Detection of Cattle Leptospirosis in Yogyakarta Based on Serology, Molecular, and Histopathological Tests

TITO SUPRAYOGA¹, KURNIASIH KURNIASIH²*, RINI WIDAYANTI³

¹Faculty of Veterinary Medicine, University of Gadjah Mada, Jl. Fauna No. 2, Karangmalang, Yogyakarta 55281, Indonesia; ²Departement of Pathology, Faculty of Veterinary Medicine, University of Gadjah Mada, Jl. Fauna No. 2, Karangmalang, Yogyakarta 55281, Indonesia; ³Departemen of Biochemistry, Faculty of Veterinary Medicine, University of Gadjah Mada, Jl. Fauna No. 2, Karangmalang, Yogyakarta 55281, Indonesia.

Abstract | Leptospirosis is a zoonotic disease caused by Leptospira sp. which has been reported around the world. In Indonesia, leptospirosis occurs in humans and animals. Humans can become infected with Leptospira sp. through direct and indirect exposure to the urine of reservoir animals. Rodent (rats) is the main reservoir for Leptospira sp., however, other mammals such as cattle can also be infected with Leptospira sp. and become a source of transmission to humans and other animals. The study aimed to detect pathogenic Leptospira sp. from slaughtered cattle in the abattoir of Yogyakarta, Indonesia. Fifteen sera and kidneys of cattle were fixed in the absolute ethanol. The sera were tested using microscopic agglutination test (MAT), and the kidneys were separated into two part. The first part was extracted, amplified and tested by polymerase chain reaction (PCR) using LipL32 gene primers. The other part was processed by histopathology. Both of these test were used to detect the leptospirosis in cattle. The MAT showed that as much as 33.3% (5/15) of collected samples were seroreactive against Bangkinang serovar. In addition, the PCR showed similar result that indicated by the representation of band product in 498 bp. Histopathology showed that the positive samples in both MAT and PCR test suffering for interstitial nephritis, perivasculitis, atherosclerosis, nephrosis, and fibrosis. This study showed that the MAT, PCR, and histopathology could be used as the detection tools for cattle leptospirosis.

Keywords | Cattle, Histopathology, Leptospira sp., MAT, PCR

INTRODUCTION

Leptospirosis is a worldwide zoonosis that has a significant impact on animal and public health. During 2018, 285 cases of leptospirosis in humans were reported in Indonesia, with a case fatality rate of 17.8% (Gasem et al., 2020). This zoonotic disease is caused by spirochete bacteria from the genus Leptospira sp. Infection of Leptospira sp. in humans results from direct exposure (with animal tissue, body fluids, or urine) and indirect exposure with soil or urine-contaminated water (Zarantonelli et al., 2018). Leptospira sp. enters the bloodstream through wounds, skin abrasions or it can also be through mucous membranes of the oral cavity and conjunctiva (Brito et al., 2018).

The main reservoir animals for most Leptospira sp. serovars are wild mammals, especially rodents. Domestic animals such as cattle, dogs, sheep, and pigs also act as reservoir animals (Deneke, 2020). Cattle and other ruminants infected with leptospirosis have reproductive problems (Yatbantoong and Chaiyarat, 2019). Cattle and other ruminants infected with leptospirosis have reproductive problems (Sunder et al., 2018), abortion, weak progenies, increased service per conception and calving interval value, weight loss, decreased growth rate, and milk production (Daud et al., 2018). Clinical manifestations of infection
with *Leptospira* sp. depending on the type of serovar infecting and the immune condition of the infected animal (Susanti, 2015).

*Leptospira* sp. will be located in the kidneys of their natural host such as cattle, bufaloe, horse, sheep, goat, pig, dog, and rodent causing little or no damage and maintaining infection in these animals. After infection occurs in the kidney, the cattle will eliminate *Leptospira* sp. in urine for up to 542 days (Daud et al., 2018; Susanti, 2015).

The prevalence of leptospirosis in beef cattle in Bantul is 18.67%, in Kulon Progo is 14.05% (Susanti, 2015), in the Progo river flow is 13.03% (Mulyani et al., 2016). Serovar Hardjo, Pomona, Icterohaemorrhagiae and, Grippotyphosa are serovars that often infect cattle (Grippi et al., 2020).

