DOI:10.3969/cjz.j.issn.1002-2694.2012.11.005

16S rRNA gene based genospecies identification of *Leptospira* strains isolated from *Apodemus agrarius* in Guizhou Province, 2011

LI Shi-jun[1], WANG Ding-ming[1], ZHANG Chui-cai[2], LI Xiu-wen[2], TIAN Ke-cheng[1], LIU Ying[1], TANG Guang-peng[1], JIANG Xiu-gao[2]

(1. *Institute of Communicable Disease Control and Prevention, Guizhou Provincial Center for Disease Control and Prevention, Guiyang* 550004, *China*;
2. *Institute of Communicable Disease Prevention and Control, Chinese Center for Disease Control and Prevention, Beijing* 102206, *China*)

ABSTRACT: In order to understand the etiologic characteristic of leptospirosis in Guizhou Province, nearly full length of 16S rRNA gene of four leptospiral strains isolated from *Apodemus agrarius* in the epidemic area of leptospirosis in Guizhou Province in 2011 were amplified with PCR and subsequently sequenced. These sequences were compared to each other and to the representative strains of 17 *Leptospira* genospecies, *Leptonema illini*, and *Turneriella parva*. Phylogenetic tree were constructed to demonstrate the evolutionary relationship of the four isolates and the representative strains of 17 *Leptospira* genospecies, *Leptonema illini*, and *Turneriella parva*. The results showed that nearly full length (1 492 bp) of 16S rRNA gene for the four isolates were amplified and successfully sequenced. The alignment showed that the nucleotide homogenies of the four strains were as high as 100%, and the four isolates were most related to strains belonged to genospecies *Leptospira interrogans* serogroup Icterohaemorrhagiae, with the homogeny of 99.9%. Phylogenetic tree indicated that the isolates from Guizhou, the representative strains of seventeen genospecies of *Leptospiraceae*, *Leptonema illini* and *Turneriella parva* formed four main clusters (pathogenic, intermediate, nonpathogenic, and other). Isolates from Guizhou and the strains from the eight pathogenic genospecies groups were included in the pathogenic clade, in which the isolates from Guizhou were most related to genospecies *Leptospira interrogans*. Our results suggest that *Leptospira* strains isolated from *Apodemus agrarius* in the epidemic area of leptospirosis in Guizhou Province in 2011 belong to genospecies *L. interrogans*, which indicate that genospecies *L. interrogans* might be the epidemic genospecies of *Leptospira* in the localities. It will contribute to the control and prevention of leptospirosis in Guizhou Province.

KEY WORDS: *Leptospira*; 16S rRNA gene; genospecies; *Apodemus agrarius*; Guizhou

Supported by the Guizhou Province Governor Special Funds for Outstanding Scientific and Technological Talent (No. Guizhou Province Specifically Co-word (2010) 90).

Corresponding author: Li Shi-jun, Email: zjumedjun@163.com; Wang Ding-ming, Email: cfswdm@gzcdc.gov.cn

Leptospirosis is one of the most widespread zoonoses and is caused by infection with pathogenic spirochetes of the *Leptospira* genus [1]. The disease in human is most frequent in developing countries, and the spectrum ranges from subclinical infection to severe symptoms of multiorgan dysfunction with high case-fatality rates, reaching a mortality rate as high as 70% in the case of severe pulmonary hemorrhage syndrome [2-5]. In contrast, most of the infected mammalian reservoirs, such as rodents, only presents mild chronic disease or are asymptomatic, and shed infectious organisms in the urine for their lifetime [6,7]. Leptospires dwell in the renal tubules of their maintenance hosts and are excreted into the environment with the urine. Humans may be infected indirectly from animals by contacting with contaminated water, soil or mud in a moist environment, or by direct contact from urine, fresh carcasses or organs [8]. Rodents are recognized as important mammal reservoirs of *Leptospira* spp [9-10]. Infection may occur early in the lifespan of the animal and the chances of infection increases with age [1,11]. After infection, the spirochetes are localized in the kidneys and excreted by urine discontinuously[1,11]. Once excreted, the bacteria can survive in a favorable environment for months or years before infecting new hosts, including humans. So, surveillance on carrier status of reservoir hosts and analysis on the characteristic of causative agents contribute to the clinic laboratory diagnosis, active surveillance, outbreak investigation and source tracking for leptospirosis.

