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RESEARCH ARTICLE

Molecular Identification of *Brucella abortus* Collected from Whole Blood Samples of Seronegative Dairy Cattle with Reproductive Disorders in Central Java, Indonesia

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ABSTRACT

Brucellosis is a zoonotic disease that led to economic losses to cattle industries worldwide including Indonesia. A rapid, precise and accurate diagnosis technique is required for early detection and for the control of *brucellosis* in animals and humans. The objective of this study was to molecularly identify and characterize *B. abortus* in the blood samples obtained from seronegative dairy cattle that have had reproductive disorders in Central Java, Indonesia, using PCR, phylogenetic and nucleotide sequence analyses of the IS*711* regions. Results showed that in seronegatively dairy cattle with reproductive disorders, *B. abortus* was successfully detected from the whole blood and serum samples without separation of the buffy-coat and without isolation of the organism by conventional PCR. The result also showed high homology level (up to 100%) and close phylogenetic relationships between nucleotide sequences of the IS*711* gene fragment of *B. abortus* local isolates compare to the isolates that have been accessed in GenBank (CP 009099, CP 023242, CP 023242, CP 023308 and LT 671513). We concluded that PCR technique is useful for routine diagnosis of brucellosis in seronegative dairy cattle with reproductive disorders.

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INTRODUCTION

Reproductive efficiency is an important component of a successful cattle operation. Factor that induces poor reproductive performances is a major concern to the economic consequences. *Brucella* is facultative intracellular gram negative bacteria that cause brucellosis in several species of animals and human (Gupta *et al*., 2014). Even though is not pathognomonic, the clinical consequences caused by *Brucella spp*. in infected animals usually correlated with reproductive problems such as abortion, infertility, retained placenta and birth of weak calves that produce large economic losses to dairy and beef farmers. Identification of infected animals among cattle population is one of the most important but challenging strategy to control the disease from spreading. In Indonesia, brucellosis is categorized as a strategic animal disease that rapidly spread among animals in the herd. Like most other ASEAN countries, the presence of brucellosis in the herd is mostly detected serologically. Serological method for brucellosis are not always sensitive and repeatedly been reported to cross-react with antigens other than *Brucella spp*. (Al-Garadi *et al*., 2011; Priyadarshini *et al*., 2013). Other than that, the methods may also produce false-positive results, especially in cattle that has been vaccinated using *B. abortus* strain 19 or may cause false-negative results in cattle with no antibodies to *B. abortus*. False-negative animal can be a source of disease transmission that interferes the brucellosis control program (Silva Moi *et al*., 2014; El-Diasty *et al*., 2018). The gold standard for the diagnosis of brucellosis is isolation. However, the standard procedure for *Brucella* isolation has many disadvantages such as false-negative results, time consuming, and requires biosafety level 3 facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and characterization (Perez-Sancho *et al*., 2013; Silva Moi *et al*., 2014). Therefore, polymerase chain reaction (PCR)-based method to detect the occurrence of the *Brucella* infection have become prevalent in the last decade because of their specificity, technical ease and lowering the costs (Mahajan *et al*., 2017). Their sensitivities, however, could be affected by the stage of

infection and the appropriate samples to be selected (Geresu and Kassa, 2016). So, far the PCR has not been proven to be effective for routine diagnosis of brucellosis without serologically testing and/or culturing the bacteria. Therefore, it needs to be furtherly studied.

Genetically, all *Brucella* species have high degree similarities at their nucleotide level (Anonymous OIE, 2009). However, unrestricted travel and trade of livestock between different provinces and island and changing ecosystem have led to the possible occurrence of genetic variability within *Brucella* species. The objective of this study was to molecularly identify and characterize *B. abortus* obtained from seronegatively dairy cattle in Central Java, Indonesia that have had reproductive disorders. The whole blood and sera samples were directly and simultaneously used without buffy-coat separation and/or bacteria isolation of the microorganism for PCR analysis.

