Original Research Paper

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SEASONAL ISOLATION AND IDENTIFICATION WITH ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF PASTEURELLA MULTOCIDA AND MANNHEIMIA HAEMOLYTICA FROM GOATS IN DRY AND WET SEASONS IN KELANTAN, MALAYSIA

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ABSTRACT This study was conducted from November 2015 to August 2016 which involves 7 districts in Kelantan, Malaysia. It investigates the rate of prevalence of pneumonic pasteurellosis caused by P. multocida and M. haemolytica in goats in wet season and more specifically during the dry season. It is also, to determine the antibacterial susceptibility of the two aforementioned bacteria. The samples of nasal swabs were collected during the wet and dry seasons in the months of November, December, and January. And April, May, June, July, and August respectively. A total of 164 nasal swabs were taken from goats, with signs of pneumonia. The number of bacterial isolation isolated from 164 samples are 191. The isolates were processed and identified using standard microbiological techniques. Conventional polymerase chain reaction (C-PCR) assay was conducted to confirm the isolates. Results indicate that 9.8% of the samples isolated were positive for P. multocida, while 11%, of the samples were positive for M. haemolytica. The prevalence rate of positive isolates of P. multocida during the wet season was 25% while in the dry season was 75%. On the other hand, for M. haemolytica the result was 44.44% during the wet season and 55.55% during the dry season. Antibacterial susceptibility test for P. multocida isolates using disk diffusion test revealed that all isolates were susceptible to Ciprofloxacin (100%), Chloramphenicol (93.8%), Ampicillin (87.5%), and Gentamicin (87.5%). Furthermore, the isolates were most frequently resistant to Penicillin (100%). In addition, M. haemolytica isolates showed that they were susceptible to Ciprofloxacin (94.4%), Chloramphenicol (94.4%), Ampicillin (72.2%) and Gentamicin (66.7%). However, the isolates were resistant to Penicillin (88.9%). In conclusion, pneumonic pasteurellosis disease due to P. multocida and M. haemolytica has become a significant issue in respiratory infection of goats, in both sexes, all ages and in several breeds, specifically during the dry season in Kelantan.

KEYWORDS : Seasonal isolation, Pasteurella multocida, Mannheimia haemolytica, antibiotic susceptibility, Goats

INTRODUCTION

Pasteurella multocida and Mannheimia haemolytica are among the most common bacterial pathogens that affect different animal types including production animals in Malaysia as well as worldwide (Amin, 1998). In many parts around the world, goats have a major role for providing a livelihood to many people (Elsheikh & Hassan, 2012). Small ruminants are high value assets for the majority of Southeast Asian nations, as they provide meat and milk. However, they are prone to respiratory diseases such as pneumonia through infection of the upper and lower respiratory tract. Usually, the causes of these infections are bacterial, viral or fungal. Factors such as climate, physiological conditions, inappropriate housing, transportation, and etiological factors play a significant role in the incidence of respiratory diseases (Kumar et al., 2014). Respiratory diseases known to have a very significant economic cost by way of lost productivity and in the socio-economic development of poor farmers. Furthermore, respiratory diseases also result in high rates of mortality and morbidity. Pneumonic Pasteurellosis (also known as respiratory mannheimiosis) being the most common example and highly prevalent in ruminants (Abera et al., 2014). Microbiological examination has shown that *P. multocida* is a gram negative, non-motile coccobacillus and it has been viewed a very heterogenic bacterial species due to various antigenic specificity. (Shayegh et al., 2009). Mannhemia haemoltica (previously Pasteurella haemolytica) is among the most common etiological agents of pneumonic Pasteurellosis. *M. haemolytica* is gram negative, a little bit hemolytic, with multiple forms and usually coccobacillus (Highlander, 2001). It has also been recognised as one of the commensals of the goat's nasal cavity, tonsils, and nasopharynx. Moreover, it is able to multiply in the nasal and nasopharyngeal, transformed and become invasive. Abrupt climate changes boost Goat's susceptibility to *P. Multocida* and *M. haemolytica* pneumonias (Abdullah et al., 2015). Any change in environmental conditions could thus affect such agents, either positively or negatively, and therefore influence livestock health (Forman et al., 2008). Seasonal influence is observed with more of the pneumonia cases being recorded in the main rainy season than in the dry season (Emikpe et al., 2013).