There were several cases of leptospirosis patients as well as death cases reported in Rongjiang, Jingping and Liping counties, Southeast of Guizhou, every year in recent decade, which were only clinically and serologically diagnosed. For example, 127 human leptospirosis cases including 28 death cases reported in Liping County from 2001 to 2008 [12]. However, *L. interrogans* were never isolated from patients in recent years, and the epidemic bacteria genospecies remains unclear.

Traditionally, several hundred serovars of *Leptospira* were classified into two species, (*Leptospira interrogans* (*L. interrogans*) and *Leptospira biflexa* (*L. biflexa*) [13], which contained pathogenic and saprophytic strains, respectively. Pathogenic *Leptospira* are classified into more than 200 serovars based on serological methods [15]. And based upon the most recent DNA-based classification, to date 17 *Leptospira* species have been described, which can be divided into pathogenic (i. e., having the potential to cause disease in humans and animals) and saprophytic (i. e., free living and considered not to cause disease) species. Some strains show unclear pathogenicity and are termed intermediates [15]. Therefore, identification of *Leptospira* genospecies is important for the control and prevention of leptospirosis in the local area.

The objective of this study was to reveal the etiologic characteristics of leptospirosis in Guizhou Province by 16S RNA gene analysis and genospecies identification of *Leptospira* strains isolated from the rodents in the local epidemic area, which will contribute to clinical laboratory diagnosis, active surveillance, outbreak investigation and source tracking for leptospirosis.

Materials and Methods

Leptospiral strains and cultivation

Four *Leptospira* strains (Table 1) used in this study were strain JP13, JP15, JP19 and LP62 isolated from *Apodemus agrarius* in the rice-field environment of epidemic region of leptospirosis in Guizhou Province, which was identified as pathogenic *Leptospira* by PCR and cultivated with liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, USA) at 28℃[14].

Tab. 1 Background information of *Leptospira* strains used in this study

Strain no.	Host	Environment	Region	Year of isolation
JP13	*Apodemus agrarius*	Rice-field	Jinping	2011
JP15	*Apodemus agrarius*	Rice-field	Jinping	2011
JP19	*Apodemus agrarius*	Rice-field	Jinping	2011
LP62	*Apodemus agrarius*	Rice-field	Liping	2011

16S rRNA gene sequencing

DNA was extracted from cultures of strains of *Leptospiraceae* using DNA Extraction Kit (SBS GenenTech, Beijing, China) according to the manufacturer's directions, and the DNA concentration were diluted to 1 ng/μL with ND-1000 Spectrophotometer (Nanodrop, USA). The 16S rRNA genes were amplified from the purified DNA using the PCR Kit (TaKaRa, Otsu, Japan). Briefly, each 50μL reaction system contained 19μL of deionized water, 25 μL of PreMix Taq, 2 μL of DNA, and 2 μL of fD1 (forward: 5′-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCAG-3′) and rP2 (reverse: 5′-CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACTT-3′) primers with concentrations of 5 pmol/μL corresponding to positions 8 and 1 492, respectively[13]. Amplification was performed on an TProfessional PCR thermocycler at 94℃ for 5 min, followed by 35 cycles of 94℃ for 15 s, 50℃ for 5 s, and 72℃ for 90 s, with a final single extension of 72℃ for 5 min, and then held at 4℃. Amplified products were characterized by electrophoresis of 1 μL of each reaction on a 1.2% agarose gel for 30 min at 85 V. The PCR products were sent to TaKaRa Company (Dalian, China) for purification, sequencing and sequence assembly.

Phylogenetic analysis

The homologies among the 16S rRNA gene

sequences obtained from leptospires were analyzed using the MegAlign program in the DNAStar software package (Inc., United States). Phylogenetic trees were constructed for the 16S rRNA gene sequences of leptospires isolated from *Apodemus agrarius* in Guizhou and representative strains of 17 *Leptospira* genospecies, *Leptonema illini* and *Turneriella parva* using the Neighbor-Joining (NJ) method [13]. The 16S rRNA gene sequences of the representative strains of 17 genospecies used for comparison with those obtained in this study were downloaded from the NCBI database (Table 2).

