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Molecular Characterisation of the GdhA$^-$ Derivative of *Pasteurella multocida* B:2

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ABSTRACT

Pasteurella multocida B:2 is an important veterinary pathogen causing fatal and acute haemorrhagic septicaemia (HS) in bovine. A live vaccine candidate, *P. multocida* B:2 GDH7 was reported to enable protection in cattle and buffaloes via intranasal (i. n.) administration. This potential vaccine was also reported to be self-transmitted from the vaccinated animal to the free-ranging animals allowing wider vaccination coverage. Prior to commercialisation, this potential vaccine requires further characterisation in accordance with the authoritative guidelines from the World Organisation for Animal Health (OIE). Hence, in this study, the potential vaccine strain, *P. multocida* B:2 GDH7 and the virulent parent strain were characterised through genomic and proteomic profiling. A crucial first step was to develop a sensitive yet simple and robust identification test to differentiate both strains which has been achieved by the development of a precise yet straightforward PCR method. In genomic profiling, Repetitive Extragenic Palindromic sequence-PCR (REP-PCR)

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was manipulated and both strains have a different display of genomic DNA band patterns. Some of the major OMPs were observed and prominent immunogens of *P. multocida*, OmpA and OmpH were observed to be expressed differently between these strains through SDS-PAGE analysis. In conclusion, a reproducible PCR detection method has enabled differentiation of both strains. Further characterisation of these strains shows a significantly different profile through genomic and proteomic profiling.

Keywords: Haemorrhagic septicaemia, live-attenuated vaccine, OMPs, *Pasteurella multocida*, REP-PCR

INTRODUCTION

Hemorrhagic septicaemia (HS) is a major disease in cattle and buffaloes which is caused by the infection of *Pasteurella multocida* B:2 or E:2. In Asia, the serotype of the bacteria responsible for the disease is *P. multocida* B:2 (Zamri-Saad, 2013). This endemic disease causes great economic losses particularly towards farmers due to high mortality rate and rapid transmission of an outbreak field to the adjacent field (Zamri-Saad & Annas, 2016). Transmission occurred from diseased animals or carriers through intranasal and oral routes (Abubakar & Zamri-Saad, 2011). Invasion of the bacteria through endothelial cells resulted in rapid infiltration of the animals' bloodstream (Galdiero et al., 2001). High occurrence of outbreak happened during monsoon or raining season due to the weakening of immune response of the animals making them susceptible to the infection (Zamri-Saad, 2013). Vaccination is an effective routine for controlling outbreak of HS especially in Asian countries due to semi-wild rearing methods of the animals (Zamri-Saad, 2013).

Vaccines for HS usually are registered prior to rainy season using oil-adjuvant vaccine or alum-precipitated vaccine. Despite both vaccines containing bacterin, only short-termed protection was detected (Zamri-Saad, 2013) and also tedious administration process, resulting in ineffective disease outbreak control. Therefore, live-attenuated vaccine was recommended to control HS outbreak efficiently such that this type of vaccine enabled mimicry of natural route of infection like the wild-type (De Alwis, 1999). Live-attenuated vaccine consisted of live organisms such as the attenuated bacteria with reduced virulence when compared to the wild-type (Zamri-Saad, 2013). A local strain of *P. multocida* B:2 from previous outbreak was attenuated into a derivative known as *P. multocida* B:2 GDH7. The mutant was generated through the disruption of the *gdhA* gene by the insertion of kanamycin cassette (Othman et al., 2017). This resulted in the arrested metabolism and thus weakened the pathogenicity of the mutant. Since currently available vaccines such as alum-precipitated vaccine and oil-adjuvant vaccine were discovered to be less effective, a new alternative is paramount. The aforementioned *gdhA* gene disruption has been found to be a promising manipulation for non-pathogenic *P. multocida* B:2 vaccine

development (Othman et al., 2012). Field studies were performed previously to show its effectiveness and apathogenicity when challenged (Rafidah et al., 2012; Rafidah & Zamri-Saad, 2013). However, the characteristics of the mutant were yet to be thoroughly scrutinized. Therefore, further information of the developed live vaccine can enable comprehension on its efficacy for HS control and prevention.

For commercialisation purposes, upscaling the potential vaccine strain *P. multocida* B:2 GDH7 with an economical media is recommended when compared to the commercial available media (Sarwar et al., 2013). This establishment will provide a more competitive and sustainable product in the market. Hence, a minimal media known as YDB media was formulated with the purpose to reduce production cost of *P. multocida* B:2 GDH7 as vaccine strain for HS (Hazwani-Oslan et al., 2017; Oslan et al., 2018). The formulated media has enabled significant reduction by about 10-fold per litre of the cell production with no significant effect on cells concentration when compared to the commercially available media (Oslan et al., 2018).

The organisation specified in the animals' health concerns, World Organisation for Animal Health or also initially known as Office International des Epizooties (OIE) has regulated the production of vaccines in order to assure safe vaccines for animal diseases control in each country (World Organisation for Animal Health [OIE], 2008, 2016). However, the profile characterisation of the attenuated strain, *P. multocida* B:2 GDH7 has yet to be fully apprehended. In this study, a conventional PCR assay was established as a detection method to distinguish *P. multocida* B:2 GDH7 from its parent strain. Hence, unique primers to the attenuated *P. multocida* B:2 GDH7 were generated. Genotypic and phenotypic profiling of a modified bacterial strain is vital in ensuring vaccine quality according to the OIE's vaccine production guidelines. Therefore, the information on the genomic and proteomic profiles of the bacterium used for vaccination will enable better understanding towards the vaccine and its efficacy prior to commercialisation.

METHODS

Bacterial Strains

Pasteurella multocida strains used in this study were listed in Table 1. All *P. multocida* strains were cultured on Brain-Heart Infusion (BHI) (Oxoid, UK) or YDB modified media agar plates or in broth. YDB is a minimal media developed by Oslan et al. (2018) to replace BHI media for the purpose of high-scale production of *P. multocida* B:2 GDH7 as a live-attenuated vaccine for HS. The bacteria were cultured in 37°C and shaken at 180 rpm if in broth.

Genomic DNA Extraction

Genomic DNA extraction of all *P. multocida* strains were performed using overnight cultures. Overnight cultures of each *P. multocida* were pelleted down by centrifugation at 6, 000 x*g* at 4°C. The pellets were used for the genome extraction

Table 1

List of bacteria used in this study

Bacteria name	Description	References
Pasteurella multocida B:2 wild-type	Isolated from an outbreak case in Malaysia, 2006	Othman (2007)
Pasteurella multocida B:2 GDH7	Δ*gdhA* mutant derived from the wild-type strain above	Othman (2007)
Pasteurella multocida B:2 JRMT12	Δ*aroA* mutant derived from the wild-type strain from Sri Lanka	Tabatabaei et al. (2002)

using MYgen™ Genomic DNA Prep Kit from Gene Express (Gene Xpress PLT, Malaysia) according to the manufacturer's protocol. The concentration of the extracted genomic DNA was measured using Implen NanoPhotometer™. The extracted genomic DNA was maintained and stored at -20°C for long-term storage.

PCR Analysis

PCR amplification was carried out with 20 µL of PCR mixture consisting of 50 ng genomic DNA as template, 10 µM of both forward and reverse primers pairs (Table 2), 1X PCR buffer, 20 µM dNTPs mix, 40 µM $MgCl_2$ and 6 U *Taq* polymerase (Thermo Fisher Scientific, US). Eppendorf Mastercycler® Nexus Thermal Cycler was used to amplify with initial denaturation at 95°C for 3 min following with 30-35 cycles of denaturation at 95°C for 30 sec, annealing of the primers at selected temperature (Table 2) for 30 sec and extension at 72°C for 1-5 min. A final extension step was added at 72°C for 10 min. The amplified PCR products were electrophoresed in 1-2% agarose gel (Vivantis, Malaysia) stained with HealthView™ nucleic acid stain (Genomics BioSci &Tech, Taiwan). The nucleic acid stain is a safe option compared to ethidium bromide.

