VIRULENCE GENES PROFILE OF HAEMORRHAGIC SEPTICAEMIA (HS)-CAUSING *PASTEURELLA MULTOCIDA* SEROGROUP B IN MALAYSIA

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ABSTRACT. Haemorrhagic septicaemia (HS) is a disease caused by *Pasteurella multocida* serogroup B in the Asian region. The disease mainly affects cattle and buffaloes causing high mortality due to septicaemia. HS is a notifiable disease in Malaysia and Veterinary Research Institute (VRI) is the main local HS vaccine producer. However, there are still sporadic outbreaks in the East Coast of Peninsular Malaysia in the year 2017. This study is conducted to characterise the virulence profile of HS-causing *P. multocida* serogroup B isolates from outbreak cases. All ten isolates showed only a single type of virulence genes profile. The nine virulence associated genes (*hgbA, ExBDtonB, ptfA, pfhA, sodA, sodC, nanB, oma87* and *nanH*) were present in 100% (10/10) of the isolates, while the remaining three genes (ompH, hgbB, and toxA) were not detected. Virulence genes profile of Malaysia *P. multocida* serogroup B isolates differ from isolates from other geographical locations. This study found no virulence gene profile variation between vaccine seed and field isolates. The sporadic HS outbreak in 2017 may be due to other contributing factors on the ground, such as vaccination schedule or delivery failure. Study and characterisation of potential vaccine candidates is needed for disease prevention.

Keywords: Pasteurella multocida, serogroup B, haemorrhagic septicaemia, virulence genes

INTRODUCTION

Haemorrhagic septicaemia (HS) is a disease caused by *Pasteurella multocida*, particularly serotype B in Asian region and serotype E in African region. The disease mainly affects cattle and buffaloes causing high mortality where the animals will show clinical signs such as fever, respiratory distress with nasal discharge, frothing from the mouth, eventually causing recumbency and death due to septicaemia (OIE Terrestrial Manual 2012). In Malaysia, HS caused significant losses to farmers during the early 1940s and in the 1980s (Joseph, 1979). HS is listed as a notifiable disease by the Department of Veterinary Services Malaysia (DVS). Since then, DVS Malaysia and Veterinary Research Institute (VRI) remain as the main producer for HS vaccine (alum precipitated and oil adjuvant) using local field isolate. However, there are still sporadic outbreaks in the east coast of Peninsular Malaysia in the year 2017.

Profiling of virulence genes is needed to understand the pathogenicity of our local *P. multocida* serogroup B isolates in the manifestation of haemorrhagic septicaemia, it is especially important when VRI produces local vaccines to protect and ensure the sustainability of local livestock industries. The virulence genes that have been studied includes lipopolysaccharides (LPS), outer membrane protein (OMP), iron acquisition related factors, bacterial adhesions and

colonisation factors, extracellular enzymes and toxin production (Ewers *et al.*, 2006 and Gharibi *et al.*, 2017). This study is conducted to characterise the virulence genes profile of *P. multocida* serogroup B isolates from outbreak cases.

MATERIALS AND METHOD

Bacterial strains

Ten *P. multocida* serogroup B isolates, which is one vaccine seed C82 and nine isolates from outbreak cases were included in this study. The isolates were revived from stored maintenance media and cultured on 5% ox blood agar by overnight incubation at 37 °C. The bacteria were confirmed by observation of the colony morphology, Gram staining and a series of biochemical tests according to Quinn *et al.*, (1994) prior to molecular analysis.

DNA extraction

The bacterial DNA was extracted from overnight culture grown on blood agar. Few colonies 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 5 µl of the supernatant was used as DNA template. All the isolates were re-confirmed as *P. multocida* serogroup B by multiplex capsular PCR typing and HS-causing type-B-specific PCR assay (Table 1) prior to analysis of the virulence genes.

Virulence genes profile by multiplex PCR

Three sets of multiplex PCR (Table 2) was performed to detect the twelve virulence genes (Table 3) in a total reaction volume of

Table 1. The primer name, sequence, amplicon sizes and reference. (Townsend *et al.*, 1998 and 2001)

Primer name	Primer sequence (5' – 3')	Amplicon (bp)
CapB	CAT-TTA-TCC-AAG-CTC-CAC-C GCC-CGA-GAG-TTT-CAA-TCC	760
KTT72 KTSP61	AGG-CTC-GTT-TGG-ATT-ATG-AAG ATC-CGC-TAA-CAC-ACT-CTC	620

