VETERINARSKI ARHIV 80 (6), 715-722, 2010

Immunochromatographic assay of *Babesia caballi* and *Babesia equi* Laveran 1901 (*Theileria equi* Mehlhorn and Schein, 1998) (Phylum Apicomplexa) infection in Philippine horses correlated with parasite detection in blood smears

Mary Jane Cruz-Flores[1], Michelle Bata[1], Bridget Co[1], Florencia G. Claveria[1]*, Rodolfo Verdida[1], Xuenan Xuan[2], and Ikuo Igarashi[2]

[1]*Biology Department, College of Science, De La Salle University-Manila, Manila, Philippines*

[2]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan*

CRUZ-FLORES, M. J., M. BATA, B. CO, F. G. CLAVERIA, R. VERDIDA, X. XUAN, I. IGARASHI: Immunochromatographic assay of *Babesia caballi* and *Babesia equi* Laveran 1901 (*Theileria equi* Mehlhorn and Schein,1998) (Phylum Apicomplexa) infection in Philippine horses correlated with parasite detection in blood smears. Vet. arhiv 80, 715-722, 2010.

ABSTRACT

Sera collected from 71 slaughtered and 33 racing horses were assayed for *Babesia* spp. infection using immunochromatographic (ICT) assay. The ICT strips which were developed at the National Research Center for Protozoan Diseases (NRCPD), Obihiro University, Hokkaido, Japan contained a recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and a recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t) for the detection of anti-horse *Babesia* spp. antibodies. The 63 sero-positive blood samples consisted of 41(57.7%) and 22 (66.7%) cases in slaughtered and racing horses, respectively. Twelve sera (19.0%) reacted with both *B. caballi* and *B. equi* antigens, 45 sera (71.0%) reacted with rBc48 antigen only, and six sera (10.0%) were positive for *B. equi* antibodies only. *Babesia caballi* infection accounted for 90.5% cases. Infection with *B. caballi* and/or *B. equi* confirmed in Giemsa-stained blood smears prepared from racing horse samples only revealed 22 (66.7%) seropositive cases. Paired pear or crescent-shaped merozoites (0.5-1.25 µm), characteristic of *B. caballi* were observed in 20 blood smears, while only two seropositive cases revealed the presence of both *B. caballi* and the Maltese cross or tetrad-shaped merozoites (0.62-0.95 µm) generally associated with *Theileria* sp. (*B. equi*) parasite. To our knowledge, this is the first immunochromatographic assay of equine babesiosis in the Philippines validated by the detection of specific etiologic agent(s) in blood smears.

Key words: *Babesia caballi, Babesia equi,* rBc48/rEMA-2t antigens, immunochromatography, blood smears, horses

*Corresponding author:

Dr. Florencia G. Claveria, Biology Department, College of Science, De La Salle University-Manila, 2401 Taft Avenue, Manila 1004, Philippines, Fax: +63 2536 0228; E-mail: florencia.claveria@dlsu.edu.ph

ISSN 0372-5480
Printed in Croatia

Introduction

Babesia equi and *Babesia caballi* are important tick-transmitted apicomplexan blood parasites of horses (FRIEDHOFF, 1988; HIRATA et al., 2003; XUAN et al., 2001; IKADAI et al., 1999), and can destroy erythrocytes with accompanying clinical manifestations like anemia, icterus, jaundice, edema, gastro-enteritis, bronchopneumonia, and abortions in infected horses (BŐSE et al., 1995; FRIEDHOFF, 1988; KUTTLER, 1988). While *B. caballi* infection is more often associated with congestion of parasites in capillaries and small blood vessels in different organs and low parasitemia in the peripheral circulation, *B. equi* infection is characterized by higher parasitemia, lysis of RBC and is fatal due to anemia (KUTTLER, 1988). In blood smears, parasites are detectable only during the acute stage, but animals that survive the infection become lifelong carriers and can be diagnosed serologically (YOUNG, 1988; HUANG et al., 2003; HIRATA et al., 2002).