Table 2

List of primers used for detection between Pasteurella multocida *B:2 strains*

Primer name	Primer sequence	Targeted region	Expected fragment size (bp)	Annealing temperature (°C)
Kan Forward	TCGAGCATCAAATGAAACTG	Kanamycin resistance gene (KR)	794[a]	51.3
Kan Reverse	TATTCAACGGGAAACGTCTT			

Table 2 *(Continued)*

Primer name	Primer sequence	Targeted region	Expected fragment size (bp)	Annealing temperature (◦C)
CD1 Forward	CCGCCTTCACTTCTGTTGAATACTC	Probable region containing kanamycin cassette (*gdhA* upstream)	475[ab]	60.7
CD1 Reverse	CCCCATTGGTAATGTGGTTAACGCG			
CD2 Forward	ATGGCGTACAATTAGTGGCTGAAGG	Probable region containing kanamycin cassette (*gdhA* downstream)	389[ab]	59.8
CD2 Reverse	CTTTCTTGACGAACAAAGTGCGGTG			

Note. [a]The expected size was derived from preliminary *in silico* analysis of primers and the targeted sequence respectively based on the parent strain's genome. [b]Addition of a kanamycin cassette with 1, 200 bp is expected to be present in *Pasteurella multocida* B:2 GDH7

REP-PCR Analysis

Repetitive element palindromic-PCR (REP-PCR) analysis in this study was based from Townsend et al. (1997) with minor modifications. PCR mixture with 25 µL containing 50 ng of genomic DNA template, 20 pmol of primer pairs REP1R-Dt (NNNNCGNCGNCATCNGGC) and REP2-IDt (NCGNCTTATCNGGCCTAC), 1.5 U *Taq* polymerase, 125 µM of dNTPs mix, 80 µM $MgCl_2$ and 1X *Taq* Buffer (Thermo Fisher Scientific, US). REP amplification was performed using Eppendorf Mastercycler® nexus thermal cycler with initial denaturation at 95◦C for 3 min following with 30 cycles of denaturation at 95◦C for 30 s, annealing at 45◦C for 1 min and annealing at 72◦C for 3 min. Final extension step was added at 72◦C for 10 min. The amplified REP-PCR products were electrophoresed using agarose gel of 2%.

Cell-Free Proteins Extraction of *Pasteurella multocida* B:2 Strains

Cell-free proteins extraction method was slightly modified from Ataei et al. (2009). Cell-free extracts (CFEs) are proteins obtained by mechanical disruption of bacterial cells to release the cytosolic proteins and the disrupted membrane proteins. A few colonies of each strain of *P. multocida* B:2 was inoculated into respective broth (5 mL) to culture overnight at 37◦C, shaken at 180 rpm. Each 16-18 h cultures was further inoculated into fresh 100 mL (1:500) of broth to grow overnight until it reached OD_{600nm} >1.2. The cultures were

harvested by centrifugation at 6, 000 x*g*, 30 min, 4°C. The cell pellets were washed with PBS and centrifuged at 6, 000 x*g*, 30 min, 4°C. The pellets were resuspended in 5 mL of cold PBS and sonicated for 5 min with 40% amplitude. The sonicated suspension was centrifuged at 6, 000 x*g* for 30 min at 4°C to pellet down the remaining unbroken cells. The CFEs of each *P. multocida* B:2 strains were quantified using Bradford's assay (Bradford, 1976) and stored in -20°C.

Outer Membrane Proteins (OMPs) Extraction of *Pasteurella multocida* B:2 Strains

Extraction of *P. multocida* B:2 strains' OMPs in this study was referred from Prasannavadhana et al. (2014) and Wheeler et al. (2009) with modifications. A few colonies of each strain of *P. multocida* B:2 was inoculated into respective broth (5 mL) to culture overnight at 37°C, shaken at 180 rpm. Each of the overnight cultures (800 µL) was further inoculated into fresh 400 mL (1:500) of broth to grow overnight until it reached OD_{600nm} >1.2. The cultures were harvested by centrifugation at 6, 000 x*g* for 30 min at 4°C. 45 mL of cold 20 mM Tris-HCl (pH 7.2) was added to wash pellet and centrifuged at 6, 000 x*g* for 30 min at 4°C. The pellets were resuspended in 5 mL of cold 20 mM Tris-HCl (pH 7.2) and sonicated for 10 min with 40% amplitude. The sonicated suspension was centrifuged at 6, 000 x*g* for 30 min at 4°C to collect the remaining unbroken cells. The supernatant containing cytoplasmic proteins, cell membrane elements and OMPs was collected and cold 20 mM Tris-HCl (pH 7.2) was added to the suspension until it reached 10 mL. The supernatant was centrifuged using Beckman Coulter Optima™ ultracentrifuge at 100, 000 x*g* for 1 h at 4°C. The resulting pellet was resuspended with 5 mL of 0.5% (w/v) sodium N-lauroylsarcosine (Sarkosyl) (Thermo Fisher Scientific, US) using sterile long-form Pasteur pipette. The sarkosyl solubilisation was done at room temperature for 20 min with intermittent resuspension of the protein pellet. The 0.5% sarkosyl was added to the suspension until it reached 10 mL and then centrifuged at 100, 000 x*g* for 1 h at 4°C to separate the soluble cytoplasmic proteins with the insoluble OMPs. The OMPs pellet was washed with 10 mL of cold 20 mM Tris-HCl (pH 7.2). The suspension was again centrifuged at 100, 000 x*g* for 1 h at 4°C. The OMPs pellet was resuspended in ≤500 µL of 20 mM Tris-HCl (pH 7.2). The OMPs of each strain of *P. multocida* B:2 were quantified with Bradford's assay (Bradford, 1976) and then stored in -20°C.

SDS-PAGE Analysis of Proteins

The proteins were analysed through SDS-PAGE method by Laemmli (1970) with slight modifications. The gel was run with 1X SDS-PAGE running buffer at 70 V for 1 h 45 min. Coomassie-blue staining method was used to visualize and to approximate the proteins bands from the respective proteomic profiles.

RESULTS AND DISCUSSION

Identification and Differentiation Between *Pasteurella multocida* B:2 Wild-type and *P. multocida* B:2 GDH7

The amplification of *gdhA* gene from *P. multocida* B:2 wild-type only was successful whereas identification and differentiation of *P. multocida* B:2 GDH7 was not as expected from previous study (Othman, 2007). Amplification of the *gdhA* gene-based primers was only observed from the wild-type strain whereas no amplifications observed from our mutant strain (Figure 1a). Similar observation was determined through the amplification with the multiplex primers as shown in Figure 1b. In addition, the specifically designed KanR primers has enabled in the amplification of the KR cassette (Table 2) of the mutant when compared to the wild-type. As shown in Figure 1c, only the mutant was observed to possess the ~800 bp DNA band when compared to the wild-type strain. This showed the presence of KR cassette only in the mutant and the positive control which was a vector harbouring the same KR cassette as the mutant.

