High prevalence of pathogenic *Leptospira* in wild and domesticated animals in an endemic area of China

Wang Yalin\(^1\), Zeng Lingbing\(^1\), Yang Hongliang\(^1,4\), Xu Jianmin\(^2\), Zhang Xiangyan\(^1\), Guo Xiaokui\(^1\), Pal Utpal\(^3\), Qin Jinhong\(^1\)*

\(^1\)Department of Medical Microbiology and Parasitology, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
\(^2\)Jiangxi Provincial Center for Disease Control and Prevention, Nanchang 330029, China
\(^3\)Department of Veterinary Medicine, University of Maryland, College Park, Maryland, 20742, USA
\(^4\)Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA 80526

**Objective:** To assess the prevalence of *Leptospira* detected in wildlife and domesticated animals in Jiangxi Province, China, in 2009. **Methods:** Urine samples from 28 buffaloes and kidney samples from 50 pigs, 50 dogs and 38 rats were collected from Fuliang and Shangrao County, Jiangxi Province, China, in October 2009. Polymerase chain reaction (PCR) and culture analyses were used to detect *Leptospira*. The cultured isolates were typed using the microscopic agglutination test (MAT).

**Results:** The results showed that rats potentially serve as the main reservoir of leptospiral infection, followed by dogs. Although 16% of rats (6/38) were positive using culture analysis, PCR analysis using the diagnostic primers G1/G2 and B64I/B64II or lipL32 showed identification as 50% and 24%, respectively, of the rat samples as positive for the presence of leptospiral DNA. **Conclusions:** PCR-based detection of leptospiral DNA in infected kidney tissues of reservoirs is more efficient when using G1/G2 primers than lipL32 primers. However, the latter primers have a potential application for detection in urine samples. The alarmingly high prevalence of leptospiral DNA in the wild rat population near human habitation underscores the utility of routine *Leptospira* surveillance, preferably using PCR methods, which are more sensitive than traditional culture-based methods.

### 1. Introduction

Leptospirosis, one of the most common and widespread zoonoses in the world, is caused by the pathogenic leptospiral species. The clinical manifestations of leptospirosis in humans and animals broadly range from flu–like episodes to dysfunction of multiple organs and sometimes death. Leptospirosis in mammals is transmitted by physical contact with infected animals or by exposure to water or soil contaminated with the urine of infected animals, whereas direct human–to–human transmission is rarely reported\(^1\). Many wild or domestic animals, mainly rodents, small marsupials, cattle, pigs and dogs, serve as reservoir hosts or carriers of leptospiral pathogens and, owing to their presence in close proximity to humans, serve as the most important sources for human infection\(^2\)–\(^4\). Infected animals may shed leptospiral pathogens via urine or other excreta intermittently or regularly for months, years or even a lifetime\(^1\). More importantly, even vaccinated animals may still shed infectious organisms in their urine\(^1\). Therefore, regular surveillance of carrier hosts for leptospiral infection and persistence is highly warranted for routine evaluations of the risk of human exposure and prevention of the disease.

China is one of the most endemic regions of global leptospirosis\(^5\). Surveillance of leptospirosis in domestic and wild animals such as buffaloes, pigs, dogs and rats has been routinely performed in many Chinese provinces\(^6\)–\(^9\). The annual infection rate in 2005 of trapped rats was...
consent was obtained from each livestock owner prior to the humanely euthanized soon after being trapped, and all contamination, the kidneys were isolated from the animal concerning experimental animals in China. Wild rats were efforts made to minimize suffering. Verbal informed County, Jiangxi Province, China, in October 2009 were harvested at mid-log-phase by centrifugation at 12,000 rpm using the cell debris, and the supernatant was subjected to further examination under a dark microscope. Leptospira species included in the antigen panel are listed in Table 1[14].

2. Materials and methods

2.1. Ethical approval

This study was reviewed and approved by the Laboratory Animal Sciences Center of the Shanghai Jiao Tong University School of Medicine and Jiangxi CDC ( Permit Number: SYXK2008–0050). The study was conducted adhering to the regulations for the administration of affairs concerning experimental animals in China. Wild rats were humanely euthanized soon after being trapped, and all efforts were made to minimize suffering. Verbal informed consent was obtained from each livestock owner prior to the sample collection.

