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Molecular characterisation of *Leptospira* strains in Pakistan

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Abstract

Introduction: Leptospirosis affects a wide range of mammals, humans, and even a few poikilothermic animal species. In Pakistan, serological studies of equine leptospirosis have reported a prevalence of over 40%, but no study has ever been conducted towards molecular detection of *Leptospira* in horses. **Material and Methods:** Blood samples from 128 horses were screened using ELISA and 41 positive samples were examined for the presence of leptospiral DNA using specific primers for 16S rRNA gene. **Results:** Out of 41 tested samples, 20 samples were found to be PCR-positive, revealing a fragment of 306 bp after gel electrophoresis. Sequencing and phylogenetic analysis of positive samples revealed circulation of pathogenic *Leptospira* spp. in Pakistani horses. No evidence of circulation of intermediate species was found in this study. **Conclusion:** This study reports the first molecular evidence of equine leptospirosis in Pakistan and lays ground for further research in this area. It also confirms the efficiency of 16S rRNA for the diagnosis of equine leptospirosis.

Keywords: horses, *Leptospira interrogans*, 16S rRNA, Pakistan.

Introduction

Leptospirosis is an infectious disease caused by spirochaete in the genus *Leptospira* along with its more than 260 serovars (24), infecting a variety of domestic and wild animals (1). It has also been reported in a few poikilothermic vertebrates such as amphibians and reptiles (11). Leptospirosis is one of the six diseases enlisted by OIE with reference to "animal diseases and climate change" (19). The disease is most commonly found in rural and urban areas of tropical and subtropical parts of the world (30). It spreads more readily in tropical regions as *Leptospira* can survive longer in warm and humid environment. Hence, there are areas in Southeast Asia, China, South and Central America, and Africa where leptospirosis is endemic (25).

Leptospirosis usually evolves as an acute or chronic disease, affecting individual animals or animal groups. In horses, leptospirosis is displayed through equine recurrent uveitis and reproductive disorders including the third trimester abortion. Most infections are asymptomatic (9). Early and accurate diagnosis is essential to cure leptospirosis (10). The serological methods are most commonly used to diagnose leptospirosis (13). The microscopic agglutination test (MAT) and ELISA have pivotal role in veterinary diagnosis (16, 26). However, serological testing is complicated because of the high degree of cross-reactivity between various *Leptospira* serovars (17). Recently, molecular techniques such as conventional and real-time PCR are recognised as specific and sensitive tests for the rapid detection of infection during early stages of the disease and often negate the need for isolation and culture of the infecting organism for a confirmatory result. These techniques can be performed reliably on various templates, including blood, urine, and kidney tissues (12). Rapid and accurate results using molecular diagnostic techniques are now replacing serological tests in areas where leptospirosis is endemic (17).

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In Pakistan, serological studies on equine leptospirosis showed a prevalence of up to 44% in some areas of the country, which is quite alarming (21). All previous studies reporting *Leptospira* in Pakistan were based on serological testing (2, 20). However, no attempts had ever been made at molecular detection of the organism in any mammalian host. Therefore, This fact encouraged us to diagnose equine leptospirosis using molecular techniques for the first time in Pakistan. Consequently, the presence of leptospiral DNA in horses was confirmed.

Material and Methods

Sample collection. A total of 128 blood samples were collected from horses of rural and urban areas of Punjab, Pakistan. The blood was aseptically collected by jugular vein puncture into EDTA-coated vacutainers (BD Biosciences, USA).

All collected samples were subjected to ELISA (Horse *Leptospira* IgG ELISA Kit, MyBiosource, USA) to screen for equine leptospirosis. Out of 128 samples, 41 samples were ELISA positive and consequently examined for the presence of leptospiral DNA by PCR.

