

Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence, and nucleic acid hybridization for detection of *Leptospira borgpetersenii* serovar hardjo in urine of cattle

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Objective—To compare sensitivity and specificity of various polymerase chain reaction (PCR) assays for detection of *Leptospira borgpetersenii* serovar *hardjo* in bovine urine and to compare results of the optimal PCR assay with results of immunofluorescence, nucleic acid hybridization, and bacteriologic culture.

Animals—6 heifers.

Procedure—Heifers were exposed to serovar *hardjo* type *hardjo-bovis* by conjunctival instillation of 10^6 leptospires on 3 successive days. Urine samples were collected before and after infection. Sensitivity and specificity of 5 PCR assays were compared, to determine the optimal assay for use with bovine urine samples. The optimal PCR assay was then compared with results of bacteriologic culture, nucleic acid hybridization, and immunofluorescence.

Results—A PCR assay with the best combination of specificity (100%) and sensitivity (91%) was selected for comparison with the other diagnostic tests. Sensitivity for nucleic acid hybridization was 55%, whereas sensitivity for bacteriologic culture and immunofluorescence was 89 to 93%.

Conclusions and Clinical Relevance—Bacteriologic culture, PCR, and immunofluorescence were sensitive for detection of *L. borgpetersenii* serovar *hardjo* type *hardjo-bovis* in urine specimens of cattle, but a single technique was not the most sensitive for each animal tested. Therefore, the use of 2 techniques in combination is warranted for maximal sensitivity for diagnosis. (*Am J Vet Res* 2000;61:316–320)

Leptospirosis is a worldwide zoonotic disease caused by infection with pathogenic *Leptospira* spp. *Leptospira borgpetersenii* serovar *hardjo* is the most common cause of leptospirosis in cattle throughout the world. Infection of cattle with serovar *hardjo* is often

subclinical but can cause abortion, stillbirth, birth of weak calves, and decreased milk production.¹⁻³

Leptospires invade the host after being deposited on mucous membranes, and, after an incubation period of variable duration (3 to 20 days), *L. borgpetersenii* serovar *hardjo* can be detected in blood of infected cattle.^{3,4} During the period of leptospiremia, leptospires enter and replicate in many tissues, including the liver, reproductive tract, spleen, kidney, eye, and CNS. Agglutinating antibodies can be detected in serum soon after the period of leptospiremia. Appearance of circulating antibodies coincides with clearance of leptospires from blood and most organs. Persistent infection of the kidney and reproductive tract may develop, and serovar *hardjo* may be shed in urine of infected cattle for months after infection.^{3,5-7} Cattle persistently infected with serovar *hardjo* are an important reservoir of infection for other animals, including humans. Identification of infected cattle is important, because treatment with antibiotics or removal of infected cattle can reduce the spread of infection within herds and to humans.^{6,8}

Several methods are available for diagnosis of leptospirosis in cattle. Serologic tests detect antibodies against leptospires in the serum of cattle and are widely available and inexpensive to perform.^{9,10} However, serologic assays are relatively insensitive for detection of serovar *hardjo* infections in cattle, compared with other serovars, and do not indicate active infection.⁵ Interpretation of serologic results also is complicated by vaccine-induced antibodies and serologic cross-reactivity between leptospiral serovars. Leptospires can be detected in urine by use of darkfield microscopy, immunofluorescence, and bacteriologic culture with specialized culture medium and prolonged incubation times. In the past decade, methods for detection of leptospiral DNA in tissues and body fluids have been developed. Nucleic acid hybridization techniques¹¹⁻¹³ and assays based on the polymerase chain reaction (PCR)¹⁴⁻²² have been described.

Several PCR assays to detect leptospires in various samples from animals and humans have been described. Each assay uses different primers, sample preparation methods, and amplification conditions.¹⁴⁻²² It is important to determine which assay is optimal for detection of serovar *hardjo* in bovine urine. Bovine urine samples are often contaminated with other bacteria and present challenges for diagnosis by bacteriologic culture and PCR. The purpose of the study reported here was to compare sensitivity and specificity of several different PCR assays for detection of

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L borgpetersenii serovar hardjo in bovine urine and to compare results of the optimal PCR assay with results of immunofluorescence, nucleic acid hybridization, and bacteriologic culture.

