

Duration of Antibodies against 24 kd Protein of *Rhipicephalus sanguineus* Extract in Dogs Infested with the Adult Ticks

Hisashi INOKUMA, Miho MUKAI, Kouichi OHNO, Yoshimi YAMAMOTO, Susumu TAKAHASHI and Takafumi ONISHI

Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

(Received 3 June 1998/Accepted 21 September 1998)

ABSTRACT. A 24 kd protein from *Rhipicephalus sanguineus* (Rs24p) which was common to larvae, nymphs, male and female whole body and salivary gland extract of males and female was detected specifically in the serum from dogs after repeated infestation with adult *R. sanguineus*. The duration of antibodies against Rs24p in dogs infested with adults was examined by Western blotting analysis. Anti-Rs24p antibody was detected in two of 4 dogs during the period of 40 days in the first infestation. In the second infestation, all dogs showed positive reaction against Rs24p, but the duration of the antibodies varied greatly among the animals.—**KEY WORDS:** anti-tick antibody, canine, *Rhipicephalus sanguineus*.

J. Vet. Med. Sci. 61(2): 179–181, 1999

Rhipicephalus sanguineus is distributed worldwide in dog. However, this tick is only found in Okinawa Prefecture, Japan [11]. *R. sanguineus* is a well known vector of many diseases of dogs. *Ehrlichia canis* and *Hepatozoon canis* are reported to be transmitted only by this tick [1, 2]. Recently, dogs positive for anti-*E. canis* antibodies [12] and dogs infected with *H. canis* [6] were reported in the western part of Japan, but no evidence of vector ticks was presented. The detection of anti-tick antibodies has been used as a biological marker of exposure to *Ixodes dammini* in humans [8–10]. Antibodies against larvae, nymphs, adults and salivary gland from adult *R. sanguineus* were detected in the serum of rabbits after repeated infestation with adult ticks [4], however there have been few information about the anti-tick antibody in dogs. In this study, the duration of antibodies against extract of *R. sanguineus* was examined in dogs infested with adults.

Eight tick naive adult male Beagle were used in the experiments. *R. sanguineus* eggs, unfed larvae, unfed nymphs, and unfed female and male adults and unfed female of *Haemaphysalis longicornis* were obtained from a colony maintained at our laboratory using rabbits (Japanese white, Kyudo, Japan). The ear bag method was used for tick infestation. Four dogs (Nos. 1 to 4) were infested with 10 pairs of adult *R. sanguineus* in the ear bags on day 0 as the first infestation. Eight to ten females successfully engorged on days 6 to 10. On day 60, each dog was infested with 10 pairs of adult *R. sanguineus* in the ear bags as the second infestation. During the second infestation, 4 to 8 females successfully engorged with feeding periods of 7 to 14 days. We put 10 pairs of ticks because 17 adults *R. sanguineus* was the mean number of ticks per infestation in the field [3]. Two dogs (Nos. 5 and 6) were infested with 20 adult female of *H. longicornis* on day 0 as the first infestation and 17 and 18 of females, respectively, engorged on days 4 to 7. Same number of ticks were infested on day 60 as the second infestation. Eighteen and 14 of ticks engorged on dogs Nos. 5 and 6, respectively with feeding periods of 4 to 9 days. Another 2 dogs, Nos. 7 and 8, were used as control

animals without tick infestation. Sera were obtained from each dog on days 0, 6, 12, 18, 24, 30 and 40 in the first infestation, and on days 0, 6, 12, 24, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160 and 170 in the second infestation.

Salivary glands were dissected from unfed females under a microscope. Eggs, unfed larvae, unfed nymphs, unfed females, unfed males and salivary glands from females were homogenized with ground glass homogenizers in 2.0 ml of phosphate buffered saline (PBS, pH 7.2) and 5 mM phenylmethylsulfonyl fluoride (PMSF, Nakalai Tesque, Japan) in an ice bath. This crude extract was centrifuged at $7267 \times g$ for 10 min at 4°C. The supernatant were used as the source of analysis. The protein concentrations of samples were determined with a kit (DC-Protein Assay Kit, Bio-Rad, U.S.A.), and stored at -20°C until use. Tick extracts were separated by 12% SDS-PAGE under reducing conditions and then transferred to the nitrocellulose membranes by electrotransferring.

