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RESEARCH PAPER

Molecular characterization of *Pasteurella multocida pfhaB1* gene fragment from buffalo and cattle isolates from Nusa Tenggara Timur Indonesia

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ABSTRACT

Almost all regions in Nusa Tenggara Timur Province Nusa Tenggara (NTT) Province Indonesia are endemic areas of Haemorragic Septicaemia (HS), which caused by *Pasteurella multocida* Serotypes B: 2. The fragment *pfhaB1* gene is one of the virulence factors of *P. multocida*. The objectives of this study were to determine the phylogenetic and homology of *P. multocida pfhaB1* gene fragment of isolated from buffalo and cattle in NTT. The *P. multocida* isolates were re-cultured and further microscopic examined the biochemical tests, PCR, sequencing, homology, and phylogenetic relatedness test. *P. multocida* was observed as gram negative, coccobacilus, no growth on MacConkey Agar, does not produce H_2S and gas, nonmotile and indole positive, does not produce urease enzymes, does not use citrate as a carbon source, does not ferment maltose and lactose but it does ferment glucose, sucrose and mannitol. The *pfhaB1* gene fragment from buffalo and cattle NTT isolates and also Katha strain vaccine showed DNA band 506 bp. *P. multocida* isolates from buffalo and cattle in NTT have 91% - 99% score homology with the comparative isolate. The isolate *P. multocida* from buffalo and cattle in NTT are in one cluster and their phylogenetic relatedness is close to isolates from Iran and India. It is concluded that the *pfhaB1* gene fragment of *P. multocida* from buffalo and cattle isolates have phylogenetic relatedness close and homolog with the other comparative isolates.

Keywords : Haemorrhagic Septicaemia; Nusa Tenggara Timur; *Pasteurella multocida; pfhaB1* gene

INTRODUCTION

Nusa Tenggara Timur (NTT) Province is one of the provinces that have high potency on livestock in Indonesia, and this is the primary income source for local people of NTT. The livestock sector has become a mainstay of the regional agricultural sector in making a significant contribution to the regional economic growth, that also supported by the population of buffalo and cattle in NTT is 936,611, include 803,450 beef cattle, 39 dairy cows and 133,122 buffaloes (Sukada *et al.,* 2016). However, the buffalo and cattle population in Nusa Tenggara Timur province are decreasing because of a disease (KPDE, 2006). Haemorragic Septicaemia (HS) is an animal disease that occurs almost every year in the Kupang Regency (Berek *et al.,* 2015).

Nusa Tenggara Timur is an endemic area of Haemorragic Septicaemia (HS), except Lembata District (Berek *et al.,* 2015). The economic loss caused by HS infection that attacks buffalo and cattle in Kupang District NTT Province is approximately IDR 16.2 Billion per year

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(KPDE, 2006). The HS disease in NTT caused by *P. multocida* serotypes B (Puspito, 2018). A fever followed by respiratory involvement and neck swelling is the dominant diagnosis of HS dise[26]. The clinical sign of HS in buffaloes is severe than in cattle (Priadi and Lily, 2001). One of the virulence genes of *P. multocida*, is a pfhA (*a Filamentous Hemagglutinin*) gene which has an important role as epidemiological marker genes for charact[3]izing *P. multocida*field strains and as adhesion factor (Ewers[10]2006). The pfhA gene has two a *Filamentous Hemagglutinin* genes which are *fhaB1* and *fhaB2* ((M[3] *et al.,* 2001; Johnsonet *et al.,* 2013; Lainson *et al.,* 2013; Guo *et al.,* 2014). The *fhaB1* and *fhaB2* genes were identified as encoding the potential virulence using signature-tagged transposon mutants[25] a mouse septicemia model and selectively captured of transcribe[12] sequences in rabbit livers (Fuller *et al.,* 2000). According to Gou *et al.* [3]014). The *fhaB1* plays a critical role in disease [3]thogenesis and the progression of FC because the virulence of the *fhaB1* mutant was showed a high degree of attenuation w[3]n the chickens were challenged intranasally and a lesser degree whe[5] challenged intramuscularly in SPF chickens with wild type strain C48-102 and The *fhaB1* mutant increased sensitivity to the bactericidal activity of the serum complement system.

