Parasitology International 61 (2012) 532–537

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

A recently identified ovine *Babesia* in China: Serology and sero-epidemiology

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ARTICLE INFO

Article history:
Received 21 February 2012
Received in revised form 22 April 2012
Accepted 23 April 2012
Available online 2 May 2012

Keywords:
Babesia sp. in Xinjiang
Merozoite antigens
ELISA
Western blotting
Sero-epidemiology

ABSTRACT

Babesia sp. in Xinjiang, transmitted by *Hyalomma*, is a large *Babesia* that is infective for small ruminants, but it has almost no pathogenicity in healthy sheep. On the basis of the sequences of the 18S rRNA and internal transcribed spacer (ITS) genes, morphological characteristics, vector tick species and pathogenicity it was identified recently as a novel *Babesia* species. In the present study, an enzyme-linked immunosorbent assay (ELISA) was developed using soluble merozoite antigens of *Babesia* sp. in Xinjiang (BXJMA) derived from *in vitro* culture. When the positive threshold was chosen as 24.65% of the specific mean antibody rate, the specificity and sensitivity were both 97.3%. There was no cross-reaction between BXJMA and positive sera from sheep infected with other Chinese ovine piroplasms or *Anaplasma ovis* in the ELISA and western blotting. Specific antibodies against *Babesia* sp. in Xinjiang could be detected 2 weeks post infection and a high level of antibodies persisted for more than 12 weeks in experimentally infected sheep. The ELISA was tested on 3857 sera collected from small ruminants in 50 prefectures of 22 provinces to evaluate the sero-epidemiology of *Babesia* sp. in Xinjiang infection, and the average positive rate was 31.66%. These data provide that the developed ELISA is a powerful tool for the sero-diagnosis of *Babesia* sp. in Xinjiang and confirm that it is a novel species.

1. Introduction

Babesia sp. in Xinjiang, a large ovine *Babesia* species, was isolated from a splenectomized sheep infested with partially engorged *Rhipicephalus sanguineus* and *Hyalomma anatolicum anatolicum* collected from sheep, goats and cattle in the Kashi prefecture, Xinjiang Uygur Autonomous Region (province) in northwestern China in 2001 [1]. The morphological characteristics of the erythrocytic stages showed that the typical piriform pair was slender. The average size was 2.42 (±0.35) μm × 1.06 (±0.22) μm [2]. The *Babesia* species has almost no pathogenicity for healthy sheep: no clinical signs were presented by such sheep post infection. Infected splenectomized sheep showed clinical signs of fever, parasitemia and mild hemolytic anemia but recovered naturally. More serious signs, such as depression, lack of appetite, recumbency, and serious anemia were presented if the splenectomized animals were immuno-suppressed with injections of 10 mg dexamethasone per day. If the parasitemia exceeded 5%, animals would die. The febrile response lasted for 3–6 days, and the maximum temperature recorded was 41.5 °C [1–3]. Experimental transmission confirmed that *H. a. anatolicum* was the vector of *Babesia* sp. in Xinjiang [2].

Liu et al. [4] performed phylogenetic analysis on ovine *Babesia* species based on 18S rRNA gene sequences. The result showed that the percent identities shared by *Babesia* sp. in Xinjiang and other ovine *Babesia* species was between 73.4% and 86.8%. On the basis of ITS gene sequences, the percent identity shared by *Babesia* sp. in Xinjiang and other Chinese ovine *Babesias* was 2.5–12.2% [5]. Although it has 94.6–94.9% percent identity with two Chinese bovine strains of *Babesia*, *Babesia* U sp. Kashi (infective to cattle and transmitted by *Hyalomma* spp.) [6] and *B. orientalis* (only infective to buffalo and transmitted by *R. haemaphysaloides*) [7], *Babesia* sp. in Xinjiang could not infect cattle by experimental inoculation [2]. It can be differentiated easily from the ovine *Babesia* species *B. motasi*, *B. ovis*, *B. crassa* and other Chinese *Babesia* spp., as reported by Bai et al. [8] and Guan et al. [9]. Therefore, the parasite was reported to be a novel ovine *Babesia* species by Guan et al. [2] on the basis of the abovementioned data.

