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In Vitro Observations on Drug Responsiveness of *Babesia bovis* and on the Emergence of Drug Resistant Parasites

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ABSTRACT

Four culture lines of *Babesia bovis* Thai strain were successfully established in this study. The results of the in vitro microtest for their sensitivities to quinuronium sulfate revealed that all the culture lines were similarly drug responsive. The 50% inhibitory concentrations for the four culture lines were found to be within the same range, varying from 1.72×10^{-6} to 2.43×10^{-6} g/ml. One resistant line TS-2R selected from the drug-treated culture had an IC_{50} of 1.71×10^{-5} g/ml which was 7 times higher than that of its original isolate TS-2.

INTRODUCTION

Babesiosis is one of the most important tick-borne diseases of domestic animals, and at least 1.3 billion domestic animals are at risk worldwide (Konigshoefer 1977). Bovine babesiosis in particular threatens the cattle industry in tropical and sub-tropical regions, and production of cattle and buffaloes in many Asian countries including Thailand has been impaired by this lethal disease (Brockelman 1990). In Thailand, the important causative agents of bovine babesiosis are *Babesia bovis* and *B. bigemina*. The disease is usually characterized by symptoms of fever, hemolytic anemia and hemoglobinuria (Ristic 1970). The prevalence of the infections in some areas is as high as 15% (Jittapalapong and Leowijak 1988).

In order to improve yields produced by local herds, productive dairy breeds from the temperate zone have been continually imported. The imported cattle and their hybrid offspring appear to be more susceptible to Babesia infection than native breeds. High mortality of infected animals has been observed despite chemotherapeutic treatment with diamidine derivatives and quinuronium sulfate (Mallick et al 1987). Although failure of quinuronium sulfate treatment has been repeatedly reported, no one has attempted to characterize the resistant strains of the parasites. The major difficulty has been the unavailability of a rapid method to determine the sensitivity of *Babesia* parasites to babesiacidal drugs. In the past, most testing and screening of babesiacidal drugs were carried out with experimental infections in splenectomized calves, which were costly and time-consuming. Nevertheless, the new babesiacidal drugs imidocarb, diminazene and amicarbalide were introduced as a result of those studies.

EMERGENCE OF DRUG RESISTANT STRAIN OF *BABESIA BOVIS*

Recent success in the continuous cultivation of erythrocytic stages of *B. bovis* by Levy and Ristic (1980) has provided a new approach for investigation of bovine babesiosis in various aspects. Such cultivation methods have been further improved so that laboratory applications such as study of isoenzyme patterns and in vitro drug sensitivity assays are now feasible (Vega et al 1985; Rodriguez et al 1986; Brockelman and Tan-ariya 1991).

We have recently developed an in vitro microtest to assess the sensitivity of *B. bovis* and *B. bigemina* in continuous culture to drug(s) in our laboratory. We report here on the use of this system to determine the patterns of susceptibility or resistance of Thai strains of *B. bovis* to quinuronium sulfate.

MATERIALS AND METHODS

Collection of parasites. Four blood samples infected with *B. bovis* used in this study were collected from three different localities in Thailand: isolates TS-2 and TS-4 from Nakhon Srithamaraj Province (South), isolate KP from Nakhon Pathom Province (Central), and isolate NA from Petchaboon Province (North). Ten ml of each blood sample was aseptically collected by jugular venipuncture from a calf naturally infected with *B. bovis*. The infection was amplified by removal of the spleen 5 days prior to the collection of blood, except for the KP blood sample which was obtained from a calf whose spleen was still intact. The parasitaemia on the day of collection ranged from 1 to 5 percent. The blood samples were directly transferred into RPMI 1640 medium containing heparin as an anticoagulant and transported while being chilled on ice packs to the Department of Microbiology, Faculty of Science, Mahidol University on the day of collection.

Preparation of drug. A 5% (w/v) sterile stock solution of quinuronium sulfate (Acaprin[a]) was diluted with distilled water to a final solution of $2x10^{-4}$ g/ml. The working solutions were then serially diluted by 2 fold with complete culture medium to obtain varying concentrations of drug ranging from 10^{-4} to 10^{-7} g/ml.

Culture medium. The culture medium was medium 199[b] with Hank's balanced salt (HBSS) which contained 25 mM of TES[c] buffer, 40% bovine serum (v/v), penicillin (100 I.U./ml) and streptomycin[d] (100 μg/ml). Because of the possibility of natural immunity of the Thai calves to *Babesia*, non-immune serum used in the preparation of complete culture media was obtained from the Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, Gainesville. Aliquotes of 50 ml were kept frozen in sterile tubes until use. The same lot of serum was used throughout experimental drug assays. Bovine erythrocytes used were from the donor calf which was maintained at the National Animal Health and Production Institute.

a. Bayer, Leverkusen
b. GIBCO, Grand Island, New York
c. Sigma, St. Louis, Missouri
d. Seromed, Biochrom KG, Berlin

Establishment of culture and maintenance. The method used in the initiation and maintenance of the continuous culture of *Babesia* was modified from that described by Vega et al (1985). Briefly, upon arrival in our laboratory, each sample was passed through a cellulose CF 11 column in order to remove leukocytes, and washed once with 40 ml of Vega y Martinez solution (VyM) by centrifugation at 1000 g for 10 min at 4^{O}C. After the supernatant and the top layer, which was about one-fifth of the packed cell volume, were discarded, the packed cells were gently resuspended with complete culture medium to make a 10% suspension. An aliquote of each 1-ml suspension was placed in a 24-well culture plate and incubated at 37^{O}C under an atmosphere of 5% CO_2 (v/v) and 95% humidified air. The medium was changed every other day, at which time approximately 5 µl of blood were taken from the cell bed for thin blood smear preparation. Percent parasitaemia of each culture was determined from Giemsa stained blood smears. Any culture showing 4-6% parasitaemia was subcultured with non-infected cells to reduce parasitaemia to 1%. The cultures that needed to be subcultured every 4 days were considered well adapted to in vitro conditions, and thus identified as continuous culture lines. These culture isolates were routinely maintained by a daily change of medium. Subculturing was performed whenever the parasitaemia of the culture reached 4-6%.