Tab. 2 GenBank accession numbers of leptospiral 16S ribosomal RNA gene sequences used in this study [13]

Clade	Species	Serovar	Strain	GenBank no.
Pathogenic	*Leptospira interrogans*	Icterohaemorrhagiae	RGAT ATCC 43642T	AY631894
	Leptospira interrogans	Australis	Ballico	AY996794
	Leptospira interrogans	Autumnalis	Akiyami A	AY996791
	Leptospira interrogans	Bulgarica	Mallika	AY996792
	Leptospira interrogans	Canicola	Hond Utrecht IV	AY996798
	Leptospira interrogans	Copenhageni	M 20	AY996790
	Leptospira interrogans	Hardjo	Hardjoprajitno	AY996796
	Leptospira interrogans	Hardjo	Lepto-0184	AY996797
	Leptospira interrogans	Pomona	Pomona	AY996800
	Leptospira interrogans	Pyrogenes	Salinem	AY996793
	Leptospira alexanderi	Manhao 3	L60T ATCC 700520T	AY631880
	Leptospira alexanderi	Manzhuang	A23	AY996803
	Leptospira alexanderi	Nanding	M 6901	AY996804
	Leptospira borgpetersenii	Javanica	Veldrat Batavia 46T ATCC 43292T	AY887899
	Leptospira borgpetersenii	Ballum	Mus 127	AY631884
	Leptospira kirschneri	Cynopteri	3522 CT ATCC 49945T	AY631895
	Leptospira kirschneri	Bim	1051	AY996802
	Leptospira kirschneri	Bim	PUO 1247	AY996801
	Leptospira noguchii	Panama	CZ 214T ATCC 43288T	AY631886
	Leptospira santarosai	Shermani	LT 821T ATCC 43286T	AY631883
	Leptospira santarosai	Georgia	LT 117	AY996805
	Leptospira weilii	Celledoni	CelledoniT ATCC 43285T	AY631877
	Leptospira genomospecies 1	Sichuan	79601T ATCC 700521T	AY631881
Intermediate	*Leptospira inadai*	Lyme	10T ATCC 43289T	AY631896
	Leptospira inadai	Aguaruna	MW 4	AY631891
	Leptospira inadai	Kaup	LT 64-68	AY631887
	Leptospira broomii	Not designated	5399T ATCC BAA-1107T	AY796065
	Leptospira fainei	Hurstbridge	BUT 6T ATCC BAA-1109T	AY631885
	Leptospira fainei	Hurstbridge	BKID 6	AY996789
Nonpathogenic	*Leptospira biflexa*	Patoc	Patoc IT ATCC 23582T	AY631876
	Leptospira biflexa	Andamana	CH 11	AY631893
	Leptospira meyeri	Ranarum	Iowa City FrogT ATCC 43287T	AY631878
	Leptospira meyeri	Hardjo	Went 5	AY631889
	Leptospira meyeri	Semaranga	Veldrat Semarang	AY631892
	Leptospira wolbachii	Codice	CDCT ATCC 43284T	AY631879
	Leptospira wolbachii	Gent	Wa Gent	AY631890
	Leptospira genomospecies 3	Holland	WaZ HollandT ATCC 700522T	AY631897
	Leptospira genomospecies 4	Hualin	LT 11-33T ATCC 700639T	AY631888
	Leptospira genomospecies 5	Saopaulo	Sao PauloT ATCC 700523T	AY631882
Other	*Leptonema illini*	Illini	3055T	AY714984
	Leptonema illini	Habaki	Habaki	AY996806
	Turneriella parva	Parva	HT NCTC 11395T	AY293856
	Turneriella parva	Parva	S-308-81	AY398688

Results

16S rRNA gene sequence analysis

Nearly full length (1 492 bp) of 16S rRNA gene for strain JP13, JP1, JP19 and LP62 were amplified by using the primer pair of rP2 and fD1 (Figure 1) and subsequently successfully sequenced. These sequences were compared to each other and to the sequence of strains from the seventeen genospecies of *Leptospiraceae* [13]. The alignment showed that the homogeny of sequences within the four strains in this study and the three strains isolated from *Rattus tanezumi* in Rongjiang in 2007 was 100%, the homogenies among the four isolates and strains from pathogenic genospecies, including *Leptospira alexanderi*, *Leptospira borgpetersenii*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira santarosai*, *Leptospira weilii*, and *Leptospira* genomospecies 1, were from 98.8% to 99.9%, with the highest percentage (99.9 %) compared with *Leptospira interrogans* serovar icterohaemorrhagiae strain RGAT ATCC 43642T, while the homogenies among the four isolates and strains of intermediate genospecies, including *Leptospira inadai*, *Leptospira broomii*, and *Leptospira fainei*, were from 95.0 % to 95.2 %, reaffirming the high degree of species conservation among spirochetes. But the homogenies of the four isolates and *Leptospira* reference strains belonging to the nonpathogenic genospecies, such as *Leptospira alexanderi*, *Leptospira borgpetersenii*, *Leptospira noguchii*, were only 88.4%-88.6%.

