

Diagnosis of Bovine Babesiosis by the Capillary-Tube Agglutination Test

By TETSURO MINAMI

Kyushu Branch, National Institute of Animal Health

Three species of *Babesia*, parasitic to cattle in Japan, have been so far reported^{6,7,8}. Two of them, *Babesia bigemina* and *B. argentina*, with high pathogenicity are distributed in Okinawa district as a main cause of piroplasmiasis in that area. The third one (referred to the Japanese *Babesia* species), which is widely distributed in Japan except Okinawa Prefecture, is less pathogenic than the former two species, but it often shows the mixed-infection with *Theileria sergenti*, *Anaplasma* etc., giving the complicated symptom of piroplasmiasis of grazing cattle.

The decisive diagnosis of babesiosis is to detect the pathogenic protozoa. The microscopic examination of stained blood-smear preparations is most frequently employed for detecting the protozoa. In cattle infected with *T. sergenti*, the protozoa can easily be detected in blood even time elapsed after infection. However, with *Babesia*, although it can be detected easily at the stage immediately after the initial infection, the microscopic detection becomes very difficult after time elapsed because the number of the protozoa in the circulating blood decreases rapidly. Therefore, with the tolerant, cattle, it is not possible to carry out microscopic examinations of protozoa and the diagnosis of pathogenicity, unless the protozoa is multiplied by inoculating the blood sample to the healthy splenectomized cattle.

With regard to the serological methods of diagnosis, it was reported that the complement-fixation test, fluorescent antibody technique, gel-precipitation test, etc. have already been applied and proved to be highly specific¹¹.

However, these methods are generally complicated in procedures requiring skillfulness, and still have problems related to the positive limit or their popularization. As to the agglutination test, various methods of test have been developed. As the indirect agglutination test, the passive haemagglutination test^{1,4} or the rapid slide agglutination test⁵ by using latex particles is being employed for diagnosis of *B. argentina* and *B. bigemina*. As the direct agglutination test, the capillary-tube agglutination test⁹, slide agglutination test³, and card agglutination test¹² are used for *B. bigemina*, and the parasitized erythrocyte agglutination test² for *B. argentina*. Procedures of these agglutination tests are generally simple, and particularly the capillary-tube agglutination (CA) test is considered as a serological method of diagnosis with high practicability.

Therefore, the author attempted to utilize the CA test in diagnosing the Japanese *Babesia* species as an initial step in the effort to simplify the serological diagnosis of bovine babesiosis in Japan.

Preparation of antigen

As *Babesia* strains, the Miyake strain of the Japanese *Babesia* species and the Kochinda strain of *B. bigemina*, both stored at the temperature below -76°C by the rapid-freezing method¹⁰ were used.

As experimental animals, the Holstein calves splenectomized of 3-5 months of age were used. Blood showing more than 15% in the ratio of parasitized erythrocytes to the total number of erythrocytes was used for the preparation

of the antigen.

The antigen was prepared by the Löhr & Ross method (original method) and the author's modified method. The CA antigen by the original method (referred to original antigen) was prepared by treating 50% suspension of washed infected erythrocytes with an ultrasonic disintegrator (MSE, 150W) at its maximum output for 5 min, and repeating washing by ultra-centrifugation at 30,000 rpm for 30 min and homogenizing. The CA antigen by the modified method (referred to modified antigen) was prepared as follows: washed infected erythrocytes were hemolyzed with 0.35% NaCl solution. After removing leucocytes and unhemolyzed erythrocytes, the lysate was centrifuged at 10,000 rpm for 15 min. The sediment, washed, was subjected to the sonic treatment at the minimum output for 10-30 sec, and then washed and homogenized repeatedly (Fig. 1). In the original antigen, all the protozoa were crushed, whereas the modified antigen differs from the original antigen in that the original form of the protozoa was mostly kept unchanged.

The CA antigens of the Japanese *Babesia* species, prepared by both methods were compared by box-titration. The modified antigen showed higher antigen and antibody titer values as well as more clear agglutination figures than the original antigen. In addition, the microscopic examination of Giemsa stained smear preparations of the modified antigen and its agglutination figures after reaction detected the protozoa in the antigen, and it was considered that the agglutination in the CA test is mostly due to the agglutination of protozoa. With the original antigen, no protozoa was observed. Therefore, it was decided to use the modified antigen, standardized by box-titration, in all future works.

