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SHORT COMMUNICATION

A sero-epidemiological survey of Chinese *Babesia motasi* for small ruminants in China

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Abstract *Babesia motasi* BQ1 (Lintan) was first isolated from *Haemaphysalis qinghaiensis* collected in Gannan Tibet Autonomous Region, Gansu province in April 2000. In this study, a total of 3,204 serum samples from small ruminants in 22 provinces located in different districts of China were tested for antibodies against merozoite antigens from cultured *B. motasi* BQ1 (Lintan) by enzyme-linked immunosorbent assay. This method can survey the prevalence of low-pathogenic Chinese *B. motasi*. The results of this survey indicated that the average positive rate was 43.5 %, and the positive rates of investigated provinces were significantly different from 6.1 to 91.0 %, and the infections had been found in all provinces investigated. Our data provide large important information regarding the current sero-prevalence of *B. motasi* in China.

Introduction

Ovine babesiosis is an economically important tick-borne disease of small ruminants, caused by protozoan parasites of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa. The disease induced by *Babesia ovis*, *Babesia motasi*, and *Babesia crassa* is generally characterized by fever, anemia, hemoglobinuria, and even death in infected sheep and goats, and widespread in tropical and subtropical areas of the world (Kuttler 1988; Uilenberg 2006). *B. ovis* and *B. motasi* are believed to be the primary agents of ovine babeisosis. *B. ovis* is a small *Babesia* species transmitted by *Rhipicephalus bursa* and has high pathogenicity for small ruminants. *B. motasi* is a large one, and the vector tick is *Haemaphysalis punctata* (Uilenberg 2006; Friedhoff 1997). Nevertheless, Uilenberg (2006) considered that *B. motasi* may consist of at least two species or subspecies from different geographical areas, namely a low pathogenicity group (in northern Europe) and a high pathogenicity group (in southern Europe and the Mediterranean basin). *B. crassa* is a large non-pathogenic *Babesia* infective for small ruminants. It is different from *B. ovis* and *B. motasi* based on morphology, serology, and molecular phylogeny (Hashemi-Fesharki and Uilenberg 1981; Papadopoulos et al. 1996; Schnittger et al. 2003).

In the 1980s, ovine babesiosis was first reported by Chen (1982) and Zhao et al. (1986) in Sichuan and Heilongjiang provinces of China, respectively, and suspected that the pathogens were *B. ovis* based on the morphological character of parasites in blood smears and clinical appearance of sick animals. However, they could not isolate parasites. Since then, several large *Babesia* strains have been isolated from field-collected blood or ticks from different regions in China by Lanzhou Veterinary Research Institute (Bai et al. 2002; Guan et al. 2002; Liu et al. 2007). These strains can be divided into two clusters, based on the 18S rRNA and ITS gene sequences, namely *B. motasi* (including *B. motasi* BQ1 (Lintan), *B. motasi* BQ1 (Ningxian), *B. motasi* (Tianzhu), *B. motasi* (Madang), and *B. motasi* (Hebei)) and a new ovine *Babesia*, *Babesia* sp. Xinjiang (Liu et al. 2007; Niu et al. 2009; Guan et al. 2009). For Chinese *B. motasi*, these strains could be further separated into two subclusters based on ITS gene sequences, low-pathogenic *B. motasi* BQ1 (Lintan), *B. motasi* (Tianzhu), and *B. motasi* (Madang) and high-pathogenic *B. motasi* BQ1 (Ningxian) and *B. motasi* (Hebei) (Niu et al. 2009). Moreover, Guan et al. (2010c) demonstrated that the antigens from in vitro

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culture of *B. motasi* BQ1 (Lintan) had cross-reaction with positive sera from *B. motasi* (Tianzhu) infected sheep but had not with those of *B. motasi* BQ1 (Ningxian) and *B. motasi* (Hebei) in ELISA. These results are coincident with Uilenberg's (2006) demonstration: there are at least two species or subspecies in *B. motasi* in China.

In addition, the strains *B. motasi* BQ1 (Ningxian) and *B. motasi* BQ1 (Lintan) could be transmitted by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis* (Bai et al. 2002; Guan et al. 2010b), and the two tick species are of wide distribution in China (Teng and Jiang 1991; Yin and Luo 2007; Chen et al. 2008; Chen et al. 2010). The prevalence of babesiosis is usually consistent with distribution of its vector ticks in the given area (Kuttler 1988). However, so far, the prevalence of *B. motasi* has not been investigated in China as there were no appropriate detection techniques. Guan et al. (2010c) developed an ELISA using merozoite soluble antigens of *B. motasi* BQ1 (Lintan) derived from in vitro culture. Specificity of the ELISA was 95.5 % when 30 % of the antibodies rate was chosen as its positive threshold. There was no cross-reaction with positive sera from *B. motasi* BQ1 (Ningxian), *B. motasi* (Hebei), *Babesia* sp. Xinjiang, *Theileria luwenshuni*, *Theileria uilenbergi*, and *Anaplasma ovis* infected sheep, but strong cross-reaction was present with *B. motasi* (Tianzhu). Thus, this technique is specific for Chinese low-pathogenic *B. motasi* and can be used to perform epidemiological investigation. In this study, we propose to carry out a large-scale sero-epidemiological survey for low-pathogenic *B. motasi* in small domestic ruminants using the ELISA to understand its exact prevalence and distribution in China.

