Serotype Detection, Molecular Characterization, and Genetic Relationship Study on *Pasteurella multocide* Local Isolate

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ABSTRAK

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Pasteurella multocide merupakan bakteri penyebab penyakit ngorok atau Haemorrhagic Septicaemia (HS) di Indonesia dengan tingkat mortalitas dan morbiditas yang tinggi pada heterogenus spesies tak terkecuali sapi sebagai sumber produk hewani yang memiliki nilai ekonomis tinggi. Kompleksitas identifikasi secara konvensional dan biokimia menjadi kendala utama dalam deteksi penyakit ini terutama karena *P. multocide* memiliki lima golongan serotipe yaitu A, B, D, E dan F, meskipun serotipe B merupakan penyebab utama kasus HS di Asia termasuk Indonesia. Untuk itu perlu dilakukan suatu penelitian yang dapat mempermudah penentuan serotipe. Tujuan penelitian ini selain untuk deteksi serotipe lima isolat *P. multocide* yang berasal dari Lampung dan Kupang, juga untuk melakukan karakterisasi molekuler dan studi kekerabatan genetik dengan teknik Polymerase Chain Reaction (PCR) dan sekuensing. Setelah dilakukan PCR terhadap gen spesifik, gen kapsular, gen 16S rRNA, sekuensing dan analisis menggunakan Bioedit, BLASTn, CLUSTALW dan MEGA7.0.25, diketahui bahwa kelima isolat terbagi menjadi dua kelompok serotipe yaitu A dan B. Isolat *P. multocide* (kode PMc) asal Lampung memiliki homologi tinggi dengan isolat ATCC 12945, sehingga dapat digunakan sebagai kontrol positif serotipe A dalam deteksi isolat *P. multocide* lain dengan PCR. Sedangkan isolat *P. multocide* asal Kupang dapat digunakan sebagai kontrol positif serotipe B karena identik dengan *P. multocide* PMTB2.1 (CP007205.2) asal Malaysia yang diisolasi dari kerbau terinfeksi HS.

Kata Kunci: *Pasteurella multocide*, Ngorok, Deteksi, Polymerase Chain Reaction (PCR), *Sekuensing*

ABSTRACT

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Pasteurella multocide is a bacteria that causes snoring disease or Haemorrhagic Septicaemia (HS) in Indonesia with high mortality and morbidity in heterogeneous species including cattle as a source of animal products with high economic value. The complexity of conventional and biochemical identification is a major obstacle in the detection of this disease because *P. multocide* has five serotypes A, B, D, E and F, while serotype B is the leading cause of HS cases in Asia including Indonesia. Therefore, it is necessary to conduct a research that can determine the serotype and molecular characterization and genetic study of five isolates of *P. multocide* from Lampung and Kupang by Polymerase Chain Reaction (PCR) technique. After PCR was performed on specific genes, capsular genes, 16S rRNA genes, sequencing and analysis using Bioedit, BLASTn, CLUSTALW and MEGA7.0.25, it was found that the five isolates were divided into two serotype groups: A and B. Isolate *P. multocide* (code: PMc) from Lampung is high homolog with ATCC isolate 12945, so it can be used as a positive control serotype A in the detection of other *P. multocide* isolates with PCR. Whereas, isolate *P. multocide* from Kupang can be used as positive control of serotype B because it is identical to *P. multocide* PMTB2.1 (CP007205.2) from Malaysia that is isolated from buffalo infected by HS.

