P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2025.014

Sero-epidemiological Study on *Leptospira* Infection in a Closed Cattle Population in Indonesia

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Article History: 24-608 Received: 05-Sep-24 Revised: 22-Dec-24 Accepted: 21-Jan-25 Online First: 03-Feb-25

ABSTRACT

Sero-epidemiological study on *Leptospira* infection was done in a closed cattle population. The epidemiology scope includes clinical symptoms, prevalence of infection, serovar of *Leptospira* caused infection, breeds infected, and its possible transfection to human. Serum samples of 980 cattle and 58 workers were collected in this study. All the serum samples were tested against Leptospirosis using the Microscopic Agglutination Test (MAT). Fourteen serovars of *Leptospira interrogans* were used as antigens, namely Icterohaemorrhagiae, Canicola, Ballum, Javanica, Celledoni, Pyrogenes, Cynopteri, Rachmati, Australis, Grippotyphosa, Hardjo, Bataviae, Tarassovi, and Dan Pomona. The data were analyzed using descriptive epidemiology. The study showed that Leptospirosis infected 62 cattle (6.32%). The infection was detected in Bali, Madura, and Onggole Cattle. The most prevalent breed of leptospirosis was in Bali cattle (7.55%). Several *Leptospira* serovars-infected cattle in the population were found, namely Tarrasovi, Hardjo, Grippotyphosa, and Batavia serovars (single infection) and Hardjo and Tarrasovi serovars (multiple infections). The most dominant serovar-infected cattle in this area was Tarrasovi (64.51%). The infection was not detected in humans, but it was shown that farm workers' exposure to *Leptospira* spp. was very low.

Keywords: *Leptospira*, Leptospirosis, Cattle, Epidemiology, Serology.

INTRODUCTION

Leptospirosis is a widespread zoonotic disease in the world caused by pathogenic spirochetes of the genus *Leptospira* (Schuller et al. 2015). This disease is mainly found in tropical and sub-tropical countries, including in Indonesia. Leptospirosis can have an economic impact on the livestock industry (Ellis 2015; Gizamba and Mukisha 2023). *Leptospira* genus is divided into 2 species, namely *L. interrogans* which is a pathogenic bacteria and *L. biflexa* which is saprophytic (Mohammed et al. 2011). There were about 300 serovars of *Leptospira*, which divided into 28 groups (Saito et al. 2013) and *Interrogans Leptospires* are the main pathogenic species of *Leptospires* that can infect in animals and humans (Adler et al. 2010).

These bacteria circulate in host reservoirs of animals including rats, other rodents, livestock and pets (Ko et al. 2009; Motto et al. 2021; Moinet et al. 2021). *Leptospira* mainly infects domestic livestock, wild animals, and humans (Carvalho et al. 2024). Domestic animals that these bacteria can infect are cows, sheep, goats, camels, pigs, dogs and cats (Ellis 2015), while wild animals that can be infected by *Leptospira*, namely skunks, raccoons, beavers, foxes and opossum (Shearer et al. 2014).

Leptospirosis should not be considered a problem of the individual animal because of the nature of the disease but as a problem of the herd. In livestock, leptospirosis causes a decrease in production, mainly related to reproductive problems (Fornazari et al. 2012). In cattle, clinical symptoms that often appear, such as abortion,

Cite This Article as: Susanti S, Adjid RMA, Noor SM, Mulyadi A, Sumirah S, Nuradji H, Ratnawati D, Dikman DM, Pamungkas D, Rachmawati F, Anastasia Y and Purba HHS, 2025. Sero-epidemiological study on *Leptospira* infection in a closed cattle population in Indonesia. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2025.014

recurring fever, stillbirth and are often associated with the Sejroe serogroup, especially Hardjo serovar (Mughini-Gras et al. 2014; Macchi et al. 2024). Leptospirosis in humans commonly comes in through skin abrasions and mucosal surfaces when handling an aborted fetus. The disease causes a wide range of clinical symptoms, from febrile illness to severe and fatal illness (kidney disorders). Transmission to humans and other animals can occur directly or indirectly through infected reservoir host animals, which will carry bacteria in their kidney tubules and release pathogenic *Leptospires* in their urine (Haake and Levett 2015; Bradley and Lockaby 2023; Chanchayanon et al. 2024).

