International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Detection of Goat Brucellosis in Several Districts Showing Bovine Brucellosis Seropositivity in Java Island, Indonesia

Mujiatun[a*], Retno Damajati Soejoedono[b], Etih Sudarnika[c], Susan Maphilindawati Noor[d], Okti Nadia Putri[e], Lussi Andriana[f], Marjono[g], Ika Sih Kartika[h], Mukromin[i], Sumirah[j], Supartono[k]

[a,g,h,i]*Veterinary Public Health, Graduate School of BAU, Bogor Agricultural University, Bogor, West Java, Indonesia, Center for Diagnostic Standards of Agricultural Quarantine, Indonesian Agricultural Quarantine Agency, Jakarta, Indonesia*

[b,c,e]*Department of Animal Disease and Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, West Java, Indonesia*

[d,j,k]*Indonesian Research Center for Veterinary Science-Bogor, Indonesian Agency for AgriculturalResearch and Development, Bogor, Indonesia*

[f]*Cilegon Agricultural Quarantine Services, Jl. Raya Transit Cikuasa, Pantai Merak, Cilegon, Banten, Indonesia*

[a]*Email: mujiatun.bbuskp@gmail.com,* [b]*Email: retnodmail@yahoo.com*

[c]*Email: etih.sudarnika@gmail.com,* [d]*Email: susan_yurismono@yahoo.com*

[e]*Email: irasumirah71@yahoo.com,* [f]*Email: lussi5088@gmail.com*

[g]*Email: mjopkl@yahoo.com,* [h]*Email: ikasihkartika@gmail.com*

[i]*Email: mukromin78@yahoo.com,* [j]*Email: irasumirah71@yahoo.com*

[k]*Email: eston21rsf@gmail.com*

Abstract

Brucellosis is a zoonotic disease that infects multiple species of animals. The species of *Brucella* that infects goats is *Brucella melitensis.*

---

* Corresponding author.

The proportion of brucellosis seropositive among goats in several districts showing bovine brucellosis seropositivity in Java Island, Indonesia was investigated in 2015. Serum samples were collected from 214 goats with judgment sampling from quarantine exits, livestock wholesalers and goat farms in Java Island. The Rose Bengal test (RBT), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) were used to detect antibodies against *Brucella*. *Brucella* was identified from seropositive samples using bacterial culture and polymerase chain reaction (PCR). From serological examination of the goat samples, only two samples showed positive results for RBT, and three were positive for CFT, but none of those samples showed positive results for ELISA. *Brucella* spp bacteria were not detected by bacterial culture and PCR from vaginal swabs or milk samples. The proportion of Brucellosis seropositive amongst tested goats was low (2.3%;95% Confidence Interval (CI); 0.0209 - 0.0251) (5/214). The present of seropositive goats indicates the previous exposure of brucellosis which genarated antibodies. However, the species of *Brucella* that had infected them remains unknown.

Keywords: *Brucella;* brucellosis; goat; serology; seropositive.

1. Introduction

Brucellosis is an infectious and widespread zoonosis caused by the genus *Brucella* [1]. *Brucella* can infect several mammals such cattle, goats and also humans. Goat brucellosis is commonly caused by *B. melitensis* which is very pathogenic to humans, and the disease is one of the most serious zoonotic diseases in the world [2,3]. Brucellosis among humans is caused by the high intensity of transmission in ruminants, high density of livestock and people, widespread marketing of non-pasteurized milk and dairy products and also consumption of raw meat [4–7].

Brucella spp. have spread among Asian countries such as the Middle Eastern countries, Mongolia, and Nepal with varied prevalence [8,6,9]. Goat brucellosis is problematic in Thailand and Malaysia because of high level of movement of goat. Isolates from these countries were found to be phylogenetically similar to isolates from India, Iran, and Israel but most closely resembled isolates from Singapore [10–12].

Animal brucellosis is well established in Indonesia, but it affects only beef, dairy cattle and pigs [13–15]. Brucellosis among cattle under extensive farming conditions revealed high seroprevalence (19.3%) in East Indonesia [16]. *Brucella suis* biotype 1 was isolated from pigs slaughtered in Kapuk, Jakarta [17]. Human brucellosis (*B. abortus*) incidence had reported in Indonesia [18].