2.2. Sample collection

A total of 164 samples collected from Fuliang and Shangrao County, Jiangxi Province, China, in October 2009 were tested in the current study. Thirty-eight wild rats, which included Apodemus agrarius and Rattus losea, were trapped by the trap–night method, with rat clips from 9 paddy fields in Fuliang County. To decrease the possibility of contamination, the kidneys were isolated from the animal’s dorsal aspect[13]. Morning urine samples from 28 buffaloes were collected from 6 paddy fields in Fuliang County, and kidney samples from 50 pigs and 50 dogs were collected from freshly slaughtered animals in 2 slaughterhouses in Shangrao County.

2.3. Reference strains and culture conditions

Fifteen prevalent pathogenic leptosomal reference strains in China are listed in Table 1. All strains were grown in Ellinghausen McCullough Johnson Harris (EMJH) liquid medium at 28 °C. The Leptospira strains were enumerated by counting the number of leptospiral cells. The cells were harvested at mid–log–phase by centrifugation at 12,000× g for 15 min at 4 °C.

2.4. Culture and isolation of Leptospira

Freshly isolated tissues (two samples from each animal) were inoculated into 5 mL of Korthoff medium with 250 μg of 5-fluorouracil. One milliliter of buffalo mid–stream urine was inoculated into 5 mL of Korthoff medium with 250 μg of 5-fluorouracil, and 10–fold serial dilutions were made in 2 additional tubes. Samples were then incubated at 28 °C and examined for spirochete growth for up to two months by examination under a dark microscope.

2.5. MAT

The isolates were typed by MAT. The procedure was performed, as detailed, with the following minor modifications[11]. Briefly, all isolates were examined and diluted, using saline, to approximately 10^7 cells/mL. The diluted cultures were then added to serially diluted standard rabbit serum (National Institute for the Control of Pharmaceutical and Biological Products, China) in 96–well flat–bottom microtiter plates and incubated at 37 °C for 2 h. The agglutination result was evaluated by dark–field microscopy at 100× magnification. Leptospira species included in the antigen panel are listed in Table 1[14].

2.6. PCR detection

Kidney samples were stored at −80 °C until use. Leptospira DNA was extracted from the kidney using the DNeasy Tissue Kit (Qiagen) according to the product manual. The urine samples were centrifuged for 5 min at 5,000 rpm to discard the cell debris, and the supernatant was subjected to further centrifugation at 12,000 rpm for 20 min to collect the pellet within 2 h of the initial collection; the pellet was stored at −20 °C until use. Leptospira DNA was extracted from the collected urine pellets by centrifugation at 12,000 rpm using the DNA Micro Kit (Qiagen). The DNA from 15 reference strains was isolated using the bacterial DNA isolation kit (Watsonbiot, China). The DNA concentration was tested by spectrophotometer.

Three primer pairs were used to detect the leptospiral DNA. Two pairs were G1/G2 and B64I/B64II, which are widely used[15]. Another primer pair was lipL32 (lipL32F: 5’–AAA CTG ATT TTG GCT AT –3’ and lipL32R: 5’–TGT TTT TCG ATT TCT CAG G–3’), which amplifies a 758 bp product. The primer pair was designed based on a sequence alignment of lipL32 sequences of Leptospira species, including 4 sequenced pathogenic leptosomal strains deposited in NCBI and 6 draft Leptospira reference strains sequenced by our research group (unpublished data). Also, the lipL32 gene sequence of Leptospira kirschneri strain MORU UT130, which G1/G2 could not amplify, was included in our primer design. The lipL32 primer pair was targeted to a specific, yet conserved region shared by the pathogenic Leptospira species above.

The limitation for PCR amplification using G1/G2 or lipL32 primers was detected using a series of diluted Leptospira DNA from 1×10^3 pg/μL to 1×10^5 pg/μL and PCR products of lipL32 and G1/G2 that were cloned into T1–vector (TranS) from 1×10^2 copies/μL to 1×10^5 copies/μL as the templates. The PCR products amplified using the lipL32 or G1/G2
primer pairs were purified and further cloned into T1-vector (TranS). The concentration of the PCR products was measured with the OD260 nm value, and then the number of copies was calculated according to its molecular weight. The dilution buffers were double distilled water (ddH2O) and the DNA from the normal buffalo urine or normal rat kidney samples, respectively.