While the detection of parasites in blood smears aids the confirmation of the specific etiologic agent, it is time-consuming. Thus, the reliability of the results can be influenced by parasite density and the worker's level of competence or familiarity with the morphological features of different species (HOMER et al., 2000; LEVINE, 1971). Other diagnostic methods, like the complement fixation test (CFT) and indirect fluorescent antibody test (IFAT), do not only require large amounts of *Babesia* antigens, but have low sensitivity and specificity; with the CFT unable to detect latent infections and it discriminates between negative and carrier animals (JAMES, 1988; HIRATA et al., 2002). Although enzyme-linked immunosorbent assay (ELISA) is more sensitive and specific, it is time consuming and labor intensive, and therefore unsuitable for field use (WEILAND and REITER, 1988; VERDIDA et al., 2005; HUANG et al., 2004). Recently a simple, quick, more reliable and inexpensive immunochromatographic test (ICT) using recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and a recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t) has gained acceptability in the diagnosis of both acute and latent babesiosis in horses (VERDIDA et al., 2005; MOHEBALI et al., 2004; HUANG et al., 2003, 2004, 2006).

Documented studies on babesiosis in the country are scanty. Based on hematological parameters and clinical manifestations, there exists only the reports of cattle infection with *B. bigemina* and *B. argentina* (=*B. bovis*) (MOLINA and MONTENEGRO, 1977; DUMAG and REYES, 1960), and dogs with *Babesia canis* (CARLOS et al., 1972; ST JOHN et al., 1931). Very recently we reported 28.0% infection of stray dogs with *Babesia gibsoni* using the ICT-*B. gibsoni* p50-truncated antigen, with 80.4% of the dogs infested with *Rhipicephalus* ticks (CRUZ-FLORES et al., 2008), that have been incriminated as competent vectors of canine babesiosis (BATTSETSEG et al., 2002; YIN et al., 1997; KUTTLER et al., 1988). We present here the first report of cases of horse babesiosis using ICT, confirmed through demonstrating the specific etiologic agent(s) in blood smears.

Materials and methods

Collection of horse blood samples, serum extraction and blood smear preparation. Blood samples were collected from 71 (24 males, 47 female) approximately 5-10 year old horses in a private slaughterhouse in Bulacan (origin of animals: pasture in Batangas), and 33 (12 males, 21 females) ages 3-17 year old horses in a racing facility in Sta. Ana, Manila. Photographs of the facilities could not be provided in compliance with the agreement made with the management. About 20 mL of blood samples were obtained from the shoulders/upper arm of each horse immediately after slaughter. With the help of the well-trained caretaker of the stable, 5-20 mL of blood was extracted from the neck/jugular vein of each racing horse. The extraction of blood from each of the racing horses was limited to a single attempt, which explains the variation in blood volumes obtained. Blood samples were transferred to properly-labeled screw-capped test tubes, kept in a cooler. These were transported to the Science and Technology Research Center, Parasitology Laboratory, De La Salle University-Manila. Sera in properly-labeled plastic microcentrifuge tubes were kept refrigerated at 4 ^{o}C, prior to assay.

Blood smears from racing horses were fixed in methanol and stained with Giemsa. Using a light photomicroscope, *B. caballi* was identified as large paired pear-shaped or crescent-shaped parasites measuring 3.0 × 2.0 μm, while the small *B. equi* parasites were identified with tetrad or Maltese cross arrangement of merozoites measuring 2.0 ×1.0 μm (LEVINE, 1971; KUTTLER, 1988).

Immunochromatographic test (ICT). The ICT is basically a strip of nitrocellulose membrane (NC) that contains a recombinant *B. caballi* 48-kDa rhoptry protein (rBc48), and a recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t) for the detection of anti-horse *Babesia* spp. antibodies. Recombinant antigens were prepared using *in vitro* cultured parasites at the National Research Center for Protozoan Diseases (NRCPD), Obihiro University, Japan. ICT strips were developed by HUANG et al. (2006) of the NRCPD. Both the ICT strips and sera from specific pathogen free (SPF) horses and *B. caballi*- and *B. equi*-infected horses were generously provided by Prof. Xuan of the same research center. ICT strips were stored in a 4 ^{o}C refrigerator, while the negative and positive serum samples were kept in the freezer, prior to use.