In Indonesia goat are commonly kept as source of meat and milk. Java Island is the source of goat breeding stock for other islands in Indonesia. Most of the farmers in Java Island have goats; however, the consumption rate of goat meat in Indonesia is very low. The goats are only the source of organic manure and as savings. Any surplus of goat stock could be exported to other countries, but *Brucella* infections are still a problem in Indonesian livestock, including goats. However, information regarding brucellosis incidence among goats in Indonesia is limited. The aim of this study was to detect of goat brucellosis in several districts reported to have seropositivity of bovine brucellosis in Java Island in 2015.

2. Material and Methods

In this study the seropositive proportion of brucellosis among goats in several districts showing bovine brucellosis seropositivity in Java Island, Indonesia was investigated. This study was performed in accordance with the regulations for Research in Animal Health in the Indonesian Law on Livestock and Animal Health (UU/18/2009 junto UU/41/2014, article 66 and 79) [19,20].

2.1. Sample Collection

Serum, vaginal swabs and goat milk samples were collected from 214 goats at Cilegon quarantine exits, Ambarawa livestock wholesalers and goat farms on Java Island in 2015. The farms that were selected were farms in Boyolali, Semarang and Purworejo. Boyolali and Semarang districts were selected according to monitoring data of Cilegon Quarantine services and surveilance data of Veterinary Disease Investigation Center-Wates that found serological positive result of goat and bovine brucellosis from those areas. Purworejo district was selected because the area is a source of goat breeding stock in Java Island and there were diseases with clinical sign similarly with brucellosis in previous time (personal communication with the District Veterinarian). Samples were collected aseptically and stored at -20°C until further use.

Serum samples were colected for serological identification. Vaginal swabs and goat milk were collected for *Brucella* identification. All samples were sampled by judgment sampling based on clinical signs of brucellosis. The animal selection criteria were female animals originating from areas where brucellosis is endemic in cattle, diseases with clinical sign similarly with brucellosis and the animals were older than 1.5 years old. Some goats had showed clinical signs of the diseases: abortus, still birth, mastitis, vaginal discharge and thinness.

2.2. Serological Examination

Serological examination was conducted parallely using RBT [1,21]; and the multispecies indirect ELISA IDVet. The volume used in RBT for detection of brucellosis in goat were 75 µl and 25 µl for serum and antigen respectively. This method was slightly different from the RBT in large ruminants where the ratio between serum and antigen is 1:1. This was meant to increase the sensitivity of RBT and minimize the differences in the results between RBT and CFT [21].

The comersial iELISA kit has sensitivity of 100% (CI 95%: 89.57%-100%) and specificity of 100% (CI 95%: 99.11%-100%). This kit was used because it is acknowledged by the World Animal Health Organization (OIE) and commercially available.

The Indonesian *Brucella abortus* Standard Sera (IBASS) that were used as the positive and negative controls were obtained from Veterinary Disease Investigation Center-Maros, South Sulawesi with a CFT titre ranging between CFT 2/128 and 3/128. Samples with positive results from one of the three serological tests RBT, CFT or ELISA, were accepted as seropositive for brucellosis.

Serologically positive animals for RBT or CFT were cultured for bacteria from vagina swabs and milk samples.

The PCR assay was performed parallelly to milk samples and suspected bacterial culture.

2.3. Bacteria Culture

Brucella identification was determined from vaginal swabs or goat milk from individuals that proved seropositive in their serum samples. Milk samples were centrifuged at 6000 - 7000 g for 15 minutes to separate the cream and pellet. The skim milk was disposed into a disinfectant then the cream and milk pellet were homogenized. One to two milliliters of homogenized milk cream and vaginal swab in Amies transport media were immediately inoculated into the basal medium trypticase soy broth (TSB) medium with 2-5% bovine calf serum (BCF) and *Brucella* selective supplement (SR083A; Oxoid) added. The culture was incubated at 37 ^{o}C with 5% CO_2 for 11 days.

On the third day the growth was observed; media that appeared cloudy were inoculated onto a solid trypticase soy agar (TSA) medium with 2-5% BCF and *Brucella* Selective Supplement (SR083A; Oxoid) added. The inoculum was incubated at 37 ^{o}C with 5% CO_2 for 5-7 days. The growth of the *Brucella* spp. colonies was checked periodically. Identification of bacteria was performed through morphology, Gram staining, and biochemical reactions (citric utilization, H_2S production, urease activity, and growing in Mc Conkey agar) [1]. The bacterial culture was performed in two different laboratories, equipments and analysts. Further identification was conducted by PCR assay.