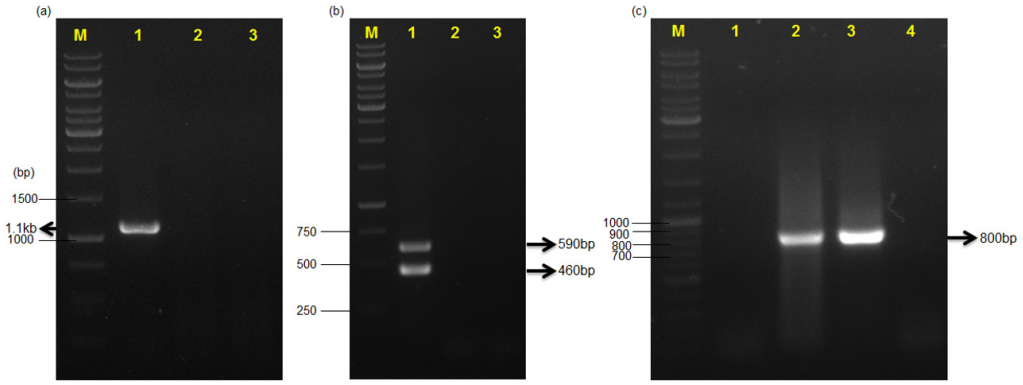

Figure 1. Identification and differentiation between *Pasteurella multocida* B:2 wild-type and *P. multocida* B:2 GDH7. (a) PCR products by using *gdhA* gene-based primers (Othman, 2007): Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7, Lane 3, Negative control. (b) PCR products by using multiplex primers (Townsend et al., 1998): Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7, Lane 3, Negative control. (c) PCR products by using KanR primers: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, Positive control; Lane 4, Negative control

Two primer pairs were designed in order to confirm the location of the kanamycin resistance cassette within the genome of *P. multocida* B:2 GDH7 (Figure 2). The primers were designed based on the genome sequence of the wild-type. The strategy in Figure 2 was derived to amplify the targeted part of the mutant's genome to locate the kanamycin gene. Amplification was successful for *P. multocida* B:2 wild-type's

native gene which acted as a negative control in this experiment (Figure 3). Therefore, the primers based on the kanamycin sequence (KanR primers) were sufficient to be used to distinguish both the wild-type and the attenuated *gdhA* derivative. The presence of the kanamycin sequence in the mutant *P. multocida* B:2 GDH7 was evidently distinct but was absent in the *P. multocida* B:2 wild-type as initially expected (Figure 1c). Furthermore, the kanamycin resistance gene (KR) sequence amplified from the mutant was confirmed to be highly similar to the kanamycin resistance gene initially inserted in this mutant strain (Othman et al., 2017) and also to the positive control of pET28 harbouring the same gene (Supplementary material 1).

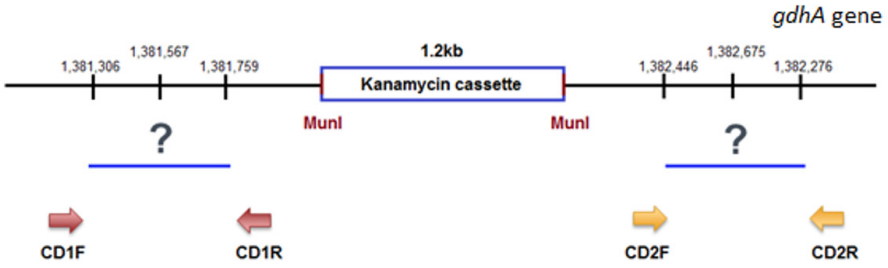

Figure 2. Primers and target size map of kanamycin sequence predicted by *in silico* analysis. The kanamycin sequence was suggested to be located in the region marked '?' after the analysis of the amplification by the respective two primer pairs above

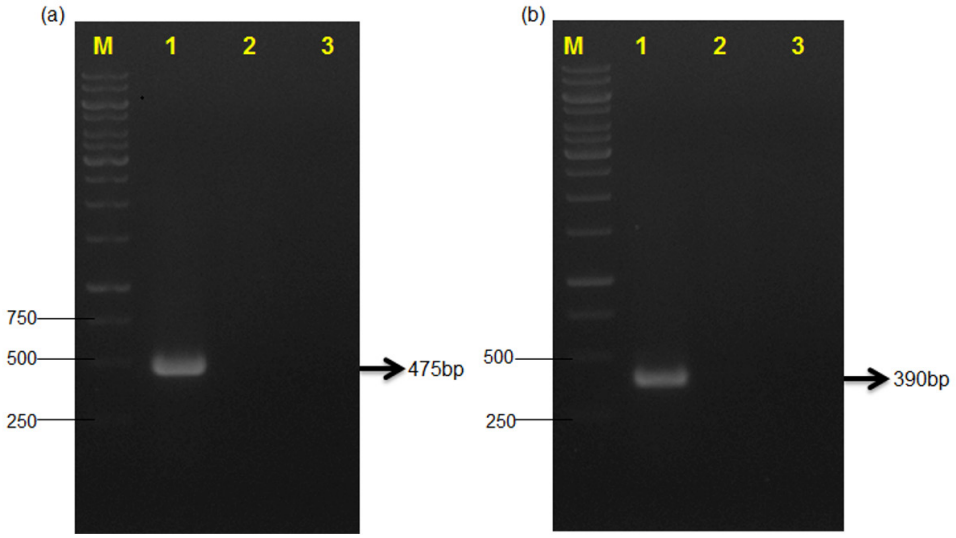

Figure 3. PCR amplification using CD1 and CD2 primers. (a) PCR products amplified by using CD1 primers: (b) PCR products amplified by using CD2 primers: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, Negative control

From the PCR amplification based on the strategy in Figure 2, only the wild-type possessed the expected amplified DNA sizes (Figure 3). The amplification by using CD1 primers has produced a DNA band with 475 bp whereas CD2 produced DNA band of 390 bp in wild-type. In contrast, no amplification was observed from the genome of *P. multocida* B:2 GDH7. This could indicate a total disruption of the *gdhA* gene in the *P. multocida* B:2 GDH7 as compared to the wild-type. None of the investigated areas of the gene were amplified when using the CD1 and CD2 primers (Table 2). Further sequencing of the mutant strain can be implemented to investigate the location of the KR cassette in the mutant genome. It was also reported that *GDH1* gene encoding the glutamate dehydrogenase was found to be a hotspot for genetic mutation in *S. cerevisiae*. Manipulation of *GDH1* gene has contributed to genome shuffling and also phenotype change of the organism (Biot-Pelletier et al., 2018). Hence, it can be proposed that KR cassette could have been reshuffled to other region of the bacterial genome. This could have contributed to the inability of kanamycin cassette localisation due to unknown vicinity sequences post-reshuffling. El-Brolosy and Stainier (2017) also commented that the genome reshuffling could have compensated the gene annotations. Hence, in this study this resulted in inability of the targeted product to be amplified from the mutant strain by using the multiplex primers (Figure 1b).

Genomic Profiling of *Pasteurella multocida* B:2 Strains

All the DNA bands profile of *P. multocida* B:2 strains in this study were seen with different patterns and allocated with different molecular weight (MW) sizes. The genomic patterns observed from REP-PCR were reproducible throughout this study. The genomic profile of wild-type strain possessed eight DNA bands comprising of sizes 2.9 kb, 2.3 kb, 1.7 kb, 1.4 kb, 1.1 kb, 850 bp, 700 bp, and 425 bp. Meanwhile, the profile of *P. multocida* B:2 GDH7 consisted of DNA bands with sizes 2.2 kb, 1.6 kb, 1.3 kb, 800 bp, and 425 bp. Only three bands were observable in the profile of *P. multocida* B:2 JRMT12 which were 1.5 kb, 1.0 kb, and 600 bp. Nonetheless, both *P. multocida* B:2 wild-type and *P. multocida* B:2 GHD7 shared a similar DNA band of 425 bp that was not observed in the profile of *P. multocida* B:2 JRMT12. The major heterogeneity between REP profiles of the bacterial strains could be represented from the pathogenicity of respective strains (Biswas et al., 2004). Saxena et al. (2006) reported two DNA bands at 1000 bp and 800 bp could be observed in every strains and isolates of *P. multocida*. However, in this study, both bands could not be observed from the *P. multocida* B:2 GDH7 mutant's genome (Figure 4). In contrast, the genome pattern of *P. multocida* B:2 wild-type possessed both of the respective DNA bands with slight difference in sizes. A plausible band of 425 bp was seen to be present in both profiles of *P. multocida* B:2 wild-type

and *P. multocida* B:2 GDH7 (Figure 4). Previously it was reported that repetitive elements are localised in particular regions of the bacterial isolates (Turni et al., 2018). Hence, this could indicate the 425 bp band was conserved only between the two strains. Nevertheless, whole genome sequencing is crucial in order to confirm this occurrence.

