Mannheimia haemolytica and *Pasteurella multocida* Pneumonia in Sheep and Goats in Fayoum Governorate : The Use of ELISA Technique

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Summary

A total of 299 cases of small ruminant animals (193 sheep and 106 goats) from different localities of Fayoum governorate were subjected to clinical examination for respiratory manifestations. Nasopharyngeal swabs and blood samples were separately collected from all studied animals. Also, a total of 138 lung tissues samples were collected from 94 sheep and 44 goats from different abattoirs. These samples consisted of 68 and 32 lungs from sheep and goats respectively showed pneumonia manifestation. The rest samples (26 and 12 respectively) were from normal healthy lungs. Swabs and lung samples were subjected for bacteriological examination and The **Enzyme linked immunosorbent assay** (ELISA) test was carried out on sera samples. Bacteriological examination revealed the isolation of M. *haemolytica* and *P. multocida* either in pure culture or mixed with other different bacteria such as *E. coli*, *Kl. pneumonae*, *P. aerogenosa* and *S. aureus*. *M. haemolytica* and *P. mltocida* was recovered in a percentage of 20.6 % and 21.3 % from studied sheep and goats respectively. The recovery rate from diseased cases was significantly higher than the apparent healthy cases, almost double the incidence. The *M. haemolytica* capsular A and T antigens, and the somatic antigens type A: 2 T: 4; T: 10 and T: 15 were detected with percentages of 58.3, 41.7, 52.8, 25.0, 16.7 and 5.5 respectively. The highest incidence was recorded with capsular antigen A (58.3 %) and somatic antigen type A: 2 (52.8 %) in both sheep and goats. *Pasteurella multocida* isolates showed capsular antigen type A (70.9%) and type D (21.8%), but 4 isolates (7.3%) were untyped. The somatic antigen of the typed strains were identified as A:2 , A:5, D:1, D:3 and D:4 with a percentage of 49.0, 17.7, 19.6, 9.5 and 3.9 respectively. The highest incidence was reported with capsular antigen A (70.9 %) and somatic antigen type A: 2 (49.0 8%) in both sheep and goats. It is worthy to denote that the 2 strains identified as D: 4 were goat's isolates. From our results, it could be concluded that identical Pasteurella strains were shared by the goats and sheep.

The recovery rate for ELISA was found to be 95.5 % and 94.7% for *M. haemolytica* and that *Pasteurella multocida* respectively, which considered highly significant. The ELISA technique as a "culture-independent methods" was proved to be easy, practical, sensitive and efficient method for quick diagnosis of *M. haemolytica* and *Pasteurella multocida* infection in sheep and goats.

Introduction

Wild and domestic animal populations are known to be sources and reservoirs of emerging diseases (27). In ruminants, respiratory disease is multifactorial and a leading cause of morbidity and mortality. Respiratory disease is a complex syndrome involving stress factors, parasitic, fungal, bacterial and viral infections (35).

Pneumonia is one of the most common respiratory problems in small ruminants throughout the world. Pneumonia occurs when infectious and non-infectious agents cause the lungs to become inflamed. Bacterial pneumonia is generally regard as the most frequent and serious cause of morbidity, mortality and economic losses associated with respiratory diseases in sheep and goats (32).

The family Pasteurellaceae "Pohl" contains Gram-negative, facultatively anaerobic and fermentative bacteria of the genera Pasteurella, Haemophilus, and Actinobacillus. Approximately 20 different species of the genus Pasteurella have been identified using phenotypic and genetic analyses. Of these species, *P. multocida* and *P. haemolytica* (now called *Mannheimia haemolytica*) are the most prominent pathogens in domestic animals causing severe diseases and major economic losses in the cattle, swine, sheep, goats and poultry (9)

Members of the Pasteurellaceae cause an array of deadly illnesses including bacterial pneumonia known as "pasteurellosis", a particularly devastating disease for sheep and goats. Cross-transmission rates of Pasteurellaceae bacteria in populations of domestic sheep and domestic goats were studied (27).

