CAPSULAR SEROGROUP OF PASTEURELLA MULTOCIDA ISOLATED IN VRI, MALAYSIA FROM YEAR 2014 TO 2016

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ABSTRACT. A total of 65 *Pasteurella multocida* were isolated and identified from various animal's samples received by Veterinary Research Institute (VRI) during the period of 2014 to 2016. These animals comprises of cattle, goat, pig, chicken, duck and rabbit. The serogroup of *Pasteurella multocida* were carried out using designation system of Carter's capsular typing and molecular serogrouping method. Based on cases submitted to VRI, the prevalence of pasteurellosis in Malaysia ranging from 1.0% to 3.2% (2014 to 2016). It is low compared to previous reports and the pattern of predominant serogroups and animal hosts were found to be changing every year. In 2014, 80% (12/15) of the isolates were *Pasteurella multocida* Carter's type D where all were isolated from goats. In 2015, the predominant serogroup changed to *Pasteurella multocida* Carter's type A with a prevalence rate of 40.6% (13/32) which were mostly isolated from duck and cattle. While for *Pasteurella multocida* Carter's type D, the prevalence in 2015 reduced to 21.9% (7/32) compared to the previous year and it was isolated from various animal species. Interestingly, in 2015 there was one isolate of *Pasteurella multocida* Carter's type B isolated from goat with no reported history of outbreak. In 2016, the prevalence of *Pasteurella multocida* Carter's type A increased to 72.2% (13/18), with a high percentage (92.3%) infection in young calves showing clinical signs with high mortality and morbidity in infected farms. Furthermore, during these 3 years of study, 3 isolates of *Pasteurella multocida* serogroup F were also identified each from pig, goat and chicken, respectively. In conclusion, this study revealed that pasteurellosis had become sporadic in Malaysia and the distribution of serogroups were diverse in all species of animal with no definitive host.

Keywords: pasteurellosis, *Pasteurella multocida*, Carter's capsular typing, molecular serogrouping, animal species.

INTRODUCTION

Pasteurellosis is a bacterial infection caused by *Pasteurella* genus that affects a wide range of animals. The most reported organism is *Pasteurella multocida* which is well known as a common commensal in the upper respiratory tract of healthy animals. *P. multocida* could causes disease when it is inhaled into the deeper portions of the respiratory tract and the animal's normal defense system becomes impaired. Pasteurellosis caused by *P. multocida* often manifested as a pneumonic or septicemic form. The disease is mainly triggered by physical or physiological stress due

to adverse environmental and climatic conditions such as bad weather, poor management, overcrowding, transportation or previous infection of pathogenic organisms (Mohamed and Abdelsalam, 2008; Always *et al*., 2012). Many strains of *P. multocida* express a polysaccharide capsule and the antigenicity of the capsule can be used to identify five serogroups A, B, D, E and F. At VRI, the *P. multocida* serogroup was identified on the basis of differences in capsular polysaccharides (Carter, 1955) and molecular serogrouping (multiplex PCR) as recommended by World Organisation for Animal Health (OIE). Pasteurellosis outbreaks in Malaysia have been well documented from the early 1970s to 1990s, especially clinical outbreaks of haemorrhagic septicaemia in cattle and buffaloes (Joseph, 1979). In 2014, Nafizah *et al*. reported that the prevalence rate of pasteurellosis is about 3.2% based on cases received in VRI. The aim of this report is to highlight the current status and diversity pattern of *P. multocida* isolates in VRI.

MATERIALS AND METHODS

Conventional typing of Pasteurella multocida

Bacteria Isolation

A total of 65 strains of *P. multocida* which were isolated in the Bacteriology Unit, VRI from 2014 to 2016 was selected for this study. The isolates were from nasal swabs or visceral organs of various animal species using conventional methods as described by Quinn *et al*. (1994). The species of animals comprises cattle, goat, pig, chicken, duck and rabbit. The samples were cultured onto 5% bovine blood agar and MacConkey agar and incubated aerobically at 37 °C for 18 to 24 hours. Suspected *P. multocida* colonies were identified and confirmed by Gram-staining and a series of biochemical tests.

Carters' capsular typing of Pasteurella multocida

P. multocida type D were identified using acriflavine test according to Carter and Subronto (1973). For the acriflavine test (Figure 1), 3 ml of brain-heart infusion (BHI) culture was centrifuged at 2000 rpm for 20 minutes. Then 0.5 ml of 1:1000 aqueous solution of acriflavine (Sigma Aldrich, USA) was added to 0.5 ml of the concentrated test culture. The isolates that exhibited flocculation precipitate after an interval of 5 minutes were identified as type D (Figure 1).

