Molecular Identification and Serogrouping of *Pasteurella multocida* Field Isolats

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Abstract. Haemorrhagic septicaemia (HS) is a serious disease affecting buffalo and cattle worldwide mainly caused by *Pasteurella multocida* (PM) serogroups B and E. In Indonesia cases of HS have been reported every year from 2005 to 2017. The bacterial identification was done primarily based on morphological and biochemical characteristics, while the serogroup has never been determined. This research was aimed to implement molecular biological techniques for identification and serogrouping of three archival PM isolates; two isolates were originated from HS cases in South Sulawesi (SulSel) and one isolate from East Nusa Tenggara (NTT). Polymerase chain reaction (PCR) amplifications of PM family-specific gene 16SrRNA, species-specific gene *kmt* and serotype-specific gene *bcbD* identified all of the isolates were PM, which was substantiated by inoculation of VITEK® 2 compact. The three PM isolates were belong to capsular serogroup B. Sequence analysis of 690 nucleotides of the capsular gene and the respective hypothetical amino acid was further confirmed their identity. Two isolates of SulSel and NTT were 100% identical to M1404 B:2 (AF169324.1), meanwhile one silent mutation was noted in the other SulSel isolate. These finding substantiate the application of PCR as a rapid, sensitive and specific method for PM identification and characterization.

Keywords: capsular gene sequencing, molecular serogrouping, *Pasteurella multocida*, PCR, VITEK

1. Introduction

Haemorrhagic Septicaemia (HS) or Septicaemia Epizootica (SE) in Indonesia is an acute and fatal disease affecting buffalo and cattle caused by *Pasteurella multocida* (PM) serogroups B and E [1]. Haemorrhagic Septicaemia is endemic in Indonesia, the incidence of HS is reported almost every year until 2017 in some areas of Indonesia [2,3]. The diagnosis of HS in Indonesia is generally performed on the basis of clinical symptoms followed by bacterial isolation through culture method, biochemical test and serological identification in 4-12 days [4,5]. This research was aimed to implement molecular biological techniques for identification and serogrouping of PM isolates less than 48 hours.

2. Materials and methods

2.1. *Pasteurella multocida*

Three bacterial isolates are the collection of Laboratory of Bacteriology Department of Animal Diseases and Veterinary Public Health Faculty of Veterinary Medicine, Bogor Agricultural University
originating from HS cases in South Sulawesi (SulSel) and East Nusa Tenggara (NTT). Isolate collection was derived from blood of cattle showing HS symptoms. The isolates identified as PM based on morphological microscopy and biochemical tests (glucose, lactose, maltose, mannose, sucrose, catalase, oxidase, indole, and nitrate) and inability to grow on MacConkey agar. The isolates were codenamed MKS2014_003, MKS2014_004 originating from SulSel and NTT2015_006 originated from NTT.

2.2. Culture and biochemical test
The bacteria collection were subcultured on blood agar (BA) medium containing 0.002 g/L neomycin and 0.0035 g/L bacitracin and incubated at 37 °C for 24 hours. Colonies grown in BA medium were Gram stained and examined under a microscope with 100× magnification, and the bacterial cell size was measured with OptiLab Viewer and Image Raster 3 [6]. Further identification was done using the identification card for gram-negative (ID-GN card) and VITEK 2® Compact system (bioMérieux) was used for all reading and interpretation of results [7,8]. The three categories of results were as follows: (i) correct identification; excellence, very good, good, acceptable, (ii) low level of discrimination, and (iii) no identification.

2.3. DNA extraction of Pasteurella multocida
DNA extraction was performed using a mini Presto™ gDNA bacteria kit [9] according to company protocol. Bacterial pellet was resuspended in 200 µl GT buffer, 20 µl of proteinase K was added and homogenized by 10 second vortexing then incubation at 60 °C for 10 minutes. After incubation, the cells were lysed using 200 µl GB buffer and vortexed for 10 second and re-incubated at 70 °C for 10 minutes. During incubation, the tube was inverted every 3 minutes until the lysate was clear. A total of 200 µl 100% ethanol was added and homogenized. The suspension was then transferred to GD Column, and centrifuged at 15,000 g for 2 minutes, the supernatant was removed and 400 µl W1 buffer was added and centrifuged at 15,000 g for 30 seconds. The supernatant was removed and 600 µl Wash Buffer was added then incubated at room temperature for 1 minute and centrifuged at 15000 g for 30 seconds. The GD Column was placed back in the 2 ml Collection Tube and centrifuged again for 3 minutes at 15000 g to dry the column matrix. The genomic DNA was eluted with 100 µl preheated Elution Buffer then incubated for 3 min and centrifuged at 15000 g for 30 seconds. The eluted DNA was stored at -20°C.