However, there is not sero-diagnostic technique available for *Babesia* sp. in Xinjiang, which hinder us to explore serological and sero-epidemiological characteristics for identifying and naming the parasite as a valid *Babesia* species in future. Therefore, in the present study, an enzyme-linked immunosorbent assay (ELISA) was developed using soluble merozoite antigens from *in vitro* culture of *Babesia* sp. in Xinjiang. The serological cross-reactions between the antigens of *Babesia* sp. in Xinjiang and sera against *Babesia*, *Theileria* and *Anaplasma* species infective to ruminants in China were explored using techniques of ELISA and western blotting. In addition, sero-epidemiological surveillance for

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doi:10.1016/j.parint.2012.04.004

Babesia sp. in Xinjiang infection was performed in 50 prefectures of 22 provinces using the ELISA. These data suggest that the ELISA can be considered as a powerful tool to use in sero-diagnosis of *Babesia* sp. in Xinjiang infection and confirm that the parasite is a novel species infective to small ruminants.

2. Materials and methods

2.1. Parasites

A monoclonal line (G5) of *Babesia* sp. in Xinjiang derived from *in vitro* culture by limiting dilution, as described previously [10], was maintained and cryopreserved in liquid nitrogen at the Lanzhou Veterinary Research Institute (LVRI), China.

2.2. Sera

Sera positive for *Babesia* sp. BQ1 (Lintan) (n = 5), *Babesia* sp. BQ1 (Ningxian) (n = 4), *Babesia* sp. Tianzhu (n = 4), *Babesia* sp. Hebei (n = 4), *Theileria luwenshuni* (n = 2), *T. uilenbergi* (n = 2), *B. bovis* (n = 2), *B. bigemina* (n = 2), *Babesia* U sp. Kashi (n = 2) and *Anaplasma ovis* (n = 3) were provided by the Vector and Vector-borne Disease (VVBD) laboratory of LVRI [11–15].

Sera collected 12 weeks post infection (wpi) [3] from two Tan mutton sheep (Nos. 3201 and 026) infected with the original strain of *Babesia* sp. in Xinjiang were used to evaluate antibody kinetics.

Sera collected from 371 4–6 month-old lambs, purchased from a *Babesia*-free region in Jingtai county, Gansu province of China before experiments, from 2005 to 2010, were used to evaluate the specificity of the ELISA, as extensive testing (light microscopic examination (ME), PCR, nested PCR (nPCR), reverse line blot (RBL) or loop-mediated isothermal amplification (LAMP)) has never found an animal positive; a mixture of these sera was used as the negative control serum. Sera (n = 112) collected 3–12 wpi from 10 sheep experimentally infected with *Babesia* sp. in Xinjiang were used to evaluate the sensitivity of the ELISA, and a mixture of these sera was used as the positive control serum [11].

Field sheep sera were collected from 3449 sheep in 36 prefectures of 17 provinces in which *Hyalomma* spp. are distributed [16–19], during March to September 2010 and 2011. The provinces sampled were: Gansu (Jiuquan, Zhangye, Wuwei, Baiyin and Gannan), Xinjiang (Shihezi, Urumchi, Aksu and Ili), Qinghai (Haibei), Inner-Mongolia (Erdos and Chifeng), Hebei (Baoding and Chengde), Ningxia (Wuzhong), Shanxi (Lvliang), Jilin (Songyuan), Liaoning (Liaoyang), Shandong (Dongying), Anhui (Hefei), Hubei (Suizhou), Shannxi (Yulin), Tibet (Lhasa), Sichuan (Panzhihua and Luzhou), Yunnan (Wenshan, Xishuangbana, Qujing, Dehong and Honghe), and Guizhou (Qiandongnan, Qiannan, Qianxinan, Anshun, Guiyang and Tongren). In addition, 408 field sera were collected from sheep in 14 prefectures of 5 provinces in which no *Hyalomma* spp. were distributed [16–19], during March to June 2011, comprising Chongqing (Jiangjin and Wanzhou), Guangxi (Chongzuo, Baise, Nanning and Guilin), Hunan (Yongzhou, Huaihua and Changde), Guangdong (Qingyuan and Zhaoqing), and Zhejiang (Jinhua, Taizhou and Lishui) provinces (Fig. 1, Table 1).