15 μL of slaughtered horse test serum plus 15 μL of phosphate buffer saline solution (pH 7.2), and 22.5 μL racing horse serum plus 7.5 μL PBS solution were loaded into the sample application pad of each ICT strip, and reactivity was evaluated 10 min post-application. In view of the very light bands formed during the initial ICT assay of racing horse sera, we opted to use a higher concentration relative to sera from slaughtered horses. Results were interpreted as follows: the presence of either purple, red, or pink coloration in the control and the two test lines indicates both *B. caballi* and *B. equi* infection; positive for *B. equi* indicates coloration only in the control line and the *B. equi*

test line; positive for *B. caballi* only indicates coloration in the control line and the *B. caballi* test line; coloration only in the control line indicates the absence of infection or seronegative results; and absence of color change or reactivity in both the test and control lines suggests invalid results.

Results

Of the total 104 serum samples examined, 63 (60.5%) were serologically positive, comprising 41 (57.7%) and 22 (66.7%) cases among the slaughtered and racing horses, respectively. Twelve sera (19.0%) reacted with both the rBc48 and rEMA-2t antigens, 45 sera (71.0%) reacted with only the rBc48 antigen, and only six (10.0%) were positive for anti-rEMA-2t antibodies (Figs. 1 and 2). The preponderance (n = 57; 90.5%) of *B. caballi* infection, including mixed infection with *B. equi* was worth noting.

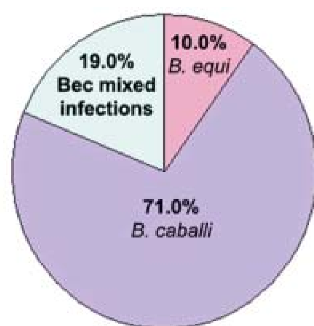

Fig. 1. Distribution of *Babesia* spp. infection in 63 (n = 104) seropositive horse sera.

A comparison of the ICT results in serum samples obtained from slaughtered and racing horses is summarized in Table 1. The percentages of infection in male and female horses were 58.3% and 61.8%, respectively. Nine (75.0%) of the male racing horses were seropositive compared to 62.0% female cases. Among the slaughtered horses, the infection rate was higher among females at 62.0% relative to 50.0% in males.

Table 1. Comparison of results of ICT assay of sera obtained from slaughtered and racing horses according to gender

Horse sera	Males			Females			Total
	sero^{+}(%)	sero^{-} (%)	total	sero^{+} (%)	sero^{-} (%)	total	
Slaughtered	12 (50.0)	12 (50.0)	24	29 (62.0)	18 (38.0)	47	71
Racing	9 (75.0)	3 (25.0)	12	13 (62.0)	8 (38.0)	21	33
Overall Total	22 (58.3)	15 (41.7)	36	42 (61.8)	26 (38.2)	68	104

Only Giemsa-stained blood films were prepared from racing horses. Of the 22 (66.7%) seropositive blood samples, 20 (91.0%) demonstrated intra-erythrocytic paired pear-shaped or crescent shaped merozoites (1.1-1.25 µm long), frequently associated with *B. caballi* parasites (Fig. 3), while only two blood smears exhibited mixed infection with *B. caballi* and the tetrad or Maltese cross arrangement of merozoites (0.5-1.25 µm) associated with *Theileria* sp. (*B. equi*) parasites (Fig. 4). None of the blood samples examined manifested only the *B. equi*-like parasites.

