Seroepidemiological Studies of Bovine Babesiosis Caused by *Babesia ovata, B. bigemina* and *B. bovis* in Peninsular Malaysia

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ABSTRACT. Cattle in Peninsular Malaysia were examined for evidence of infection with *Babesia ovata, B. bigemina* and *B. bovis* by an enzyme-linked immunosorbent assay for detection of antibody to the three *Babesia* species. All of the test samples when assayed with *B. ovata* antigen, resulted in low value indicating low probability of cattle infected with *B. bigemina*, 74.4% were positive for *B. bovis* and 72.6% were positive for both *Babesia* species. In addition, a serological survey with regard to age difference was carried out on a milk production farm. High reactivity antibody to *B. bigemina* and *B. bovis* was detected in calves less than 1 month of the age. The reactivity decreased in calves 1–3 months of the age. Then, the reactivity increased for both *Babesia* species in 6 months old calves. These results suggested that cattle infected with *B. bigemina* and *B. bovis* were widespread throughout Peninsular Malaysia and that both parasites might exist as an enzootical parasite.—KEY WORDS: *Babesia bigemina, B. bovis, B. ovata*, ELISA, seroepidemiological survey.

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Bovine babesiosis is a serious problem in many parts of the world. There are a number of the *Babesia* species which cause the disease [1]. In Japan, infection with *B. ovata* a species closely related to *B. bigemina* are widespread. The parasite appears to be serologically and immunologically distinct [2, 10]. *B. ovata* has been detected only in eastern part of Asia including Japan and South Korea [8].

A number of epidemiological survey on bovine babesiosis caused by *B. bigemina* and *B. bovis* has been reported using a variety of serological test in the tropical and subtropical zones [4, 7, 11]. Recently, an enzyme linked immunosorbent assays (ELISA) has become available for a serological diagnosis and survey, since a number of serum samples can be tested in short period of time with high sensitivity. In Malaysia, the existence of cattle infected with *B. bigemina* or *B. bovis* has been noted in a local area [9]. However, no serological survey of babesiosis of the complete Peninsular Malaysia have been reported. Future control strategies to bovine babesiosis would be facilitated if the epidemiology and distribution of *Babesia* infection were known for the entire Peninsular Malyasia.

This paper describes an antibody prevalence study for *B. ovata, B. bigemina* and *B. bovis* in 9 states of Peninsular Malaysia using merozoite antigen in an ELISA assay. The existence of three *Babesia* parasites, together with prevalence pattern of antibody with respect to age difference, will be discussed.

MATERIALS AND METHODS

Stock of Babesia: The Miyake stock of *B. ovata* was used in the present study. It was isolated in 1967 from grazing cattle in Tokyo, and maintained by serial passage in cattle [2]. The Kochinda stock of *B. bigemina* was used. It was isolated in 1966 from Japanese black cattle, introduced from Okinawa Prefecture into mainland Japan, and maintained in the same way [2]. The Miyama stock of *B. bovis* was isolated in 1971 from cattle in Okinawa and maintained in the same way [2].

Antigen preparation: The merozoites were purified by a modified nitrogen decompression method [10]. The isolated merozoites were resuspended in an appropriate volume of PBS. Following sonication, this suspension was divided into aliquots and stored at −80°C until use. The antigen for use was prepared by mixing the antigen suspension with the same volume of 0.4 or 4% Triton X-100 in PBS. The mixture was left to stand for 3 hr at 4°C, and then used as antigen for ELISA.

ELISA for reference sera: Positive reference serum for *B. ovata, B. bigemina* and *B. bovis* merozoite antigen as well as negative reference serum were obtained from known experimental animals (Dr. Nakamura, NIAH, Tsukuba, Japan).

Experimental design: The serological survey was conducted in three phases. The first experiment involved the determination of serum antibody to *B. ovata* followed by the prevalence study of *B. bigemina* and *B. bovis* in 9 states of Peninsular Malaysia. The third part of the experiment, involved the prevalence of the antibody with respect to age against the three *Babesia* species on dairy farm. In addition, blood smear were made from EDTA samples, fixed in methanol, stained with Giemsa and examined for intraerythrocytic forms of *Babesia* parasites.