Materials and methods

A cloned line (G7) of *B. motasi* BQ1 (Lintan), derived from in vitro culture by limiting dilution, provided by the Vector and Vector-borne Disease (VVBD) laboratory of Lanzhou Veterinary Research Institute (LVRI), was original isolated from a splenectomized sheep infested by adult *H. qinghaiensis* collected in the field. This parasite was cultured in vitro as described previously (Guan et al. 2002; Guan et al. 2010a). Briefly, parasites were cultured in sheep erythrocytes in RPMI-1640 (Lonza, Belgium) supplemented with 20 % of heat-inactivated fetal bovine serum (FBS) in 24-well plates. Cultures were incubated in a humidified 5 % CO_2 atmosphere at 37 °C. To produce large quantities of cultured parasites, the cultures were shifted into 75-cm^2 flasks with erythrocytes (2.5 %) and FBS (20 %) (Fig. 1a).

Merozoite antigens of *B. motasi* BQ1 (Lintan) were prepared according to method previously described by Guan et al. (2010c) with few modifications. Briefly, the cultures were centrifuged (900×*g*, 10 min, 20 °C) when percentage of parasitized erythrocytes reached 5–8 % in 75-cm^2 flasks, and the pellets of erythrocytes were re-transferred into flasks added fresh erythrocytes up to 1 ml to continue cultivation. Then, supernatant was shifted into 50-ml tubes and centrifuged (3,000×*g*, 30 min, 4 °C) for harvesting the free merozoite pellets (Fig. 1b). To remove contaminants of hemoglobin and fragments of erythrocytes, the pellet was washed three times in phosphate-buffered saline, pH7.2, (14,000× g, 15 min, 4 °C). After the third washing, the pellet was resuspended in PBS and subjected to five freeze–thawing cycles, sonicated on ice for 30 min (5-s sonication with 5-s interval), centrifuged (13,000×*g*, 10 min, 4 °C), and the supernatant was used as antigen. The protein concentration was measured with the kit of BCA protein assay (71285–3, Novagen). Reactivity and specificity of the antigens were tested using ELISA and western blot (Guan et al. 2010c).

Standard positive and negative control sera were provided by the VVBD laboratory of LVRI (Guan et al. 2010c). The field sera from sheep (*n*=3,204) were randomly collected from 44 counties which covered 22 provinces of China, from March to September during 2010–2012, and stored at −20 °C until use (Guan et al. 2012). In these samples, 2,922 sera were collected from 39 prefectures of 20 provinces in which the recorded vector ticks of *B. motasi*, such as *H. longicornis*, *H. qinghaiensis*, and/or *H. punctata* are distributed, comprising Gansu (Lanzhou, Wuwei and Gannan), Xinjiang (Altay, Aksu, and Ili), Qinghai (Haibei), Hebei (Baoding), Ningxia (Wuzhong), Shanxi (Lvliang), Jilin (Songyuan), Liaoning (Liaoyang), Shandong (Dongying), Anhui (Hefei), Hubei (Suizhou),

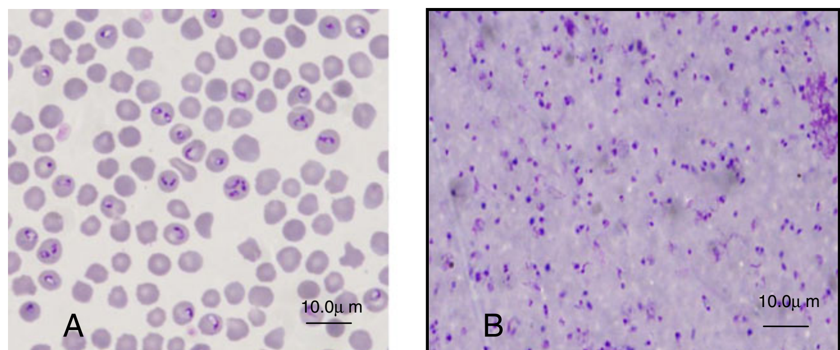

Fig. 1 Micrograph of smear of *B. motasi* BQ1 (Lintan). **a** The blood smear of *B. motasi* BQ1 (Lintan) from in vitro culture, bar=10.0 μm. **b** Free merozoites purified from supernatant

