Evaluation of a monoclonal antibody–based dot-blot ELISA for detection of Leptospira spp in bovine urine samples

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**Objective**—To evaluate the efficacy of a novel monoclonal antibody (MAb)-based dot-blot ELISA for detection of Leptospira antigens in urine samples of cattle.

**Sample Population**—Blood and urine samples of 45 test cattle from 5 farms in Chonburi province and 20 control cattle from 2 farms in Khon Kaen province in Thailand.

**Procedure**—Blood and urine samples were assayed (microscopic agglutination test and urine antigen test) for Leptospira infection by use of an MAb-based dot-blot ELISA, and results for the ELISA were compared with those for dark-field microscopy (DFM), microbial culture, and a polymerase chain reaction (PCR) assay.

**Results**—All urine samples with positive results for DFM, microbial culture, PCR assay, or > 1 of these tests also had positive results when tested by use of the MAB-based dot-blot ELISA, except for 1 sample that had positive results only for the PCR assay. Detection limits of the dot-blot ELISA were 10³ leptospires/mL of urine and 9.3 ng of Leptospira that had positive results only for the PCR assay. Results for the ELISA were in agreement with results for dark-field microscopy (DFM) (95.45%, 100%, 91.77%, 100%, and 95.83%, respectively), microbial culture (100%, 61.54%, 66.62%, 28.57%, and 100%, respectively), and PCR assay (95.45%, 100%, 91.77%, 100%, and 95.83%, respectively).

**Conclusions and Clinical Relevance**—The MAb-based dot-blot ELISA is suitable as a tool for detecting leptospires in urine samples of cattle.

Leptospirosis is a worldwide zoonotic disease caused by spirochetes of the genus Leptospira. Leptospires infect all species of mammals, including humans. Among domesticated animals, cattle, dogs, and pigs are the species most commonly infected. Clinically, leptospirosis may be mild, which is difficult to diagnose, or the disease may prove fatal. Animals infected with host-adapted serovars of Leptospira spp become maintenance hosts. Examples of leptospires that adapt to the hosts and cause those animals to become maintenance hosts are Leptospira interrogans serovar hardjo (bovine), L. interrogans serovar bratislava (swine), and L. interrogans serovar australis (ovine). Certain leptospires, such as L. interrogans serovar pomona and L. interrogans serovar icterohaemorrhagiae, are not host-adapted to cattle and pigs and cause illness in these animals accompanied by signs of severe clinical disease that sometimes results in death. Host-adapted serovars have not been implicated in infections in humans, but there are variations in virulence among the serovars that infect humans.

Most cattle develop chronic leptospirosis after infection; they become carriers and shed Leptospira organisms in their urine. Only rarely do animals develop acute illness, which can be fatal (especially in young animals). In these instances, clinical signs include abortion, stillbirths, weak newborns, decrease in milk yield, mastitis, icterus, and hemoglobinuria. Differential diagnoses for leptospirosis in cattle include babesiosis, anaplasmosis, toxicosis attributable to the ingestion of rape or kale, postpartum hemoglobinuria, acute hemolytic anemia (in calves), other causes of abortion, agalactia, brucellosis, and infection with Coxiella burnetii (ie, Q fever).

The most widely accepted serologic test for diagnosis of leptospirosis is the microscopic agglutination test (MAT). A 4-fold increase in the MAT titer in paired sera or a single MAT titer of ≥ 1:400 is diagnostic of leptospirosis in humans, whereas a titer of ≥ 1:100 is evidence of past exposure. In cattle, however, the minimum MAT titers for Leptospira infection in several studies have varied from 1:10 to 1:40. The direct and definite means of diagnosing leptospirosis is by documentation of the organisms in samples. Microbial culture, dark-field microscopy
(DFM), and polymerase chain reaction (PCR) assays have been used on urine samples to detect cattle that are carriers of *Leptospira* organisms and to diagnose leptospirosis in dogs.\(^\text{13-16}\) A monoclonal antibody (MAb)–based dot-blot ELISA has been developed as an antigen detection assay for diagnosis of leptospirosis in humans.\(^\text{27}\)

In the study reported here, the efficacy of an MAb–based dot-blot ELISA\(^\text{27}\) as a potential tool for the detection of *Leptospira* organisms in urine samples obtained from test and control cattle in 2 provinces in Thailand was evaluated. Results for the ELISA were compared with results for other detection methods, such as DFM, microbial culture, and a PCR assay.

