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Evaluation of a Polymerase Chain Reaction for the Diagnosis of Leptospirosis in Cattle

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ABSTRACT

Bovine leptospirosis is a highly prevalent infection worldwide causing serious losses in cattle production and serving as a source for human infection. Diagnosis and assessment of prevalence of this infection in bovine herds is difficult due to limitations of current procedures. The present report describes the adaptation of a polymerase chain reaction (PCR) protocol for detection of leptospiral DNA in bovine urine. The amplification products corresponded to a segment of the *Leptospira* 16S rRNA gene detected using two sets of primers (A/B and C/D). A total of 547 urine samples from *Bos taurus* (n=327) and *Bos indicus* (n=220) were collected from animals in Andean and Coastal regions of Ecuador, either by furosemide-induced urination or from bladders at the slaughterhouse. The results of this research showed a PCR positivity of 13.52% using primers A/B. *Bos taurus* samples obtained by urination and those obtained from bladder showed a significant difference in PCR positivity (*P= 0.036*). Differentiation of *Leptospira* species was preformed by DNA sequencing of the amplified products. Three amplicons showed 90 and 98% sequence identity with *L. borgpetersenii* and 98% identity with *L. inadai*. The results of this study suggest that PCR could be an excellent approach for epidemiological studies.

RESUMEN

La leptospirosis bovina es una infección altamente prevalente a nivel mundial causante de serias pérdidas en la producción ganadera y sirviendo como una fuente infecciosa en los humanos. Tanto el diagnóstico como la valoración de la prevalencia de esta infección en manadas de bovinos son difíciles debido a las limitaciones de los procedimientos actuales. El presente reporte describe la adaptación de un protocolo de la Reacción en Cadena de la Polimerasa (PCR) para la detección de ADN de leptospira en orina bovina. Los fragmentos amplificados correspondieron al segmento del gen 16S rRNA de *Leptospira*, el mismo que fue detectado mediante la utilización de dos sets de cebadores (A/B y C/D). Se colectaron un total de 547 muestras de orina de *Bos taurus* (n=327) y *Bos indicus* (n=220) correspondientes a la Sierra y Costa ecuatoriana respectivamente; las muestras se tomaron por micción inducida con furosemida o a partir de vejigas recolectadas directamente del matadero. Los resultados de esta investigación mostraron una positividad en la PCR del 13.52% utilizando los primers A/B. Las muestras obtenidas de *Bos taurus* por micción inducida y aquellas obtenidas directamente de vejiga mostraron una diferencia significativa de positividad de la PCR (*P= 0.036*). La diferenciación entre especies de *Leptospira* fue realizada mediante secuenciamiento del ADN de los productos amplificados. Tres de los amplicones mostraron una identidad de secuencia de entre el 90 y 98% con *L.borgpetersenii* y 98% de identidad con *L.inadai*. Los resultados de este estudio sugieren que la PCR podría ser un excelente acercamiento para estudios epidemiológicos.

Evaluation of a Polymerase Chain Reaction for the Diagnosis of Leptospirosis in Cattle

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ABSTRACT

Bovine leptospirosis is a highly prevalent infection worldwide causing serious losses in cattle production and serving as a source for human infection. Diagnosis and assessment of prevalence of this infection in bovine herds is difficult due to limitations of current procedures. The present report describes the adaptation of a polymerase chain reaction (PCR) protocol for detection of leptospiral DNA in bovine urine. The amplification products corresponded to a segment of the *Leptospira* 16S rRNA gene detected using two sets of primers (A/B and C/D). A total of 547 urine samples from *Bos taurus* (n=327) and *Bos indicus* (n=220) were collected from animals in Andean and Coastal regions of Ecuador, either by furosemide-induced urination or from bladders at the slaughterhouse. The results of this research showed a PCR positivity of 13.52% using primers A/B. *Bos taurus* samples obtained by urination and those obtained from bladder showed a significant difference in PCR positivity (*P= 0.036*). Differentiation of *Leptospira* species was preformed by DNA sequencing of the amplified products. Three amplicons showed 90 and 98% sequence identity with *L. borgpetersenii* and 98% identity with *L. inadai*. The results of this study suggest that PCR could be an excellent approach for epidemiological studies.

KEYWORDS

Leptospirosis; Diagnosis; PCR; 16S rRNA; *Lesptospira borgpetersenii; Bos taurus*; *Bos indicus*.