Amplification of REP profiles generated multiple DNA bands with different sizes due to multiple locations of REP sequences distinctly located within the bacterial genome. Thus, the multiple DNA bands observed post-gel electrophoresis depicted the genomic distance between the binding sites of the REP-based primers and the adjacent repetitive elements within the genomic content (Ishii et al., 2009). As shown in Figure 4, each strain's genome profile comprised multiple DNA bands with various molecular weights showing the REP sequences location variety within each genome. Despite the similar ancestral background, the genome fingerprints between the strains in this study were seen to be completely diverse to each other. This confirmed that attenuation of the mutant not only altered the bacterial metabolism (Kamal et al., 2017) but could also have altered the genome organization. Moreover, the attenuated mutant was observed capable of providing mass protection towards buffaloes when challenged with the wild-type strain in field tests (Rafidah et al., 2012;

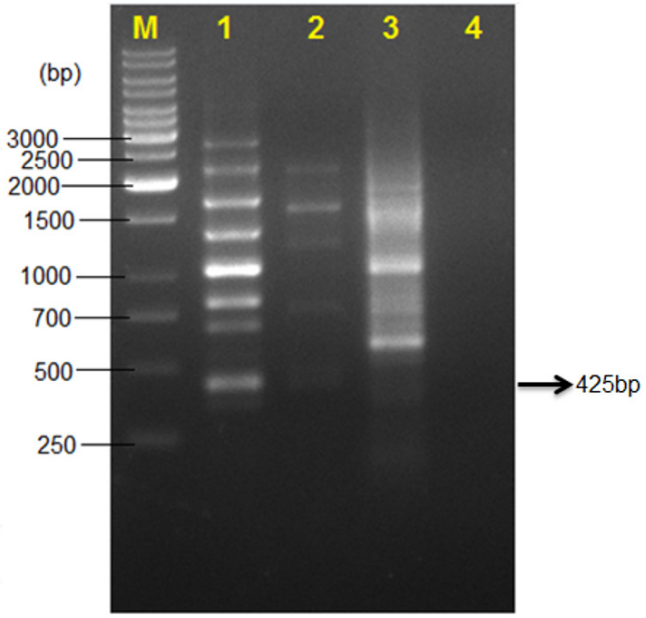

Figure 4. REP-PCR assay for genotyping analysis of *Pasteurella multocida* B:2 strains. Comparison between *P. multocida* B:2 wild-type, *P. multocida* B:2 GDH7, and *P. multocida* B:2 JRMT12: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, *Pasteurella multocida* B:2 JRMT12; Lane 4, Negative control

Rafidah & Zamri-Saad, 2013). Opportunistic pathogens evolved rapidly with the highest rate of homologous recombination events in order to adapt to the host environment (González-Torres et al., 2019). Homologous recombination and antibiotic selection was found to induce mutagenesis and this lead to genome instability and genome rearrangement (Darmon et al., 2014; Oliveira et al., 2015). However, contribution of the mutant strain's genome instability towards its apathogenicity remains unknown.

Fingerprint typing analysis towards another mutant strain, *P. multocida* B:2 JRMT12, was also conducted to compare the genetic organisation of the mutant with the rest of the bacterial strains in this study. From the REP-PCR profile, all of the strains possessed significant differences of their genomic content (Figure 4). In addition, the genomic profile of *P. multocida* B:2 JRMT12 was observed to contain more DNA bands when compared to *P. multocida* B:2 GDH7 and the wild-type strains. The excessive presence of multiple DNA bands in the profile of *P. multocida* B:2 JRMT12 observed causes the profile to smear when compared to other strains' profiles. Similar to *P. multocida* B:2 GDH7, this occurrence also could be contributed from the attenuation process of *P. multocida* B:2 JRTM12. Hence, the attenuation of the *aroA* mutant could also contribute to its genome reorganisation (Tabatabaei et al., 2002).

Proteomic Profiling of *Pasteurella multocida* B:2 Strains' Cell-Free Extracts

The CFE profiles were observed with proteins ranging from 12 kDa to 115 kDa (Figure 5). Conspicuous protein bands were observed with differences in the respective CFE profiles of each bacterial strain. The CFE of wild-type strain possessed conspicuous band of 28 kDa whereas *P. multocida* B:2 GDH7 strain possessed a 30 kDa band. These bands are high likely to be identified as OmpH (Ataei et al., 2009). As expected, there were no significant different observed between the CFE profiles of *P. multocida* B:2 GDH7 cultured in BHI or YDB broth media. However, the protein bands above 15 kDa of the latter sample were observed as faint bands. The minimum nutrients provided by YDB media could possibly limit the expressions of these proteins as compared to proteins expressed with the commercial media. Moreover, the CFE profile of the reference mutant strain, *P. multocida* B:2 JRMT12 was shown to possess more proteins as compared to the other strains. It was also interesting to note that the were no visible protein bands of sizes >15 kDa for CFE from *P. multocida* B:2 wild-type when compared to the others. Overall, the prominent protein bands of each CFE profile were distinctively visible and each CFE profiles were distinctive towards the specific strains.

From these findings, the colony morphology (unpublished data) and

genomic pattern changes are high likely contributed by genomic alterations of the mutants as compared to the virulent strain. This was observed in a study of attenuated *Mycobacterium tuberculosis* strain H37Ra, when compared against its virulent strain in order to observe the respective genomic organization (Zheng et al., 2008). The spontaneous attenuation introduced towards *M. tuberculosis* H37Ra strain had caused multiple mutations within its genome. The multiple mutations of the attenuated strain had caused genetic variations that affected the metabolism and growth related genes. The attenuation also contributed to colony morphology differences of the mutant with the virulent strains (Zheng et al., 2008). The latter observation was also recorded in this study with *P. multocida* B:2 GDH7 and its wild-type strain. Thus, the attenuation against *P. multocida* B:2 GHD7 has possibly contributed to its colony morphology changes (unpublished data) and consequently towards the genomic alteration. Nevertheless, a subsequent thorough comparative genomic analysis is required for further information between the genomic organisations of both strains.

In *P. multocida*, cell-free extracts (CFEs) are less commonly profiled as compared to outer membrane proteins (OMPs). In a report by Ataei et al. (2009), the CFEs profile of a Sri Lankan strain *P. multocida* B:2 (*P. multocida* B:2 strain 85020) showed numerous protein bands with sizes that ranged from 16 to 90 kDa with a conspicuous protein band at 30 kDa. The 30 kDa was also observed in the OMP profile and identified as OmpH from mass spectrometry (MS) analysis. However, this protein was proven to be non-immunogenic due to no response in immunoblotting with the vaccinated animal sera (Ataei et al., 2009). In contrast, this study obtained different sizes of conspicuous protein bands in the respective strains' CFEs profile (Figure 5) as compared to Ataei et al. (2009).

