

RESEARCH PAPER



Genetic diversity of *Leptospira interrogans* circulating isolates and vaccine strains in China from 1954–2014

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ABSTRACT

Leptospirosis is one of the most important but neglected, infectious tropical diseases worldwide. *Leptospira interrogans* is now recognized as a leading cause of the disease. Little is known of the genetic diversity and phylogenetic characteristics of *L. interrogans* within China. To better understand the transmission and genetic diversity of *L. interrogans* populations, we characterized 271 isolates and seven vaccine strains from China during 1954–2014 using multilocus variable-number tandem repeat analysis (MLVA). 110 different *L. interrogans* MLVA profiles (MTs) were identified, of which five were predominant, reflecting a high level of genetic diversity in *L. interrogans* population in China. Different from that of circulating isolates, seven vaccine strains have different MT, of which some are phylogenetically away from the circulating isolates. The results showed that Icterohaemorrhagiae, Hebdomadis, and Canicola ranked as the top three serogroups among *L. interrogans* strains tested. The cluster analysis demonstrate the clonal links between rodent and human isolates, suggesting the rodent species played a key role in the transmission of leptospirosis to humans, and contributed to the circulation of the pathogen in humans. Taken together, these findings should provide insight into a better knowledge of the epidemiology and molecular evolution of *L. interrogans* in China. Furthermore, the results should facilitate the selection of candidate vaccine strains in the future.

ARTICLE HISTORY

Received 19 July 2018
Revised 12 September 2018
Accepted 22 September 2018

KEYWORDS

Phylogenetic; *Leptospira interrogans*; China; transmission; murine; human

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic species of the *Leptospira* genus.¹ A wide variety of mammalian hosts can act as infection reservoirs.^{2,3} Humans usually get the disease following environmental exposure to *Leptospira*, which were released in the urine of infected animals.^{3,4} Infection occurs during occupational or recreational exposure to contaminated soil and/or water with organisms entering the accidental human host via abrasions or less commonly via the conjunctiva.⁵ It is estimated that there are 1.03 million human leptospirosis cases and 58,900 deaths each year; suggesting that the disease has become as a leading zoonotic cause of morbidity and mortality worldwide.⁶


In China, the earliest leptospirosis case could be traced to the 1930s and it becomes more frequently reported after 1955.⁷ Ten outbreaks of leptospirosis with incidences of more than 10 cases per 100,000 populations and with > 2.5 million cases and > 20,000 deaths reported over the past sixty years.⁷ Improvements in sanitation, and vaccination of high-

risk populations, have played crucial role in reducing the disease burden since 1980s in China, and the incidence of leptospirosis has recently been significantly decreased. However, small localized outbreaks still occur in some epidemic regions in China.^{8–10} Furthermore, due to the variety of geographic and climatic conditions in China, pathogenic leptospiral serovars are far more diverse than in other countries. Over 70 serovars in 18 serogroups of pathogenic *Leptospira* have been isolated and identified from different hosts,¹¹ of which some serogroups (i.e. Manhao) are only found in China.¹⁰ With more recent genetic classification based on DNA relatedness, *L. interrogans* is now recognized as a leading cause of the disease, although other pathogenic species have also been found in China.^{11,12} However, little is known of the genetic diversity and phylogenetic characteristics of *L. interrogans* within China.

The current licensed *Leptospira* vaccine in China is a multivalent, inactivated whole cell vaccine.^{11,13} Three highly virulent (serogroups Icterohaemorrhagiae, Grippotyphosa,

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 supplementary material data can be accessed [here](#).

and Autumnalis) and four low-virulence strains (serogroups Canicola, Pomona, Australis, and Hebdomadis) are used as vaccine strains, which are the major *L. interrogans* serogroups and covered more than 80 % serogroup coverage of circulating strains in the country.¹³ Considering the inherent side-reactions and serogroup-specific immunity from inactivated vaccines, it is suggested that universal recombinant vaccines should be developed in the future. Meanwhile, it is crucial to select the appropriated vaccine strains for understand the genetic diversity of *Leptospira* isolates and vaccine strains.

