NOTE *Parasitology*

Epidemiological Investigation of Equine Piroplasmosis in China by Enzyme-Linked Immunosorbent Assays

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ABSTRACT. The objective of this study is to investigate the seroprevalence of equine piroplasmosis in China. A total of 1990 sera were collected from clinically healthy horses in various districts located in ten different provinces of China and examined by using indirect enzyme-linked immunosorbent assays (ELISAs) with recombinant *Theileria equi* (*T. equi*) merozoite antigen 2 (rEMA-2) and *Babesia caballi* (*B. caballi*) 48-kDa rhoptry protein (rBc48), respectively. The results showed that 1,018 (51.16%) and 229 (11.51%) samples were positive for *B. caballi* and *T. equi* infection, respectively. The number of samples with mixed infection was 152 (7.64%). These results indicated that equine piroplasmosis was widespread in China.

KEY WORDS: *B. caballi*, ELSIA, serological prevalence, *T. equi.*

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Equine piroplasmosis is a tick-borne disease of horses, donkeys, mules and zebra. This disease is caused by two kinds of hemoprotozoan parasites, named as *Babesia caballi* (*B. caballi*) and *Theileria equi* (*T. equi*), both of which are transmitted by species of Ixodid ticks [5]. Clinically, the acute phase of the disease is characterized by fever, anorexia, weight loss, tachypnea and congestion of mucous membranes, but chronic infection usually presents unconspicuous clinical symptoms. Persistently-infected horses that recover from the acute infection usually carry the parasites for lifelong and serve as the reservoir hosts for transmission to other susceptible animals [21]. There are no vaccines available for this disease [12]. The control measures of equine piroplasmosis require effective diagnostic approaches that can detect carrier or chronically infected animals.

Equine piroplasmosis has a worldwide distribution. In recent years, many surveillance studies of equine piroplasmosis have been reported in many countries all over the world, such as Korea, Mongolia, Venezuelan, Tunisia, Sudan, Italy, Hungary, Saudi Arabia, Mexico and Texas of U.S.A. [1, 3, 7, 10, 13–18]. In China, *B. caballi* and *T. equi* were documented in Heilongjiang Province as early as 1943 [25]. Although both *B. caballi* and *T. equi* infections have been identified in China, a comprehensive survey of the infections has never been conducted [4, 22, 24].

Equine piroplasmosis causes serious health effects to horses, especially with respect to agricultural production including low working capacity, high cost of the control measures and impact on the transport of goods and international trade [12]. Therefore, there is an urgent need to determine the prevalence of the disease in China to facilitate the control of the infection.

The immunodominant merozoite surface protein of *T. equi* (EMA-2) is expressed throughout life cycle of the parasite, in both vector tick stage and in mammalian host stage [20]. The 48-kDa merozoite rhoptry protein of *B. caballi* (Bc48) is recognized earliest by the host immune system and throughout infection [2]. Indirect enzyme-linked immunosorbent assay (ELISAs) using recombinant EMA-2 and Bc48 (rEMA-2 and rBc48) is highly sensitive and specific for detecting antibodies in infected horses and widely used in serological surveillance of equine piroplasmosis [6, 11, 12, 19].

The rEMA-2 and rBc48 were expressed in *Escherichia coli*(*E. coli*) and purified as Glutathione S-transferase (GST) fusion proteins as described previously. The recombinant GST was also expressed and purified as a control [9]. The ELISA was performed as described previously [23]. Briefly, 96-well plates were coated overnight at 4 °C with 2 μg/m*l* purified recombinant proteins in coating buffer (carbonate-bicarbonate buffer, PH 9.6), respectively. The plates were washed once with washing buffer (phosphate buffer saline with tween-20) and then blocked with blocking buffer (3% skimmed milk dissolving in phosphate buffer saline) for 2 hr at 37°C. After washing once with the washing buffer, 100 μl of horse sera diluted in blocking buffer were added into

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Table 1. Prevalence of equine piroplasmosis in ten provinces or areas of China