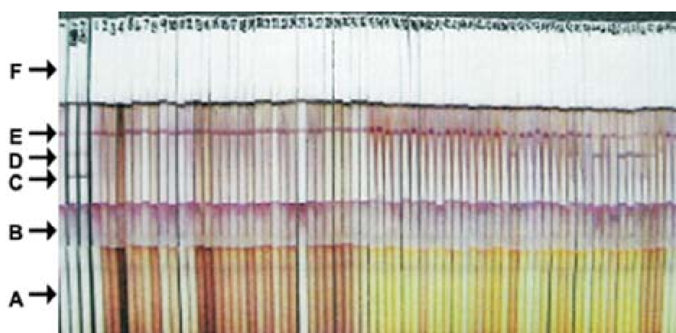

Fig. 2. Immunochromatographic test strips posttests. Lane 1: negative control: ICT strip reacted with serum sample from uninfected specific pathogen free (SPF) horse. Positive control: lane 2, reacted with serum from *B. equi*-infected horse and lane 3, reacted with serum from *B. caballi*-infected horse. Test strips: Lanes 1-33, reacted with racing horse sera. The ICT strip contains the following components: a sample pad, where the test serum was introduced (A); a conjugate pad, on which the rEMA-2t and rBc48 gold colloid particles were adsorbed (B); Bc test line, where rBc48 was immobilized for the detection of anti-*B. caballi* antibodies (C); Be test line, where rEMA-2t was immobilized for the detection of anti-*B. equi* antibodies (D); control line, where rabbit anti-rEMA-2t IgG antibodies were immobilized (E); and an absorbent pad, which absorbed the excess liquid test serum (F).

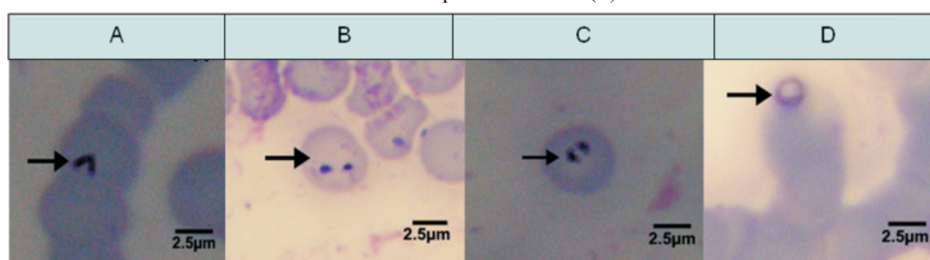

Fig. 3. Blood smears positive for *Babesia caballi* parasites. Note joint paired merozoites (A: 1.25 × 0.25 µm), pear-shaped (B: 1.1 µm), crescent-shaped (C: length: 1.0 µm), and ring form (D). ×1000

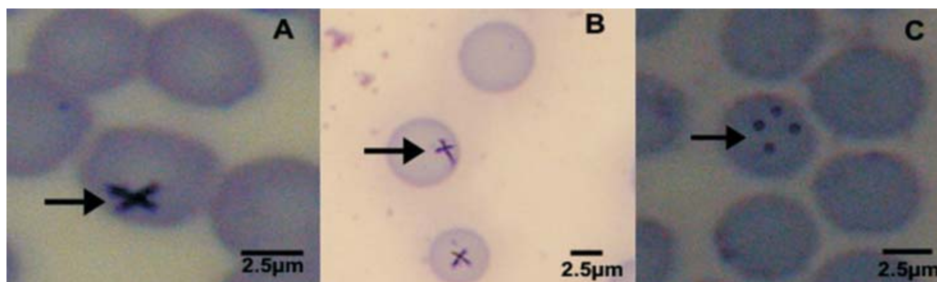

Fig. 4. *Babesia equi* showing Maltese cross (A: 0.69-1.15 × 0.27 µm; B: 0.50 -1.5 µm), and in tetrad form (C: 0.36 µm) merozoites. ×1000.

Discussion

Present findings reinforce the sensitivity and specificity of recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and the recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t) antigens in the diagnosis of horse *Babesia* spp. as earlier documented by HUANG et al. (2004). Several studies have documented a cosmopolitan and essentially identical distribution pattern of *B. caballi* and *B. equi*, influenced by using similar genera of tick vectors (KUTTLER, 1988; FRIEDHOFF, 1988). In the current survey, although both species were identified, the dominance of *B. caballi* infection was worth noting. *Babesia caballi* (1.0 to 1.25 µm) parasites were larger compared to the small *B. equi*. However, merozoites of *B. caballi* measured smaller compared to the 3.0 by 2.0 µm earlier reported by KUTTLER (1988) and LEVINE (1971). The Maltese cross arrangement of four merozoites of *B. equi* parasites is similar to *Theileria* sp., and is used as a differential feature from *B. caballi*, a species morphologically similar to bovine *B. bigemina* (KUTTLER, 1988; SCHEIN, 1988). To our knowledge, this is the first immunochromatographic assay of equine babesiosis in the country, validated by the detection of specific etiologic agent(s) in blood smears. In view of the role of ticks as intermediate hosts of *Babesia* spp., future studies should look into ticks infesting horses, including tick surveillance and species identification.