2.4. Family identification
Polymerase Chain Reaction (PCR) for Pasteurellaceae identification was performed using primers targeting 16S-rRNA; forward 5'-CATAGATTGAGCCTACTAAATG-3' and reverse 5'-GTCAGTACATTCCAAGG-3' [10]. The amplification reaction was carried out using the Myfx™ Mix (BIO LINE), 20 mM primer and RNase free water with final volume of 25 µl. The PCR cycle condition was as follows: 50 °C for 2 minutes followed by 35 cycles with temperature 95 °C for 1 minute, at 95 °C for 15 seconds, at 55 °C for 15 seconds, at 72 °C for 30 seconds, and final stage at 72 °C for 10 minutes. The PCR product was electrophoresed using 1% agarose gel containing 5% ethidium bromide and observed using a UV transilluminator.

2.5. Identification of species
Polymerase Chain Reaction (PCR) for Pasteurellaceae identification was performed using primers targeting alpha / beta hydrolase gene (kmt); Forward 5'-ATCCGCTATTACCCAGTGG-3' and reverse 5'-GCTGTAAACGAACTCGCCAC-3' [1]. The amplification reaction was carried out using the Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 20 mM primer and RNase free water with final volume of 25 µl. The PCR cycle condition was as follows: 50 °C for 2 minutes followed by 35 cycles with temperature 98 °C for 10 minutes, 98 °C for 10 seconds, temperature 55°C for 30 seconds, 72 °C for 30 seconds, and final stage at 72 °C for 10 minutes. The
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PCR product was electrophoresed using 1% agarose gel containing 5% ethidium bromide and observed using a UV transilluminator.

2.6. Serogroup identification and sequencing
Polymerase Chain Reaction (PCR) for Pasteurellaceae identification was performed using primers targeting bchD gene; forward 5’-CATTATCCAAGCTCACC-3’ and reverse (12863-12880) 5’GCCCGAGAGTTCTCAACC-3’[1]. The amplification reaction same as in identification of species. The PCR product that has the expected length is purified and sequenced (1-Base, Malaysia).

2.7. Analysis of nucleotides and amino acids
The nucleotide (nt) sequence of bchD genes were blasted using BLASTN tool (NCBI) to determine the sequence identity and homology to other isolates submitted to GenBank database. Sequences analysis was performed using BioEdit version 7.2.5 package (http://www.mbio.ncsu.edu/bioedit/bioedit.html), CLC Sequence Viewer 7.7. (Qiagen Bioinformatics). Phylogenetic tree was constructed by maximum likelihood method using MEGA 7 software version 7.0.14 (MEGA, PA, USA)[11].

3. Results

3.1. Culture and biochemical test
Small colonies with flat edge, greyish or semi-transparent, non-haemolytic was observed. The cells was Gram negative, cocobacil with 0.4 × 1.29 µm in size, this is similar to that expressed by OIE 2012. Biochemical testing using VITEK® 2 compact system identified all the three isolates as PM with 95% probability value as indicated on Table 1. The abbreviation of biochemistry listed in the table can be seen in the GN Well Contents VITEK®2 System guide.

