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An outbreak of the desert sub-type of zoonotic visceral leishmaniasis in Jiashi, Xinjiang Uygur Autonomous Region, People's Republic of China

Jun-Yun Wang [a], Chun-Hua Gao [a], Yue-Tao Yang [a], Hai-Tang Chen [a], Xue-Hua Zhu [a], Shan Lv [a], Sheng-Bang Chen [b], Su-Xiang Tong [c], Peter Steinmann [a,d,e], Kathrin Ziegelbauer [d,e], Xiao-Nong Zhou [a,*]

[a] National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention; WHO Collaborating Center for Malaria, Schistosomiasis and Filariasis, Shanghai 200025, People's Republic of China
[b] Gansu Provincial Center for Disease Control and Prevention, Lanzhou 730020, People's Republic of China
[c] Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region, Urumqi 830002, People's Republic of China
[d] Swiss Tropical and Public Health Institute, 4002 Basel, Switzerland
[e] University of Basel, Basel, Switzerland

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ABSTRACT

Few outbreaks of the desert sub-type of zoonotic visceral leishmaniasis (VL) have been described worldwide. In 2008, the incidence rate of VL in Jiashi County, Xinjiang Uygur Autonomous Region in the western part of the People's Republic of China, increased more than twenty-folds compared to the average annual incidence rate. The majority of the cases (96.6%) occurred among <2 year-old infants. For the first time in the desert area of Xinjiang, the parasites were isolated from bone marrow aspirates, using the NNN medium culture approach. The genetic analysis of the ITS-1 nucleotide sequence indicated that three isolates from eastern Jiashi County were genetically closely related and belonged to the *Leishmania infantum* group. However, they differed from an isolate from Kashi city which was classified as a member of the *Leishmania donovani* group.

1. Introduction

Visceral leishmaniasis (VL), also called kala-azar, is a vector-borne parasitic disease caused by the protozoan *Leishmania donovani* complex [1,2]. The disease is endemic in 61 countries and is responsible for the annual loss of an estimated 1.81 million disability adjusted life-years (DALYs) and 57,000 lives [3]. Prior to the initiation of a national control program in 1951, VL was one of the major parasitic diseases in the People's Republic of China, endemic in 17 provinces, cities and autonomous regions. Based on the available epidemiological data, about 530,000 VL cases were estimated in China in 1951 [4,5]. Following great efforts in the frame of the national control program, the disease has been largely brought under control in the eastern regions of the country, and VL is currently only endemic or occurs sporadically in 43 counties in six provinces or autonomous regions in western China, namely Xinjiang, Gansu, Sichuan, Shaanxi, Shanxi and Inner Mongolia [6–11]. However, VL recently reemerged in western China. More than 2629 new VL cases were diagnosed in the endemic regions of western China between 1990 and 1999 [7].

Two epidemiological types of VL can be distinguished in western China based on the ecosystem and epidemiological characteristics, i.e. geographical and landscape characteristics, age distribution of patients, *Leishmania* species, vector sandfly species and their ecology, and source of infection [6,7,12]. The first type is an anthroponotic type endemic in the oases of the plains of Kashi prefecture, Xinjiang Uygur Autonomous Region. Most cases occur in young people, with 8.3–38.9% of the cases involving under 5 year-old, and few infant cases. Some of the patients may develop post-kala-azar dermal leishmaniasis. Concurrent or consecutive cases often occur in the same household. The canine infection rate is very low (0–0.3%) [4]. The widely distributed peridomestic *Phlebotomus longiductus*, an endemic species in Xinjiang, is the main vector. Among all identified sandfly species, *P. longiductus* was the most prevalent species in Xinjiang (97.6–100%). It feeds on both humans and cattle, and has been found to be naturally infected with *L. donovani* [13]. Previous experiments also demonstrated that *L. donovani* can circulate between *P. longiductus* and rats [14,15].

The second type of VL is zoonotic and has been divided into two subtypes, namely a mountainous and a desert sub-type [14]. The mountainous sub-type occurs in the western mountainous and hilly regions of China in the provinces of Gansu, Sichuan, Shaanxi and Shanxi.