In recent years, it is reported that multilocus variable-number tandem repeat (VNTR) analysis (MLVA) method can provide high discrimination for genotyping of endemic *Leptospira* prevalence and have favourable consistency with sequencing method.¹⁴ Isolates of *Leptospira interrogans* and *L. borgpetersenii* in East Asia were analyzed by MLVA using 4 loci, demonstrating that there may be variability in the range of genetic diversity among different *Leptospira* serogroups, which may be attributed to maintenance host animals and environmental factors.¹⁵ Pavan and his colleagues also investigated the genetic diversity and epidemiological relationships of *L. interrogans* isolates from Argentina using MLVA by the

loci VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31.¹⁶ Therefore, to better understand the transmission and genetic diversity of *L. interrogans* populations, in this study, we explored the genetic diversity and phylogenetic transmission of *L. interrogans* circulating isolates and vaccine strains in China in the past sixty years using a MLVA method. Furthermore, the modes of transmission, implicated reservoir hosts, prevalent serogroups, and regional distribution of infection were also analyzed.

Results

In total, nine strains were from five countries outside China and seven vaccine strains as well as 271 circulating isolates of *L. interrogans* from China used in the study were from the 11 provinces with the most widespread prevalence of leptospirosis in China (Figure 1). All the seven vaccine strains (CMCC-1, CMCC-2, CMCC-3, 56,601, 56,606, 56,608, 56,609) were human originated. Among the 271 circulating isolates, 76 originated from humans (28%) while 195 were non-human originated (72%) (Supplementary Table 1). Serogroup of the isolates was determined using regular serological methods as