In preliminary experiment, many common bands were observed among the supernatants of extracts from eggs (EE), unfed larvae (LE), unfed nymphs (NE), unfed males (ME), salivary gland of males (MSGE), unfed females (FE) and salivary gland of unfed females (FSGE) by SDS-PAGE analysis and silver staining (Fig. 1). All samples reacted with the serum from dog No. 1 just before the first infestation and from the same dog on 12 days after the second infestation. Two common bands with molecular weights of 24 and 62 kd were detected in LE, NE, ME, MSGE, FE and FSGE (Fig. 2A). However, one of the bands (62 kd) was found in strips reacted strongly with pre-serum (Fig. 2B). These findings suggested that the 24 kd protein (Rs24p) is a common antigen among larvae, nymphs, males, females and salivary glands of adults, but not eggs, and the protein reacted with sera from dogs infested with adult *R. sanguineus* repeatedly. The source of Rs24p is not known but the salivary gland is one possible candidate. Martin Hernandez *et al.* [5] performed a similar experiment using rabbits and *R. sanguineus*, and they found two bands

in the 27.5–18.5 kD range in LE, ME, MSGE, ME and FSGE with sera from rabbits infested with adult ticks. As the larval extract is the most readily available source of antigens for analysis, we examined the duration of antibodies against Rs24p in dogs by using larval extract.

To evaluate the duration of antibodies in dogs infested with adults, the specific band against Rs24p was examined in strips reacted with sera from dogs infested with the ticks.

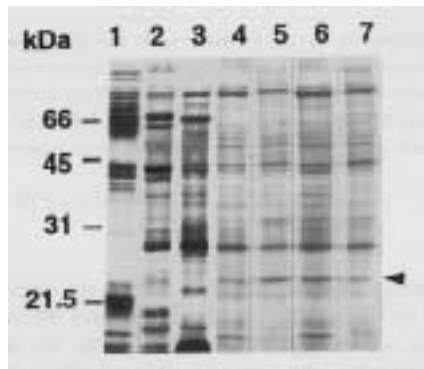


Fig. 1. Antigens from extracts of *Rhipicephalus sanguineus* eggs (lane 1), larvae (lane 2), nymphs (lane 3), males (lane 4), male salivary glands (lane 5), females (lane 6) and female salivary glands (lane 7) were separated by 12% SDS-PAGE, and silver staining was performed. An arrow shows molecular weight of 24 kD.

The results are summarized in Table 1. Two of 4 dogs infested with *R. sanguineus* showed positive but faint bands against Rs24p on days 6 and 12 at the end of the first infestation, and the bands continued until days 40 and 18, respectively. In the second infestation, positive bands were observed in all 4 dogs, but the duration of the anti-Rs24p antibody showed wide variation among the dogs. The positive band was observed on day 0 at the beginning of infestation and lasted until day 160 in dog No. 1, while the positive band appeared on day 12 at the end of the infestation and continued until day 30 in dog No. 4. In all dogs, the reactivity of the positive bands was reduced with time.

There were no positive bands at 24 kD with serum from dogs repeatedly infested with *H. longicornis* (Table 1) or no-tick control. Recently, Parmar *et al.* [7] found that both *Hyalomma anatolicum* and *Boophilus microplus* had a common antigen. In the western part of Japan, some tick species such as *H. longicornis*, *H. flava*, *H. campanulata*, *I. ovatus*, and *Amblyoma testudinarium* are commonly seen in dogs [11]. More studies are needed to confirm the specificity of the protein from larval extract before it can be used as a biological marker for tick exposure.

REFERENCES

1. Craig, T.M. 1990. pp.778-785. In: Infectious Diseases of the Dog and Cat (Greene, C.E. ed.), WB Saunders Co., Philadelphia.
2. Groves, M.G., Dennis, G.L., Amyx, H.L. and Hursll, D.L.

