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First detection of *Babesia gibsoni* infection in Philippine stray dogs by immunochromatographic test (ICT)

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ABSTRACT

A total of 46 stray dogs in an impounding facility comprising 17 males and 29 females were diagnosed using the *Babesia gibsoni* P50 truncated antigen immunochromatographic test (P50t-ICT). Thirteen dogs (28.0%) were serologically positive. There was no cross-reactivity with serum samples from *Babesia canis* (= *B. vogeli*)-infected dogs and none of the ICT strips showed invalid results, which reinforce the sensitivity and specificity of the P50t antigen and the reliability and accuracy of the P50t-ICT. Thirty-seven (80.4%) dogs had mixed tick infestations principally of the genus *Rhipicephalus* and *Boophilus*. From 11 seropositive and 20 seronegative dogs a total of 80 *Rhipicephalus* ticks were pooled. Among the 33 seronegative dogs, 48.5% had infestation with *Rhipicephalus* sp. only, 12.1% with *Boophilus* sp. only, 12.1% with mixed *Rhipicephalus* sp. and *Boophilus* sp., and 19.6% were un-infested. This paper documents the first account of serological detection of *B. gibsoni* in stray dogs and their infestation mainly with *Rhipicephalus* sp. suggestive of their role as putative key vectors of *B. gibsoni* in Philippine stray dogs.

Key words: *Babesia gibsoni*, P50t-ICT, Philippine stray dogs, ticks

Introduction

Babesiosis is a tick-borne parasitic infection of wild and domestic animals, including canines (MCCOSTER, 1979). The larger *B. canis* PIANA and GALLI-VALERIO 1895 and the smaller *B. gibsoni* PATTON, 1910 constitute two major species infecting canines in many parts of the world (BIRKENHEUER et al., 2003; ZAHLER et al., 2000; YAMANE et

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al., 1993a; KUTTLER, 1988; EWING and BUCKNER, 1965). Canines are susceptible to *Babesia* spp. with young dogs often developing more serious disease than older ones (MUHLNICKEL et al., 2002; DE WAAL, 2000; BOSE et al., 1995). Clinical manifestations include weakness, depression, lethargy, anorexia, malaise, anemia, fever, splenomegaly, and haemoglobinuria (WULANSARI et al., 2003; MEINKOTH et al., 2002; CONRAD et al., 1991).

Animals suspected of infection with *Babesia* spp. are conventionally diagnosed through examination of blood smears alongside disease manifestations, a common practice in places that do not have the capability of other more specific tests. In chronic cases where there is low parasitemia, the determination of the specific etiologic agent merely based on blood smears tends to be problematic. Today, several serological assays have been developed, such as the indirect fluorescence antibody test (IFAT) which is sufficiently sensitive, however, it has certain drawbacks, such as possible cross-reactivity between antigens of closely related *B. gibsoni* and *B. canis* or with normal dog erythrocytes, as well as subjectivity in the quantification of the intensity of fluorescence (BIRKENHEUER et al., 2003; YAMANE et al., 1993b). Likewise, the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), which are indisputably sensitive and specific, are considered somewhat impractical for field surveys because they are time consuming and labor intensive (VERDIDA et al., 2004; FUKUMOTO et al., 2001a; FUKUMOTO et al., 2001b; BOSE et al., 1995). Recently, a simpler and more practical approach, the immunochromatographic test (ICT) or the dipstick assay, has been gaining greater acceptability in the diagnosis of protozoan infections relative to other serological/molecular diagnoses (VERDIDA et al., 2005; MOHEBALI et al., 2004; RICHARDSON et al., 2002; TJITRA et al., 1999; MILLS et al., 1999). In the Philippines, studies on animal babesiosis are scarce and are largely based on Giemsa-stained blood smears and/or clinical manifestations and hematological parameters. To our knowledge, only the cattle *B. bigemina* and *B. argentina (= B. bovis)* (MARTINI, 1909a and 1909b; YUTUC, 1956; DUMAG and REYES, 1960; MOLINA and MONTENEGRO, 1977), and the canine *B. canis* (ST. JOHN et al., 1931; CARLOS et al., 1972) have been reported.We herein document the first report of *B. gibsoni* infection in impounded stray dogs in Manila, Philippines using ICT containing a highly purified *B. gibsoni* truncated immunodominant surface antigen.

