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ORIGINAL ARTICLE

Comprehensive surveillance of the antibody response to *Borrelia burgdorferi* s.l. in small ruminants in China

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Abstract

Borrelia burgdorferi sensu lato, the etiological agent of Lyme disease, is tick transmitted and has a wide range of mammalian reservoirs in nature, including both wild and domestic animals. To understand the seroprevalence of *B. burgdorferi* s.l. in small ruminants will add value to the risk analysis of Lyme disease. The current study was intended to map the potential endemic regions of Lyme disease by large-scale investigation of sera from sheep and goats. In this study, a total of 2,758 serum samples from sheep and goats in 21 provinces located in 40 different districts of China were tested for antibodies against *B. burgdorferi* s.l. by enzyme-linked immunosorbent assay. The results of this survey indicated that the overall prevalence of *B. burgdorferi* s.l. infection ranges from 5.3 – 63.5 % (mean: 26.3%), and the infection was found in all provinces investigated. Generally, the positive rate declined from the south (south – 34.7%, southwest – 32.4%) towards the north of China (north – 18.4%, northeast – 16.5%, northwest – 17.2%). A significant difference was also observed in the infection rate between south and north (33.2% vs. 17.4%, $P<0.001$). This study presents a comprehensive investigation of the serological distribution of *B. burgdorferi* s.l. in small ruminants in China.

Key words

Borrelia burgdorferi s.l., Seroprevalence, Lyme disease, Small ruminants

INTRODUCTION

Lyme disease, or Lyme borreliosis, which is caused by *Borrelia burgdorferi* sensu lato (s. l.), a group of genetically diverse spirochetes, is one of the most prevalent tick-borne zoonoses, and can cause a complex multisystem disorder in human patients [1]. *B. burgdorferi* s.l. was recovered initially in 1982 by Burgdorfer from a tick vector, *Ixodes scapularis*, in the USA, and the pathogen was discovered in *I. persulcatus* in 1986 in the Heilongjiang province of China [2, 3]. Lyme disease is widely prevalent in the USA and Europe and also occurs in Russia and some Asian countries [4, 5, 6]. Besides causing a multisystemic disease in humans, Lyme disease affects a wide range of wild and domestic animals, including dogs, horses, cattle, sheep, goats, cats, rodents, birds and deer [7, 8]. Numerous serologic surveys on the prevalence of *B. burgdorferi* s.l. in reservoirs have been carried out in different countries [9, 10, 11]. Several surveys have also shown that *B. burgdorferi* s.l. infection in domestic animals is common in some regions of China [12, 13, 14, 15], but there have been no systematic studies on the distribution of antibodies against *B. burgdorferi* in small ruminants (sheep and goats) in China.

Increased understanding of the epidemic situation of Lyme disease will contribute to the development of models for prediction of human Lyme borreliosis risk. Yang et al. [16] developed an enzyme-linked immunosorbent assay (ELISA) using soluble antigens of *B. burgdorferi* s.l. The specificity and sensitivity of the ELISA were 90.0% and 90.1% when 15% of the antibody rate was chosen as its positive threshold. There was no cross-reaction with positive sera from sheep infected with *Mycoplasma*, *Brucella*, *Toxoplasma*, *Babesia*, *Theileria* or *Anaplasma*. Thus, this technique can be used to perform epidemiological investigation. The presented study aimed to carry out a large-scale sero-epidemiological survey of *B. burgdorferi* s.l. in small ruminants in various locations in China, which could serve as an indication for the presence of the pathogen in the areas investigated.

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MATERIALS AND METHOD

***Borrelia burgdorferi* s.l. strains.** The reference strain *B. burgdorferi* sensu stricto (s.s.) B31 was purchased from the American Type Culture Collection (ATCC) and was stationary cultivated at 33 °C under microaerophilic conditions in BSK-H medium (Sigma-Aldrich, St. Louis, MO), supplemented with an antibiotic mixture containing SXT25, FOS50 and AK30 (Oxoid Ltd, Basingstoke, UK) [17]. Cultures were incubated for at least one week. Growth was detected by examining the culture supernatant using dark-field microscopy and collection of the spirochetes in the log-phase (approximately 10^8–10^9/ml) of growth.