Key Words: *Pasteurella multocide*, Snoring Disease, Detection, Polymerase Chain Reaction (PCR), Sequencing

INTRODUCTION

Pasteurella multocide (*P. multocida*) is a pathogen gram-negative bacteria for livestock animals and causes specific pasteurellosis with the main symptoms: fever and respiratory disorder causing sound like snoring due to the neck swelling that extends to chest area resulting in suppression of respiratory cavity. Therefore, pasteurellosis on cattle and buffalo or Septicaemia Epizootic (SE)/ Haemorraghic Septicaemia (HS) in Indonesia is well known as snoring disease. In addition to being acute, its high morbidity and sepsis are extremely deadly to the infected animals. HS is a mayor epizootic disease on cattle and buffalo in the developing countries with high average morbidity and mortality (El-Jakee et al. 2016). HS can acutely infect cattle or buffalo that leads to economic losses in the Southeast Asia including Indonesia. Therefore, this disease is a

crucial disease from the economy point of view (Moustafa et al. 2015). The first infection in Indonesia was reported in Balaraja, Tangerang in 1884 and last several years, it was reported to extend to the South Kalimantan in 2003; South Bengkulu, South Tapanuli, Riau, Jambi and Kaur in 2005; East Nusa Tenggara and Muko-muko Bengkulu in 2006.

P. multocide is divided into five capsule serotypes: A, B, D, E, and F. The serotype causes HS to cattle or buffalo is serotype B:2 or Asian serotype and serotype E:2 or African serotype (Moustafa et al. 2015). *P. multocide* with Serotype A and some serotypes D capsule leads to fowl cholera in poultry (Dziva et al. 2008). *P. multocide* along with the *Bordetella bronchiseptica* causes acute atrophic rhinitis to pig related to high strain toxigenicity, because the *P. multocide* play a dominant role leading to the development of anthropic rhinitis lesi (Magyar et al. 2013; Kubatzky 2012; Shayegh et al. 2009) as well as it is related to pneumonia and septicemia diseases (De Oliveira et al. 2015). Liu et al (2017) reported that prevalence of *P. multocide* Serotype A is the most common in China before 1990 followed by serotype B and D.

Those five different *P. multocide* capsule serotypes causing the detection and differentiation processes are a different from indirect haemagglutination test developed. A conventional method to detect and diagnose Pasteurella infection on the bacterial observation uses microscopy with the staining. Moreover, isolation using in vitro on the selective medium followed by phenotypic and serology characteristic are conducted (Wilson & Ho 2013) that takes a long time. Method of detection and differentiation of serotype A and D of *P. mulcotide* widely used in the veterinary laboratory, especially in the developing countries is a non-serology test (Arumugam et al. 2011b). Some DNA-based methods that have been developed to determine the type of molecular are PCR-based, Restriction endonuclease analysis/REA, ribotyping, colony hybridization test, Filled Alternation Gel Electrophoresis (FAGE) and Real-Time PCR (Ranjan et al. 2011). The other DNA-based methods that are also developed are DNA hybridization (Mutters et al. 1985), endonuclease restriction analysis (Kim & Nagaraja 1990), pulsed-field gel electrophoresis (PFGE) and Capsular Polymerase Chain Reaction (PCR) (Townsend et al. 2001). The PEGE is determined as the golden standard, but it requires complicated planning and expensive equipment compared to the Capsular PCR which is simpler to be applied. Therefore, in this study, it was developed a technique of detection and differentiation of *P. mulcotide* using Capsular PCR which can detect capsular gen of *P. multocide* to four isolates from Lampung and one isolate from Kupang.

MATERIALS AND METHODS

Bacteria isolation

P. multocide isolate used in this study were five sample isolates from Lampung (4 isolates) and Kupang (1 isolate). Those five isolates have been re-detected morphologically and bio-chemically at Laboratory of Bacteriology, Indonesian Research Center for Veterinary Science (IRCVS), Bogor. Isolate of *P. multocide* ATCC12945 (serotype A) (Furian et al. 2014) and NCTC 11668 (serotype B) from America and IRCVS collection, respectively were used as control positive, while the *Brucella sp* isolate as a negative-control.