**Materials and Methods**

**Animals**—Samples were obtained from 65 cattle. There were 45 test cattle (25 bulls and 20 cows) from 5 farms in Chonburi province (approx 95 km east of Bangkok); these farms had a recent history of abortion. These 45 cattle had not been vaccinated against leptospires and were seronegative (MAT titer \(\geq 1:50\)) when tested for leptospirosis. Additionally, 20 control cattle (10 bulls and 10 cows) from 2 farms in Khon Kaen province (approx 450 km northeast of Bangkok) were included in the study. These farms did not have a history of abortion, and the 20 control cattle all were seronegative (MAT titer < 1:50) when tested for leptospirosis.

Farms and cattle were selected on the basis of a database of the National Institute of Animal Health (NIAH), Department of Livestock Development, Ministry of Agriculture and Cooperation, Thailand. Because Chonburi and Khon Kaen are major cattle-producing provinces in Thailand, we believed that the farms included in the study were representative of other farms in Thailand.

**Collection of samples**—Blood and urine samples were collected from the 65 cattle. Personnel of the NIAH collected initial blood samples from test cattle within 2 weeks after abortions were reported on the farms. Blood and urine samples were collected from test and control cattle approximately 3 months after the date of collection of the first blood samples.

Blood samples were collected from each cow or bull via venipuncture of the coccygeal vein with an 18-gauge needle. Samples were collected into plain evacuated glass tubes. Blood samples were centrifuged and the sera harvested. Serum samples were immediately added to a tube that contained 5 mL of brain-heart infusion broth. The same samples were used as a negative-control strip. The dot-blot ELISA was performed as described elsewhere\(^\text{27}\) with a few modifications. In brief, 2 primers (5’–GGC GGC GCC TCT AAA CATG–3’ and 5’–TCC CCA TTG AGC AAG ATT–3’) generated from 16S rDNA of *L. interrogans* serovar canicola strain Moulton\(^\text{27}\) were used to amplify the *Leptospira* DNA. Amplification of DNA was performed in a 25-µL reaction volume that contained 5 µL of template DNA, 2µM deoxynucleoside triphosphates, 1X PCR buffer, 50µM of each primer, and 0.01 U of Taq DNA polymerase/µL. The PCR assay was performed in a thermal cycle\(^\text{b}\) by use of the following cycling characteristics: 30 cycles (denaturation at 94°C for 3 minutes, annealing at 63°C for 1.5 minutes, and extension at 72°C for 2 minutes) with an additional extension at 72°C for 10 minutes. Amplicons were detected by use of ethidium bromide staining and UV transillumination.

**PCR assay of urine samples**—*Leptospira* DNA in the urine samples was extracted in preparation for the PCR assay.\(^\text{27}\) Two primers (5’–GGC GGC GCC TCT AAA CATG–3’ and 5’–TCC CCA TTG AGC AAG ATT–3’) generated from 16S rDNA of *L. interrogans* serovar canicola strain Moulton\(^\text{27}\) were used to amplify the *Leptospira* DNA. Amplification of DNA was performed in a 25-µL reaction volume that contained 5 µL of template DNA, 2µM deoxynucleoside triphosphates, 1X PCR buffer, 50µM of each primer, and 0.01 U of Taq DNA polymerase/µL. The PCR assay was performed in a thermal cycle\(^\text{b}\) by use of the following cycling characteristics: 30 cycles (denaturation at 94°C for 3 minutes, annealing at 63°C for 1.5 minutes, and extension at 72°C for 2 minutes) with an additional extension at 72°C for 10 minutes. Amplicons were detected by use of ethidium bromide staining and UV transillumination.