MATERIALS AND METHODS

Study area

The study was conducted in Kelantan state in the northeast of Peninsular Malaysia during the period from Nov. 2015 until Aug. 2016. Kelantan state is located 453 km from Kuala Lumpur, the capital of Malaysia. The state is geographically positioned at 6.1254° N latitude and 102.2381° E longitude. It has been divided into ten districts. The wet season in this state is from November, to January when the Northeast Monsoon blows. On the other hand, the dry season comprises of, April, May, June, July and August.

Animals

The live animals used in this research were goats of various breeds, ages and of both sexes. A total of 164 live goats were intentionally selected based on the norms used within this research, which involve wet conditions and, more particularly, the dry season. This study included all the goats, which had signs of pneumonia.

Sample collection

Samples of nasal swabs collected were 61 during the wet season and 103 during the dry season. A total of 164 samples of nasal swabs were collected aseptically, from goats that showed infection and signs of pneumonia. The signs included: difficulty in breathing and irregular breathing pattern, nasal discharge (mucous/ purulent), coughing, and sneezing. Details of age, sex and breed were recorded. First samples were collected during the wet season (November, December, and January). Second samples were collected during the dry season (April, May, June, July, and August). In the field study, when collection samples and before starting the sampling, body condition check-up and case history record were performed for each case. All the swabs used throughout the study were sterile Amies transport swab in a tube with Charcoal or without Charcoal media. During the preparation of the animal for sampling, and before compiling nasal swabs from the animals, gross debris was swept out from the nares with a sanitary wipe. After ensuring that the external nares were cleaned and disinfected, a sterile transport swab was inserted deep (approximately 16-20 cm) and rotated into the nares of each animal. Subsequently the swab was inserted into a media tube and closed tightly. All the swabs after sampling were kept in the deep fridge cold box and closed carefully, and directly transported to the laboratory.

Bacteriological examination

Bacterial culture and isolation

Preparation was aseptically made before starting the culture, by performing all the procedures of the culture inside the Biological Safety Cabinet (Class II) in order to avoid any contamination. The primary culture was from nasal swabs. The procedure was as follows: each swab was removed from its tube, and smeared over the surface of the agar plate, which contained 5% sheep blood agar. The plates were

then labelled and aerobically incubated overnight at 37°C for 18- 24 hours. The growth pattern of *P.multocida* and *M.haemolytica* on blood agar was observed and noted. After that, the Secondary culture was performed. A selected typical (single) colony was sub cultured and streaked on Mac-Conkey agar plate and another on sheep blood agar, and incubated at 37°C for another 18- 24 hours to isolate and ensure the organism would be in pure culture.

Identification of bacterial isolates

The suspected colony of *P. multocida* and *M. haemolytica* was studied separately. Firstly, the typical colonies of *P. multocida* obtained from blood agar were non-haemolytic, rounded, intermediate in size, sometimes small, sweetish odour, gray in appearance and glistening. Growth test on Mac-Conkey agar was negative, and showed no visible growth Secondly, typical *M. haemolytica* colonies exhibited a smooth surface, entire edge, low convex shape, translucent appearance, and medium sized. The colour was greyish with β- distinct zone of hemolysis on blood agar . It was grown on MacConkey agar, and was observed to be of small, red pinpoint shape. Following the completion of the study and the notation of all characteristic of each pathogen grown on Blood and MacConkey agar, the subcultures were done on Nutrient slant agar to obtain pure cultures for further tests. The isolates of *P. multocida* and *M. haemolytica* were subjected to further identification. Suspected bacteria colonies were achieved by observation of colonial morphology under microscopy and isolates were determined by using some biochemical test which including: Catalase, Oxidase, Triple Sugar Iron (TSI), Citrate, (Sulfide), indole, and Motility (SIM), Methyl Red and Voges-proskauer (MR-VP). Store the suspected bacterial isolates were in nutrient slant agar and storage was in a refrigerator at 2-4°C. Re-subculture was done every two to three weeks to refresh the colonies.

