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Re-emergence of canine *Leishmania infantum* infection in mountain areas of Beijing

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Abstract

Canine Leishmaniasis (CanL) is an endemic infectious disease in China, causing visceral Leishmaniasis (VL) and resulting in important public health problem. However, in the last 3 y, endemic trends have changed considerably and spatial–temporal aggregation areas have shifted from northwestern to central China. Although Beijing was an endemic area for CanL in the last century, this disease has not been reported in Beijing since control programs were implemented in the 1950s. In the present study, PCR and immunochromatographic (ICT) were used to estimate prevalence of *Leishmania* infection in domestic dogs living in Beijing, a VL re -emergencearea. In total, 4420 canine blood samples were collected at vet clinics in 14 districts of Beijing. Overall prevalence (percentage of dogs seropositive and/or PCR positive) of CanL infection in Beijing was 1.22% (54/4420). However, prevalence of CanL in the western mountain areas was 4.68% (45/961), significantly higher than that (0.26%, 9/3459) of the plains. In addition, multilocus sequence typing (MLST) of seven enzyme-coding genes was used to examine phylogenetic relationships of CanL strains. Forty-one *Leishmania infantum* isolates were well separated from the other strains and divided into five major clades (A to E) by MLST analysis. All clades were closely related to strains from Sichuan Province and Gansu Province. A phylogenetic tree, based on the MLST, revealed that *L. infantum* in Beijing was genetically related to strains from western endemic of Mountain type VL in China. In conclusion, CanL has re-emerged in Beijing, and almost 5% of dogs living in Beijing's mountain areas were infected with *L. infantum*. The phylogenetic tree based on MLST effectively distinguished species of *Leishmania* and reflected geographical origins. Because dogs are considered a natural reservoir, comprehensive control measures including surveillance, phylogenetic analyses and management should be implemented to mitigate or eliminate Leishmaniasis.

Keywords Canine Leishmaniasis, Re-emergence, Prevalence, MLST

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Introduction

Visceral Leishmaniasis (VL), also known as kala-azar, is a vector-borne zoonotic disease caused by more than 20 protozoan parasites of the genus *Leishmania*, posing serious threats to human and animal health. There are many clinical manifestations of VL, including prolonged irregular fever, spleen enlargement, anemia, emaciation, leukopenia, and increased serum globulin levels [1].

Currently, this zoonosis is prevalent in 88 countries across East Africa, South Asia, South America and the Mediterranean. The worldwide annual incidence of VL is estimated at 200,000–400,000 cases globally, with approximately 60,000 VL deaths due to failure in timely treatment [2]. Before the founding of People's Republic of China, the prevalence of Leishmaniasis both in humans and dogs was extremely high in north region of the Yangtze River, involving 16 provinces and autonomous regions. However, after a nationwide comprehensive prevention and control program from 1949–1958, the disease was eliminated gradually in major epidemic areas, including Beijing [1]. Nevertheless, 200 to 500 VL human cases are reported in China annually, sparsely distributed in rural areas of Xinjiang Uygur Autonomous Region, Gansu and Sichuan [3].

Clinically and geographically, VL infection was classified into three main forms: anthroponotic visceral Leishmaniasis (AVL), desert-type zoonotic visceral Leishmaniasis (DT-ZVL), and mountain-type zoonotic visceral Leishmaniasis (MT-ZVL) [1]. Both AVL and DT-ZVL are predominantly endemic in Xinjiang, whereas MT-ZVL is prevalent in the extension region of Loess Plateau, including Gansu, Sichuan, Shaanxi, and Shanxi provinces. However, the incidence of MT-ZVL has increased rapidly in the last 5 years, accounting for 94.44% of the total number of VL human cases in China [4]. Re-emergence of MT-ZVL has expanded into central China, including the Yanshan-Taihangshan mountain areas (Henan, Hebei and Beijing) [5], suggesting it is a serious public health problem in China.

In MT-ZVL endemic areas, domestic dogs infected with CanL act as parasite reservoirs in transmission to humans or dogs. Canine *Leishmania* (CanL) is caused by *Leishmania infantum* infection and transmitted by *Phlebotomus chinensis* [6]. Beijing was once an endemic area of VL and CanL. *L. infantum* was also isolated in wild animals in the hilly area of Beijing [1]. In 2022, human and dog populations in Beijing were 22 million and 2 million, respectively. A large number of CanL cases and one VL human were confirmed in Beijing in the last 3 years. However, there are almost no reports regarding prevalence of *Leishmania* infection in humans and dogs since implementation of the control program in the 1950s.

Serological antibody tests and PCR have been extensively used to investigate canine infection with *L. infantum* [7]. In addition, molecular biology tools are widely used to assess epidemiology, transmission, and pathogenicity of parasites. Multifocal enzyme electrophoresis (MLEE) is currently the gold standard for identification and typing of *Leishmania* [8]. However, the use of MLEE is limited by the disadvantages of being time-consuming, technically demanding, and the need for large numbers of parasites to be cultured [9]. This approach has, therefore, been replaced by more efficient and convenient methods such as multifocal sequence typing (MLST). The latter has widely been used in molecular epidemiological studies of bacterial pathogens, also applied to *Leishmania* including 10 loci for the *Leishmania donovani* complex [10] and four or five markers for *L. viannia* spp. [11]. These reports clarified that MLST had high resolution on *Leishmania* species but also discussed the need to analyze more candidate genes in a broader range of Leishmaniasis species. Based on published targets for the subgenus *Leishmania*, we combined seven enzyme-coding genes (*falat, enol, pgm, spdsyn, g6pdh, icd* and *mpi*) to distinguish Chinese *Leishmania* isolates and to study phylogenetic relationships.

The objective of this study was to determine the prevalence of *L. infantum* infection in domestic dogs living in Beijing and investigate phylogenetic relationships among Chinese *Leishmania* isolates. Understanding the prevalence and phylogenetic relationship of *Leishmania* infection in domestic dogs should provide evidence to support disease management strategies, benefitting both animal and human health.