The use of different bacterial strains in the respective experiments could contribute to the differences observed in each CFEs profile. Although targeting similar bacterial serotypes, but the strain of *P. multocida* B:2 used by Ataei et al. (2009) was of Sri Lankan origin. Differences of the CFEs profiles were also observed from other serotypes of *P. multocida* B:2 isolated from Indian strain (Chaudhuri et al., 2001). Thus, it can be assumed that different strains had represented different proteomic profiles. This is likely due to differential environment adaptation in each geographical region of respective bacterial strains. Even so, the CFE profiles cannot be further scrutinised other than the presence of the conspicuous bands. Therefore, OMP profiling was performed to further examine the proteomic profiles of each bacterial strain in this study.

Proteomic Profiling of *Pasteurella multocida* B:2 Strains' OMPs

Similar to the CFE profiles, the OMP profiles of each strain was also seen to be distinct with each other (Figure 6). The OMP profiles consisted of protein bands with sizes

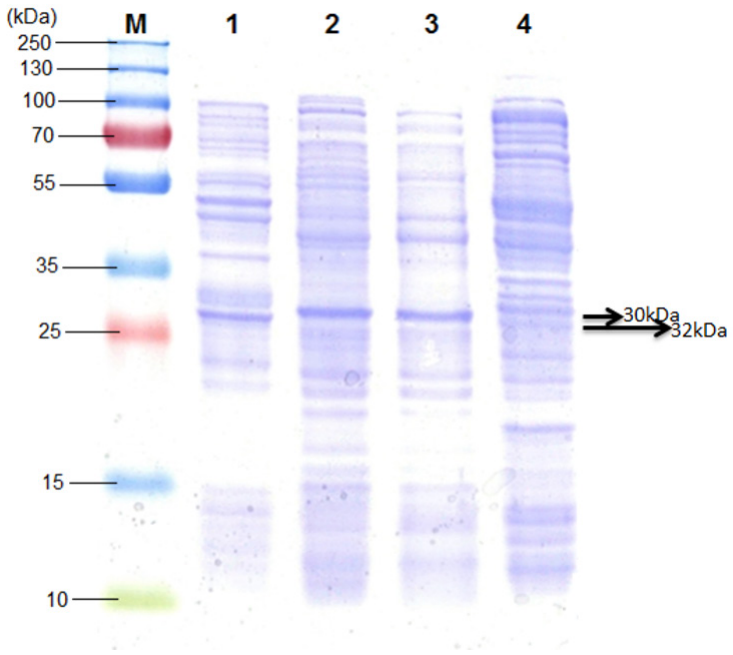

Figure 5. CFE profiles of each *Pasteurella multocida* B:2 strains represented on a Coomassie blue-stained SDS-PAGE gel. Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7 grown in BHI media; Lane 3, *Pasteurella multocida* B:2 GDH7 grown in YDB media; Lane 4, *Pasteurella multocida* B:2 JRMT12; Lane M, protein ladder

ranging from 14 kDa to 90 kDa. The high molecular weights (HMW) protein bands were faintly observed in the OMP profile of mutant strain *P. multocida* B:2 GDH7 as compared to the other two strains. However, the pattern of the HMW proteins of the two latter strains was also positioned at a different location when compared to one another. The HMW proteins of *P. multocida* B:2 GDH7 were observed between 55 kDa to 70 kDa size. Meanwhile, the HMW proteins of *P. multocida* B:2 wild-type were seen to be higher than 70 kDa in which the wild-type possessed two protein bands when compared to *P. multocida* B:2 GHD7's HMW proteins.

In each of the bacterial OMP profiles, they comprised two distinct and highly expressed protein bands within the range of about 22 kDa to 32 kDa. The sizes of these two protein bands were different to each other such that *P. multocida* B:2 wild-type possessed a visibly dense protein band at about 28 kDa and 23 kDa. Both *P. multocida* B:2 GDH7 grown in BHI and YDB have similar OMP profile with distinct bands of about 30 kDa and 22 kDa, proving that using a minimal media did not have significant effect on OMP expression. Simultaneously, two bands of about 34 kDa and 32 kDa were observed from the OMP profile of *P. multocida* B:2 JRMT12. Proteins with

lower molecular weight (LMW) were also observed from the OMP profiles. Both *P. multocida* B: wild-type and *P. multocida* B:2 JRMT12 shared the same protein band size of about 14 kDa whereas *P. multocida* B:2 GDH7 OMP profiles possessed protein band of about 15 kDa. Furthermore, all OMP profiles shared one similar protein size at around 18 kDa.

OMP typing or profiling was used in this study to characterise between *P. multocida* B:2 strains (Figure 6). It is understood that most of the virulent factors that include OMPs, resides on the surface of *P. multocida* due to their association with hosts' cells colonisation and invasion. (Boyce et al., 2006). The OMPs were expressed in iron-rich environment in order to understand on the proteins that contributed to the pathogenicity of the wild-type *P. multocida* B:2 and the apathogenicity of both mutants, *P. multocida* B:2 GDH7 and *P. multocida* B:2 JRMT12 strains. Hence, from the SDS-PAGE analysis, respective strains' OMP profiles showed notable differences and also similarities with each other. In this study, it was identified that two conspicuous bands were present with different sizes in each OMP profile. These OMPs could be determined as OmpA or OmpH which are known as major OMPs in *P. multocida* (Prasannavadhana et al., 2014; Wheeler, 2009).

Various strains of *P. multocida* B:2 were targeted for OMP profiling to determine

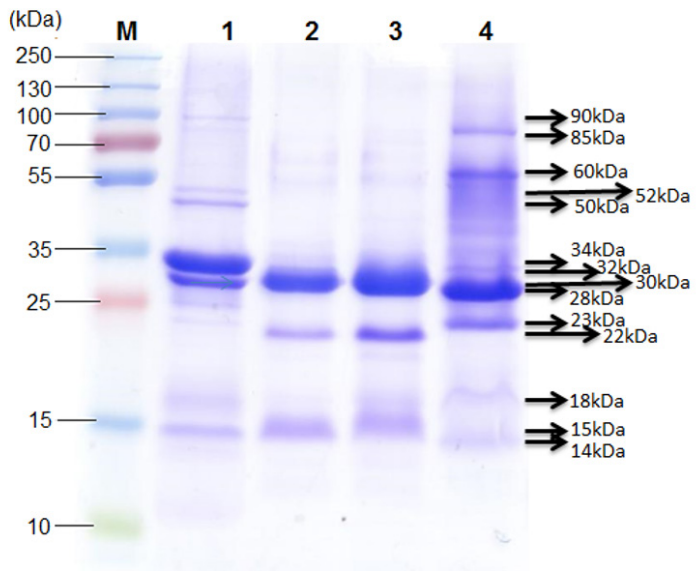

Figure 6. OMP profiles of each *Pasteurella multocida* B:2 strains represented on a Coomassie blue-stained SDS-PAGE gel. Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7 grown in BHI media; Lane 3, *Pasteurella multocida* B:2 GDH7 grown in YDB media; Lane 4, *Pasteurella multocida* B:2 JRMT12; Lane M, protein ladder

the strain specific markers as a preliminary typing analysis for isolates differentiation (Munir et al., 2007). In 2007, an OMP profile of *P. multocida* serotype B:2 isolated from Pakistan showed presence of six polypeptide bands ranging from 15 kDa to 91 kDa (Munir et al., 2007). Furthermore, two predominant bands were observed with 32 kDa and 39 kDa and with less conspicuous bands, 15 kDa, 44 kDa, 72 kDa, and 91 kDa. Four Malaysian outbreak isolates of *P. multocida* B:2 were targeted for OMP profiling that showed six protein bands ranging from 26 kDa to 100 kDa (Tan et al., 2010). Each outbreak strains possessed slight differences in respective OMP profiles. Moreover, major OMP, OmpH was identified from protein size of 37 kDa after immunoblotting analysis (Tan et al., 2010).