Genomic DNA extraction. Genomic DNA was extracted from the blood samples using PureLink® Genomic DNA minikit (Thermo Fisher Scientific Inc. USA) according to the manufacturer's instructions. Concentration of extracted DNA was measured using Nanodrop-1000 spectrophotometer (Thermal Scientific, USA).

PCR amplification of *Leptospira* 16S rRNA gene. The amplification of *Leptospira* 16s rRNA gene was performed using primers Lp-F: 5'-GCGCGTCTTAAACATGCAAG -3' and Lp-R: 5'-CTTAACTGCTGCCTCCCGTAG-3' (7). PCR amplification was performed with 50 µL volume containing: 1 µg of template DNA, 1 µM of each forward and reverse primers, 2 mM $MgCl_2$, 200 µMdNTP, 5 µL of 10 × PCR buffer, and 1 unit of Taq DNA polymerase (Invitrogen, USA). A master cycler gradient was used with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 58°C, and extension at 72°C for 1 min, followed by a final extension for 5 min at 72°C.

Agarose gel electrophoresis. PCR product was analysed on 1% (wt/vol) agarose gel. DNA ladder of 1Kb was used as a molecular marker. The expected size of amplicons was 306 bp.

Sequencing and phylogenetic analysis of 16S rRNA gene. Amplified fragments were gel-purified using an EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc, USA) according to manufacturer's instructions. Sequencing of DNA fragments was performed using dideoxy chain-termination method. The 16S rRNA gene sequences of the isolates of *Leptospira* were retrieved from NCBI database. Phylogenetic tree was constructed based on partial nucleotide sequence of 16S rRNA gene using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (www.megasoftware.net). The tree topologies were evaluated using bootstrap test of phylogeny with maximum likelihood obtained after 1000 replicates of the dataset.

Results

Out of 41 tested samples, 20 (30.76%) samples were found to have DNA of *Leptospira*. Primers designed for leptospiral 16S rRNA amplified 265 bp fragment, and was visualised by gel electrophoresis (Fig. 1). Blast sequence analysis of the sequences recorded showed 100% resemblance with *Leptospira interrogans* serovar Icterohaemorrhagiae, and serovar Australis, revealing the circulation of pathogenic *Leptospira* in Pakistan. Phylogenetic tree (Fig. 2) revealed that *L. interrogans* detected in our study was closely related to sequences reported from India, a country neighbouring to Pakistan. The dendrogram shows that the samples studied in this experiment and already reported sequences of intermediate species lie in different clades, providing no evidence of circulation of intermediate *Leptospira* species in Pakistan.

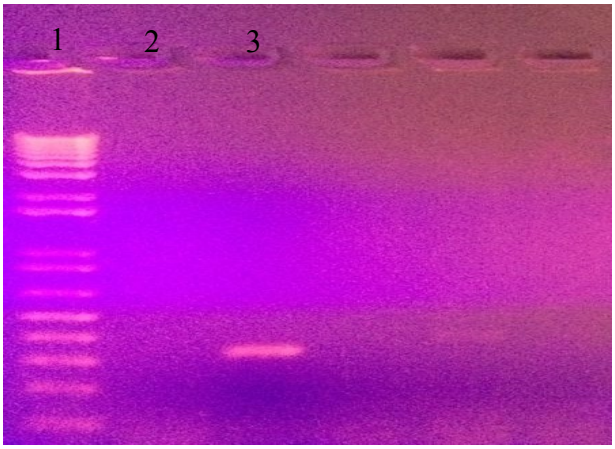

Fig 1. Agarose gel electrophoresis of PCR analysis products for 16S rRNA (pathogenic *Leptospira*, 306 bp). Line 1: DNA ladder (1Kb), Line 2: negative sample, Line 3: Positive sample (306 bp)

Discussion

Leptospirosis is the most widespread zoonosis in the world with higher prevalence in tropical countries. Its clinical signs are nonspecific, making its diagnosis a challenge (29). Despite high rates of seropositivity among animals in Pakistan (2, 20), to date, there have been no data on the molecular studies of leptospirosis in horses or in any mammalian host. This investigation reports the first molecular evidence of equine leptospirosis in Pakistan, where previous serological reports have revealed a prevalence of over 40% (21).