Materials and Methods

Cattle—Six mixed-breed heifers that lacked serum antibodies against *L borgpetersenii* serovar hardjo detectable by the microscopic agglutination test were used.¹⁰ Heifers were housed individually and exposed to *L borgpetersenii* serovar hardjo by conjunctival instillation of 10⁹ leptospores on 3 successive days. Three heifers were exposed to *L borgpetersenii* serovar hardjo type hardjo-bovis strain 320 isolated from a bull in the United States, and 3 heifers were exposed to *L borgpetersenii* serovar hardjo type hardjo-bovis strain 355, a bovine isolate from Northern Ireland.⁴

Sample collection—Urine samples were collected from heifers on 3 occasions prior to exposure to leptospores, 3 times/wk from days 5 to 12 after exposure, and 2 times/wk from days 15 to 53 after exposure. Urine was collected 15 minutes after IV administration of 500 mg of furosemide.⁹ The vulva of each heifer was washed with clean water immediately prior to urine collection. Urine was processed immediately for leptospiral culture, immunofluorescence, nucleic acid hybridization, and PCR.

Preparation of urine for PCR—Urine samples were transferred into 50-ml plastic disposable centrifuge tubes and centrifuged at 17,000 × g for 30 minutes. The supernatant was decanted, and the pellet was processed for PCR as described by Gerritsen.²² Briefly, the pellet of urine was washed with 1 ml of 1 mM ethylenediaminetetraacetic acid and 1 ml sterile distilled water. Samples were centrifuged for 10 minutes at 15,000 × g in a microcentrifuge, and the pellet was washed and resuspended in 60 μl sterile distilled water and boiled for 10 minutes at 100 C. After processing, samples were stored at -20 C until used for PCR. Addition of *Leptospira biflexa* serovar patoc to urine samples as suggested in the original description²² of the method did not improve the sensitivity of any of the PCR assays and, therefore, was not used in this study.

PCR assays—PCR assays were performed in a total volume of 50 μl. Final concentrations of constituents of the reaction buffer^c were: 10 mM Tris/HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100. Final MgCl₂ concentration and amplification conditions were different for each assay (Table 1). Reaction mixtures contained 200 μM of each dNTP, 50 pmol of each primer, 10 μl DNA sample, and 2.5 U *Taq* polymerase.^c Samples were covered with 50 μl of mineral oil, and amplification was performed in a thermocycler.^d The last extension step of each assay was extended to 10 minutes.

Southern blotting and hybridization of PCR products—To improve sensitivity and ensure specificity of the PCR assays, amplified products were blotted and hybridized with probes specific for the leptospiral sequences. Ten microliters of each PCR product was electrophoresed in

an ethidium bromide-stained 2% agarose gel. Fragments were transferred to nylon membranes^e by Southern blotting, and DNA was denatured and crosslinked to the membranes by UV irradiation, by use of standard protocols.²³ Oligonucleotide probes used in the PCR assays described by Wagenaar¹⁸ (probe A) and Gravekamp¹⁴ (sequence: 5'AA/GATGATCGGC ATT/CACGTTT/CGCA/GCCGTT3') were end-labeled with digoxigenin-ddUTP as instructed by the manufacturer, by use of a DIG-oligonucleotide 3'-end labeling kit.^f The probe for the PCR assay described by Mérien¹⁶ was labeled with digoxigenin-dUTP by PCR with a nested primer set. The probe for the PCR assay described by Gerritsen²² was labeled by random priming on the whole plasmid or by random priming on the insert,¹⁷ by use of a digoxigenin-DNA labeling kit^g according to the manufacturer's instructions. Blots were prehybridized at hybridization temperatures (Table 1). Double-stranded probes were heated at 100 C for 10 minutes and rapidly cooled on ice. Prehybridization, hybridization, washing, and chemiluminescent detection^f were performed, as instructed by the manufacturer. To ensure specificity, PCR results were recorded as positive only if the amplified fragment was of the proper size and hybridized with the appropriate probes.

Sensitivity of PCR assays on seeded urine samples—Heifer urine samples collected prior to exposure to leptospores were seeded with serial 10-fold dilutions of cultured *L borgpetersenii* serovar hardjo type hardjo-bovis cells. Seeded urine samples were processed for PCR as described.