References

BATTSETSEG, B., S. LUCERO, X. XUAN, F. CLAVERIA, N. INOUE, A. ALHASSAN, T. KANNO, I. IGARASHI, H. NAGASAWA, T. MIKAMI, K. FUJISAKI (2002): Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polymerase chain reaction. Vet. Parasitol. 107, 351-317.

BŐSE, R., W. K. JORGENSESN, R. J. DALGLIESH, F. T. FRIEDHOFF, A. D. DE VOS (1995): Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61-64.

CARLOS, E. T., E. R. CARLOS, F. T. CALALAY, C. C. CABILES (1972): *Babesia canis:* clinical observations in dogs in the Philippines. Phil. J. Vet. Med. 11, 81-91.

CRUZ-FLORES, M. J., F. G. CLAVERIA, R. VERDIDA, X. XUAN, I. IGARASHI (2008): First detection of *Babesia gibsoni* infection in Philippine stray dogs by immunochromatographic test (ICT). Vet. Arhiv 78, 149-157.

DUMAG, P. U., P. V. REYES (1960): Occurrence of endoglobular parasites morphology resembling *Babesia argentina* in Santa Gertrudes bulls and Shorthorn cattle at the Bongabon stock farm quarantine station, Bongabon, Nueva Ecija. Phil. J. Anim. Indust. 21, 221-224.

FRIEDHOFF, K. T. (1988): Transmission of *Babesia*. In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 23-52.

HIRATA, H., H. IKADAI, N. YOKOYAMA, X. XUAN, K. FUJISAKI, N. SUZUKI, T. MIKAMI, I. IGARASHI (2002): Cloning of a truncated *Babesia equi* gene encoding an 82-kilodalton protein and its potential use in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 40, 1470-1474.

HIRATA, H., X. XUAN, N. YOKOYAMA, Y. NISHIKAWA, K. FUJISAKI, N. SUZUKI, I. IGARASHI (2003): Identification of a specific antigenic region of the P82 protein of *Babesia equi* and its potential use in serodiagnosis. J. Clin. Microbiol. 41, 547-551.

HOMER, M. J., I. AGUILAR-DELFIN, S. R. TELFORD III, P. J. KRAUSE, D. H. PERSING (2000): Babesiosis. Clin. Microbiol. Rev. 13, 451-469.

HUANG, X., X. XUAN, N. YOKOYAMA, L. LU, H. SUZUKI, C. SUGIMOTO, H. NAGASAWA, K. FUJISAKI, I. IGARASHI (2003): High-level expression and purification of a truncated merozoite antigen-2 of *Babesia equi* in *Escherichia coli* and its potential for immunodiagnosis. J. Clin. Microbiol. 41, 1147-1151.

HUANG, X., X. XUAN, L. LU, S. ZHANG, N. YOKOYAMA, N. SUZUKI, I. IGARASHI (2004): Development of an immunochromatographic test with recombinant EMA-2 for the rapid detection of antibodies against *Babesia equi* in horses. J. Clin. Microbiol. 42, 359-361.

HUANG, X., X. XUAN, R. VERDIDA, S. ZHANG, N. YOKOYAMA, L. XU, I. IGARASHI (2006): Immunochromatographic test for simultaneous serodiagnosis of *Babesia caballi* and *B. equi* infections in horses. Clin. Vaccine Immunol. 13, 553-555.

IKADAI, H., X. XUAN, I. IGARASHI, S. TANAKA, T. KANEMARU, H. NAGASAWA, K. FUJISAKI, N. SUZUKI, T. MIKAMI (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.

JAMES, M. K. (1988). Immunology of Babesiosis. In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 119-130.

KUTTLER, K. L. (1988): Worldwide Impact of Babesiosis. In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 1-22.

LEVINE, N. D. (1971): Taxonomy of the piroplasms. Trans. Am. Micros. Soc. 90, 2-33.

MOHEBALI, M., M. TARAN, Z. ZAREI (2004): Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick rK39 test and direct agglutination. Vet. Parasitol. 121, 239-245.