A report on *P. multocida* serotype B's OMP profiling by Somshekhar et al. (2014) has stated minor differences between the Indian field isolates and the vaccine strain (*P. multocida* B:2 P52) with polypeptide bands ranging from 16 kDa to 123 kDa. In addition, a prominent band of 32 kDa was identified as any of the two major OMPs for all bacterial strains of the study. Meanwhile, protein bands of 46 kDa and 123 kDa were only observed in the vaccine strain's OMPs but not found in the rest of field isolates' OMPs. Nevertheless, *P. multocida* B:2 P52 is a local strain isolated from previous outbreak in India and is used as formalin-killed bacterin vaccine production for HS outbreak control in India (Chaudhuri et al., 2001). Hence, the OMP profiles of the respective bacteria should not have major differences to each other. In contrast, this study used a genetically manipulated *P. multocida* B:2 (*P. multocida* B:2 GDH7) to profile its OMPs and to be compared to the profile of its parent strain, isolated from previous outbreak in Malaysia (Othman, 2007). Another notable report of *P. multocida* B:2 OMP profiling in 2014 by Prasannavadhana et al. (2014) had shown two major OMPs with bands sizes of 33 kDa and 38 kDa. These bands then were confirmed by mass spectrometry analysis as OmpH at 33 kDa and OmpA at 38 kDa. Minor polypeptide bands were also observed in the OMP profile with 14-15 kDa bands on the gel. Therefore, the conspicuous bands identified in this study can be predicted as either major OmpH or OmpA.

A low molecular protein known as Omp16 was also found to be expressed in capsular types A, D and F of *P. multocida* but with protein size heterogeneity across avian, bovine, porcine, and ovine isolates (Wheeler, 2009). Moreover, this protein was also expressed in all of the previous studies of *P. multocida* B:2 with protein sizes ranging from 14 kDa to 19 kDa. Thus, this protein is a highly conserved protein in the OMP profile of *P. multocida*. However, unlike the previous reports, in this study, two low molecular weight proteins were observed at 14 kDa/15 kDa, and 18 kDa. This low molecular weight proteins pattern was also observed in *P. haemolytica* isolated from bovine but of unknown identity and function (Davies et al., 1992). Moreover, these proteins were only reported

in selected strains of *P. multocida* B:2 such as *P. multocida* P52 and four isolates of *P. multocida* serotype B isolated from England and Wales (Aiswarya et al., 2017; Davies et al., 2003). Furthermore, several isolates of *P. multocida* of capsular type A were also found with this protein pattern (Davies et al., 2003). The reason behind the occurrence of this protein pattern was not known due to limited information of these proteins aside from the reported immunogenic Omp16. Although, the band was observed in multiple serotypes and isolates of *P. multocida*, it could have an importance in each pathogenesis.

Proteins of HMW were frequently reported in iron-limited conditions due to the proteins association in the iron uptake and regulation in iron deprived environment (Wheeler, 2009). However, a protein that is present in either iron rich or iron-limited conditions which is 89 kDa TbpA was reported to be present only in bovine and ovine isolates (Wheeler, 2009). TbpA was also detected in *P. multocida* B:2 with the size of 87 kDa and 89 kDa (Prasannavadhana et al., 2014). Aside from that, another HMW found in OMP profile *P. multocida* to be constitutively expressed in both conditions is Omp87 of 87 kDa.

The reason behind the obvious differences between the OMP profiles of each *P. multocida* B:2 strains is still unclear. However, further proteomic analysis such as mass spectrometry is highly able to provide clarification on this phenomenon. Still, one can assume that the differences observed between the conspicuous bands in all strains of *P. multocida* B:2 used in this study was most likely due to the differences of genetic makeup between the strains. The attenuation performed in both mutant strains might have altered the genomic content. And the alteration was shown through the different patterns of each OMP profile. However, genetic make-up differences cannot be represented solely by the bacterial OMP (Davies, 2004).

It was understood that the differences between the OMP profiles between each *P. multocida* strains is represented by the rapid evolution of the respective OMPs when compared to housekeeping genes due to introduction of diversified selection pressures (Davies, 2004). Therefore, media selection of *P. multocida* B:2 GDH7 might also influenced the unique pattern of its OMP profile when compared to the parent strain. It could also be postulated that the conspicuous bands exhibited in respective profiles were different due to the up-regulation and down-regulation of the selected conspicuous bands and its similar sizes of protein bands.

The differences of protein expression might also be reflected from the attenuation process of the mutants than the wild-type. An initial OMPs determination analysis was performed on the genome of *P. multocida* B:2 PMTB2.1, which was known as the parent strain used in this study. The selected housekeeping gene *gdhA*, disrupted in *P. multocida* B:2 GDH7, was found to be located in the outer membrane of the bacteria. Hence, alteration of this outer membrane protein gene could have shifted

the biogenesis of the membrane in order to survive the post-attenuation (Bernardini et al., 1993; Bochner, 2009; Godlewska et al., 2009; Martorana et al., 2014). This phenomenon could also address the weakening of both mutants' pathogenicity and their ability in giving protection when vaccinated to the animals (Rafidah et al., 2012; Tabatabaei et al., 2002). Aside from GdhA, the proteins TbpA, OmpA and also OmpH were identified in the initial OMPs determination analysis of *P. multocida* B:2 PMTB2.1 genome. Hence, the presence of these proteins might explain their contributions towards the virulence of the wild-type and the apathogenicity of the mutant strains.

CONCLUSION

This study has enabled characterisation between the *Pasteurella multocida* B:2 wild-type and its attenuated derivative, *P. multocida* B:2 GDH7. It was revealed that kanamycin-based primers enabled discrimination between the mutant strain *P. multocida* B:2 GDH7 and wild-type strain *P. multocida* B:2 by using conventional PCR analysis. The conventional detection method may enable simple but accurate identification and maintenance of the potential live attenuated vaccine that can be applied to ensure the consistency and the quality of the vaccines during commercialisation process. The genotypic and proteomic profiles of each strain were distinguished in this study. The differences in both genomic and OMP profiles were uniformed and consistent that could be represented as differences contributing to the apathogenicity of each strain in this study.

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REFERENCES

Abubakar, M. S., & Zamri-Saad, M. (2011). Clinico-pathological changes in buffalo calves following oral exposure to *Pasteurella multocida* B:2. *Basic and Applied Pathology*, *4*(4), 130–135. https://doi.org/10.1111/j.1755-9294.2011.01113.x

Aiswarya, V., Mathakiya, R. A., Bhanderi, B. B., & Roy, A. (2017). Characterization of *Pasteurella multocida* isolates of buffalo origin from Gujarat state of India by outer membrane protein profile analysis. *Buffalo Bulletin*, *36*(2), 313-322.