Antimicrobial susceptibility determination

Antibiotics sensitivity determinations were performed after identifying the isolates of *P. multocida* and *M. haemolytica* by microbiological and biochemical characteristic examination. The determinations were via using the Kirby-Bauer disc diffusion method, after being cultured on Mueller-Hinton agar (Oxoid). Total (n = 34) isolates, which showed positive reaction in the tests and highly expected to be *P. multocida* and *M. haemolytica*. Sixteen isolates of *P. multocida* and eighteen of *M. haemolytica* were used in this investigation. Isolates were recovered from nasal cavity from June 2015 to August 2016 during both wet and dry seasons in Kelantan. Mueller-Hinton agar (MHA) is the ideal medium for routine susceptibility testing employing Kirby-Bauer disc diffusion method for non-fastidious bacteria (both aerobe and facultative anaerobe). All the isolates of *P. multocida* and *M. haemolytica* were tested against antimicrobials. Antimicrobial disks used in the sensitivity test include: Ciprofloxacin 5 µg, Gentamicin 10 µg, Oxytetracycline 30 µg, Chloramphenicol 30 µg, Penicillin 10 µg, Ampicillin 10 µg, Enrofloxacin 5 µg, and Sulfamethoxazole 25 µg. Reading and interpretating the results of disk diffusion assay and comparing with standards to determine the susceptibility of the organisms to the drug are according to the Manual of Antimicrobial Susceptibility Testing (M A S T). Each result is interpreted and categorized as in the following: Susceptible (S), intermediately susceptible (IS) and resistant (R).

DNA Extraction and Polymerase Chine Reaction (PCR)

DNA extraction has been done for all suspected colonies of *P. multocida* and *M. haemolytic* that show ideal reaction in the bacteriological and biochemical examination. This procedure carried out according to previously described by Kumar et al., (2015). The total bacterial genomic DNA from each pathogen (*P. multocida* and *M. haemolytica)* isolate is extracted by using the method of DNeasy Blood & Tissue Kit (Qiagen, Germany). As per manufacturer's instructions (Qiagen, Germantown, MD, USA). The concentration of the DNA is measured and determined spectrophotometrically by using Nanophotometer™ P- Class (IMPLEN, Germany). While, The purity of DNA is assessed by computing the ratio of A_{260} / A_{280} nm. The ratio A_{260}/ A_{280} nm, of DNA is between 1.7 and 2.0, indicating that the DNA is pure.

Primers and PCR Amplification

The oligonucleotide primers used in this study are mentioned in Table-1. Primers targeting KMT1 and Rpt2 genes of *P. multocida* and *M. haemolytica* were obtained from a previously study Deressa et al., (2010) and Kumar et al., (2015). The PCR reaction is completed in a final reaction volume of 25 µl in a thermal cycler. This study used a master mix ready-to-use solution containing: PCR reaction buffer, 0.06U/µl of Taq DNA polymerase, 3mM of MgCl2 and 400µM of each dNTPs.

PCR Assay

Polymerase chain reaction was preformed on Bio-Rad C1000 Thermal Cycler. The steps and conditions of thermal cycling for both primer pairs has been done as following: Preheating the lid at 100 ºC for 5-6 min, Pre-denaturation of DNA at 95 ºC for 4 min, denaturation of DNA at 95 ºC for 1 min, annealing the primers at (50 ºC, for P.multocida) and (48 ºC, for M. haemolytica) both for 1min. Extension at 72 ºC for 1min, cycling (Repeat steps 3-5, 35X) and Final extension was carried out at 72 ºC for 10 min.

Gel Analysis of the PCR Products

The products of PCR were analyzed on 1% agarose gel, stained with Midori green-stain. Two DNA ladders 100 bp for samples of *P. multocida* and 1 kb for *M. haemolytica*. were used to determine the size of the amplified fragments. The results were then analyzed under t-test in SPSS Statistics.

Results and Discussion

The result of Bacteriological examination test of the nasal swabs

All the nasal swabs during the wet and dry season were examined respectively. 77 colonies were isolated from 61 samples examined during the wet season, the months of (Nov-Dec-Jan). On the other hand, 114 colonies were isolated from 103 samples examined during the dry season, the months of (Apr-May-Jun). The results of relationship between season and the prevalence of *P. multocida* has been shown in (Table 1). While for *M. haemolytica* in (Table 2). The results of the overall prevalence of among goats, in both season shown that positive goat infected was 9.8% for *P. multocida* and 11% for *M. haemolytica* of total 164 animals tested.