Leptospiral culture—Four formulations of semi-solid leptospiral culture medium were used. Ellinghausen-McCullough-Johnson-Harris semi-solid leptospiral culture medium²⁴ containing 100 μg of 5-fluorouracil/ml⁸ and 1% rabbit serum was used. Three formulations of Tween 80/Tween 40/lactalbumin hydrolysate semi-solid medium²⁵ containing 0.4% rabbit serum and 0 μg, 100 μg, or 200 μg of 5-fluorouracil/ml also were used. Medium was inoculated, incubated, and examined as described.²⁶ Because of limitations in time and resources, culture was only performed on alternate urine samples collected from each heifer after infection.

Immunofluorescence assay—Urine was processed and stained with a fluorescein-labeled rabbit anti-hardjo serum as described.²⁷ Leptospores were identified by typical shape and specific fluorescence when examined by incident-light fluorescence microscopy.

Nucleic acid hybridization—Urine samples were processed, and the DNA was hybridized with a radiolabeled *L borgpetersenii* serovar hardjo type hardjo-bovis specific repetitive sequence element probe as described.¹³

Results

Sensitivity of PCR-assays in seeded urine samples—The reported sensitivity of 50 leptospores/ml urine was obtained using PCR assays described by Gerritsen,²² Gravekamp,¹⁴ Mérien,¹⁶ and Wagenaar¹⁸ on

Table 1—Conditions for polymerase chain reaction (PCR) amplification and hybridization of DNA from *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis

PCR assay	MgCl ₂	Denaturation	Annealing	Extension	Cycles	Hybridization
Gerritsen ²²	2.0 mM	1 min; 94 C	1 min; 55 C	3 min; 72 C	36	68 C
Gravekamp ¹⁴	2.0 mM	1 min; 94 C	1 min; 55 C	2 min; 72 C	35	55 C
Mérien ¹⁶	2.0 mM	1 min; 94 C	90 s; 63 C	2 min; 72 C	30	68 C
Hookey ¹⁵	3.5 mM	40 s; 94 C	17 s; 58 C	1 min; 72 C	32	ND
Wagenaar ¹⁸	2.0 mM	1 min; 95 C	2 min; 55 C	3 min; 72 C	35	55 C

ND = Not done.

Table 2—Specificity (%) and sensitivity (%) of various PCR assays for detection of *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis in *Leptospira*-negative and *Leptospira*-positive urine in cattle

PCR assay	Negative			Positive		
	+	-	Spec	+	-	Sens
Gerritsen ²²	0	18	100	27	6	82
Gravekamp ¹⁴	0	18	100	30	3	91
Mérien ¹⁶	2	16	89	29	4	88
Wagenaar ¹⁹	7	11	61	ND	ND	ND

+ = Positive results. - = Negative results. Spec = Specificity. Sens = Sensitivity. ND = Not done.

urine samples seeded with known numbers of cultured serovar hardjo cells. Sensitivity of the assay described by Hookey¹⁵ was 5,000 leptospores/ml of urine, which was considerably lower than the other assays and lower than the published value for this assay. Attempts to improve the sensitivity of this assay by modification of the reaction mixture and amplification conditions were unsuccessful. Therefore, the primers described by Hookey¹⁵ were not used further in this study.

Specificity of PCR assays in bovine urine—Eighteen urine samples collected from the heifers prior to exposure to serovar hardjo were used to determine specificity of the PCR assays (Table 2). The number of false positive results (7/18) obtained by use of the assay described by Wagenaar¹⁸ was unacceptable for use of this assay in bovine urine samples, and, therefore, this assay was not evaluated further. The specificities of assays that used primers described by Gerritsen²² and Gravekamp¹⁴ were equal and higher than the specificity obtained by use of primers described by Mérien.¹⁶

Sensitivity of PCR assays—Thirty-three urine samples that contained serovar hardjo, as determined by culture, were used to determine sensitivity of PCR assays that used primers described by Gerritsen,²² Gravekamp,¹⁴ and Mérien¹⁶ (Table 2). Sensitivity of the assays ranged from 82 to 91%.