Sera and collection sites. Standard positive and negative control sera for *B. burgdorferi* s.l. were prepared from experimental animals; the quality of these aliquots has been proved by previous validation [16]. Blood samples (n=2,758) were collected from the jugular veins of sheep and goats in

plain tubes, according to ethical guidelines, from March – September 2010–2012. These animals were healthy and distributed in 40 counties which covered 21 provinces of China. Clotted blood samples were separated by centrifugation and the sera stored at –20 °C until use. The provinces sampled were: Gansu (Yuzhong and Zhangye), Xinjiang (Aletai and Aksu), Qinghai (Qilian), Inner-Mongolia (Chifeng), Hebei (Baoding), Ningxia (Wuzhong), Shanxi (Lvliang), Jilin (Songyuan), Liaoning (Liaoyang), Shandong (Dongying), Anhui (Hefei), Shannxi (Yulin), Tibet (Lhasa), Sichuan (Panzhihua and Luzhou), Yunnan (Fuyuan, Honghe, Yanshan, Ruili, Jinghong and Menghai), Guizhou (Qinglong, Dushan, Rongjiang, Yuping and Ziyun), Chongqing (Jiangjin and Wanzhou), Guangxi (Lingui, Jingxi, Pingxiang and Tianyang), Hunan (Yongzhou, Xinhuang and Linli), Guangdong (Qingyuan and Zhaoqing), and Zhejiang (Hangzhou) provinces (Fig. 1, Supplementary Tab. 1).

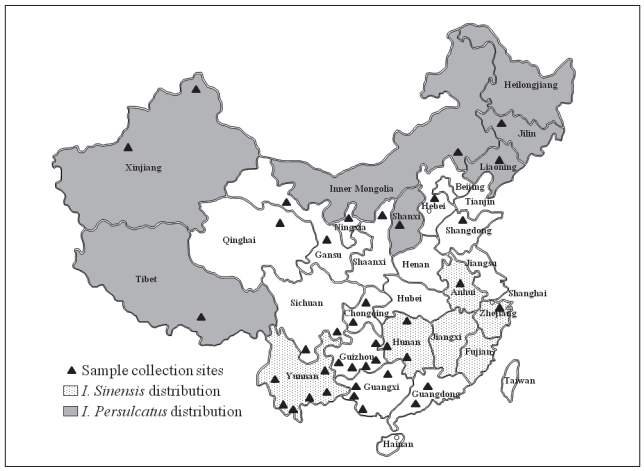

Figure 1. Sample collection sites in China

Preparation of antigens. Preparation of *B. burgdorferi* s.l. whole antigens was conducted as described previously by Yang et al. [16]. Briefly, the *B. burgdorferi* s.l. cultures were harvested into 50 ml tubes and centrifuged at 13,000 *g* for 30 min. The pellets of *B. burgdorferi* s.l. were washed three times with phosphate-buffered saline, pH 7.2 (PBS). The collected *B. burgdorferi* s.l. were re-suspended with PBS and subjected to five freeze–thawing cycles, sonicated for 30 min (5 s sonication with 5 s interval), centrifuged (13,000 *g*, 15 min) and the supernatant, containing soluble protein, was used as the antigen. The protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Merck KGaA, Darmstadt, Germany). Reactivity and specificity of the antigens were verified using ELISA and western blot, and the samples were stored at –20 °C until use [16].

Antibody testing. An ELISA procedure described previously by Yang et al. [16] (modified from Chauvin et al. [18]) was employed. Briefly, microtiter plates were coated at 37 °C for 1 h and then at 4 °C overnight with 100 µl/well of *B. burgdorferi* s.l. whole antigen solution (25 µg/ml in 0.1 M carbonate–bicarbonate buffer, pH 9.6). The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated with 200 µl/well of a blocking solution (2% gelatin in PBST) for 30 min at 37 °C. After drying the plate, 100 µl/well of the samples, blank (PBST), standard positive and negative controls (dilution of 1:200 in PBST + 0.5% gelatin), were distributed in duplicate and the plates incubated at 37 °C for 1 h. After washing as above, 100 µl/well of monoclonal anti-goat/sheep IgG–peroxidase antibody produced in mice (A-9452, Sigma-Aldrich, St. Louis, MO, USA) and diluted 1:4000 in PBST was added and the plates were again incubated at 37 °C for 1 h. The washes were repeated as described above, and subsequently 50 µl of TMB (T0440–1L, Sigma-Aldrich, St. Louis, MO) was added to each well and incubated for 15 min at room temperature. The reaction was stopped with 50 µl of 2 M H_2SO_4. Absorbance values were read at a wavelength of 450 nm in an automatic ELISA reader (microplate reader Model 680, Bio-Rad Laboratories Inc, CA, USA). The results are expressed as the percentage of the specific mean antibody rate (AbR%), determined using the formula: AbR%=(Sample mean OD – Negative control mean OD) / (Positive control mean OD – Negative control mean OD)×100%. The cutoff value was chosen as 15%, as previously reported [16].

Statistical analysis. The results were analyzed using a chi-square test in Predictive for Analytics Software (PASW Statistics 18.0, SPSS Inc, Chicago, IL, USA). A difference was considered statistically significant when $P<0.05$.