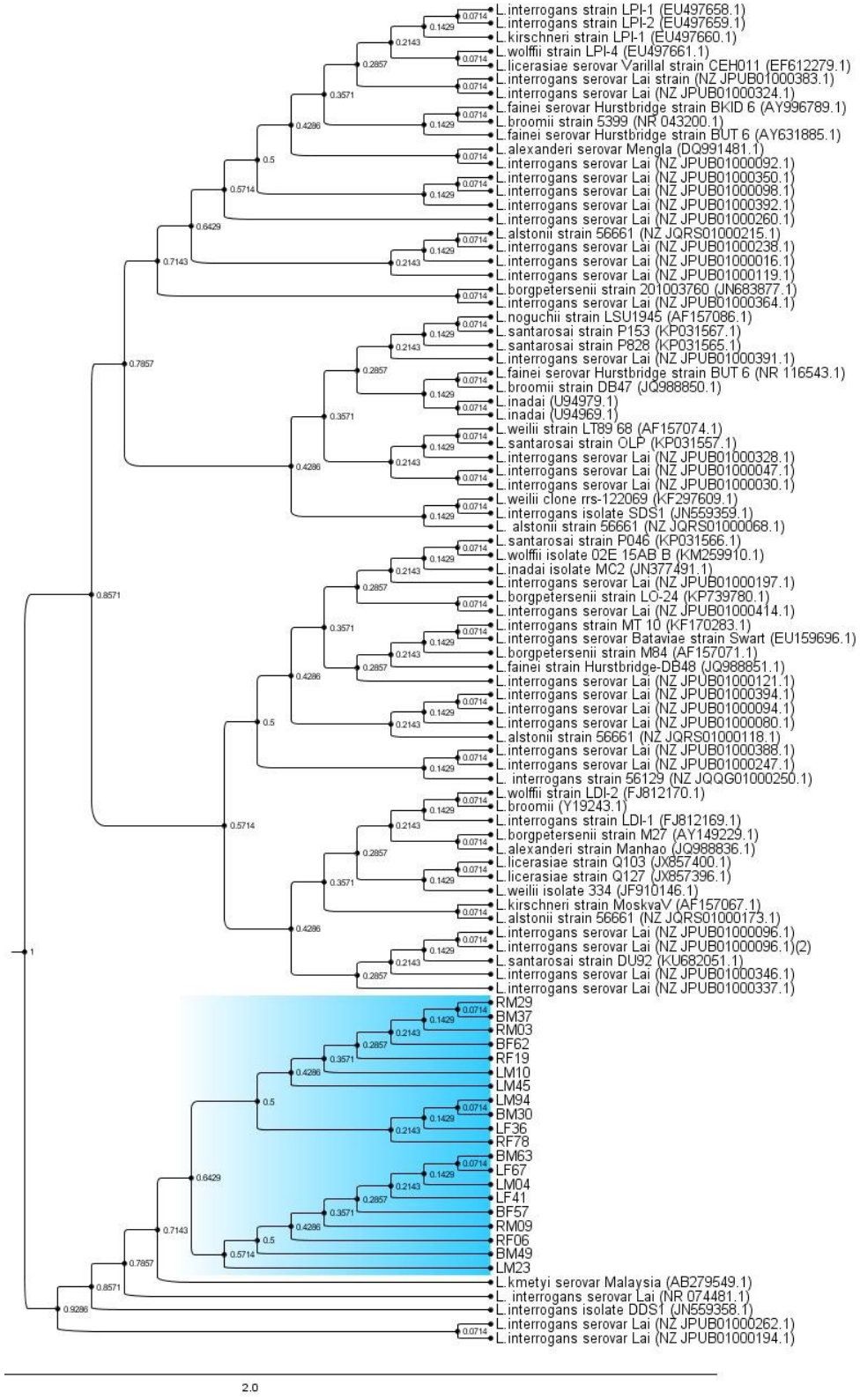

Fig. 2. Maximum likelihood phylogenetic tree based on the nucleotide sequence of *Leptospira* spp. 16S rRNA in horses. Dendrogram was constructed with 1000 replication using MEGA 6.0 software. The sequences determined in this study are highlighted. GenBank accession numbers of already reported strains are shown in parenthesis