2.4. Polymerase Chain Reaction (PCR)

The PCR was conducted on milk samples and bacteria cells that had grown from the culture. Samples were mixed with 200 µl phospate buffered saline (PBS) before DNA extraction (QIAamp DNA mini kit, Qiagen with Qiacube robotic extraction). The DNA from the extraction was collected and stored in a freezer at -20°C. The PCR primary pairs B-F : 5’ TCA GGC GCT TAT AAC CGA AG 3’ and B-R : 5’ ATC TGC GCA TAG GTC TGC TT 3’ with a product length of of PCR 261 bp for the target *Brucella* spp. PCR amplifications (Qiagen Hot StarTaq Master Mix Kit, Cat No 203445) were modified from previous work [22]: 2 µl cDNA, 5 µM (0.5 µl) primers, 12.5 µl Qiagen Hot StarTaq Master Mix, ddH2O (total 25.0 µl). The PCR programs: 15 min at 95 ^{o}C (pre-denaturation); followed by 45 cycles of 1 min, 95 ^{o}C (denaturation), 1 min at 57 ^{o}C (annealing) and 2 min at 72 ^{o}C (extension); 7 min at 72 ^{o}C (final extension). The amplification process used the Thermal Cycle Applied Biosystem (ABI) Veriti. Visualization of the PCR results used 2% agarrose electrophoresis, 120 volts, 200 mA, 50 minutes (Owl Model OSP-300)

2.5. Data Analysis

The data resulting from this study were analyzed descriptively to describe the positive of brucellosis based on the serological test results (proportion of seropositive).

3. Result

3.1. Serological Result

Table 1 shows the results of the RBT, CFT and indirect ELISA to detect antibody against *Brucella* spp. in goat samples.

Table 1: Serological test results for RBT, CFT and indirect-ELlSA for the detection of *Brucella* spp.

Source of animals	Serological assay	Sample interpretation		
		Positive	Negative	% Positive
Quarantine Exit	RBT	1	21	4.48
	CFT	1	21	4.48
	ELISA	0	28	0
Livestock Market	RBT	1	39	2.50
	CFT	2	38	5.00
	ELISA	0	40	0
Farm	RBT	0	152	0
	CFT	0	152	0
	ELISA	0	152	0
Total		5	209	2.3

The serological test results of the 214 serum samples demonstrated two RBT positive samples (+ and ++), three CFT positive samples (1/4, 1/8 and 1/16) and no ELISA positive samples. There were 5 sample out of 214 goat serum samples which were seropositive for brucellosis (2.3%, CI 95%; 0.0209 - 0.0251) (5/214). This indicates that the seropositve goats had been exposed to brucellosis and generated antibodies.

The RBT and CFT methods are most commonly tests used for serological testing of brucellosis in small ruminant [2,23], and the official tests used by the European Union countries [23]. These are also the standard tests according to World Animal Health Organization [24]. The RBT and CFT are used simultaneously to increase the possibility of the detecting individuals that are infected and to improve disease control in areas where eradication efforts have not yet been finalized [21,25,26]. Parallel examination between RBT and ELISA will improve diagnostic sensitivity in goat herds having high risk of *B. melitensis* [27].

Brucellosis screening tests on sheep using the ELISA techniques improved the diagnosis of *Brucella* [28]; however, in this study, brucellosis was not detected in the serum samples tested using ELISA. This was different from previous study in which ELISA demostrated a higher sensitivity (97-100%) than that of combination between CFT and SAT, or CFT and RBPT, or CFT and skin delayed type-hypersensitivity (SDTH) (75-88%) [28]. Indirect and competitive ELISA and Fluorescence Polarization Assay (FPA) are promising tests, but need to be evaluated and standardized before being used in large scale. RBT and CFT are the serological tests mostly used for large scale surveillance/eradication purposes [29].

3.2. Bacterial culture and PCR result

Table 2 shows the results of the culture and PCR. It shows that none of milk or vaginal swab samples were positive of any *Brucella* spp.

Table 2: Bacterial culture compared with PCR results of seropositive samples for *Brucella* spp.