Shannxi (Yulin), Tibet (Lhasa), Sichuan (Panzhihua and Luzhou), Yunnan (Wenshan, Xishuangbana, Qujing, Dehong, and Honghe), Chongqing (Jiangjin and Wanzhou), Hunan (Yongzhou, Huaihua, and Changde), Guangdong (Qingyuan and Zhaoqing), Zhejiang (Jinhua, Taizhou, and Lishui), and Guizhou (Qiandongnan, Qiannan, Qianxinan, Anshun, and Guiyang). In addition, 282 field sera were collected from sheep in five prefectures of two provinces in which no vector ticks of *B. motasi* were distributed, Inner Mongolia (Chifeng) and Guangxi (Chongzuo, Baise, Nanning, and Guilin) (Chen et al. 2010; Yin and Luo 2007; Chen et al. 2008; Teng and Jiang 1991; Chu et al. 2008; Zheng et al. 2011) (Table 1).

Standard enzyme-linked immunosorbent assays (ELISAs) described previously by Guan et al. (2010c) were performed in the present study. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl of merozoites antigen at a concentration of 3 μg/ml. On blocking, 150 μl of 2 % gelatin in carbonate buffer was used. Sera were diluted at 1:200 with the PBST, and blank (PBST), standard positive and standard negative sera as control. Second antibody, HRP-conjugated monoclonal anti-sheep/goat IgG antibody (A-9452, Sigma) was diluted at 1:2,000 with the PBST. Positive signal was present 50 μl of TMB, and the reaction was terminated by 50 μl of 0.1 M H_2SO_4. The optical density (OD) was measured with an ELISA automat (Microplate reader 680, BIO-RAD) at a wavelength of 450 nm. The results were represented as percentage of antibody mean rate (AbR %) calculated with the formula: AbR %=(Sample mean OD−Negative control mean OD)/(Positive control mean OD−Negative control mean OD)×100 %. The cutoff value was chose 30 % as previously reported (Guan et al. 2010c).

Results

Sero-epidemiology of *B. motasi* BQ1 (Lintan) in small ruminants from China is presented in Table 1. A total of 3,204 sera were randomly collected form 22 provinces in China and examined by the ELISA technique. The overall positive sera was 43.6 % (n=1,446). The sero-prevalence was varied from 1.6 to 91.0 %. The positive ratio was significantly higher in Inner Mongolia (91 %), Hunan (81.7 %), Guangdong (82.4 %), and Shandong (76.9 %), and the lowest sero-prevalence was obtained in Jilin province (1.6 %), in the northeast of China.

Discussion

Since Chen (1982) and Zhao et al. (1986) reported ovine babesiosis in Sichuan and Heilongjiang provinces in the 1980s, this disease had been reported in several provinces of China, such as Yunnan, Shanxi, Henan, and Gansu, and

Table 1 ELISA results of *B. motasi* antibodies detection in field-collected sera from 22 provinces in China

Province	Prefecture (no. of positive sera/no. of sera)	No. of sera	No. of positive sera	Positive ratio (%)
Inner Mongolia	Chifeng	134	122	91.0
Guangdong	Qingyuan (30/36)	74	61	82.4
	Zhaoqing (31/38)			
Hunan	Yongzhou (25/27)	82	67	81.7
	Huaihua (23/29)			
	Changde (19/26)			
Shandong	Dongying	91	70	76.9
Liaoning	Liaoyang	195	143	73.3
Chongqing	Jiangjin (20/30)	58	42	72.4
	Wanzhou (22/28)			
Sichuan	Panzhihua (20/32)	63	42	66.7
	Luzhou (22/31)			
Shanxi	Lvliang	50	31	62.0
Xinjiang	Altay (38/90)	464	267	57.5
	Aksu (86/187)			
	Ili (143/187)			
Guangxi	Chongzuo (13/18)	148	78	52.7
	Baise (30/60)			
	Nanning(9/31)			
	Guilin (26/39)			
Anhui	Hefei	144	74	51.4
Yunnan	Wenshan (9/29)	160	70	43.8
	Xishuangbanna (13/32)			
	Qujing (12/35)			
	Dehong (20/32)			
	Honghe (16/32)			
Gansu	Lanzhou (4/91)	337	116	34.4
	Wuwei (15/62)			
	Gannan (97/224)			
Qinghai	Haibei	96	33	34.4
Guizhou	Qiandongnan (9/29)	157	53	33.8
	Qiannan (11/30)			
	Qianxinan (7/34)			
	Anshun (15/32)			
	Guiyang (11/32)			
Ningxia	Wuzhong	80	22	27.5
Zhejiang	Jinhua (3/17)	65	13	20.0
	Taizhou (7/29)			
	Lishui (3/19)			
Hebei	Baoding	396	63	15.9
Tibet	Lhasa	113	13	11.5
Shaanxi	Yulin	74	7	9.5
Hubei	Suizhou	37	3	8.1
Jilin	Songyuan	186	3	1.6
Total		3,204	1,393	43.5

