Original Article

Molecular Typing of *Leptospira interrogans* Strains Isolated from Rattus tanezumi in Guizhou Province, Southwest of China*

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Abstract

Objective To identify and type three leptospires isolated from *Rattus tanezumi* in Guizhou Province by using three molecular techniques (PFGE, MLVA, and MLST), reveal the molecular characteristic of causative agents of local leptospirosis and evaluate these three molecular methods based on their detection resolution and efficiency.

Methods Three *Leptospira* strains were isolated from the kidney of *Rattus tanezumi* and cultured with EMJH medium. PFGE, MLVA, and MLST assays were applied to type the three strains isolated from *Rattus tanezumi* in Guizhou Province.

Results PFGE, MLVA, and MLST typing showed that the three leptospiral isolates matched with leptospiral serogroup Icterohaemorrhagiae serovar Lai. The findings of the genotyping methods were consistent. MLVA and MLST defined genotypes, whereas PFGE allowed the recognition of additional subgroups within the genotypes, and the findings of molecular typing were also consistent with those of traditional techniques.

Conclusion Three leptospiral isolates from Guizhou Province matched with leptospiral serogroup Icterohaemorrhagiae serovar Lai, and PFGE, MLVA, and MLST, as reliable molecular techniques for identifying and typing of *Leptospira interrogans*, would contribute to the active surveillance, outbreak investigation and source tracking for leptospirosis in Guizhou Province.

Key words: L. interrogans; PFGE; MLVA; MLST; Rattus tanezumi

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INTRODUCTION

Leptospirosis is a worldwide zooanthroponotic disease caused by pathogenic leptospires[1-2], which evokes severe diseases in humans, but only causes mild chronic or asymptomatic infection in many host animals[3-4]. For example, pulmonary diffuse hemorrhage, a serious clinical form of leptospirosis, is fatal to about 25% of patients[5], while most of the

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infected mammalian reservoir animals, such as rodents, only present mild chronic conditions which sometimes are asymptomatic and shed infectious organisms in the urine for their lifetime[6-7]. Therefore, identification and typing of leptospires isolated from reservoir animals contributes to the active surveillance, outbreak investigation and source tracking for leptospirosis.

Guizhou Province is an old natural epidemic focus of *L. interrogans*[8-10]. The prevalence of human leptospirosis was for the first time etiologically confirmed in this province in 1958[10]. There were several cases of leptospirosis as well as death cases reported in Rongjiang County, Southeast of Guizhou, which were clinically and serologically diagnosed as leptospirosis only recently. However, *L. interrogans* have not been isolated from patients so far, and thus the epidemic bacteria type remains unclear.

Traditionally, several hundred serovars of *Leptospira* are classified into two species, *L. interrogans* and *L. biflexa*[11], which contain pathogenic and saprophytic strains, respectively. Based upon DNA-DNA hybridization data, the genus is now classified into 17 species, several of which contain both pathogenic and nonpathogenic serovars. Microscopic agglutination test (MAT) and cross-agglutinin absorption test (CAAT) are, traditionally, used to identify leptospires. However, these techniques are laborious and time-consuming, requiring the maintenance of a collection of more than 200 reference strains and corresponding rabbit antisera. Several sequence-based molecular methods have been applied for the identification of leptospires[12-13], however, they do not yet have wide application. Molecular techniques such as pulsed-field gel electrophoresis (PFGE)[14-16], multiple locus variable number tandem repeat analysis (MLVA)[17-19] and multilocus sequence typing (MLST)[7,20] have been applied for the identification of leptospiral isolates in recent years.

In this study, PFGE, MLVA, and MLST are applied to identify and type three leptospiral isolates from *Rattus tanezumi* in Guizhou Province, Southwest of China. It aims: i) to reveal the molecular characteristic of causative agents of leptospirosis in Guizhou over recent years; and ii) to evaluate the aforesaid molecular techniques used for the identification of pathogenic leptospires , which will facilitate active surveillance, outbreak investigation and source tracking of leptospirosis.