* Corresponding author. Tel.: +86 21 64738058; fax: +86 21 64332670.
E-mail addresses: wangjy0@yahoo.com.cn (J.-Y. Wang), gaochunhua1976@yahoo.com.cn (C.-H. Gao), yangyuetao@yahoo.com.cn (Y.-T. Yang), cht906@sohu.com (H.-T. Chen), zhuxuehua1956@163.com (X.-H. Zhu), lvshan000@126.com (S. Lv), chenshengbang@hotmail.com (S.-B. Chen), tongsuxiang@163.com (S.-X. Tong), peter.steinmann@unibas.ch (P. Steinmann), kathrin.ziegelbauer@stud.unibas.ch (K. Ziegelbauer), ipdzhouxn@sh163.net (X.-N. Zhou).

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Patients are mostly children less than ten years old, and infants are commonly infected, and those patients are distributed as a sporadic pattern geographically. High *Leishmania* spp. infection rates in dogs (up to 69.6%) were detected in Gansu province by PCR [16]. Elimination and prohibition of dogs markedly reduced the number of human cases, thus dogs likely are the principal source of infection for the mountainous sub-type [7]. The vector of this form is *P. chinensis*, a zoophilic species that also feeds on man. During daytime, these sandflies rest mainly in caves and other shelters but they enter villages after sunset [13].

The desert sub-type is endemic in the northwestern desert regions of China, including Xinjiang, western Inner Mongolia and northern Gansu [14]. These regions were uncultivated deserts for a long time before they were populated by immigrants who introduced agricultural activities; consequently, autochthonous infantile kala-azar occurs, and the region is considered to be a natural nidus of kala-azar-wild animals presumably being the source of infection. The wild species, *P. wui* and *P. alexandri*, are the vector infesting in the specific landscapes, such as dry desert region or the stony desert, respectively [15].

It has been noticed for a long time that both anthroponotic type and desert sub-type of VL are present in Jiashi County, Kashi Prefecture, Xinjiang Uygur Autonomous Region, but the distribution of these two types of VL did not overlap geographically [7,11]. They were separated by a wide range of saline–alkali land, anthroponotic type of VL is endemic in the west of Baren town, the capital town of Jiashi County, and desert sub-type of VL is distributed in the east of Baren town (Fig. 1). The disease is sporadic in the eastern region and less than ten cases of VL occurred annually during 1996–2007, according to the data from passive VL case surveillance conducted by Xinjiang Centre for Disease Control and Prevention.

The number of infant VL cases dramatically increased in the eastern part of Jiashi County, Xinjiang, in September and October 2008. Considering the fact that outbreaks of desert sub-type VL are rare and seldom reported, in spite of outbreaks of other type of leishmaniasis have been reported worldwide [17–21], an emergency response was initiated in October 2008 and lasted until December of that year, with an emphasis on active VL surveillance and the detection of the causative agent. Here, we present the main findings and the epidemiological features of this VL outbreak in order to highlight this neglected disease in a little-known context.

2. Methods

Two main data sources of VL available in Jiashi County were used in the epidemiological investigation, including (i) passive surveillance data and (ii) active surveillance data. All patients with suspected clinical features of VL were admitted to hospitals at township, county, provincial levels and were subsequently diagnosed based on clinical assessment and a positive recombinant K39 (rK39) antigen-based immunochromatographic strip test, or the Kalazar Detect (batch JL1019; InBios, Seattle, WA). The rapid test was performed following the manufacturers' instructions [22]. Parasite examination, such as stained smears and pathogen culture analysis, was conducted for part of cases. In addition, bone marrow aspirates were obtained and cultured in NNN medium from several clinically diagnosed cases for isolation and genetic analysis of causative agent using molecular method, i.e. PCR.

2.1. Passive surveillance

The routine passive surveillance of VL in the area was based on clinical examination results. All cases were diagnosed in accordance with the National Criteria for Visceral Leishmaniasis Diagnosis in China and reported via the web-based National Diseases Reporting Information System (NDRIS) operated by the Chinese Center for Disease Control and Prevention (China CDC), according to the National Regulation on the Control of Communicable Diseases [23]. Passive surveillance data covered personal and clinical information, including the patient's name, age, gender, address, dates of symptom onset and diagnosis.