Microscopic agglutination test (MAT) is the gold standard for diagnosing leptospirosis recommended by the World Health Organization (WHO) (Fraga et al., 2015). The principle of this test is based on the reaction between the serovar antigen *Leptospira* sp. with antibodies from serum that can be observed using a dark field microscope to see the presence of agglutination (Levett, 2001). Polymerase chain reaction (PCR) can detect pathogenic *Leptospira* sp. quickly and precisely. The advantage of using PCR technology is that it has a high test sensitivity. Various sequences have been targeted including the 16S rRNA gene, the *LipL32* gene, which encodes the membrane lipoprotein *Leptospira* sp. and genes coding for the immunoglobulin-like proteins (*Lig*) *Leptospira* sp, which are important as virulence factors for these bacteria (Fraga et al., 2015; Marquez et al., 2017). *LipL32* was consistently found in pathogenic *Leptospira* sp. (Podgoršek et al., 2020).

The study aimed to detect pathogenic *Leptospira* sp. circulating in slaughtered cattle from Yogyakarta abattoir through serological microscopic agglutination tests (MAT), molecular polymerase chain reaction (PCR) and histopathology. It is expected to provide information on the existence of pathogenic *Leptospira* sp. that infect cattle in Indonesia. Further, this data provide benefits in efforts to prevent the incidence and transmission of leptospirosis in humans and animals.

**MATERIALS AND METHODS**

**Ethic approval**

All stages of the research were approved by the Ethical Commitee of Gadjah Mada University (number: 0045/EC-FKH/Int./2020). A total of fifteen sera and kidneys from slaughtered cattle were collected from the abattoir in Yogyakarta. The data of collected specimens were embedded in Table 1.

**Time and Place of Study**

The study was conducted from April 2019 until April 2020. The study was conducted in several places. The microscopic agglutination test (MAT) was carried out in the Laboratory of Balai Besar Penelitian dan Pengembangan Vektor dan Reservoir Penyakit (B2P2VRP), Salatiga, Central Java, Indonesia. The PCR was conducted in the Laboratory of Biotechnology, Disease Investigation Centre, Wates, Yogyakarta, Indonesia. The histopathology was conducted in the Department of Pathology, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia.

**Microscopic Agglutination Test (MAT)**

All the sera were tested using microscopic agglutination test (MAT) to detect the presence of *Leptospira* sp. antibodies from serum that can be observed using a dark field microscope to see the presence of agglutination (Levett, 2001). The MAT test used several *Leptospira* sp. antigens including serovar Bangkinang, Grippophyphosa, Icterohaemorrhagiae, Canicola, Pyrogenes, Hardjo, Hebdomadis, Pomona, Djasiman, Robinsonsi, Bataviae, Mini, Sarmin, Manhao, and Rama. The positive reaction of antigen-antibody was indicated by the titre ≥1:80 of each serovar.

**Polymerase Chain Reaction (PCR) and Histopathology**

The kidney samples were separated into two parts. The first was tested using polymerase chain reaction (PCR), and the other using histopathology. For the PCR, the kidneys were stored inside the absolute ethanol. Further, the organ...
was DNA extracted with gSYNC™ DNA Extraction Kit (Geneaid) then amplified. LipL32 forward primer 5’-GGA CGG TTT AGT CGA TGG AA -3’ and LipL32 reverse primer 5’- GGG AAA AGC AGA CCA ACA GA -3’ were used to amplify LipL32 gene (Ikaratri, 2020). The PCR used 35 cycles with 95°C of first denaturation for 5 minutes, then 94°C of denaturation for 30 seconds, 58°C of annealing for 30 seconds, 72°C of elongation for 1 minute, followed by 72°C of final elongation for 7 minutes, and holding 4°C. On the other hand, the histopathology was performed for the other part of kidney using routine staining (Slaoui and Fiette, 2011).

**Analysis data**

The data was represented as positive and negative for the MAT and PCR. However, the data of histopathology was reported as the histopathological finding. Further, the collected data were analysed descriptively.