Results

Distribution of canine Leishmaniasis in Beijing, based on serology and PCR

A total of 4420 domestic dogs from 14 of 16 districts of Beijing were randomly selected and blood samples were collected for serological tests (immunochromatographic, ICT) and PCR detection (Fig. 1). The overall prevalence (percentage of dogs seropositive and/or PCR-positive) of CanL infection in Beijing was 1.22% (54/4420; 95% CI: 0.89–1.54%). The prevalence was 0.68% (30/4420; 95% CI: 0.44–0.92%) by ICT and 0.93% (41/4420; 95% CI: 0.65–1.21%) by PCR, respectively. Geographically, the prevalence was highest at 9.0% (23/256; 95% CI: 5.5–12.5%) in Mentougou District, followed by 4.7% (11/232; 95% CI: 2.0–7.4%) in Shijingshan District, 3.2% (6/190; 95% CI: 0.7–5.7%) in Yanqing District, 2.3% (3/128; 95% CI:0–5.0%) in Huairou District, 1.4% (4/289; 95% CI: 0.03–2.7%) in Changping District, 1.3% (2/155; 95% CI: 0–3.1%) in Fangshan District, and 0.9% (5/563; 95% CI: 0.1–1.7%) in Haidian District. In

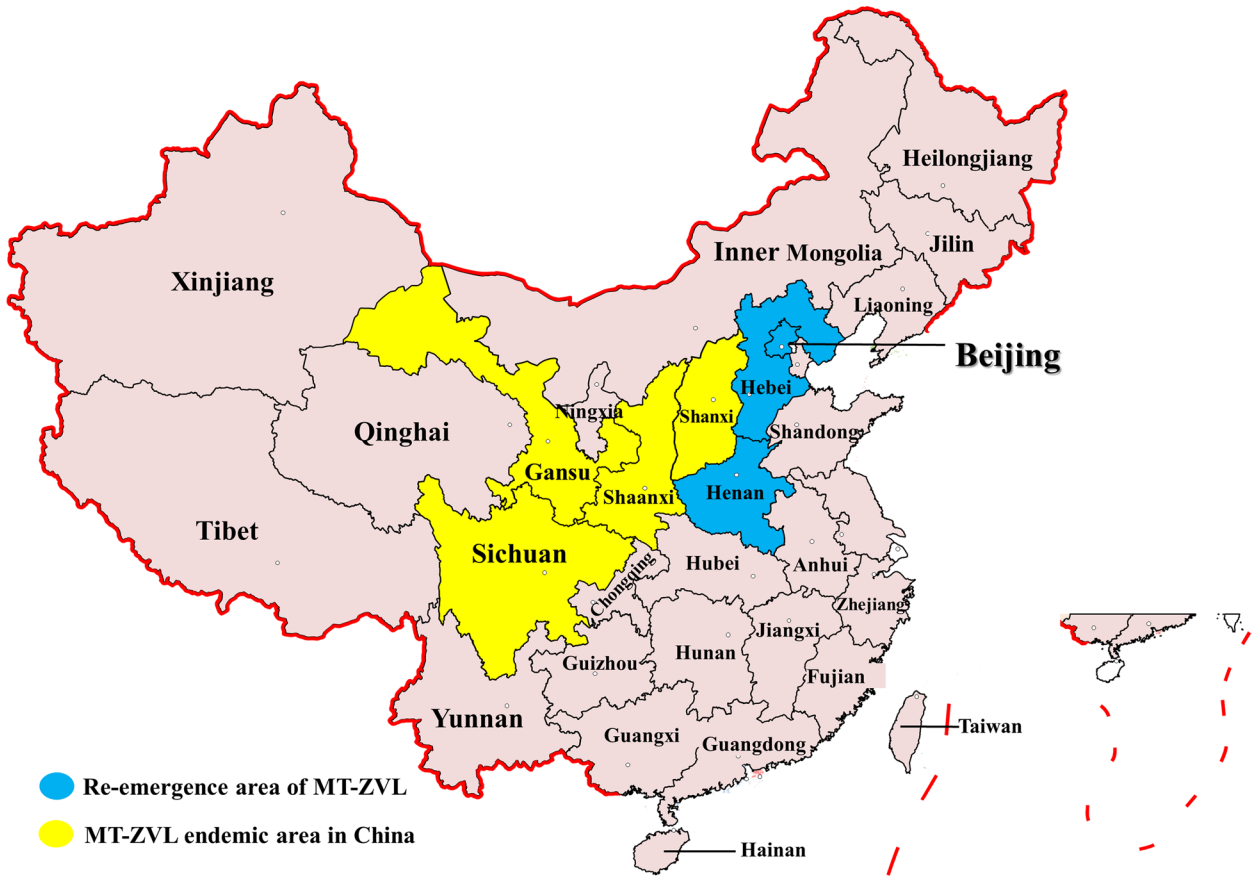

Fig. 1 Map of China with locations of sampled sites. MT-ZVL: mountain-type zoonotic visceral Leishmaniasis

all other districts, none of the total of 2607 dogs was seropositive or PCR-positive (Fig. 2, Table 1).

Nine of the 3459 domestic dogs from plains of Beijing were infected with CanL, for a prevalence of 0.26% (95% CI: 0.09–0.43%). In contrast, 45 of 961 dogs from mountain areas of Beijing were infected with CanL, for a prevalence of 4.7% (95% CI: 3.34–6.02%) ($p < 0.01$) (Table 1). Regional distribution had a significant effect on CanL infection in domestic dogs. In the 9 infected dogs from plain areas, 5 (56%, 95% CI: 23–88%) had travelled to the western mountains in the last 24 months.

Twenty (37%, 95% CI: 24–50%) out of 54 positive dogs presented one or more clinical signs of CanL (i.e., skin lesions, cachexia, onychogryphosis, alopecia, periorbital rings of alopecia, and lymph node enlargement) whereas the other 34 dogs were asymptomatic (63%, 95% CI: 50–76%). Results for clinical status, serology (including ELISA and dipstick test), and PCR detection are presented in Table 2. A total of 17 dogs were test-positive by both methods of which 10 had clinical signs. Thirty dogs were positive by ICT with 16 (53%, 95% CI: 35–71%) of them having clinical signs. Four dogs with clinical signs of Leishmaniasis were ICT-negative. The presence of parasite DNA was detected in 41 dogs, with 14 (34%, 95% CI: 19–49%) of these dogs having clinical signs.

MLST and phylogenetic analyses distinguished species of *Leishmania* and reflected geographical origins

The unrooted phylogenetic tree presented in Fig. 3 was constructed based on the joint sequence of *alat*, *enol*, *pgm*, *spdsyn*, *g6pdh*, *mpi* and *icd* using the Neighbor-joining method. The 41 *Leishmania* isolates from Beijing were grouped as a population into the *L. donovani* complex and well separated from other strains. Furthermore, isolated strains were divided into five major clades (A to E) with other strains retrieved from the database. MTG-ZZ5 formed a group (clade A) with *L. chagasi* MCER/BR/1981/M6445. The phylogenetic tree detected strain WMS-7 divided into a distinct cluster (clade B). Thirty-six isolates closely related to *L. infantum* JPCM5 were divided into clade C. Two other groups (clade D and E), consisting of LSS-17, NN-22 and XBD-21, were separated from the other Chinese isolates.