Sample code	Seropositive*	Type of sample					
		Milk			Vaginal swab		
		PCR	Bacterial culture	PCR culture	PCR	Bacterial culture	PCR culture
13	CFT	N	A	NP	NP	A	NP
17	RBT	N	A	NP	NP	A	NP
IN22	RBT	N	S	N	NP	A	NP
B5	CFT	N	A	NP	NP	A	NP
P1	CFT	N	A	NP	NP	A	NP

*Serological test showing positive result

N = Negative; NP= not performed; A=Atypical (Biochemical Test); S=Suspect

Figure 1 demonstrates the PCR results of milk samples and suspected bacterial culture from the seropositives goats. All the samples were negative.

M 1 2 3 4 5 6 7 8 M

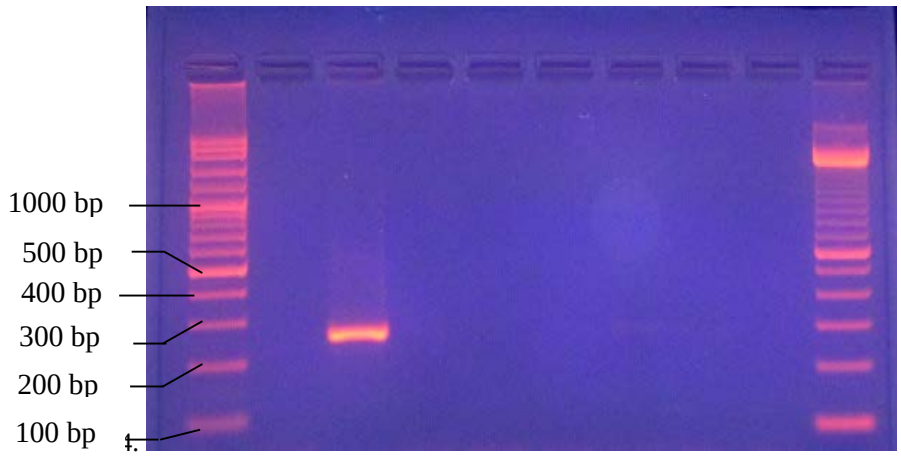

Figure 1: Gel electrophoresis image of amplicon produced from *Brucella* spp primers (261 bp).

M = molecular marker 100 bp; 1= negative control; 2= positive control *Brucella abortus* S99; 3= milk no 13; 4= milk no 17; 5= milk no IN22; 6= milk no B5; 7= milk no P1; 8= culture no IN 22.

None of the vaginal swabs or milk samples from seropositive animals showed positive result in PCR and bacterial culture. This is likely because the numbers of microbes in the milk or vaginal swabs collected were very low, thus were not detected. *Brucella* is a fastidious bacterium which is difficult to grow and the growth is

slow [1]. It is also probable that a low number of bacteria was being intermittently shed into milk [23]. This might explain why only 44% of the milk samples were cultured and PCR positive from naturally infected animals of *B. abortus* [30].

Another explanation could be that the immune system of the animal has killed the bacteria, as investigated on previous study that toll-like receptors (TLR) 9 in the innate immune system of mammals has an important role in clearing *Brucella* infection [31].

The previous study demonstrated that infections that had been eliminated would trigger immunity [32]. Generally, infections in the long term is a cause of the disease, but there is a possibility of elimination of the bacteria. In infected animals, the humoral immune response is as effective as the cellular immune response (cell mediated immune mechanism) [23].

Brucella is an intracellular bacterium which can survive in macrophages and dendritic cells [33]. However, the bacteria could also be destroyed by macrophages if there is a failure when associating with the membrane endoplasmic reticulum (ER) in forming a replicative vacuole [34,35].

Macrophages or Antigen Presenting Cells (APC) which have fragmented antigens would present the antigen fragments to the lymphocyte T helper cells (Th cells) through the Major Histocompatibility Complex (MHC) class II molecule which is located on the surface of the macrophage. The Th cell interacts with the APC through the Cluster of Differentiation (CD4) and T-cell Receptor (TCR) belonging to the Th. Then there will be activation of the Th cell, the Th cell proliferates and secretes cytokine (interleukin-1/IL-1) which would activate naive B cells into plasma cells that are ready to produce specific antibody against the *Brucella* antigen [36].

3.3. The Origin of Seropositive Goats

Figure 2 demonstrates the map of the district origin of seropositive samples in Java Island. The seropositive samples of goat brucellosis in this study were from Ambarawa livestock wholesalers and Cilegon quarantine exit. The seropositive goats in Cilegon quarantine exit originated from Ngawi District that would be transported to Sumatra Island.