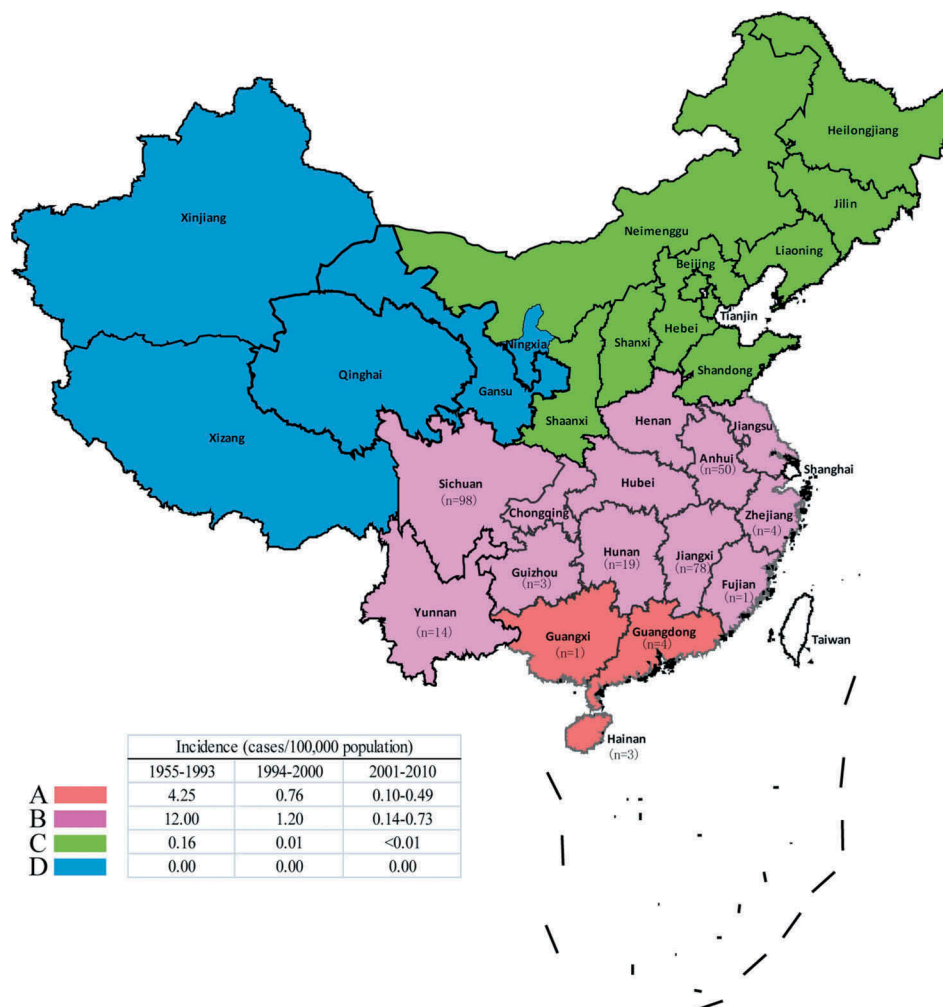


Figure 1. Geographic distribution of 271 *L. interrogans* isolates and seven vaccine strains from China. The number of isolates recovered from each province included in this study are listed. The provinces colored by pink and orange represent leptospirosis in epidemic areas B and A, respectively and the incidence data was also cited from previous studies.⁷

previously described.¹² Of the 271 circulating isolates in China, serogroup Icterohaemorrhagiae (48%), Autumanlis (11%) and Hebdomadis (8%) ranked as the three most prevalent strains. Geographically, Sichuan (35%), Jiangxi (28%) and Anhui (18%) ranked as the three provinces which contributed most to isolate number in China (Figure 1). Icterohaemorrhagiae was the most prevalent serogroup circulating in Anhui (80%), Sichuan (58%) and Jiangxi (44%). Serogroup Autumanlis (16%) and Hebdomadis (18%) were also prevalent in Sichuan province. Grippotyphosa (58%) was the most common serogroup circulating in Hunan province. Serogroups Icterohaemorrhagiae (22%), Hebdomadis (19%) and Canicola (8%) ranked as the three serogroups which contributed most to the pathogen's circulation in humans. Furthermore, Icterohaemorrhagiae was the predominant serotype (53%) circulating in non-humans. Most of the non-human isolates were derived from murine sources. Details of species strain, serogroup, serovar, host, geographic source of isolation and year of isolation are listed in Supplementary Table 1.

The MLVA results showed that the 271 circulating isolates and seven vaccine strains can be resolved into 110 MTs (designated MTs1-110), suggesting high genetic diversity among *L. interrogans* population in China (Figure 2). 82 MTs were represented by single strain including six vaccine strains (CMCC-1, CMCC-2, CMCC-3, 56,606, 56,608, and 56,609) while 15 MTs were represented by two strains. 22 MTs were represented by at least three isolates, of which 5 contained at least 10 isolates (MT1, MT2, MT3, MT4, and MT5). Vaccine strains 56,601 and other two isolates belonged to MT20. Furthermore, different from that of Chinese isolates, nine international reference strains had unique MT types, respectively (Supplementary Table 1). All nine *L. interrogans* reference strains outside of China were located on the most peripheral positions of the MST tree (Figure 2), and were distantly related to strains from China.

MT1 was the most predominant genotype among the isolates analyzed in this study, which was consisted of one strain from Sichuan province and the other 40 strains came from Anhui province. Interestingly, the strain from Sichuan province was separated from human and the other 40 strains from *Apodemus agrarius*, implicating that MT1 isolate may have spread from Anhui to Sichuan province (Figure 2(a,b)). MT2 consists of one strain from Hunan province with the other 23 strains coming from Sichuan province. All MT2 strains were separated from animal hosts, most (92%, 22/24) of which was *Apodemus agrarius* (Figure 2(a,b)). MT3 consisted of 13 strains from Jiangxi province and surprisingly four other animal groups except human (cattle, canine and in the rodents *Rattus rattoides* and *Rattus losea*) were found in this MT (Figure 2(a,b)). Similar to MT2, MT4 consisted of 10 strains from Hunan province along with two subspecies of murine in addition to an isolate of unknown origin. MT5 consisted of 10 isolates from Jiangxi province. Three murine subspecies existed in this MLVA subtype. Furthermore, there were 17 more MTs (MT6-MT22) scattered on the MST tree, whose number isolate ranged from 3 to 8 (Supplementary Table 1).