MATERIALS AND METHODS

Sample collection: In this study, samples were from field cases that were individually reported during period of March to May, 2018. A total of 50 dairy cattle that had reproductive disorders such as abortion (10), repeat breeding (32), retention of placenta (6) and uterine prolapsed (2) but previously had no antibodies to *B. abortus* were bled from coccygeal vein using 2 types of vacutainer tubes. Plain vacutainer and EDTA-coated tubes (Beckton Dickensen) were respectively used for whole blood and sera separation. Both whole blood and sera samples were subjected to molecular detection of genes and species specific of *B. abortus.*

DNA purification: Positive control (live freezed-dried vaccine, Pusat Veteriner Farma, Surabaya, Indonesia that has been reconstituted with distilled water), whole blood and serum samples were extracted using GeneJET Genomic DNA Purification Kits described in the protocol provided by the manufacturer (Thermo Fisher Scientific, Waltham, Massachusetts, America). Briefly, 200 µL of either positive control solution, whole blood or serum were pipetted into a sterile 1.5 ml eppendorf tube and then 400 µL of lysis solution and 20 µL of Proteinase K solution were added into it, the solution was mixed thoroughly by vortexing and incubated the sample mix at 56°C for 10 min. After incubation, the prepared lysate was transferred into a GeneJET Genomic DNA Purification Column inserted in a collection tube and centrifuge for 1 min at 6000xg.After centrifugation, discard the flow-through, place back the purification column into a new collection tube, add 500 µL of wash buffer I and centrifuge for 1 min at 8000xg. Discard the flow-through, place back the purification column into a new collection tube, add 500 µL of wash buffer II and centrifuge for 3 min at 12000xg. Discard the collection tube and insert the GeneJET Genomic DNA Purification Column into a sterile 1.5 ml eppendorf tube, add 200 µL of elution buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g.

DNA amplification and detection of PCR products: Molecular detection of *B. abortus* was done using gene-specific *bcsp31* primer (Bailey *et al*., 1992) and species-specific IS*711* primer (Doust *et al*., 2007).

PCR was carried out in a total volume of 50 µl, the DNA amplification was obtained by adding 25 µl of GoTaq®Green Master Mix 2x (Promega, Madison, WI 53711-5399 USA), 2,5 µl of 10 pMol primer forward and 2,5 µl of 10 pMol primer reverse (Table 1), 2.5 µl of 50 nMol MgCl, 2, 5 µL of Template DNA and Nuclease free water to 50 µl.The PCR mixtures were then subjected to the cycling condition as in Table 2 in Personal Combi Thermocycler Biometra (37079 Goettingen Germany).The PCR amplification product(s) were separated on a 1.5% agarose gel, stained after electrophoresis with ethidium bromide and visualized using ultraviolet transillumination. For sequencing, PCR products were purified using High Pure PCR Product Purification Kit (Roche Life Science, Mannheim, Germany). Forward and reverse sequences for each sample were aligned and used in phylogenetic analysis. The sequences were compared to other previously published sequences. The sequence identities of nucleotide, as well as the estimation of the evolutionary divergence between sequences were analyzed using DNA Baser and Mega7 software, respectively (Kumar *et al*., 2016). The same tool was used to perform Neighbor-Joining analysis.

RESULTS

In this study, the presence of *B. abortus* was detected by conventional PCR from four out of 50 dairy cattle. Sample either in the form of whole blood or sera from the same animal produce the same result. Gel electrophoresis of amplicon confirmed that 2 pairs of primers used, specifically amplified bcsp31 and IS*711* brucella fragment genes in the position of 223 bp and 498 bp respectively (Fig. 1 and 2).

The clinical status of the dairy cattle at the time of sampling varied from abortion (10), repeat breeding (32), retention of placenta (6) and uterine prolapsed (2), Three of dairy cattle that showed positive by PCR had experienced abortion and one cattle suffered from sub-infertility. The incidence of abortion that give positive results of brucellosis occur at different term of pregnancy (2.5 months, 4 months and 7.5 months).

The results of homology analysis in this study (Fig. 3) showed high degree of homology (98-100%) between IS711 gene nucleotide sequence of *B. abortus* local isolates and *B. abortus* from foreign countries. Whereas based on phylogenetic analysis, multiple nucleotide alignment among *B.abortus* isolates based on IS711 regions has close relationships with isolates from Zimbabwe (CP 009099) and water buffalo from Italy (CP 023242, CP 023308 and LT 671513).

