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Molecular and serological evidence for *Anaplasma* infection in yaks

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Abstract: Bovine anaplasmosis caused by *Anaplasma marginale* is considered to be an important disease in China; large-scale investigations have not been carried out since the 1990s, however, and whether or not yaks can be infected by *A. marginale* has never been investigated. In this study, 2 yaks were successfully infected by the inoculation of blood infected with *A. marginale* under experimental conditions. Furthermore, an investigation of 482 field serum samples from yaks using an enzyme-linked immunosorbent assay (ELISA) with the recombinant major surface protein 5 (MSP5) showed that 84.4% of the results were positive. In consideration of previously determined ecological and biological data related to *A. marginale*, it is assumed that yaks are potential natural reservoir hosts of *A. marginale* in the northwest of China.

Key words: Yak, *Anaplasma marginale*, ELISA, MSP5

Introduction

Bovine anaplasmosis, an important vector-borne disease caused by *Anaplasma* spp., is endemic in tropical and subtropical areas of the world (1). The acute phase of bovine anaplasmosis is characterized by fever, anemia, general weakness, pallor of the mucous membranes, weight loss, abortion, decreased milk production, and mortality (1,2). Currently, *A. marginale* is the only pathogen recognized to cause anaplasmosis of cattle in China (3,4). With regard to diagnosis, a complement fixation test (CFT) and indirect fluorescent antibody test (IFAT) were developed in the 1990s (5,6). However, these methods were not applied widely for serological investigation in the field. Investigation has been performed only by microscopic examination of blood smears, and the results showed that the rate of *A. marginale* infection in cattle ranged between 20% and 79.31% in 26 provinces in China (3). Recently, an indirect enzyme-linked immunosorbent assay (ELISA) based on recombinant major surface protein 5 (MSP5) was established and a primary evaluation was reported (7,8). In this study, the MSP5-based ELISA was used to assess the current prevalence of anaplasmosis in yaks in China.

To our knowledge, *A. marginale* infection in yaks has never been reported in China. In order to investigate whether the yak is susceptible to *A. marginale*, 2 yaks (Numbers 32 and 33), both 2 years old and free of *Babesia*, *Theileria*, and *Anaplasma*, were inoculated with 5 mL of blood infected with 20% *A. marginale* parasitemia originating from cattle in Henan Province. After the inoculation, clinical signs, rectal temperature, and blood smears were observed

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daily for 1 month. Serum and EDTA-anticoagulated blood samples were prepared on postinfection days 0, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, and 150 in order to detect antibodies and the nucleic acid of the pathogen using ELISA and *polymerase chain reaction* (PCR), respectively. The DNA was extracted using a genomic DNA extraction kit (Gentra, QIAGEN, Germany). The infected animals did not show any clinical signs or changes in body temperature during the whole period of observation (data not shown); however, *A. marginale*-like pathogens were observed in blood smears from the 20th day after infection in both yaks, indicating a mild infection in the experimental yaks.

Antibodies against MSP5 were monitored using the ELISA method (7). The results showed that specific antibodies were detectable on the 10th day after inoculation, and the antibody response lasted for more than 1.5 months (Figure). To further confirm the infection, the blood of the inoculated yaks was examined by PCR. The primers (MSP45/MSP43) and program were used as described previously (9). The reaction occurred in a final volume of 50 µL, which contained 1.0 mM of each primer, 5 µL PCR buffer, 4 µL dNTPs, 0.25 µL TaKaRa Taq (5 U/mL) (TaKaRa, China), and 1 µL of the DNA sample. The PCR products were ligated into pGEM-T Easy vectors (Promega, USA) and then transformed into *Escherichia coli* JM 109. The positive clones were subjected to sequencing by the BigDye Terminator Mix supplied by TaKaRa. The NCBI sequence blast using the BLASTn tool revealed the presence of *Anaplasma* sequences with a maximum of 99.7% similarity to *A. marginale* sequences, which confirmed successful infection. The sequence was deposited in GenBank under the accession number HM640938.

In order to evaluate the disease under field conditions, 482 serum samples collected from yaks in Xinjiang, Inner Mongolia, and Gansu Province in September of 2005 were investigated using ELISA (7). The ELISA protocol was as follows: 96-well polystyrene ELISA plates (Nunc, Denmark) were coated with 100 µL of recombinant MSP5 antigen (5 µg/mL) per well and incubated overnight at 4 °C. All subsequent incubation steps were preceded by washing 3 times with phosphate-buffered saline (PBS; 137 mM NaCl, 2.67 mM KCl, 3.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) containing 0.1% Tween 20. After washing the plates, nonspecific binding sites were blocked by the addition of 200 µL of blocking solution (0.5% fish gelatin in 0.1 M carbonate buffer, pH 9.6) to each well followed by incubation for 30 min at 37 °C. The serum samples were diluted at a ratio of 1:200 in PBS containing 0.5% fish gelatin, and 100 µL/well was added prior to incubation at 37 °C for 1 h. The secondary antibody, horseradish peroxidase-conjugated rabbit antibovine IgG (Jackson ImmunoResearch Laboratories, Inc., Lot Number 50511), was diluted at a ratio of 1:20,000; 100 µL was added to each well and incubated at 37 °C for 1 h. In the next step, 100 µL of substrate solution (0.4 mg/mL o-phenylenediamine (OPD) in phosphate/citrate buffer, pH 4.0, containing 0.08% hydrogen peroxide) was added to each well and allowed to develop for 30 min at room temperature.