Table 2. Multiplex PCR protocols and targeted virulence genes. (Gharibi *et al.*, 2017)

Multiplex PCR	Target virulence genes
1	*ptfA, hgbA, sodA, pfhA*
2	*ExBDtonB, toxA, , ompH, nanH*
3	*oma87, hgbB, nanB, sodC*

Table 3. The virulence genes, primer sequences and amplicon sizes. (Ewers *et al.*, 2006 and Gharibi *et al.*, 2017)

Gene	Virulence associated genes	Primer sequence (5′ – 3′)	Amplicon (bp)
Outer membrane proteins			
oma87	Porin	ATG-AAA-AAA-CTT-TTA-ATT-GCG-AGC TGA-CTT-GCG-CAG-TTG-CAT-AAC	948
ompH	Porin	CGC-GTA-TGA-AGG-TTT-AGG-T TTT-AGA-TTG-TGC-GTA-GTC-AAC	438
Iron acquisition genes			
hgbA	Iron uptake	TGG-CGG-ATA-GTC-ATC-AAG CCA-AAG-AAC-CAC-TAC-CCA	419
hgbB	Iron uptake	ACC-GCG-TTG-GAA-TTA-TGA-TTG CAT-TGA-GTA-CGG-CTT-GAC-AT	788
ExBDtonB	Iron metabolism	GGT-GGT-GAT-ATT-GAT-GCG-GC GCA-TCA-TGC-GTG-CAC-GGT-T	1144
Adhesins			
ptfA	Type IV fimbriae	TGT-GGA-ATT-CAG-CAT-TTT-AGT-GTG-TC TCA-TGA-ATT-CTT-ATG-CGC-AAA-ATC-CTG-CTG-G	488
pfhA	Hemagglutinin	AGC-TGA-TCA-AGT-GGT-GAA-C TGG-TAC-ATT-GGT-GAA-TGC-TG	275
Extracellular enzyme			
sodA	Superoxide dismutases	TAC-CAG-AAT-TAG-GCT-ACG-C GAA-ACG-GGT-TGC-TGC-CGC-T	361
sodC	Superoxide dismutases	AGT-TAG-TAG-CGG-GGT-TGG-CA TGG-TGC-TGG-GTG-ATC-ATC-ATG	235
toxA	Dermonecrotoxin	CTT-AGA-TGA-GCG-ACA-AGG-TT GGA-ATG-CCA-CAC-CTC-TAT-A	865
Sialidases			
nanB	Sialidase	GTC-CTA-TAA-AGT-GAC-GCC-GA ACA-GCA-AAG-GAA-GAC-TGT-CC	554
nanH	Sialidase	GAA-TAT-TTG-GGC-GGC-AAC-A TTC-TCG-CCC-TGT-CAT-CAC-T	360

25 µl using MyTaq™ Mix (Bioline, UK), 0.5 µl of each primer and 5.0 µl of DNA template. All multiplex PCR amplification reactions were performed in a thermal cycler (Eppendorf, Germany) with 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 SYBR™ Safe DNA Gel Stain (Invitrogen, USA) and visualized using gel documentation system (Major Science, USA). The molecular size of PCR products were compared with a 100bp DNA HyperLadderTM (Bioline, UK).

RESULTS

All ten *P. multocida* serogroup B isolates showed only a single type of virulence genes profile. The information of the isolates and results were listed in Table 4. The nine virulence associated genes *hgbA, ExBDtonB, ptfA, pfhA, sodA, sodC, nanB, oma87* and *nanH* were present in 100% (10/10) of the isolates (Figure 1). These genes were responsible to code for all five main components of the *P. multocida* serogroup B virulence traits, which are outer membrane protein (*oma87*), iron acquisition gene (*hgbA* and *ExBDtonB*), adhesins (*ptfA* and *pfhA*), extracellular enzyme (*sodA* and *sodC*) and sialidases (*nanB* and *nanH*). Remaining three genes, which were *ompH, hgbB*, and *toxA* was not identified in all of the isolates.