2.2. Active surveillance

Active surveillance was conducted by a national emergency response team between October and December 2008 in each household in Jiashi County from which VL cases had previously been reported. All children under 5 years old in these households were registered by name and a personal identification number was assigned. An enrolment form containing demographic data, medical history and present complaints was completed during an interview with their parents. Subsequently, a general physical examination was conducted with particular focus on fever associated with clinical splenomegaly or wasting symptoms which are considered significant indicators for VL. Individuals with indicative clinical features were admitted to hospitals at township, county or provincial level. Definitive diagnosis was based on the clinical assessment and a positive rK39 dipstick test [22]. Individuals with positive results both in the physical examination and the dipstick test were considered true VL cases and referred for standard treatment. All relevant information regarding these VL cases was also reported to NDRIS by local hospitals according to standard procedures [23].

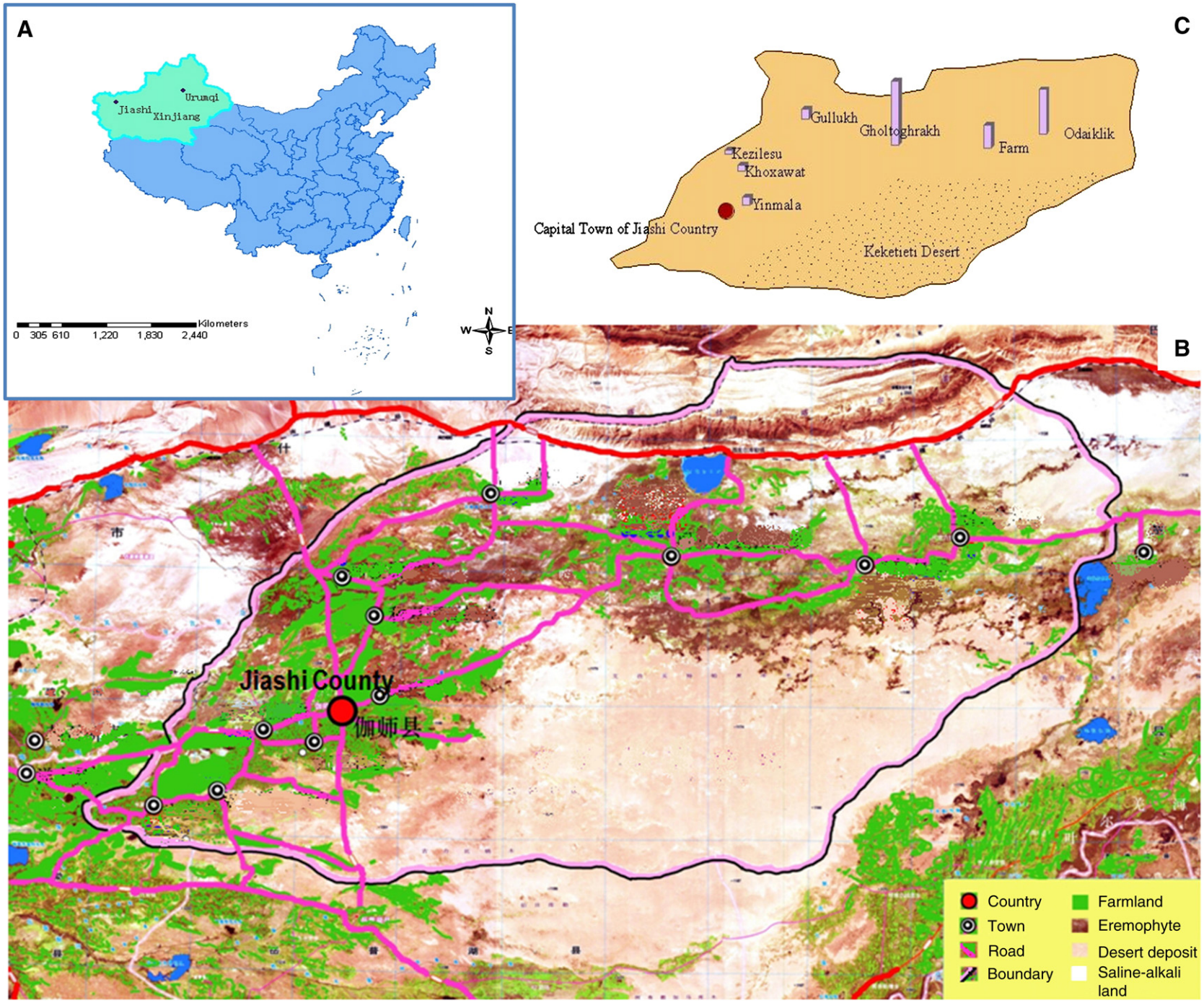
2.3. Isolation and analysis of causative agent