| NO | Biochemistry | HASIL | NO | Biochemistry | HASIL | NO | Biochemistry | HASIL |
|----|--------------|-------|----|--------------|-------|----|--------------|-------|
| 2  | APPA         | -     | 4  | PyrA         | -     | 7  | dCEL         | -     |
| 10 | H2S          | -     | 12 | AGLTp        | -     | 14 | GGT          | -     |
| 17 | BGLU         | -     | 19 | dMAN         | +     | 21 | BXYL         | -     |
| 23 | ProA         | -     | 27 | PLE          | -     | 31 | URE          | -     |
| 33 | SAC          | -     | 35 | Dtre         | -     | 37 | MNT          | -     |
| 40 | ILAtk        | +     | 42 | SUCT         | -     | 44 | AGAL         | -     |
| 46 | GlyA         | -     | 48 | LDC          | -     | 56 | CMT          | +     |
| 58 | O129R        | -     | 61 | IMLTa        | -     | 64 | ILATa        | -     |
| 3  | ADO          | -     | 5  | IARL         | -     | 9  | BGAL         | -     |
| 11 | BNAG         | -     | 13 | dGLU         | +     | 15 | OFF          | -     |
| 18 | dMAL         | -     | 20 | dMNE         | +     | 22 | Balap        | -     |
| 26 | LIP          | -     | 29 | TyrA         | +     | 32 | dSOR         | -     |
| 34 | dTAG         | -     | 36 | CIT          | -     | 39 | 5KG          | -     |
| 41 | AGLU         | -     | 43 | NAGA         | -     | 45 | PHOS         | +     |
| 47 | ODC          | +     | 53 | IHISa        | -     | 57 | BGUR         | -     |
| 59 | GGAA         | -     | 62 | ELLM         | +     |

3.2. Molecular detection of Pasteurella multocida
A set of primers specific to Pasteurellaceae 16S-rRNA gene detected approximately 500 bp amplicon in all of the three isolates as shown in figure 1A. Further identification using primers specific to kmt
gene amplified fragment of 460 bp in size (figure 1B), which further distinguished the three isolates as *Pasteurella multocida*.

![Figure 1. A. The 16S-rRNA gene amplification results. Polymerase chain reaction product size of 504 bp. B. Primer kmt gene amplified 460 bp. The amplicons were electrophoresed in 1.5% agarose gel. Lanes: M - Molecular size marker 1Kb Kappa (Thermo Fisher Scientific), Line1- (MKS2014_003), line 2- (MKS2014_004) and line 3- (NTT2015_006).](image)

3.3. Serogrouping and sequencing

Amplification using primer set specific to bchD gene detected 760 bp amplicon that can be seen in figure 2. Nucleotide sequence analysis of 690 bp of MKS2014_003 and NTT2015_006 displayed 100% identity to serogroup B M1404 B:2 reference isolate (AF169324.1) in compare to lower 99% identity shown by MKS2014_004. However, 100% identity of putative bases obtained 230 amino acids (aa).

![Figure 2. Results DNA amplification using bchD primer genes for identification of serogroup B. M - Molecular size marker 1Kb Kappa (Thermo Fisher Scientific), line 1- (MKS2014_003), 2-(MKS2014_004), 3-(NTT2015_006).](image)

The bchD protein is a capsular gene that has an important role in virulence factor. Capsule has been implicated in virulence in *Pasteurella multocida* as encapsulated strains have been shown to be more virulent [12]. Mutations occur in the bchD gene shown by 1 aa change at position 617 as shown in figure 3. Substitution of aa not accompanied by changes in translation for Glysine (Gly/G). Based on the alignment analysis, substitution was occurred in nucleotide sequence, whereas the corresponding amino acid (glycine) was not changed, indicating a silent mutations.
Figure 3. Comparison of nt and putative aa sequences of MKS2014_004 highlights C to T.

4. Discussion
On the test results using VITEK®2 Compact 95% probability value is a very good grading category for the identification of gram negative bacteria, especially in this case PM [13]. The kmt gene is a gene that marks the part of outer protein membrane (omp) and is expressed as part of that conserved based on some alignment made using the BioEdit programme. Part of this gene is specifically used against PM, while on Pasteurella sp. others have to use different omp marker genes such as Superoxide dismutase (sodA) gene to identify P. canis, P.dagmatis, and P.stomatics [14].

The bcbD gene is part of the serologically distinguishable PM cell capsule based on capsule antigen in serogroup B. Capsule antigen is capable of giving the difference to the disease caused to each type of capsule being controlled by each gene. The genes that control these antigens are virulent, this is it can be reinforced with the location of genes located on the surface so as to disrupt the process of phagocytosis host to PM. As a result the virulence factor is able to easily defeat the host defence and cause Haemorrhagic Septicaemia [15].