The diagnosis of Leptospirosis is based on two principles: isolation of organisms and detection of anti-*Leptospira* antibodies. Isolation by culture requires a long time (6-8 weeks) causing delays in diagnosis, antibiotic therapy and does not contribute to early diagnosis of the disease (Pinto et al. 2022). To confirm the diagnosis serological tests are performed in the laboratory with MAT tests using live antigens (Hernández-Rodríguez et al. 2011). Serological test of MAT is the gold standard for immunological diagnosis and detects both immunoglobulin G and M (Suprayoga et al. 2021).

In Indonesia, leptospirosis in livestock is categorized as a strategic infectious animal disease as this disease is economically considered to be detrimental to farmers (Kementerian Pertanian 2023). However, information of leptospirosis epidemiology in livestock (cattle, sheep, buffalo, goats) in this country is very difficult to obtain. This is because there are not many research institutions that conduct research for the disease for various reasons. Besides that, the general occurrence of this zoonotic disease is often discussed and carried out an investigation/testing if there was human case caused by a bacterial infection of *Leptospira.*

This study aims to identify epidemiology of the disease in a closed cattle population, including its possible transfection to humans who worked at the same environment.

MATERIALS AND METHODS

Informed consent/ethical approval

All experiments (animal handling and procedures) were approved by the Animal Ethics Committee of the Indonesian Agency for Agricultural Research and Development (Balitbangtan/BB Litvet/Rm_A/07.03/ 2019).

Location and time of research

Research activities were done at local cattle farm which located in Pasuruan, East Java Province of Indonesia. Duration of the study period was May-December 2019. The sample was analyzed at Bacteriological Laboratory of Indonesia Research Center for Veterinary Science (IRCVS), Bogor, West Java, Indonesia.

Materials and design research

This study used sample serums of humans (farm workers) and cattle from local farm environment. A total number of humans (farm workers) were used 58 people (male and female). A total number of cattle were used 980 cattle (bulls and cows).

Sample and collection sample

Research material was collected as serum samples from cattle and humans from the same farm environment. Cattle blood was collected using needles and vacutainer tubes. Meanwhile, the collection of human blood was carried out by using a syringe by the local health department employees. Blood sampling in animals and humans was conducted aseptically at the specified blood sampling point. The blood serum was taken from the jugular vein of cattle. The vacutainer tubes containing blood were labeled with the identifying specimen number, specimen type, species, breeder name, location, and collection date. Afterwards, it was left in an oblique position for a while, about 5-10min.

Sample delivery from site to laboratory

During the transportation of serum sample to the laboratory, the sample was placed in an ice box with a temperature of 4-8°C, and the ice box was protected from the sun. The serum fluid from serum samples was separated from the blood clots by carefully pouring the serum fluid into the tube (1.5mL screw tube) so that only the serum fluid entered the tube. The tubes were given the identity (sample number, type of sample, species, animal breed, owner's name, location, and date of collection) and then tested. If it was not possible to do the test on the same day, then the serum was stored in the refrigerator at a temperature of 4-8°C to be carried out for the following day test. If the testing time was still be conducted in the next few days, the tube was stored at -20°C until further processed for testing purposes.

Detection of *Leptospira* using Microscopic Agglutination Test (MAT)

The Serological tests of MAT were used to detect antibodies towards *Leptospira*, both serum from animals and humans origin were used as evidence of infection (Sykes et al. 2022, Bağatir and Aktaş 2024). This test was included in the scope of diagnostic testing of Indonesia Research Center for Veterinary Science (IRCVS) that has been certified with ISO 17025. The antigens used in this test were live antigens from *Leptospira interrogans* serovar icterohaemorrhagiae, ballum, pyrogenes, cynopteri, javanica, celledoni, canicola, rachmati, australis, pomona, grippotyphosa, hardjo, bataviae, and tarassovi were obtained from Royal Tropical Institute, KIT Biomedical Research, Amsterdam (Nederlands). These serovars were cultured and maintained in EMJH (Ellinghausen-McCullough-Jonson-Harris) liquid media at room temperature. The antigen was used 5-9 days old which grown in a liquid EMJH medium and incubated at 28-30°C. Antigen concentrations were approximately $2x10^8$ *Leptospires* per mL.