MATERIALS AND METHODS

Leptospiral Strains and Cultivation

Leptospira interrogans strains used in this study were isolated from the kidney of *Rattus tanezumi* (belonging to Icterohaemorrhagiae Serovar-groups identified by MAT) in Guizhou Province, Southwest of China, and cultivated at 28 °C in Ellinghausen-McCullough-Johns on-Harris (EMJH) liquid medium[21-22].

PFGE Analysis

DNA for PFGE was prepared by the method as previously described[15-16]. Briefly, agarose blocks containing leptospiral DNA were prepared with 5 mL cultures in EMJH and then digested with 30 units of Not I restriction enzyme (New England Biolabs, USA) for 2 h at 37 °C. Plug slices containing the digested DNA were placed in the wells of a 1% agarose gel (Seakem Gold, Invitrogen Life Technologies) in 0.5 × Tris-borate-EDTA (TBE). The fragments were separated by PFGE and with the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) at a temperature of 14 °C with buffer recirculation, 120° constant angle, 6 V/cm and with ramped pulsed times of 2-35 s for 18 h. The DNA size marker Salmonella serotype Braenderup H9812 was digested with 50 U Xba I (New England Biolabs), as is recommended by PulseNet[17]. The gels were stained with ethidium bromide (1.0 μg/mL) and then photographed under ultraviolet (UV) trans-illumination with the Gel Doc 2000 system (Bio-Rad). The images were captured on Gel Doc 2000 system (Bio-Rad Laboratories) and converted to TIFF files for analyses. The PFGE patterns were analyzed by using BioNumerics software package (Ver.4.0; Applied Maths, Inc., Austin, TX, USA). The Dice band-based coefficient was used for the cluster analysis. The isolates were identified by comparing their PFGE patterns with a reference library of fingerprint patterns consisting of 15 serogroups and 15 Serovar of leptospiral reference strains of China.

MLVA Analysis

DNA was extracted from cultures of three strains of *Leptospiraceae* by using DNA Extraction Kit (SBS Genetech, Beijing, China) according to the manufacturer's directions and the DNA concentration was diluted to 1 ng/μL with ND-1000 Spectrophotometer (*Nanodrop, USA*). The VNTR loci used in this method were previously described[23]. The

primers were synthesized by Invitrogen. PCR amplification was performed in 50 μL final volumes. All reactions contained 21 μL of PCR Pre Mix (SBS Genetech, Beijing, China), 2 μL of forward and reverse primers of VNTR locus (5 pmol/μL), 10 μL DNA (1 ng/μL), and 15 μL of H_2O. The PCR reactions ran on a PE9700 thermal cycler (Applied Biosystems) under the following conditions: 94 °C for 10 min, varying cycles of 94 °C for 30 s, annealing at varying temperatures (Table 1) for 30 s, extension at 72 °C for 1 min with a final single extension of 72 °C for 10 min, and then held at 4 °C. Amplified products were characterized by electrophoresis of 1 μL of each reaction on a 2% agarose gel for 4 h at 100 V. Each VNTR locus could be identified by being assigned a size by the GENESCAN software (Applied Biosystems). The copy number of the repeats of each VNTR locus was deduced from the band sizes of the amplified products. This size was then converted into an allele designation, which in turn formed the allele string for the seven loci. The allele string was constructed in the following order: VNTR4-VNTR7-VNTR27-VNTR29-VNTR30-VNTR36-VNTR50. Bionumerics software package, Version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium), was used for the analysis of the data with the categorical coefficient and for the unweighted-pair group method with average linkages clustering parameters. The reference strains used for clustering were the ten strains of the Chinese epidemic serotype, which had been successfully analyzed[23]. The polymorphism indices of individual and grouped VNTRs were calculated by using Nei's diversity index.