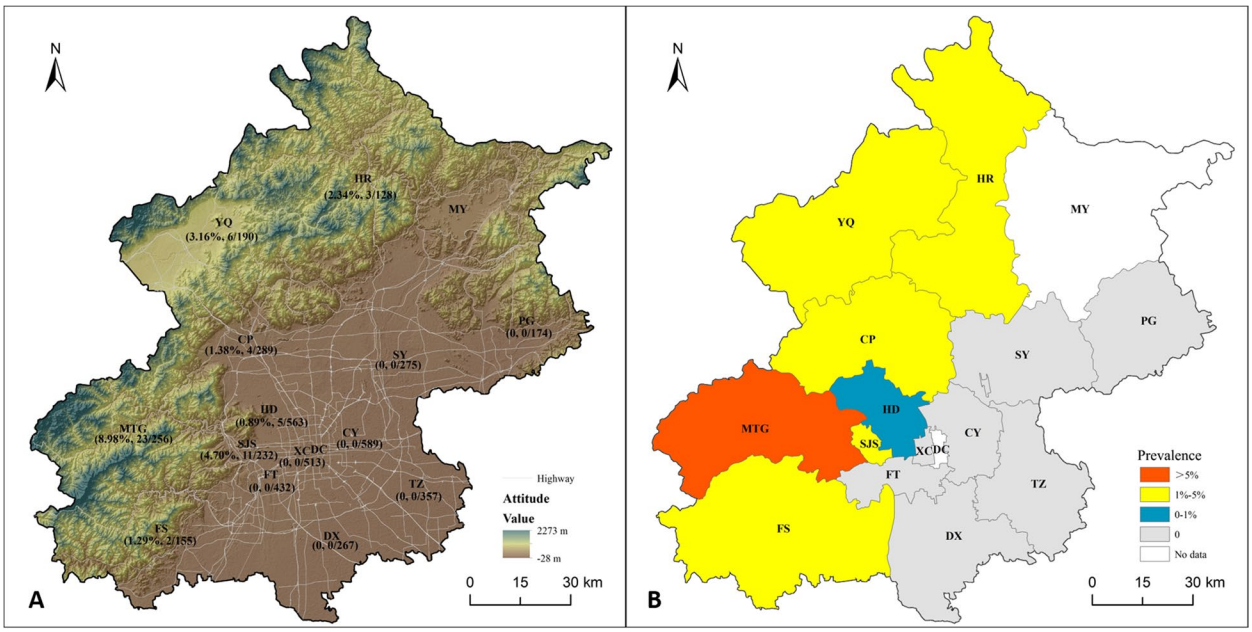

Fig. 2 Prevalence and geographical locations of canine Leishmaniasis in domestic dogs from Beijing from 2021 to 2022. DC, Dongcheng District; XC, Xicheng District; CY, Chaoyang District; HD, Haidian District; FT, Fengtai District; SJS, Shijingshan District; CP, Changping District; DX, Daxing District; FS, Fangshan District; MTG, Mentougou District; MY, Miyun District; PG, Pinggu District; SY, Shunyi District; TZ, TongZhou District; YQ, Yanqing District; HR Huairou District. Maps were created using ArcGIS 10.8

Allelic profiles and sequence types (ST) of all strains of *Leishmania* were listed in Table 3. Analysis of the minimum spanning tree results in Fig. 4 demonstrated the presence of ST1 as the major genetic contributor to these populations, with the ST1 type accounting for 46.3% of all isolates. Other STs such as ST14, ST25 and ST26 were also predicted to be genetic sponsors of other ST members in the clonal complex.

Phylogenetic relationships between isolates in this study and those from other parts of Asia were investigated. In the neighbor-joining tree, the five clades of the Beijing isolate formed six subclades with the reference strain, and the branches clustered in the tree reflected the geographic origin of Leishmaniasis (Fig. 5). Among them, Clade A formed a clade with strains from Sichuan Province, whereas Clades B-E formed a clade with the

Table 1 Serology and PCR detection results of canine Leishmaniasis in domestic dogs from different districts of Beijing

District	ICT (%)	PCR (%)	Total (%)	Total Sample No	Prevalence (%)
Mentougou	14 (5.47)	18 (7.03)	23 (8.98)	256	8.98
Shijingshan	5 (2.16)	8 (3.45)	11 (4.70)	232	4.70
Yanqing	4 (2.11)	5 (2.63)	6 (3.16)	190	3.16
Huairou	1 (0.78)	2 (1.56)	3 (2.34)	128	2.34
Fangshan	2 (1.29)	2 (1.29)	2 (1.29)	155	1.29
Pinggu	0 (0)	0 (0)	0 (0)	174	0
Changping	2 (0.69)	3 (1.04)	4 (1.38)	289	1.38
Shunyi	0 (0)	0 (0)	0 (0)	275	0
Haidian	2 (0.36)	3 (0.53)	5 (0)	563	0.89
Fengtai	0 (0)	0 (0)	0 (0)	432	0
Xicheng	0 (0)	0 (0)	0 (0)	513	0
Chaoyang	0 (0)	0 (0)	0 (0)	589	0
Tongzhou	0 (0)	0 (0)	0 (0)	357	0
Daxing	0 (0)	0 (0)	0 (0)	267	0
Total	30 (0.68)	41 (0.93)	54 (1.22)	4420	1.22

Table 2 Clinical status, serology and PCR detection of canine Leishmaniasis in domestic dogs of Beijing

Group No	ICT	PCR	Clinical status		
			No. symptomatic dogs	No. asymptomatic dogs	Total No. dogs
1	+	+	10	7	17
2	+	-	6	7	13
3	-	+	4	20	24
4	-	-	0	4366	4366
Total			20	4400	4420

reference strains from Gansu Province. The Xinjiang strains were grouped into two separate clades, while the Shandong strain was closely related to the Indian and Nepalese strains. The reported reference strains from Asia and the Beijing isolate were well separated from the reference strains from other regions, and Clades A to E in this study were more closely related to Sichuan and Gansu, which are endemic areas of MT-ZVL.

Discussion

VL is an important endemic infectious disease in China. Currently, two epidemiological types of VL, anthroponotic (AVL) and zoonotic (ZVL), are present in China [1], with AVL endemic only in the oases of the plains of Kashi prefecture, Xinjiang Uygur Autonomous Region. Most cases occur in juveniles and adult people. In addition, ZVL can be divided into two subtypes, namely mountainous and desert sub-type; the latter is endemic in the northwestern desert regions of China, including Xinjiang and western Inner Mongolia. This region is considered a natural nidus of kala-azar-infected wild animals that are presumably the source of human infection. The mountainous subtype, caused by *Leishmania infantum*, occurs in the western mountainous and hilly regions of Gansu, Sichuan, Shaanxi, and Shanxi provinces of China, where *Ph. chinensis* was the vector and dogs were reservoirs with high prevalence [4]. Elimination and prohibition of dogs in the endemic area markedly reduced the number of human cases, indicating dogs were likely the principal source of infection for the MT-ZVL [1]. Notably, a longitudinal national study from 2004 to 2019 demonstrated that epidemic trends changed significantly and spatial–temporal aggregation areas shifted from northwestern to central China [12]. This trend was not only observed in historical endemic areas (Gansu, Sichuan, Shaanxi and Shanxi provinces), but also in nonendemic areas of Henan and Hebei provinces, indicating re-emergence of MT-ZVL in these two provinces [4]. From 2019–2021, the incidence of MT-ZVL rocketed rapidly and generated new hotspots in central China, including re-emergence in the Yanshan-Taihangshan mountain areas (Henan, Hebei and Beijing) [5]. During the last 3 years, cases of MT-ZVL accounted for 94.44% of the total number of reported cases in China, indicating a huge threat to residents of Yanshan-Taihangshan mountain areas, including Beijing. Therefore, evaluation of prevalence of CanL is of great importance to understand epidemiology of the mountainous sub-type of ZVL in Beijing and to prevent transmission of human VL. In the present study, blood samples from domestic dogs in various districts of Beijing were collected and prevalence of *L. infantum* infection in these dogs was evaluated.