During the preliminary examination, the serum was diluted with PBS ratio of 1:25. Afterwards, the 50µL of diluted serum specimens were added in 96 well round bottomed microplates and then, added 50µL of *Leptospira interrogans* serovar and incubated for 2 hours at 28-30°C. The mixture serum-antigen was transferred to the slide (uncovered with a cover glass) and read with a phase contrast microscope at 100x magnification.

Serum titration

Samples that showed a reaction of 50% or more agglutination on the preliminary examination were then diluted with PBS in the ratio of 1:50, 1: 200, 1: 800 and 1: 3200. A total of 50µL of each serum was then dropped in microplate holes. Furthermore, each of these dilutions was added with 50µL of *Leptospira interrogans* antigen, incubating at 28-30°C for 2 hours. Agglutination analysis was done by using a phase contrast microscope (Olympus BH2). The reading endpoint was 50% or more agglutination (estimated from the number of free leptospires, i.e. as much as 50% or less) and the titer was defined as the highest end of serum dilution in the serum-antigen mixture indicating 50% or more agglutination.

Statistical analysis

The data was analyzed by descriptive epidemiology. The variables measured were presented in percentage form. The percentages were calculated for continuous numerical variables (Sahak et al. 2019).

RESULTS

Number of cattle

The total number of cattle involved in this study is shown in Table 1.

Table 1: Breed cattle composition and number of cattle

Breed of Cattle	Sample Number	%
Madura	192	19.60
Bali	159	16.22
OC	610	62.24
POBA	19	1.94
Total	980	100.00

Noted: OC (Ongole Crossbreed), POBA (crossing between Ongole crossbreed and Bali cattle).

There were 980 serum samples were tested with MAT to detect the presence of antibodies against *Leptospira*. The MAT test used live antigens, which was the most widely used serological test and as a reference for other serological tests.

Serological test of samples by MAT

In this study, 14 serovars as antigens were used as suggested by OIE (OIE 2021). The results of the serological test of the samples are seen in Table 2.

Table 2: Results of cattle serum tested by MAT for *Leptospira*

No.	MAT Results	Breed of Cattle				Total (%)
		Madura (%)	PO (%)	Bali (%)	POBA (%)	
1.	Positive	7 (3.6)	43 (7.05)	12 (7.55)	0 (0)	62 (6.33)
2.	Negative	185 (96.4)	567 (92.95)	147 (92.45)	19 (100)	918 (93.67)
	Total	192 (100)	610 (100)	159 (100)	19 (100)	980 (100)

The data show that as many as 62 samples, or 6.33% of 980 samples tested, reacted with *Leptospira* antigen.

Fig. 1 shows that the sample was negatively detected when bacteria *Leptospira* did not undergo agglutination between each other. Meanwhile, the sample was positively detected when bacteria *Leptospira* underwent agglutination between each other (Fig. 2).

Serovar of *Leptospira* infection in cattle

The study showed that the serovars of *Leptospira* that infected cattle were; Tarrasovi, Hardjo, Grippotyphosa, and Batavia (Table 3).

Table 3: Serovar of *Leptospira* infected the cattle

No.	Serovar *Leptospira interrogans*	Number of Infected animals	%
1.	Tarrasovi	40	64.51
2.	Hardjo	18	29.03
3.	Grippothyposa	2	3.23
4.	Batavia	1	1.61
5.	Hardjo and Tarrasovi	1	1.62
	Total	62	100%