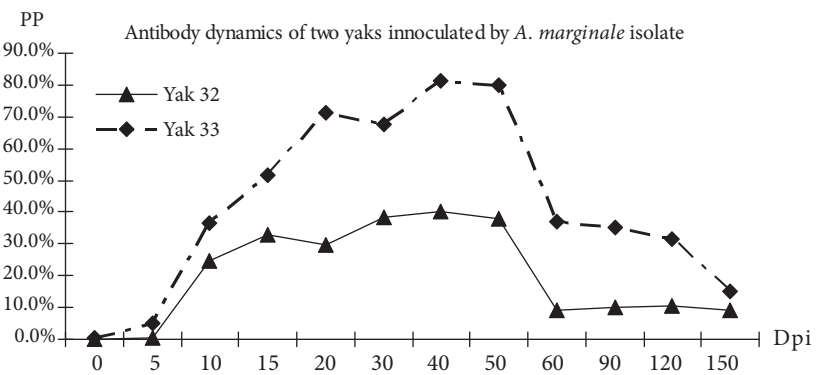

Figure. The IgG antibody dynamics of 2 yaks experimentally infected with Anaplasma marginale.

The reaction was stopped by the addition of 50 µL of 2 M H_2SO_4, and the optical density (OD) value was read at 492 nm with an ELISA reader (Stat Fax 2600, USA).

The results were presented as the percentage of the OD value, which was calculated using the formula below.

$$OD\% = \left(\frac{\text{Mean OD of sample} - \text{Mean OD of standard negative}}{\text{Mean OD of standard positive} - \text{Mean OD of standard negative}}\right) \times 100$$

The test was considered to be valid only when the mean OD value of the standard positive divided by the mean OD value of the standard negative was greater than 2. A percentage of the OD value greater than 20% was judged to be positive, while those lower than 20% were rated as negative.

The results showed that 407 out of 482 yaks (84.4%) were positive (Table). The rate of seropositivity in naturally infected yaks was 81.9%, 92.6%, and 85.6% in Xinjiang, Inner Mongolia, and Gansu Province, respectively (Table). This is the first serological evidence that yaks can be infected naturally by *Anaplasma* spp.

Bovine anaplasmosis was considered to be an important disease in China in the 1990s, when much effort was expended on the prevention and control of the disease (5,6). With the use of chemical treatments, changes in herding management, tick control programs, and improvements in animal health, outbreaks of the disease have not been reported frequently during the last 10 years, although occasional cases in cattle still occur from region to region (10-12). However, no infection in yaks has been reported so far. In this study, 2 yaks were successfully infected by inoculation of blood infected with *A. marginale*. The infection evolved in a mild form in terms of the body temperature and clinical signs. The limited data do not suggest that the yak is more resistant to *Anaplasma* infection than cattle; however, it is clear that yaks might be a potential reservoir of *A. marginale*.

Table. Information on the source and serum positivity levels of the field samples tested for *Anaplasma* species.

Province	Animals tested	Seropositive animals (%)
Xinjiang	282	231 (81.9%)
Inner Mongolia	68	63 (92.6%)
Gansu	132	113 (85.6%)
Total	482	407 (84.4%)

This result encouraged us to investigate the infection of yaks with *Anaplasma* under field conditions. In testing serum samples from yaks in some regions of potential *Anaplasma* endemicity (Table), we observed a surprisingly high portion of positive serum samples. However, we did not have proper field blood samples with which to confirm the infection on the basis of nucleic acid detection. It is known that the reservoir hosts of *A. marginale* include cattle and many other ruminants, and that approximately 20 species of ticks are capable of transmission of *A. marginale*, as reviewed by Kocan (13). Although we were unable to isolate *Anaplasma* pathogens from the yaks investigated, the distribution of biological transmission vectors of *A. marginale* and the spread of the *A. marginale* pathogens in cattle in the regions under study have been documented. In previous studies, *A. marginale* was isolated successfully from ticks collected in Gansu Province, and the experiments showed that *Rhipicephalus* (*Boophilus*) *microplus* transmits *A. marginale* from stage to stage (transstadial) or within a stage (intrastadial); *Haemaphysalis longicornis* and *Hyalomma detritum* transmit *A. marginale* intrastadially (14,15). With regard to tick infestation of yaks, 9 species in 4 genera have been recorded: *Ixodes ovatus*, *Dermacentor abaensis*, *D. nuttalli*, *D. silvarum*, *Haemaphysalis warburtoni*, *H. formosensis*, *H. qinghaiensis*, *H. tibetensis*, and *R.* (*Boophilus*) *microplus* (16). It is clear that some of these species are the vectors of *A. marginale*. However, this is indirect evidence that the yak is a potential natural reservoir host of *A. marginale*; further molecular and pathological investigations must be carried out in order to confirm this hypothesis.

Persistently infected reservoir hosts of *A. marginale* are potentially important factors in the epidemiology and spread of anaplasmosis because they could serve as a source of infective blood for mechanical spread by various routes and for biological transmission by ticks. This study showed that the yaks in China are potentially infected with *Anaplasma* pathogens. This should warn both local government and animal holders to consider yaks as a potential source for outbreaks of the disease.

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