Table 1. Examination of anti-*B. burgdorferi* s.l. antibodies from field samples using ELISA

Geographical region		No. of samples		Positive rate (%)
Area	Province	Tested	Positive	
North	Hebei	348	33	9.5%
	Shanxi	45	17	37.8%
	Inner-Mongolia	134	47	35.1%
	Subtotal	527	97	**18.4%**
Northeast	Jilin	186	13	7.0%
	Liaoning	195	50	25.6%
	Subtotal	381	63	**16.5%**
East	Zhejiang	52	5	9.6%
	Anhui	144	40	27.8%
	Shandong	91	21	23.1%
	Subtotal	287	66	**23.0%**
Central South	Hunan	82	36	43.9%
	Guangxi	109	26	23.9%
	Guangdong	74	30	40.5%
	Subtotal	265	92	**34.7%**
Southwest	Yunnan	159	38	23.9%
	Guizhou	156	65	41.7%
	Chongqing	58	29	50.0%
	Sichuan	63	40	63.5%
	Tibet	113	6	5.3%
	Subtotal	549	178	**32.4%**
Northwest	Shannxi	74	5	6.8%
	Gansu	361	49	13.6%
	Ningxia	81	7	8.6%
	Qinghai	98	16	16.3%
	Xinjiang	135	52	38.5%
	Subtotal	749	129	**17.2%**
Total		2758	625	22.7%

RESULTS

In the present investigation, antibodies against *B. burgdorferi* s.l. were detected in 625 of 2,758 small ruminants; the mean seroprevalence was 26.3% (range: 5.3–63.5%). Positive animals were found in all provinces, with prevalence ranging from 5.3% – 63.5% (Tab. 1). If the positive rates were dissected in view of the six administrative regions in China, they were: 16.5% in the northeast, 18.4% in the north, 17.2% in the northwest, 32.4% in the Southwest, 23.0% in the east and 34.7% in central-south China. The highest seroprevalence (34.7%) was found in central- south China, which is a subtropical region. The lowest seroprevalence (16.5%) was found in northeast China, which is a temperate and frigid region. There was a significant difference in seroprevalence between these two regions ($P<0.01$) in *B. burgdorferi* s.l. infection.

DISCUSSION

Lyme disease, which is caused by *B. burgdorferi* s.l., is one of the most prevalent tick-borne zoonoses [19]. *B. burgdorferi* s.l. circulates in an enzootic cycle between the primary vertebrate reservoir and the ticks [20, 21]. Several species of rodents, birds and domestic animals, especially dogs, horses and cattle, serve as reservoir hosts in the natural life cycle of *B. burgdorferi* s.l. [8, 22]. They can become naturally infected by *B. burgdorferi* s.l. and remain infective for a long period. Since Ai et al. [3] reported Lyme disease and isolated the pathogens in Heilongjiang province in 1986, *B. burgdorferi* s.l. has been isolated in several provinces of China, including Fujian, Jilin, Liaoning, Beijing, Inner Mongolia, Xinjiang, Gansu, Hebei, Sichuan, Chongqing, Anhui, Zhejiang, Hunan, Guizhou, Guangdong, Guangxi and Taiwan [23, 24, 25, 26, 27, 28].

In epidemic areas, small ruminants are at greater risk of exposure than humans because they are more exposed to the vector ticks. Serologic survey for antibodies to *B. burgdorferi* s.l. in small ruminants may provide useful information on the geographical distribution of *B. burgdorferi* s.l. in China. However, until now, few epidemiological studies have been carried out on this pathogen in China. Thus, in the presented study, a large-scale sero-epidemiological investigation was conducted in 21 provinces using an ELISA developed with whole cell antigen, to evaluate the prevalence of *B. burgdorferi* s.l. infection in China. The cross-reactivity of the whole cell antigen of *B. burgdorferi* s.s. with the antibodies of other genospecies has been well demonstrated by immunoblot and ELISA [16]. Although it was considered that whole cell antigen preparations lack specificity because of the presence of cross-reactions with common bacterial antigens, such as heat shock proteins, flagellar antigens, and others [29, 30, 31, 32], the results are expressed as the percentage of the specific mean antibody rate (AbR%) in this study, and there was no cross-reaction with positive sera from sheep infected with *Mycoplasma*, *Brucella*, *Toxoplasma*, *Babesia*, *Theileria* and *Anaplasma* when a 15% antibody rate was chosen as the positive threshold.