Several studies on the *P[8]multocida* have been reported by researchers, for instance Kalhoro *et al.* (2015) found that the *P. multocida* type 1 B-6 was a gram-negative, short rods, arranged singly, and bipolar. Coccobacillary in shape and recorded as non-motile and non-spore forming. Kumar *et al.* (2008) have identified a capsule of *P. multocida* fro[5] buffalo and cattle isolates by PCR method are 760 bp. According to Tatum *et al.* (2005) showed a high degree of att[5]uation intranasally when Turkeys we[5] challenged by *P. multocida fhaB2* gene mutant fragment in an avian strain P-1059 (A:3) and to a lesser degree when intravenously administered. However, the study on the identification and characterization of *pfhaB1* gene fragment on *P. multocida isolated* from buffalo and cattle in NTT until now was not reported p[1]voously. Therefore, the objectives of this study were to determine the phylogenetic and homology of *P. multocida pfhaB1* gene fragment of isolated from buffalo and cattle in NTT, Indonesia.

MATERIALS AND METHODS

Bacterial Isolate

Pasteurella multocida isolate bacteria from Buffalo and Cattle of NTT Province Indonesia was taken from Center of Veterinary (*Balai Besar Veteriner*) Denpasar, Indonesia were obtained from HS cases that occurred in NTT at 2016 and Katha strain vaccine from PUSVETMA Surabaya Indonesia as the positive control.

Pateurella multocida Reculture and Biochemical Test

[14]

Pasteurella multocida isolate from NTT were re-cultured on Blo[11] Agar and incubated at 37 °C for 24 hours and followed by biochemical tests include: Triple Sugar Iron Agar (TSIA), Sulfid Indol Motility (SIM), Urea, Simmon's Citrate [21]gar (SCA), MacConkey, Glucose, Maltose, Mannitol, Lactose and Sucrose) in Laboratory of Microbiology, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia.

DNA Extra[28]on

The DNA extraction was carried out using extraction KIT Gene JET Genomic DNA Purification produced by Thermo Fisher Scientific, Jakarta, Indonesia. Bacteria were taken from

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several colonies, while Katha strain vaccine was centrifuged and washed several times to get a pellet. Bacteria and vaccine pellet were added with 180 *µl* of Degestio Solution and incubated at 50 0C for 30 min. The suspension was added with 200 *µl* lysis solution and mixed by vortex for 15 seconds. Then suspension was added with 400 *µl* of ethanol 50%, and tube extraction was put into purification DNA column and centrifuged at 6000 rpm for 1 min. Pellets were washed using 500 *µl* of washing buffer I then centrifuged at 8000 rpm for 1 min. The second washing used was 500 *µl* of buffer II and centrifuged at 12000 rpm for 3 min. Then pellet was added with 200 *µl* of E buffer and incubated in the room temperature for 2 min and centrifuged at 8000 rpm for 1 min. DNA extraction microtube was moved to a 1.5 ml microtube from the column of DNA purification. The concentration of DNA was measured by Nano Drop Spectrophotometer produced by Maestrogen, Hsinchu City 30091, Taiwan.

DNA Amplification and Electrophoresis

The sequence of primers for detection of *pfhaB1* gene fragment in this study was 5' GAC GGG GTA GAT TTA TCC TC 3' (Forward primer) and 5' CCA GGC AGA TCA AAA TAC CC 3' (Reverse primer) that were designed in this study based on complete CDS from GenBank (AF237928.1) with the DNA band target is 506 bp. One *microliter* forward primer, 1 *µl* of reverse primer, 12.5 *µl* of master mix, 3 *µl* of of DNA extract and 2.5 *µl* of H_2S were added into a sterile microtube and a PCR was then further amplified using thermocycler with first denaturation temperature at 95 °C for 3 min followed by the second denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 70 °C for 1 min, final extension at 72 °C for 10 min. PCR was done for 35 cycles. Product of PCR was visualized using 2% concentration agarose electrophoresis gel for 50 min. The DNA was visualized using transilluminator UV at a wavelength of 302 nm.

Sequencing

The PCR products were sent to CV Biotek Prima Indoplus, Sidoarjdo, Indonesia for sequencing to detection nucleotide structure of *pfhaB1* genes fragment and was further sent to Genetika Science, Jakarta, Indonesia for sequencing to compare the result of sequencing from *pfhaB1*genes fragments.

Multiple Alignment Analysis

The sequencing results was reprocessed using the Bioedit 8.0 Ver program to get the consensus of the fragment gene *pfhaB1* from buffalo and cattle NTT isolates and also Katha strain vaccine. The nucleotide sequences obtained were further translated to an amino acid by toggle translation software on the Bioedit 8.0 Ver program.