Bone marrow aspirates were obtained from 19 individuals younger than two years old who were suspected to have VL based on the clinical examination and were positive in the rk39 dipstick test. All 19 individuals originated from Gholtoghrakh township (Fig. 1C), the center of this outbreak and the source of more than half of the recent cases. Smears were prepared from each sample, stained with Giemsa and examined microscopically. Only nine out of the 19 samples were inoculated into NNN medium and incubated at 24 °C [24]. Cultures were examined once every ten days for the appearance of promastigotes in the NNN medium for sixty days.

ITS-1 amplification and analysis was conducted in order to compare the *Leishmania* spp. isolates from the samples collected in the outbreak areas together with an isolate (reference code: MHOM/CN/80/801) obtained previously from an area of anthroponotic VL in Kashi city. The following procedures were employed [25]. DNA was extracted from cultured *Leishmania* promastigotes as described previously [26,27]. The nucleotide sequence of the ITS-1 region was amplified using extracted DNA as template and previously published primers (LITSR: 5′ ctg gat cat ttt ccg atg, and L5.8 S: 5′ tga tac cac tta tcg cac tt) and conditions [28]. Two microliters of extracted DNA were used as template for each PCR reaction, and a reaction without DNA was run as negative control. Amplicons were visualized using a final concentration of 10 μg/ml ethidium bromide after electrophoresis in a 1% agarose gel.

The PCR products were processed using the Qiagen PCR cleanup kit (Qiagen, Valencia, CA) and sequenced by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. DNA sequences were analyzed using GENEDOC (Free Software Foundation, Inc. Boston, USA) and aligned using Clustal X [29]. Amended sequences were

Fig. 1. Location and topography of Jiashi County, Xinjiang Uygur Autonomous Region, People's Republic of China. (A) Location of Jiashi county and Xinjiang Uygur Autonomous Region in the People's Republic of China. (B) Topography of Jiashi County showing the saline–alkali land located in the eastern part of the capital. (C) Origin of visceral leishmaniasis cases, showing the geographic distribution of 258 cases of desert sub-type zoonotic VL in seven townships (yellow color) and ten cases of anthroponotic VL (green color). ● Origin of three *Leishmania* spp. isolates obtained in the frame of this study.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Kezilesu
Khoxawat
Yinmala
Capital Town of Jiashi Country
Keketieti Desert
B
Jiashi County
伽师县
Country
Town
Road
Boundary
Farmland
Eremophyte
Desert deposit
Saline-alkali land

submitted to the GenBank (Bankit) for comparison with stored sequences.

