Sokoto Journal of Veterinary Sciences, Volume 23 (Number 2). June, 2025

RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v23i2.2

Stephen *et al.*/*Sokoto Journal of Veterinary Sciences*, **23**(2): 77 - 84.

Molecular detection of *Leptospira interrogans* serovar Hardjo from cattle and goats in Maiduguri, Nigeria

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Publication History:
Received: 06-08-2024
Revised: 26-03-2025
Accepted: 06-04-2025

Abstract

Leptospirosis is an important zoonotic disease of livestock with a worldwide distribution. The prevalence of Leptospirosis is often associated with infertility, abortion, retention of fetal membrane, decreased milk production, and early neonatal mortality in livestock. *Leptospira interrogans* serovars Pomona, Canicola, Bratislava, Grippotyphosa, Hardjo, and Icterohemorrhagiae are the most common causes of infection in various hosts. This study aimed to determine the genetic characteristics of *Leptospira interrogans* serovar Hardjo associated with reproductive losses in cattle and goats in Maiduguri, Nigeria. A total of 376 blood samples (188 each from cattle and goats) were collected, and 30 sera consisting of 12 ELISA-positive and 18 negative sera were subjected to phenol-chloroform DNA extraction and conventional PCR amplification, targeting the LipL32 structural gene of *L. interrogans*. All seropositive samples tested negative for PCR, while one seronegative sample tested positive for PCR. The PCR-positive sample yielded a 423bp amplicon size, and sequence analysis of the positive DNA showed 98.4% to 99.6% similarity with other *Leptospira* Hardjo sequences deposited in the GenBank. This study is, to the best of our knowledge, the first report on the genetic characteristics of the field serovar of *Leptospira interrogans* serovar Hardjo circulating in cattle and goats with reproductive problems in northeastern Nigeria. Further studies are required to elucidate the full spectrum of the molecular epidemiology of *Leptospira* Hardjo in Nigeria.

Keywords: *Leptospira interrogans* serovar Hardjo, Molecular detection, PCR, Ruminants, Nigeria

Introduction

Leptospirosis is a globally recognised zoonotic disease caused by a host-dependent spirochete of the genus *Leptospira*, order *Spirochaetales*, and family *Leptospiraecea* (Faine, 1994). *Leptospira* can be found throughout the tropical and temperate regions and is recognised as the most widespread zoonosis worldwide (Budihal & Perwez, 2014). Leptospira organisms include the pathogenic *Leptospira interrogans* and the saprophytic Leptospira biflexa groups (Benacer *et al.,* 2013). Rodents are the major reservoir hosts for pathogenic Leptospires, while the saprophytic or environmental species are naturally present in water and soil (Mohammed *et al.,* 2011). The disease is due to pathogenic *L. interrogans* serovar Pomona, Canicola, Bratislava, Grippotyphosa, Hardjo and Icterohemorrhagiae (Tulu, 2020). A recent study in Uganda has found *L. interrogans* Australis and *L. kirschner* Grippotyphosa in pigs and *L. borgpetersenii* Tarassovi in small ruminants (Alinaitwe *et al.,* 2024). Transmission of *Leptospira* results from direct or indirect exposure to the urine or other body fluids of infected animals (Shafighi *et al.,* 2014), and the organisms enter the body through the mucous membrane of the eyes, nose, vagina, or broken skin (Thayaparan *et al.,* 2013).

Leptospira interrogans serovar Hardjo type Hardjobovis is responsible for bovine leptospirosis, which causes acute or chronic persistent infection of the kidneys and female reproductive tract (Shafighi *et al.,* 2014). The disease, which manifests acute, subacute, or chronic signs, is a major cause of infertility and poor reproductive performance in farm animals (Walker, 2005). The age, gender, herd size, and the presence of other domestic animals such as sheep and dogs on farms are associated with an increased risk of exposure to leptospirosis in cows (Yupiana *et al.,* 2020).