The aim of this investigation was to detect the presence of leptospiral DNA in horse blood samples and to analyse genetic diversity of the *Leptospira* in the country. Conventional diagnostic tests, such as MAT, are mostly serological and, as such, confirm the disease best at a late acute phase, when antibiotic treatment is already less effective (28). Diagnosis of leptospirosis through culture is not practical in diagnostic laboratories because of the complexity of reagents, several weeks of growth time, and contamination

problems in culture media (8). All these challenges drew our attention towards the use of molecular techniques for the diagnosis of equine leptospirosis.

The purification, sequencing and phylogenetic analysis of the ribosomal 16S gene amplicons revealed that the species involved in infection was *Leptospira interrogans* (Fig. 2). This confirms that the primer set for standard PCR was specific to pathogenic *Leptospira* spp. The 16S rRNA is the most common molecular marker for the detection of *Leptospira* (23), because of its low variability and highly conserved region (15). PCR with the use of these primers is a simple and specific tool for identification of the organisms from different species. Several researchers used 16S rRNA for identification of pathogenic leptospirae (18).

Molecular detection rates were lower in this study as compared to previously reported serological studies in animals (2, 20) because the present study explored blood samples for the extraction of leptospiral DNA, where bacteria are found only after 3-10 d exposure. This septicaemic phase is followed by an immune phase (4-30 d) which is characterised by an increased titre of antibodies correlated with the elimination of bacteria from blood (5, 17, 18).

Prevalence of leptospiral serovars varies depending on particular geographical regions (4). Titres of various serovars have been reported in horses, but Icterohaemorrhagiae, Bratislava, Copenhageni, and Pomona are among the most prevalent serovars (27). Pakistan is a tropical country (22) and in tropical regions serogroup Icterohaemorrhagiae has been reported to be the most frequent infection (3). Blast sequence analysis performed in this study showed 100% resemblance with *L.* Icterohaemorrhagiae, and *L.* Australis, revealing the circulation of pathogenic *Leptospira* in Pakistan. Pakistan is bordered by India, Iran, and China and sero-epidemiological studies in these countries used *L. interrogans* as an antigen for the detection of MAT antibodies, confirming the prevalence of this species in these regions (14). Moreover, *L. interrogans* has been reported to be prevalent in 60% of leptospirosis cases in china (31). The mapping results of phylogenetic analysis revealed that *L. interrogans* detected in our study is closely related to *L. interrogans* serovar Icterohaemorrhagiae, Automunalis, and Copenhangeni which have also been detected in Iran (6). The phylogenetic tree (Fig. 2) reveals that *L. interrogans* from our study was closely related to the sequences reported from India. Moreover, it reveals that no intermediate species of *Leptospira* were found in this study. This analysis demonstrates that *L. interrogans* is the prevalent pathogenic species in this region and the use of 16S rRNA sequences can be the basis for leptospiral detection in Pakistan.

Being the first ground-breaking study on molecular detection of equine leptospirosis in Pakistan, our research has opened a gateway to future investigation in this area. A further study involving human and environmental samples to explore molecular epidemiology will help to design effective strategies for leptospirosis prevention.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: Formal consent from the owners of the horses was obtained prior to the collection of blood samples. The study design was submitted to and approved by the Ethical Review Committee for the Use of Animals, UVAS, Lahore, Pakistan.

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