Table 1. Information of VNTR Loci Proposed for MLVA of Leptospiral Isolates

VNTR Locus	Primers (5'-3')	Unit Length (bp)	Annealing Temperature (°C)
VNTR-4	F: AAGTAAAAGCGCTCCCAAGA R: ATAAAGGAAGCTCGGCGTTT	34	58
VNTR-7	F: GATGATCCCAGAGAGTACCG R: CCCTCCACAGGTTGTCTTG	46	59
VNTR -27	F: AGTTCGTCGGGTGAGC R: GATTTCTTTCGGTGGC	46	54
VNTR -29	F: GGGTGCCGGGTTGT R: TGCCACATCTCATCCATTAC	47	50
VNTR -30	F: TAGGTTCGGCGTTTAGTA R: TTAGATGTTTCGCTTTGG	46	54
VNTR -36	F: GGCGTCGAAGACAAA R: CTCTACCAGGAGATTATCAAA	38	54
VNTR -50	F: CTTGTTGGATCACAATACGAACTATA R: GGTAAGGGACAAAGTAAGTGAAGC	46	59

MLST Analysis

DNA was extracted from cultures of three strains of *Leptospira* by using DNA Extraction Kit (SBS Genetech, Beijing, China) according to the manufacturer's directions and the DNA concentration was diluted to 1 ng/μL with ND-1000 Spectrophotometer (*Nanodrop*, *USA*). Six loci were selected based on performance of primers as previously described (which can also be obtained from the sharing website: http://leptospira.mlst.net)[20]. Primer for mreA was redesigned in this study. These loci were pntA, sucA, fadD, tpiA, pfkB, and glmU, which are located on Chromosome I with the exception of fadD. Primer sequences are shown in Table 2. Amplifications were performed in 50 μL total volumes of PCR reaction mix containing 10 μL (1 ng/μL) of genomic DNA, 4 μL of each primer (5 pmol), 4 μL dNTP (TaKaRa, Otsu, Japan), 0.8 μL TaKaRa Taq™, 0.2 μL of Pyrobest DNA Polymerase (TaKaRa, Otsu, Japan) and 5 μL of 10 × buffer. PE9 700 thermal cycler (Applied Biosystems) was used to perform PCR with an initial denature step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 10 seconds, 52 °C (mreA, pfkB, pntA, sucA, and tpiA), or 50 °C (fadD and glmU) for 15 seconds, 72 °C for 50 seconds, then 72 °C for 7 min. PCR product size ranged from 555 bp to 638 bp. The sequence's start and end points used to define each MLST locus are shown in Table 2. MLST was performed for the isolates by using these 7 loci. Following the standard MLST protocol, each allele was assigned a different allele number and the allelic profile (string of seven integers) was used to define the sequence type (ST). A *Leptospira* MLST website was established to provide public access to these data, and to provide the resource to other investigators who can use this to assign the ST of further strains. This can be accessed at http://leptospira.mlst.net.

RESULTS

Genotyping of the Leptospiral Isolates Based on PFGE

By PFGE, each isolate produced a fingerprint pattern consisting of twelve or thirteen bands (Figure 1). The twelve-band (Strain G30 and G31) pattern produced a PFGE type (LepNot I.003), and the thirteen-band pattern (Strain G32) produced another PFGE type (LepNot I.002). The cluster analysis demonstrated that both PFGE types were of high similarity (clustering together at >95%) to Serogroups Icterohaemorrhagiae Serovar Lai (Figure 2).

Table 2. Information of Loci Proposed for MLST of Leptospiral Isolates

Gene	Size of PCR Product (bp)	Primer 5'-3'	Annealing Temperature (°C)
PntA	638	F: TGCCGATCCTACAACATTA R: AAGAAGCAAGATCCACAACTAC	52
SucA	560	F: AGAAGAGGCCGGTTATCATCAG R: CTTCCGGGTCGTCTCCATTTA	52
PfkB	560	F: CCGAAGATAAGGGGCATACC R: CAAGCTAAAACCGTGAGTGATT	52
TpiA	534	F: AAGCCGTTTTCCTAGCACATTC R: AGGCGCCTACAAAAAGACCAGA	52
MreA	602	F: AAAGCGGCCAACCTAACACC R: CGATCCCAGACGCAAGTAAG	52
GluM	557	F: GGAAGGGCACCCGTATGAA R: TCCCTGAGCGTTTTGATTT	50
FadD	577	F: AGTATGGCGTATCTTCCTCCTT R: TTCCCACTGTAATTTCTCCTAA	50

Genotyping of the Leptospiral Isolates Based on MLVA

MLVA was applied to type the three isolates of Rattus tanezumi in Guizhou Province. Seven VNTR loci (VNTR4, VNTR7, VNTR27, VNTR29, VNTR30, VNTR36, and VNTR50) based primers were used to amplify the chromosome DNA of leptospiral isolates, and all of the seven loci were successfully amplified from the three isolates (Figure 3).