Primer

Standard OIE primer was used to detect specific species (kmt gene) and capsular gene (*hya*D-*hya*C and *bcb*D) to those five *P. multocide* isolates in this study. CAPA is a pair of primer to detect amplicons of the capsular gene of *hya*D-*hya*C and *bcb*D (serotype A), while the CAPB is a pair of primer to detect B/bcbD capsular amplicon (serotype B). Amplification of 16S rRNA gene was conducted using a pair of 16S PM1 primer designed from five gene references from GenBank on online devices of Primer3Plus (http://www.bioinformatics.nl/primer3plus). Structure of nucleotide base of forward primer of 16S PM1 was 5'-AGG-CCT-TCG-GGT-TGT-AAA-GT-3 and reverse: 5'-CCA-TGC-AGC-ACC-TGT-CTC-TA-3'with the size of the product by 642 base pairs. Before the construction, specivity of primer was tested first using the same online software device (Thornton & Basu 2011).

Detection and characterizing of specific species molecular (kmt Gene) of *P. multocide* using PCR

Phenotypic and bio-chemical detection of those five *P. multocide* isolates was performed. Nuclear acid-based detection was started using extraction of *P. multocide* DNA as template PCR using specific primer of KMT1T7: 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3' and KMT1SP6: 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3' (Townsend et al. 2000).

Reconstitution of *P. multocide* isolate was conducted by dissolving frozen dry isolates in the Brain Herat Infusion (BHI) then was incubated at 38°C for 24 hours. Isolates culture from incubation in the blood agar medium was then harvested for DNA isolation using Qiagen® kit.

Concentration of isolated DNA was then measured using NanoDrop 1000 *Spectrophotometer* $OD_{260/280nm}$ and stored in the micro tube at 20°C as stock template

of each PCR reaction. The kmt gene was detected by reacting a mixture of 22.5 µl Platinum PCR SuperMix High Fidelity Invitrogen, 0.5 µl primer KMT1T7, 0.5 µl primer KMT1SP6, and 1.5 µl DNA samples. PCR reaction was performed using these following programs: pre-denaturation (at 94°C for 2 minutes); denaturation (at 94°C for 30 seconds); annealing (at 55°C for 30 seconds); elongation (at 68°C for 30 seconds); post elongation (at 68°C for 1 minute). The reaction was performed for 35 cycles and then the PCR product was electrophoresed in the gel agarose 2% at 100 volt, 200 mA for 70 minutes and showed the tape at around 460 base pairs under UV exposure and was documented using the geldoc.

Detection and characterization of capsular gene molecule of *P. multocide* using PCR

Once the isolate in previous PCR reaction was confirmed as *P. multocide*, then further detection of the capsular serotype was performed. Mix reaction consisting of 22.5 µl of Platinum PCR SuperMix High Fidelity invitrogen, 0.5 µl primer of capsular forward, 0.5 µl primer of capsular reverse and 1.5 µl DNA samples. PCR condition and the electrophoresis on agarose gel were similar to the detection program of the kmt gene. Amplification of capsular gene of A/*hya*D-*hya*C used primer of CAPA: forward 5'-TGC-CAA-AAT-CGC-AGT-CAG-3' and reverse 5'-TTG-CCA-TCA-TTG-TCA-GTG-3' with product 1044 bp (Townsend et al. 2001). Amplification of capsular B/bcbD gene used primer of CAPB: forward 5'- CAT-TTA-TCC-AAG-CTC-CAC-C-3' and reverse 5'- GCC-CGA-GAG-TTT-CAA-TCC -3'with the product at around 760 bp.

Detection and characterization of molecule gene 16S rRNA of *P. multocide* using PCR

Amplification of 16S rRNA gene was performed to determine the genetic relationship (Mizrahi-Man et al. 2013) of six local isolate of *P. multocide* from various countries at the GenBank. PCR reaction was performed by mixing 12.5 µl Hot Star Taq, 1 µl primer forward, 1 µl primer reverse, 1 µl DNA and 9.5 µl RNAse free water in micro tube. PCR condition was programed to pre-denaturation (at 95°C for 15 minutes); denaturation (at 94°C for 1 minute); annealing (at 53°C for 45 seconds); elongation (at 72°C for 1 minute); and post-elongation (at 72°C for 10 minutes). The reaction was conducted as 32 cycles and the PCR product was then electrophoresed in the agarose gel 2% at 100 volt, 200 mA for 50 minutes and the desired result was 642 bp under UV exposure or gelDoc camera (Lee at al. 2014).