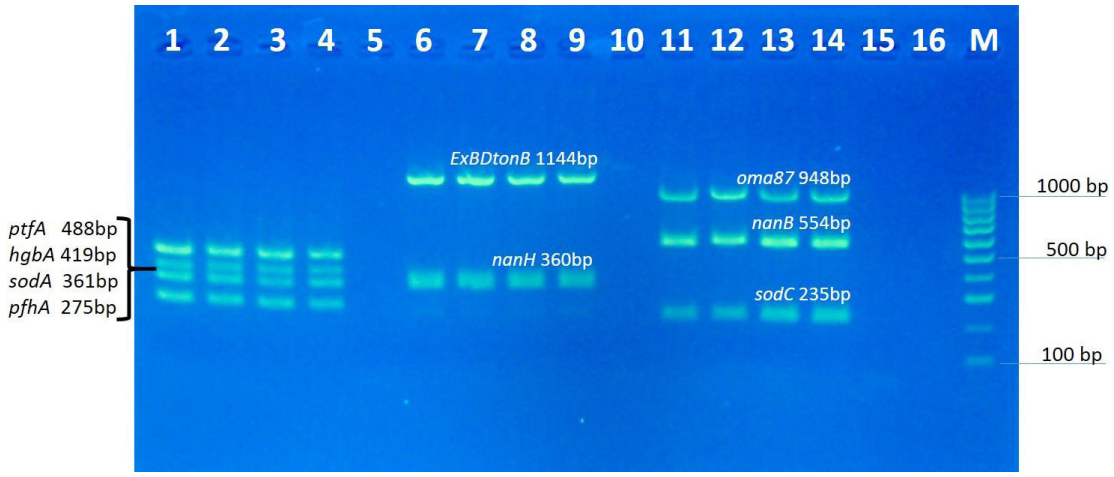

Figure 1. Agarose gel electrophoresis for four *P. multocida* serogroup B isolates in three different sets of multiplex PCR. Lane 1-4, multiplex PCR 1 (*ptfA, hgbA, sodA, pfhA*); lane 6-9, multiplex PCR 2 (*ExBDtonB, toxA, ompH, nanH*); lane 11-14, multiplex PCR 3 (*oma87, hgbB, nanB, sodC*); lane 16, negative control; lane M, marker, 100bp DNA ladder.

Table 4. Virulence gene profile of *P. multocida* serogroup B isolates.

Sample ID	Year	Host	Location	*ptfA*	*hgbA*	*sodA*	*pfhA*	*ExBDtonB*	*toxA*	*ompH*	*nanH*	*oma87*	*hgbB*	*nanB*	*sodC*
C82	N.A.	N.A.	N.A.	+	+	+	+	+	-	-	+	+	-	+	+
5712	2017	Cattle organ	Marang, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
5954	2017	Cattle organ, bone	Marang, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
6371	2017	Buffalo organ	Kuala Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
6389	2017	Cattle culture	Dungun, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
6497	2017	Buffalo bone	Marang, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
7586	2017	Buffalo organ	Dungun, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
7587	2017	Cattle organ	Dungun, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
8039	2017	Cattle bone	Kuala Berang, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+
10055	2018	Cattle culture	Kemaman, Terengganu	+	+	+	+	+	-	-	+	+	-	+	+

DISCUSSION

In this study, only a single type of virulence profile was observed from all ten *P. multocida* serogroup B isolates (Table 4). The virulence genes profile of Malaysia *P. multocida* serogroup B isolates differ from isolates from other geographical location such as England, Pakistan and Kazakhstan (Davies, 2004; Gharibi *et al.*, 2017; Orynbayev *et al.*, 2019) in terms of the absence of *ompH* gene, which encodes for outer membrane protein that function as a protective antigen. Detection of *ompH* gene was reported to be associated with *P. multocida* serogroup A isolated from cattle and birds. Malaysia *P. multocida* serogroup B isolates contain only two out of three iron acquisition genes of interest, which are *hgbA* and *ExBDtonB* that is needed for iron uptake into the periplasmic space. The absence of *hgbB* gene, one of the iron acquisition genes in this study was in agreement with Laxmi *et al.*, 2014, where the gene was found to be more prevalent in avian isolates, compared to large ruminant isolates. The absence of *toxA* gene encoding for dermonecrotoxin was in agreement with Harper *et al.*, 2006, Laxmi *et al.*, 2014 and Orynbayev *et al.*, 2019. Furian *et al.*, 2013 also reported negative for *toxA* gene in *P. multocida* of avian origin. Prevalence of the gene *toxA* was observed mainly in *P. multocida* serogroup D, which is responsible for pathogenesis of atrophic rhinitis in pig and serogroup A for respiratory disease in sheep and goat.