The amino acid sequences were alignment analysed with the comparative fragment gene pfhA from GenBank include: putative filamentous hemagglutinin/AF237928.1, *Bos taurus*/USA Kentucky/CP015571.1, Cattle/Denmark /CP026859.1, Bovine/ Iran/CP017961.1, Bos Taurus/USA Kansas /CP015558.1, Bos Taurus /USA Tennessee /CP015572.1, Cattle /China /CP014618.1, Calf/ India/ CP023305.1 using ClustalW software on Bioedit 8.0 Ver program.

Homology and Phylogenetic Tree

The nucleotide sequences of *pfhaB1* genes fragment from buffalo and cattle in NTT and Katha strain vaccine were blasted using online BLAST program (Needle man and Wunch) (Pertsemlidis and Jhon, 2001)and a phylogenetic tree was analyzed using MEGA (Molecular Evolutionary Genetics Analysis) with the comparative fragment gene pfhA (Kumar *et al.,* 2016) .

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RESULTS

Biochemical Tests of *Pasteurella multocida*

Pasteurella multocida isolates were confirmed by microscopic test and biochemical test. Biochemical profiles of *P. multocida* indicated that gram-negative, coccobacillus, no growth on MacConkey Agar, were not produced H2S and gas, non-motile and indole positive, it was not produced urease enzymes, were not used citrate as a carbon source. The bacterias were not fermented maltose and lactose, but this fermented glucose, sucrose, and mannitol. The biochemical test result can be seen in Table 1.

Amplification of Fragment *pfhaB1* gene of *Pasteurella multocida*

Two isolates of *P. multocida* bacteria from NTT and Katha strain vaccine have been detected *pfhaB1* gene fragment by PCR method with primers: Forward 5' GAC GGG GTA GAT TTA TCC TC 3' and Reverse 5' CCA GGC AGA TCA AAA TAC CC 3'. The result showed a DNA band of *pfhaB1* genes fragment PCR product of 506 bp. The DNA product of *pfhaB1* genes fragment was equal with Katha strain vaccine as positive control and target DNA band (Figure 1).

Table 1. Biochemical Test of *Pasteurella multocida* from buffalo and cattle NTT isolates

Biochemical Test	Buffalo	Cattle
MCA	No growth	No growth
TSIA	Slat and butt (+) H_2S (-) gas (-)	Slat and butt (+) H_2S (-)gas (-)
SIM	Non motile and indole positive	Non motile and indole positive
SCA	-	-
Urease	-	-
Maltose	-	-
Lactose	-	-
Glucose	+	+
Sucrose	+	+
Mannitol	+	+

+: Positive - : Negative

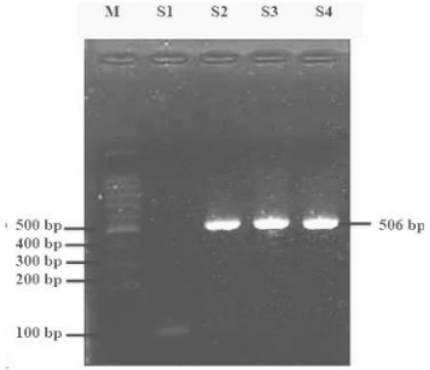

Figure 1. Electrophoresis gel of PCR products of *pfhaB1* gene *Pasteurella multocida* with the primer *pfhaB1* FWD and *pfhaB1* Rev. Symbol M is Marker, S1 is Negative control, S2 is Katha strain vaccine as positive control, S3 is *pfhaB1* gene from cattle isolate, and S4 is *pfhaB1* gene from buffalo isolate. The agarose gel showed a DNA band of *pfhaB1* genes of 506 bp.

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Sequencing and Multiple Alignments of *pfhaB1* Genes Fragment

The results of sequencing of Pasteurella multocida isolates of the *pfhaB1*gene fragment after being analyzed using the Bioedit program have the same nucleotide sequences. The results of sequencing confirmed that the *P. multocida* isolate of fragment *pfhaB1* gene from buffalo harboured three different amino acids sequences with cattle isolate and eight different amino acids sequences with Katha strain vaccine (Figure 2).

Homology and Phylogenetic Tree of *Pasteurella multocida pfhaB1* Gene Fragment

The phylogenetic relatedness of *P. multocida pfhaB1* gene fragment from buffalo (MK806389) and cattle isolates in NTT (MK806391) was close and in the same cluster. Although both of them in the different cluster with *pfhaB1* gene fragment from Katha strain vaccine, bovine from Iran (CP01796.1) and calf from India (CP014618.1) were close. The phylogenetic relatedness can be seen in Figure 3.