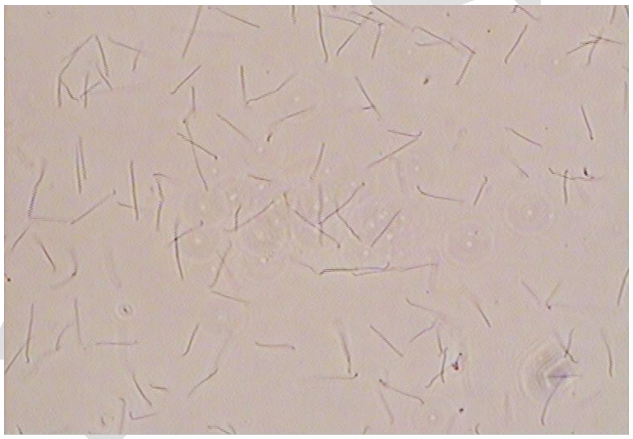

Fig. 1: The appearance of bovine serum samples was negative by MAT using phase contrast microscope (400x magnification).

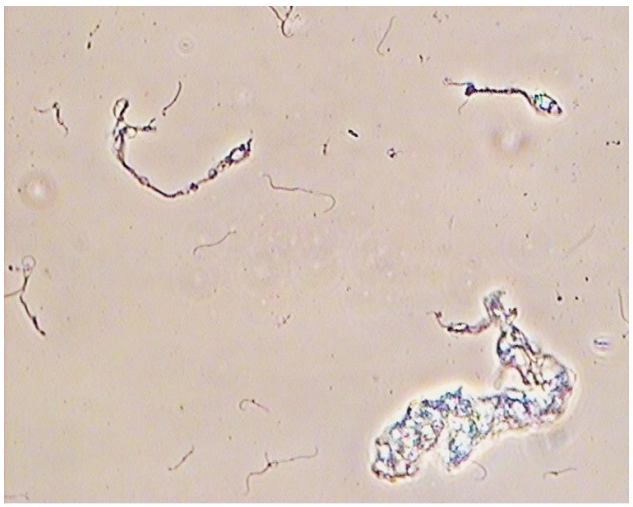

Fig. 2: The appearance of bovine serum samples was positive by MAT using phase contrast microscope (400x magnification).

Table 3 shows *Leptospira* infection in cattle was caused by the serovar Tarrasovi, Hardjo, Grippothyposa, and Bataviae and by combination of Hardjo and Tarrasovi. From this data, it can be shown that the most dominant serovar was Tarrasovi. This is following previous research by Yatbantoong and Chaiyarat (2019), which stated that 92.2% of *Leptospira* antibodies were detected with the highest prevalence of *L. interrogans* serovar Tarassovi.

DISCUSSION

Leptospirosis is a zoonotic disease that spread geographically, caused by pathogenic spirochetes of the genus *Leptospira* (Sykes et al. 2022). The situation of this disease in developing countries, like Indonesia, is a major challenge because humans and animals live in close relationships (Ellis 2015). Infected animals by *Leptospira* can carry the bacteria for long periods, shedding and contaminating environment through urine, birth material or abortion (Samrot et al. 2021; Di Azevedo and Lilenbaum 2020; Bierque et al. 2020). Infection in humans can occur through injured skin, mucous membranes including the oral cavity and conjunctiva, or transplacental infections that can result in fetal death or neonatal infection (Sykes et al. 2022).

The incidence of Leptospirosis in livestock and their relation to their occurrence in humans is still very limited. Due to these conditions, this study was conducted to enrich information with the prevalence of leptospirosis in Indonesia. This research was conducted on a local breed of cattle population in a farm area with the aim to determine the epidemiology of the disease in a closed condition. Epidemiological studies are carried out by looking at clinical symptoms, seroprevalence of infection, infected breeds, *Leptospira* serovar that causes infection, and possible transfection to humans (Galan et al. 2021; Wainaina et al. 2024).

The total number of cattle in this local farm was 1,000 cattle. Nevertheless, there were only 980 cattle (98%) sampled. This was caused by many factors, such as; difficulty to sampling serum, wild cattle, cow with late stage of pregnancy status, too young of age and so on. However, the number of samples obtained was counted to be sufficient to illustrate the epidemiology of the disease in the cattle population (Hajian-Tilaki 2011).