Reaction procedures

The CA test was carried out following the Löhr & Ross method⁹. Bovine serum was taken after the experimental infection of *Babesia*, and heated at 56°C for 30 min to inactivate.

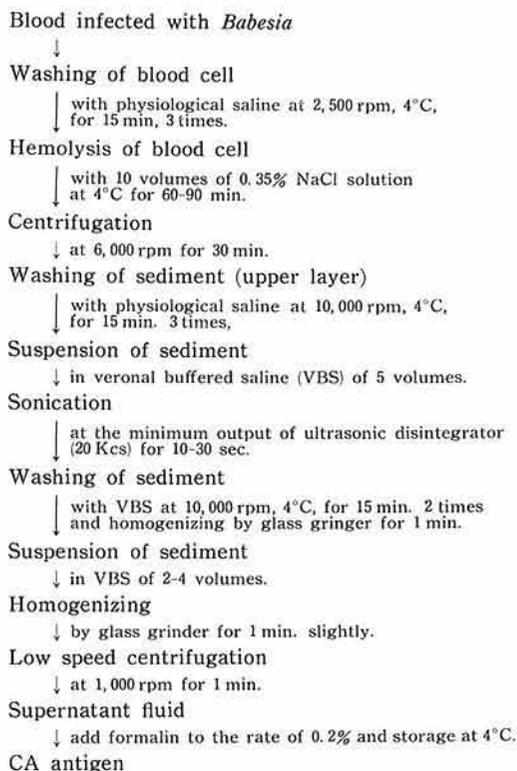


Fig. 1. Modified preparation method of *Babesia* CA antigen

Original serum or that made to twofold serial dilutions with veronal buffered saline (VBS) were used.

Procedure of the CA test is as follows: Firstly, the CA antigen is well mixed, sucked into a glass capillary tube 90 mm in length and 0.5 mm of inside diameter to about 10 mm high, and the antigen clinging outside the capillary tube was wiped off. Secondly, the inactivated serum was sucked into the capillary tube from the same opening used for the antigen to the height of 60 mm, and outerwall of the tube was cleaned again. Finally, the tube was placed vertically with that opening upwards.

The reaction was run at room temperature (22-25°C). When strong reaction occurred, the rating was made after 1-2 hrs. When the reaction was weak or doubtful, observation of agglutination figures in the capillary tube was made after allowing to stand still overnight.

The rating made by naked eyes based on the time required for the occurrence of agglutination reaction and on the extent of forming agglutination figures was as follows (Plate 1).

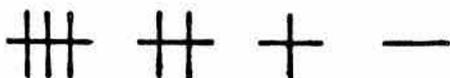
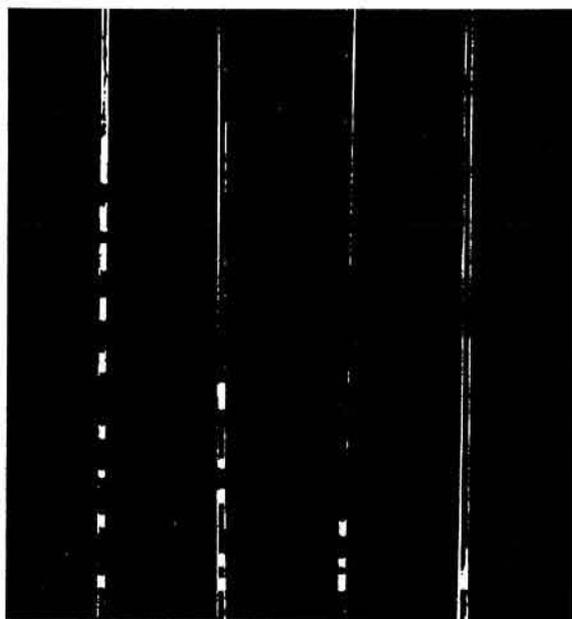


Plate 1. Rating criteria in CA reaction

- +++ : Apparent agglutination clumps were recognized in more than 1/2 of the reaction mixture after 1-2 hrs.
- ++ : Apparent clumps were recognized in less than 1/2 of the reaction mixture after 1-2 hrs.
- + : Agglutination reaction did not appear or was doubtful after 1-2 hrs, but apparent clumps or a large number of fine clumps were recognized after keeping the reaction mixture stand still overnight.
- ± : A small number of fine clumps were observed.
- : No clumps were observed at all.