The copy number of the repeats of each VNTR locus was deduced from the band size of the amplified products. This size was then converted into an allele designation, as shown in Table 3. The cluster analysis indicated that all the three leptospiral isolates matched with serovar-groups Icterohaemorrhagiae Serovar Lai strain Lai (Figure 4).

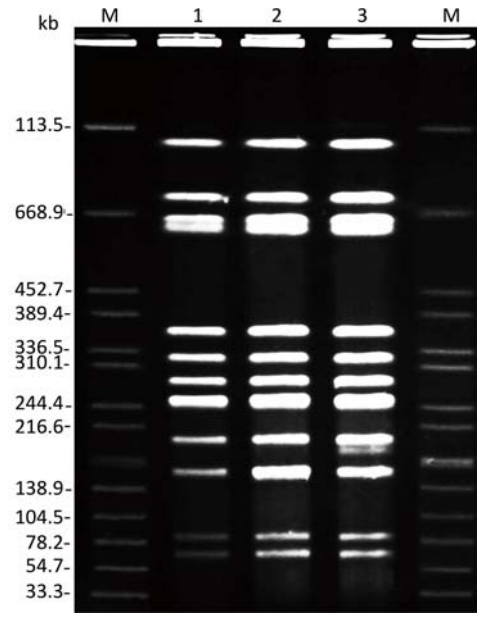

Figure 1. PFGE profile of *Leptospira* strains isolated form *Rattus tanezumi* in Guizhou Province. M: DNA Marker. 1: Strain G30 (PFGE type LepNot I.003). 2: Strain G31 (PFGE type LepNot I.003). 3: Strain G32(PFGE type LepNot I.002).

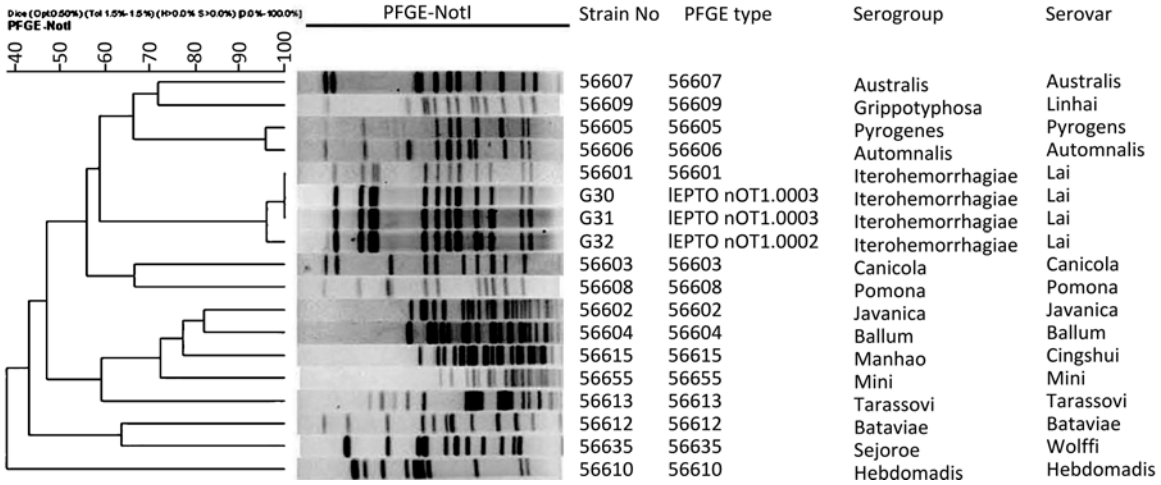

Figure 2. Dentrogram of PFGE profile showing *Leptospira* strains isolated from *Rattus tanezumi* in Guizhou and *L. interrogans* strain Lai clustering together at >95%.