The homology score of *P. multocida pfhaB1*gene fragment from buffalo isolate of NTT to pfhA gene fragment bovine from Iran (CP01796.1) and Calf from India (CP014618.1) each were 99%. While homology score of *P. multocida pfhaB1* genes fragment from Buffalo NTT isolate to *Bos taurus*from USA Kentucky (CP015571.1), cattle from Denmark (CP026859.1), *Bos taurus*from USA Kansas (CP015558.1), *Bos taurus* from USA Tennessee (CP015572.1) each were 93% and homology score of fragment *pfhaB1* genes *P. multocida* from buffalo NTT isolate to cattle from China (CP014618.1) was 90%. The homology score of buffalo isolate can be seen in Table 2.

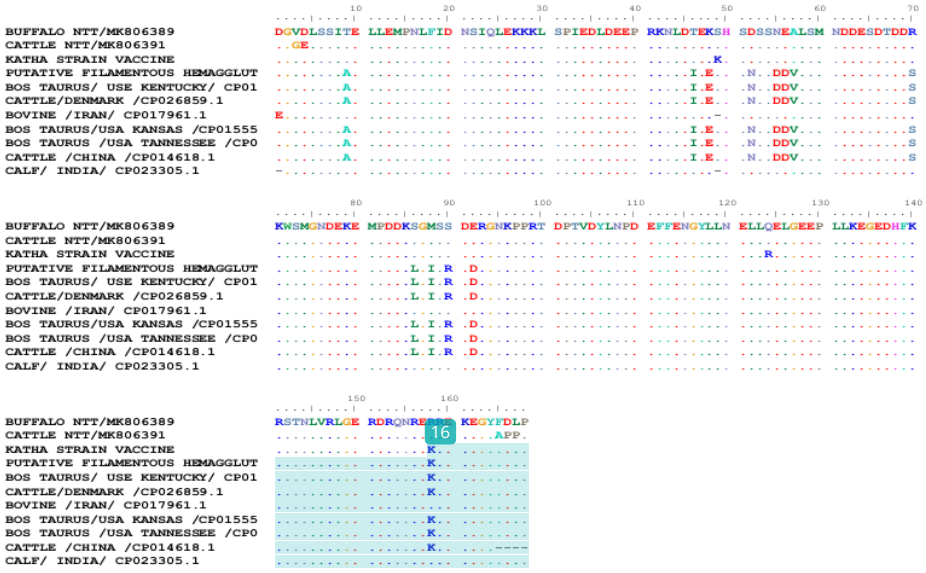

Figure 2: Amino acids sequences *pfhaB1* gene fragment a coding pfhA protein buffalo NTT/ MK806389, cattle NTT/ MK806391, Katha strain vaccine, Bos Taurus/USA Kentucky/CP015571.1, Cattle/Denmark /CP026859.1, Bovine/Iran/CP017961.1, Bos Taurus/USA Kansas/CP015558.1, Bos Taurus /USA Tannessee /CP0 15572.1, Cattle /China/CP014618.1, Calf/India/CP023305.1.

Aceh Journal of Animal Science (2019) 4(2): 70-79
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The homology score of *P. multocida pfhaB1* gene fragment from cattle NTT to pfhA gene fragment from bovine from Iran (CP01796.1) and calf from India (CP014618.1) each were 97%, while homology score of isolate *P. multocida pfhaB1* genes fragment from Cattle NTT isolate to Bos Taurus from USA Kentucky (CP015571.1), cattle from Denmark (CP026859.1), *Bos Taurus* from USA Kansas (CP015558.1), *Bos taurus* from USA Tennessee (CP015572.1) each was 91%, and homology score to cattle from China (CP014618.1) was 90%. The homology score of cattle isolate can be seen in Table 2.

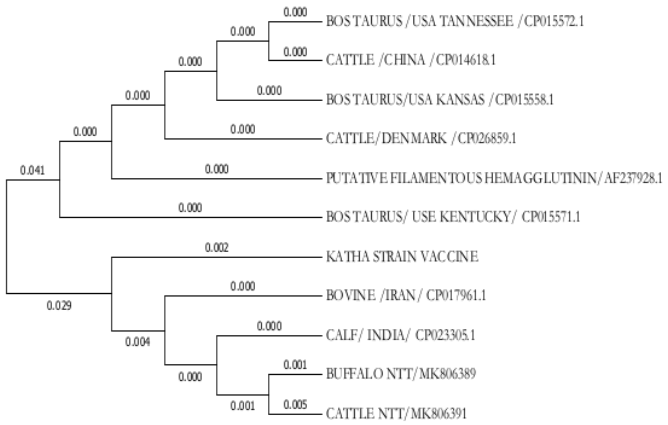

Figure 3. The phylogenetic of fragment *pfhaB1* genes sequences from Buffalo and Cattle isolate of Nusa Tenggara Timur (NTT) as well as Katha strain vaccine compared to the GenBank data base using MEGA with 1000 boostrep.