In order to reveal the phylogenetic relationship of isolates from Jiashi county and Kashi city with *Leishmania* spp. isolates from other parts of the world, ITS-1 sequences of 18 different *Leishmania* spp. isolates were downloaded from GenBank and included in the analysis, i.e. seven isolates of *L. donovani* (accession nos.: MHOM/LK/2002/L60b, AM901448; MHOM/KE/75/H9, EU326228.1; MHOM/IN/80/DD8, AJ000292.1; MHOM/ET/67/HU3, AJ634373.1; MHOM/SD/93/9S, AJ634372.1; MCAN/MA/2002/AD3, AM901453.1 and MHOM/IQ/1981/SUKKAR2, AM901452.1), ten isolates of *L. infantum* (accession nos.: MHOM/IR/04/IPI-UN10, GQ444144.1; MHOM/IT/94/ISS1036, AJ634353.1; MHOM/MT/85/BUCK, AJ634350.1; MHOM/TN/80/IPT1, AJ000289.1; MHOM/UZ/2007/OBA, FM164419.1; MHOM/CN/78/D2, AJ000303.1; MHOM/CN/54/Peking, AJ634345.1; MHOM/FR/80/LEM189, AJ634351.1; MHOM/ES/92/LLM373, AJ634352.1; and MHOM/BR/74/pp75, EU326227) and one *L. tropica* isolate (accession no.: MHOM/SU/60/OD, EU326226.1). Phylogenetic and molecular evolutionary analyses were conducted in MEGA version 4.0 [30]. A phylogenetic tree was constructed using the Maximum Parsimony method [31]. Evolutionary distances were calculated using the method proposed by Jukes and Cantor [32]. Bootstrap analysis [33] was performed with 1000 replicates.

2.4. Ethical considerations

The protocol for passive VL surveillance and the outbreak investigation had been reviewed and approved by the Ethical Review Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai. Oral informed consent was obtained from the legal guardians of the enrolled children.

3. Results

3.1. Epidemiological characteristics

3.1.1. Geographical distribution

Jiashi County (39.1°–40.0°N, 76.2°–78.0°E) is located in Kashi prefecture in the southwestern part of Xinjiang, an autonomous region in western China. The county covers an area of 6715 km^2 with a wide desert area in the south (Fig. 1). The open basin drops from southwest to northeast. The population is 357,181, based on the 2007 census. It has a typical arid desert climate, with an annual mean temperature of 12.7 °C, dropping from 26 °C in summer to below −5 °C in winter and a mean annual rainfall of 45 mm.

A total of 268 VL cases from Jiashi County were recorded in the NDRIS from January 2008 to May 2009. The cases originated from 12 of the 13 townships in the county, and 96.3% of the cases (258/268) occurred in seven townships located in eastern Jiashi where the desert sub-type of zoonotic VL is known to be endemic. The average incidence rate at township level was 0.128%, ranging from 0.024% to 0.39% (Fig. 1C, Table 1). The other ten cases occurred in five townships to the West of the capital, where anthroponotic VL is endemic. No cases were reported from one township in the area where anthroponotic VL is endemic.

Table 1
Origin of VL cases reported from Jiashi County between January 2008 and May 2009.

Type of endemic area	Township	Population	No. of cases	Incidence (%)
Desert sub-type of zoonotic VL	Gholtoghrakh	37,906	149	0.3931
	Odaiklik	14,671	40	0.2726
	Farm	9794	14	0.1429
	Gullukh	22,189	13	0.0586
	Yinmala	29,278	16	0.0546
	Khoxawat	45,354	16	0.0353
	Kezilesu	41,215	10	0.0243
Sub-total	7	200,407	258	0.1287
Anthroponotic type VL	Kezleboy	34,557	2	0.0058
	Mixia	27,294	1	0.0037
	Jiangbaz	31,299	3	0.0096
	Xiaptle	29,156	2	0.0069
	Baren	29,661	2	0.0067
Sub-total	5	151,967	10	0.0066
Total	12	352,374	268	0.0761

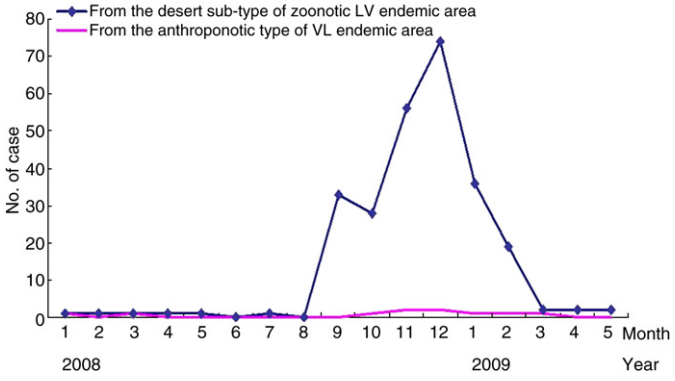

Fig. 2. Temporal distribution of VL cases in Jiashi County from January 2008–May 2009.