The definitive diagnosis for identifying Leptospires from clinical specimens is by culture and isolation (Bharti *et al.,* 2003). However, culture is difficult to propagate and requires several weeks of incubation, as well as specialised culture (Weyant *et al.,* 1999). The popular microscopic agglutination test (MAT) is also a subjective technique that is expensive, difficult to implement and requires expertise to analyse the test results (Faine *et al.,* 1999). Serological methods such as the enzyme-linked immunosorbent assay (ELISA) have been used for the routine diagnosis of Leptospires, and several IgG and IgM antibody/antigen capture kits are commercially available (Levett, 2001). The polymerase chain reaction (PCR) based assays are also available for the detection and characterisation of pathogenic *Leptospira*, enabling superior sensitivity and early diagnosis (Ahmed *et al.,* 2012). Despite the emergence and widespread applications of these sensitive and specific PCR assays for the rapid detection and characterisation of pathogenic leptospires worldwide, only one study has reported the application of PCR in the diagnosis of leptospirosis among cattle in Nigeria (Udechukwu *et al.,* 2023; Stephen *et al.,* 2024). In this study, a conventional PCR targeting the LipL32 structural gene of *L. interrogans* in ELISA-positive sera of cattle and goats with reproductive problems in Maiduguri, Nigeria, was carried out.

Materials and Methods

Study area and study population

This study was conducted in smallholder farms in Maiduguri, the capital and largest urban centre in Borno state, Nigeria. The study area lies within the semi-arid zone of northeastern Nigeria between latitudes 10.2°N and 13.4°N and longitudes 9.8°E and 14.4°E. The climate of Maiduguri is hot and dry for most of the year, with a short rainy season from June to October, followed by a long period of dry season. The mean temperatures range from 13-41℃, and the annual rainfall is between 9-198mm with a sunshine of 7-9 hours daily and a relative humidity between 19-78% (Musa & Pindar, 2005). The mainstay of economic activity in Maiduguri and its environs is arable crop farming, pastoral nomadism, and fishing. Most of the livestock in the state are herded by smallholder pastoralists in small groups between 2-50 in the outskirts of town. The predominant system of production is semi-intensive, where the animals are left to graze during the day and provided with limited housing and some feed supplementation during the night.

Sample size and sample collection

The sample size for this study was estimated according to Thrusfield (2005) based on an expected prevalence of 13.1% for goats (Agunloye, 2002) and 8.4% for cattle (Ngbede, 2013), 95% CI, and 5% desired absolute precision. Thus, $n = Z_{1-\alpha/2}^{2}. p (1 - p)/d^{2}$ = 175 goats and 118 cattle. However, 188 goats and 188 cattle were sampled to increase precision and minimise sampling error. Samples were randomly collected from a cross-section of cattle herds and goat flocks with reports of reproductive problems. Each animal was gently restrained to collect 5ml of whole blood from the jugular vein into plain vacutainer tubes. The samples were further processed by centrifugation at 1107×g for 5 minutes at 25℃ and kept frozen at -20℃ until used for the ELISA and PCR.

Enzyme-linked immunosorbent assay screening

A commercial ELISA KIT (Leptospira hardjo IgG/IgM test System, Biopanda Reagents, United Kingdom was used in this study. A serum IgG/IgM antibody-capture

ELISA test kit with a sensitivity of 96.9%, specificity of 90.4%, positive and negative predictive values of 88.7% and 97.4%, respectively, was used to initially screen all the 376 samples collected from smallholder farms for the presence of Leptospira Hardjo antibodies (Biopanda Reagents Ltd, Belfast, United Kingdom) according to manufacturer's instructions (Stephen *et al.,* 2022). All 12 positive sera (n = 8 cattle and n = 4 goats) and 18 randomly selected suspected sera from the ELISA were subjected to DNA extraction, PCR amplification, and sequencing to confirm and characterise *Leptospira interrogans.*

DNA isolation and molecular detection of L. interrogans by polymerase chain reaction

DNA was extracted using the phenol-chloroform method described by Javadi *et al.* (2014). The oligonucleotide primer sequences used in this study are shown in Table 1. The total volume of the prepared master mix for the detection of *Leptospira interrogans* was 20µl consisting of a commercial "Hot Start" PCR premix (Bioneer Alameda, USA) 10 µl; others included 3 µl of nuclease free water, 1µl of forward primer, 1µl of reverse primer and 5 µl of template DNA. The reaction mix was vortexed for 5secs, loaded into the thermocycler, and run using the optimised protocol under the following conditions: pre-denaturation at 94℃ for 5 mins; denaturation at 94℃ for 30 secs; annealing at 63℃ for 30 secs; extension at 72℃ for 30 secs; and final extension at 72℃ for 5 mins (Levette *et al.,* 2005). A positive serum of a clinical sample obtained from the National Veterinary Research Institute (NVRI) was used as an internal control against the test sera. All amplification reactions were performed in a Perkin Elmer thermocycler (Perkin Elmer Cetusz, Norwalk, USA), and the products were cooled to room temperature before gel electrophoresis.