TEXT ORGANIZATION

1. **Introduction**
2. **Materials and Methodology**
3. **Results**
4. **Discussion**

INTRODUCTION

Leptospirosis is a zoonotic worldwide disease caused by pathogenic species of the genus *Leptospira* [1]. In cattle this infection causes infertility, abortion, stillbirths, reduced milk production, and even death [2]. *Leptospira interrogans* serovar Hardjo type Hardjo-bovis is the primary cause of acute and chronic leptospirosis in cattle, in addition causes persistent infection of kidneys and female reproductive tract [3]. Contact with urine is probably the most common transmission route [3]. Human infections among dairy farmers are common where the disease is known as milker's fever [4].

Leptospiral isolation is costly, very difficult, and often unsuccessful [5, 6]. The standard laboratory diagnosis of bovine leptospirosis is performed using the microscopic agglutination test (MAT); this technique is time consuming, cumbersome, and requires trained personnel [6, 7]. Serologic tests do not allow early leptospiral diagnosis, especially in infections caused by serovar Hardjo type Hardjo-bovis [2, 8]. Although MAT provides some information about infecting serovars, cross-reactivity among serovars is of concern [2]. MAT also is unable to differentiate between natural infection and vaccine induced titres [2, 9]. In addition a false negative assay may result from the absence of specific serovars in the assay.

It is necessary to improve diagnostic procedures for animal leptospirosis. Molecular techniques such as PCR have the potential to improve leptospirosis diagnosis [10, 11]. The aim of this research was to evaluate a PCR protocol for the investigation of leptospirosis infection in cattle from dairy farms and at slaughterhouses. A PCR protocol which amplifies a segment of the leptospiral 16S rRNA gene [12] was chosen for evaluation and to also allow the possibility of detecting infecting species.

MATERIALS AND METHODOLOGY

Urine Samples from Cattle

Four field veterinarians were instructed to collect urine samples (from asymptomatic animals) using diuretic furosemide as previously described [13]. A total of 269 *Bos taurus* urine samples from 29 farms close to Quito (Andean region) were collected in sterile tubes. Additionally, bladder urine samples from *Bos taurus* (n=58) and *Bos indicus* (n=220) were collected at the local slaughterhouse by cystocentesis. Species identification of the cattle was based on phenotypic features. *Bos taurus* samples came from Andean farms whereas *B. indicus* came from the Coastal region (tropical). Information about the origin of the animals was obtained from slaughterhouse personnel.

DNA Extraction

A modification of a previously published protocol was used [14]. Urine samples (approximately 50 ml) were transported to the laboratory on ice and immediately centrifuged at 3,287 X *g* for 15 minutes; the pellet was resuspended in 1 ml of 1X PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, H_2O, 1.4 mM KH_2PO_4, (pH 7)] transferred to a 1.5 ml tube and centrifuged for 5 minutes at 9,660 X *g*; followed by a second wash of the pellet preformed with 1ml of 1X PBS. After removal of the supernatant, 700 µl of a solution containing 2% CTAB, 1.4 mM NaCl, 20 mM EDTA, 100 mM HCl (pH 8) was added to each sample and incubated at 65°C for 2 hours with vortexing every 15 minutes. Samples were cooled to room temperature and 700 µl of Chloroform/isoamyl alcohol (24:1) were added, homogenized, and centrifuged at 9,660 X *g* for 5 minutes; the supernatants were transferred to a new sterile tube, 50 µl of sodium acetate and 1000 µl of 100% ethanol were added and stored at -20°C overnight for DNA precipitation. Samples were centrifuged at 13,148 X *g* for 10 minutes, and supernatants were removed, pellets were washed with 1 ml of ethanol 75% and centrifuged for 10 minutes at 13,148 X *g*. The supernatants were discarded and pellets were allowed to dry at room temperature for approximately 15 minutes, and finally resuspended in 50 µl of TE buffer and stored at -20 °C until used.

Urine Experimentally Mixed with *Leptospira*

In order to test the level of detection and the presence of PCR inhibitors, a log phase culture of *Leptospira interrogans* serovar Saxkoebing in EMJH medium (*Leptopira* Medium Ellinghausen-McCullough/ Johnson-Harris) [15] (1.52 x 10^8 cells/ml as determined by Petroff-Hausser Chamber count) was subjected to 8 10-fold dilutions in urine from a PCR negative cow. The DNA was extracted from 1 ml of each dilution as described previously, the final DNA pellet was re-suspended in 25µl of TE buffer and 2.5 µl of the re-suspended pellet were used in each PCR reaction.