Tabel 1: Primer base sequence for *Brucella abortus* used in this study

Primer	Forward (5'→ 3')	Reverse (5' → 3')	PCR Product	Ref.
Brucella spp. (*bcsp*31)	TGGCTCGGTTGCCAATATCAA	CGCGCTTGCCTTTCAGGTCTG	223 bp	Baily *et al.* 1992
Brucellaabortus (IS*711*)	TGCCGATCACTTAAGGGCCTTCAT	GACGAACGGAATTTTTCCAATCCC	498 bp	Doust *et al.* 2007

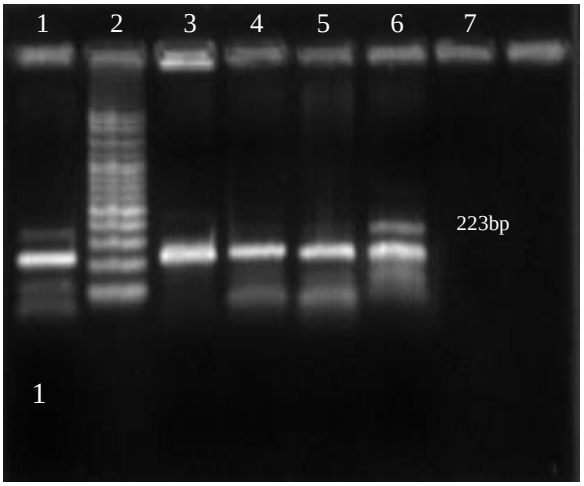

Fig. 1: *Brucella spp*. detection by PCR technique (223bp). Lane 1: positive control, lane 2: Vivantis DNA marker, lane 3-6: *Brucella spp.* positive field samples from the whole blood, lane 7: negative control.

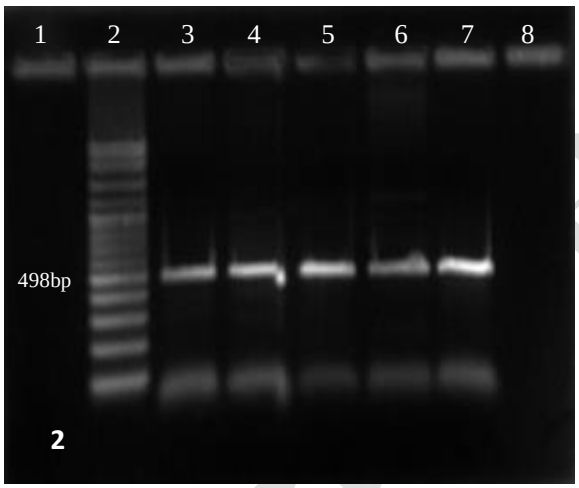

Fig. 2: *Brucella abortus* detection by PCR technique (498 bp). Lane 2: Vivantis DNA marker plus 100 bp, lane 3: positive control, lane 4-7: *B.abortus* positive field samples isolated from sera samples), lane 8: negative control.

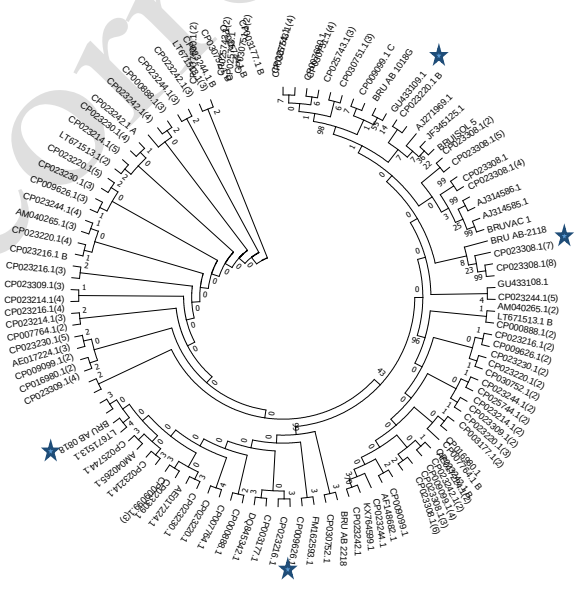

Fig. 3: Phylogenetic trees from *Brucella abortus* isolated from field cases in this study. The tree resulted from the comparative alignment of the 498 bp IS*711* sequence from *Brucella abortus* genome.