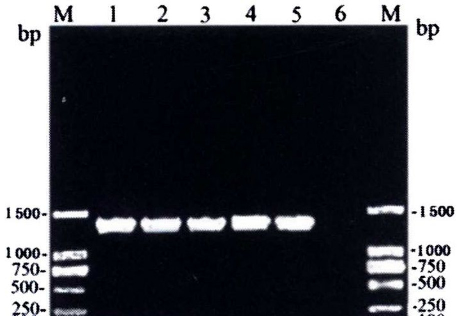

Fig. 1 PCR detection results of leptospiral 16S rRNA gene with the amplification fragment at the sides of 1 492 bp

M: Marker; 1: Strain 56601; 2: Strain JP13; 3: Strain JP15; 4: Strain JP19; 5: Strain LP62; 6: Negative control.

Phylogenetic tree of 16S rRNA gene

Phylogenetic analysis of 16S rRNA gene sequences showed that leptospiral strains isolated from *Apodemus agrarius* in Jinping (strain JP13, JP15 and JP19), Liping (strain LP62) in 2011 and the representative strains of 17 *Leptospira* genospecies, *Leptonema illini* and *Turneriella parva* formed four main clusters (pathogenic, intermediate, nonpathogenic, and other) of species (Figure 2). The isolates of Guizhou as well as the strains from the eight pathogenic species (*Leptospira alexanderi*, *Leptospira borgpetersenii*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira santarosai*, *Leptospira weilii*, and *Leptospira* genomospecies 1) group could be divided into the pathogenic clade, in which the seven strains from Guizhou were most related to genospecies *Leptospira interrogans*, such as *Leptospira interrogans* serovar icterohaemorrhagiae strain RGAT ATCC 43642T, *L. interrogans* serovar Autumnalis, strain Akiyami A and so on. The intermediate clade comprised species *Leptospira inadai*, *Leptospira broomii*, and *Leptospira fainei*, while species *Leptospira biflexa*, *Leptospira Meyer*, *Leptospira wolbachii*, *Leptospira* genomospecies 3, *Leptospira* genomospecies 4, and *Leptospira* genomospecies 5 formed the nonpathogenic clade. In addition, *Leptonema illini* and *Turneriella parva* were included in another clade separated clearly from the pathogenic, intermediate and nonpathogenic clade.

Discussion

The present study demonstrates that nearly full length of 16S rRNA gene of the four leptospires isolated from the kidney of *Apodemus agrarius* were sequenced in Jinping and Liping counties, Guizhou Province, Southwest China. Homogeny analysis and phylogenetic tree indicated the four isolates belonged to genospecies *L. interrogans*, which is consistent with the identification results of Microscopic Agglutination Test (MAT).

There were several cases of leptospirosis patients as well as death cases reported in Guizhou Province in every year of recent years. For example, according to the China National System for Disease Control and Prevention, twelve human leptospirosis cases with one death case were reported in Guizhou in 2011. However, these reported cases