Provinces or areas	No. of samples examined	No. of *B. caballi*-positive samples	No. of *T. equi*-positive samples	Mixed infection
Hebei	53	15 (28.3%)	16 (30.19%)	2 (3.77%)
Guangdong	266	84 (31.58%)	23 (8.65%)	23 (8.65%)
Shaanxi	192	85 (44.27%)	2 (1.04%)	2 (1.04%)
Xinjiang	350	271 (77.43%)	34 (9.71%)	30 (8.57%)
Jiangsu	227	80 (35.24%)	9 (3.96%)	4 (1.76%)
Yunnan	298	200 (67.15%)	112 (37.58%)	92 (30.87%)
Beijing	149	51 (34.23%)	3 (2.01%)	2 (1.34%)
Guizhou	120	24 (20%)	9 (7.5%)	3 (2.5%)
Ningxia	201	156 (77.61%)	8 (3.98%)	3 (1.49%)
Gansu	134	52 (38.81%)	13 (9.7%)	4 (2.99%)
Total	1990	1018 (51.16%)	229 (11.51%)	152 (7.64%)

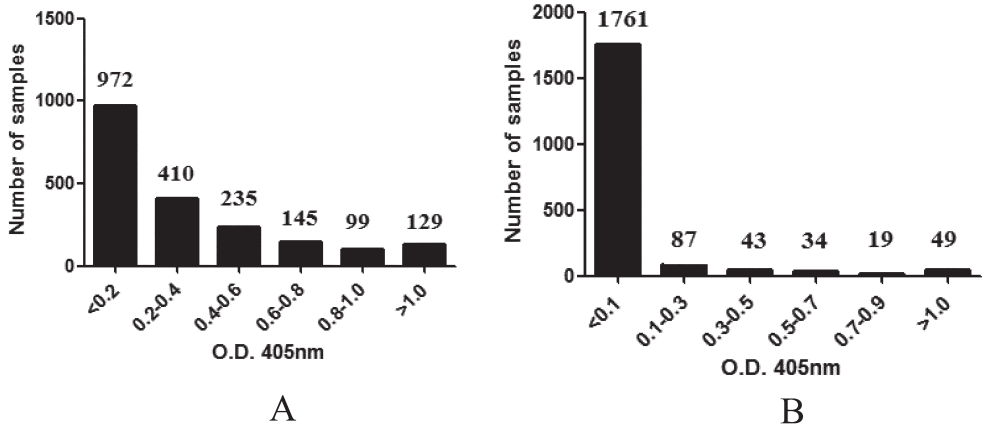

Fig. 1. A, Distribution of ELISA results using rBc48 represented by OD value. The ELISA cutoff value was determined as 0.2. B, Distribution of ELISA results using rEMA-2 represented by OD value. The ELISA cutoff value was determined as 0.1.

the wells and incubated at 37°C for 1 hr. The plates were then washed six times with washing buffer, subsequently filled with HRP conjugated goat anti-horse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) diluted in the blocking buffer and incubated at 37 °C for 1 hr. The plates were then washed six times with washing buffer, and 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was used as a substrate. After incubation at room temperature for 30 min, the absorbance at 405 nm was measured using a microplate reader (Benchmark Plus, BioRad, Hercules, CA, U.S.A.) The ELISA results for each sample were calculated by substracting the mean absorbance values of rGST from the mean absorbance values of rEMA-2 or rBc48. The samples were considered positive for *T. equi* infection, if the calculated absorbance value with rEMA-2 was equal to or greater than 0.1 [8]. The samples with OD value more than 0.2 were considered as positive for *B. cabali* infection in the ELISA with rBc48 [24].

In this study, a total of 1990 serum samples were collected from ten different provinces or areas of China, including Hebei, Beijing, Shaanxi, Guangdong, Xinjiang, Ningxia, Yunnan, Guizhou, Gansu and Jiangsu, and some of them are vital localities for the horse industry in China.