2.3. Preparation of the soluble antigens

An *in vitro* culture of *Babesia* sp. in Xinjiang (G5) in sheep erythrocytes was maintained in RPMI-1640 medium supplemented with 20% FBS in 75 cm^2 flasks. Harvesting of free merozoites and preparation of soluble merozoite antigens from *Babesia* sp. in Xinjiang (BXJMA) were conducted as described previously by Guan et al. [11] with some modification. Briefly, the cultures were harvested into 50 ml tubes when parasitaemia reached 10–20%, and centrifuged at 900 *g* for 10 min. The supernatants were collected into 50 ml tubes for harvesting free merozoites by centrifugation at 3000 *g* for 15 min. The pellets of merozoites were washed 3 times with phoshphate-buffered saline, pH 7.2 (PBS). The collected merozoites were re-suspended with PBS and subjected to 5 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 antigen (BXJMA). The protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Cat No. 71285-3, Novagen, USA) and the samples were stored at −20 °C until use.

2.4. Western blotting procedure

Proteins from BXJMA or lysates of uninfected sheep erythrocytes were separated by 12% SDS-PAGE and electroblotted onto nitrocellulose (NC) membranes of 0.45 mm pore size (RPN303E, Amersham). The NC sheet was cut into strips 0.25 cm in width (each strip containing about 16 μg proteins) and blocked with 10% skimmed milk powder in 0.1 M Tris-buffered saline (pH 7.6) with 0.1% Tween 20 (TBST), overnight at 4 °C. The NC strips were probed with each test serum. Following four washes with TBST, secondary antibodies, monoclonal anti-goat/sheep IgG–alkaline phosphatase conjugate, produced in mice (A8062, Sigma) or anti-bovine IgG (whole molecule)–alkaline phosphatase conjugate, produced in rabbits (A0705, Sigma), were applied. After five washes with TBST, positive signals were revealed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (B1911, Sigma) [11,20].

2.5. ELISA procedure

An ELISA procedure described previously by Chauvin et al. [21] and Guan et al. [11] was employed in this study. Briefly, 96-well microplates (Nunc) were coated at 37 °C for 1 h and then at 4 °C overnight with 100 μl/well of BXJMA at a concentration of 8 μg/ml in a coating buffer (0.1 M carbonate–bicarbonate buffer, pH 9.6). The plates were washed three times with PBS containing 0.1% Tween 20 (PBST) and incubated with 150 μl/well of a blocking solution (2% gelatin in PBST) for 30 min at 37 °C. After drying the plate, the samples, blank (PBST), standard positive and negative control (dilution of 1: 200) were distributed in duplicate and the plates incubated at 37 °C for 1 h. After washing as above, a peroxidase conjugate of monoclonal anti-goat/sheep IgG clone GT-34 (A-9452, Sigma) diluted at 1:2000 or a peroxidase conjugate of anti-bovine IgG (whole molecule)–peroxidase antibody produced in rabbits (A-5295, Sigma) diluted at 1:20,000 with PBST was added to each well and the plates were again incubated at 37 °C for 1 h. The plates were washed five times as described above, and subsequently 50 μl of TMB (T0440-1L, Sigma, USA) was added to each well and incubated at room temperature for 15 min. The reaction was stopped by the addition of 50 μl of 0.1 M H_2SO_4 and the optical density (OD) was measured with an ELISA reader (microplate reader Model 680, Bio-Rad, USA) at a wavelength of 450 nm. 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%. Cutoff points, specificity and sensitivity of the ELISA were evaluated by receiver operating characteristic (ROC) analysis using MedCalc statistical software (version 11.4; http://www.medcalc.be) with 371 sera from sheep not infected with *Babesia* and 112 sera from sheep infected with *Babesia* sp. in Xinjiang.

3. Results

3.1. Evaluation of positive threshold value, specificity and sensitivity of the ELISA

The positive threshold value, specificity and sensitivity were determined by testing 371 negative sera and 112 positive sera. The AbR was calculated for each serum sample and MedCalc statistical software