Serum which shows agglutination figures higher than + was referred as CA test positive, and that lower than ± as negative. Antibody titer was expressed by the maximum

dilution times of serum showing the reaction higher than +.

Specificity of the CA test

To examine the specificity of CA antigen of the Japanese *Babesia* species, CA antigens were prepared by the modified method from blood infected with *B. bigemina* and normal bovine blood, and the cross CA test was carried out using sera collected from healthy cattle and the animals experimentally infected with the Japanese *Babesia* species, *B. bigemina*, *B. argentina*, *T. sergenti*, *Anaplasma marginale*, *A. centrale*, and *Eperythrozoon wenyonii*.

Results indicated that the CA antigen of the Japanese *Babesia* species did not react with any other serum than *Babesia*-infected serum, and that the normal antigen did not react with any serum. Among the species of *Babesia*, a cross-reaction was observed, like other serological reactions. However, the degree of reaction was apparently strong for a same species, with titers of 4-1,024 times. On the contrary between different species, no reaction or weak reaction with titers of 2-16 times, even when cross-reactions occurred, were observed (Table 1).

The CA antibody of the Japanese *Babesia* species could be detected within about 5 days after the appearance of the protozoa, and its titer value reached the maximum, 128-1,024 times, after 3-10 days. Although the parasitic protozoa disappeared from blood generally 3-21 days after infection, the CA antibody could be detected over a period more than 1 year. These results were considered almost the same as the case of *B. bigemina* reported by Löhr & Ross⁹⁾ (Fig. 2).

The CA antigen for 2-219 days at 4°C after formalin was added, was examined. It was demonstrated that even after 7 months of storage, the antigen maintained its activity without any loss, showing a strong reaction to the antibody of the same species.

In conclusion, the CA test can be a rapid and simple method of serological diagnosis of the Japanese *Babesia* species, which is widely

Table 1. Specificity of CA antigen

Serum		CA antibody titer		
Species	Cattle number	The Japanese <i>Babesia</i> species antigen	<i>B. bigemina</i> antigen	Normal* antigen
The Japanese <i>Babesia</i> species	{ 1	4	—	—
	{ 2	32	4	—
	{ 3	128	8	—
	{ 4	1,024	16	—
<i>B. bigemina</i>	{ 5	—	8	—
	{ 6	2	32	—
	{ 7	16	128	—
	{ 8	16	256	—
<i>B. argentina</i>	{ 9	1	2	—
	{ 10	—	2	—
	{ 11	—	—	—
<i>T. sergenti</i>	{ 12	—	—	—
	{ 13	—	—	—
<i>A. marginale</i>	{ 14	—	—	—
	{ 15	—	—	—
<i>A. centrale</i>	{ 16	—	—	—
	{ 17	—	—	—
<i>E. wenyonii</i>	{ 18	—	—	—
	{ 19	—	—	—
Normal serum	{ 20	—	—	—
	{ 21	—	—	—
	{ 22	—	—	—

* CA antigen prepared from normal bovine blood.