Comparison of PCR results with results of other diagnostic tests—The PCR assay described by Gravekamp¹⁴ was found to have the best combination of specificity (100%) and sensitivity (91%) and was used to compare the sensitivity of PCR, immunofluorescence, bacteriologic culture, and nucleic acid hybridization for detection of serovar hardjo. To determine sensitivity of the different assays, we defined criteria for results that would be considered true positives; a true criterion standard assay that detects 100% of samples containing leptospores is not available. Positive results from urine samples collected from heifers after urinary shedding was first detected in that heifer were considered true positive results and used to calculate sensitivity. Results were consistently positive with 1 or more assays in these samples with 1 exception: for 1 heifer, results of PCR assay for a urine sample collected 7 days after exposure were positive, but results of other tests were negative. Results for further samples were negative until 18 days after exposure, when multiple test results were positive. For the purposes of analysis, the PCR-positive result from this heifer on day 7 was considered a false positive.

Table 3—Results of bacteriologic culture, immunofluorescence, nucleic acid hybridization, and PCR for detection of *Leptospira borgpetersenii* serovar hardjo in urine from experimentally infected cattle

Heifer	First positive test	Culture	IF	Hybrid	PCR
1	PCR	5/6*	10/11	7/11	11/11
2	PCR	6/7	11/12	9/12	12/12
3	PCR, Cult, IF	6/6	11/11	9/11	11/11
4	IF	4/5	9/10	2/10	5/10
5	PCR, Cult	7/7	10/12	3/12	11/12
6	PCR, IF	5/6	11/11	7/11	11/11

*No. positive/No. tested.
IF = Immunofluorescence assay. Hybrid = Nucleic acid hybridization.
Cult = Bacteriologic culture.

Leptospira borgpetersenii serovar hardjo was first detected in the urine of heifers from 15 to 21 days after exposure by use of various assays; the assay that first detected leptospores differed among heifers (Table 3). Sensitivity of bacteriologic culture (89%; 33 positive results for 37 *Leptospira*-positive samples), PCR (91%; 61 positive results for 67 *Leptospira*-positive samples), and immunofluorescence (93%; 62 positive results for 67 *Leptospira*-positive samples) were similar; sensitivity of nucleic acid hybridization (55%; 37 positive results for 67 *Leptospira*-positive samples) was considerably less than that of the other techniques. In general, sensitivity of the assays was consistent from heifer to heifer (Table 3). However, results of PCR were positive in only 5 of 10 samples from 1 heifer and results of hybridization were positive in only 2 of 10 samples from 1 heifer and 3 of 12 samples from a different heifer.

Test results were in agreement for 92% of samples tested by use of culture and immunofluorescence, 88% of samples tested by use of culture and PCR, 91% of samples tested by use of PCR and immunofluorescence, 77% of samples tested by use of culture and nucleic acid hybridization, 71% of samples tested by use of immunofluorescence and nucleic acid hybridization, and 76% of samples tested by use of PCR and nucleic acid hybridization.

Discussion

The PCR assay described by Gravekamp et al¹⁴ had the best combination of specificity (100%) and sensitivity (91%). The PCR assays described by Mérien et al¹⁶ and Wagenaar et al,¹⁸ which used primers derived from ribosomal gene sequences, had higher numbers of false positive results than the other PCR assays. Although the primer sequences were selected from the highly variable regions of the ribosomal gene, there seems to be a cross-reaction between these sequences and DNA from other microorganisms found in bovine urine. However, the assay described by Mérien et al¹⁶ had good sensitivity in urine samples that contained leptospores. For samples that have a low risk of contamination with other microorganisms, such as blood and CSF, this PCR assay may be useful.

The PCR and nucleic acid hybridization assays had low sensitivity in urine samples from 1 and 2 heifers, respectively. Culture and immunofluorescence assays for samples from these heifers revealed intact, viable leptospores; these results indicate that sensitivity of

DNA-based diagnostic assays varies from animal to animal. Presumably, there were substances in the urine of these heifers that inhibited recovery of the DNA, inhibited the PCR reaction, or inhibited binding of the DNA to a solid support. The recently described magnetic immunocapture method of sample processing may be of benefit for urine samples containing inhibitory substances.²⁸

The assay that first detected leptospire differed among heifers, but PCR was 1 of the first tests to yield positive results in 5 of 6 heifers; therefore, PCR was a sensitive method for detection of *L borgpetersenii* serovar hardjo under these carefully controlled experimental conditions. Further analysis and comparison of PCR, culture, and immunofluorescence is warranted in field samples that are often collected and handled under less than optimal conditions. Another variable that may influence results of such field studies is the degree of laboratory expertise in performance of the various assays. For routine use in our laboratory, immunofluorescence is faster, less prone to laboratory error, and easier to perform than PCR.