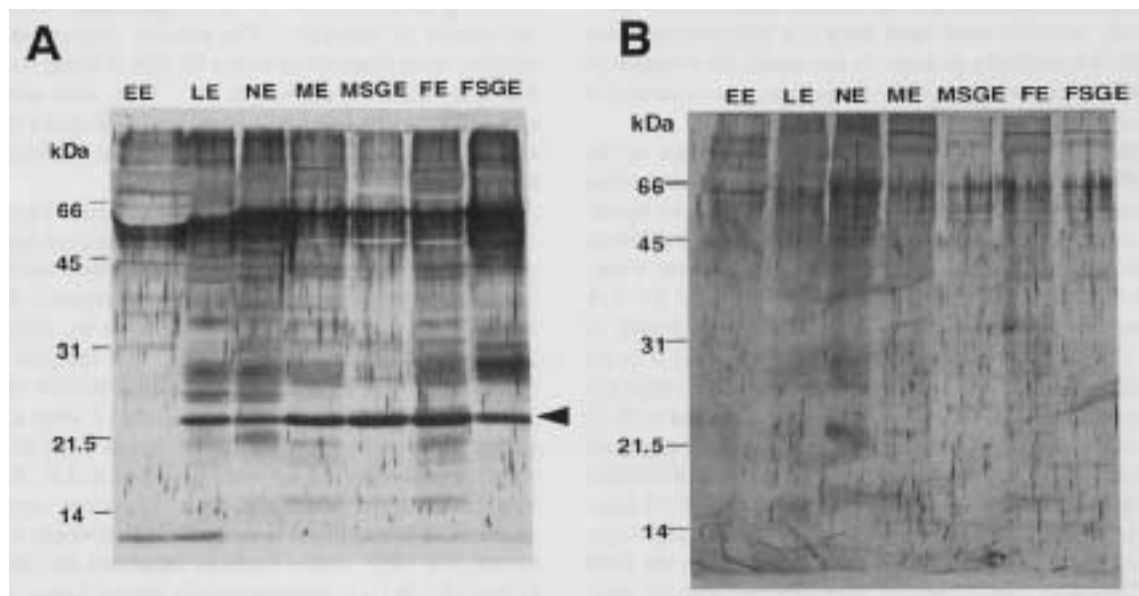


Fig. 2. Immunological reactions to extracts of *Rhipicephalus sanguineus* eggs (EE), larvae (LE), nymphs (NE), males (ME), male salivary glands (MSGE), females (FE) and female salivary glands (FSGE) were examined with serum from dogs repeatedly infested with the ticks by Western blotting. The transferred proteins were stained with dog serum (see below) followed by a secondary antibody, alkaline phosphatase-conjugated anti-dog IgG (American Qualex, U.S.A.) and developed with alkaline phosphatase conjugate substrate kit (Bio-Rad, U.S.A.). (A) Serum from a dog (No.1) on day 12 of the second infestation with 10 pairs of adult *R. sanguineus*. (B) Serum from the same dog before tick infestation. An arrow shows molecular weight of 24 kD.

Table 1. Duration of antibodies against 24 kd protein from larval extract of *Rhipicephalus sanguineus* in dogs infested with adult *R. sanguineus*, *Haemaphysalis longicornis* or without tick infestation

Tick species	First infestation								Second infestation																		
and dog No.	0	6	12	18	24	30	40	0	6	12	18	24	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170
<i>R. sanguineus</i>																											
1	-	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	NT	NT
3	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	NT	NT	NT	NT
4	-	-	-	-	-	NT	-	-	+	+	+	+	+	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
<i>H. longicornis</i>																											
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Without tick																											
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT: Not tested. +: Positive. -: Negative.

1975. *Am. J. Vet. Res.* 36: 937-940.
3. Inokuma, H., Tamura, K. and Onishi, T. 1995. *J. Vet. Med. Sci.* 57: 567-568.
4. Martin Hernandez, R., Cuellar del Hoyo, C., Olmeda Garcia, A.S. and Rodrogez Rodriguez, J.A. 1994. *Med. Vet. Entomol.* 8: 238-244.
5. Martin Hernandez, R., Cuellar del Hoyo, C., Olmeda Garcia, A.S. and Rodrogez Rodriguez, J.A. 1995. *Med. Vet. Entomol.* 9: 358-364.
6. Murata, T., Amimoto, A., Shiramizu, K., Hara, Y., Inoue, M., Kanoe, M., Uzuka, U., Taura, Y. and Nakama, S. 1993. *J. Jpn. Vet. Med. Assoc.* 46: 395-397 (in Japanese with English summary).
7. Parmar, A., Grewal, A.S. and Dhillon, P. 1996. *Vet. Immunol. Immunopathol.* 51: 345-352.
8. Schwartz, B.S., Ribeiro, J.M.C. and Goldstein, M.D. 1990. *Am. J. Epidemiol.* 132: 58-66.
9. Schwartz, B.S., Ford, D.P., Childs, J.E., Rothman, N. and Thomas, R.T. 1991. *Am. J. Epidemiol.* 134: 86-95.
10. Schwartz, B.S., Nadelman, R.B., Fish, D., Childs, J.E., Forseter, G. and Wormser, G.P. 1993. *Am. J. Trop. Med. Hyg.* 48: 50-57.
11. Yamaguchi, N., Tipton, V.J., Keegan, H.L. and Toshioka, S. 1971. *Brigham Young Univ. Sci. Bul. Ser.* 15: 1-226.
12. Yamamoto, S., Honda, M., Ashida, Y., Nishimura, Y., Niizeki, H. and Rikihisa, Y. 1994. *J. Jpn. Vet. Med. Assoc.* 46: 395-397 (in Japanese with English summary).