Materials and methods

Collection site of dog blood samples and serum extraction. Blood samples were collected from 46 stray dogs in an impounding facility in Barangay Salawag, Dasmariñas, Cavite. Only the animal gender was noted, while age and health status were not determined. With due respect to the wishes of the management of the impounding facility, photos of the

study site could not be provided in this paper. About 20 mL of blood was extracted from each dog and transferred to properly-labeled capped tubes and transported inside a cooler to the Science and Technology Research Center, Parasitology Laboratory, De La Salle University-Manila. Whole blood samples were processed at 5000 rpm for 10 min using a RC5 sorval refrigerated centrifuge (USA), and sera collected were kept in the refrigerator at 4 ºC, prior to assay. Owing to the considerably thick consistency of the dogs' blood, several attempts to prepare Giemsa-stained blood smears to obtain supplementary data on parasitemia were unsuccessful.

Collection of ticks. Ticks were plucked out of the skin including those in the ears and in between the toes. Ticks on the ground near the cages were also pooled. The ticks collected were properly noted and placed in properly-labeled bottles containing denatured alcohol (95% ethanol + 4% methanol + 1% pyridine). The ticks were identified at the genus level (ROBERTS and JANOVY, 2000; FRIEDHOFF, 1988) and were properly documented.

Immunochromatographic test (ICT): The ICT is essentially a nitrocellulose membrane (NC)-based serological strip that contains a highly purified *B. gibsoni* recombinant truncated P50 (P50t) antigen and anti-P50t IgG antibody, developed by the National Research Center for Protozoan Diseases (NRCPD), Obihiro University, Hokkaido, Japan (VERDIDA et al., 2005). An ICT strip comprises the following components: a sample application pad, where the test serum sample is introduced; a conjugate pad, on which the P50t-gold colloid conjugates are adsorbed; the test and control lines where the P50t antigen and anti-P50t IgG antibodies are immobilized, respectively; and an absorbent pad, which absorbs the superfluous test serum/fluid that diffused through the membrane (Fig. 1). The *B. gibsoni* ICT strips were generously provided by Prof. Xuan and Mr. Verdida of the NRCPD, Japan. Dr. Xuan generously provided the sera from specific pathogen free (SPF) dogs (Nihonnosan, Japan) and from *B. gibsoni*- and *B. canis* (=*B. vogeli*)-infected dogs. The ICT strips were kept in a 4 degrees C refrigerator, while the negative and positive serum samples were kept in the freezer, prior to use.

The test and control serum samples were processed as follows: 15 µl of test serum added to 15 µl of phosphate buffer solution (pH 7.2) was applied on the sample application pad, and the results were obtained 10 min post-application. Results were interpreted as follows: positive result or presence of *B. gibsoni* infection should show either a purple, red or pink coloration in both the test and control lines; control line turned red denotes seronegative (sero-) results; and absence of color change or reactivity in both the test and control lines reflects invalid results.

Results

The 46 stray dogs diagnosed comprised 17 (37%) males and 29 (63%) females. Thirteen dogs were infected, representing an overall 28% infection (Fig. 1). Interestingly, infection was higher in females at 41.4% (12/46 cases) compared to 5.9% (1/46) in males. Some test lines in the 13 *B. gibsoni* seropositive (sero+) cases exhibited lighter bands. There was no cross-reactivity with serum samples from *B. canis* (= *B. vogeli*)-infected dogs and none of the ICT strips showed invalid results.

Larvae, nymphs and partially and fully engorged adult ticks were collected from both *B. gibsoni* sero- and sera+ dogs. Thirty-seven (80.4%) of the 46 dogs had mixed of ticks infestations principally with the genus *Rhipicephalus* and *Boophilus*. Nine dogs (19.6%) were free of tick ectoparasites. A total of 80 *Rhipicephalus* sp. of ticks (Fig. 2) were pooled from 11 sero+ and 20 sero- (= 30 ticks) dogs. In two sero+ dogs, only *Boophilus* sp. were found. Interestingly, among the 33 uninfected dogs, 16 (48.5%) had infestation with *Rhipicephalus* sp. only, 4 (12.1%) with mixed *Rhipicephalus* sp. and *Boophilus* sp. and 4 (12.1%) with *Boophilus* sp. only.