3.1.2. Temporal distribution

Of the 258 VL cases detected from January 2008 to May 2009 in the seven eastern townships, 96.5% (249/258) occurred in the period of September 2008–February 2009, with a peak in December 2008. The ten cases in the western area where anthroponotic VL is endemic occurred over a period of seven months (Fig. 2).

3.1.3. Population distribution

Out of the 268 cases, 93.3% (250/268) occurred in individuals aged between three months and one year, and 96.6% (259/268) in under two years old. Among the 258 cases in the seven eastern townships, 95.7% (247/258) were noted in children less than one year old while another 5 cases were seen in children aged between one and two years. The ratio of males to females was 1:0.928 (139:129).

3.2. Isolation of causative agent

Only one of the 19 samples (5.3%) was considered positive upon microscopic examination of at least 2000 visual fields by experienced experts while all blood samples tested positive with the rk39 dipstick test. Out of the 9 samples inoculated into the NNN medium, promastigotes were detected in 3 samples (including the microscopically positive sample) after 40 days (reference codes: MHOM/CN/08/JIASHI-1, MHOM/CN/08/JIASHI-2 and MHOM/CN/08/JIASHI-5).

3.3. Genetic analysis of isolates

The ITS-1 region could be amplified from three *Leishmania* spp. isolates from Jiashi county and the sample previously isolated in Kashi city. A 313 bp nucleotide was obtained from the three isolates from Jiashi county (MHOM/CN/08/JIASHI-1, MHOM/CN/08/JIASHI-2 and MHOM/CN/08/JIASHI-5), while a 316 bp nucleotide resulted from the isolate from Kashi city (MHOM/CN/80/801). The alignment analysis of the ITS-1 sequence

Table 2
Alignment of the variable parts of the ITS-1 sequences amplified from *Leishmania* spp. isolates from Jiashi county and Kashi city, Xinjiang Uygur Autonomous Region.

Strain	Position (25–38)	Position (68–72)	Position (88)
Jiashi (313 bp)	CCCAAAAAA – CAT	A – TG	C
Kashi (316 bp)	CC–AAAAAAAACAT	ATATG	T

"–" represent absence of nucleotide.