The positive rates in the ELISAs using the two antigens are shown in Table 1, and the distribution of OD values is shown in Fig. 1. Totally, 1,018 (51.16%) and 229 (11.51%) equine samples were positive for *B. caballi* and *T. equi* infection, respectively. Mixed infections were detected in 152 (7.64%) serum samples. Although there were no samples collected in northeast of China in this study, another study conducted in 2003 reported that, out of 111 equine samples, 38 (34%) and 36 (32%) samples were positive for *T. equi* and *B. caballi* infection, respectively [22]. These results indicated that equine piroplasmosis is widespread in China, and therefore, the impact on the horse industry caused by this disease should be considered. The seroprevalence of *B. caballi* and *T. equi* infection in different locations is shown in Fig. 2. The highest prevalence of *B. caballi* was found in Ningxia province (77.61%), and the lowest infection rate was noted in the Guizhou province (20%). Likewise, the highest infection rate of *T. equi* was found in Yunnan province (37.58%), and the lowest prevalence was noted in

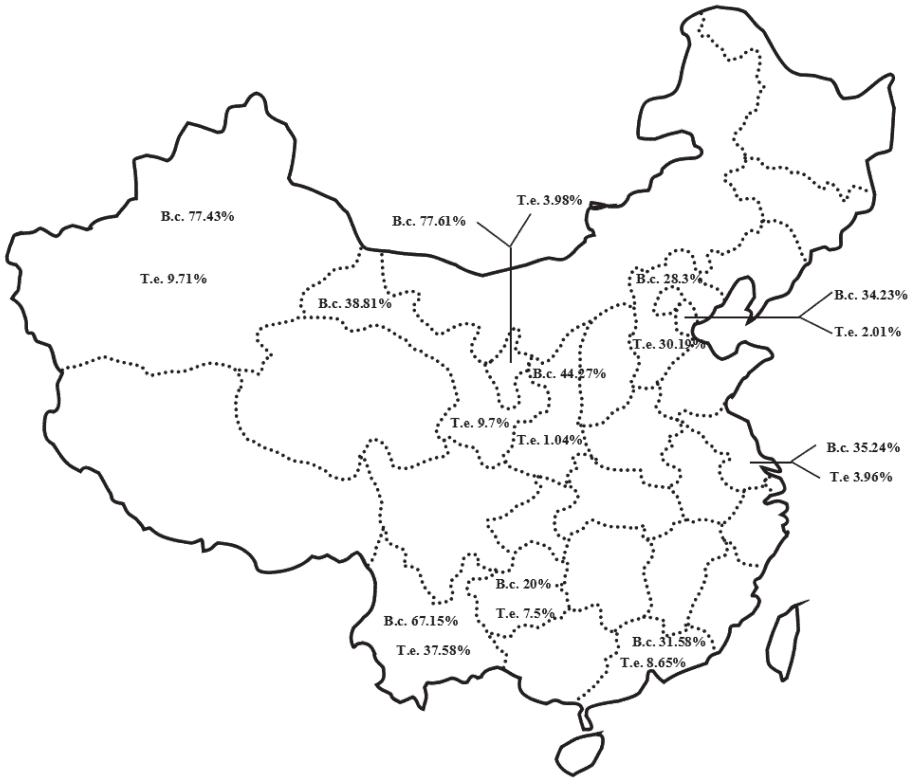

Fig. 2. The seroprevalence of *B. caballi* and *T. equi* infections and sampling locations.

Shaanxi province (1.04%). Notably, the seroprevalence of *B. caballi* was significant higher than that of *T. equi* in nine provinces, except for Hebei. Differences of the positive rate might be caused by the difference in distribution of tick vectors or the use of horses.

In 2002, a surveillance study of equine piroplasmosis in Xinjiang province reported that, out of the 70 samples collected from three farms in Xinjiang, 28 (40.0%) and 17 (24.3%) were positive for *T. equi* infection and *B. caballi* infection respectively [24]. Out of all the 350 sera, 34 (9.71%) samples were positive for *T. equi*, while 271 (77.43%) were positive for *B. caballi* in the present study. The positive rate of *B. caballi* infection was predominant over *T. equi*. This difference might be caused by the differences of sampling time and sample number. The dominant positive rate of *B. caballi* infection was also reported in a study conducted in Mongolia (adjacent to Xinjiang province), in which the positive rate for *T. equi* and *B. caballi* infection out of 250 samples was 49 (19.6%) and 129 (51.6%), respectively.

Serological surveillance of *B. caballi* and *T. equi* infections has been conducted in many countries. However, extensive survey of the prevalence about this disease in China has never been performed. There were few reports, and most of them focused on the northern China, rather than southern China. Our data demonstrate the successful applications of ELISAs with rBc48 and rEMA-2 as antigens for investigating the epidemiology of equine piroplasmosis caused by *B. caballi* and *T. equi* infections in horses in China. To our knowledge, this study is the first comprehensive survey for equine piroplasmosis in China. Our data may be regarded as important information that would contribute to establish prevention and control measures against equine piroplasmosis in China.