16S rRNA gene sequencing

Amplification products of 16S rRNA were sent to 1st Base Singapore to be sequenced using Sanget method.

Data analysis

Data of sequencing were analyzed using BIODET program to determine consensus and contig sequences, then blast nucleotide was done using BLASTn program (NCBI) to determine the identity structure and sequence homology resulted to other isolates in the GenBank. The sequence was compared to five highest isolates of BLASTn with multiple alignments using ClustalW program. Phylogenic tree was built using Neighbor joining program MEGA 7.0.25 method (Peng et al. 2017).

RESULTS AND DISCUSSION

Specific gene of *P. multocide* was detected using electrophoresing the PCR result of those five isolates in agarose gel. DNA of those five isolates was isolated using Qiagen® kit and then PCR was performed in accordance with the formula and condition described in the materials and methods. Amplification of specific gen (kmt) of *P. multocide* using showed the amplicons at around of 460 bp (Figure 1). It showed molecularly that those five isolates in this study were *P. multocide*. Solongo et al. (2015) performed genetic analysis of *P. multocide* isolated from local cattle of Mongolia and showed similar result. PCR condition was performed in accordance to the properties of forward and reverse oligonucleotide. Before the PCR process, melting temperature checking of forward and reserve primers was performed according to master mix that will be used in order to obtain the optimal result (Lorenz 2012).

Capsular gene of those five local isolates of *P. multocide* was detected using primer of CAPA and CAPB. Amplification result showed that two of five sample isolates were *P. multocide* serotype A that coded capsular gene A (PMa and PMc) showed with amplicons appearance at around 1044 base pair (Figure 2). Whereas, those three other isolates were isolate *P. multocide* serotype B (PMb, PMd, and PMk) that showed amplicons at 750 base pair (Figure 3).

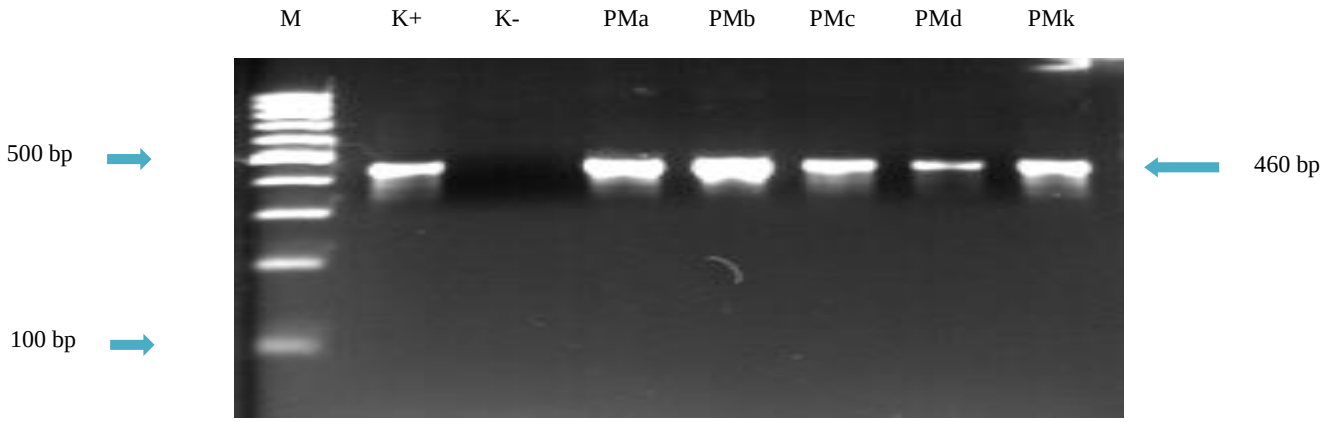

Figure 1. Detection of kmt gene of *P. multocide* using PCR.
PCR product of 460 *base pairs* (bp) specific to *P. multocida*
M: Marker, **K+:** Control positive (NCTC11668), **K-:** Control negative (*B. abortus*),
PMa, PMb, PMc, PMd: *P. multocide* Isolate from Lampung,
PMk: *P. multocide* Isolate from Kupang.