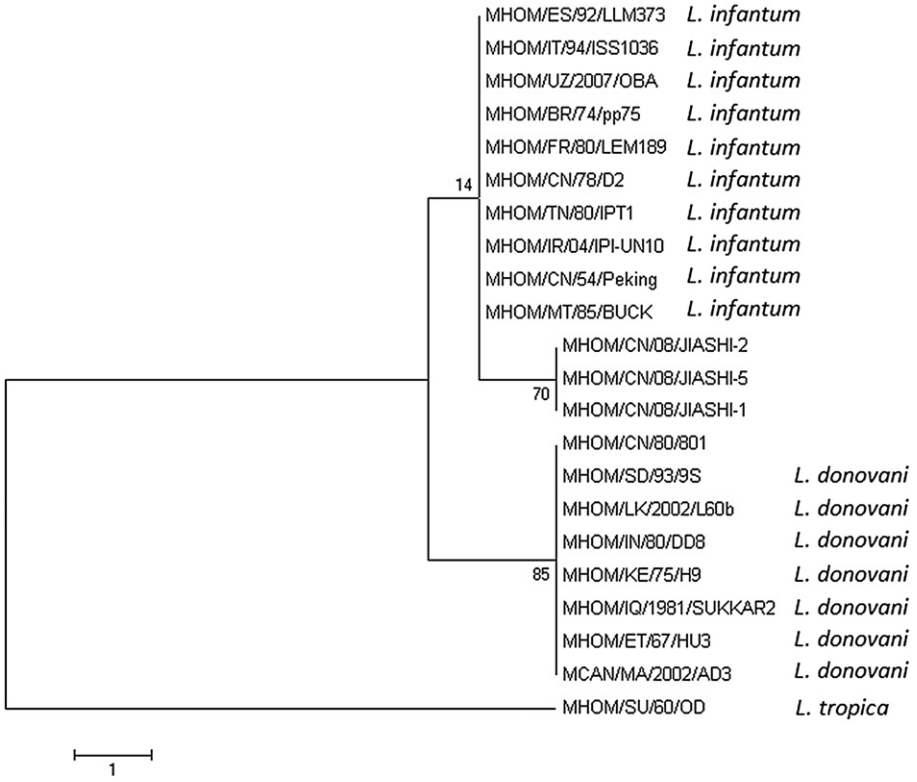

Fig. 3. Dendrogram based on *Leishmania* spp. ITS-1 sequence comparison. The dendrogram was constructed using the Maximum Parsimony method. Bootstrap analysis was performed with 1000 replicates. Strain accession numbers were: MHOM/CN/08/JIASHI-1 GQ367486; MHOM/CN/08/JIASHI-2, GQ367487 and MHOM/CN/08/JIASHI-5, GQ367488 (these three isolates collected and analyzed through this study). MHOM/CN/80/801, GQ367489 (collected in 1980 and analyzed in this study). The 7 isolates of *L. donovani* were: MHOM/IN/80/DD8, AJ000292; MHOM/LK/2002/L60b, AM901448; MHOM/IQ/1981/SUKKAR2, AM901452; MCAN/MA/2002/AD3, AM901453; MHOM/SD/93/9S, AJ634372; MHOM/ET/67/HU3, AJ634373 and MHOM/KE/75/H9, EU326228. The 10 isolates of *L. infantum* are: MHOM/IT/94/ISS1036, AJ634353; MHOM/MT/85/BUCK, AJ634350; MHOM/ES/92/LLM373, AJ634352; MHOM/FR/80/LEM189, AJ634351; MHOM/CN/54/Peking, AJ634345; MHOM/IR/04/IPI-UN10, GQ444144; MHOM/UZ/2007/OBA, FM164419; MHOM/TN/80/IPT1, AJ000289; MHOM/CN/78/D2, AJ000303 and MHOM/BR/74/pp75, EU326227. A single isolate of *L. tropica* (MHOM/SU/60/OD, EU326226) was used.

suggested 100% agreement among the three isolates from Jiashi County, but differed from the sample of Kashi city with 98% agreement (Table 2).

The ITS-1 sequence of the 3 isolates (MHOM/CN/08/JIASHI-1, MHOM/CN/08/JIASHI-2, MHOM/CN/08/JIASHI-5) collected in Jiashi county (accession nos.: GQ367486, GQ367487 and GQ367488) and the isolate (MHOM/CN/80/801) from Kashi city (accession no.: GQ367489) were submitted to GenBank (Bankit). The Maximum Parsimony phylogenetic tree including three isolates from Jiashi County, one from Kashi city and another 18 isolates was constructed based on 1000 bootstrap replicates (Fig. 3) suggested that the isolates from Jiashi County belonged to the *L. infantum* group. However, there were distinct differences to other *L. infantum* strains from Europe, the Middle East and North Africa. The isolate from Kashi city was classified into the *L. donovani* group.

4. Discussion

A number of leishmaniasis outbreaks in different parts of the world have been reported over the past decades [17–21]. Dogs were the source of VL outbreaks in the Namangan region in the Fergana valley in Uzbekistan where parasites were assigned to a genetically distinct cluster clearly separated from *L. infantum* populations in the Middle East and North Africa [34]. However, there are few reports of outbreaks of the desert sub-type of zoonotic VL such as that reported here.