PCR Assay

The PCR protocol used in this paper was previously described [12]. Primers A/B (Figure 1) allowed the amplification of a 331bp fragment of the leptospiral 16S rRNA gene. An internal set of pimers C/D (Figure 1) were used for PCR confirmation. The PCR assay conducted, consisted of 40 cycles: denaturation at 94 °C for 3 minutes, annealing at 63°C for 1.2 minutes, and elongation at 72°C for 30 seconds. PCR products were analyzed by electrophoresis in 1.2 % agarose gels. Each PCR reaction was run with positive (DNA from *L. interrogans* culture) and negative control (no DNA template).

An analysis of the primer sequences was performed in order to identify potential mismatch problems; nucleotide sequences of all primers were compared to homologous leptospiral sequences in GenBank using BLAST (blastn) (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Positive Samples DNA Sequencing

Nine amplicons were sent to Macrogen (Seoul, South Korea) for DNA sequencing. Amplicons corresponded to both set of primers (A/B and C/D). Sequences were compared to those in GenBank using BLAST. Phylogenetic analysis was carried out using Mega 4.1 package.

Statistical Analysis

Correlation Contingency analysis was preformed; using Fisher's exact test (two-tailed test). P-value <0.05 were considered significant.

RESULTS

PCR Amplification

Amplification of DNA extracted from artificially contaminated urine samples indicated that PCR using primers A/B and C/D was able to detect spirochetes even in a 10^9 fold dilution suggesting that DNA extracts from urine did not contain any inhibitory compound. Polymerase chain reaction analysis using primers A/B of 547 urine samples showed 13.52% positivity. There was a significant difference of positivity (*P= 0.036*) between *Bos taurus* samples obtained by urination (22.3%) and those obtained directly from the bladder (8.6%). No significant difference in positivity existed between *Bos taurus* bladder (8.6 %) and *Bos indicus* bladder samples (4.1 %) (*P= 0,321*), (Table 1).

Performance of Primers A/B and C/D

Primers C/D were originally designed to confirm leptospiral origin of PCR products [12] (the product of C/D is internal to the amplicon obtained with A/B), however we found that some samples that were negative to primers A/B were positive for primers C/D. Therefore, a subset of 58 urine samples collected from bladder was used to compare sensitivity of both set of primers (Table 2). Five samples (corresponding to 8.6% of the total number of samples) were positive when primers A/B were used, however this value increased to 9 (15.51% of the total number of samples) when primers C/D were used *(P= 0.508),* however the same 5 samples that were positive with primers A/B were also positive when primers C/D were used. Additionally 57 *B. taurus* urination samples previously tested for A/B primers PCR , were also tested for primers C/D (Table 2); 32 of 57 (56.14%) DNA samples which were positive to PCR with A/B primers were also positive with C/D primers. In order to investigate the discrepancies of PCR reactions using the two set of primers, primer sequences were compared to the leptospiral homologous sequences deposited in the GenBank. Primer A was found to have some mismatches with sequence of *L. meyeri* Ranarum [GenBank:Z21648]; primer B had mismatches with sequences of: *L. inadai* serovar Lyme 10 ATCC [GenBank: AY631896], *L. fainei* Hurstbridge 1 [GenBank:AY995712], *L. meyeri* serovar Semaranga Veldrat [GenBank:FJ154599] and *L. inadai* serovar Aguaruna [GenBank:AY631891]; primer C had mismatches with corresponding sequences of: *L. inadai* serovar Lyme 10 ATCC [GenBank: AY631896], *L. fainei* Hurstbridge [GenBank:AY995712], *L. meyeri* RANARUM ICF 16S ribosomal RNA [GenBank:Z21648], *L. meyeri* serovar Semaranga Veldrat [GenBank:FJ154599] and *L. inadai* serovar Aguaruna [GenBank:AY631891]; there were no mismatches found with primer D (Figure 1).

DNA Sequencing

Readable sequences were retrieved from 4 amplicons out of 9, three of which showed 90-98% homology to *L. borgpetersenii* and one 98% to *L. inadai* (Figure 2).