Gel electrophoresis

Agarose gel was prepared using 1.5g of Agarose powder poured into a beaker containing 100µl of Tris Acetate EDTA (TAE) buffer. To visualise the amplified PCR products, eight microlitres (8µl) of amplified product was used for gel electrophoresis. These were loaded into wells on the prepared agarose gel after mixing with loading dye. Electrophoresis was performed in TAE buffer at 100 volts for 40 mins. After this, the gel was visualised in a gel imaging system (Gel Doc system Bio-Rad, U.S.A) using UV light and the image was then captured using the monitor-mounted camera attached to the Gel Doc System. The desired band of the right amplicon size was then observed and recorded. The desired band size of LipL32 for the amplification of *Leptospira interrogans* is 423bp adopted from Levett *et al.* (2005).

Sequencing reaction

The sequencing reaction mix was prepared in a 0.2ml thin-wall tube. All reagents were kept on ice while preparing the sequencing reactions, which consisted of, dH2O (7µl), DNA template (3µl), forward primer (1µl), reverse primer (1µl) and Quick start master mix (8µl), making a total of 20µl. Consolidated liquid at the bottom of the tube was briefly centrifuged before amplification. This reaction mixture was then loaded onto the cycler and run under the following conditions: 96℃ for 20 secs, denaturation at 50℃ for 20 secs, annealing at 60℃ for 4 mins, extension for 30 cycles and held at 4℃. The re-suspended sample was transferred into the appropriate well of the sample plate. Each of the re-suspended samples was overlaid with one drop of light mineral oil, and the reaction was run in a sequencer (Beckman Coulter Analysis System, Brea, California, U.S.A) using the standard protocol described by Haake *et al.* (2004).

Phylogenic analysis

Nucleotide sequences were edited using BioEdit software (v7.2.5). Subsequently, these sequences were aligned with those of reference strains and retrieved from GenBank. The aligned sequences were used in constructing a phylogenetic tree based on the Neighbour-Joining method using MEGA v11 software. Evolutionary distances were computed using the Kimura 2-Parameter model, with results expressed as base substitutions per site.

Results

Detection of Leptospira interrogans

The results of the molecular detection of *L. interrogans* using conventional PCR showed that only one sample from cattle was positive for the organism (Plate I), while all the goat sera tested negative (Plate II).

Phylogenetic analyses, Amino acid substitutions and Sequence identity matrix

Leptospira species sequences from this study (Jashila) formed a major cluster and a sub-cluster with Leptospira species sequences from Spain (MH375336) and pathogenic Leptospira species from Corsica, France (MN527296), respectively (Figure 1), with more than 94% similarity in each case (Table 2). Furthermore, amino acid similarity showed that S, A, E, M, and K at positions 1, 3, 4, 5, and 26, respectively, from this study had been substituted with P, S, L, K, and E amino acids in the other sequences from GenBank (Figure 2).