Human cases of desert sub-type kala-azar have been reported in desert areas in the northern part of Gansu Province, western part of the Inner Mongolia Autonomous Region, and Xinjiang Uygur Autonomous Region [35]. In Xinjiang, desert sub-type kala-azar is mainly found along the northwestern and eastern fringe of the Tarim basin, in the middle and lower reaches of the Yerqiang river and in the upper and middle reaches of the Tarim river, e.g. in Maigaiti, Bachu, Atushi, Jiashi, Keping, Awati, Aksu, Kuche, Shaya, Luntai, Korla and Weili counties. Desert sub-type kala-azar is also found in the Bachu and Tarim reclamation areas, and in the desert areas along the Peacock river valley and Turfan-Hami basin [7,14,36]. The geographical distribution of desert sub-type kala-azar covers the desert zone between 37.5°–42°N and 76°–105°E where the soil property is of desert–forest–grassy marshland type. The vegetation is mainly of the desert–forest landscape consisting of *Populus diversifolia* and *Tamarix ramosissima*, *Halimodendron halodendron* and newly reclaimed desert oasis [37]. Many originally uncultivated desert regions have recently been populated and agricultural activity has increased. Autochthonous kala-azar mainly occurs in infants in these areas, and the existence of wild animal reservoir hosts is suspected but they have yet to be identified. *P. wui* and *P. alexandri* are the main vectors in the dry desert region and the stone desert, respectively [4,13,15,38]. Up to now, little information of the animal hosts was available for the desert sub-type kala-azar. It is difficult to investigate the species of *Leishmania* spp., since the parasite is difficult to be cultured and isolated directly from the human host, because the majority of cases of desert sub-type kala-azar are infants under two years of age and their parents are reluctant to let their kid be examined by bone marrow aspirates. Therefore, it is important to understand the species status of the parasites isolated from the outbreak areas of Jiashi County, and the transmission patterns of desert sub-type kala-azar, in order to formulate reasonable and feasible control strategy in these regions.

Previous observations suggest that differences exist between the anthroponotic and the desert sub-type kala-azar, mainly with regard to the following four aspects [2]. Firstly, age-incidence patterns: infants dominate among individuals with the desert sub-type disease, while the majority of anthroponotic cases occur among young adults

[7]. Second, seasonal distribution: most desert sub-type kala-azar cases occur between October and December, whereas the peak of case onset for the anthroponotic type is from April to May, with a secondary peak from September to October [4,15]. Third, causative agents: bone marrow smears detect a lower proportion of *Leishmania* spp. parasites in patients with the desert sub-type disease compared to the anthroponotic type, and the culture success rate is also lower [15]. Finally, vectors: the anthroponotic type disease is transmitted by the peridomestic *P. longiductus*, while the desert sub-type of zoonotic VL is transmitted by the wild *P. wui* [38].

In the present study, most VL patients were less than two years old, and most cases were diagnosed between September 2008 and February 2009. The parasite detection rate in bone marrow samples was very low using either of the employed methods, i.e. microscopy and culture. The results of a vector surveillance study conducted in September 2008 in the outbreak area suggest that *P. wui* indeed were the locally predominated species (unpublished data). Based on the available evidence, this outbreak was attributed to the desert sub-type of zoonotic VL. For the first time in Xinjiang, the parasite was successfully isolated from bone marrow samples using a NNN culture approach. Results of the genetic analysis showed that the ITS-1 nucleotide sequences of three isolates from the outbreak area are similar to one another and are clustered into the *L. infantum* group in the phylogenetic tree, but differed from an isolate from Kashi city with an agreement of 98%. The latter sample was classified into the *L. donovani* group according to the phylogenetic tree.

It is particularly difficult to determine the involved *Leishmania* species in the case of the desert sub-type VL since the isolation and culture of the parasite is difficult due to the young age of most infected individuals and the reluctance of parents to consent to bone marrow aspiration. However, the exact identification of the species is important to better understand the transmission pattern of desert sub-type kala-azar and to formulate effective and locally acceptable control strategies.

We were unable to ascertain the causes of the outbreak. However, we speculate that the groundwater level might be implicated, based on the fact that the incidence of VL increased from west to east (Fig. 1) and that sandfly density positively correlated to soil moisture, in correspondence with the hypsography of the county which drops from west to east, mirrored in the flow of underground water [37,39]. Possible changes in climate condition and its contributions to the outbreak should be investigated in the future [6,40,41].

5. Disclaimers

The opinions expressed by the authors contributing to this journal do not necessarily reflect the opinions of the Chinese Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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Jun-Yun Wang (M.Sc.) is the Vice Chief of the Department of Filariasis, Leishmaniasis and Echinococcosis, National Institute of Parasitic Diseases, China CDC in Shanghai, People's Republic of China. He has been engaged in research on the epidemiology and diagnosis of leishmaniasis for more than 20 years.