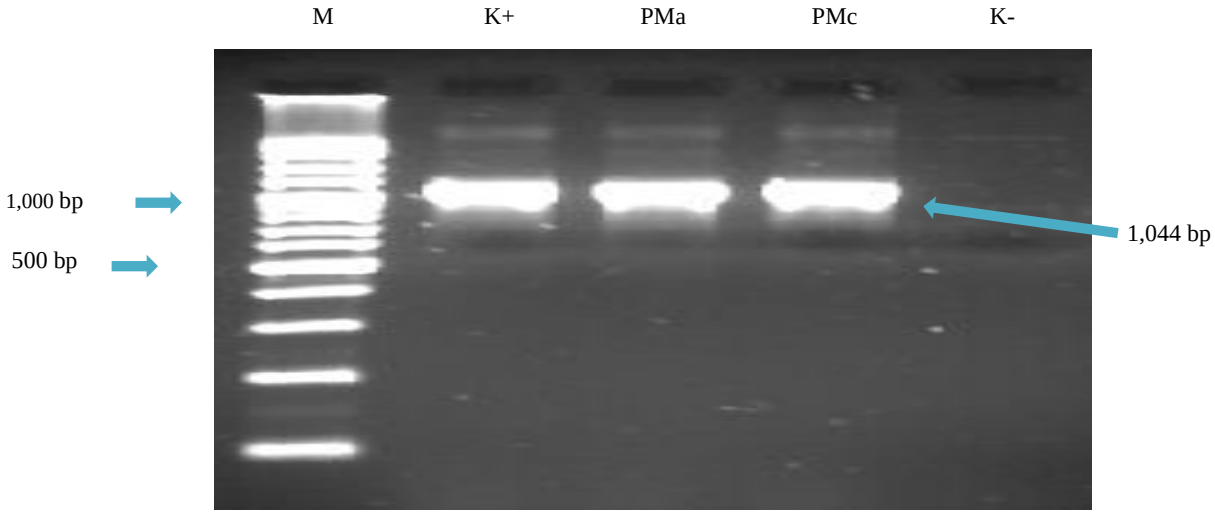

Figure 2. Detection of A capsular gene (*hyaD-hyaC*) of *P. multocide* using PCR.
Amplicons of serotype B of *P. multocide* on around 1.044 *base pairs* (bps)
M: Marker, **K+:** Control Positive (ATCC12945), K-: Kontrol Negative (*B. abortus*)
PMa, PMc: *P. multocide* Isolate from Lampung.

The two PCR processes informed that *P. multocide* in this study were serotype A and B. all isolate samples from cattle in Lampung and Kupang. Serotype A on cattle generally related to pneumonia (Frank 1989), while the serotype B related to the hemorrhagic septicemia/HS (Ranjan et al. 2011). Kupang as the capital of East Nusa Tenggara province known as beef cattle production center in eastern Indonesia, is an endemic area of HS case or known as snoring disease. The snoring disease on cattle happens almost every year in Kupang (Berek et al. 2015) leading to decrease in cattle population in latest several years (Priyanto 2016). While the HS outbreak in South Lampung was reported in 1984 and 1989 (Putra 2006). However, this study has not been able to determine that *P. multocide* from Kupang always serotype B. It shows that it needed further studies with more samples from more area in East Nusa Tenggara, except Kupang.