morbidity and mortality ranged from 12.55 to 39.42 % and 0.7 to 27.22 %, respectively (Yin et al. 1997; Li et al. 2006; Yang et al. 2009; Zhang and Song 2004). It caused serious problems for livestock industry of small ruminants. But till now, few epidemiological studies were carried out on this disease in China. Guan et al. (2008) showed that the positive rate, determined by a LAMP method for *B. motasi* BQ1 (Lintan), was 14.3 % (52/365) in Gannan Tibet Autonomous Region of Gansu Province in China (Guan et al. 2008). In 2010, 974 sera collected from Gansu province were tested for the antibody of *B. motasi* BQ1 (Lintan) by the ELISA, with soluble merozoite antigens of *B. motasi* BQ1 (Lintan) from culture in vitro. The average positive rate of *Babesia* sp. BQ1 (Lintan) was 66.84 % (Guan et al. 2010c). Recently, Guan et al. (2012) developed an ELISA for *Babesia* sp. Xinjiang infection using merozoite antigen from in vitro culture and a sero-epidemiological survey was performed for infection of *Babesia* sp. Xinjiang in 3,856 of small ruminants which covered 22 provinces in China. Results showed that the average positive rate was 31.66 %. However, epidemiological data of *B. motasi* is absent in China. Thus, in the present study, a large-scale sero-epidemiological investigation was conducted in 22 provinces using the ELISA developed with merozoite antigen derived from in vitro culture of *B. motasi* BQ1 (Lintan) to evaluate the prevalence of low-pathogenic *B. motasi* in China. The results showed that the infections had been found in all provinces investigated, although the sero-positive rates demonstrated significant difference between some provinces. Sero-positive rates varied from 1.6 to 91.0 %, and the average prevalence was 43.5 %, which indicated low-pathogenic *B. motasi* is common prevalence in China.

It was well considered that *H. punctata* was the vector ticks of *B. motasi* (Freidhoff 1997; Uilenberg 2006), and Guan et al. proved that *H. qinghaiensis* and *H. longicornis* could also transmit *B. motasi* BQ1 (Lintan) to sheep by experiment (Guan et al. 2010b). Experimental transmission of *H. punctata* for *B. motasi* BQ1 (Lintan) has not been studied. However, these evidences indicate that *B. motasi* at least can be transmitted by *H. punctata*, *H. qinghaiensis*, and *H. longicornis*, although other tick species still need to be proved as vector ticks of *B. motasi* via experimental transmission in future. In China, *H. qinghaiensis*, *H. longicornis*, and *H. punctata* are of wide distribution except in Fujian, Guangxi, Hainan, and Inner Mongolia provinces on the basis of current literature (Chen et al. 2010; Yin and Luo 2007; Chen et al. 2008; Teng and Jiang 1991; Chu et al. 2008; Zheng et al. 2011). However, in the present study, the positive rates for of *B. motasi* in Guangxi and Inner Mongolia were 52.7 and 91.0 %, respectively. This could be explained as following: (1) the vectors might be introduced into these areas by the natural migration of the wildlife; (2) with the expanding scope of animal trade, sheep and goats carried *B. motasi* may be purchased from an endemic areas; (3) the fact that the vector of *B. motasi* might exist in Fujian, Hainan, and Inner Mongolia has not been identified. Thus, investigation of the vector ticks and detection of *B. motasi* in ticks would be performed to interpretate the real reasons for this condition in future.

To our knowledge, this is the first large-scale investigation of the occurrence of ovine babesiosis caused by *B. motasi* in small ruminants in China using serologic tests. These data might provide important information about the incidence of *B. motasi* infections in small ruminants and would be very beneficial for management and control programs of babesiosis. The ELISA approach, used in this study, could be used to survey the prevalence of low-pathogenic Chinese *B. motasi*. However, the positive sera against to high-pathogenic Chinese *B. motasi*, such as *B. motasi* Hebei and *B. motasi* BQ1 (Ningxian), do not react with the soluble merozoite proteins from *B. motasi* BQ1 (Lintan). Future studies should develop an ELISA with the recombination proteins or molecular approach which would be used to conduct the prevalence of these parasites in China.

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Conflict of interest None declared.

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