MOLINA, J. Q., M. M. MONTENEGRO (1977): Anaplasmosis in two Philippine cows. Phil. Vet. Med.16, 181-187.

SCHEIN, E. (1988): Equine babesiosis. In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 197-208.

ST. JOHN, J. H., J. S. SIMMONS, F. H. REYNOLDS (1931): A survey of Mongrel dogs in Manila, Philippine Islands, for helminthic and blood protozoan parasites. Phil. J. Sci. 44, 309-311.

VERDIDA, R., X. XUAN, S. FUKUMOTO, X. HUANG, J. ZHOU, I. IGARASHI, F. CLAVERIA, H. NAGASAWA (2005): Development of a practical immunochromatographic test with recombinant P50 for the diagnosis of *Babesia gibsoni* infection in dogs. Parasitol. 131, 769-774.

WEILAND, G., I. REITER (1988): Methods for the Measurement of the Serological Response to *Babesia*. In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 143-162.

XUAN, X., A. LARSEN, H. IKADAI, T. TANAKA, I. IGARASHI, H. NAGASAWA, K. FUJISAKI, Y. TOYODA, N. SUZUKI, T. MIKAMI (2001): Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705-709.

YIN, H., W. YU, J. LUO (1997): Babesiosis in China. Trop. Anim. Hlth. Prod. 29, 11S-15S.

YOUNG, A. S. (1988): Epidemiology of Babesiosis In: Babesiosis of Domestic Animals and Man (Ristic, M., Ed.). CRC Press, Inc., Boca Raton, Florida. pp. 81-98.

Received: 14 October 2009
Accepted: 9 July 2010

CRUZ-FLORES, M. J., M. BATA, B. CO, F. G. CLAVERIA, R. VERDIDA, X. XUAN, I. IGARASHI: Imunokromatografski test za dokaz invazije vrstama *Babesia caballi* i *Babesia equi* Laveran 1901 (*Theileria equi* Mehlhorn i Schein, 1998) (Phylum Apicomplexa) u filipinskih konja u usporedbi s dokazom parazita u krvnim razmascima. Vet. arhiv 80, 715-722, 2010.

SAŽETAK

Uzorci seruma 71 zaklanog konja i 33 športska konja bili su pretraženi na prisutnost protutijela za babezije imunokromatografskim testom (ICT). Testovi razvijeni u Nacionalnom istraživačkom centru za protozojske bolesti u sklopu Sveučilišta Obihiro u Hokaidu u Japanu sadržavali su rekombinantni protein od 48-kDa (rBc48) vrste *B. caballi* i rekombinantni krnji merozoitski antigen 2 (rEMA-2t) vrste *B. equi* za određivanje protutijela za vrste roda *Babesia*. Od ukupno 63 serološki pozitivna konja, 41 (57,7%) pripadao je skupini kojoj je krv bila uzeta pri klanju, a 22 (66,7%) bila su iz skupine športskih konja. Dvadeset uzoraka seruma (19,0%) bilo je pozitivno na oba antigena (*B. caballi* i *B. equi*), 45 uzoraka (71,0%) samo na antigen rBc48, dok je svega šest uzoraka (10%) bilo pozitivno na protutijela za vrstu *B. equi*. Protutijela za vrstu B. *caballi* bila su dokazana u 90,5% pretraženih uzoraka. Pretragom krvnih razmazaka obojenih po Giemzi babezije su bile dokazane u svega 22 (66,7%) športska konja. Kruškaste tvorevine (0,5-1,25 μm), karakteristične za merozoite protozoona *B. caballi* bile su dokazane u 20 razmazaka krvi. Samo u dva serološki pozitivna uzorka dokazana je vrsta B. *caballi* i merozoiti razmješteni u obliku malteškoga križa (0,62-0,95 μm) što je i karakteristika protozoa iz roda *Theileria* (*B. equi*). Ovim istraživanjem prvi put je dokazana prikladnost imunokromatografskoga testa za određivanje protutijela za babezije konja na Filipinima, a rezultati su uspoređeni s nalazom uzročnika u krvnim razmascima.

Ključne riječi: *Babesia caballi, Babesia equi,* rBc48/rEMA-2t, imunokromatografija, krvni razmasci, konji