Amplification of 16S rRNA gene using PCR method to *P. multocide* isolate was performed to determine the relationship of local *P. multocide* with several isolate from abroad. Subunit gene of ribosome

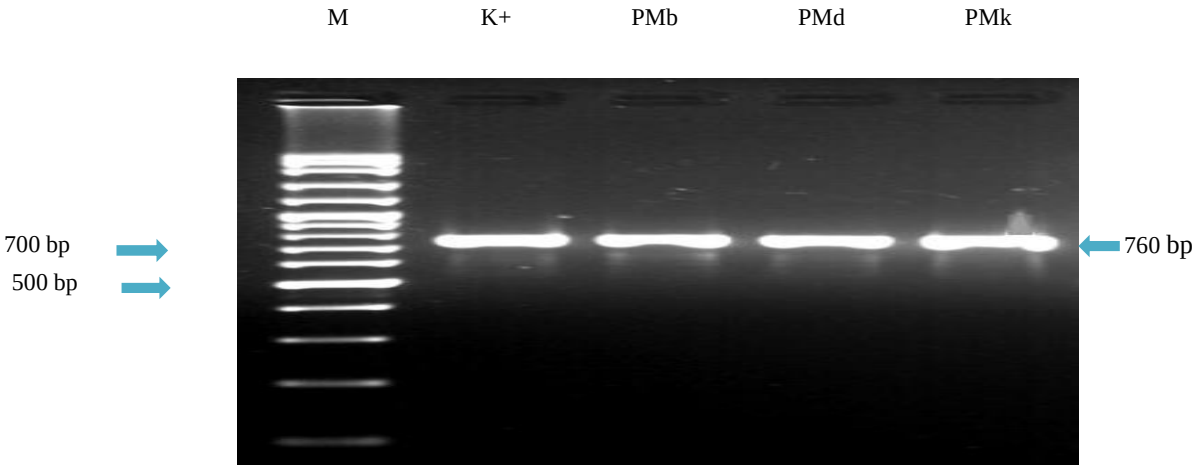

Figure 3. Detection of B capsular gene (*bcb*D) of *P. multocide* using PCR. Amplicons of B *P. multocide* serotype on around 760 *base pairs* (bps)
M: Marker, **K+:** Control positive (NCTC11668),
PMb, PMd: *P. multocide* Isolate from Lampung,
PMk: *P. multocide* Isolate from Kupang.

16S (16S rRNA), especially has been widely used in studying and characterizing bacteria community coverage on various ecology including the host related to the community like human microbiome endogen (Arumugam et al. 2011a) or the host that unrelated to humans. rRNA sequence, especially 16S rRNA represents the most important targets in the study of bacterial and ecological evolution, including to determine the phylogenic among taxa and exploration of environment bacteria diversity. The 16S rRNA gene was selected because that gene is distributed universally that allow phylogenic relationship analysis between the distant taxa (Větrovský & Baldrian 2013).

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Amplification result of 16S rRNA gene of *P. multocide* local isolate was 642 base pairs (Figure 4) which then was sequenced and analyzed using BIODET program to determine the contig sequence. The BLASTn program on NCBI site was used to blast to determine isolate homolog to the other isolates at the GenBank.