Discussion

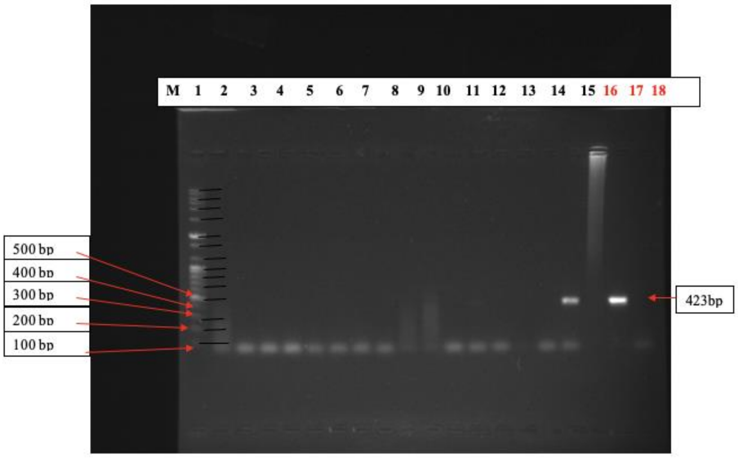


Plate I: Gel electrophoresis of Amplified PCR Product for Cattle. Lanes 1-13 are test samples, Lane 14 is a positive product, Lane 15 is a negative sample, Lanes 16 is a positive control and Lane M is a 100bp molecular marker

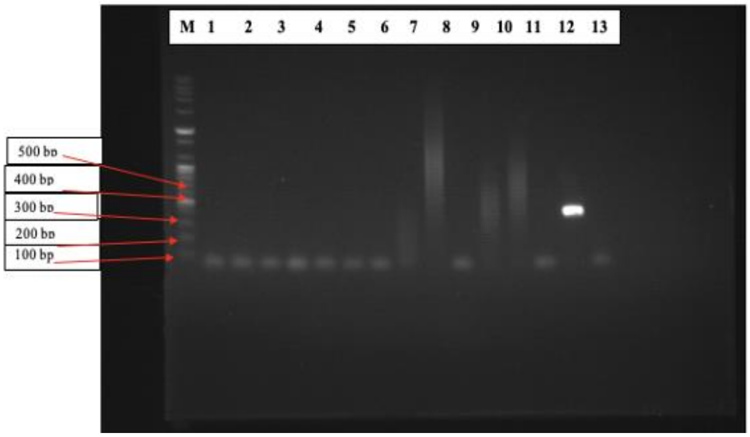


Plate II: Gel electrophoresis of amplified PCR Product for Goat. Lanes 1-11 are negative samples, Lane 12 is a positive control, Lane 13 is a negative sample, Lane M= 100bp molecular weight marker

Leptospirosis is an emerging zoonosis and neglected tropical disease of global importance causing reproductive problems in farm animals and human disease in people with occupational exposure (Skykes *et al.,* 2022). Despite the emergence and widespread use of sensitive and specific molecular tests for the rapid detection and characterization of pathogenic leptospires worldwide (Rayahu *et al*., 2021), there remains a lack of information about the organism in the study area. Therefore, in this study, both Leptospira interrogans seropositive and seronegative samples were subjected to conventional PCR and Sanger sequencing. The PCR results showed that Leptospira interrogans was not detected in any of the ELISA-positive caprine and bovine sera; however, one ELISA-negative bovine serum tested positive. The failure to detect genetic material in sera may be associated with host cellular immunity mediated by macrophages and humoral antibodies, which ingest the leptospires (Gangani *et al.,* 2023). These anti leptospires may be non-specifically directed against antigens to all leptospires or specifically directed to a particular serovar or serogroups. Thus, the failure to detect *Leptospiral* DNA in ELISA-positive sera may be due to an active immune response effectively clearing the organisms in seropositive reactors with long-standing exposures (Ricardo *et al.,* 2008). However, it was interesting to find that one ELISA-negative serum sample tested positive for *Leptospira* Hardjo DNA by conventional PCR. This case might be actively incubating the pathogen in the early stage of the infection before seroconversion (Panteleo *et al.,* 2022). This finding is consistent with Siphran *et al*. (2011), who reported the detection by PCR of pathogenic Leptospira species in blood from patients with culture-negative leptospirosis, confirming the superior sensitivity of PCR in the early diagnosis of infections.