Further analysis showed that isolates from human and animals located together in many MTs (Figure 2(b)), such as MT1 (human and murine), MT3 (human, cattle, canine, and murine), MT10 (human and murine) and MT19 (human, cattle, and weasel), suggesting that there were direct transmissions of *L. interrogans* pathogens from animals to humans. The numbers shown on the MST branch indicate MLVA distance between adjacent MTs based on the seven VNTR alleles. Strains in one cluster mean that there are no differences among these isolates. Number n means that the adjacent MTs differed from each other by n VNTR alleles. All MLVA distances on the MST tree were analyzed. If not more than 2 alleles difference were found between adjacent MTs, it is regarded that direct or indirect transmission events happened between the two MTs. Based on the MLVA distance data shown on the branches of the phylogenetic tree, we could demonstrate strain clonality between rodent and human isolates, suggesting rodent species played a key role in the transmission of leptospirosis to human in China. Canine and cattle are also important reservoirs for transmission of the pathogen to humans (Figure 2(b)). Transmission between different kinds of animal can also be found (Figure 2(b)).

Congruence of the phylogenetic MTs and serogroups was weak for the strains used in this study (Figure 2(c)). One MT may contain several different serogroups (MT1, MT2, MT3, MT4, MT10, MT11, MT17, MT20, and MT22) and one serogroup may also exist in different gene clusters (e.g., Icterohaemorrhagiae, Grippotyphosa and Canicola). Strains of serogroup Icterohaemorrhagiae represent nearly half of the total stains. These strains are scattered on one side of the MST tree and not very far from each other, which seems like a result of a founder effect. The same situation also happened to serogroup Grippotyphosa and Australis.

Discussion

Leptospirosis has a broad geographical distribution due to wide range of serogroups and large spectrum of mammalian hosts.^{4,17,18} The morbidity and mortality of the disease in different countries are not the same, which is depending on the location, climate, economic development degree.¹⁹ In some developing countries with a tropical climate, the incidence of Leptospirosis are still high.^{20,21} The expansion of urban slums also provides conditions for the spread of the disease.²²⁻²⁵ These factors contribute to distinct epidemiology and genetic diversity of *Leptospira* in different countries and regions. To better control and prevent this disease, it is very important to understand the genetic diversity of *L. interrogans* circulating in China.

Based on the incidences of leptospirosis and latitude and longitude, four epidemic areas of leptospirosis (designated as A, B, C, and D) were divided in China.⁷ Areas A and B, located in Southern China, are the areas with the most widespread prevalence of leptospirosis. With numerous rivers and lakes, a moist climate, and rice-planting tradition, these areas confer advantages for the spread and prevalence of *L. interrogans*. The incidences of leptospirosis in areas C (≤ 0.01) and D (0.00) was much lower than in areas A and B. All 278 *L. interrogans* strains (including 7 vaccine strains) tested in this study were collected