To-date, isolates of *B. burgdorferi* s. l. have been classified into tens of different genomic species, of which four species (*B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, and *B. valaisiana*) have been identified in China [23, 33]. Of these, three (*B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*) are known to be pathogenic [23]. Domestic animals, such as sheep and goats, are important hosts of Borreliae. In the case of small ruminants, the course of the disease is chronic, subclinical, and sometimes without any pathological signs [8]. Serologic surveys of *B. burgdorferi* s.l. infection in small ruminants have been conducted in several provinces in China and in other countries. The seroprevalence reported in small ruminants in China was 22.2% in Anhui [15], 19.1% in Jiangxi [14], 30% in Hebei [12], and 22.5% in Xinjiang [13]; the seroprevalence was 22.1% in Turkey [9], 23.8% in Egypt [11], and 18.4% and 16.5% in Slovakia [10]. In the presented study, the seroprevalence of *B. burgdorferi* s.l. infection in small ruminants (mean: 26.3%) was comparable with the results of other reports in China and with the seroprevalence detected in other countries. However, significant differences in seroprevalence were found in different provinces, ranging from 5.3% – 63.5% (Tab. 1). Similar results have been found in Anhui province: the positive rate was 22.2% in the plains area, but 61.8% in the mountain district [15].

The seroprevalence of *B. burgdorferi* s.l. infection in small ruminants is therefore variable. The value may be dependent on the distribution of tick vectors, age of the animals, weather conditions, living conditions and husbandry practices. From a geographical point of view, the seroprevalence varied according to location, with the south of China showing higher seroprevalence rates (34.7% for central- south and 32.4% in the southwest), and decreased towards the north of China (18.4% – north, 16.5% – northeast, 17.2% – northwest). A significant difference was observed in the infection rate between the south and north of China (33.2% vs. 17.4%, $P<0.001$). It is well known that Lyme disease is associated with tick vectors; great diversity in the prevalence of *B. burgdorferi* s.l. in ticks across various locations in China has been reported by Yang et al. [34]. The positive rate of *B. burgdorferi* s.l. in ticks was lower in the north (26.6%) than in the south (61.2%, $P<0.01$) [34], which is in accordance with the results in small ruminants in this study. The seroprevalence of *B. burgdorferi* s.l. infection in the south of China was also higher than that observed in the east of China (33.2% versus 23.0%, $P<0.01$). In the south of China, the seroprevalence in the central-south was slightly higher than that observed in the southwest (34.7% vs. 32.4%, $P>0.05$). In the north of China, the seroprevalence in the north (18.4%) was slightly higher than that observed in the northwest (17.2%) and northeast (16.5%) ($P>0.05$). In this study, the results also showed great diversity in seroprevalence across the various locations studied (Tab. 1).

To date, at least 169 strains of *B. burgdorferi* s.l. have been isolated from ticks, animals and human patients distributed in 16 provinces in China [23, 24, 25, 26, 27, 28]. In these provinces, positivity of anti-*B. burgdorferi* s.l. antibodies in small ruminants has been demonstrated in 14 provinces (Xinjiang, Inner-Mongolia, Gansu, Jilin, Liaoning, Hebei, Beijing, Sichuan, Chongqing, Anhui, Guizhou, Guangdong, Hunan, Zhejiang); no serum samples were collected from the other two provinces (Heilongjiang and Taiwan). In the presented study, positivity was also found in another eight provinces (Shanxi, Shandong, Guangxi, Yunan, Shannxi, Ningxia, Qinghai, Tibet), which indicated the presence of *B. burgdorferi* s.l. in small ruminants in those province. It has been shown that *I. persulcatus* are competent vector ticks of *B. burgdorferi* s.l. throughout the world [35].

Previous studies have also indicated that *I. sinensis* has the ability to transmit spirochetes to animals during larvae–nymph and nymph–adult periods [36]. Ogden et al. revealed that sheep serve as a vehicle to transfer infection among co-

feeding ticks [37]. The co-feeding might play an important role in the horizontal transmission of *B. burgdorferi* s.l. from ticks to hosts and may pose a threat of transfer of the disease to human beings. *I. persulcatus* belongs to the *I. ricinus* species complex and occurs in Heilongjiang, Jilin, northern Inner Mongolia, Liaoning, Tibet, Xinjiang and Shanxi; *I. sinensis* occurs in Fujian, Jiangxi, Hunan, Yunnan, Zhejiang and Anhui [38] (Fig. 1). However, in the current study, positive results for *B. burgdorferi* s.l. were also found in Hebei, Shandong, Guangxi, Guangdong, Guizhou, Chongqing, Sichuan, Shannxi, Gansu, Ningxia, and Qinghai (Tab. 1). This could be explained by the following:

1) the vectors of *B. burgdorferi* s.l. exist in these provinces but have not been identified;
2) the expanding scope of the animal trade;
3) the vectors might have been introduced into these areas by the natural migration of wildlife;
4) other vector tick species may be involved in the transmission of *B. burgdorferi* s.l.

Thus, investigation of the vector ticks is required in order to determine the explanation for this situation.

To the best of the authors' knowledge, this is the first large-scale investigation of the occurrence of Lyme disease caused by *B. burgdorferi* s.l. in small ruminants in China using serologic tests. These data will provide important information about the prevalence of *B. burgdorferi* s.l. infections in small ruminants and will be very beneficial for the management and control programmes for Lyme disease.

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