DISCUSSION

This report demonstrates that a previously described PCR protocol could be successfully used in diagnosis of bovine leptospirosis. Additionally, the amplification of 16S rRNA gene allowed the identification of infecting leptospiral species. Other reports describing results of PCR application in urine using a different set of primers and a DNA extraction kit for urine found 35% positivity in cattle in USA [16]. In the present manuscript a 22% positivity was found in similar samples (*Bos Taurus* induced urination) in dairy cattle. The prevalence of leptospirosis in cattle is often investigated by use of the microscopic agglutination test (MAT) [6, 7, 17]. MAT yields limited information about infecting serovars [6]. Even though nucleotide data do not supply evidence of infecting serovars, [1, 2] amplicons containing 16S rRNA gene sequences allow the identification of the infecting species. In the present study three nucleotide sequences showed high sequence homology *to L. borgpetersenii* ribosomal genes suggesting infection by serovar Hardjo type Hardjo-bovis, the most common *Leptospira* found in cattle around the world [18, 19]. It is likely that the advent of new generation sequencing procedures will produce inexpensive high quality sequence very useful for epidemiological studies. The16S rRNA sequences are conserved among members of leptospiral species therefore this approach may allow to recognize the infecting specie but may not be able to identify clonal relationships.

The protocols presented here for DNA extraction are inexpensive, do not leave any inhibitory residue in the sample and produce stable DNA extracts, this procedure has been used previously for the detection of *Leptospira* in urine samples [20]. Urine samples from induced urination showed a significant higher positive results (*P= 0.036*) than samples obtained directly from bladder. It is possible that urine from induced urination may contain a larger number of spirochetes due to carryover from female genital tract, which is often colonized by *Leptospira* [2, 21]. Also the used of diuretic might actually enhance the flushing of spirochetes into the urine, whereas static urine in the bovine bladder may lead to death of the spirochetes and potential breakdown of DNA. However, urine samples from induced urination were not collected randomly so it is likely that chosen animals were showing some signs of leptospirosis. A higher number of positive samples (from bladder) were observed in samples form *B. taurus* than *B. indicus* even though it was not statistically significant. These observations may require additional research because *B. taurus and Bos indicus* are raised in different environments, highlands and lowlands respectively.

The discrepancy in reactivity observed in PCR using primers A/B and C/D might be partially explained by mismatches of the primers with DNA sequences from leptospiras in the field, however many of these mismatches found seemed to be located in non-critical regions [22, 23]. Based on the results presented here, it is advisable to use both sets of primers in order to capture all positive samples.

ABBREVIATIONS

PCR: Polymerase Chain Reaction

DNA: Deoxyribonucleic acid

PBS: Phosphate-buffer saline

CTAB: Cetyl trimethylammonium bromide

EDTA: Ethylenediaminetetraacetic acid

TE: Tris- EDTA buffer

EMJH: Ellinghausen-McCullough-Johnson-Harris medium

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FIGURES

Figure **1**. Mismatches found between primer sequences and leptospiral 16S rRNA gene sequences found in GenBank. Asterisk indicate nucleotide identity and horizontal line indicate gap. DNA sequences of leptospiral species and their respective accession numbers are indicated.

a) Primer A

	5'																			3'
Primer A	**G**	**G**	**C**	**G**	**G**	**C**	**G**	**C**	**G**	**T**	**C**	**T**	**T**	**A**	**A**	**A**	**C**	**A**	**T**	**G**
Leptospira inadai (Lyme) [GenBank:AY631896]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira fainei [GenBank: AY995712]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira borgpetersenii [GenBank: FJ154586]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira santarosai [GenBank:FJ154589]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Leptospira kirschneri [GenBank:DQ991477]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira weilii [GenBank:U12677]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira Noguchi [GenBank:AY631886]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L. meyeri (Ranarum) [GenBank:Z21648]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**C**
Leptospira interrogans [GenBank: FJ812169]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira meyeri (Semaranga) [GenBank:FJ154599]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira inadai (*Aguaruna*) [GenBank:AY631891]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

b) Primer B

	3'																				5'
Primer B	**A**	**A**	**T**	**C**	**T**	**T**	**G**	**C**	**T**	**C**	**A**	**A**	**T**	**G**	**G**	**G**	**G**	**G**	**G**	**A**	**A**
Leptospira inadai (Lyme) [GenBank:AY631896]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**A**	*	*
Leptospira fainei [GenBank: AY995712]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**A**	*	*
Leptospira borgpetersenii [GenBank: FJ154586]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira santarosai [GenBank:FJ154589]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira kirschneri [GenBank:DQ991477]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira weilii	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

[GenBank:U12677]																					
Leptospira Noguchi [GenBank:AY631886]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L. meyeri (Ranarum) [GenBank:Z21648]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira interrogans [GenBank: FJ812169]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira meyeri (Semaranga) [GenBank:FJ154599]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**C**	*	**A**	*	*
Leptospira inadai (*Aguaruna*) [GenBank:AY631891]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	**A**	*	*

c) Primer C 5' 3'