Table 2: PCR cycle protocol for amplification of *Brucella abortus*

Gene target	PCR product	Cycle condition			Cyclus number
		Step	Temp.	Time	
BCSP31	223 bp	Initial denaturation	95°C	5 min.	1
		Denaturation	94°C	1 min	
		Annealing	55°C	1 min	40x
		Extension	72°C	1 min	
		Final extension	72°C	5 min	1
Brucella sp (IS711)	498 bp	Initial denaturation	95°C	5 min.	1
		Denaturation	95°C	30 sec	
		Annealing	58°C	30 sec	35x
		Extension	72°C	30 sec	
		Final Extension	72^{0}C	5 min	1

DISCUSSION

Brucellosis is a zoonotic disease that has been classified in a group of high-risk pathogens. A rapid, precise and accurate diagnosis technique is needed for early detection of infection and for the control of *brucellosis* in animals and humans (Mahajan *et al*., 2017). PCR is a very potential and reliable molecular diagnosis method that has been widely used for detecting many infectious diseases. According to Ducrotoy *et al*. (2017), in the case of detecting brucella infection at various stages, a combination between types of biological samples and appropriate diagnostic methods must be considered. The results of this study have revealed that using PCR both whole blood without buffy-coat separation and serum samples are suitable for direct testing without previous isolation of the organism (*B. abortus*). The use of commercially available kits in this study help improve efficiency in *Brucella* DNA extraction by reducing sample processing time and reducing the risk of cross contamination between samples.This finding support previous research done by AL-Garadia *et al*. (2011), Khamesipour *et al*. (2013) and Karthik *et al*. (2014).

The antibody level and the number of the bacteria in the circulation were affected by the infection status of the animals at the time of specimen collection (Moussa *et al*., 2011; Raghava *et al*., 2017). *Brucella* is a facultative intracellular microorganism. In the early stage of infection, high concentrations of *brucella* were in the blood circulation. As a consequences, a good quantity of DNA can be easily extracted and give positive result for PCR analyses. While during chronic condition most *brucella* will hide intracellularly which make the diagnosis more challenging and make the whole blood become sample of choice (Islam *et al*., 2018). All dairy cattle in this study were seronegative but were positive molecularly. This condition was probably related to the number of *brucella* in the circulation was not enough to trigger off the antibody level that can be detected serologically but still enough to be amplified by PCR.

In this study, time difference between the disease incidence and the time of blood collections for each brucellosis positive animals were 1 week to 6 weeks. The degree of *brucella i*nfection in the blood was affected by the susceptibility of the animal against the organism (Manthei *et al*., 2015). A high persistence of bacteremia was demonstrated in highly susceptible animal. Whereas, low persistence of bacteremia correlated with animals that have varying degree of resistance was observed. In

experimentally infected cattle with virulent *B abortus*, the bacteria could be found in the blood circulation for as long as ninety-seven weeks (Manthei and Carter, 2010).

Based on phylogenetic analysis (Fig. 3) 1 positive sample in this study (BRU_AB_1018) shared high similarity (98% homology) with the representation of the *Brucella abortus* genome strain accession number CP009099 isolated from bovine in Zimbabwe (Ledwaba *et al*., 2014). Two other positive samples (BRU_AB_2118 and BRU_AB_2218) has 100% homology with *Brucella abortus* strain 9810 chromosome 1 and *B. abortus* strain 84573 chromosome 2 isolated from water buffalo in Italy (Paradiso *et al*., 2017). Whereas another positive sample (BRU_AB_0818) was related to *Brucella abortus* strain Wisconsin genome assembly, chromosome 2 (Anonymous NCBI, 2018). Previous research has reported that based on omp2a gene, *B. abortus* isolated from beef cattle in South Sulawesi and East Nusa Tenggara, Indonesia, were closely related to isolates from France (AY008719) and India (FN552417, FN552432, FN552430) (Ratnasari *et al*., 2014). This difference is likely to be due to the amplification of different nucleotide gene fragments. In order to prove the existence of genetic diversity among *Brucella sp*. in Indonesia further research involving higher amounts of *Brucella* positive samples from different regions of Indonesia needs to be carried out immediately, so that the economic losses incurred can be minimized and public health can be guaranteed.

Conclusions: It could be concluded that PCR has successfully confirmed the presumptive diagnosis of brucellosis based upon the clinical consequences of having reproductive disorders in seronegative dairy cattle using whole blood samples. The application of the PCR technique for routine diagnosis of brucellosis in seronegative animals with reproductive disorders was therefore advisable.

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Authors contribution: S played a valuable role in blood sampling in the field. RW had responsibility in preparing and extracting the DNA from whole blood and serum samples. HW had role in doing the PCR analysis and sequencing. All researchers together discussed and compiled manuscripts for journal publications.

REFERENCES

Al-Garadi MA, Khairani-Bejo S, Zunita Z, *et al.,* 2011. Detection of *Brucella melitensis* in blood samples collected from goats. J Anim Vet Adv 10:1437-44.