**Results and discussion**

The MAT test showed that 5/15 (33.3%) samples were positive against *Leptospira* sp. (serovar Bangkinang) (Table 2). The result was categorised as positive if the titre antibody representing between 80. In this study, the titre antibody of the collected specimen between 80 until 320. These results indicated high level of titre antibody. The PCR demonstrated similar results with the MAT (Table 2). The positive samples of PCR was indicated by the appearance of band in of 498 bp according to the target *Leptospira* sp. (Figure 1). The histopathology showed that the positive sample in MAT and PCR demonstrated interstitial nephritis, glomerulonephritis, perivasculitis, atherosclerosis, nephrosis, and fibrosis (Table 2 and Figure 2). The glossary of MAT, PCR, and histopathology was embedded in Table 2.

**Table 2:** The result of the serological, molecular and histopathology.

| No. | Sample code | MAT | PCR | Histopathology                          |
|-----|-------------|-----|-----|-----------------------------------------|
| 1   | SPG 1       | -   | -   | No histopathological changes            |
| 2   | SPG 2       | +   | +   | Interstitial nephritis                   |
| 3   | SPG 3       | -   | -   | No histopathological changes            |
| 4   | SPG 6       | +   | +   | Interstitialis nephritis                 |
| 5   | SPG 7       | +   | +   | Interstitial nephritis, glomerulonephritis |
| 6   | SPG 8       | -   | -   | No histopathological changes            |
| 7   | SPG 10      | -   | -   | No histopathological changes            |
| 8   | SPG 11      | -   | -   | No histopathological changes            |
| 9   | SPG 12      | -   | +   | Interstitial nephritis, perivasculitis, glomerulonephritis |
| 10  | SPG 13      | -   | -   | No histopathological changes            |
| 11  | SPG 14      | -   | -   | No histopathological changes            |
| 12  | SPG 15      | -   | -   | No histopathological changes            |
| 13  | SPG 16      | +   | +   | Interstitial nephritis, atherosclerosis, nephrosis |
| 14  | SPG 17      | -   | -   | No histopathological changes            |
| 15  | SPG 18      | +   | -   | Fibrosis                                |

*Leptospira* sp infection will be increasing IgM antibodies at the beginning of the infection, followed by IgG antibodies that will last a long time. The MAT test will detect either IgM nor IgG, and this test can be used after 6-10 days of infection. This test is based on the agglutination reaction due to the reaction between *Leptospira* sp. antigen, and antibody in the patient’s serum, which is observed using a dark field microscope (Grippi et al., 2020). Case reports regarding Bangkinang serovar infection in cattle have never been reported. In 1999 Brenner et al., reported the first isolation of *Leptospira interrogans* serovar Bangkinang but in cases of human leptospirosis in Indonesia.
that commonly infect cattle are Hardjo, Grippotyphosa, Icterohaemorrhagiae, and Pomona.

The use of PCR can help in the early diagnosis of leptospirosis. Amplification of specific target genes for the detection of leptospirosis has been carried out by previous investigators. Guedes et al. (2019) used primers targeted at the 16S rRNA gene that produced a 330 bp product to identify *Leptospira* sp. that infects cattle in the Brazilian Amazon. Sumanta et al. (2015) used primers targeted at the 16S rRNA gene to detect leptospirosis in mice in Yogyakarta, Indonesia. Primers targeted at the secY and 16S rRNA genes could identify the *Leptospira interrogans* serogroup Sejroe serovar Hardjo (Cosate et al., 2017).

The outer protein membrane (OMPs) plays an important role in the pathogenesis process of *Leptospira* sp. *LipL32* is an outer membrane protein and is a specific virulence factor most commonly found in pathogenic *Leptospira* sp. and not found in non-pathogenic or saprophytic *Leptospira* sp. (Nagraik et al., 2020). Positive results of PCR testing using primers targeted at the *LipL32* gene in this study indicate that *Leptospira* sp. infecting these cattle is a type of pathogenic *Leptospira* sp. *LipL32* gene can be used as a genetic marker to detect leptospirosis caused by *Leptospira* interrogans (Pinna et al. 2018). Latifah et al. (2017) stated that the *LipL32* gene was found in pathogenic *Leptospira* sp. The primer design that targets the *LipL32* gene can amplify pathogenic *Leptospira* sp, namely *Leptospira interrogans* serovar Bataviae, *Leptospira interrogans* serovar Australis and *Leptospira interrogans* serovar Javanica by producing a product of 786 bp.