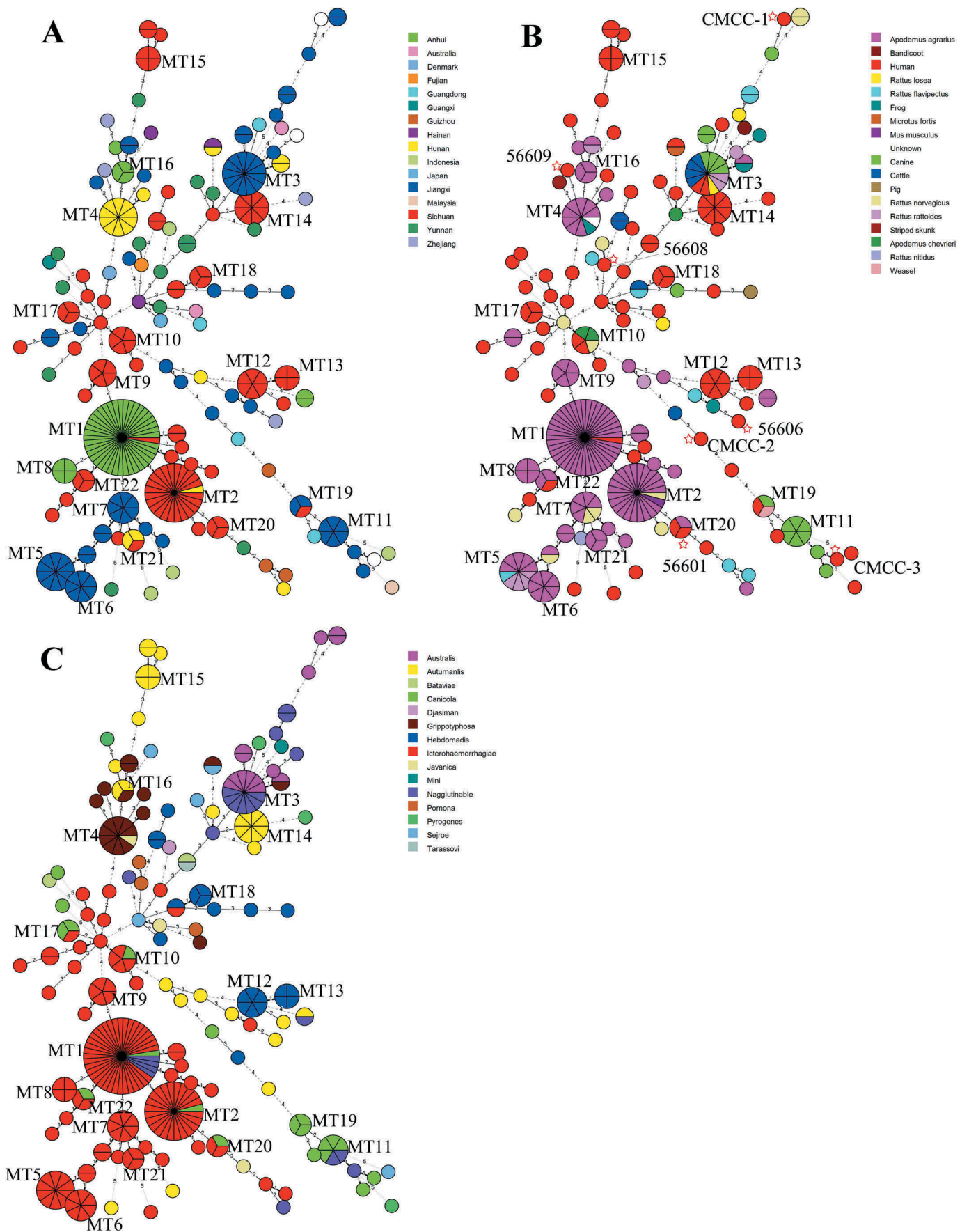


Figure 2. Minimum spanning tree of multilocus variable number tandem-repeat analysis (MLVA) for *L. interrogans* isolates in China. The clustering used the categorical coefficient and eBURST priority rule of the highest number of single-locus changes. Each circle represents an MLVA type (MT). Thick lines, types differ by a single MLVA locus; thin lines, double-locus variants; dotted lines, 2 types differing by > 2 MLVA loci. The size of the circle reflects the number of isolates with a given MT. Only MT containing at least three isolates were labeled in the figure. The color codes for source of isolation, host and serogroups are respectively shown as Figure 2(a,b and c). Pie charts within a circle are used to indicate the proportion of isolates in a given MT. Vaccine strains are labeled with star and strain name respectively in Figure 2B.

from areas A and B. Furthermore, of 34 Chinese serogroup reference strains represented the diversity revealed by serogroup typing of > 300 isolates.¹³ Thus, these strains used should provide a good representation of geographic and genetic diversity of *L. interrogans* population in China.