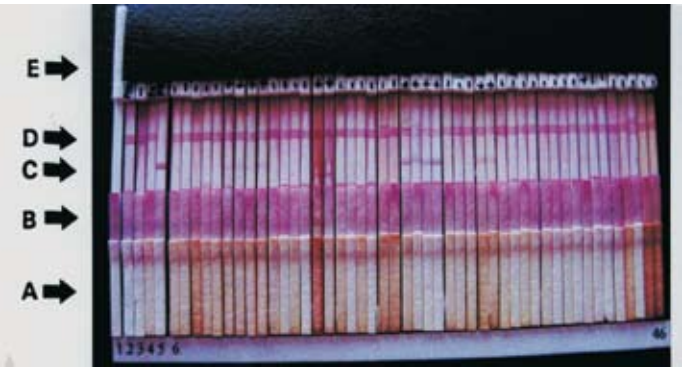

Fig. 1. *B. gibsoni* immunochromatographic assay. Lane 1: A pre-tested ICT strip showing a sample pad (A); a conjugate pad, on which the P50t-gold colloid conjugates are adsorbed (B); the test (C) and control (D) lines on which the P50t antigen and the anti-P50t IgG antibodies are immobilized; and an absorbent pad (E) which absorbs the extra liquid test sera that diffused through the membrane (E). ICT strips reacted with phosphate buffer solution (lane 2), with serum samples from SPF dog (lane 3) and *B. canis/B. vogeli*-infected dog (lane 4) as negative control; andreacted with serum from *B. gibsoni*-infected dog (lane 5) as positive control. ICT strips reacted with sera collected from 46 stray dogs (lanes 6-46). Note 13 seropositive relative to seronegative results/bands in test sera and negative control.

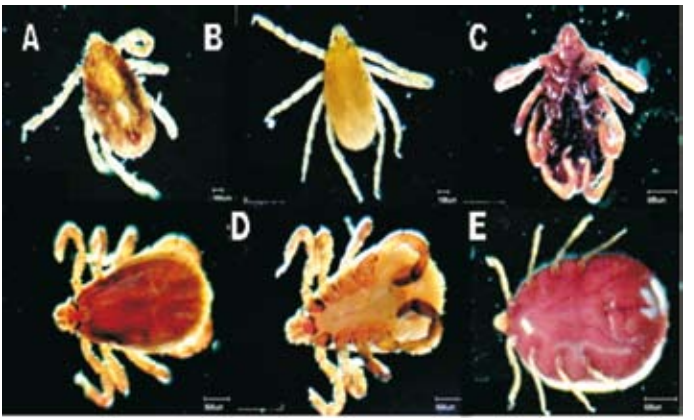

Fig. 2. *Rhipicephalus* sp. ticks. A. Larva. B. Nymph. C. Adult female. D. Adult male showing dorsal and ventral body surfaces. E. Engorged female tick.

Discussion

Based on clinical manifestations and blood smear examinations, only *B. canis* has been reported in Philippines dogs (CARLOS et al., 1972; ST. JOHN et al., 1931). The present finding documents the first account of *B. gibsoni* infection in Philippine stray dogs. In view of the lack of supplementary data on parasite density, we can only infer the possibility of dogs having either acute or chronic infection. The heavy and light reactive bands in test lines of the 13 sero+ cases are likewise suggestive of differences in the dog's status or grade of infection. Using the P50t-ICT, 44.4% clinical cases of dogs manifesting anemia were positively diagnosed for *B. gibsoni* (VERDIDA et al., 2005) compared to 39.3% infection determined by PCR in field dogs that likewise had anemia (FUKUMOTO et al., 2001a). We obtained a much lower 28% infection in the present study relative to those earlier reported by VERDIDA et al. (2005) and FUKUMOTO et al. (2001a), the difference of which may have been due to the homogeneity of the dog population used in their studies, which were all established clinical cases manifesting anemia. The present percentage of infection however is higher compared to the 5.8% in stray dogs (BIRKENHEUER et al., 2003) and 8.8% in 80 roaming dogs (INOKUMA et al., 2004), which reinforces the sensitivity and specificity of the P50t antigen used. Moreover, as none of the ICT strips showed invalid results, the reliability and accuracy of the P50t-ICT in the diagnosis of *B. gibsoni* is further established.