**MAb–based dot-blot ELISA**—The urine samples stored at –20°C were thawed and mixed well. Six milliliters of each sample was centrifuged (12,000 X g for 20 minutes at 25°C). Five milliliters of the supernatant was then carefully removed with a pipette, and the sediment was resuspended in the remaining fluid and boiled for 30 minutes. A slot-blot device\(^\text{c}\) was used to place duplicate aliquots (200 µL/ aliquot) of the boiled urine samples on 2 nitrocellulose membrane strips. A positive-control antigen (ie, a homogenate of *L. interrogans* serovar icterohaemorrhagiae) and a negative-control antigen (ie, *Escherichia coli* K-12 homogenate) were also placed on a separate nitrocellulose membrane that was used as an antigen-control strip. The dot-blot ELISA was performed as described elsewhere\(^\text{27}\) with a few modifications. In brief, strips were air-dried and treated with blocking solution for 10 minutes. Then, one of the sample strips and the antigen-control strip were washed several times and allowed to react with MAB LD5, a monoclonal antibody specific for pathogenic *Leptospira* spp.\(^\text{27}\) The duplicate sample strip, which was used as a negative-control strip, was left untreated in the washing solution. After incubation on a rocking platform for 20 minutes at 25°C, all nitrocellulose membrane strips were washed and allowed to react serially with biotinylated rabbit anti-mouse immunoglobulin, streptavidin–enzyme conjugate, and substrate. The reaction was stopped by rinsing the strips with distilled water. A positive-control reaction was evident as a dark purplish-blue spot, whereas a positive reaction was evident as a light purplish-blue spot, which was dis-
tions (10^7 cells/mL to 1 cell/mL) of results for DFM and Urine samples from the 20 control cattle had negative results for DFM. Twelve urine samples from 5 bulls and 7 cows had positive results for Leptospira spp when tested by use of the MAT, 15 (50%) were reactive against 2 or more Leptospira serovars. Serum samples of all 20 control cattle did not react against any of the 26 Leptospira serovars used in the MAT.

**Leptospira culture and DFM of urine samples**—Twelve urine samples from 5 bulls and 7 cows had positive results for Leptospira organisms when evaluated by use of DFM. Of these 12, 6 urine samples from 4 bulls and 2 cows yielded growth of Leptospira spp. Urine samples from the 20 control cattle had negative results for DFM and Leptospira culture.

**PCR assay of urine samples**—Serial concentrations (10^3 cells/mL to 1 cell/mL) of *L. interrogans* serovar bataviae and serial concentrations of genomic DNA (50 ng to 5 fg) were subjected to PCR procedures. The minimum number of *Leptospira* cells and the amount of genomic DNA that could be amplified by the PCR assay were 100 cells and 500 fg, respectively. Size of the PCR amplicon detected was approximately 331 bp. Twenty-two of 45 (48.9%) urine samples from test cattle and 0 of 20 urine samples from control cattle had positive results for *Leptospira* spp when tested by use of the PCR assay. Of the 22 samples with positive results for the PCR assay, 21 had positive results and 1 had negative results when tested by use of the MAb–based dot-blot ELISA.

**MAb–based dot-blot ELISA**—*Leptospira* antigen was detected by use of the MAb–based dot-blot ELISA in 21 urine samples of the test cattle. All samples that had positive results for *Leptospira* spp when tested by use of DFM or microbial culture had positive results when tested by use of the MAb–based dot-blot ELISA. Nine samples (6 bulls and 3 cows) had positive results when tested by use of the dot-blot ELISA but negative results when tested by use of DFM or microbial culture. Urine samples of all 20 control cattle had negative results for *Leptospira* spp when tested by use of the dot-blot ELISA. The lowest amount of *Leptospira* whole cells and homogenate detectable by use of the dot-blot ELISA was 1,000 cells and 9.3 ng, respectively.