It is recently reported that 17 different STs were found in 120 serogroup icterohaemorrhagiae strains belonged to three pathogenic *Leptospira* species (*L. interrogans*, *L. borgpetersenii*, and *L. kirschneri*) in China, suggesting a high level of genetic diversity in the serogroup Icterohaemorrhagiae strains.¹² In this study, the MLVA analysis rendered 110 genotypes amongst *L. interrogans* strains circulating in China in the past sixty years, further providing the evidence to support high genetic diversity in *L. interrogans* population. Whole genomic analysis suggested that the same pathogenic *Leptospira* species may be undergo similar evolution from the most recent common ancestor.²⁶ The genetic heterogeneity among *L. interrogans* population also reflect that different mechanism may involved with intraspecies evolution.

The results showed that all nine *L. interrogans* reference strains outside of China were located on the most peripheral positions of the MST tree (Figure 2), and were distantly related to strains from China. Thus, the emergence and re-emergence of *L. interrogans* in different regions of China was not caused by the introduction of exotic strains, but by the persistence of local reservoirs of infection. Basing the MST tree as a source of geographic information suggests that Jiangxi and Sichuan provinces played key roles in the circulation of *L. interrogans* in China. Though up to 50 isolates were also recovered from Anhui province. Almost all of these clustered together in MT1 with small links occurring between Anhui and other provinces. But another situation was found in Jiangxi and Sichuan province (Figure 1(a)). MT3, MT5, MT6, MT7, and MT11 were found in Jiangxi with up to 26 small size (1 or 2 isolates) MTs also found. Most of these MTs are phylogenetically far from each other and when scattered on the MST tree show a tight connection with other provinces, such as Sichuan, Hunan, Zhejiang, and Guangdong. The situation in Sichuan is the same as Jiangxi. MT2, MT9, MT10, MT12, MT13, MT15, MT17, MT18, and MT22 were found in Sichuan with up to 24 small size (1 or 2 isolates) MTs also found in this province. Most of these MTs are phylogenetically far from each other and when scattered on the MST tree show a tight connection with other provinces, such as Jiangxi, Yunnan, Hunan, Anhui, and Guangdong. Jiangxi and Sichuan are located on the Yangtze River Basin. Numerous rivers and lakes, a moist climate, a large agricultural population base and rice-planting are all advantageous for the spread and prevalence of *L. interrogans* within and out of these two provinces. A possible explanation is that circulating isolates derived from a single source, for example water contaminated by a strain of *L. interrogans*. Another explanation is that the pathogen may spread among these species over a short period.

The cluster analysis indicated that rodent species played a key role in the transmission of leptospirosis to humans in China. Their large numbers and extensive distribution in nature are important reasons.^{1,27} When water and soil are contaminated by murine urine or excrement, they can

become a pathogen source. Leptospirosis case investigation reports and deaths recorded by China CDC investigators submitted from 2003–2017 were reviewed (earlier data were not available from the website). Most of the leptospirosis cases in China occurred from July to December with a peak in September (Supplementary Figure 1).⁷ This is the time of rice planting and harvest in Southern China. Rats are also more active in these months.²⁸ Canine and cattle are also important reservoirs for the transmission of the pathogen to humans. Farmers may get infected from the domestic animals they feed or come into close contact with. That is why in rice-planting seasons in southern China, many cases of *L. interrogans* were reported in farmers.

Consistent with the previous finding¹⁴, we also found week congruence between typing results of MLVA methods and serological tests for the 287 *L. interrogans* isolates. The two methods were associated with different types of genetic variation. MLVA methods can also show variation in repeated sequences, which are extensively located in the genome of pathogen.^{14,15} Serological tests were involved with the evolution of the membrane proteins. Molecular mutations on the membrane proteins can be a result of positive or negative selection.^{1,10} However, mutations detected by MLVA methods may be indicative of neutral selection.^{14,15,29} Therefore, in order to understand the epidemiology and genetic evolution of pathogenic *Leptospira*, it is suggested that a combined molecular (MLVA or multilocus sequence typing) and serological analysis may be a better approach for the typing of this pathogen.