Brucellosis seropositive results in this study were detected in samples collected from the livestock wholesalers and the quarantine exit, but not from the goats in smallholding farms. This trend is caused by the socio-economic behaviour of the farmers in Java Island. They usually have only a small-scale farm (from 1 to 30 goats/farmers), clean the pens daily, burn the leftover leaves and grass from feed, sell the sick and barren animals to the livestock market or to livestock traders. Large livestock markets are places where livestock trade happens. Goats coming from various places throughout Java Island are aggregated in large flocks. They are bought by farmers and or other livestock traders, and then brought to other farms, other islands, or the slaughter house. This condition is in line with the results of previous studies that had identified keeping large flocks of livestock, having contact with sheep and foraging on pastures, not using disinfectants and having contact with other herds of goats were the risk factors for *Brucella* seropositive results [37,38].

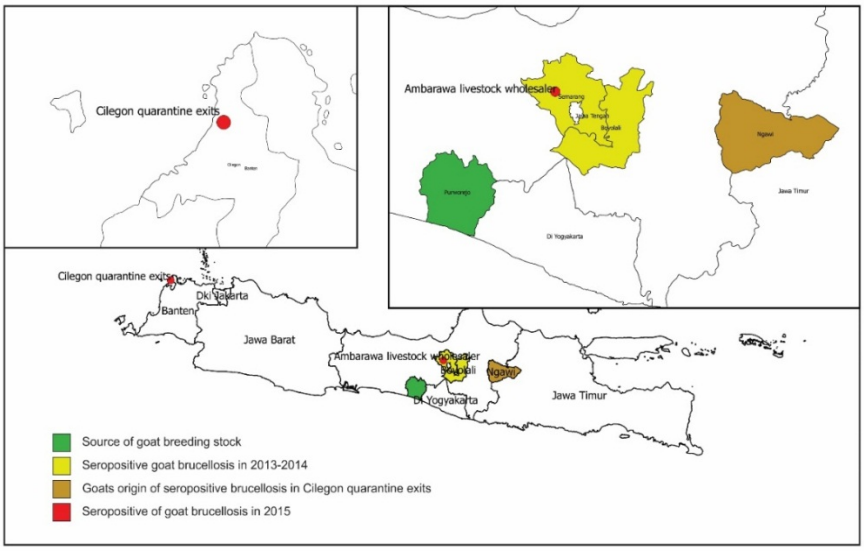

Figure 2: Map of seropositive samples origin of goat brucellosis in Java Island

Based on observations in the field, farmers on this island kept their goats and cattle close to each other. At times, they even put them in the same pen, and the livestock traders collected goats and cattle. They sold in one place in holding pens or at the livestock market. This condition was possibly the source of *Brucella* spp. infection in goats. The results of this study demonstrate that the goats in Java Island which are a source of breeding stock for goats in Indonesia were not detected to harbor *B. melitensis.*

4. Conclusions

Goat brucellosis in several districts showing bovine brucellosis seropositivity in Java Island, Indonesia has a low proportion of brucellosis seropositivity (2.3%, CI 95%; 0.0209 - 0.0251) (5/214) indicating the exposure of *Brucella* spp. among goats might occur; however, the source of infection remains unknown. Therefore, improving brucellosis surveillance in small ruminants is necessary to prevent the spread of goat brucellosis among goats in Indonesia. Quarantine actions must be improved to control goat brucellosis through transportation and animal movement. We recommend that all goats be tested parallelly with RBT and CFT or ELISA before being transported to another island. The seropositive goat for RBT and/or CFT and/or ELISA test must be slaughtered.

Acknowledgements

This research was partially supported by a scholarship from the Ministry of Agriculture-Republic of Indonesia. The authors would like to express their deepest gratitude to the Indonesian Agricultural Quarantine Agency and the Research Center for Veterinary Science, Indonesian Agency for Agricultural Research and Development for facilitating this research; the Veterinary Disease Investigation Center-Maros for the standard sera, the Veterinary Disease Investigation Center-Subang and the Veterinary Disease Investigation Center-Wates for the sera and surveillance data.

References

[1] V. J. Alton GG, Jones LM, Angus RD, "Techniques for the Brucellosis Laboratory." Institut National de la Recherche Agronomique. INRA Press., Paris, pp. 1–190, 1988.

[2] OIE, "Caprine and ovine brucellosis (excluding Brucella ovis)," OIE Terr. Man. 2012, no. May, pp. 968–977, 2012.