Leptospirosis is characterized by the development of vasculitis, endothelial damage, and inflammatory infiltrates composed of monocytes, plasma cells, and neutrophils. While in the kidney, interstitial nephritis is the major finding accompanied by an intense cellular infiltration composed of neutrophil and monocyte (Yadeta et al., 2016). Leptospirosis induces renal dysfunction such as acute tubulointerstitial nephritis and acute tubular necrosis and also vasculitis but rare (Wu and Wu, 2019).

Pathological reactions in the kidneys due to *Leptospira* sp. infection are caused by a direct reaction caused by the organism and also due to the body’s immune response to the infection. When *Leptospira* sp. invasion occurs, bacterial components such as lipopolysaccharides, peptidoglycan, and outer membrane proteins (glycoproteins) will activate the Toll-like receptor-dependent pathway. Toll like receptor activation in the proximal tubular will produce pro-inflammatory cytokines and chemokines (include inducible nitric oxide (iNOS), monocyte chemoattractant protein-1 (CCL2/MCP-1), regulated upon activation normal T-cell expressed and secreted (RANTES), and tumor necrosis factor most commonly found in pathogenic *Leptospira* sp.
factor (NTF-α) then trigger the inflammatory process. The cell-mediated immune response through the release of cytokines and chemokines will cause the migration of neutrophils to the glomerulus and interstitial tissue cause glomerulonephritis and tubulointerstitial nephritis. 

Leptospira sp. will induce the pathway for fibrosis in tubular cells by activating the transforming growth factor-β1/Smad pathway. This activation will result in increased extracellular matrix production in the renal tubular cells (Tanaka et al., 2017; Wu and Wu, 2019).

Histopathological changes in this study were similar to the histopathological findings made by previous investigators. Prakoso et al. (2020) found changes in the kidney organs of cattle infected with Leptospira sp. including chronic interstitial nephritis, hemorrhage, renal vascular congestion, and renal tubular nephrosis. Research conducted by Ajayi et al. (2020) stated that kidneys infected with Leptospira sp. showed changes in the form of tubular necrosis, glomerular, and tubular atrophy of the kidneys, hemorrhage, and interstitial fibrosis. The inflammatory reaction is dominated by the infiltration of lymphoplasmacytic cells. Meanwhile, research conducted by Magalhães et al. (2020) stated that the histopathological changes of the bovine kidney originating from Triangulo Mineiro infected with Leptospira sp. is the occurrence of hyaline in the renal tubules, congestion, and hydropic degeneration of the renal tubules.

CONCLUSION

Based on serological and molecular tests, it was found Leptospira interrogans serovar Bangkinang at the Yogyakarta slaughterhouse. Further, the positive sample with leptospirosis showed several histopathological changes including interstitial nephritis, glomerulonephritis, perivasculitis, atherosclerosis, tubular nephrosis, and fibrosis in positive bovine kidneys.

AUTHOR’S CONTRIBUTION

Tito Suprayoga was conducted the sample collection, performed the examination and analyzed the data. Kurniasih Kurniasih and Rini Widayanti was performed the molecular and serological tests. All author contributed during writing the draft of manuscript and approved the final version of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.
Khbou MK, Haouala K, Benzart M (2016). High frequency of seropositivity of Leptospira in cattle in North Tunisia. Vet. Med. Sci., (3): 13–21. https://doi.org/10.1002/vms3.52

Latifah, I, Abdul-Halim A, Rahmat M, Nadia FM, Ubi ZE, Asmah H, Nasir MA (2017). Isolation by culture and PCR identification of LipL32 gene of pathogenic Leptospira spp. In wild rats of Kuala Lumpur. Malays. J. Pathol., 39(2): 161–166. http://www.mjpah.org.my/2017/v39n2/LipL32-gene.pdf