Serum sample: A total of 271 serum samples were collected from 9 states in Peninsular Malaysia, comprising 30 to 31 samples from each states. The samples were provided by the serum bank of the Veterinary Research

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Institute in Malaysia. Serum as well as EDTA blood sample were collected from Fresian Sahiwal animals from a dairy farm (Sungai Siput, Milk Collecting Centre, Perak).

ELISA procedure: The ELISA procedure was carried out as previously described in detail [10]. Briefly, each antigen was diluted with 0.05% carbonate/bicardonate buffer (pH 9.6) and dispensed in volume of 0.1 m*l* into the wells of polystyrene microplate (ELISA plate S, MS-8496F; Sumitomo Bakelite, Japan). The antigen coated plates were incubated overnight at 4°C and washed five times with PBS containing 0.02% Tween 20. The serum samples were diluted 1:100 with PBS containing 0.15% Tween 20 and added to each well in volume of 0.1 m*l*. Then, the plates were incubated for 1 hr at 37°C and washed as described above. Rabbit antibovine IgG horseperoxidase conjugate (H- & L-chain specific; Cappel Laboratories, U.S.A.) diluted 1:8000 with PBS containing 0.15% Tween 20 was added in 0.1 m*l* volume to each well. The plates were again incubated for 1hr at 37°C. After washing as described above, 0.1 m*l* ABTS (2,2'-azino-di-3-ethyl-benzthiazoline sulfonate, 0.5 mg/m*l*) in sodium/citric phosphate buffer (pH 4.0) containing 0.03% hydrogen peroxidase was added to each well and the plates were incubated for 1 hr at 37°C. The enzyme reaction was stopped by adding 1% SDS to each well. The optical density (OD) was measured with a microplate reader (MR5000, Dynatech, U.S.A.) at 405 nm. The serum sample with OD of 0.37 and above for the *B. bigemina*, and that of 0.23 and above for *B. bovis*, were tentatively classed as antibody positive according to Shimizu *et al.* [10].

RESULTS

Prevalence of antibody against B. ovata/bigemina/bovis in Peninsular Malaysia: The relationship between the OD value in ELISA assay to *B. ovata* and *B. bigemina* antigens for 271 field sera collected from 9 states of Peninsular Malaysia are shown in a scattergram in Fig. 1. All of the samples reacted more intensely with *B. bigemina* antigen as compared to *B. ovata* antigen. Figure 2 showed the scattergram of the relationship between the OD value in ELISA assay to *B. bigemina* and *B. bovis* antigen. Almost all of the samples examined intensively reacted to one/both antigens. Overall, 97.8% samples were positive to *B. bigemina* antigen, 74.4% were positive to *B. bovis* antigen, and 72.6% were positive to both (Fig. 3). All samples collected from 7 states except for Perlis and Selangor were positive for one/both *Babesia* species.

Antibody prevalence with respect to the age against B. bigemina and B. bovis on a dairy farm: All of the serum sample tested from calves less than 1 month of age and adult had relatively high OD value to *B. bigemina* and *B. bovis*, whereas the sample tested from calves 1–3 month of age had low OD value (Fig. 4). However, no *Babesia* parasites were detected by blood smear examination of samples collected in the present study. All of the serum samples had low activity to *B. ovata* irrespective of the animal age.