[3] J. A. Assenga, L. E. Matemba, S. K. Muller, J. J. Malakalinga, and R. R. Kazwala, "Epidemiology of Brucella infection in the human, livestock and wildlife interface in the Katavi-Rukwa ecosystem, Tanzania.," BMC Vet. Res., vol. 11, no. 1, p. 189, 2015.

[4] Y. M. Hegazy, A. Moawad, S. Osman, A. Ridler, and J. Guitian, "Ruminant Brucellosis in the Kafr El Sheikh Governorate of the Nile Delta , Egypt : Prevalence of a Neglected Zoonosis," vol. 5, no. 1, 2011.

[5] F. F. Norman, B. Monge-Maillo, S. Chamorro-Tojeiro, J.-A. Pérez-Molina, and R. López-Vélez, "Imported brucellosis: A case series and literature review," Travel Med. Infect. Dis., vol. 14, no. 3, pp. 182–199, 2016.

[6] H. G. Garcell, E. G. Garcia, P. V. Pueyo, I. R. Martín, A. V. Arias, and R. N. Alfonso Serrano, "Outbreaks of brucellosis related to the consumption of unpasteurized camel milk," J. Infect. Public Health, vol. 9, no. 4, pp. 523–527, 2016.

[7] M. K. Aworh et al., "Human brucellosis: seroprevalence and associated exposure factors among abattoir workers in Abuja, Nigeria - 2011," vol. 8688, pp. 1–9, 2013.

[8] D. S. Jackson, D. V. Nydam, and C. Altier, "Prevalence and risk factors for brucellosis in domestic yak Bos grunniens and their herders in a transhumant pastoralist system of Dolpo, Nepal," Prev. Vet. Med., vol. 113, no. 1, pp. 47–58, 2014.

[9] B. Zolzaya et al., "Representative seroprevalences of human and livestock brucellosis in two Mongolian provinces," Ecohealth, vol. 11, no. 3, pp. 356–371, 2014.

[10] P. H. Bamaiyi, L. Hassan, and Z. A. M, "Updates on Brucellosis in Malaysia and Southeast Asia," Malaysian J. Vet. Res., pp. 71–82, 2014.

[11] P. H. Bamaiyi, L. Hassan, S. Khairani-bejo, M. Zainalabidin, and M. Ramlan, "The prevalence and distribution of Brucella melitensis in goats in Malaysia from 2000 to 2009," vol. 119, pp. 232–236, 2015.

[12] C. Inchaisri, P. Prasomsri, T. Boonserm, H. Hogeveen, and K. Ajariyakajorn, "A stochastic simulation model for brucellosis eradication in goat flocks in an area with high flock prevalence but low animal

prevalence Prod age," Small Rumin. Res., vol. 136, pp. 227–237, 2016.

[13] P. T. Koesharjono, C., Van Peenen , P.F.D., Joseph, S.W., Sulianti Saroso, J., Irving C.S., Durte, "Serological Survey of Pigs from a Slaughterhause in Jakarta, Indonesia," NAMRU, vol. 2, no. 2, pp. 1–11, 1971.

[14] E. D. Setiawan, "Studi tentang Beberapa Sifat Biologik Brucella abortus Isolat Lapang." Program Pasca Sarjana, Institut Pertanian Bogor, Bogor, pp. 1–152, 1992.

[15] A. Sudibyo, "Studi Brucellosis dan Karakterisasi Protein Antigenik Brucella abortus Isolat Lapang pada Sapi Perah." Program Pasca Sarjana, Institut Pertanian Bogor, Bogor, pp. 1–113, 1994.

[16] H. Muflihanah, M. Hatta, E. Rood, P. Scheelbeek, T. H. Abdoel, and H. L. Smits, "Brucellosis seroprevalence in Bali cattle with reproductive failure in South Sulawesi and Brucella abortus biovar 1 genotypes in the Eastern Indonesian archipelago," 2013.

[17] J. W. B. Van Der Giessen and A. Priadi, "Swine brucellosis in Indonesia Swine brucellosis in Indonesia," vol. 2176, no. June, 2016.

[18] H. Danusantoso, Halim, Joseph, S.W., and Sidarta, "A Review of Brucellosis in Indonesia with Report of Recent Case," Southeast Asian J. Trop. Med. Public Health, vol. 3, no. 3, pp. 314–318, 1972.