P. multocida type A were identified using hyaluronidase decapsulation test (Figure 2) that used a hyaluronidase producing *Staphylococcus aureus* according to Carter and Rundell (1975). *P. multocida* strain were culture on blood agar to provide lines of growth approximately 3-5 mm apart. A hyaluronidase-producing strain of *Staphylococcus aureus* were streaked at right angles to the *Pasteurella* streak lines and incubated overnight. The hyaluronidase effect with type A culture manifested as a diminution in the size of the *Pasteurella* colonies in the region adjacent to the *Staphylococcus aureus* streaks (Figure 2).

P. multocida type B were identified using specific B serogroup hyperimmune sera prepared in rabbits. Positive reaction

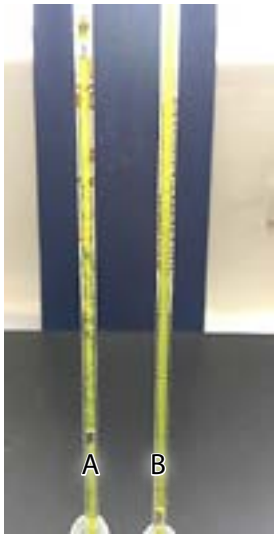

Figure 1. Acriflavine test: Tube A, positive reaction of heavy flocculation precipitate using inoculum of *P. multocida* serogroup D and acriflavine solution. Tube B, negative reaction.

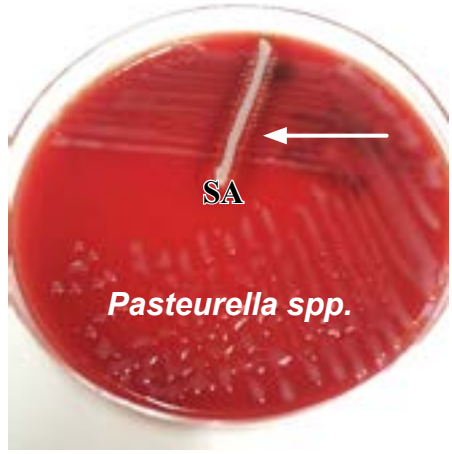

Figure 2. Hyaluronidase decapsulation test: Diminution in the size (arrow) of the *Pasteurella* colonies in the region adjacent to the *Staphylococcus aureus* (SA) streak.

of slide agglutination test were identified as type B.

Molecular serogrouping of *Pasteurella multocida*

DNA Extraction

P. multocida colonies grown overnight on blood agar were transferred into a 1.5 ml Eppendorf tube containing 100 µl nuclease-free water. The homogenous suspension was placed in a thermal block at 95 °C for 10 minutes. After cooling to room temperature, the samples were centrifuged at 13,000 rpm for 2 minutes and 10 µl of the supernatant was used as DNA template.

Multiplex PCR

Multiplex PCR was performed as described by Townsend *et al*. (2001) with slight modification in a total reaction volume of 50 µl containing 5× MyTaq Buffer, MyTaq DNA Polymerase (Bioline UK), 5 sets of primers (CapA, CapB, CapD, CapE and CapF) and 10.0 µl of DNA template (Table 1). The amplification programme consists of initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 7 minutes. Amplified DNA was analysed by electrophoresis on 1.5% agarose gel stained with GelRed stain (Biotium, USA).

RESULTS

From the results obtained, the prevalence of pasteurellosis in Malaysia based on cases submitted to VRI (Table 2) ranged from 1.0% to 3.2% (2014 to 2016). The prevalence is low compared to previous reported prevalence which was 27.8% in year 2012 and 11.46% in Selangor (Always *et al.*, 2012; Nafizah *et al.,* 2014). The low prevalence rate in current report may due to passive sampling based on cases received in VRI.

Capsular typing is crucial for practical classification to different serotypes associated with specific disease conditions from a diagnostic or epidemiological point of view. Currently, VRI adopts serogroup designation system of Carter's capsular typing and molecular typing method as described by Townsend *et al*. (2001). These two methods were applied as a routine diagnostic tool in serogrouping of *P. multocida*.

Table 1. The capsule serogroup, primer sequences and amplicon sizes.

Capsule serogroup	Primer sequence (5' – 3')	Amplicon (bp)
CapA	TGC-CAA-AAT-CGC-AGT-CAG TTG-CCA-TCA-TTG-TCA-GTG	1044 bp
CapB	CAT-TTA-TCC-AAG-CTC-CAC-C GCC-CGA-GAG-TTT-CAA-TCC	760 bp
CapD	TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG	657 bp
CapE	TCC-GCA-GAA-AAT-TAT-TGA-CTC GCT-TGC-TGC-TTG-ATT-TTG-TC	511 bp
CapF	AAT-CGG-AGA-ACG-CAG-AAA-TCA-G TTC-CGC-CGT-CAA-TTA-CTC-TG	851 bp

Table 2. Distribution of *P. multocida* from different animal hosts in Malaysia (2014 to 2016).