Beijing once was an endemic area of CanL and *L. infantum* was also isolated in a wild raccoon dog (*Nyctereutes procyonoides*) in the hilly area of Beijing; however, there has been no report of canine VL since implementation of the control program in the 1950s [1]. In the present study, the highest prevalence (8.98%) of CanL in Beijing was detected in a typical mountain area-Mentougou District, and the average prevalence in the mountain area of Beijing was 4.68%. The high prevalence of CanL in the mountains of Beijing indicated a potential threat to the security of Beijing, as western mountains are popular tourist sites. In 2020, an outbreak of MT-CanL occurred in nonendemic Yangquan City, adjacent to Beijing and also in the Yanshan-Taihangshan mountain areas, revealing high positive rate (5.97%) of *Leishmania* specific antibodies in domestic dogs [13]. The true prevalence of CanL in Yangquan could be considerably higher as serological detection was not sensitive enough to detect infection in asymptomatic dogs. In endemic areas of China, a relatively high positive rate of CanL has been reported, including 59.43% in Jiuzhaigou of Sichuan, 41.90% in Heishui of Sichuan, and 77.21% in Wenxian of Gansu [14, 15, 16]. Although prevalence of CanL in Beijing was lower than the endemic areas, high density of residents and dogs provided a relatively large number of sensitive animals and might introduce an epidemic of canine or human *Leishmania*. Notably, a higher prevalence (> 30.0%) of CanL in the stray dogs of mountain areas of Beijing was observed (data not shown). The parasites might be transmitted from wild animals to stray dogs, and subsequently to domestic dogs or human, consistent with transmission characteristics of MT-ZVL [4]. During transmission of parasites between hosts, the lifecycle of *Ph. Chinensis* has a vital role, allowing the parasite to spread among local wild animals and maintaining *Leishmania* in its natural habitat, while concurrently transmitting it to humans and dogs and causing VL.

In epidemiological studies on CanL, serological antibody tests and PCR detection have been extensively used to investigate canine infection with *L. infantum* [7]. The

Fig. 3 Phylogenetic reconstruction based on the seven gene markers of the MLST scheme. The 41 isolates in this study were constructed as a Neighbor-joining unrooted tree with the 20 reference strains. Sequencing data of seven genes (*alat*, *enol*, *pgm*, *spdsyn*, *g6pdh*, *mpi* and *icd*) were used to build this phylogenetic tree, which was calculated using the maximum likelihood method based on the Tamura-3 parameter model. Bootstrap values were calculated from 1000 replications

standard test for Leishmaniasis is the rK39 strip test; however, a serological antibody test only detects symptomatic dogs but cannot detect asymptomatic carriers [17]. Therefore, an alternative detection method for asymptomatic canines is warranted. The sensitivity of ITS-1 nested PCR in symptomatic dogs was estimated to be 73.9% [18] and subsequently confirmed [19].

Application of high-throughput molecular techniques advances reliable identification and classification of parasites [20]. MLST is an efficient method for understanding epidemiology, transmission and phylogeny of infectious diseases [21]. It is expected to become the new gold standard method for identification of *Leishmania* spp in the future [22], although only a few studies have

Table 3 Allelic profiles and sequence types (STs) of all strains of *Leishmania* included in the study

Strain/isolate	*alat*	*enol*	*pgm*	*spdsyn*	*g6pdh*	*mpi*	*icd*	ST
W-692	1	1	1	1	1	1	1	1
HH-1	2	1	1	1	1	1	1	2
BMY-1	1	1	1	1	1	1	1	1
HBH-3	1	2	1	1	1	1	1	3
YSL-2	1	1	1	1	1	1	1	1
LML-3	3	1	1	1	1	1	1	4
BTJ-23	1	1	1	1	1	1	1	1
DH-4	1	1	1	1	1	1	1	1
BXY-21	1	1	1	1	1	1	1	1
HH-5	1	1	1	1	1	1	1	1
YZ-18	1	1	1	1	1	1	1	1
SSH-6	1	1	1	1	1	1	1	1
RCDX-20	1	1	1	1	1	1	1	1
HB-3	4	3	1	1	1	1	1	5
SH-22	1	1	1	1	1	1	1	1
XH-4	5	4	1	1	2	1	1	6
WY-7	1	1	1	1	1	1	1	1
ZQY-2	1	1	1	1	1	1	1	1
ZQY-1	6	1	1	1	1	1	1	7
ZQ-26	1	1	1	1	1	1	1	1
XH-6	1	1	1	1	1	1	1	1
WM-7	7	1	1	1	1	1	1	8
WMS-9	8	1	1	1	1	1	1	9
BZHW-12	1	1	1	1	1	1	2	10
CPH-13	9	5	2	1	1	1	1	11
2018–14	1	1	2	1	1	1	2	12
GDH-15	10	1	1	1	1	1	3	13
LJF-11	1	1	1	1	1	2	1	14
LNN-17	1	6	2	2	3	2	2	15
2018–13	1	1	2	1	1	2	1	16
CMH-18	1	7	1	1	1	2	1	17
LSS-17	1	1	1	1	4	3	1	18
XBD-21	1	1	1	1	1	3	3	19
NN-22	11	8	1	1	1	3	3	20
MTG-D5	1	1	1	1	1	1	1	1
MTG-C14	1	1	1	1	1	1	1	1
MTG-ZZ4	1	1	1	1	1	1	1	1
MTG-C9	7	1	1	1	1	1	1	8
MTG-D1	1	1	1	1	1	1	1	1
MTG-ZZ5	1	1	1	1	1	1	4	21
WMS-7	1	1	1	1	1	1	5	22
Pasteur	1	1	1	1	1	1	6	23
M6445	1	1	1	1	1	1	7	24
JPCM5	1	1	1	1	5	1	1	25
LdCL	12	9	1	1	6	4	8	26
AG83	12	9	1	3	5	4	8	27
BPK282A1	12	9	1	3	5	4	8	27
TR01	13	10	3	3	7	5	1	28
Friedlin	14	11	3	4	8	6	9	29

Table 3 (continued)

Strain/isolate	*alat*	*enol*	*pgm*	*spdsyn*	*g6pdh*	*mpi*	*icd*	ST
U1103	15	12	1	5	9	1	10	30
UA301	16	13	3	6	10	1	11	31
M2904	17	14	3	5	11	1	1	32
M4147	18	15	3	5	12	1	12	33
PSC-1	19	15	3	5	13	1	13	34
216–34	20	16	4	7	14	7	14	35
LEM1108	21	17	5	8	15	8	15	36
LEM3045	22	18	6	9	16	9	16	37
LEM452	23	19	7	10	17	10	5	38
LEM423	24	20	8	11	18	11	17	39
Scaffold3675	25	13	9	12	10	12	1	40
Parrot Tar II	26	21	10	13	19	13	1	41

used MLST to characterize Leishmaniasis parasites isolated from canine sources. In our study, seven previously reported housekeeping genes (*alat*, *enol*, *pgm*, *spdsyn*, *g6pdh*, *mpi* and *icd*) were selected based on the MLST approach to investigate genetic diversity of 41 *Leishmania* isolates from canine source in Beijing, and to explore phylogenetic relationships and evolution of Chinese Leishmaniasis isolates by linking genetic variation in *Leishmania* of Beijing canine origin to previous reports in China. A substantial number of haplotypes were detected for each marker and several unique STs identified among strains.