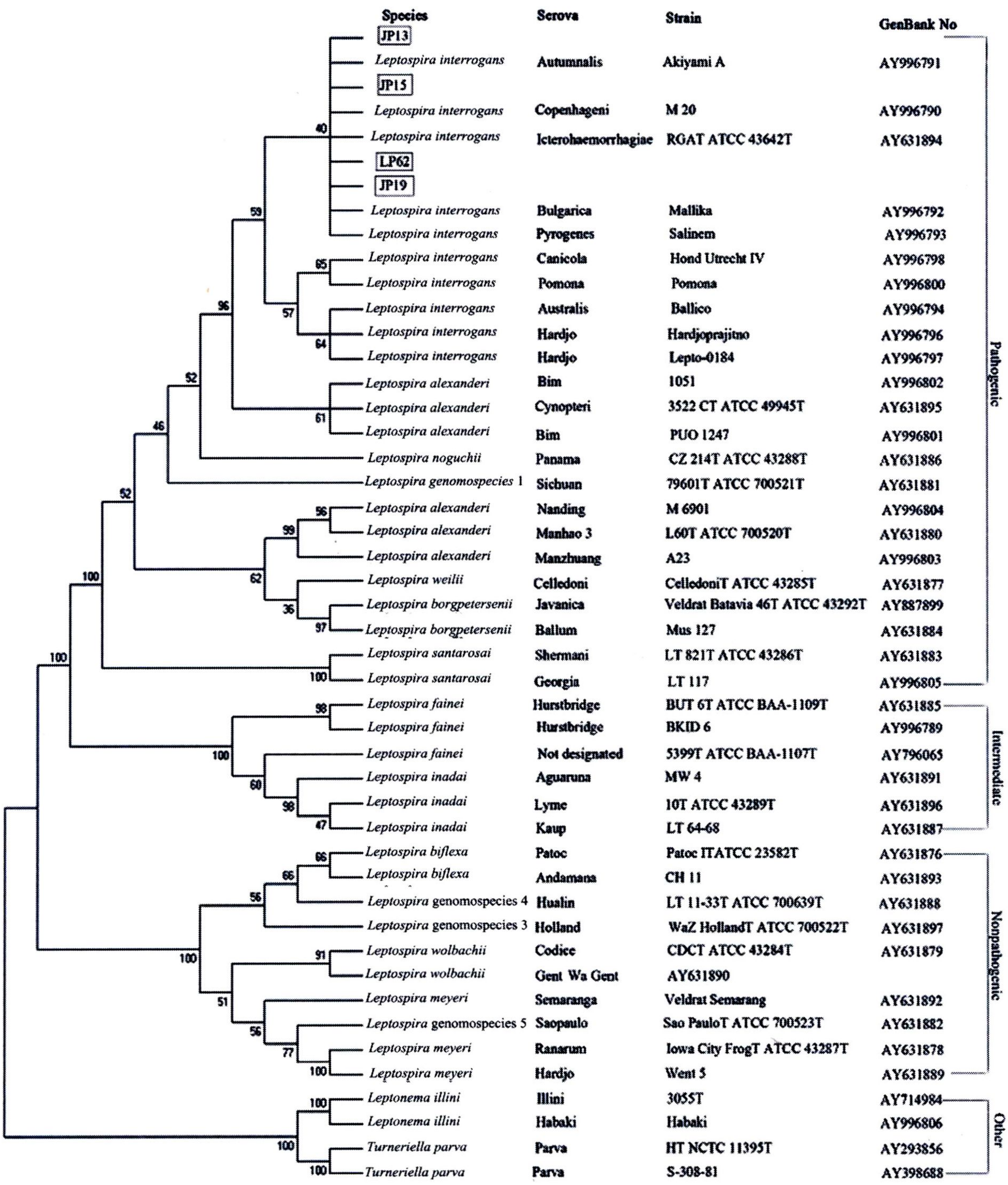

Fig. 2 Phylogenetic tree of leptospiral isolates and *Leptospiraceae* 16S rRNA gene sequences

were only clinically and serologically diagnosed, and the source of infection and the characteristic of epidemic bacteria remain unclear.

Guizhou has been proved to be the old foci of leptospirosis in China [15-17]. Liping, Rongjiang and Jinping counties, belonging to Qiandongnan Prefecture, were the high-incidence region of leptospirosis in Guizhou Province. For example, 14 126 human leptospirosis cases with 534 deaths were reported in Qiandongnan Prefecture from 1958 to 2005. Investigation on the epidemiology of leptospirosis in Liping county, a county in southeast Guizhou, revealed that a total of 127 leptospirosis cases with 28 deaths were reported from 2001 to 2008 [12]. And China National System for Disease Control and Prevention revealed that the incidence peak time was from September to October, but these reported patients were only clinically diagnosed.

Rodents are recognized as important mammal reservoirs of *Leptospira* spp [9,10]. A study performed in 1992 in Guizhou Province revealed that the animal carrier, *Apodemus agrarius*, was a very important reservoir host of leptospirosis, with a carrier rate of 7.36%, accounting for 95.84% of all the checked rats. The geographic distribution of host animal in local area had a close relation with cases of leptospirosis aggregate distribution [16]. However, few studies on the carrier status in recent year were reported. To trace the source of infection, three strains (JP13, JP15 and JP19) of *Leptospira* were isolated from *Apodemus agrarius* in Jinping County and one strain (LP62) from Liping County. Detection results suggest that *Apodemus agrarius* may be the main carrier of *Leptospira* in the localities.