Table 1. Relationship between seasons , the incidence rate and the prevalence of P.multocida

Variable		***Positive & (%)***	***Chi square value***	***Df***	***P-value***
Season	Wet season	4 (25%)	1.129	1	0.217a
	Dry season	12 (75%)			

[a]=chi square test

Table 4.9. Relationship between seasons , the incidence rate and the prevalence of *M.haemolytica*

Variable		**No of Positive (%)**	***Chi square value***	***Df***	***P-value***
Season	***Wet season***	***8(44.44%)***	0.455	***1***	***0.607a***
	Dry season	***10(55.55%)***			

Results of antibiotic susceptibility testing for *P. multocida* and *M. haemolytica*

Reading and explaining the results of antibiotic sensitivity tests were done for all Pasteurellosis cases isolates (*P. multocida* & *M. haemolytica*) respectively following the . basis of Clinical and Laboratory Standard Institute (CLSI) guidelines. The results of zone diameters were interpreted based on the Performance Standards for Antimicrobial Susceptibility Testing as detailed in (Table 3). Sixteen isolates of *P. multocida* isolated of goats with naturally pneumonia infected goats were subjected to a panel of six microbial that are available. The results of antimicrobial susceptibility tests of all isolates indicates that all the *P. multocida* isolated were highly sensitive to CIP 5 (100%), C 30 (93.8%) , AMP 10 (87.5%), and CN 10 (87.5%). On the other hand, it has shown Intermediate susceptiblity to OT 30 (50%) and resistance to P 10 (100%). (Table 4). Eighteen isolates of *M. haemolytica* whish isolated were also subjected to six microbial. The isolates of *M. haemolytica* in both season have appeared substantially sensitive to C 30 (94.4%), CIP 5 (94.4%), AMP 10 (72.2%) and 66.7% to CN 10. While it showed intermediate susceptibility to OT 30 (38.9%) and Resistant to P 10 (88.9%) (Table 5).

Table 3. Zone diameters and interpretation criteria of testing disc antimicrobial agents (inhibition zone diameter in mm) according to CLSI Standard used in this study.

M. haemolytica						
NO	Antimicrobial Agent	Symbol	Disc potency	Zone diameter in (mm)		
				Resistant	Intermediate	Susceptible
1.	Ciprofloxacin	CIP	5 µg	≤ 15	16-20	≥ 21
2.	Gentamicin	CN	10 µg	≤ 12	13-14	≥ 15
3.	Oxytetracycline	OT	30 µg	≤ 15	16-25	≥ 26
4.	Chloramphenicol	C	30 µg	≤ 12	13-17	≥ 18
5.	Penicillin	P	10 µg	≤ 28	-	≥ 29
6.	Ampicillin	AMP	10 µg	≤ 13	14-16	≥ 17

Table 4. Results of Antimicrobial susceptibility test for P. multocida, isolated from nasal cavity of goats infected with pneumonia during wet and dry season.

P.multocida					
NO	Antimicrobial tested	Valid			Total
		Susceptible	Intermediate	Resistant	
1	CIP 5	16 (100%)	-	-	
2	C 30	15 (93.8%)	1 (6.3%)	-	
3	AMP 10	14 (87.5%)	-	2 (12.5%)	
4	CN 10	14(87.5%)	2 (12.5%)	-	
5	OT 30	5 (31.3%)	8 (50%)	3 (18.8%)	
6	P 10	-	-	16 (100%)	
					16 (100%)

Table 5. Results of Antimicrobial susceptibility test for M. haemolytica, isolated from nasal cavity of goats infected with pneumonia during wet and dry season.

M. haemolytica					
NO	**Antimicrobial tested**	**Valid**			**Total**
		Susceptible	**Intermediate**	**Resistant**	
1	**CIP 5**	**17 (94.4%)**	**-**	**1 (5.6%)**	
2	**C 30**	**17 (94.4%)**	**1 (5.6%)**	**-**	
3	**AMP 10**	**13 (72.2%)**	**-**	**5 (27.8%)**	
4	**CN 10**	**12 (66.7%)**	**3 (16.7%)**	**3 (16.7%)**	
5	**OT 30**	**7 (38.9%)**	**11 (61.1%)**	**-**	
6	**P 10**	**2 (11.1%)**	**-**	**16 (88.9%)**	
					18 (100%)