Table 3. Results Obtained for Three Isolated Leptospires Analyzed by the MLVA Method

Strain No	Copy Number of the Repeats of Each VNTR Locus						
	VNTR-4	VNTR-7	VNTR-27	VNTR-29	VNTR-30	VNTR-36	VNTR-50
G30	5	4	7	1	2	16	7
G31	5	4	7	1	2	16	7
G32	5	4	7	1	2	16	7

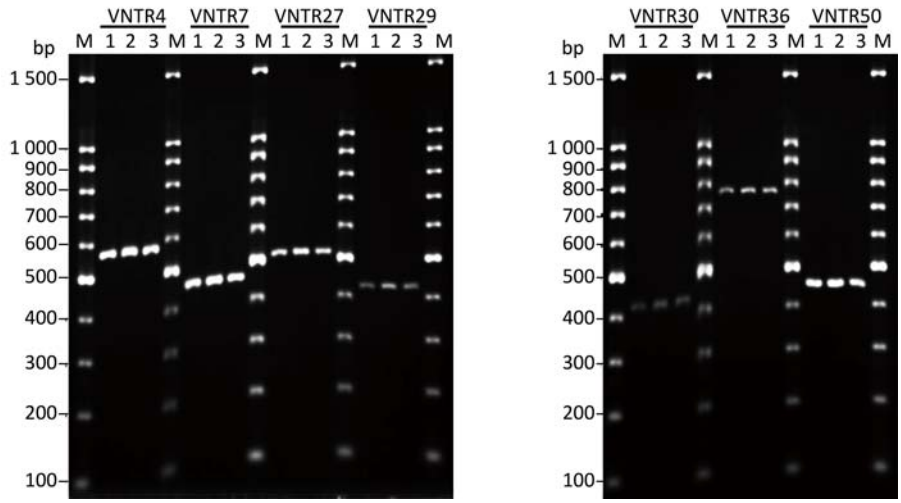

Figure 3. PCR products from the seven selected VNTR loci of various *Leptospira* isolates. M: marker. 1: *Leptospira* isolate G30 2: *Leptospira* isolate G31 3: *Leptospira* isolate G32.

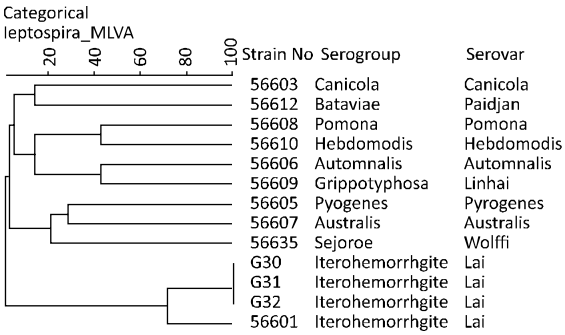

Figure 4. Dentrogram of MLVA showing *Leptospira* isolates and *L. interrogans* strain Lai clustering together at 100%.

Genotyping of the Leptospiral Isolates Based on MLST

Seven loci (pntA, sucA, fadD, tpiA, pfkB, mreA, and glmU) based primers were used to amplify the chromosome DNA of leptospiral isolates, and all of the seven loci were successfully amplified from the three isolates. MLST was performed for the isolates by using the 7 loci. Following the standard MLST protocol which can be accessed at http:// leptospira.mlst.net, an allele number was assigned to all the allele of different leptospiral strains and the allelic profile (string of seven integers for the three leptospiral strains was 1-1-1-1-1-1-1) was defined as sequence type (ST) 1. According to the ST profile, all of the three leptospiral strains were defined as Leptospiral serovar-groups Icterohaemorrhagiae Serovar Lai analyzed by using the resource at http:// leptospira.mlst.net.