**Comparison with other diagnostic methods**—Statistical analysis was used to determine the effectiveness of the MAb–based dot-blot ELISA for the detection of *Leptospira* antigen. Comparison revealed that the diagnostic sensitivity, specificity, efficacy (accuracy), positive predictive value, and negative predictive value for the ELISA were in agreement with results for DFM (100%, 72.72%, 80%, 57.14%, and 100%, respectively), microbial culture (100%, 61.54%, 66.62%, 28.57%, and 100%, respectively), and PCR assay (95.45%, 100%, 91.77%, 100%, and 93.83%, respectively).

**Discussion**

Test cattle were selected on the basis of criteria that included being on a farm with a recent history of abortion attributable to any cause, lack of vaccination against leptospirosis, and an MAT titer ≥ 1:50. A low titer (ie, 1:50) was chosen as the cutoff point because *Leptospira* antibody titers of infected animals decrease to low values within a few months, and the use of a higher titer may have been an inaccurate reflection of the prevalence of infection in the herds. There have been instances in which infected animals had low titers.
but were still excreting leptospires in the urine.\textsuperscript{31} Specific agglutinin titers (ie, MAT titers) do not develop in animals that are not infected; thus, even a low antibody titer is an indicator of past or current infection.\textsuperscript{32}

In the study reported here, analysis of the second samples obtained from test cattle at various farms 3 months after the abortions revealed a noticeable decrease in MAT titers (Table 1). Antibodies to \textit{Leptospira} spp decrease dramatically after animals have recovered from leptospirosis. The predominant serovars identified in urine samples of the cattle were \textit{L. interrogans} serovar hardjo, \textit{L. meyeri} serovar ranarum, and \textit{L. borgpetersenii} serovar sejroe. This finding is similar to that of another report\textsuperscript{33} in which the most common serovars of \textit{Leptospira} detected serologically among cattle in Thailand were ranarum, sejroe, and sarmin. Serovar hardjo was the most common serovar detected in cattle in Malaysia and Brazil.\textsuperscript{34,35} Our finding that 15 of 30 (50\%) of the MAT-positive sera reacted against 2 or more \textit{Leptospira} serovars agrees with other reports for Thailand\textsuperscript{36} and Malaysia.\textsuperscript{37} However, cross-reacting antibodies can be stimulated by a single infecting serovar.\textsuperscript{38}

\textit{Leptospira} organisms often persist in the kidneys and genital organs of cattle without the host having any clinical signs of disease. Infected bulls and cows often excrete \textit{Leptospira} organisms in the urine.\textsuperscript{39} Therefore, definitive diagnosis of \textit{Leptospira} infection is based on detection of the organism or its products (ie, DNA or antigens) in samples.\textsuperscript{11,19,10} In the study reported here, we adopted a MAb-based dot-blot ELISA\textsuperscript{24} to detect pathogenic \textit{Leptospira} antigen in the urine of cattle. The MAb that we used as the antigen detection reagent in the assay have specificity to epitopes of a 35- to 36-kd protein found only in pathogenic leptospires.\textsuperscript{27} Results for the antigen detection assay were evaluated and compared with results for other existing detection methods (ie, DFM, microbial culture, and PCR assay).

Direct DFM is the most rapid method for detecting \textit{Leptospira} spp in samples such as urine. However, the sensitivity of DFM is limited to 10,000 organisms/mL of urine;\textsuperscript{19,35} thus, it is not a particularly sensitive assay. Inexperience can also result in false-positive and false-negative results. In the study reported here, 12 of 45 (26.7\%) urine samples had positive results for \textit{Leptospira} spp when tested by use of DFM. Other spirochetes (eg, \textit{Leptomonas illini}) that could also be found in the urine of cattle, especially bulls, were not detected. They can be readily differentiated from leptospires on the basis of their greater length and width.\textsuperscript{40}