Discussion

The successful isolation of both bacterial pathogens from the diseased goats in wet and dry season in Kelantan state indicates the importance and magnitude of the disease pasteurellosis in the state of Kelantan. The aetiopathogen confirmation by isolation from nasal carriage at wet and more specific in the dry season was successfully attempted. It appears that this is the first systematic study in Kelantan state, focusing on these important pathogens that affect goats widely as demonstrated in the results. It is important to mention that in the past years, most of the studies on pneumonia in small ruminants showed that pulmonary pasteurellosis occurrence is in winter time (Weiser et al., 2003). The current study indicates the importance of the disease in the dry season. Therefore, further studies should be focusing on why and how it occurred widely in the summer dry time. Several studies have shown that, the factors such as crowding, dust, damp and humid weather, or stress, singly or all can increase the disease existence (Weiser et al., 2003). On the other hands, does these factors are making the same impact in dry weather. While no studies on this fact at the present time it's advisable to have a future planned study on this new fact. This investigation accordingly, has succeeded to highlights the severe effect of the dry weather on increasing the rate of infection and the incidence of pulmonary pasteurellosis in goats and wide prevalence of the disease. It was very important to study and enhance more knowledge regarding the rate of incidence and prevalence of these pathogens in goats at the dry season as well as the wet season. These results, indicate that the incidence and prevalence of *P. multocida* and *M. haemolytica* in goats are of high rate specifically in the dry season. The high incidence of disease in the dry season, exhibited a positive correlation with the area, suggesting the climate condition plays a role in respiratory problems in the dry season at the current time of weather change. The incidence of nasal carriage of *P. multocida* and *M. haemolytica* in goats at the wet and dry season in Kelantant was highly potential to consider that Pasteurella pathogens availability as normal inhabitants of nasal cavity and upper respiratory system. Promote to the disease when climatic changes affect the residence of the bacteria to be more virulent (Al-Sultan et al., 1982). The results of nasal carriage assay for those two phatogens by nasal swabs detection were comparable with the findings of Al-Tarazi & Dagnall (1997). Antibiotics are important remedies in modern farm animal production, as well as for small breeders in Kelantan and other places. The use of these antibiotics should be based on an accurate diagnosis since there is an increasing incidence of bacterial resistance to antibiotics in humans. This phenomenon was attributed to the use of anti-microbial drugs in food-producing animals. Also, there is a concern about possible residues in animal products. Determing the antibiotic that resistant and susceptibility of *P. multocida* and *M. haemolytica* in the wet and dry season can lead to pave a way to the veterinarian to treat the cases of pasteurellosis pneumonia. Results of antimicrobial susceptibility test for *P. multocida* and *M. haemolytica* shown that the high rate of susceptibility was for Ciprofloxacina and Chloramphenicol. This finding is similarly recorded in other studies and reports of Emikpe et al. (2014), Guler et al. (2013) and Mohamed Ragab, (2015). On the other hand, the isolates of *P. multocida* and *M. haemolytica* were most frequently resistant to Penicillin (10 µg), this is a similar finding with Jamali et al. (2014).

Conclusion

This investication has shown that *P.multocida* and *M. haemolytica* to be a widespread disease in infected goats, with pneumonic pasteurellosis at the wet and dry season in Kelantan state. Nasal swab sampling was appropriate for the isolation and identification by bacteriological, biochemical and molecular examination. *P. multocida* and *M. haemolytica* are affecting the goats in all stages of its life, and in both sexes. In this state of Malaysia, the nasal carriage of *P. multocida* and *M. Haemolytica* of infected goats was possible in both season, but in varying proportions. The high percentage of incidence and prevalence of this disease due to *P. multocida* and *M. haemolytica* in the dry season can also be the same in other regions of the country. A high prevalence of *P. multocida* and *M. haemolytica* in Goats at dry season is leading for more thinking and focusing on the impact of climate (dry) when the animals with respiratory problems, and get more knowledge of the relationships between the incidence of this disease and the effect of climate. The study, has presented a fact that *P. multocida* and *M. haemolytica* were highly sensitive to Ciprofloxacin (5 µg), at both seasons in Kelantan state. On the other hand, most of the isolates were resistant to Penicillin (10 µg). Nowadays it is an important to know that some bacterial pathogens are showing significant changes regarding, resistant to some antibiotic, although, same of these pathogens were sensitive in the meantime.

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