[19] Republik Indonesia., "Undang-Undang Republik Indonesia Nomor 18 Tahun 2009 tentang peternakan dan Kesehatan Hewan." Kementerian Hukum dan Hak Asasi Manusia, Jakarta, pp. 1–108, 2009.

[20] Republik Indonesia, "Perubahan atas Undang-Undang Nomor 18 tahub 2009 tentang Peternakan dan Kesehatan Hewan." Kementerian Hukum dan Hak Asasi Manusia, Jakarta, pp. 1–28, 2014.

[21] J. M. Blasco et al., "Efficacy of different Rose Bengal and complement fixation antigens for the diagnosis of Brucella melitensis infection in sheep and goats.," The Veterinary record, vol. 134, no. 16. pp. 415–420, 1994.

[22] M. Batson, "Step PCR Assay for identification of classical Brucella strains," CSIRO (AUS): Australian Animal Health (AAHL), 2006, pp. 1–9.

[23] EC [European Commission], "Brucellosis in Sheep and Goats," Sci. Committe Anim. Heal. Anim. Welf., pp. 1–20, 2001.

[24] Oie, "Bovine Brucellosis," OIE Terr. Man. 2009, no. May, pp. 1–35, 2009.

[25] G. Alton, "Brucella melitensis," in Animal Brucellosis, K. Nielsen and J. Duncan, Eds. Boca Raton, Florida: CRC Press, 1990, pp. 383–409.

[26] J. M. Blasco, "Diagnosis of Brucella melitensis infection in small ruminants," in Prevention of Brucellosis in the Mediterranean Countries, M. Plommet, Ed. Wegeningen: Pudoc Scientific, 1992, pp. 272–277.

[27] M. Fiasconaro et al., "Field evaluation of fluorescence polarization assay, and comparison with competitive ELISA for the detection of antibodies against Brucella melitensis in sheep in Sicily, Italy," Small Rumin. Res., vol. 130, pp. 252–255, 2015.

[28] Z. Bercovich, L. Güler, T. Baysal, B. E. . Schreuder, and F. . van Zijderveld, "Evaluation of the currently used diagnostic procedures for the detection of Brucella melitensis in sheep," Small Rumin. Res., vol. 31, no. 1, pp. 1–6, 1998.

[29] B. Garin-bastuji, J. M. Blasco, C. Mar, and D. Albert, "The diagnosis of brucellosis in sheep and goats , old and new tools &," vol. 62, pp. 63–70, 2006.

[30] O. Leary, M. Sheahan, and T. Sweeney, "Brucella abortus detection by PCR assay in blood , milk and lymph tissue of serologically positive cows," vol. 81, pp. 170–176, 2006.

[31] S. C. Oliveira, L. A. de Almeida, N. B. Carvalho, F. S. Oliveira, and T. L. S. Lacerda, "Update on the role of innate immune receptors during Brucella abortus infection," Vet. Immunol. Immunopathol., vol. 148, no. 1–2, pp. 129–135, 2012.

[32] T. A. Ficht, "Intracellular survival of Brucella: Defining the link with persistence," Vet. Microbiol., vol. 92, no. 3, pp. 213–223, 2003.

[33] J. Celli, C. De Chastellier, D. Franchini, J. Pizarro-cerda, E. Moreno, and J. Gorvel, "Brucella Evades Macrophage Killing via VirB-dependent Sustained Interactions with the Endoplasmic Reticulum," vol. 198, no. 4, 2003.

[34] G. Rajashekara et al., "Brucella: functional genomics and host–pathogen interactions," Anim. Heal. Res. Rev., vol. 7, no. 1–2, pp. 1–11, 2006.

[35] T. Starr, T. W. Ng, T. D. Wehrly, L. A. Knodler, and J. Celli, "Brucella intracellular replication requires trafficking through the late endosomal/lysosomal compartment," Traffic, vol. 9, no. 5, pp. 678–694, 2008.

[36] D. Harlow, E. D., & Lane, A laboratory manual. New York: Cold Spring Harbor Laboratory, 1988.

[37] A. M. Al-majali, "Seroepidemiology of caprine Brucellosis in Jordan," vol. 58, pp. 13–18, 2005.

[38] F. J. Reviriego and M. A. Moreno, "Risk factors for brucellosis seroprevalence of sheep and goat flocks in Spain," vol. 44, pp. 167–173, 2000.