Based on the tree constructed from our database obtained by high-throughput sequencing and other sequences of the worldwide *Leishmania* complex, comparison of seven housekeeping genes effectively separated *Leishmania* species complexes worldwide. Furthermore, the Chinese *Leishmania* isolates (Clade A to E) were sisters to other members of the worldwide *L. donovani* complex. Canine-derived *Leishmania* complex species from Beijing, China, were isolated into five clades, of which Clade A was most closely related to *L. chagasi* from Brazil, whereas clade C species were associated with *L. infantum* in Europe, and Clade B isolate WMS-7, Clade D isolate LSS-17 and Clade E isolates NN-22 and XBD-21 were isolated from other *L. donovani* complex reference strains with strong bootstrap support. Previous studies indicated differences between the *L. donovani* complex in Asia and North America, and our results corresponded to previous studies [23]. In our study, the level of SNP variation was low, but existing differences in tandem sequences had considerable genetic diversity. When genetic sequences of Chinese *Leishmania* strains were compared to those of WHO reference strains, neighbor-joining or maximum parsimony methods identified several clusters. Indeed, these clusters were observed in other tree topologies using various models, suggesting that the derived population is robust and not affected by choice of evolutionary method underlying tree construction.

The eBURST typing results demonstrated that all isolates were classified as clonal complexes without any monomers, perhaps due to high mutation rates between isolates and reference strains rather than recombination. The STs in this study were redefined because there is no systematic database; based on minimal spanning tree results, ST1 was the major genetic contributor to the population. In the clonal complex analysis, identical STs were related, and ancestors were reliably represented by eBURST. Because eBURST reduces differences among *Leishmania* strains, it is considered a useful clustering tool for analysis of relevant MLST data [24].

The region of strain isolation is a more important predictor of genetic relationships than the clinical type of disease [25]. Phylogenetic analyses of Chinese *Leishmania* species demonstrated that isolates within a clade were subdivided according to geographic origin, suggesting that intraspecific differentiation of strains was related to the geographic distribution of Leishmaniasis [26]. Our evolutionary tree analysis between isolates of this study and the previously reported subgenus *Leishmania* in China indicated an association between intraspecific differentiation and the geographic distribution of species. In the evolutionary tree results, Clade A was more closely related to isolates from Sichuan, and Clades B to E were closely related to most of the reference strains from Gansu. From 2019 to 2021, clusters of high Leishmaniasis incidence areas were mainly maintained in eastern and southeastern Shanxi Province, the border of Shanxi and Shaanxi Provinces, and the border of southern Gansu and northern Sichuan regions [5]. Although southern Gansu and northern Sichuan still had high prevalence

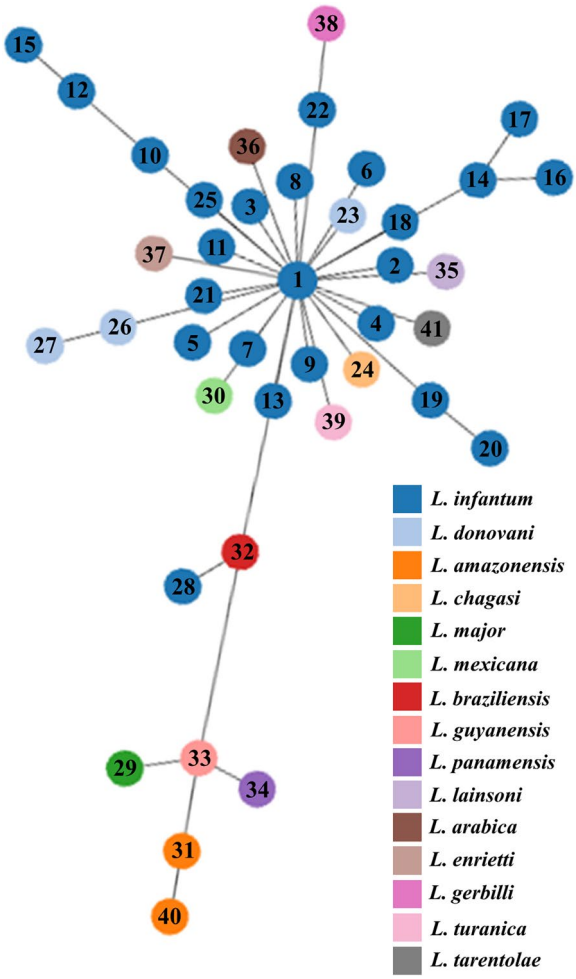

Fig. 4 Minimal spanning tree constructed by ST typing of isolates and reference strains. The eBURST algorithm was employed to evaluate relationships between 41 STs, corresponding to all *Leishmania* strains included in our analysis using seven gene markers of the MLST scheme. Clonal complexes showing relationships between STs are further classified according to *Leishmania* species, as shown by dark blue nodes (*Leishmania infantum*) and light blue nodes (*Leishmania donovani*)

areas in the traditional sense, we inferred that a new high prevalence area of CanL has also been formed in Beijing. Regarding geographical distribution of mountain ranges in China, Qinling Mountains in northern Sichuan are connected to the Qilian Mountains where Gansu is located, and also adjacent to the Taihang Mountains, whereas the Mentougou District in Beijing, the focus area of this study, is an extension of the Taihang Mountains (mainly a hilly environment). Compared to areas in southern Gansu and northern Sichuan, the hilly and deciduous environment provides favorable habitats for a large number of wild animals and sand flies, including lower elevation, higher temperature, longer activity periods, wetter and looser soil, and thus greater vector capacity for *Leishmania* transmission. Meanwhile, since Beijing was affected by the worst haze in 2012 [27], China has vigorously promoted ecological protection measures such as returning farmland to forests, promoting an ecological environment for reproduction and transmission of *Leishmania* protozoa in these areas and consistent with the Mentougou District in Beijing having developed into a high incidence area for CanL.