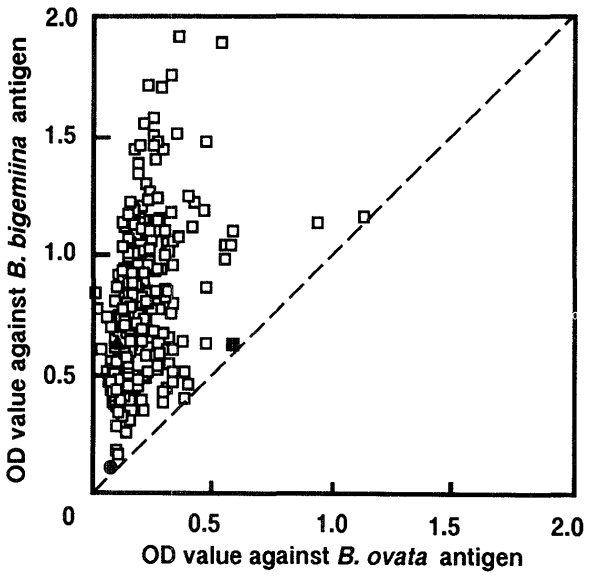

Fig. 1. Relation between the ELISA OD value for 271 cattle sera collected from Peninsular Malaysia against *B. ovata* and *B. bigemina* antigens: ■, *B. ovata* positive reference serum; ▲, *B. bigemina* positive reference serum; ●, Negative reference serum.

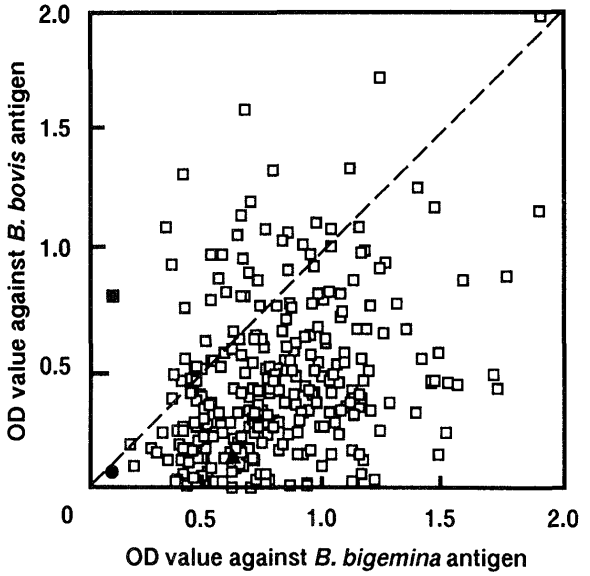

Fig. 2. Relation between the ELISA OD value for 271 cattle sera collected from Peninsular Malaysia agaisnt *B. bigemina* and *B. bovis* antigens: ▲, *B. bigemina* positive refernece serum; ■, *B. bovis* positive reference serum; ●, Negative reference serum.

DISCUSSION

All of the serum samples examined in the present study had low activity against *B. ovata* merozoite antigen,

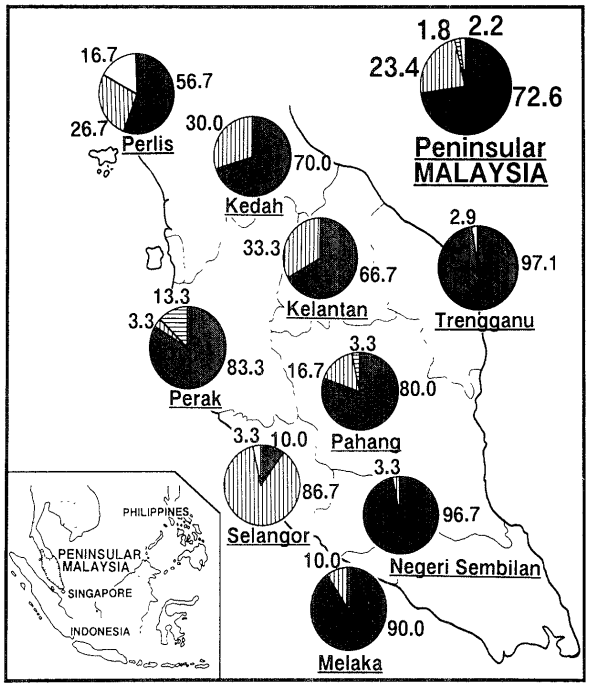

Fig. 3. Distribution of the serologically *B. bigemina* and *B. bovis* positive cattle in Peninsular Malaysia. Shaded, vertical line, horizontal line and blank area showed the percentage of antibody positive to both *B. bigemina* and *B. bovis*, *B. bigemina* only, *B. bovis* only and antibody negative to both *Babesia*, respectively.