Vaccination of at-risk populations remains the most viable strategy to control leptospirosis. An inactivated whole-cell vaccine including seven major *L. interrogans* serogroups is still used in the Chinese Expanded Program on Immunization.^{11,13} Not surprising, seven vaccine strains have different MT, which were different from other circulating isolates. It is possibly associated with these vaccine strains belonged to old circulating isolates and replaced by new type isolates. Vaccine strains, such as CMCC-1, 56,606 and 56,609, are phylogenetically away from the circulating isolates from human (Figure 2(b)). According to this study, candidate vaccines should be selected around MT17, MT12, MT18 or MT14, which was the gathering location of circulating isolates in human on the MST tree. Furthermore, considering the short-lived serogroup-specific immunity acquired from current inactivated vaccines, it is necessary to develop a universal recombinant leptospirosis vaccine.¹³ The data of genotyping for circulating isolates and vaccine strains will contribute to the choices of vaccine strain and efficacy evaluation of leptospirosis vaccine product.

Conclusions

Systematic large-scale studies were carried out that provided detailed information on the epidemiologic and phylogenetic characteristics of *L. interrogans* prevalence in China. This study described the genetic diversity and phylodynamic transmission of *L. interrogans* in China, with special focus on modes of transmission. Our results implicated: reservoir hosts; prevalent serovars; and regional distributions of the

pathogen. The results of phylogenetic analysis indicated that 278 isolates circulating in China were resolved into 110 MTs. Five major MTs were found. Cluster analysis suggested that rodent species played a key role in the transmission of leptospirosis to humans. Our results should facilitate the selection of candidate vaccine strains, which contribute to the development of new *Leptospiravaccine*.

Materials and methods

Bacterial strains and culture

Two hundred and seventy-one *L. interrogans* representative of isolates from eleven provinces during 1954–2014 and seven vaccine strains were used in this study (Figure 1). The related epidemic information for each isolate including: host, source of isolation and year of isolation were collected. Furthermore, nine *L. interrogans* reference strains from other countries were also included during this study (Supplementary Table 1).

All isolates were grown in *Leptospira* Medium Base Ellinghausen McCullough Johnson Harris (EMJH) supplemented with 10% rabbit serum to late log phase and harvested by centrifugation²⁷. Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) according to the manufacturer's instructions. Serogroup of the isolates were identified using a microscopic agglutination test as previously described.¹²

A map of the distribution of isolates showing the geographic origin and number of isolates per province in China was developed (Figure 1). We also reviewed leptospirosis case investigation reports and deaths by Chinese CDC investigators that were submitted from 2003–2017 (previous data are not available from the website). These data can be downloaded from website of China CDC (www.chinacdc.cn).

MLVA analysis

Seven VNTR loci, including VNTR4, VNTR7, V27, V29, V30, V36, and V50, were selected, and the primers for each VNTR loci were used as previous described.^{29,30} PCR were performed using a 50 µl volume, containing: 1× PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.2 µM forward and reverse primer, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 2.5 ng DNA template, and double-distilled water to make up the final volume. Thermocycling parameters included an initial denaturation at 94°C for 10 min followed by 25 cycles of 94°C for 30 s, a specific annealing temperature for each VNTR loci for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR product was then sequenced by using an ABI 3730XL system (Applied Biosystems) to identify the size of each fragment.

Data analysis

Allelic sizes were then converted into repeat copy numbers using the formula: repeat number = [fragment size (bp) – flanking regions (bp)]/repeat unit size (bp). The raw repeat copy numbers were then converted into integers. The assignment of an MLVA type was based on the combination of repeat counts for VNTR4,

VNTR7, V27, V29, V30, V36 and V50. MLVA data, expressed as allelic profiles for isolates, were analyzed using Bionumerics software v7.5 (Applied-Maths). A minimum-spanning tree based upon multilocus, variable number of tandem repeat analysis, was constructed.

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

Funding

This work was supported by National Natural Science Foundation of China (No. 81471968, 81601812 and 81801436); National Science and Technology Major Project of the Ministry of Science and Technology of China (No. 2013ZX09304101, 2017ZX10303405-002 and 2018ZX10714002); Program of High Level Creative Talents Cultivation of Guizhou Province [No. 4021(2016) of Qian Ke He Talent].

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