This study is the first documented report on the molecular detection and characterization of *Leptospira* Hardjo in northeastern Nigeria. There are no characteristics of the bovine-associated *Leptospira interrogans* in northeastern Nigeria. The result of the present study differs from the findings of Lekhal *et al.* (2022), who identified and isolated Leptospira data on the molecular interrogans in *Rattus rattus* with 39 positive samples out of 52 samples. The study provides valuable insights into the genetic relationships and sequence variations among *Leptospira* species, highlighting notable clustering characteristics and high genetic similarity among strains. Sequence analysis performed on the positive PCR product further showed that the candidate DNA of the *L. interrogans* detected in the present study was 98.6 to 99.6% similar to GenBank sequences from other geographic locations globally. The *Leptospira* sequences in our study clustered with sequences from Spain and a sub-cluster with pathogenic *Leptospira* species from Corsica, France, suggesting that certain genetic characteristics may be associated with virulence. This observation aligns with research on the evolutionary history of *Leptospira*, which shows that pathogenic species emerged from environmental saprophytes and have adapted to various hosts and environments (Giraud-Gatineau *et al.,* 2024). The observed clustering patterns can provide insights into the evolutionary history of *Leptospira*, similar to studies on *Leptospira santarosai*, which revealed high genetic diversity within this species, with clonal groups strongly associated with geographical areas (Chinchilla *et al.,* 2023). This finding highlights the importance of phylogenetic analysis in tracing the spread and evolution of pathogenic strains. Both clusters exhibited over 94% sequence similarity, indicating close genetic relationships among these *Leptospira* sequences. This level of similarity is consistent with studies using MLST schemes, which resolve isolates into distinct sequence types with nucleotide diversity ranging from 2.3% to 3.6% (Ahmed *et al.,* 2011). High-resolution typing methods like PFGE and VNTR markers further support the idea that closely related strains can be differentiated based on genetic differences (Zilber *et al.,* 2014; Naze *et al.,* 2015).

Table 1: Oligonucleotide primer sequences

Primers	**Oligonucleotide sequence**	**Amplicon size**
LipL32/270F	(5´-CGCTGAAATGGAGGTCGTATGATT-3')	270bp
LipL32/692R	(5´-CCAACAGATGCAACG AAGATCCTTT-3´)	692bp

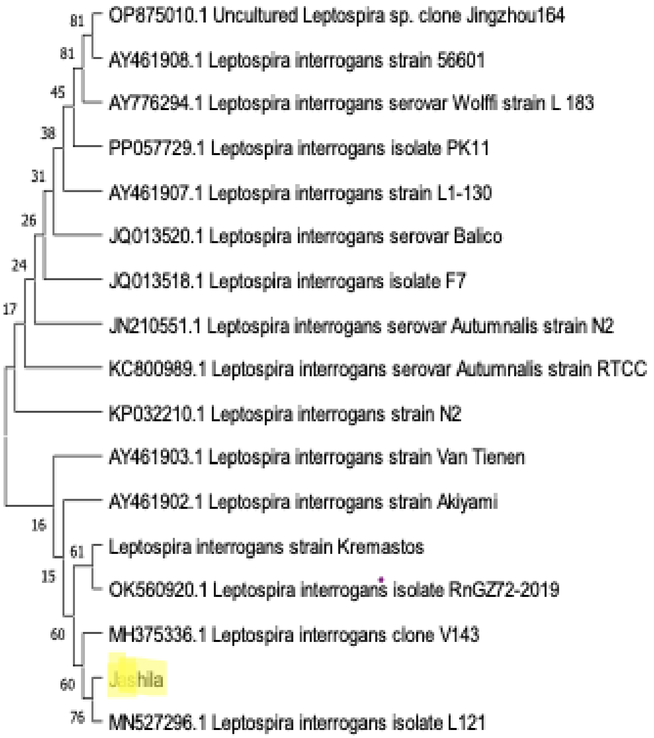


Figure 1: Phylogenetic analysis using the Neighbour-Joining method between sequences from this study (coloured yellow) and other sequences of Leptospira retrieved from the GenBank

The study identified specific amino acid substitutions at key positions: S→P1, A→S3, E→L4, M→K5, and K→E26. These substitutions indicate genetic variations that might influence the characteristics of different *Leptospira* strains. Amino acid substitutions can alter the function of virulence factors, such as the virulence-modifying (VM) proteins found in pathogenic *Leptospira*, which are known to play a role in disease pathogenesis by acting as carbohydrate-binding cytotoxins (Hamond *et al.,* 2022). Changes in these proteins could affect their ability to interact with host cells or evade the immune system. For example, changes in the ricin B-like lectin domains of VM proteins could influence their carbohydrate-binding capabilities (Chaurasia *et al.,* 2022).