Animal hosts	Year			Total
	2014	2015	2016	
Cattle	-	8	12	20
Goat	15	9	2	26
Pig	-	3	-	3
Chicken	-	4	3	7
Duck	-	7	1	8
Rabbit	-	1	-	1
Total	15	32	18	65

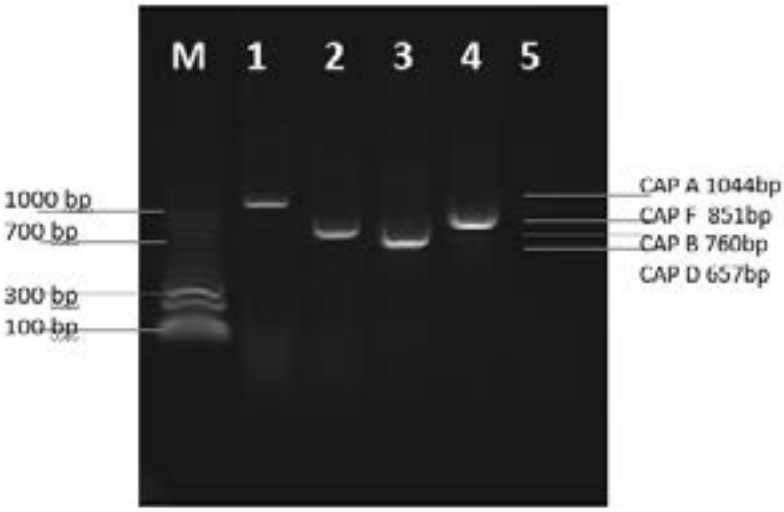

Figure 3. Lane M, 100 bp ladder; lane 1, *Pasteurella multocida* serogroup A; lane 2, *Pasteurella multocida* serogroup B; lane 3, *Pasteurella multocida* serogroup D; lane 4, *Pasteurella multocida* serogroup F; lane 5, negative control.

Table 3. Distribution of different capsular serogroups of 65 *P. multocida* strains determined by molecular serogrouping (multiplex PCR) method.

Animal hosts	*P. multocida* and its serogroups					Total
	A	B	D	F	U	
Cattle	18	-	1	-	1	20
Goat	1	1	18	1	5	26
Pig	-	-	1	1	1	3
Chicken	1	-	-	1	5	7
Duck	7	-	1	-	-	8
Rabbit	-	-	-	-	1	1
Total	27	1	21	3	13	65

U = Untypeable strains

About 20.0% (13/65) of the *P. multocida* isolates tested in this study were unable to be serogroup using both conventional and molecular method (Figure 3), therefore they were classified as untypeable (Table 3). These non-capsulated strains were isolated from almost all animal hosts. In the study by Arumugam *et al*. (2011), 22% of isolates were also classified as untypeable. Although the pathogenicity of specific serotypes of *P. multocida* strains were well described by many studies (Harper *et al*., 2006), the presence of these non-capsulated strains in disease with mixed infections should not be neglected, because the pathogenicity is unknown and it is difficult to distinguish between *P. multocida* and untypeable infection.

The pattern of predominant serogroup and animal hosts were found to be changing every year. In 2014, 80.0% (12/15) of the isolates were *P. multocida* Carter's type D where all were isolated from goats. In 2015, the predominant serogroup changed to *P. multocida* Carter's type A with a prevalence rate of 40.6% (13/32) which were mostly isolated from duck and cattle. While for *P. multocida* Carter's type D, the prevalence in 2015 reduced to 21.9% (7/32) compared to the previous year and it were isolated from various animal species.

Interestingly, in 2015 there was one isolate of *P. multocida* Carter's type B found in goat. However, there was no reported outbreak of pasteurellosis following this finding. On the other hand, *P. multocida* serogroup B causing haemorrhagic septicaemia (HS) has dropped significantly with no positive cases been diagnosed in VRI since 2006 (VRI Annual Report, 2005). This might suggest that vaccination againt HS is effective in control and prevention of the disease in Malaysia.