P. multocida and *M. haemolytica* are commonly found in the upper respiratory tract of healthy sheep and goats (32). *M. haemolytica* and *Pasteurella multocida* were the most important bacterial *causes* of pneumonia in sheep and goats (22). Pasteurella spp. was isolated from goats and free-ranging sheep from a bordering area at Idaho, Oregon, and Washington (USA). *Pasteurella haemolytica* (now called *Mannheimia haemolytica*) organisms were isolated from one goat and one of two sheep found in close association (40). *Pasteurella multocida* is a highly diverse group of bacteria recognized as important pathogens (43)

Sheep respiratory infections appear as differing clinical syndromes, mild, acute and chronic infections. A mild but chronic respiratory problem in lambs under 1 year old is thought to be caused by different microbial agents probably in association with Pasteurella . Acute bacterial pneumonia usually results from infection with Pasteurella of biotype A. (34). In goat herds, pneumonia increases production costs associated with expensive treatments. Although pneumonia often occurs in kids, illness and deaths also occur in adult animals (32).

This study was designed to clarify the role played by both pathogens *Mannheimia haemolytica* and *Pasteurella multocida* in sheep and goats population (diseased, apparent healthy and slaughtered) in Fayoum governorate and to carry out serological identification for the isolated strains. Also to apply the "Enzyme linked immunosorbent assay" (ELISA) test on sheep and goats serum samples to compare and evaluate results with that of the conventional culture method, aiming the probability of its application as quick diagnostic tools.

Material and Methods

A total of 299 cases of small ruminant animals (193 sheep and 106 goats) from different localities of Fayoum governorate were subjected to clinical examination for respiratory manifestations such as nasal discharge, cough or lung sound and mainly elevated body temperature.

Nasopharyngeal samples were collected by means of sterile cotton swabs from all sheep and goats cases. Blood samples were also separately collected from all studied animals (193 sheep and 106 goats) using sterile vacutainer tubes without anticoagulant. Each sample was labeled with animal number (or identification characters) animal species and date of sample collection.

Also, a total of 138 lung tissues samples were collected from different abattoirs at Fayoum governorate. These samples were obtained from 94 sheep and 44 goats cases, out of which 68 and 32 lungs from sheep

and goats respectively showed pneumonia manifestation. The rest samples (26 and 12 respectively) were from normal healthy lungs.
All samples were quickly transferred, using ice box, to the laboratory for bacteriological and serological examinations.

In the laboratory, swabs were firstly wetted in sterile peptone water. Swabs and loopfull from the deep area of each lung sample (after sterilizing the surface) were subjected for bacteriological examination by inoculating plates of nutrient agar, Mc Conkey's agar , 5 % sheep blood agar , brain heart infusion agar supplemented with 10 % citrated sheep blood and tryptose agar . All plates were incubated at 37 c for 24 h. – 48 h. The resultant separated colonies were purified and identified morphologically and biochemically according to (14), (10), (30) and (29).
The blood samples were centrifuged to separate the serum which was then stored at -20 °C to be used for ELISA test.

The isolated and identified strains of *Mannheimia haemolytica* and *Pasteurella mutocida* were subjected for more characterization. Full biotyping of *Mannheimia haemolytic* ***was*** carried out (5) and serotyping using the rapid plate agglutination test (23) was employed. Capsular typing of P. *multocida strains* was carried out using the indirect haemagglutination test (6) and somatic antigen typing by gell diffusion precipitin test (25).

The **Enzyme linked immunosorbent assay** (ELISA) test was carried out according to (42) (26) and (21) using flat bottom wells plates which were coated with 100 µl of either *P. multocida* or *M. haemolytic* antigens (20 µg soluble antigen in carbonate buffer pH 9.6, each). Plates were incubated overnight at room temperature. Blocking was performed with 0.1% bovine serum albumin (BSA), then 100 µl of the collected sheep and goats sera, diluted at 1:100 in PBS were added and the plates were kept for 2 hours at 37 °C in a shaking water bath. After washing the plates 5 times with PBS containing 0.05 % tween 20, 100 µl of alkaline phosphatase labeled anti-sheep IgG (for sheep sera) and anti-goat IgG (for goats sera) antibodies (Kirkegaard & Perry Laboratories, Inc.) diluted 1:3000 in PBS, were added. Plates were then kept for 1 hour at 37 °C in a shaking water bath. The chromogen paranitrophenyl phosphate, at 1mg per 5 ml substrate buffer, pH 9.8 was added. Within 30 minutes, the absorbance of the coloured reaction was read using "Titertek Mulishan ELISA reader" at 405

nm. The cut off value (positive threshold value) was determined as double fold of the mean value of negative sera.