DISCUSSION

The present study demonstrates that the three leptospires isolated from the kidney of Rattus tanezumi in Guizhou Province in Southwest China, are identified as *L. nterrogans* serovar-groups Icterohaemorrhagiae Serovar Lai by PFGE, MLVA and MLST analyses. It is consistent with the results of identification with traditional methods (MAT) (data not shown), and also with the clinical and serological diagnosis of patients in recent years, which implies that Serovar Lai was the current epidemic serotype of *L. interrogans* in this province. Our results would contribute to the active surveillance, outbreak investigation and source tracking for local leptospirosis. Moreover, PFGE, MLVA and MLST were

reaffirmed as reliable molecular techniques for leptospires typing and identification in this study.

Guizhou is an old foci of leptospirosis in China[8-10], as the prevalence of human leptospirosis was etiologically confirmed in this province as early as in 1958[10]. For example, 14 126 human leptospirosis cases with 534 deaths were reported in Qiannan Prefecture between in 1958 and 2005, and Apodemus agrarius was a very important reservoir host of leptospirosis with a carrier rate of 7.63%, which accounted for 95.84% of all the checked rats. The geographic distribution of host animals had a close relation with cases of leptospirosis aggregate distribution[10]. Investigation on the epidemiology of leptospirosis in Liping County in Southeast Guizhou, revealed that a total of 127 leptospirosis cases with 28 deaths were reported from 2001 to 2008[9]. However, the etiological characteristics (e.g. serovar-group and serovar, genospecies) of the pathogen and pathogenic leptospires, in Guizhou Province in recent years remain unknown. To track the source of infection, investigation on the animal carrier of leptospires was conducted in high incidence counties of leptospirosis in recent years, and three leptospires were isolated from the kidney of Rattus tanezumi in Rongjiang County. We identified the three leptospires with different molecular techniques as *Leptospira* Serovar-groups Icterohaemorrhagiae Serovar Lai (belonging to *L. interrogans* genospecies), which suggests that *Leptospira* Serovar-groups Icterohaemorrhagiae Serovar Lai (or genospecies *L. interrogans*) may be the epidemic serotype (genospecies) in this province in recent years. This is consistent with the result of previous studies that *Leptospira* Serovar-groups Icterohaemorrhagiae Serovar Lai were the dominant cause of leptospirosis in Guizhou province[10], which would contribute to the active surveillance, outbreak investigation and source tracking for local leptospirosis.

The findings of the present study have revealed that the three molecular methods (MLVA and MLST and PFGE) have identified the three leptospires as *Leptospiral* serovar-groups Icterohaemorrhagiae Serovar Lai, while PFGE has identified independent polymorphisms. By PFGE, the three isolates produce different fingerprint patterns consisting of twelve or thirteen bands (Figure 1). The twelve-band (Strain G30 and G31) pattern produces a PFGE type (LepNot I.003), and the thirteen-band pattern (Strain G32) produces another PFGE type (LepNot I.002), suggesting that PFGE allows recognition of subtypes within Serovar Lai. In addition, the rapidity of PCR makes MLVA appropriate for preliminary genotyping of clinical isolates, while MLST needs the gene sequencing after PCR. Moreover, both PFGE and MLVA analyses need expensive professional analytic software (eg: Numerics 4.0), which poses difficulties for general laboratories in China. However, the MLST analysis can be finished through the sharing public *Leptospira* MLST website (http:// leptospira.mlst.net). Based on the above analyses, PFGE is the best method for discriminating strains among the tested typing methods , demonstrating a strong correlation with the other techniques, while MLVA or MLST has its respective advantages for different purposes.

Furthermore, in the MLST assay, the primer for mreA used in previous studies (which can be also obtained from the sharing website: http://leptospira.mlst.ne) is not applicable to Chinese strains, and thus, the primer pair for mreA was redesigned in this study. Therefore, this is a technological improvement for *Leptospira* MLST method.

Our study on molecular identification and typing of *Leptospira* isolates has provided a scientific basis for control and prevention of Leptospirosis in Guizhou province. Besides, it has also taken the lead to compare PFGE, MLVA and MLST assays based on the resolution and efficiency of detection results. Additionally, identification of *Leptospira* isolates by using different molecular techniques also has accumulated experimental data for application of molecular techniques for the identification of clinical *Leptospira* isolates.

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