Ataei, S., Burchmore, R., Christopher Hodgson, J., Finucane, A., Parton, R., & Coote, J. G. (2009). Identification of immunogenic proteins associated with protection against haemorrhagic septicaemia after vaccination of calves with a live-attenuated *aroA* derivative of *Pasteurella multocida* B:2. *Research in Veterinary Science*, *87*(2), 207–210. https://doi.org/10.1016/j.rvsc.2009.01.007

Bernardini, M. L., Sanna, M. G., Fontaine, A., & Sansonetti, P. J. (1993). OmpC is involved in invasion of epithelial cells by *Shigella*

flexneri. *Infection and Immunity*, *61*(9), 3625–3635. https://doi.org/10.1128/IAI.61.9.3625-3635.1993

Biot-Pelletier, D., Pinel, D., Larue, K., & Martin, V. J. J. (2018). Determinants of selection in yeast evolved by genome shuffling. *Biotechnology for Biofuels*, *11*(1), 282. https://doi.org/10.1186/s13068-018-1283-9

Biswas, A., Shivachandra, S. B., Saxena, M. K., Kumar, A. A., Singh, V. P., & Srivastava, S. K. (2004). Molecular variability among strains of *Pasteurella multocida* isolated from an outbreak of haemorrhagic septicaemia in India. *Veterinary Research Communications*, *28*(4), 287–298. https://doi.org/10.1023/B:VERC.0000026656.77847.5b

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews*, *33*(1), 191–205. https://doi.org/10.1111/j.1574-6976.2008.00149.x

Boyce, J. D., Cullen, P. A., Nguyen, V., Wilkie, I., & Adler, B. (2006). Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics*, *6*(3), 870–880. https://doi.org/10.1002/pmic.200401342

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chaudhuri, P., & Goswami, P. P. (2001). Cloning of 87 kDa outer membrane protein gene of *Pasteurella multocida* P52. *Research in Veterinary Science*, *70*(3), 255–256. https://doi.org/10.1053/rvsc.2001.0470

Darmon, E., & Leach, D. R. F. (2014). Bacterial genome instability. *Microbiology and Molecular Biology Reviews*, *78*(1), 1–39. https://doi.org/10.1128/MMBR.00035-13

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*, *150*(12), 4199–4210. https://doi.org/10.1099/mic.0.27409-0

Davies, R. L., MacCorquodale, R., & Caffrey, B. (2003). Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Veterinary Microbiology*, *91*(2–3), 169–182. https://doi.org/10.1016/S0378-1135(02)00300-0

Davies, R. L., Parton, R., Coote, J. G., Gibbs, H. A., & Freer, J. H. (1992). Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype-A1 under different growth conditions. *Journal of General Microbiology*, *138*(5), 909–922.

De Alwis, M. C. L. (1999). *Haemorrhagic septicaemia*. Australian Centre for International Agricultural Research.

El-Brolosy, & M. A, Stainier D. Y. R. (2017). Genetic compensation: A phenomenon in search of mechanisms. *PLoS Genetics*, *13*(7), e1006780. https://doi.org/10.1371/journal.pgen.1006780

Galdiero, M., De Martino, L., Pagnini, U., Pisciotta, M. G., & Galdiero, E. (2001). Interactions between bovine endothelial cells and *Pasteurella multocida*: Association and invasion. *Research in Microbiology*, *152*(1), 57–65. https://doi.org/10.1016/S0923-2508(00)01168-2

Godlewska, R., Wiśniewska, K., Pietras, Z., & Jagusztyn-Krynicka, E. K. (2009). Peptidoglycan-associated lipoprotein (*Pal*) of Gram-negative bacteria: Function, structure, role in pathogenesis and potential application in immunoprophylaxis:

Minireview. *FEMS Microbiology Letters*, *298*(1), 1–11. https://doi.org/10.1111/j.1574-6968.2009.01659.x

González-Torres, P., Rodríguez-Mateos, F., Antón, J., & Gabaldón, T. (2019). Impact of homologous recombination on the evolution of prokaryotic core genomes. *MBio*, *10*(1), e02494-18. https://doi.org/10.1128/mBio.02494-18

Hazwani-Oslan, S. N., Tan, J. S., Saad, M. Z., Halim, M., & Ariff, A. B. (2017). Improved cultivation of *gdhA* derivative *Pasteurella multocida* B:2 for high density of viable cells through *in situ* ammonium removal using cation-exchange resin for use as animal vaccine. *Process Biochemistry*, *56*, 1–7. https://doi.org/10.1016/j.procbio.2017.02.016

Ishii, S., & Sadowsky, M. J. (2009). Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution: Minireview. *Environmental Microbiology*, *11*(4), 733–740. https://doi.org/10.1111/j.1462-2920.2008.01856.x

Kamal, N. M., Zamri-Saad, M., Masarudin, M. J., & Othman, S. (2017). Interaction between *Pasteurella multocida* B:2 and its derivatives with bovine aortic endothelial cell (BAEC). *BMC Veterinary Research*, *13*(1), 1-8. https://doi.org/10.1186/s12917-017-1109-1

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0

Martorana, A. M., Motta, S., Di Silvestre, D., Falchi, F., Dehò, G., Mauri, P., Sperandeo, P., & Polissi, A. (2014). Dissecting *Escherichia coli* outer membrane biogenesis using differential proteomics. *PLOS One*, *9*(6), 100941. https://doi.org/10.1371/journal.pone.0100941

Munir, R., Shahwar, D., Farooq, U., Nawaz, I., Shahzad, I., & Khanum, A. A. (2007). Outer membrane protein profiling of *Pasteurella multocida*. *Pakistan Veterinary Journal*, *27*(1), 1–4.

Oliveira, P. H., Prazeres, D. M. F., Monteiro, G. A., & Pedro H., Oliveira, G. A. M. (2015). DNA instability in bacterial genomes: Causes and consequences. In M. Poptsova (Ed.), *Genome analysis: Current procedures and applications* (pp. 261-284). Caister Academic Press.

Oslan, S. N. H., Tan, J. S., Saad, M. Z., Halim, M., Mohamed, M. S., & Ariff, A. B. (2018). Influence of amino acids and vitamins on the growth of *gdhA* derivative *Pasteurella multocida* B:2 for use as an animal vaccine. *Bioprocess and Biosystems Engineering*, *42*(3), 355–365. https://doi.org/10.1007/s00449-018-2040-y

Othman, S., Parton, R., & Coote, J. (2012). Interaction between mammalian cells and *Pasteurella multocida* B:2. Adherence, invasion and intracellular survival. *Microbial Pathogenesis*, *52*(6), 353–358. https://doi.org/10.1016/j.micpath.2012.03.005

Othman, S. S., Zamri-Saad, M., Abdul Rahim, R., & Zunita, Z. (2017). Pasteurella multocida *B:2 GDH7* (Malaysian Patent No. MY-168662-A). IP UPM Malaysia. http://www.upmip.upm.edu.my/action.do?view=ipdetail&cluster=1&ipid=887&ipdetailid=870&projectleaderid=47&page=search&search=pasteurella&searchkey=1

Othman, S. S. (2007). *Contstruction of an attenuated Pasteurella multocida B:2 by mutation in the gdhA gene* [Master's thesis, University Putra Malaysia]. Universiti Putra Malaysia *Institutional Repository.* http://psasir.upm.edu.my/id/eprint/11652/1/FPV_2007_11_A.pdf

Prasannavadhana, A., Kumar, S., Thomas, P., Sarangi, L. N., Gupta, S. K., Priyadarshini, A., Nagaleekar, V. K., & Singh, V. P. (2014). Outer membrane proteome analysis of Indian strain of *Pasteurella multocida* serotype B:2 by MALDI-TOF / MS analysis. *Scientific World Journal*, *2014*, 617034. https://doi.org/10.1155/2014/617034

Rafidah, O., & Zamri-Saad, M. (2013). Effect of dexamethasone on protective efficacy of live *gdhA* derivative *Pasteurella multocida* B:2 vaccine. *Asian Journal of Animal and Veterinary Advances*, *8*(3), 548–554. https://doi.org/10.3923/ajava.2013.548.554

Rafidah, O., Zamri-Saad, M., Shahirudin, S., & Nasip, E. (2012). Efficacy of intranasal vaccination of field buffaloes against haemorrhagic septicaemia with a live *gdhA* derivative *Pasteurella multocida* B:2. *Veterinary Record*, *171*(7), 175. https://doi.org/10.1136/vr.100403

Sarwar, N., Muhammad, K., Rabbani, M., Younus, M., Sarwar, M., Ali, M. A., Hanif, K., & Kamran, M. (2013). Optimization of physico-chemical factors augmenting *in vitro* biomass production of *Pasteurella multocida*. *Journal of Animal and Plant Sciences*, *23*(4), 1085–1088.