The method that is the criterion-referenced standard for detection of cattle that are carriers of \textit{Leptospira} organisms is microbial culture, and urine is the most commonly used sample. Cultured isolates can be further characterized and used for other purposes such as epidemiologic analysis or drug susceptibility monitoring. Included in the main disadvantages of the culture method are inoculation failure, contamination, the requirement for complex media and frequent subculturing, tedium, and a considerably long time (weeks to several months) for sufficient growth of organisms.\textsuperscript{8,18} Six of 12 urine samples that had positive results when tested by use of DFM also had \textit{Leptospira} growth during microbial culture. \textit{Leptospira} spp were detected in those cultures by approximately the third week, and no additional cultures yielded leptospires thereafter despite the fact we maintained the cultures for 8 to 10 weeks, as was suggested in another report.\textsuperscript{8}

Unfortunately, pure cultures could not be established and maintained; thus, serogrouping and serovar typing were not performed for the \textit{Leptospira} spp.

Use of PCR assays for detection of leptospires in urine appears promising as an approach for early diagnosis of leptospirosis and can be useful in the study of long-term shedding in humans and domestic animals.\textsuperscript{41} There have been PCR tests developed to differentiate pathogenic and nonpathogenic leptospires.\textsuperscript{52,55} The PCR-based detection of \textit{Leptospira} spp is a highly sensitive technique, compared with the sensitivity for DFM.\textsuperscript{54} However, PCR assays are expensive and require special facilities. False-positive and false-negative results have been reported,\textsuperscript{39} and a standardized protocol has not been established. In the study reported here, PCR assays yielded positive results for all samples that also had positive results when tested by use of the MAb–based dot-blot ELISA and for 1 sample that had negative results when tested by the use of DFM, microbial culture, and the MAb–based dot-blot ELISA. The finding of 1 urine sample that had negative results for the dot-blot ELISA but positive results for the PCR assay could have been attributable to a more sensitive nature of PCR–based tests or a false-positive result caused by contamination of the PCR assay or a non-specific product migrating at the same size as the expected PCR amplicon. The finding that none of the samples from the control cattle had positive results when tested by use of the PCR assay tends to rule out the latter possibility.

All of the samples that had positive results when tested by use of DFM or microbial culture (or both methods) also had positive results when tested by use of the MAb–based dot-blot ELISA. Moreover, 9 samples that had negative results for both DFM and microbial culture had positive results for the dot-blot ELISA. Possible explanations include that living leptospires were intermittently shed into the urine or the cattle were not excreting detectable numbers of intact or living leptospires at the time of testing and thus had negative results for DFM and microbial culture, whereas dead or disintegrated organisms were detected by use of the MAb–based dot-blot ELISA. Samples that had negative results for DFM and microbial culture but positive results for the dot-blot ELISA and PCR assay imply that the MAb–based dot-blot ELISA is extremely accurate. In addition, the sensitivity, specificity, positive predictive value, and negative predictive value obtained from the comparisons between results for the MAb–based dot-blot ELISA and the other tests revealed that results for the dot-blot ELISA were in fair agreement with results for microbial culture, good agreement with results for DFM, and excellent agreement with results for the PCR assay. Furthermore, the limit of detection for the dot-blot ELISA (1,000 cells/mL of
urate) was the only order lower than the best obtainable limit (100 cells/mL of urine) observed with a combined immunomagnetic capture and fluoroimmunoassay, which is specific only for serovar hardjo. The fastidious nature of *Leptospira* organisms is also a reason for the use of the antigen test or PCR assays, which will detect viable and nonviable organisms, as opposed to microbial culture that only detects viable organisms.

Analysis of results of the study reported here revealed that the sensitivity, simplicity, and low cost of a novel MAb-based dot-blot ELISA make it appropriate as a test for the detection of *Leptospira* organisms in urine samples of cattle. Similar to other assays, a positive result does not definitively indicate disease attributable to leptospirosis; thus, the results need to be interpreted in light of clinical signs and the medical history.

References