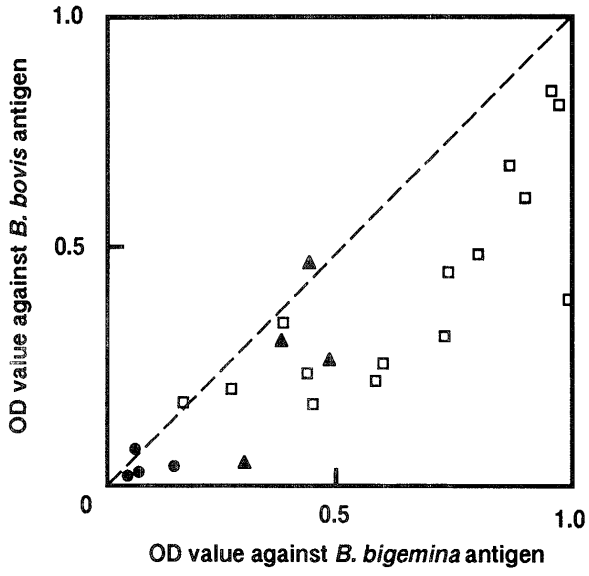

Fig. 4. Relation between the ELISA OD value for sera collected from cattle a different ages on a dairy farm in Peninsular Malaysia against *B. bigemina* and *B. bovis* antigens: ▲, Less than 1 month of age; ●, 1–3 months of age; □, 6 months and above of age (adult).

indicating low probability of infection with *B. ovata* parasite. In general, *Babesia* species may be transmitted by ticks transovarialty and/or stage to stage [1]. The tick vector species of *B. ovata* is *Haemaphysalis longicornis* which transmits the protozoan by the latter pattern. In Japan, theileriosis (*Theileria sergenti*) and babesiosis (*B. ovata*) are both transmitted by *H. longicornis* [3]. Interestingly, many cases of babesiosis caused by other *Babesia* species except for *B. bigemina* and *B. bovis* were reported in Peninsular Malaysia [9]. Kamio *et al.* [5] reported during a survey of the vector tick of theileriosis in Peninsular Malaysia that *H. bispinosa* closely related species *H. longicornis* was detected on cattle, and described that *Theileria* parasites in Peninsular Malaysia might be transmitted by the *H. bispinosa*. Therefore, more vector tick data will needed to be obtained to clarify the transmission of *Babesia* species.

The present study showed that antibody activity against *B. bigemina* and *B. bovis* antigen was high in all samples. In general, the distribution of the both parasites is correlated with the distribution of the tick vector species *Boophilus microplus*. Recently, Kamio *et al.*[5] reported that almost of all the ticks collected from cattle in Peninsular Malaysia were *B. microplus*. The present data would support their report.

Overall, 97.8% of cattle tested were antibody positive to *B. bigemina* or/and *B. bovis* antigen. Moreover, sample from calves less than 1 month of age and adult cattle aged 6 months and above had high OD value, whereas OD value fall in sample from calves 1–3 months of age. Similar changes in antibody activity have been reported in *Babesia*-endemic areas [6, 11]. In those areas, the initial decrease in antibody levels followed the disappearance of maternal antibody, which is gradually replaced by antibody produced in response to infection via natural tick challenge, and in adults the antibody level against the *Babesia* parasites maintain high values. Therefore, it was considered that enzootic stability of both *B. bigemina* and *B. bovis* existed in Peninsular Malaysia.

In conclusion, results from the present study presents evidence that cattle infected with *B. bigemina* and *B. bovis* were almost ubiquitous in all over Peninsular Malaysia. Recently, in Malaysia, importation of cattle from oversea countries, especially Austrialia, are on the increase. However, the present control measures such as regular deticking and prophylactic treatment are inadequate as outbreaks of clinical babesiosis occur and cause heavy mortalities especially in imported susceptible cattle from Australia. In the present study, a serological survey of imported beef cattle was also carried out. Many animal that had no infection with both *Babesia* were identified (data are not shown). Further systematic studies are necessary to clarify the impact of babesiosis on local animals as well as on imported animal with respect to production, epidemiolgy of the disease and the control measures.

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