Such substitutions might reflect evolutionary adaptations to different hosts or environments, potentially influencing the pathogenicity or transmission efficiency of *Leptospira* strains.

Pathogenic *Leptospira* have evolved unique genetic features, such as sialic acid biosynthesis and pathogen-specific porphyrin metabolism, which enhance their ability to infect mammals (Fouts *et al.,* 2016).

The findings align with previous research demonstrating the genetic diversity within Pathogenic *Leptospira* species. Whole genome sequencing studies have shown significant variation in genome sizes and genetic content across different *Leptospira* species, which supports the observed sequence and amino acid variations in this study (Xu *et al.,* 2016). Research on the genetic diversity of *Leptospira* strains circulating in humans and animals highlights the genus's subdivision into 68 genomic species, including both pathogenic and saprophytic types (Garcia-Lopez *et al.,* 2023). This diversity underscores the potential for sequence and amino acid variations, supporting the observed genetic

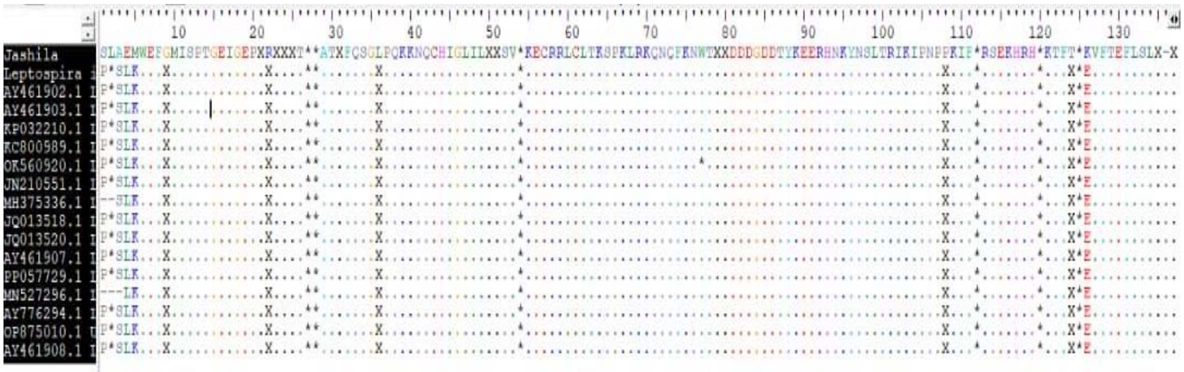
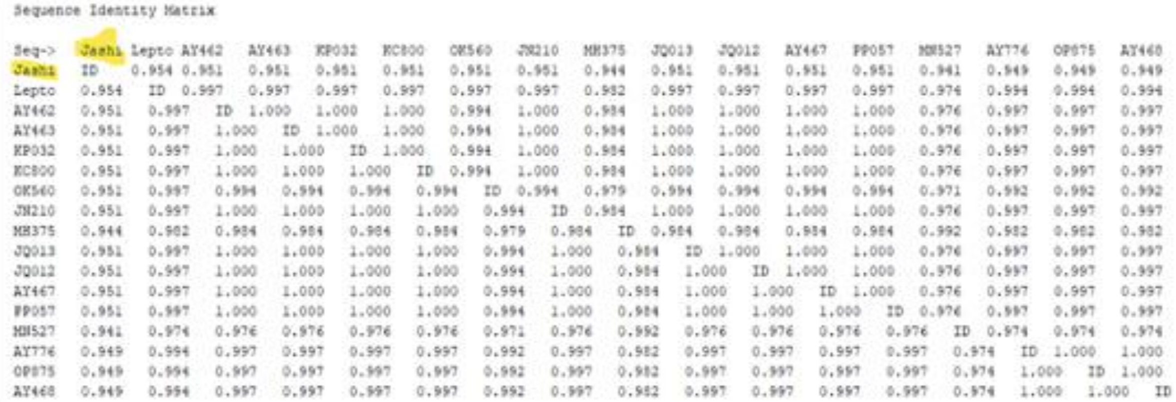


Figure 2: Amino Acid Substitutions of sequences from this study (coloured yellow) and other sequences from other parts of the world