Levett PN (2001). Leptospirosis. Clin. Microbiol. Rev., 14(2): 296–326. https://doi.org/10.1128/CMR.14.2.296-326.2001

Magalhaes GM, de Alvarenga PB, Medeiros–Ronchi AP, Moreira TA, Gundim LF, Gomes DO, Lima AMC (2020). Leptospirosis in slaughtered cows in the Triângulo Mineiro, Minas Gerais: prevalence. Serological profile and renal lesions. Biosci. J., 36(2): 539-545. https://dx.doi.org/10.14393/BJ-v36n2a2020-42397

Marquez A, Djeloudjzi Z, Lattard V, Kodjo A (2017). Overview of laboratory methods to diagnose leptospirosis and to identify and to type leptospires. Int. Microbiol., 20(4): 184–193.

Martins G, Loureiro A, Hamond C, Pinna MH, Sremont S, Mulyani GT, Sumiarto B, Artama WT, Hartati S, Juwari, Nagraik R, Kaushal A, Gupta S, Kumar D (2020). PCR based detection of bovine carriers of Leptospira strains from naturally infected cattle in Uruguay. Biosci. J., 36(2): 539-545. https://dx.doi.org/10.14393/BJ-v36n2a2020-42397

Prakoso YA, Widyarini S, and Kurniasih K (2020). Metode diagnosis penyakit leptospirosis dengan uji microscopic agglutination test. Media Bina Ilmiah, 14(2): 2077–2086. https://doi.org/10.33758/mbi.v14i2.335

Slack AT, Dohnt MF, Symonds ML, Smythe LD (2005). Development of a Multiple–Locus Variable number of tandem repeat Analysis (MLVA) for leptospirosis interrogans and its application to leptospirosis interrogans serovar Australian isolates from Far North Queensland, Australia. J. Immune Based Ther. Vaccines, 7: 1–7

Slaoui M, Fiete L (2011). Histopathology procedures: From tissue sampling to histopathological evaluation. Method Mol. Biol. 691(4): 69–82. https://doi.org/10.1007/978-1-60761-849-2_4

Sumanta H, Wilbawa T, Hadi susanto S, Nuryati A, Kusnanto H (2015). Genetic variation of Leptospirosis isolated from rats caught in Yogyakarta Indonesia, Asian Pac. J. Trop. Med., 8(9): 710–713. https://doi.org/10.1016/j.aptm.2015.07.029

Susanti (2015). Microscopic Agglutination Test untuk diagnosis leptospirosis pada sapi potong di kabupaten bantul dan kalonprogo. J. Sain Vet., 33(1): 16–22

Tanaka K, Tanabe K, Nishii N, Takie K, Sugiyama H, Wada J (2017). Sustained tubulointerstitial inflammation in kidney with severe leptospirosis. Int. Med., 56(10): 1179–1184. https://doi.org/10.18805/ijar.B-3186

Susanti (2015). Microscopic Agglutination Test untuk diagnosis leptospirosis pada sapi potong di kabupaten bantul dan kalonprogo. J. Sain Vet., 33(1): 16–22

Yadeta W, Michael BG, Abdela N (2016). Leptospirosis in animal and its public health implication: A review. World Appl. Sci. J., 34(6): 845–853. https://www.idosi.org/wasj/wasj34(6)16.pdf

Yarhantoong N, Chaiyarat R (2019). Factor associated with leptospirosis in domestic cattle in Salakphra wildlife sanctuary, Thailand. Int. J. Environ. Res. Publ. Health, 16: 1042. https://doi.org/10.3390/ijerph16061042

Zarantonelli L, Suanes A, Meny P, Buroni F, Nieves C, Menendez C, Mortola A, Picardeau M, Quintero J, Rios C, Rodriguez V, Romero A, Varela G, Revero R, Schelotto F, Riet-Correa F, Buschiazzo A (2018). Isolation of pathogenic Leptospira strains from naturally infected cattle in Uruguay for human leptospirosis. PLoS Negl. Trop. Dis., 12(9): e006694. https://doi.org/10.1371/journal.pntd.006694