Urine samples collected after shedding was first detected were defined as true positive samples for calculation of sensitivity values. Isolation of serovar hardjo from a urine sample is the only unequivocal method of determining that a urine sample contains the organism; however, because of time and resource constraints, not all urine samples were examined by bacteriologic culture. Although it was not possible to prove that each urine sample that was considered a true positive sample did, in fact, contain serovar hardjo, we believe each sample was positive, because each urine sample collected after shedding began yielded positive results with at least 1 assay (often, results of several assays were positive) and because intermittent shedding for at least 56 days after infection was unlikely.^{6,7}

One urine sample from 1 heifer yielded positive results by PCR 7 days after exposure. For calculation of assay sensitivity, that result was considered a false positive. It is possible that the positive PCR result was correct, but it seems unlikely because we have not detected *L borgpetersenii* serovar hardjo in the urine of cattle < 10 days after conjunctival exposure.^{27,29-31} In addition, after a heifer begins shedding serovar hardjo in the urine, shedding is usually consistently detected by use of several techniques.^{6,7} Therefore, it is not likely that a single sample would yield positive results, and several subsequent samples would yield negative results before shedding was next detected.

The urine sample preparation method that was used²² was rapid, simple, and reproducible. A method for magnetic immunocapture of leptospire in bovine urine samples was recently described that may further enhance sample preparation for PCR assays.²⁸

In the second part of the study reported here, the optimal PCR assay was compared with other diagnostic assays for detection of serovar hardjo in bovine urine; PCR and culture were of equivalent sensitivity. Results of other studies have indicated that PCR is more sensitive than bacteriologic culture¹⁷ or that bacteriologic culture is more sensitive than PCR.²⁸ These differences probably reflect the rigor of the culture

methods that were used, variation in laboratory expertise for culturing this fastidious organism from clinical samples, and the variable presence of contaminating bacteria in the urine samples, which interferes with isolation of leptospire.

In a different study, nucleic acid hybridization was more sensitive than bacteriologic culture for detection of serovar hardjo in bovine urine.²⁷ However, in that study²⁷ urine samples were collected from cattle during a vaccine efficacy trial²⁹ in which the vaccine failed to protect cattle from infection. In that and subsequent studies,^{30,31} culture of *L borgpetersenii* serovar hardjo from the urine of vaccinated cattle was found to be extremely difficult. In the study reported here, nonvaccinated cattle were used, and the sensitivity of bacteriologic culture was high.

Each of the evaluated assays has advantages and disadvantages for routine use in diagnosis of infection in cattle. Isolation of serovar hardjo is a definitive, sensitive diagnostic test, but bacteriologic culture is too expensive and slow (8 to 20 weeks) for routine use. Immunofluorescence, hybridization, and PCR are all rapid techniques that do not require viable leptospire. Immunofluorescence is quite sensitive when performed by experienced workers but, using generally available conjugates, is not serovar-specific. However, specially prepared conjugates can be used to identify specific serovars.³² Immunofluorescence procedures depend on structural and antigenic integrity of the organism, and these factors may be compromised in clinical specimens. Sensitivity of the nucleic acid hybridization was lower than that of the other techniques we evaluated, and this technique does not have advantages, compared with PCR assays. The PCR is sensitive but subject to contamination of samples, equipment, and reagents, resulting in false positive reactions.¹⁴ In addition, the PCR assays we evaluated are not useful to identify specific serovars. Because none of these assays had a sensitivity of 100% and there is variation among samples and cattle, 2 assays should be used to maximize the sensitivity of detection of *L borgpetersenii* serovar hardjo in bovine urine.

^aSupplied by Dr. William Ellis, Veterinary Research Laboratory, Stormont, Northern Ireland.

^bLasix, Hoechst-Roussel, Somerville, NJ.

^cPromega, Madison, Wis.

^dPerkin-Elmer, Nowalk, Conn.

^eHybond-N membrane, Amersham International, Arlington Heights, Ill.

^fLumigen PPD, Boehringer Mannheim, Mannheim, Germany.

^gSigma Chemical Co, St Louis, Mo.

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