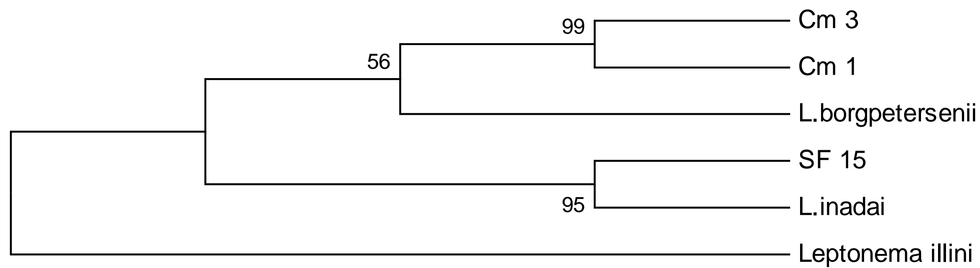
Primer C	**C**	**A**	**A**	**G**	**T**	**C**	**A**	**A**	**G**	**C**	**G**	**G**	**A**	**G**	**T**	**A**	**G**	**C**	**A**	**A**
Leptospira inadai (Lyme) [GenBank:AY631896]	*	*	*	*	*	*	**G**	*	*	*	*	*	**G**	*	*	*	*	*	*	*
Leptospira fainei [GenBank: AY995712]	*	*	*	*	*	*	**G**	*	**A**	*	*	*	**G**	*	*	*	*	*	*	*
Leptospira borgpetersenii [GenBank: FJ154586]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira santarosai [GenBank:FJ154589]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira kirschneri [GenBank:DQ991477]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira weilii [GenBank:U12677]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira Noguchi [GenBank:AY631886]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L. meyeri (Ranarum) [GenBank:Z21648]	*	*	*	?	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira interrogans [GenBank: FJ812169]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira meyeri (Semaranga) [GenBank:FJ154599]	*	*	*	*	*	*	*	*	**A**	*	*	*	?	*	*	*	*	*	*	
Leptospira inadai (*Aguaruna*) [GenBank:AY631891]	*	*	*	*	*	*	**G**	*	*	*	*	*	**G**	*	*	*	*	*	*	*

d) Primer D 3' 5'

Primer D	**T**	**A**	**C**	**G**	**G**	**G**	**A**	**G**	**G**	**C**	**A**	**G**	**C**	**A**	**G**	**T**	**T**	**A**	**A**	**G**

Leptospira inadai (Lyme) [GenBank:AY631896]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira fainei [GenBank: AY995712]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira borgpetersenii [GenBank: FJ154586]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira santarosai [GenBank:FJ154589]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira kirschneri [GenBank:DQ991477]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira weilii [GenBank:U12677]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira Noguchi [GenBank:AY631886]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
L. meyeri (Ranarum) [GenBank:Z21648]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira interrogans [GenBank: FJ812169]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira meyeri (Semaranga) [GenBank:FJ154599]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Leptospira inadai (*Aguaruna*) [GenBank:AY631891]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure 2. Phylogenetic analysis of DNA sequences of three amplicons obtained from cattle urine.



Amplicons were sequenced and DNA sequences were subjected to Maximum Parsimony analysis, numbers correspond to bootstrap values. Sequences obtained from urine samples are Cm 1 (submission number 1374057), Cm 3(submission number 1374049), and SF 15 (submission number 1374060). Sequences of *L. .borgpetersenii* [GenBank:FJ154600], *L. inadai* [GenBank:AY631896], and *Leptonema illini* [GenBank:AY714984].

TABLES

Table **1**. Leptospirosis positivity using PCR primers A/B in Cattle Urine.

	Bovine Species and Type of Sample

	B. indicus Bladder	*B. taurus* Bladder	*B. taurus* Urination
Number of samples	220	58	269
Number positive samples	9	5	60
Percentage of positivity	4.1	8.6	22.3

Urine samples were obtained either by induced urination from cattle in farms located close to Quito or from bladders at the local slaughterhouse.

Table **2**. Performance of primers A/B, and C/D.

Type of sample	**Number of samples**	**Positive A/B**	**Positive C/D**	**Positive A/B, C/D**
Bladder	58	5	9	5
Urination	57	57	32	32
Total	115	62	41	37

A subset of 58 urine samples collected from *Bos taurus* at the slaughterhouse in Quito, as well as 57 urination samples from *Bos taurus* dairy cattle, that were tested with the two sets of primers