Several species belonging to the genus *Boophilus*, *Dermacentor*, *Rhipicephalus* and *Haemaphysalis* have been identified as natural vectors of *Babesia* spp.While the genus *Boophilus* is commonly associated with cattle babesiosis (NORVAL et al., 1983; YIN et al., 1997), species of ticks belonging to the genus *Dermacentor* and *Rhipicephalus* are

linked with equine, ovine and canine *Babesia* sp. transmission, with *Rhipicephalus* spp. having been incriminated as the more competent or efficient vectors of canine *Babesia* spp. (BATTSETSEG et al., 2002; YIN et al., 1997; KUTTLER et al., 1988). In the present work, the ticks collected from *B. gibsoni*-infected and uninfected stray dogs were principally those of the genus *Rhipicephalus* and *Boophilus*. In Okinawa, Japan, imported dogs carry 100% infestation with *R. sanguineus* (INOKUMA et al., 1998). Among Japanese reared dogs however, *H. longicornis* is most frequently found (INOKUMA et al., 2003), a finding consistent with the identification of *H. longicornis* as the principal vector of *B. gibsoni* infection in China (SHEN et al., 1997). The dominance of *Rhipicephalus* tick species is suggestive of the overriding putative role of *Rhipicephalus* spp. in the transmission of *B. gibsoni* in Philippine stray dogs. The frequency of *Boophilus* sp. of ticks in both sero+ and sero- dogs is interesting in view of their association with cattle babesiosis. Furthermore, considering the lack of information on the specific tick vector(s) of canine babesiosis in the country, present preliminary data are meaningful. We are presently working on tick species identification, and hope to carry out similar studies covering other dog populations and breeds in the country. In conclusion, this paper documents the first confirmation of *B. gibsoni* infection in Philippine stray dogs using the P50t-ICT, alongside their infestation principally with *Rhipicephalus* tick species.

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SAŽETAK

Istraživanje je provedeno kako bi se imunokromatografskim testom (P50t-ICT) dokazala prisutnost protozoona *Babesia gibsoni* u pasa lutalica na Filipinima. Ukupno je bilo pretraženo 46 pasa, 17 mužjaka i 29 ženki, na prisutnost antigena p50 protozoona *Babesia gibsoni*. Trinaest pasa (28%) bilo je serološki pozitivno. Nije ustanovljen nijedan slučaj križne reaktivnosti s uzorcima seruma pasa prirodno invadiranima protozoonom *Babesia canis* (*Babesia vogeli)*. Test se pokazao prikladnim, osjetljivim, specifičnim, pouzdanim i točnim. U 37 pasa (80,4%) dokazane su mješovite infestacije krpeljima rodova *Rhipicephalus* i *Boophilus*. Na 11 serološki pozitivnih i 20 serološki negativnih pasa pronađeno je ukupno 80 krpelja iz roda *Rhipicephalus*. Od 33 serološki negativna psa krpelji roda *Rhipicephalus* dokazani su u 48,5%, roda *Boophilus* u 12,1%, a jedna mješovita infestacija vrstama tih rodova dokazana je također u 12,1% pasa. U 27,3% pasa krpelji nisu dokazani. Ovaj rad prvi put prikazuje mogućnost seroloških dokaza infekcije vrstom *B. gibsoni* u pasa lutalica i njihovu infestaciju pretežito krpeljima roda *Rhipicephalus* kao ključnih prijenosnika *B. gibsoni* u pasa lutalica na Filipinima.

Ključne riječi: *Babesia gibsoni*, P50t-ICT, psi lutalice, Filipini, krpelji