The *oma87* gene was present in all isolates, which is similar to studies by Davies *et al.*, 2004 and Gharibi *et al.*, 2017. All strains of *P. multocida* serogroup B in this study contains all two adhesion genes (*ptfA* and *pfhA*) that code for adhesion protein that play a major role in pathogenesis whereby the bacteria can easily attach, invade and colonise the host's epithelial cells. Besides, they also contain two genes that code for sialidases (*nanB* and *nanH*) that remove sialic acid conjugated to glycoproteins and glycolipids of host cells to be use as carbon source to infect host cells and inhibit host immune system (Furian *et al.*, 2013). All the isolates also possess two out of three genes encoded for extracellular enzyme (*sodA* and *sodC*), which is superoxide dismutases that have antioxidant function that was merely discussed in other studies.

Generally, there are two types of HS vaccines produced and used in Malaysia, which are alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV). This study found no virulence gene profile variation between vaccine seed and field isolates. Some unsuccessful prevention of HS in our country may be due to other contributing factors on the ground, such as vaccination schedule or delivery failure. Guidelines for vaccination regime is outlined in haemorrhagic septicaemia, OIE Terrestrial Manual 2012. Continuous study to characterise local *P. multocida* serogroup B isolates used for vaccine production is crucial to ensure production of quality and effective vaccine for HS disease outbreak prevention. This is because the bacteria might lose its pathogenic character due to single genetic events, such as point mutation, insertion, or deletion of DNA after some time.

CONCLUSION

This study found no virulence gene profile variation between vaccine seed and field isolates. The sporadic HS outbreak in 2017 may be due to other contributing factors on the ground, such as vaccination schedule or delivery failure. In the future, the study and evaluation of new potential vaccine candidates using other genotypic fingerprinting methods such as 16S DNA sequence is recommended.

REFERENCES

1. Davies, R.L. (2004). Genetic diversity among Pasteurella multocida strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16s rRNA gene. *Microbiology (Reading).* doi: 10.1099/mic.0.27409-0.PMID:15583172.
2. Ewers, C., Lübke-Becker, A., Bethe, A., Kiebling, S., Filter, M. and Wieler, L.H. (2006). Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. *Veterinary Microbiology,* 114(3-4): 304-317.
3. Furian, T.Q., Borges, K.A., Rocha, S.L.S., Rodrigues, E.E., do Nascimento, V.P., Salle, C.T.P. and Moraes, H.L.S. (2013). Detection of virulence-associated genes of *Pasteurella multocida* Isolated from cases of fowl cholera by multiplex-PCR. *Pesquisa Veterinária Brasileira* 33(2):177-182.
4. Gharibi, D., Hajikolaei, M.R.H., Ghorbanpour, M. and Barzegar, S.K. (2017). Virulence gene profiles of *Pasteurella multocida* strains isolated from cattle and buffalo. *Veterinarski Arhiv,* 87(6): 677-690.
5. 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.
6. Joseph, P.G. (1979). Hemorrhagic septicaemia in Peninsular Malaysia. *Kajian Veterinar,* 11: 65-79.
7. Laxmi, N.S., Adyasha, P., Santosh, K., Prasad, T., Santosh, K.G., Viswas, K.N. and Vijendra, P.S. (2014). Virulence genotyping of *Pasteurella multocida* isolated from multiple hosts from India. *The Scientific World Journal,* Volume 2014: 1-10.
8. OIE. (2012). *Chapter 2.4.12. Haemorrhagic septicaemia.* In: OIE Terrestrial Manual. France: World Organisation for Animal Health.
9. Orynbayev, M., Sultankulova, K., Sansyzbay, A., Rystayeva, R., Shorayeva, K., Namet, A., Fereidouni, S., Ilgekbayeva, G., Barakbayev, K., Kopeyev, S. and Kock, R. (2019). Biological characterisation of *Pasteurella multocida* present in the Saiga population. *BMC Microbiology,* 19(37): 1-10.
10. Quinn, P., Carter, M.E., Markey, B.A. and Carter, G.R. (1994). *Clinical veterinary microbiology.* London: Wolfe Publishing.
11. Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M. and Dawkins, H.J.S. (1998). Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology,* 36(4): 1096-1100.
12. Townsend, K.M, Boyce, J.D., Chung, J.Y., Frost, A.J. and Adler, B. (2001). Genetic organisation of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. *Journal of Clinical Microbiology,* 39(3): 924-929.

ACKNOWLEDGEMENT. The authors would like to thank the Director-General of Department of Veterinary Services Malaysia, Director of Veterinary Research Division DVS and Director of Veterinary Research Institute (VRI) for the permission to publish this study. Special thanks also to the staff of the Bacteriology Section, for their contribution in this study. This study was financially supported by VRI Ipoh.