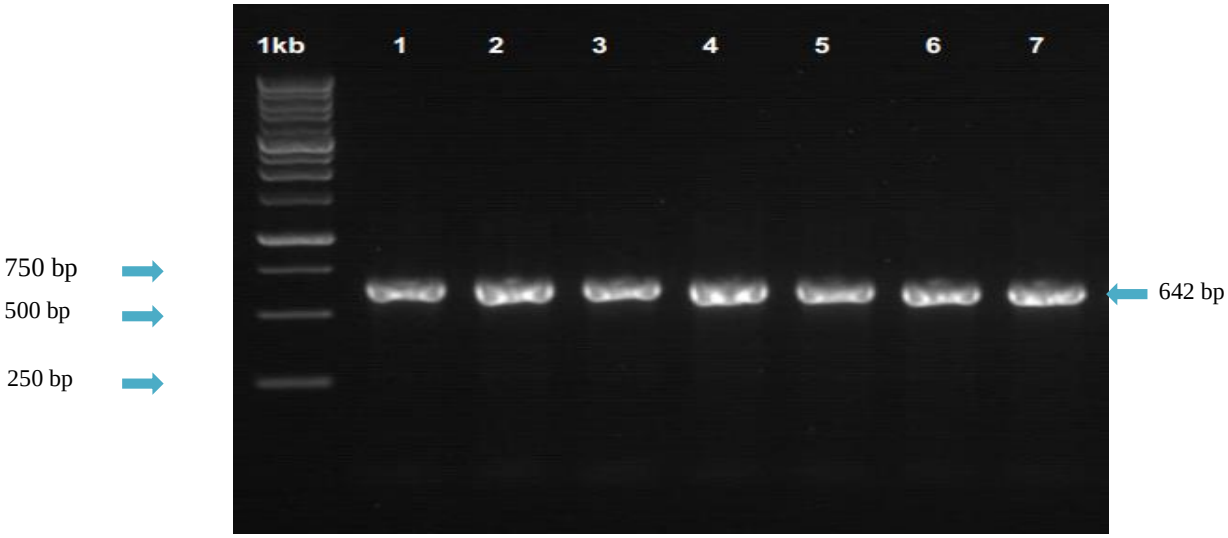

Figure 4. Detection of 16S rRNA gene of *P. multocida*
Amplicons of 16S rRNA gen of *P. multocide* on around 642 *base pairs* (bps)
1kb: Marker, 1: NCTC11668, 2: ATCC12945
3: PMa, 4: PMb, 5: PMc, 6: PMd, (Isolate from Lampung) 7: PMk (Isolate from Kupang).

Table 1. Matrix of nucleotide difference (642 nucleotides) of 16S rRNA gene using MEGA 7.0.25 program

Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
PM_797_India																
PM_FUP9_Egypt	0.000															
PM_Japan	0.000	0.000														
PM_NCTC10322_Scotland	0.000	0.000	0.000													
PM_GDZQ201401_China	0.004	0.004	0.004	0.004												
PM_TB2.1_Malaysia	1.190	1.190	1.190	1.190	1.213											
PM_Razi_Iran	1.190	1.190	1.190	1.190	1.213	0.000										
PM_NCTC10322_Canada	1.190	1.190	1.190	1.190	1.213	0.002	0.002									
PM_ATCC12945	1.190	1.190	1.190	1.190	1.213	0.002	0.002	0.000								
PM_NCTC11668	1.190	1.190	1.190	1.190	1.213	0.002	0.002	0.000	0.000							
PM_a_Lampung	1.179	1.179	1.179	1.179	1.202	0.004	0.004	0.002	0.002	0.002						
PM_b_Lampung	1.179	1.179	1.179	1.179	1.202	0.004	0.004	0.002	0.002	0.002	0.000					
PM_c_Lampung	1.190	1.190	1.190	1.190	1.213	0.002	0.002	0.000	0.000	0.000	0.002	0.002				
PM_d_Lampung	1.190	1.190	1.190	1.190	1.213	0.000	0.000	0.002	0.002	0.002	0.004	0.004	0.002			
PM_k_Kupang	1.190	1.190	1.190	1.190	1.213	0.000	0.000	0.002	0.002	0.002	0.004	0.004	0.002	0.000		
Brucella_abortus	0.220	0.220	0.220	0.220	0.225	1.195	1.195	1.195	1.195	1.195	1.184	1.184	1.195	1.195	1.195	

Description: PM: *Pasteurella multocide*; a,b,c,d, k: code for samples (local isolate).