Saxena, M. K., Singh, V. P., Kumar, A. A., Chaudhuri, P., Singh, V. P., Shivachandra, S. B., Biswas, A., & Sharma, B. (2006). REP-PCR analysis of *Pasteurella multocida* isolates from wild and domestic animals in India. *Veterinary Research Communnications*, *30*(8), 851–861. https://doi.org/10.1007/s11259-006-3321-5

Somshekhar, S. H., Veeregowda, B. M., Suryanarayana, V. V. S., Leena, G., Dhama, K., & Chakraborty, S. (2014). Outer membrane protein (OMP) profiles of *Pasteurella multocida* isolates associated with haemorrhagic septicaemia by SDS-PAGE and Western blot analysis. *Asian Journal of Animal and Veterinary Advances*, *9*(8), 513-518. https://doi.org/10.3923/ajava.2014.513.518

Tabatabaei, M., Liu, Z., Finucane, A., Parton, R., & Coote, J. (2002). Protective immunity conferred by attenuated *aroA* derivatives of *Pasteurella multocida* B:2 strains in a mouse model. *Infection and Immunity*, *70*(7), 3355–3362. https://doi.org/10.1128/IAI.70.7.3355

Tan, H. Y., Nagoor, N. H., & Sekaran, S. D. (2010). Cloning, expression and protective capacity of 37 kDa outer membrane protein gene (*ompH*) of *Pasteurella multocida* serotype B:2. *Tropical Biomedicine*, *27*(3), 430–441.

Townsend, K. M., Dawkins, H. J., & Papadimitriou, J. M. (1997). REP-PCR analysis of *Pasteurella multocida* isolates that cause haemorrhagic septicaemia. *Research Veterinary Science*, *63*(2),151-155. https://doi.org/10.1016/s0034-5288(97)90009-6

Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitriou, J. M., & 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.

Turni, C., Singh, R., & Blackall, P. J. (2018). Genotypic diversity of *Pasteurella multocida i*solates from pigs and poultry in Australia. *Australian Veterinary Journal*, *96*(10), 390–394. https://doi.org/10.1111/avj.12748

Wheeler, R. (2009). Outer membrane proteomics of *Pasteurella multocida* isolates to identify putative host-specificity determinants. *Bioscience Horizons*, *2*(1), 1–12. https://doi.org/10.1093/biohorizons/hzp002

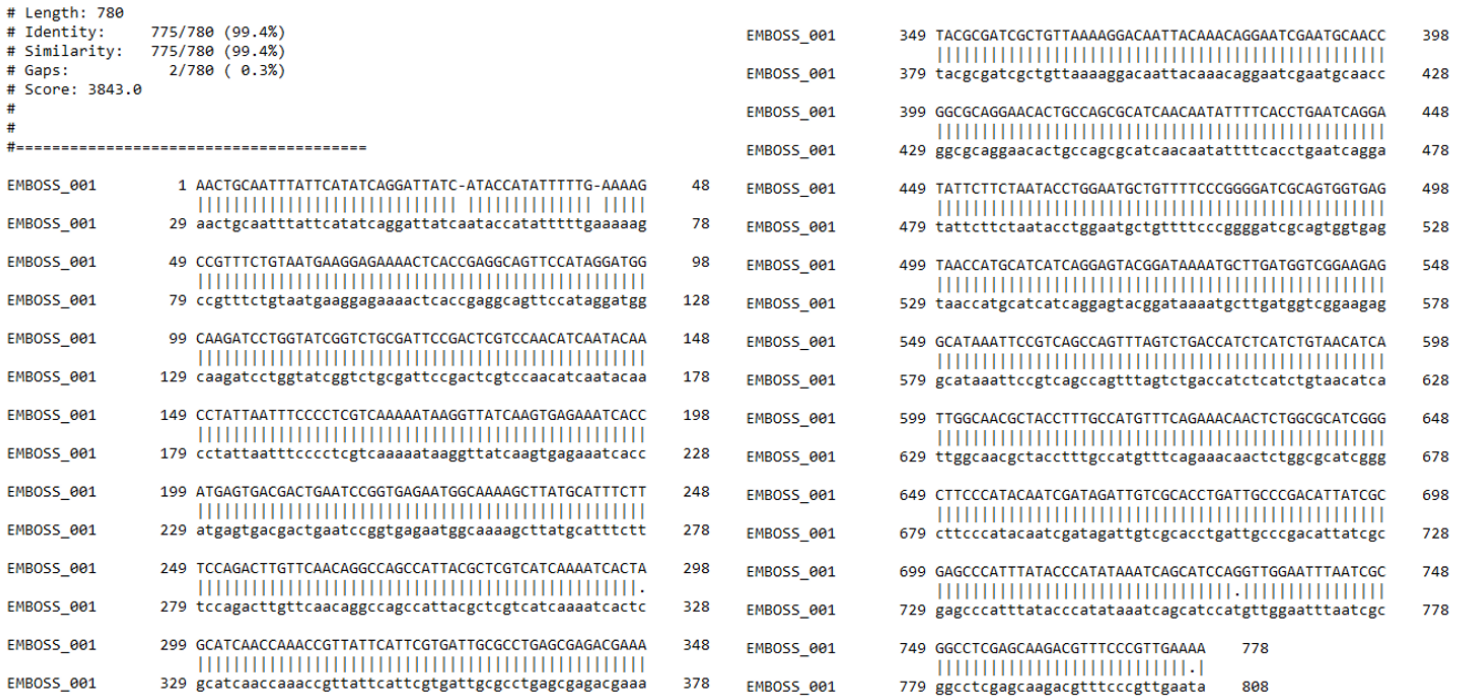
World Organisation for Animal Health. (2008). *Principle of veterinary vaccine production*: *OIE terrestrial manual*. OIE. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2008/pdf/1.01.06_VACCINE_PRODUCTION.pdf

World Organisation for Animal Health. (2016). *1.1.8. Principles of veterinary vaccine production: Manual of diagnostic tests and vaccines for terrestrial animals 2016*. OIE. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.08_VACCINE_PRODUCTION.pdf

Zamri-Saad, M. (2013). *Haemorrhagic septicaemia of cattle and buffaloes in Asia*. UPM Press.

Zamri-Saad, M., & Annas, S. (2016). Vaccination against haemorrhagic septicaemia of bovines: A review. *Pakistan Veterinary Journal*, *36*(1), 1–5. https://doi.org/10.1097/QCO.0b013e3283638104

Zheng, H., Lu, L., Wang, B., Pu, S., Zhang, X., Zhu, G., Shi, W., Lu, Z., Wang, H., Wang, S., Zhao, G., & Zhang, Y. (2008). Genetic basis of virulence attenuation revealed by comparative genomic analysis of mycobacterium tuberculosis strain H37Ra versus H37Rv. *PLOS One*, *3*(6), 2375. https://doi.org/10.1371/journal.pone.0002375



Supplementary material 1. Sequence homology of the amplified and sequenced kanamycin sequence from *Pasteurella multocida* B:2 GDH7. The sequence of the kanamycin gene region was assembled into a contig sequence prior to homology analysis. The figure above showed the sequence alignment result against the kanamycin gene of pUC4k vector. The top strand was the sequenced sample whereas the bottom strand of the alignment was the kanamycin of pUC4k