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 correspondent rabbit antisera. Based upon DNA-DNA hybridization data, the genus is now classified into 17 species [13]. Morey RE et al[13] determined nearly full-length 16S rRNA gene sequences of approximately 1 430 bp from well-characterized type strains and representative serovars of *Leptospira* species for species identification of leptospires, and concluded that 16S rRNA gene sequencing was a powerful method for identification in the clinical laboratory and offers a simplified approach to the identification of *Leptospira* species. In this study, the four strains of *Leptospira* were identified as genospecies *L. interrogans* by using 16S rRNA gene sequencing analysis. It is consistent with the species identification result for the three *Leptospira* isolated from *Rattus tanezumi* in Rongjiang County in 2007 [18] and is also consistent with the antibody detection results of local leptospirosis patients in recent years.

In the present study, four leptospires isolated from *Apodemus agrarius* in Jinping and Liping counties in 2011 were identified as *L. interrogans* which belongs to the genospecies of pathogenic clade, which is consistent with MAT detection results for the *Leptospira* antibody of the patients in the local area. Our results suggest that *Apodemus agrarius* may be the main carrier of *Leptospira* in Jinping and Liping counties, and *L. interrogans* may be the epidemic genospecies of *Leptospira* in the local area, which will contribute to the clinical laboratory diagnosis, active surveillance, outbreak investigation and source tracking for leptospirosis in Guizhou Province.

Acknowledgement

We acknowledge the contribution of Jingping County CDC, Liping County CDC and Rongjiang County CDC for rodent trapping.

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Received: 2012-09-16; Revision accepted: 2012-10-11

贵州省 2011 年黑线姬鼠钩端螺旋体分离株 16S rRNA 基因序列分析及基因种鉴定*

李世军[1],王定明[1],张翠彩[2],李秀文[2],田克诚[1],刘　英[1],唐光鹏[1],蒋秀高[2]

摘　要:目的　了解贵州省钩端螺旋体病(简称钩体)病原学特征,分析 2011 年贵州省钩体病疫区黑线姬鼠钩体分离株 16S RNA 基因序列并对其进行基因种鉴定,为贵州省钩体病的有效预防和控制提供科学依据。**方法**应用 PCR 扩增几乎全长的钩体 16S rRNA 基因片段,并将扩增产物进行双向序列测定,从 NCBI 数据库下载钩体 17 个基因种代表菌株及伊尼利螺旋体和短小螺旋体 16S rRNA 基因序列,采用生物信息软件比较分离株和各基因种代表株间的核苷酸序列,分析其亲缘进化关系,确定分离株基因种。**结果**　通过 PCR 扩增和基因测序技术获得 4 株钩体分离株 16S rRNA 基因核苷酸序列(1492 bp),4 株钩体分离株的核苷酸同源性为 100%,与 17 个钩体基因种中的问号钩体(*L. interrogans*)基因种黄疸出血群代表菌株的同源性最高(99.9%),系统进化树分析显示,钩体分离株与 17 个基因种代表菌株及伊尼利螺旋体和短小螺旋体形成致病性、非致病性、未知致病性和其它分支,贵州 4 株分离株分属于致病性基因种分支,其中与致病性钩体 8 个基因种中的问号钩体基因种亲缘关系最近。**结论**贵州省 2011 年钩体病疫区黑线姬鼠钩体分离株均属致病性钩体的 *L. interrogans* 基因种,该基因种菌株可能为当地流行菌株,该结果将为贵州省钩体病的预防和控制提供科学依据。

关键词:钩端螺旋体;16S rRNA;基因种;黑线姬鼠;贵州
中图分类号:R378　　**文献标识码**:A　　**文章编号**:1002－2694(2012)10－1081－07

* 贵州省优秀科技教育人才省长专项资金项目(No. 黔省专合字(2010) 90 号)资助
通讯作者:王定明,Email: cfswdm@gzcdc.gov.cn
作者单位:1. 贵州省疾病预防控制中心传染病防治研究所,贵阳　550004;
2. 中国疾病预防控制中心传染病预防控制所,北京　102206