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REFERENCES

1. Alanazi, A. D., Alyousif, M. S. and Hassieb, M. M. 2012. Seroprevalence study on *Theileria equi* and *Babesia caballi* antibodies in horses from Central province of Saudi Arabia. *J. Parasitol.* **98**: 1015–1017. [Medline] [CrossRef]
2. Böse, R. and Daemen, K. 1992. Demonstration of the humoral immune response of horses to *Babesia caballi* by western blotting. *Int. J. Parasitol.* **22**: 627–630. [Medline] [CrossRef]
3. Cantú-Martínez, M. A., Segura-Correa, J. C., Silva-Páez, M. L., Avalos-Ramire, R. and Wagner, G. G. 2012. Prevalence of Antibodies to *Theileria equi* and *Babesia caballi* in Horses From Northeastern Mexico. *J. Parasitol.* **98**: 869–870. [Medline] [CrossRef]
4. Chahan, B., Zhang, S., Seo, J. Y., Nakamura, C., Zhang, G., Bannai, H., Jian, Z., Inokuma, H., Tuchiya, K., Sato, Y., Kabeya, H., Maruyama, S., Mikami, T. and Xuan, X. 2006. Seroepidemiological evidence for the possible presence of *Babesia* (*Theileria*)

equi and *Babesia caballi* infections in donkeys in western Xinjiang, China. *J. Vet. Med. Sci.* **68**: 753–755. [Medline] [CrossRef]
5. de Waal, D. T. 1992. Equine piroplasmosis: a review. *Br. Vet. J.* **148**: 6–14. [Medline] [CrossRef]
6. Gong, Z. L., Liu, G. Y., Xie, J. R., Chai, H. P., Zhang, L. Y., Li, Z. X., Tian, Z. C., Wang, L. and Liu, J. G. 2010. An indirect ELISA for the detection of *Babesia caballi* in equine animals. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **28**: 200–204. [Medline]
7. Hornok, S., Edelhofer, R., Földvári, G., Joachim, A. and Farkas, R. 2007. Serological evidence for *Babesia canis* infection of horses and an endemic focus of *B. caballi* in Hungary. *Acta Vet. Hung.* **55**: 491–500. [Medline] [CrossRef]
8. Huang, X., Xuan, X., Yokoyama, N., Xu, L., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K. and Igarashi, I. 2003. High-level expression and purification of a truncated merozoite antigen-2 of *Babesia equi* in Escherichia coli and its potentia for immunodiagnosis. *Clin. Microbiol.* **41**: 1147–1151. [CrossRef]
9. Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Suzuki, N. and Mikami, T. 1999. Cloning and Expression of a 48-Kilodalton *Babesia caballi* Merozoite Rhoptry Protein and Potential Use of the Recombinant Antigen in an Enzyme-Linked Immunosorbent Assay. *J. Clin. Microbiol.* **37**: 3475–3480. [Medline]
10. Laus, F., Veronesi, F., Passamonti, F., Paggi, E., Cerquetella, M., Hyatt, D., Tesei, B. and Fioretti, D. P. 2013. Prevalence of Tick Borne Pathogens in Horses from Italy. *J. Vet. Med. Sci.* **75**: 715–720. [Medline] [CrossRef]
11. Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M., Yokoyama, N. and Igarashi, I. 2013. Prevalence and genetic diversity of equine piroplasms in Tov province, Mongolia. *Infect. Genet. Evol.* **16**: 178–185. [Medline] [CrossRef]
12. OIE. 2008. *Manual of Diagnosis Tests and Vaccines for Terrestrial Animals* 2.5.8: 884–893.
13. Rosales, R., Rangel-Rivas, A., Escalona, A., Jordan, L. S., Gonzatti, M. I., Aso, P. M., Perrone, T., Silva-Iturriza, A. and Mijares, A. 2013. Detection of *Theileria equi* and *Babesia caballi* infections in Venezuelan horses using Competitive-Inhibition ELISA and PCR. *Vet. Parasitol.* **196**: 37–43. [Medline] [CrossRef]