Results

Results of clinical examination showed that out of the 193 examined sheep and 106 goat cases, 153 and 84 respectively were considered diseased with elevated body temperature, dullness, nasal discharge, cough and/or respiratory manifestations and some times abnormal lung sounds. The rest of cases (40 sheep and 32 goats) were apparent healthy cases the body temperature of diseased cases was 40 °C or over.

In the slaughter house, lung samples were collected from cases (68 sheep and 32 goats) showed severe congestion and/or hepatization accompanied with bronchial exudates and some times pulmonary oedema. The rest samples were normal healthy lungs.

Bacteriological examination for nasopharyngeal swabs and lung tissues samples resulted in the isolation of M. *haemolytica* and *P. multocida* either in pure culture or mixed with other different bacteria such as *E. coli*, *Kl. pneumonae*, *P. aerogenosa* and *S. aureus*

The recovery rate of both *M. haemolytica* and *P. multocida* from nasopharyngeal swabs and lung tissue samples (diseased and apparent healthy cases) from sheep and goats are shown in Table (1) and Table (2) respectively. The capsular and somatic antigen type of *M. haemolytica* and *P. multocida* isolated strains are given in Table (3) and Table (4) respectively.

Results of the **Enzyme linked immunosorbent assay** (ELISA) test proved that the mean value of negative sera gave an optical density (O.D) reading of 0.24, so the cut off value (positive threshold value) was considered as 0.48. Tested sera showed O.D 0.48 or more were considered positive. The number of positive sera was23 with *M. haemolytica* (14 sheep and 9 goats), 36 with *P. multocida* (23 sheep and 13 goats) and was compared with results of conventional culture results (Table, 5). Two sheep sera and one goat serum, both from apparent healthy cases, gave O.D readings less than 0.48 and thus were considered negative. The recovery rate

of *M. haemolytica* and *P. multocida* was 95.8 % and 94.7 % respectively as shown in Table (5).

Table (1): The recovery rate of Pasteurella (*M. haemolytica* and *P. multocida*) from apparent healthy, diseased and slaughtered sheep samples.

Samples	Apparent healthy			Diseased			Total		
	Examined Samples	Positive		Examined Samples	Positive		Examined Samples	Positive	
		No.	%		No.	%		No.	%
Nasopharyngeal swabs	40	5	12.5	153	34	22.2	193	39	20.2
Lungs	26	4	15.4	68	16	23.5	94	20	21.3
TOTAL	66	9	13.6	221	50	22.6	287	59	20.6

Table (2): The recovery rate of Pasteurella (*M. haemolytica* and *P. multocida*) from apparent healthy, diseased and slaughtered goats samples.

Samples	Apparent healthy			Diseased			Total		
	Examined Samples	Positive		Examined Samples	Positive		Examined Samples	Positive	
		No.	%		No.	%		No.	%
Nasopharyngeal swabs	22	3	13.6	84	20	23.8	106	23	21.7
Lungs	12	2	16.7	32	7	21.9	44	9	20.5
TOTAL	34	5	14.7	116	27	23.4	150	32	21.3

Table (3): Capsular and somatic antigen typing of *M. haemolytic* isolated from sheep and goats samples.