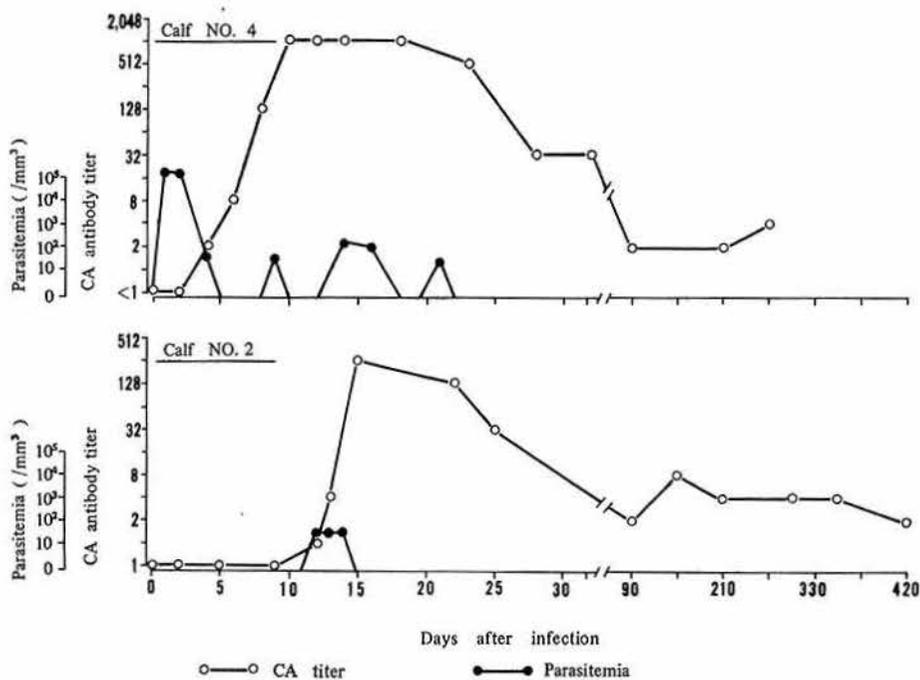


Fig. 2. Development of CA antibody in calves infected with the Japanese *Babesia* species

distributed in Japan, with high specificity and applicability for the diagnosis at the chronic stage of babesiosis. Results of the cross-tests showed that the discrimination from *B. bigemina* and *B. argentina* was almost possible, but further detailed examination using serum collected at given intervals of time will be needed.

References

- 1) Curnow, J. A. & Curnow, B. A.: An indirect haemagglutination test for the diagnosis of *Babesia argentina* infection in cattle. *Austral. Vet. J.*, 43, 286-290 (1967).
- 2) Curnow, J. A.: *In vitro* agglutination of bovine erythrocytes infected *Babesia argentina*. *Nature*, 217, 267-268 (1968).
- 3) Curnow, J. A.: The use of a slide agglutination test to demonstrate antigenic differences between *Babesia bigemina* parasites. *Austral. Vet. J.*, 49, 290-293 (1973).
- 4) Goodger, B. V.: Preparation and preliminary assessment of purified antigens in the passive haemagglutination test for bovine babesiosis. *Austral. Vet. J.*, 47, 251-256 (1971).
- 5) Goodger, B. V. & Mahoney, D. F.: A rapid slide agglutination test for the herd diagnosis of *Babesia argentina* infection. *Austral. Vet. J.*, 50, 250-254 (1974).
- 6) Hamakawa, M. et al.: Bovine *Piroplasma* in Ryukyu Islands. Preliminary report. Abstract of 73th meeting of the Japanese Society of Veterinary Science (1972).
- 7) Ishihara, T.: Bovine piroplasmosis in Japan. *JARQ*, 3, 23-31 (1968).
- 8) Ishihara, T.: Bovine babesiosis and theileriosis in Japan. *Bull. Nat. Inst. Anim. Hlth*, No. 62, 128-146 (1971) [In Japanese].
- 9) Löhr, K. F. & Ross, J. P. J.: Ein Kapillarröhrchen-Agglutinationstest zum Nachweis von *Babesia bigemina*-Antikörpern. *Z. Tropenmed. Parasitol.*, 20, 287-292 (1969).
- 10) Minami, T., Ishihara, T. & Yamabe, K.: Cryopreservation of *Trypanosoma*, *Theileria*, *Babesia*, and *Anaplasma* after rapid-freezing. *Bull. Nat. Inst. Anim. Hlth*, No. 73, 20-26 (1976). [In Japanese with English summary], *Nat. Inst. Anim. Hlth Quart.*, 16, 186-187 (1976).
- 11) Ristic, M.: Babesiosis and Theileriosis. Immunity to Parasitic Animals Vol. 2, 831-870, Appleton-Century-Crofts, New York (1970).
- 12) Todorovic, R. A. & Kuttler, K. L.: A babesiosis card agglutination test. *Amer. J. Vet. Res.*, 35, 1347-1350 (1974).