) ، (D) ، (B_6) فهى على التوالى ٣٠% ، ٣٠% ، ٢٠% أما عن الفئران المحصنة باللقاح المثبط لميكروب الباستريلا تريهالوزى ضد اختبار التحدى المناعى بالعترات الضارية لميكروب الباستريلا أنوع (A) ، (D) ، (B_6) فهى على التوالى ٢٥% ، ٢٥% ، ٢٠% ، وقد أثبتت هذه الدراسة وجود تفاعلات متبادلة ولكنها تحدث حماية محدودة بين العترات المختلفة لميكروب الباستريلا فى الأغنام ولذلك يعتبر اللقاح المثالى هو الذى يحتوى على عترات الباستريلا مالتوسيدا أنوع (A) ، (D) ، (B_6) والمانهيماهيموليتكا (A) والباستريلا تريهالوزى (T).