Table 2. Homology of nucleotide sequences of *pfhaB1* genes fragment with pfhA genes fragment from GenBank.

No	Comparative isolate	Homology Score %										
		1	2	3	4	5	6	7	8	9	10	11
1	Putative Filamentous Hemagglutinin	100										
2	Buffalo NTT / MK806389	93	100									
3	Cattle NTT/ MK806391	91	98	100								
4	Katha Strain Vaccine	93	99	97	100							
5	Bos Taurus/ USA Kentucky/ CP015571.1	100	93	91	93	100						
6	Cattle/Denmark /CP026859.1	100	93	91	93	100	100					
7	Bovine /Iran/ CP017961.1	92	99	97	99	92	92	100				
8	Bos Taurus/USA Kansas /CP015558.1	100	93	91	93	100	100	93	100			
9	Bos Taurus /USA Tannessee /CP015572.1	100	93	91	93	100	100	93	100	100		
10	Cattle /China /CP014618.1	97	90	90	90	97	97	90	97	97	100	
11	Calf/ India/ CP023305.1	92	99	97	98	92	92	99	92	92	89	100

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DISCUSSION

The local *P. multocida* isolates of buffalo and cattle NTT used were obtained from HS cases that occurred in NTT at 2016 collected by Center of Veterinary (*Balai Besar Veteriner*) Denpasar, Bali Indonesia. Based on the results of biochemical tests showed that isolate buffalo and NTT cattle were *P. multocida*bacteria. These results were in agreement with the findings by Ara *et al.* (2016). In the past study was showed the buffalo NTT isolate is *P. multocida* type B because it was detected fragment bcbd gene (Puspito, 2018). The results of her study are in accordance with Dziva *et al.* (2008), which states that the cause of HS in Asia is *P. multocida*type B.

In contrast to bcbd gene investigated, in this study, the virulence features *pfhaB1*genefragment encoding pfhA protein from the buffalo, the cattle NTT isolates and Katha strain vaccine as positive control all have the *pfhaB1* gene fragment which was shown in the DNA PCR product by DNA band at 506 bp with a clear band and thick. The results of the gene amplification indicate that the results of the amplification process of the *pfhaB1* genes fragment reached the desired gene target (Figure 1).

In this study, the sequence of primer for detection *pfhaB1* gene fragment were Forward 5' GACGGG GTA GAT TTA TCC TC3' and Reverse 5' CCA GGC AGA TCA AAA TAC CC 3' t hat was designed in this study based on complete CDS from GenBank. The *pfhaB1* gene fragment was also successfully identified in chickens by Guo *et al.* (2014) with different primers (*pfhaB1* F2: 5 'GGG TAA GAA GGC GAA AGA G3' and *pfhaB1* R2: 5 'TTC TGT CTC AGT ATT GAC C 3) and showed a DNA band 645 bp. Based on the result, both of *pfhaB1* in this study and Guo *et al.* 2014 study can be concluded the DNA band of a DNA base on the primers used. The PCR method can increase DNA sequences to around 106up to 107times. In each PCR cycle, the amount to target DNA will be double (Joshi and Deshpande, 2011). After the DNA band was found, the PCR product was further to sequencing. In this study, the PCR product was sent to CV Biotek Prima Indoplus and Genetica science for sequencing. The result both of them showed of sequencing no difference in nucleotide sequences. The nucleotide sequences of *P. multocida pfhaB1* gene fragment from buffalo and cattle NTT was submitted the GenBank with the ACC Number MK806389 and MK806391.