The Xinjiang region of China has long been an endemic area for Leishmaniasis [28], and in this study the reference strain of *Leishmania* in Xinjiang was separated into two separate clades and not distributed in the same clade as the Beijing isolate. In rural areas of Xinjiang, large-scale renovation of old housing buildings supported by poverty-alleviation campaigns has greatly improved human housing conditions and mitigated sand fly breeding around homes, reducing the frequency of population contact with sand flies and thus reducing the chance that the Beijing isolates originated from the Xinjiang region [29]. The prevention and control of Leishmaniasis is more complex than expected for several reasons: Firstly, CanL has been found in the densely populated Haidian and Shijingshan districts of Beijing, indicating an urban trend of Leishmaniasis, which has also been reported in Europe and South America [30]. Secondly, Leishmaniasis is spreading from low to high latitudes in China due to global warming, and this trend has been reported in Italy [31]. Thirdly, it has been reported that although Leishmaniasis has been controlled in China, the incidence of Leishmaniasis has climbed with development of society and improvement of the ecological environment [29]. Leishmaniasis is a zoonotic disease in which dogs and wildlife are reservoir hosts, so stray dog management should be an important part of the Leishmaniasis control program and the responsibility of the Centers for Disease Control and Prevention, veterinary services, and public safety departments [29].

In this study, we provided further strong evidence of Chinese *Leishmania* compared to isolates worldwide, which strongly suggested that Chinese isolates had a more complex evolutionary history, and that there was some geographic relationship between Chinese isolates, providing a basis for further understanding of the phylogeny and evolutionary history of Chinese Leishmaniasis parasite species. In addition, when uncloned cultures are used for DNA isolation, sequence variation may exist between various cells in a given population. Direct isolation of *Leishmania* DNA from clinical samples would avoid this potential complication in the analyses. Further studies should be conducted based on the current dataset of Chinese *Leishmania* isolates to further understand the taxonomy and evolution of Leishmaniasis parasites. A

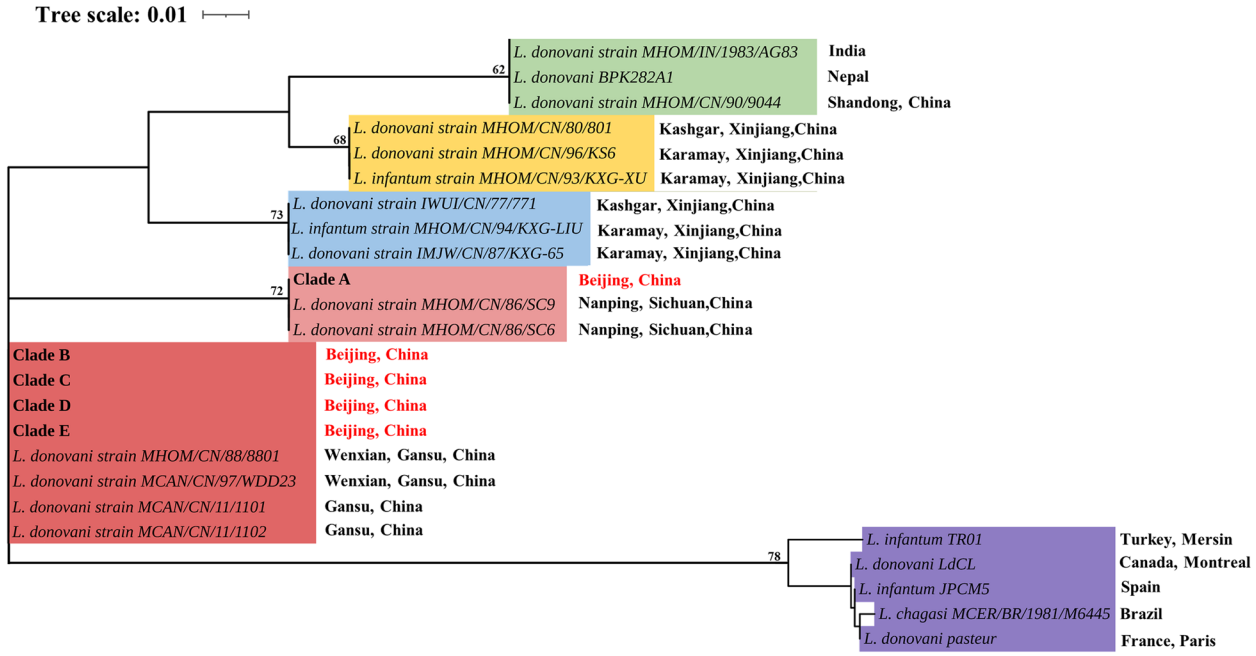

Fig. 5 Neighbor-joining unrooted trees constructed between the five clades of the isolates and reference strains of the *Leishmania donovani* complex in Asia and worldwide. Sequencing data of three genes (*g6pdh*, *mpi* and *icd*) were used to create this unrooted trees. The Tamura-3 parameter model was used, Numbers above branches correspond to bootstrap values based on 1,000 replicates

systematic comparison of many isolates worldwide would be ideal to elucidate the evolutionary profile of *Leishmania* species.

Materials and methods

Areas

Beijing is the capital and national central city of the People's Republic of China (PRC), with an area of 16,410 km^2 and a permanent population of 21.53 million. It is located in the north of the North China Plain in China, east longitude of 115.7–117.4 and north latitude of 39.4–41.6, and adjacent to Tianjin and Hebei Province. The annual average temperature in Beijing is 12.5℃, the annual average maximum and minimum temperature are 17.2 and 7.1℃, respectively, and annual average rainfall is > 600 mm. Beijing's climate is a typical semi-humid continental monsoon climate in the north temperate zone, with hot and rainy summer, cold and dry winter and short spring and autumn. Average elevation in Beijing is 43.5 m, and the highest elevation in Mentougou District of Beijing is 2303 m. This good seasonal habitat provides supportive conditions for *Phlebotomus chinensis*. In 2021, the number of registered domestic dogs in Beijing was > 2 million.

Animals and sampling

This study was conducted in accordance with ethical guidelines of China Agricultural University (CAU; Beijing, China). Prior to the beginning of the study, ethical approval (Aw61112202-2–2) was granted by the Departmental Committee of the College of Veterinary Medicine, CAU. Regarding sample calculation, a minimal sample size of 136 was estimated (95% confidence interval [CI], 5% margin of error), based on studies conducted in other endemic countries with a 10% prevalence [14]. The survey randomly included 2420 domestic dogs from veterinary clinics of 11 districts of Beijing. Sampling occurred from September 2021 to September 2022. All tested dogs were > 5 months (going through at least one sandfly season during May to September) and examined for clinical symptoms of CanL, including dermatological lesions, ocular changes, weight loss, apathy, lymph node, and spleen enlargement. Blood (5 mL) was collected by venipuncture into EDTA-coated polypropylene tubes for parasite DNA extraction, whereas an additional 2 mL was collected for sera separation. Details of the *Leishmania* isolates used in this study are presented in Table 4 and sampling sites are presented in Fig. 2.