Anonymous OIE, 2009. OIE Terrestrial Manual: Bovine Brucellosis. version adopted by the world assembly of delegates of the OIE in May 2009.

Anonymous, NCBI, 2018.

Bailey GG, Krahn JB, Drasar BS, *et al.,* 1992. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J Trop Med Hyg 95:271-5.

Doust SRH, Ahmadi Z, Ahamdi A, *et al.,* 2007. Detection of *Brucella abortus* by alkB and IS*711* based primers. J Res Med Sci 12:62-7.

Ducrotoy M, Bertu WJ, Matope G, *et al.,* 2017. *Brucellosis* in Sub-Saharan Africa: Current challenges for management, diagnosis and control. Acta Tropica 165:179-93.

El-Diasty M, Wareth G, Melzer F, *et al.,* 2018. Isolation of *Brucella abortus* and *Brucella mellitensis* from seronegative cows is a serious impediment in brucellosis control. Vet Sci 5:28-31.

Geresu MA and Kassa GM, 2016. A review on diagnostic methods of Brucellosis. J Vet Sci Techno 7:1-8.

Gupta VK, Nayakwadi S, Kumar A, *et al.,* 2014. Markers for the molecular diagnosis of brucellosis in animals. Adv Anim Sci 2:31-9.

Islam Md S, Islam Md A, Khatun Mst M, *et al.,* 2018. Molecular detection of Brucella spp. from milk of seronegative cows from some selected area in Bangladesh. J Pathogen Article ID 9378976 1-8.

Karthik K, Rathore R, Thomas P, *et al.,* 2014. Serological and molecular detection of *Brucella abortus* from cattle by RBPT, STAT and PCR and sample suitability of whole blood for PCR. Asian J Animal Vet Adv 9:262-9.

Khamesipour F, Doosti A and Taheri H, 2013. Molecular detection of Brucella spp. in the semen, testis and blood samples of cattle and sheep. J Pure Appl Microbiol 7:495-500.

Kumar S, Stetcher G, Tamura K, *et al*., 2016. Mega 7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 3:1870-4.

Ledwaba B, Mafolo J, vanHeerden H, *et al*., 2017. Genome sequences of Brucellaabortus and Brucellasuis strains isolated from bovine in Zimbabwe. Genome Announc 2:e01063-14.

Mahajan V, Banga HS, Filia, G, *et al*., 2017. Comparison of diagnostic tests for the detection of bovine brucellosis in the natural cases of abortion. IJVR 18:183-9.

Manthei KA and Carter RW, 2010. Persistence of Brucella abortus infection in cattle. Vet Res 21:183-90.

Manthei KA, Hill MC, Burke JE, *et al.,* 2015. Persistence of *Brucella abortus* infection in cattle. Proc Natl Acad Sci USA 112:4292-7.

Moussa IM, Omnia ME, Amin AS, *et al.,*2011. Evaluation of the currently used polymerase chain reaction assays for molecular detection of *Brucella* species. Afr J Microbiol Res 5:1511-20.

Paradiso R, Orsini M, Camma C, *et al.,* 2017. Complete genome sequencing of eight *Brucella abortus* biovar 1 strains isolated from water buffalo. Genome Announc 6:e00179-18.

Perez-Sancho M, Garcia-Seco T, Arrogante L, *et al*., 2013. Development and evaluation of an IS*711*-based loop mediated isothermal amplification method (LAMP) for detection of *Brucella* spp on clinical samples. Res Vet Sci 95:489-94.

Priyadarshini A, Sarangi LN, Palai TK, *et al.,*2013. *Brucellosis* in cattle and occupationally exposed human beings: A serosurveys in Odisha, India J Pure Applied Microbiol 7:3255-60.

Raghava S, Gowda MHM, Shome R, *et al.* 2017. Epidemiological and molecular characterization of Brucella species in cattle. Asian J Anim Sci 11:123-31.

Ratnasari R, Suwarno S, Rantam FA, *et al*., 2014. Homology and phylogenetic analysis of nucleotide sequence of *omp2a* gene of *Brucella abortus* local isolates. J Vet Adv 4:374-80.

Silva Moi JP, Araujo Franca S, Paixao TA, *et al*., 2012. Laboratorial diagnosis of animal *brucellosis*. R Bras Ci Vet 19:117-26.