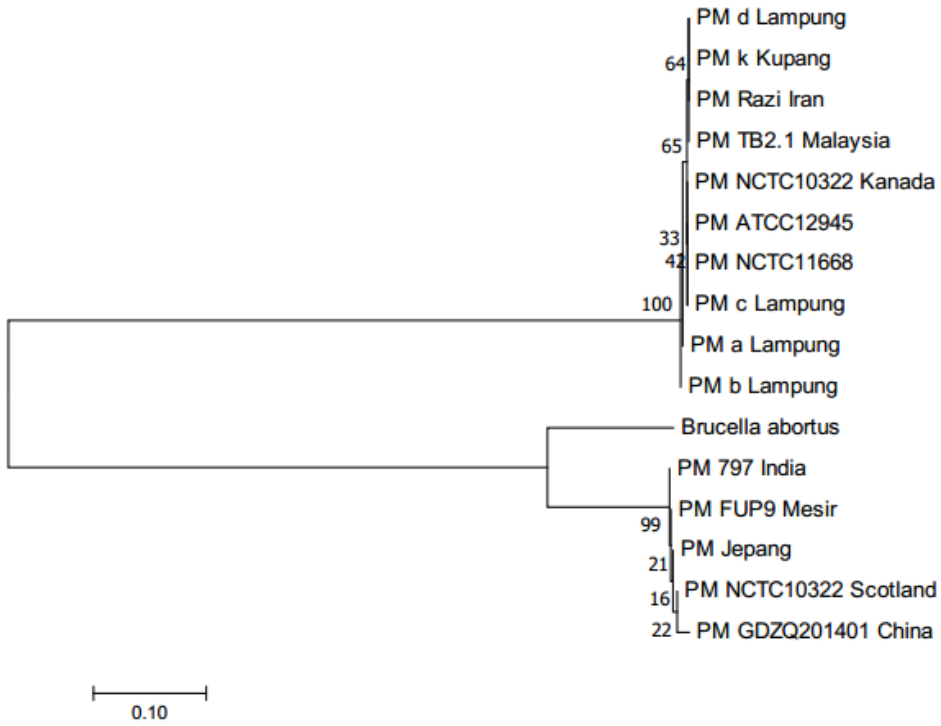

Figure 5. Phylogram of Evolutionary History of 16S rRNA (642 nt) gene nucleotide of local isolate P. mulcotide compared to isolates from other countries from GenBank using *Neighbor-joining* that counting by 2 parameters Kimura method on MEGA 7.0.25 program.

The multiple alignment of the sequence of 16S rRNA gene nucleotide of *P. multocide* of sequencing resulted in this study with the sequencing from China, Japan, India, Scotland and Egypt from the GenBank using ClustalW program showed the existence of the nucleotide.

Genetic distance counting by 2 parameters Kimura method (Table 1) showed genetic distance difference between the *P. multocide* isolates from Lampung and Kupang and *P. multocide* isolates from several countries in the world. The average genetic distance between *P. multocide* species using neighbor joining method of 642 bases of nucleotides of the 16S rRNA gene showed that level of the nucleotide of *P. multocide* isolate in the results of the study have no relationship with isolates from India, Egypt, Japan, Scotland and China. *P. multocide* isolate from Lampung and Kupang had relationship with the *P. multocide* isolate from Malaysia, Iran and Canada (Figure 5).

Matrix value of the genetic distance between each isolate presented in Table 1 was used to build phenogram tree (Figure 5). That phenogram tree shows the genetic distance between all isolates. In other words, all *P. multocide* isolates aligned is divided into two main branches, so that the *P. multocide* isolate from Lampung and Kupang is in the same branch with the *P. multocide* from Malaysia, Iran, and Canada, Pascoe et al. (2017) said that genetic structure of bacteria population can be associated with geographical location of isolation. On several species, there is a strong correlation between geographic distance and genetic distance that may be caused by different evolutionary evolution. Sheppard et al. (2014) stated that frequency of certain genotype isolated from the different host is a basic of host identification related to clonal complex. This indicates that nucleotide sequence is different in two main groups of phenogram tree that shows the thing that may occur due to a different host, geographic and isolate. It does not rule out the possibility of the different nucleotide sequence was affected by lineage development due to deviations of a single or multiple genes on Multi Locus Sequence Typing gene (Bisgaard et al. 2013).

CONCLUSION

This study and analysis informed that five bacteria isolates have been identified as *P. multocide* serotype A and serotype B. Four isolates from Lampung consisted of each two isolates either serotype A or serotype B. While, isolates from Kupang was *P. multocide* serotype B. PMc isolate had high homologue with the ATCC 12945, so that it can be used as positive control of serotype A. On the other hand, *P. multocide* isolate

from Kupang can be used as positive control of serotype B, because it is identic to *P. multocide* PMTB2.1 (CP007205.2) isolated from buffalo infected with HS in Malaysia.

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