Blood sample DNA preparation and *Leishmania* species identification

DNA samples were extracted from blood samples with TIANamp Blood DNA Kit (TianGen Biotech, Beijing, China), according to the manufacturer's instructions. The DNA concentration and purity were estimated by measuring absorbance at 260 and 280 nm using

Table 4 Details of *Leishmania* isolates used in this study

Name	Strain	Species	Location	Host	Disease
Isolates in Beijing					
MTG-ZZ5	MCAN/CN/2021/ZZ5	*L. infantum*	Beijing, China	Canine	
SSH-6	MCAN/CN/2022/SSH6	*L. infantum*	Beijing, China	Canine	
BTJ-23	MCAN/CN/2021/BTJ23	*L. infantum*	Beijing, China	Canine	
LML-3	MCAN/CN/2021/LML3	*L. infantum*	Beijing, China	Canine	
YSL-2	MCAN/CN/2021/YSL2	*L. infantum*	Beijing, China	Canine	
HH-1	MCAN/CN/2021/HH1	*L. infantum*	Beijing, China	Canine	
MTG-D1	MCAN/CN/2021/D1	*L. infantum*	Beijing, China	Canine	
XH-6	MCAN/CN/2022/XH6	*L. infantum*	Beijing, China	Canine	
YZ-18	MCAN/CN/2021/YZ18	*L. infantum*	Beijing, China	Canine	
LNN-17	MCAN/CN/2021/LNN17	*L. infantum*	Beijing, China	Canine	
2018–14	MCAN/CN/2018/201814	*L. infantum*	Beijing, China	Canine	
2018–13	MCAN/CN/2018/201813	*L. infantum*	Beijing, China	Canine	
CPH-13	MCAN/CN/2021/CPH13	*L. infantum*	Beijing, China	Canine	
CMH-18	MCAN/CN/2021/CMH18	*L. infantum*	Beijing, China	Canine	
LJF-11	MCAN/CN/2021/LJF11	*L. infantum*	Beijing, China	Canine	
BZHW-12	MCAN/CN/2021/BZHW12	*L. infantum*	Beijing, China	Canine	
RCDX-20	MCAN/CN/2021/RCDX20	*L. infantum*	Beijing, China	Canine	
W-692	MCAN/CN/2021/W692	*L. infantum*	Beijing, China	Canine	
GDH-15	MCAN/CN/2021/GDH15	*L. infantum*	Beijing, China	Canine	
MTG-C14	MCAN/CN/2021/C14	*L. infantum*	Beijing, China	Canine	
ZQ-26	MCAN/CN/2021/ZQ26	*L. infantum*	Beijing, China	Canine	
HH-5	MCAN/CN/2021/HH5	*L. infantum*	Beijing, China	Canine	
MTG-C9	MCAN/CN/2021/C9	*L. infantum*	Beijing, China	Canine	
WM-7	MCAN/CN/2022/WM7	*L. infantum*	Beijing, China	Canine	
ZQY-1	MCAN/CN/2022/ZQY1	*L. infantum*	Beijing, China	Canine	
ZQY-2	MCAN/CN/2022/ZQY2	*L. infantum*	Beijing, China	Canine	
BXY-21	MCAN/CN/2021/BXY21	*L. infantum*	Beijing, China	Canine	–
SH-22	MCAN/CN/2021/SH22	*L. infantum*	Beijing, China	Canine	–
HBH-3	MCAN/CN/2021/HBH3	*L. infantum*	Beijing, China	Canine	–
BMY-1	MCAN/CN/2021/BMY1	*L. infantum*	Beijing, China	Canine	–
WMS-9	MCAN/CN/2022/WMS9	*L. infantum*	Beijing, China	Canine	–
MTG-D5	MCAN/CN/2021/D5	*L. infantum*	Beijing, China	Canine	–
XH-4	MCAN/CN/2022/XH4	*L. infantum*	Beijing, China	Canine	–
WY-7	MCAN/CN/2021/WY7	*L. infantum*	Beijing, China	Canine	–
DH-4	MCAN/CN/2021/DH4	*L. infantum*	Beijing, China	Canine	–
HB-3	MCAN/CN/2022/HB3	*L. infantum*	Beijing, China	Canine	–
LSS-17	MCAN/CN/2022/LSS17	*L. infantum*	Beijing, China	Canine	–
NN-22	MCAN/CN/2021/NN22	*L. infantum*	Beijing, China	Canine	–
XBD-21	MCAN/CN/2021/XBD21	*L. infantum*	Beijing, China	Canine	–
WMS-7	MCAN/CN/2021/WMS-7	*L. infantum*	Beijing, China	Canine	–
MTG-ZZ4	MCAN/CN/2021/MTG-ZZ4	*L. infantum*	Beijing, China	Canine	–
Reference strains					
9044	MHOM/CN/90/9044	*L. donovani*	Shandong, China	Human	VL
SC6	MHOM/CN/86/SC6	*L. donovani*	Nanping, Sichuan, China	Human	VL
SC9	MCAN/CN/86/SC9	*L. donovani*	Nanping, Sichuan, China	Canine	–
KXG-LIU	MHOM/CN/94/KXG-LIU	*L. infantum*	Karamay, Xinjiang, China	Human	CL
KXG-XU	MHOM/CN/93/KXG-XU	*L. infantum*	Karamay, Xinjiang, China	Human	CL
KXG-65	IMJW/CN/87/KXG-65	*L. donovani*	Karamay, Xinjiang, China	Sand fly	–

Table 4 (continued)

Name	Strain	Species	Location	Host	Disease
771	IWUI/CN/77/771	*L. donovani*	Kashgar, Xinjiang, China	Sand fly	–
801	MHOM/CN/80/801	*L. donovani*	Kashgar, Xinjiang, China	Human	VL
KS6	MHOM/CN/96/KS6	*L. donovani*	Kashgar, Xinjiang, China	Human	VL
8801	MHOM/CN/88/8801	*L. donovani*	Wenxian, Gansu, China	Human	VL
1101	MCAN/CN/11/1101	*L. donovani*	Gansu, China	Canine	–
1102	MCAN/CN/11/1102	*L. donovani*	Gansu, China	Canine	–
WDD23	MCAN/CN/97/WDD23	*L. donovani*	Wenxian, Gansu, China	Canine	–
Friedlin	–	*L. major*	–	–	–
BPK282A1	MHOM/NP/03/BPK282A1	*L. donovani*	Nepal	Human	–
LdCL	–	*L. donovani*	Montreal, Canada	–	–
Pasteur	–	*L. donovani*	Paris, France	–	–
AG83	MHOM/IN/1983/AG83	*L. donovani*	India	Human	–
JPCM5	MCAN/ES/98/LLM-724	*L. infantum*	Spain	–	–
TR01	–	*L. infantum*	Mersin, Turkey	–	–
M6445	MCER/BR/1981/M6445	*L. chagasi*	Brazil	–	–
M2904	MHOM/BR/75/M2904	*L. braziliensis*	–	Human	–
UA301	–	*L. amazonensis*	–	–	–
Scaffold3675	–	*L. amazonensis*	–	–	–
U1103	MHOM/GT/2001/U1103	*L. mexicana*	–	Human	–
M4147	–	*L. guyanensis*	–	–	–
LEM1108	MPSA/SA/83/JISH220	*L. arabica*	–	–	–
LEM3045	–	*L. enrietti*	–	–	–
Parrot Tar II	–	*L. tarentolae*	–	–	–
216–34	–	*L. lainsoni*	–	–	–
LEM452	–	*L. gerbilli*	–	–	–
LEM423	–	*L. turanica*	–	–	–
PSC-1	MHOM/PA/94/PSC-1	L. panamensis	–	–	–

NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was stored at -20 °C until further use. The conserved internal transcribed spacer (ITS)-1 gene between the genes encoding for SSU rRNA and 5.8S rRNA was used as the target gene [7]. Nested PCR primers were designed with ITS-1 as the target gene. Among them, LITSR (5'-CTGGATCATTTTCCGATG-3') and ITS1 (5'-TGA TACCACTTATCGCACTT-3') were primers for the first round of amplification, whereas 2-ITSf (5'-CAT TTTCCGATGATTACACC-3') and 2-ITSr (5'-CGT TCTTCAACGAAATAGG-3') were products of the second round of amplification. In addition, deionized water with corresponding template volume was the negative control. The same reaction conditions were used for the first and second rounds: 94℃ for 5 min; 94℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s, for a total of 30 cycles, followed by 72℃ for 5 min. Target fragments with a length of 250–330 bp were considered positive products. After 8 μL of PCR products were subject to 1% agarose gel electrophoresis, remaining products were stored at -20℃. Then, PCR products with positive gel imaging were sequenced, and sequencing results were compared to *Leishmania* sequence data in GenBank using the Basic Local Alignment Search Tool (BLAST).

Detection of *Leishmania* antibodies by serology

RK39 immunochromatographic (ICT) strips (INBIOS, Washington, USA) were used to detect *Leishmania* antibodies in serum, according to manufacturer's instructions.

PCR amplification and gene sequencing

Based on previous studies performed on Chinese *Leishmania* strains that allowed retrieval of available polymorphic sequences from GenBank, including seven enzyme-coding genes (alanine aminotransferase [*alat*], enolase [*enol*], phosphoglucomutase [*pgm*], spermidine synthase [*spdsyn*], glucose-6-phosphate dehydrogenase [*g6pdh*], isocitrate dehydrogenase [*icd*] and mannose phosphate isomerase [*mpi*]) were used in this study. Primers sequences are in Table 5.

Table 5 Primers used for gene sequencing and PCR

Target gene	Primer name and sequence	Y (T_A)℃	Gene length(bp)	Product size (bp)	Chromosome	Reference
alat	ALAT.F: GTGTGCATC AACCCMGGGAA	55	1494	589	12	Marco et al., 2015 [8]
	ALAT.R: CGTTCAGCT CCTCGTTCCGC					
enol	ENOL.F: GCTGCCGAT CCTGATGGAGG	55	12,090	431	14	Marco et al., 2015 [8]
	ENOL.R: ACCCGTTCT CCATGCACAGC					
pgm	PGM.F: CAGAGAAGC TGACGTCCCAG	61	1770	529	21	Marco et al., 2015 [8]
	PGM.R: GACGGGTTC ACGAAGAAGCG					
spdsyn	SPDSYN.F: CGAACCTGT CGCTGACGTG	55	903	394	4	Marco et al., 2015 [8]
	SPDSYN.R: GAYTCG CCCTGGTTGCACAC					
g6pdh	G6PDH.F: GTGGACTTC AAGCGTCTYGAC	61	1689	573	34	Lauthier et al., 2020 [21]
	G6PDH.R: TCGGCGGAG GCRGTGTACTG					
mpi	MPI.F: TAAGTACAAGGA YCCRAACCACAAG	61	1266	441	32	Lauthier et al., 2020 [21]
	MPI.R: GCCATGATCTCR ACACCGTC					
icd	ICD.F: TCAACCTVAAG ATGTGGAAGAG	61	1308	702	10	Lauthier et al., 2020 [21]
	ICD.R: GTCCAGGCGTAG ATGGAGG					

The PCR reactions were performed in a 25 µL reaction mixture (1 µM of each primer, 12.5 µL 2 × Rapid Taq Master Mix, 8.5 µL ddH2O and 1 µL of 10 ng/µL DNA) (Vazyme., NanJing, China). Amplification was conducted in a BioRad T100™ Thermal Cycler with an initial 10 min denaturation step at 94 °C. Then, DNA samples were processed through 30 cycles of 1 min at 95℃, 1 min at the annealing temperature (TA), 90 s at 72℃, followed by a terminal elongation step of 10 min at 72℃. Amplification products were examined by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. After successful amplification of targeted regions, PCR products of the expected size were sequenced directly in both strands.

Multilocus sequencing typing (MLST)

The MLST method was used to construct a phylogenetic tree with each single gene sequence of *alat, enol, pgm, spdsyn, g6pdh, icd* and *mpi* with one or no heterozygous genes obtained by gene sequencing. The PCR products of the successfully amplified target regions were subjected to Sanger sequencing. Chromatograms of nucleotide sequences of both forward and reverse directions were examined visually and analyzed using Chromas v.2.6.6 and edited in BioEdit Sequence Alignment Editor v.7.0.5.3 (Tom Hall, Carlsbad, USA). Single nucleotide polymorphism (SNP) variations were identified. Chromatograms were revised to detect ambiguous sites (heterozygosity) evidenced by two overlapped peaks in one nucleotide position. Sequences were trimmed and consensus sequences for each gene were obtained through assembly of both strands. Multiple alignment of the nucleotide sequences was done with ClustalW in Molecular Evolutionary Genetics Analysis (MEGA) v.11.0.13 software [32]. Sequences were retrieved from NCBI and TriTrypDB databases and comparisons to homologous sequences used the Basic Local Alignment Search Tool (BLASTn) algorithm, and sequences of a total of 30 *Leishmania* reference strains (as shown in Table 4) were used for phylogenetic analysis. Analysis of sequences of seven housekeeping genes were done by MLSTest software [33] and each unique sequence was assigned an allele number which combined into an allelic profile. Each strain was characterized by a profile of seven allele numbers and assigned a sequence type (ST) number.

In the homozygous strains ST was identical to the haplotype number (H), whereas for heterozygous strains, it was a combination of the two possible alleles. Since only a few MLST reference strains have been reported in China, three major loci (*g6pdh*, *mpi* and *icd*) were selected for Neighbor-joining unrooted tree construction using the protocol optimization algorithm analysis in MLSTes software.

Statistical analyses

A database was established using EpiData 3.1, and statistical analyses performed using IBM SPSS 23.0. Categorical data were described by cases (n) and rate (%) and a $\chi 2$ test used to compare PCR and ICT results for detection of CanL. For all analyses, $p < 0.05$ was considered significant.

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Authors' contributions
GL, ZX, YJ and HB conceived the study. YJ, YW and GL organized the sampling plan. YL, GL and WH were responsible for the collection of samples in this study. YW and MG obtained the sequence and analyzed the results. GL, JK and HB were responsible for data statistics and analysis. GL, LW, JK and HB drafted the manuscript and all authors critically contributed to its final version. All authors read and approved the final manuscript.

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Availability of data and materials
Data will be shared upon reasonable request by readers.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
There is no conflict of interest to declare.

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