14. Ros-García, A., M'ghirbi, Y., Hurtado, A. and Bouattour, A. 2013. Prevalence and genetic diversity of piroplasm species in horses and ticks from Tunisia. *Infect. Genet. Evol.* **17**: 33–37. [Medline] [CrossRef]
15. Salim, B., Bakheit, M. A., Kamau, J. and Sugimoto, C. 2013. Current Status of Equine Piroplasmosis in the Sudan. *Infect. Genet. Evol.* **16**: 191–199. [Medline] [CrossRef]
16. Scoles, G. A., Hutcheson, H. J., Schlater, J. L., Hennager, S. G., Pelzel, A. M. and Knowles, D. P. 2011. Equine piroplasmosis associated with Amblyomma cajennense ticks, Texas, USA. *Emerg. Infect. Dis.* **17**: 1903–1905. [Medline] [CrossRef]
17. Seo, M. G., Yun, S. H., Choi, S. K., Cho, G. J., Park, Y. S., Kwon, O. D., Cho, K. H., Kim, T. H., Jeong, K. S., Park, S. J., Kwon, Y. S. and Kwak, D. 2011. Seroprevalence of equine piroplasms in the Republic of Korea. *Vet. Parasitol.* **179**: 224–226. [Medline] [CrossRef]
18. Sloboda, M., Jirků, M., Lukešová, D., Qablan, M., Batsukh, Z., Fiala, I., Horin, P., Modry, D. and Lukes, J. 2011. A survey for piroplasmids in horses and Bactrian camels in North-Eastern Mongolia. *Vet. Parasitol.* **179**: 246–249. [Medline] [CrossRef]
19. Tanaka, T., Xuan, X., Ikadai, H., Igarashi, I., Nagasawa, H., Fujisaki, K., Mikami, T. and Suzuki, N. 1999. Expression of *Babesia equi* merozoite antigen-2 by recombinant baculovirus and its use in the ELISA. *Int. J. Parasitol.* **29**: 1803–1808. [Medline] [CrossRef]
20. Ueti, M. W., Palmer, G. H., Kappmeyer, L. S., Scoles, G. A. and Knowles, D. P. 2003. Expression of Equi Merozoite Antigen 2 during Development of *Babesia equi* in the Midgut and Salivary Gland of the Vector Tick Boophilus microplus. *J. Clin. Microbiol.* **41**: 5803–5809. [Medline] [CrossRef]
21. Ueti, M. W., Palmer, G. H., Scoles, G. A., Kappmeyer, L. S. and Knowles, D. P. 2008. Persistently infected horses are reservoirs for intrastadial tick-borne transmission of the apicomplexan parasite *Babesia equi*. *Infect. Immun.* **76**: 3525–3529. [Medline] [CrossRef]
22. Xu, Y., Zhang, S., Huang, X., Bayin, C., Xuan, X., Igarashi, I., Fujisaki, K., Kabeya, H., Maruyama, S. and Mikami, T. 2003. Seroepidemiologic studies on *Babesia equi* and *Babesia caballi* infections in horses in Jilin province of China. *J. Vet. Med. Sci.* **65**: 1015–1017. [Medline] [CrossRef]
23. Xuan, X., Nagai, A., Battsetseg, B., Fukumoto, S., Makala, L. H., Inoue, N., Igarashi, I., Mikami, T. and Fujisaki, K. 2001. Diagnosis of equine piroplasmosis in Brazil by serodiagnostic methods with recombinant antigens. *J. Vet. Med. Sci.* **63**: 1159–1160. [Medline] [CrossRef]
24. Xuan, X., Chahan, B., Huang, X., Yokoyama, N., Makala, L. H., Igarashi, I., Fujisaki, K., Maruyama, S., Sakai, T. and Mikami, T. 2002. Diagnosis of equine piroplasmosis in Xinjiang province of China by the enzyme-linked immunosorbent assays using recombinant antigens. *Vet. Parasitol.* **108**: 179–182. [Medline] [CrossRef]
25. Yin, H., Lu, W. and Luo, J. 1997. Babesiosis in China. *Trop. Anim. Health Prod.* **29**: 11S–15S. [Medline] [CrossRef]