The pathogenesis of PM genes as well as their virulence mechanisms is not much is known [16]. In addition to the capsules some of the more virulence factors of PM are it is known that Pasteurella Multocida Toxin (PMT) is intracellular activate the transducer signals and transcription factors which are then able to release cytokines that are capable of causing inflammation. The virulence factor is Lipopolysaccharide (LPS) and OMP can be detected by host receptors in the form of Toll-Like Receptor (TLR), which is also capable of activating factors transcription and then release the next inflammatory cytokine [17].

Based on the genetic organization of PM strain M1404 B: 2 (AF169324.1) the bcbD genes is among other capsule genes arranged sequentially between lipopolysaccharide B and lipopolysaccharide A with a length of 14 kb, whereas the length of bcbD is 760 bp [12]. Thus this can be the basis of the emergence of DNA bands on the identification of PM serogroup B.

A silent T to C mutation was demonstrated in MKS2014_004 isolate. Silent mutation generally does not cause changes in gene expression, but an exception has been shown that silent mutation can increase or decrease the stability of mRNA, as demonstrated by a rare codon such as a CAC codon on the His5 gene found in E. coli [18]. However, whether or not the silent mutation found in MKS2014_004 has an effect of mRNA stability cannot be established in this study.

5. Conclusion
Three bacterial isolates were identified as PM serogroup B. Two isolates MKS2014_003 and NTT2015_006 were 100% identical to M1404 B: 2 (AF169324.1), meanwhile one silent mutation was noted in MKS2014_003 isolate. These findings reinforce the application of PCR as a rapid, sensitive and specific method for PM identification and serogrouping. Further research related to isolation and molecular identification and characterisation of PM field isolate is necessary to elucidate their diversity.
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References
[1] [OIE] Office Internationale des Epizooties (FR) 2012 Haemorrhagic Septicaemia. Terrestrial manual. Chapter 2.4.11
[2] Natalia L and Priadi A 2006 Disease septicaemia epizootica: Disease research and control efforts on cattle and buffalo in Indonesia Digital Library Research and Development Board of Agriculture National Workshop. Availability of Science and Technology in the Control of Stable Diseases in Large Ruminant Livestock p 53–67
[3] Inarsih, Zulkifli, Novriyenti A, Oktavia E, Anindita K and Azfirman 2016 Buletin informasi kesehatan hewan 17(51) 1–5
[4] Putra A A G 2006 The situation of contagious animal diseases is strategic in large ruminants: surveillance and monitoring Proceedings of a national workshop on the availability of science and technology in strategic disease control in large ruminants (Denpasar: Regional Veterinary Investigation and Test Center VI) 31–49
[5] Sumadi, Pasaribu F H, Pudjiatmoko T, Mariana S R, Irawati T and Amijaya D 2005 Buletin Pengujian Mutu Obat Hewan 1 1–5.
[6] Kawaroe M, Prartono T, Sunuddin A and Saputra D 2016 Hayati 23(2) 62–66
[7] Valenza G, Ruoff C, Voge U, Frosch M and Abele-Horn M 2007 Journal of clinical microbiology 45(11) 3493–97
[8] Gherardi G, Angeletti S and Panitti M 2012 Elsevier 72 20–31
[9] Ahmed W A, Mohammed R J and Khala I A 2017 Advances in Microbiology 7 304–314
[10] Bootz F, Kirschnek S, Nicklas W, Wyss S K and Homberger F R 1998 Laboratory animal science 48(5) 542–546
[11] Kumar S, Stecher G and Tamura K 2016 Molecular Biology and Evolution 33 1870–74
[12] Boyce J D, Chung J Y and Adler B 2000 Veterinary Microbiology 72(1-2) 121–134.
[13] Pincus D H 1988 Microbial identification using the biomérieux Vitek® 2 system.Inc. (Hazelwood, MO: 1–32)
[14] Król J, Bania J, Florek M, Pliszczak-Król A and Staroniewicz Z 2011 Journal of Veterinary Diagnostic Investigation 23(3) 532–537
[15] John D, Boyce and Adler B 2000 Infection And Immunity 68(6) 3463–68
[16] Bosch M, Garrido M E, de Rozas A M P, Badiola I, Barbé J and Llagostera M 2004 Veterinary microbiology 99(2) 103-112
[17] F. Kubatzky K F 2012 Springer-verlag 361 53–72
[18] Chevance F F V, Guyon S L and Hughes K T 2014 PLOS Genetics 10(6) 1–14