The PCR reaction was performed by Taq DNA polymerase (Fermentas) in a buffer containing 2 mM Mg2+ using the DNA from the collected kidney and urine samples in the same amount as template. For the G1/G2 and B64I/B64II primers, the PCR conditions consisted of one cycle for 2 min at 94 °C; followed by 35 cycles of 1 min at 94 °C, 30 seconds at 55 °C, 1 min at 72 °C, with a final elongation step of 10 min at 72 °C. The PCR conditions for lipL32 are the same as described above, except that the annealing temperature and time were 50 °C and 1 minute, respectively.

2.7. Statistical analysis

Software (SAS, VS 6.12) was used to perform chi–square tests to assess significant differences between groups and to examine the concordance between culture isolation, MAT, and PCR methods using Cohen’s unweighted kappa correlation.

3. Results

3.1. Efficiency of leptospiral detection using PCR assays with G1/G2 and lipL32 primers

All 15 leptospiral reference strains, which are prevalent in China and listed in Table 1, were detected by PCR with the G1/G2 or lipL32 primer pairs. The G1/G2 or lipL32 primer pairs did not amplify saprophytic strains (data not shown).

When using the leptospiral DNA as a template, the detection limit for PCR amplification using the G1/G2 or lipL32 primers was 10^{-12} pg of leptospiral DNA in ddH2O. Accordingly, when using the cloned PCR products of G1/G2 or lipL32 as templates, the detection limits for PCR amplification using the G1/G2 or lipL32 primers were one copy of recombinant plasmid each, which is roughly equivalent to a single cell of the pathogen (Figure 1). However, when leptospiral DNA was diluted with DNA isolated from normal rat kidneys, the sensitivity of PCR-based detection using the G1/G2 or lipL32 primers was reduced to 10^{-2} pg (4/6), respectively. In PCR (lipL32)-positive samples, the sensitivity for PCR (G1/G2, B64I/B64II) was 100%.

Table 1

Leptospira strains endemic in China.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serotype</th>
<th>Strain</th>
<th>Strain number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Lai</td>
<td>Lai</td>
<td>56601</td>
</tr>
<tr>
<td>Javanica</td>
<td>javanica</td>
<td>M10</td>
<td>56602</td>
</tr>
<tr>
<td>Canicola</td>
<td>canicola</td>
<td>Lin</td>
<td>56603</td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum</td>
<td>Pishu</td>
<td>56004</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>pyrogenesis</td>
<td>4</td>
<td>56605</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>autumnalis</td>
<td>4</td>
<td>56606</td>
</tr>
<tr>
<td>Australis</td>
<td>australis</td>
<td>65–9</td>
<td>56607</td>
</tr>
<tr>
<td>Pomona</td>
<td>pomona</td>
<td>Luo</td>
<td>56608</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Linhai</td>
<td>Lin 6</td>
<td>56609</td>
</tr>
<tr>
<td>Hebolomadis</td>
<td>hebdomadis</td>
<td>P 7</td>
<td>56610</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Wolffi</td>
<td>L.183</td>
<td>56635</td>
</tr>
<tr>
<td>Mini</td>
<td>Mini</td>
<td>Nan 10</td>
<td>56655</td>
</tr>
<tr>
<td>Bataviae</td>
<td>paidjian</td>
<td>L.37</td>
<td>56612</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>tarassovi</td>
<td>65–52</td>
<td>56613</td>
</tr>
<tr>
<td>Manhao</td>
<td>cingshui</td>
<td>L.105</td>
<td>56615</td>
</tr>
</tbody>
</table>

*The strain numbers are designated according to the National Institute for Control of Pharmaceutical and Biological Products.

Table 2

Culture and PCR-based detection of leptospirosis in wild reservoirs and domesticated animals collected in Fuliang and Shangrao County, Jiangxi Province, in October 2009.

<table>
<thead>
<tr>
<th>Reservoirs</th>
<th>Leptospira carrier rate (positive number /total number) using different methods of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture</td>
</tr>
<tr>
<td>Rat (Apodemus agrarius)</td>
<td>4/23</td>
</tr>
<tr>
<td>Rat (Rattus losea)</td>
<td>2/15</td>
</tr>
<tr>
<td>Pig</td>
<td>0/50</td>
</tr>
<tr>
<td>Dog</td>
<td>0/50</td>
</tr>
<tr>
<td>Buffalo</td>
<td>0/28</td>
</tr>
</tbody>
</table>

1 In culture–positive samples, the sensitivity for PCR using G1/G2, B64I/B64II and PCR using lipL32 was 100 % and 67 % (4/6), respectively. 2 In PCR (lipL32)–positive samples, the sensitivity for PCR (G1/G2, B64I/B64II) was 100%.
were positive. Serotyping attempts using MAT identified only 16% (6/38) of rat kidney samples were positive, while none of the samples collected from pigs, dogs or buffaloes could be detected by the three methods indicated that Cohen’s kappa coefficient of agreement between PCR and culture was 0.41, while PCR using the lipL32 or G1G2 and B64I/B64II primers was 0.61, indicating a moderate agreement between these methods. However, the carrier rate of *Leptospira* in wild rats as determined by PCR with the G1/G2 and B64I/B64II primers was significantly higher than the rate determined using the lipL32 primer pair (P<0.01).