Isolated Strains	Capsular antigen type				Somatic antigen type							
	A		T		Type A		Type T					
					A :2		T :4		T:10		T:15	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
36	21	58.3	15	41.7	19	52.8	9	25.0	6	16.7	2	5.5

Table (4): Capsular and somatic antigen typing of *P. multocida* isolated from sheep and goats samples.

olated d rains	Capsular antigen type						Somatic antigen type									
	A		D		Un-typed		Type A				Type D					
							A:2		A:5		D:1		D:3		D:4	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
55	39	70.9	12	21.8	4	7.3	25	49.0	9	17.7	10	10.6	5	9.8	2	3.9

Table (5): Comparison between results of conventional culture and serum ELISA methods

Microorganism	Conventional culture positive	ELISA positive	Recovery rate
M. haemolytica	24	23	95.8 %
P. multocida	38	36	94.7 %

Discussion

Respiratory infections and pneumonia caused by *M. haemolytica* and *P. umltocida* can lead to significantly decreased growth performance. These two pathogens cause outbreaks of acute pneumonia in sheep and goats of all ages. Respiratory infections from these pathogens are associated with poor management practices, occur as a secondary infection, or occur as a consequence of severe stress. Transportation stress, viral infections (e.g., parainfluenza-3 virus), lung parasites, prior bacterial infections, overcrowded pens, poor housing conditions, sudden environmental changes, and other stressful conditions increase sheep and goats' susceptibility to *P. multocida* and *M. haemolytica* pneumonias (32).

In Egypt, plenty information are available on pneumonia and respiratory infections in cattle (39; 17; 36; 20 and 4), while only scanty researchers could be traced on pneumonia and respiratory infections in sheep and goats (24; 16 and 33).

Results of bacteriological examination revealed the recovery of *M. haemolytica* and *P. umltocida* with a percentage of 20.6 % and 21.3 % from studied sheep and goats respectively (Tables, 1 and 2). The recovery rate from diseased cases was significantly higher than the apparent healthy cases, almost double the incidence. These results were in agreement with that recently reported by (24) and (33) while higher recovery percentage was cited by (16).

Serological typing of the capsular and somatic antigen component of the isolated pasteurella strains were tabulated (Tables, 3 and 4). The *M. haemolytica* capsular A and T and the somatic antigens type A: 2 T: 4; T: 10 and T: 15 were detected with a percentages of 58.3, 41.7, 52.8, 25.0, 16.7 and 5.5 respectively (Table, 3). The highest incidence was recorded with capsular antigen A (58.3 %) and somatic antigen type A:2 (52.8 %) in both sheep and goats. This result agreed with that reported by (33); (1) and (3), while disagreed with (37) and (13). The prevalence of Type A is most important as is associated with a severe form of pneumonia. Sheep and goats that survive an acute stage may recover or become chronically infected with reduced lung capacity (32)

Pasteurella multocida is a highly diverse group of bacteria recognized as important pathogens (43). *Pasteurella multocida* isolates

showed capsular antigen type A (70.9%) and type D (21.8%), but 4 isolates (7.3%) were untyped. The somatic antigen of the typed strains were identified as A:2 , A:5, D:1, D:3 and D:4 with a percentage of 49.0, 17.7, 19.6, 9.5 and 3.9 respectively. The highest incidence was reported with capsular antigen A (70.9 %) and somatic antigen type A:2 (49.0 8%) in both sheep and goats. It is worthy to denote that the 2 strains identified as D: 4 were goats isolates These results mostly agreed with that cited by (43), (11) and (18).

From our results, it could be concluded that identical Pasteurella strains were shared by the goats and sheep. This result comes in harmony with that obtained and proved by (40) who clarified that P. *haemolytica* organisms which were isolated from one goat and one of two sheep found in close association and had identical electrophoretic patterns of DNA fragments, and also cited that *Pasteurella multocida* isolates cultured from the goat and one of the sheep had identical electrophoretic profiles. Although the direction of transmission could not be established, evidence suggests transmission of strains from goats to sheep. Goats may serve as a reservoir of Pasteurella strains that may be virulent in sheep; therefore, goats in sheep habitat should be managed to prevent contact with sheep. Sheep which have nose-to-nose contact with goats should be removed from the habitat.

Globally, results from bacterial culture attempts have been the primary source of information on host-associated bacteria, but studies have shown that culture-based results (morphological, biochemical and serological examinations) significantly underestimate bacterial diversity in biological samples. The use of advanced techniques of diagnosis such as ELISA, PCR, Electron Microscopy scanning and DNA gene sequences analysis ... etc, was nowadays termed " culture-independent diagnostic methods" and were recently employed (38; 7; 2 and 41). The latter (41) using a culture-independent method reported that "Pasteurellaceae" bacteria were the most diverse phylogenetic group often associated with respiratory disease in live sheep and concluded that culture-independent methods were even able to directly detect pasteurella toxin in swab and lung tissue.