The occurrence of clinical differences with many reported abroad are related to Leptospirosis, it is likely due to many factors such as the cattle breed. However, in this research that has been done, within one year there were no clinical symptoms that were evident, such as miscarriage, infertility, calf death and so on. This condition may be closely related to the management of cultivation carried out, including the adequacy of the amount of feed and nutrients, as well as the environment that did not cause "stress" pressure on animals. Clinically the symptoms due to leptospirosis infection in this local breed cattle that are not apparent possibly may support breed resistant.

Clinical symptoms of leptospirosis in cattle can vary from mild, invisible infection to acute infection that cause death. Most animal infections do not show clinical symptoms, but clinical disease also sometimes occurs and may be fatal (Sykes et al. 2022). In cattle, this disease causes reproductive failure such as fetal death, abortion, premature birth and the birth of weak calf and low of weight calf (Loureiro and Lilenbaum 2020). Leptospirosis in cattle is generally caused by *L. interrogans* serovar hardjo infection. Cattle are known as maintenance hosts for serovar hardjo and infection with serovar is usually subclinical (Schafbauer et al. 2019). Acute leptospirosis in cattle is rare and is mostly related to incidental serovars such as Pomona, Grippotyphosa and Icterohaemorrhagiae (Dehkordi et al. 2011). Clinical signs of acute bovine leptospirosis include haemolytic anaemia, hemoglobinuria, high fever, and jaundice (Koizumi and Yasutomi 2012; Sohm et al. 2023). Acute infections most often occur in calves/young cows (Adugna 2016). In pregnant or lactating cows, abortion usually occurs in the last trimester of pregnancy. Infertility and a sudden decrease in milk production can affect up to 50% of cows at once and trigger a decrease in herd milk production, this decrease can last up to 8 weeks but individual cows' milk production will return to normal within 1-14 days (Yadeta et al. 2016). The severity of clinical symptoms depends on the infectious *Leptospira* serovar and the immunity of infected animal (Ellis 2015).

The cattle farm consisted of 3 breeds of local cattle, namely Madura, Bali and Ongole Crossbreed (PO) and a few POBA (cross breed between PO and Bali cattle) as shown in Table 1. Each breed of cattle was housed separately and in groups with open railings (made of iron pipes) cage with the capacity of around 20-30 cattle. The distance between the cattle of one type and the other ranges from 15-20 meters. This condition increases the possibility of spreading the disease between cattle and cages, within the same breeds of cattle. Transmission of leptospirosis occurs through the environment, air, employees and wild animals, such as rats (Daud et al. 2018; Sunaryo and Priyanto 2022).

In this study, 14 serovars as antigens were used as suggested by OIE (OIE 2021). The MAT test can be used in serological examination, seroinvestigation and seroprevalence in leptospirosis cases (Susanti 2015; Balamurugan et al. 2018; Daud et al. 2018). The results of the serological test of the samples are shown in Table 2. Table 2 show the prevalence of Lepotospirosis in Bali (12 cattle=7.55%) was higher that Ongole crossbreed/ OC (43 cattle=7.05%) and Madura (7 cattle=3.6%). This situation was certainly a natural thing as PO-cattle has the highest population, which had the population of 610 cattle compared to other breeds of cattle (Table 1). The highest prevalence of *leptospira* infection in cattle is Balinese cattle, followed by PO and Madurese cattle, namely 7.55, 7.05 and 3.6% respectively. This prevalence rate does not seem to differ; however, it is questionable as Leptospirosis is highly contagious, but the results show low prevalence rate. So, it may lead to hypothesis which supports the statement that the types of local cattle are more resistant than other cattle breeds. So far, no study has been done for breed resistance for Leptospirosis in cattle. Therefore, research on this aspect needs to be developed involving local breed and imported breed of cattle. The results of previous research by Mulyani et al. (2016) showed that the most dominant *Leptospira* serovar in beef cattle in Kulon Progo Regency (DIY, Indonesia) was hardjo (38.0%) as the dominant serovar.

Serogroups found in cattle were Pomona (3.2%), Sejroe (3.1%) and Icterohaemorrhagiae (0.6%) was also reported by Fávero et al. (2017). Cows are the main reservoir for serovar hardjo (Mughini-Gras et al. 2014). Livestock are recognized as maintenance hosts for serovar Hardjo, as well as other Sejroe serogroup members who cause chronic disease with subclinical and persistent infections in their reproductive tract (Lilenbaum and Martins 2014).