In 2016, prevalence of *P. multocida* Carter's type A increased to 72.2% (13/18), with a high percentage (92.3%) isolated from cattle. This occurrence was found to be different and need to be highlighted because there is increasing trend of *P. multocida* Carter's type A infection that causes respiratory disease in cattles. Moreover, it causes high mortality and morbidity with clinical signs in young calves in infected farms. This is compared to a study by Nafizah *et al*. (2014), where the prevalence of pasteurellosis in cattle and poultry was only 9.3% from 2009 to 2013.

Furthermore, in this 3-year study, 3 isolates of *P. multocida* serogroup F were also identified each from pig, goat and chicken. This shows that serogroup F can affect various animal hosts as was reported in turkey, rabbit and pig (Jaglic *et al*., 2005; Zhong *et al*., 2017).

CONCLUSION

Pasteurellosis has become sporadic in Malaysia and the distribution of serogroup is diverse in all species of animal with no definitive host. Control and prevention of pasteurellosis depend much on herd management by reducing the stress factors and planned vaccination programmes. Continuous surveillance is needed to monitor this respiratory disease as the economic impact due to treatment cost and mortality will definitely affect the livestock industry.

REFERENCES

1. Always N.S., Abdul Rahim M. and Murugaiyah M. (2012). Prevalence of *Mannhaemia haemolytica* and *Pasteurella multocida* in goats from selected farms in Selangor, Malaysia. In: *7th Proceedings of the Seminar on Veterinary Science*, p 112.
2. Arumugam N.D., Ajam N., Blackall P.J., Asiah N.M., Ramlan M., Maria J., Yuslan S. and Thong K.L. (2011). Capsular serotyping of *Pasteurella multocida* from various animal hosts – a comparison of phenotypic and genotypic methods. *Tropical Biomedicine,* **28(1):** 55-63.
3. Carter G.R. (1955). Studies on *P. multocida*. A haemagglutination test for identification of serological types. *Amer. J. Vet. Res.,* **16:** 481-484.
4. Carter G.R. and Rundell S.W. (1975). Identification of type A strains of *P. multocida* using staphylococcal hyaluronidase. *Veterinary Record* **96:**343.
5. Carter G.R. and Subronto P. (1973). Identification of Type D strains of *Pasteurella multocida* with acriflavine. *American Journal of Veterinary Research* **34:** 293-294.
6. Furian T.Q., Borges K.A., Pilatti R.M., Almeida C., Nascimento V.P. do, Salle C.T.P. and Moraes H.L. de S. (2014). Identification of the capsule type of *Pasteurella multocida* isolates from cases of fowl cholera by multiplex PCR and comparison with phenotypic methods. *Brazilian Journal of Poultry Science,* **16(2):** 31-36.
7. Harper M., Boyce J.D. and Adler B. (2006). *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *Federation of European Microbiological Societies Microbiol Lett*, **265:** 1–10.
8. Jaglic Z., Kucerova Z., Nedbalcova K., Pavlik I., Alexa P., Bartos M. (2005). Characterisation and comparison of *Pasteurella multocida* isolated from different species in the Czech Republic: capsular PCR typing, ribotyping and dermonecrotoxin production. *Vet. Med. Czech,* **50(8):** 345-354.
9. Joseph P.G. (1979). Hemorrhagic septicaemia in Peninsular Malaysia. *Kajian Veterinar.* **11:** 65-79.
10. Mohamed R.A. and Abdelsalam E.B. (2008). A review on pneumonic pasteurellosis (respiratory mannheimiosis) with emphasis on pathogenesis, virulence mechanisms and predisposing factors. *Bulgarian Journal of Veterinary Medicine,* **11(3)**:139-160.
11. Nafizah M., Roseliza R., Khoo E., Khoo L.L., Rosnah Y., Zamila Z., Saifu Nazri R. and Ramlan M. (2014). Prevalence of pasteurellosis from various animals species diagnosed in VRI from year 2009 to 2013. *Malaysian Journal of Veterinary Research,* **5(1):**216-217.
12. Peng Z., Liang W., Wang Y., Liu W., Zhang H., Yu T., Zhang A., Chen H. and Wu B. (2017). Experimental pathogenicity and complete genome characterisation of a pig origin *Pasteurella multocida* serogroup F isolate HN07. *Veterinary Microbiology,* **198:** 23–33.
13. Quinn P., Carter M.E., Markey B.A. and Carter G.R. *Clinical Veterinary Microbiology*. London: Wolfe Publishing, 1994.
14. Townsend K.M., Boyce J.D., Chung J.Y., Frost A.J. and Adler B. (2001). Genetic organization of *Pasteurella multocida* cap Loci and development of a multiplex capsular PCR typing system. *J. Clin. Microbiol.* **39(3):** 924-929.
15. Veterinary Reseach Institute (VRI) Malaysia, (2006). *Annual Report 2005*.

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