In our study, we tried the use of **Enzyme linked immunosorbent assay** (ELISA) test, the mean value of negative sera readings was

determined and the cut off value (positive threshold value) was considered as double the latter value (42; 26 and 21). The recovery rate was found to be 95.5 % and 94.7% for *M. haemolytica* and that *Pasteurella multocida* respectively, which considered highly significant. It is important to mention that two sheep sera and one goat serum, both from apparent healthy cases, gave O.D readings less than the proposed positive threshold value and were considered negative.

Thus, we can conclude that the ELISA technique as a "culture-independent methods" was proved to be easy, practical, sensitive and efficient method for quick diagnosis of *M. Haemolytica* and Pasteurella *multocida* infection in sheep and goats.

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الالتهاب الرئوى بالمانهيما هيمولتيكا والباستريلا مالتوسيدا فى الاغنام والماعز بمحافظة الفيوم : استخدام اختبار الاليزا

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الملخص

تم فحص اجمالى ٢٩٩ حالة من المجترات الصغيرة (١٩٣ أغنام و ١٠٦ ماعز) من اماكن مختلفة بالفيوم اكلينيكيا للاعرض التنفسية ثم جمع المسحات الأنف بلعومية وعينات الدم من كل الحيوانات . وأيضا ١٣٨ عينة رئة من ٩٤ اغنام (٦٨ بها أعراض الالتهاب الرئوى و٢٦ سليمة) و٤٤ من الماعز (٣٢ بها أعراض الالتهاب الرئوى و١٢ سليمة) بالمجازر المختلفة بالفيوم. تم فحص المسحات وعينات الرئة بكتريولوجيا واجراء اختبار الاليزا لعينات السيرم واوضحت النتائج عزل المانهيما هيمولتيكا والباستريلا مالتوسيدا فى صورة فردية واخرى مختلطة مع الميكروب القولونى والكلبسيلا الرئوية والسيدوموناس ايروجينوزا والميكروب العنقودى الذهبى. وكانت نسبى عزل الباستريلا (هيمولتيكا والمالتوسيدا) ٢٠,٦ % و ٢١,٣ % من الاغنام والماعز على التوالى وكانت نسبة العزل فى المجترات المريضة اعلى بكثير من السليمة . تم تحديد فى المانهيما هيمولتيكا الانتجن الكبسولى A و T والجسمى A:2 وT:4 وT:10 و T:15 بنسبة ٥٨,٣ و ٤١,٧ و٥٢,٨ و٢٥,٠ و١٦,٧ و ٥,٥% على التوالى وكانت اعلى نسبة عزل هى الانتيجن الكبسولى A (٥٨,٣) والانتيجن الجسمى A:2 (٥٢,٨) فى كلا من الاغنام والماعز. اظهرت الباستريلا مالتوسيدا الانتيجن الجسمى A بنسبة ٧٠,٩ % و D بنسبة ٢١,٨ % وكانت أربعة معزولات غير مصنفة (٧,٣ %). وتم تصنيف الانتيجن الجسمى للعترات كالتالى: A:2 و A:5 وD:1 و D:3 وD:4 بنسبة ٤٩,٠ و١٧,٧ و١٩,٦ و٩,٥ و٣,٩% على التوالى.وكانت اعلى نسبة هى A (٧٠,٩) و A:2 (٤٩,٠٨) فى كلا من الأغنام والماعز. ومن الجدير بالذكر ان D:4 عزلت من الماعز. وأوضحت النتائج ان نسبة حساسية وفاعلية اختبار الاليزا مقارنة بالعزل البكتريولوجى هى 95.5 و 94.7 % لتحديد تواجد المانهيما هيمولتيكا والباستريلا مالتوسيدا على التوالى وهى نسبة عالية خاصة أنها طريقة لا تعتمد على الزرع البكتريولوجى، واسرع، وذو حساسية عالية فى تشخيص المانهيما هيمولتيكا والباستريلا مالتوسيدا فى الأغنام والماعز ونوصى بتعميمها.