The main hosts of *Leptospira* serovar Icterohaemorrhagiae are brown rats (*Rattus norvegicus*),

serovar Hardjo (cattle and sheep), serovar Canicola and serovar Bratislava (pigs and possibly dogs) (Ellis 2015). Table 3 shows that there is one sample that reacted positively to more than one *Leptospira* serovars (serovar hardjo and tarrasovi). This may occur due to cross-reaction between various *Leptospira* serovars or cattle have been infected with more than one *Leptospira* serovars (Chirathaworn et al. 2014).

Serovars of Icterohaemorrhagiae, Pomona, and Grippotyphosa can also be associated with bovine leptospirosis (Lilenbaum and Martins 2014). In each region only a small number of serovars are found, certain animal species will be infected by serovars that are maintained by these species or by serovars that are maintained by other animal species in the area (Ellis 2015). In this study 58 serum samples from farm workers on this farm were also tested with MAT, but none of them detected any antibodies to *Leptospira* bacteria. In previous research (Binti et al. 2018), it was reported that the seroprevalence rate of leptospirosis in cattle breeders (northeast Malaysia) could reach 72.5%.

Higher concentrations of pathogenic *Leptospira* in a farm will cause a greater risk of infection to cattle farmers who come into contact with contaminated environment (Binti et al. 2018; Dreyfus et al. 2021). In this research it has been proven that 62 cattle have been infected by *Leptospira interrogans* which potentially infects to human within same environment. However, none of humans were proved to have antibodies against the disease. This is evidence that there was no transfection or propagation of *Leptospira* from cattle to humans. This can happen due to good health management, such as treatment of infected animals, limiting contact between farm workers and livestock through the implementation of optimal biosecurity. Leptospirosis in cattle farms can be controlled through an integrated approach with increased biosecurity, antibiotic treatment and vaccination of cattle herds (Mughini-Gras et al. 2014).

Human cases with Leptospirosis in Indonesia were 274 and 18 people died related to Leptospirosis infection in Kulon Progo in 2011 (Mulyani et al. 2014). While in Demak district in 2014 there were 19 cases found, in 2015 was found 12 cases and in 2016 was found 7 cases with the majority (66%) cases occurred in men (Kuswati and Nurjazuli 2016). World Health Organization (2003) stated that Leptospirosis is especially risky for people who work outdoors with animals such as farmers, breeders, veterinarians and military personnel. Factors that play a role in the level of pathogenicity of *Leptospira* disease in local cattle, including the propagation of infection in humans, seemed to be related to livestock management. From results of our study, it seems that factors that play a role in the level of pathogenicity of *Leptospira* disease in local cattle, including the propagation of infection in humans, related to livestock management.

Conclusion

The *Leptospira* infection in cattle has occurred in a closed population / farm at relatively low level (6.63%). Several *Leptospira* serovars infected cattle in the population were found, namely single infections of Tarrasovi, Hardjo, Grippotyphosa, and Batavia serovars, and multiple infections by the Hardjo and Tarrasovi serovars. The most dominant serovar infected cattle in this area was Tarrasovi. *Leptospira* infections were detected in Bali, Madura, and PO cattle, with the highest prevalence in Bali cattle. It was hypothesis that a local cattle breed is more resistant than other cattle breeds. Factors that play a role in the level of pathogenicity of Leptospirosis in local cattle, including the propagation of infection in humans, seemed to be related to livestock management.

Conflict of interest: The authors declare that they have no conflict of interest regarding the publication of this article.

Acknowledgment: This research was funded by Indonesian government budget at the Indonesia Research Center for Veterinary Science financial year 2019. The authors would like to thanks to all relevant parties who have helped in conducting the research implementation both in field and laboratory.

Author's contribution: SS, RMAA, SMN, AM, HN contributed to design the experiment. SS, RMAA, SMN, AM, HN, SS, DR, DMD, DP, FR, YA, HHSP conducted research, collected data, analyzed data and finalized the manuscript. All authors approved and finalized the manuscript.

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