The DNA sequences of *pfhaB1*genes fragment were further translated to amino acid using google translation software on Bioedit 8.0 Ver program and the results obtained from the translation of proteins from the nucleotides of each of *pfhaB1*amino acid fragment as coding pfhA protein of *P. multocida* (Figure 2). A single gap in the protein alignment corresponds to a group of three gaps in the DNA alignment. This means that the DNA will be aligned in a manner that respects codon–codon boundaries and analogous codon positions will, therefore, always line up (Wernersson and Pedersen, 2003). In this study, it was found 506 nucleotide sequences of *pfhaB1* genes fragment so that 506 nucleotides were translated into 168 amino acids from buffalo NTT, cattle NTT, and Katha strain vaccine. It was reached with the desired gene target. The amino acid sequence of the translational proteins from the nucleotides of each *pfhaB1* genes fragment was aligned using ClustalW software on BioEdit 8.0 Ver Program (Figure 2). By sequence alignment of *pfhaB1* gene fragment sequences, we identified the *pfhaB1* gene fragment of buffalo NTT isolate have three different amino acid sequences with Katha strain

vaccine (amino acids 49, 124, 158). While the *pfhaB1* gene fragment of cattle NTT isolates has eight different amino acid sequences with Katha strain vaccine (amino acids 3,4,49, 124, 158, 165, 166, and 167). However, both of the *pfhaB1*gene fragment buffalo NTT and cattle NTT isolates shows the general nature of the *pfhaB1*gene fragment from the isolate still has a high level of homology. The difference amino acids sequences in these isolates is a result of mutations. Mutations are caused by physical changes to the hereditary material and, because DNA is a long sequence of base pairs organized into physically unlinked chromosomes, there are many possible ways it can change (Louwe and William, 2010). In this case, there has been a point and inversions mutation. Point mutation is mutation change only a single letter and leads to so-called 'single nucleotide polymorphisms' in populations while inversions is mutation various sizes that change the orientation of a stretch of DNA (Louwe and William, 2010). Although, the *pfhaB1*gene fragment from two isolates showed mutations but still has the general nature of the *pfhaB1*gene fragment from the isolate because it has a high score of homology.

The similarity of *pfhaB1* gene fragment of *P. multocida* from Buffalo NTT (MK806389) and Cattle NTT (MK806391) was 99%, and they both were in the same cluster (Figure 3). Although in the different cluster, phylogenetic relatedness between the *pfhaB1*gene of *P. multocida* of buffalo NTT isolate and the *pfhaB1* genes of *P. multocida* cattle NTT isolates were close to Katha strain vaccine, the similarity of them were 99% and 97%, respectively. The result of homology can be the reason that the Katha strain vaccine can be promoted as prevention of HS disease in Nusa Tenggara Timur Indonesia.

Homology both *P. multocida pfhaB1* gene fragment from buffalo and cattle NTT were (have similarity) 99% and 97% compared to the bovine pfhA gene fragment from Iran (CP017961.1) and calf from India (CP023305.1). Gou *et al.* (2014), in their study, showed that *P. multocida* pfhA gene fragment is divided into two types namely *pfhaB1* and *pfhaB2*, so it is possible that *P. multocida* of pfhA gene fragment of bovine from Iran and Calf from India is *pfhaB1*. The *pfhaB1*gene fragments from buffalo NTT and cattle NTT also have high homology score with *P. multocida* of pfhA genes fragment from Bos taurus isolates from USA Kentucky (CP015571.1), Cattle from Denmark (CP026859.1), *Bos taurus* from USA Kansas (CP015558.1)), *Taurus bosses* from USA Tennessee (CP015572.1) and Cattle from China (CP014618.1)were 90-93%. These results indicate that the *pfhaB1* gene fragment from buffalo NTT and cattle NTT isolates with comparative isolates are homolog.

The homology score can be said homolog if the percentage of homology more than 60% (Dale, 2003). This study showed that the percentage of homology of the fragment *pfhaB1* gene as coding of pfhA protein from Buffalo NTT and Cattle NTT with Katha strain vaccine and pfhA gene fragment from comparative isolates were more than 90% so that the *pfhaB1*gene fragment from buffalo NTT and cattle NTT were homolog. In addition, based on the homology score is it possible that the pfhA gene fragment from the comparative isolates is *pfhaB1*. This is caused the *P. multocida* from Nusa Tenggara Timur Indonesia possibility from this country or otherwise through export and import activities of buffalo and cattle between countries (Puspito, 2018). The 1% difference at the homology score caused there are gene changes caused by mutations, deletions, and insertions on the DNA sequences (Loewe and William, 2010).

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CONCLUSIONS

The phylogenetic relatedness of the fragment *pfhaB1* gene of *P. multocida* from Buffalo and Cattle NTT was close to Katha strain vaccine, Bovine from Iran, Cattle from China, and Calf from India, and also the *pfhaB1*gene fragment from buffalo NTT and cattle NTT were homolog with the comparative isolates

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