Table 2: Percentage similarity between sequences from this study (coloured yellow)and sequences retrieved from Genbank



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Sequence Identity Matrix

Seq->  Jashi Lepto AY462  AY463  KP032  KC800  OK560  JN210  MH375  JQ013  JQ012  AY467  PP057  MN527  AY776  OP875  AY468
Jashi   ID   0.954 0.951  0.951  0.951  0.951  0.951  0.951  0.944  0.951  0.951  0.951  0.951  0.941  0.949  0.949  0.949
Lepto  0.954  ID   0.997  0.997  0.997  0.997  0.997  0.997  0.982  0.997  0.997  0.997  0.997  0.974  0.994  0.994  0.994
AY462  0.951 0.997  ID    1.000  1.000  1.000  0.994  1.000  0.984  1.000  1.000  1.000  1.000  0.976  0.997  0.997  0.997
AY463  0.951 0.997 1.000   ID    1.000  1.000  0.994  1.000  0.984  1.000  1.000  1.000  1.000  0.976  0.997  0.997  0.997
KP032  0.951 0.997 1.000  1.000   ID    1.000  0.994  1.000  0.984  1.000  1.000  1.000  1.000  0.976  0.997  0.997  0.997
KC800  0.951 0.997 1.000  1.000  1.000   ID    0.994  1.000  0.984  1.000  1.000  1.000  1.000  0.976  0.997  0.997  0.997
OK560  0.951 0.997 0.994  0.994  0.994  0.994   ID    0.994  0.979  0.994  0.994  0.994  0.994  0.971  0.992  0.992  0.992
JN210  0.951 0.997 1.000  1.000  1.000  1.000  0.994   ID    0.984  1.000  1.000  1.000  1.000  0.976  0.997  0.997  0.997
MH375  0.944 0.982 0.984  0.984  0.984  0.984  0.979  0.984   ID    0.984  0.984  0.984  0.984  0.992  0.982  0.982  0.982
JQ013  0.951 0.997 1.000  1.000  1.000  1.000  0.994  1.000  0.984   ID    1.000  1.000  1.000  0.976  0.997  0.997  0.997
JQ012  0.951 0.997 1.000  1.000  1.000  1.000  0.994  1.000  0.984  1.000   ID    1.000  1.000  0.976  0.997  0.997  0.997
AY467  0.951 0.997 1.000  1.000  1.000  1.000  0.994  1.000  0.984  1.000  1.000   ID    1.000  0.976  0.997  0.997  0.997
PP057  0.951 0.997 1.000  1.000  1.000  1.000  0.994  1.000  0.984  1.000  1.000  1.000   ID    0.976  0.997  0.997  0.997
MN527  0.941 0.974 0.976  0.976  0.976  0.976  0.971  0.976  0.992  0.976  0.976  0.976  0.976   ID    0.974  0.974  0.974
AY776  0.949 0.994 0.997  0.997  0.997  0.997  0.992  0.997  0.982  0.997  0.997  0.997  0.997  0.974   ID    1.000  1.000
OP875  0.949 0.994 0.997  0.997  0.997  0.997  0.992  0.997  0.982  0.997  0.997  0.997  0.997  0.974  1.000   ID    1.000
AY468  0.949 0.994 0.997  0.997  0.997  0.997  0.992  0.997  0.982  0.997  0.997  0.997  0.997  0.974  1.000  1.000   ID
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differences among *Leptospira* strains.

This information is needed to facilitate tracking of the point of entry and distribution of the clonal strain or serovars of this pathogen circulating in livestock in the country, which could be useful in the planning of control measures against Leptospirosis in Nigeria. Certainly, this should be the direction of future studies in this area. Further studies should be conducted to elucidate the molecular epidemiology of *Leptospira interrogans* in the wider livestock-producing areas of this country.

Acknowledgement

The authors acknowledge the contributions of colleagues in the Department of Theriogenology, Faculty of Veterinary Medicine, University of Maiduguri. We are also grateful to our collaborators at the Department of Immunology, University of Maiduguri Teaching Hospital and DNA Labs Kaduna, for their support in the laboratory aspect of this work.

Funding

No funding was received.

Conflict of Interest

The authors declare that there is no conflict of interest.

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