4. Discussion

The samples tested in this study were collected in October 2009 in Fuliang and Shangrao County, Jiangxi Province, China. The rule to select where and when to collect the samples is based on data collected by the Jiangxi CDC, which has studied the prevalence of pathogenic *Leptospira* from 2006 to 2009. Because Jiangxi Province is one of the most leptospirosis–infected areas in China, four surveillance spots are located there. The Jiangxi CDC has investigated the infection rates of leptospirosis twice a year in wild and domestic animals in May and October using the culture method. According to the data, Fuliang and Shangrao counties were the most endemic areas among four surveillance spots, as demonstrated by high carrier rates of *Leptospira* among rats and dogs. The infection rates of captured rats were much higher in October than in May. Therefore, samples were collected in Fuliang and Shangrao County in October 2009, which follows the rice harvest.

As the data showed, rats served as a major *Leptospira* reservoir in Jiangxi Province. Other than rats, which might also be the most important source of leptospirosis transmission, dogs are likely to be a potential source of leptospirosis transmission. By contrast, few buffaloes and pigs tested positive for leptospirosis, and therefore they may not have an important role in transmission.

Previous studies in China have always relied on the culture method to determine leptospirosis prevalence, and, because of the low sensitivity of this method, the true carriage rate could be underestimated. Because of its high sensitivity and specificity, PCR–based detection was recommended by the World Health Organization, and the G1/G2 primer sets have been widely used in the PCR–based detection of leptospirosis[16]. However, because the G1/G2 primer pair fails to detect *Leptospira kirschneri* infection[3, 15, 17–19], additional primers, such as the B64I/B64II primer pair, must be used to complement G1/G2 primer–based PCR assays. We found that lipL32 is one of the highly transcribed leptosomal genes that are also highly conserved across pathogenic leptosomal species and that lipL32 primers could be used for PCR–based diagnosis of leptospirosis[20, 21]. lipL32 is an outer membrane protein that is produced not only during laboratory cultivation but also during mammalian infection[22] and has already been reported to be helpful in the detection of leptospirosis[3, 23].

Previous studies identified that PCR using primers G1/G2 was able to detect 10 pg of leptosomal DNA[19]. In our study, we further optimized the PCR conditions and showed that the detection limit of the G1/G2 and lipL32 primer sets could be substantially reduced to 10−2 pg of genomic DNA isolated from the cultured *Leptospira interrogans* strain Lai isolate. However, compared to lipL32, the G1/G2 primers were more sensitive in the detection of leptosomal DNA in host tissues.
Unlike our results indicating superior performance of G1/G2 primers, a previous investigation found insignificant differences in PCR–based detection when lipL32, lipL21 or G1/G2 primers were used\[3]. Use of primers that target different regions of the target genes and the different experimental conditions could account for the variation in the results. Additionally, the G1/G2 primer pair had different sensitivities in urine and blood samples\[19]. While the sensitivity of the lipL32 primers in tissues was lower than that of G1/G2, their sensitivities are similar in urine. Therefore, the lipL32 primers are a potential tool in urine detection.

Our research has focused on the prevalence of pathogenic *Leptospira* in animal hosts, both wild and domesticated, in the Jiangxi Province of China. Also, our results further underscored the utility of more sensitive detection methods, such as PCR assay, for the detection of leptospirosis in place of traditional, less efficient methods. The alarmingly high prevalence of leptospiral DNA in wild and domesticated animals near human habitation should remind us to pay close attention to *Leptospira* surveillance. Although the PCR primers lipL32 and G1/G2 display a similar sensitivity in their ability to amplify pure leptospiral DNA isolated from cultured cells, an assessment of infected samples suggests that the G1/G2 primers are superior in detecting infected tissue samples, whereas the lipL32 primers have an advantage in urine detection.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgments**

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**References**