Drug sensitivity assay. The assay for responsiveness of *B. bovis* Thai isolates to quinuronium sulfate was carried out in the same manner as previously described (Brockelman and Tan-ariya 1991) except that stock *Babesia* cultures were not synchronized before use. Cultures which showed 4-5% parasitaemia, with most of the parasites appearing in paired pear-shaped stage, were selected for the experiment. These cultures were pooled and centrifuged at 500 g for 10 min at 4^{O}C. The packed cells were resuspended with complete culture medium to make a 50% suspension. The percent parasitaemia of the culture material was adjusted to approximately 0.5% by diluting with a 50% suspension of washed uninfected erythrocytes. The percent cell suspension of the culture material was subsequently reduced to 20% with complete medium and was stored at 4^{O}C until use.

For each experiment, 50 µl of complete culture medium with varying concentrations of drug were dispensed into wells of a 96-well microtiter plate. Four replicates were used for each treatment. As a control, complete culture medium without drug was also dispensed into four wells. An aliquote of 50 µl of prepared culture material was then added to each well. Therefore, the final cell suspension was 10%. To increase humidity over the plate, wells of rows 1 and 12 were filled with 150 µl of sterile distilled water. The microtiter plates were gently shaken by hand and were incubated in a humidified 5% (v/v) CO_2 incubator at 37^{O}C for 44 h without changing the medium.

Selection of drug resistant parasites. After drug assay, wells containing high drug concentrations (i.e., $2.5x10^{-6}$ and $5.0x10^{-6}$ g/ml) were aspirated and the remaining cell beds were individually transferred into wells on a 24 well culture plate. One hundred µl of 10% cell suspension of non-infected erythrocytes were added to each well. The culture plate was kept in a 5% CO_2-incubator. The medium was changed every- other day. The presence of parasites was determined from thin blood smears made on days 7 through 14. Any culture showing parasites was continuously maintained until its parasitaemia reached 4-5%. These treated culture lines were re-exposed to quinuronium sulfate at the previous corresponding dose.

The parasites surviving from the second treatment were re-assayed for their responsiveness to quinuronium sulfate after their parasitaemia had reached a level of 4-6%.

Evaluation.

Culture establishment. Growth and development of newly established culture isolates were observed from Giemsa-stained thin blood smears. Successful establishment of a culture was indicated by the appearance of developing schizonts and paired pear-shaped stages after one week. The ability of the parasite to adapt itself to continuous cultivation was expressed as percentage of parasitaemia determined by counting the number of infected cells per 5000 erythrocytes in thin blood smears.

Drug response. Drug responsiveness of the parasites was measured as percentage parasitaemia determined from Giemsa-stained thin blood smears after 48 h exposure to varying drug concentrations. In addition, abnormal looking and dead parasites were also noted. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of drug which reduced parasite population growth to one-half that of the control (without drug). IC_{50}s were calculated from linear regression analysis (linear data were achieved when percent inhibition was above 20% through 80-90%): $Y = a + bX$, where Y is the percent inhibition of parasite population growth (50%) and X is the concentration of drug. Percent inhibition was calculated as $100(a-b)/a$, where a = number of parasites in the medium without drug, and b = number of parasites in the medium containing drug. Drug sensitivities were compared among isolates by comparing their IC_{50}s.

RESULTS

Establishment of continuous cultures. In vitro continuous culture of *B. bovis* Thai strains was successfully achieved for the first time in this study. Well developed parasites were obtained from all initial cultures after 2-7 days of cultivation depending on the starting percent parasitaemia of the infected blood samples. The parasites from fresh cultures appeared as ring, schizont and pair pear-shaped stages after 48-72 hours of culture. The parasites multiplied at a very low rate at the beginning of cultivation, but much more rapidly after 2 weeks of adaptation to culture conditions. Approximately 4-5-fold increases in percent parasitaemia were observed in most of the cultures within 48 hours (Table 1).

We noticed that growth of the parasites varied with percent of starting parasitaemia as a result of subculturing. Good growth of the parasites is necessary for evaluation of drug sensitivity; therefore, four culture lines of TS-4 with different starting parasitaemia were set up and observed for 48 h. Results (Table 2) clearly show that growth of the cultures started with parasitaemias of 1%, 2% and 4% were not as good as that started with 0.5% as the rate of increase was inversely related to starting parasitaemia.

In another experiment, the growth of isolate TS-4 during the first 48 h on the 24-well culture plate and 96-well microtiter plate were compared. Results revealed no significant differences between growth rates observed on these two different types of tissue culture plate if their starting parasitaemias were adjusted within the range of 0.4-0.6%. Thus, we decided that the in vitro microtest for drug sensitivity of *Babesia* in this study should be carried out with a starting parasitaemia of 0.4-0.6%.

Table 1 Growth of *Babesia bovis* Thai isolates during 48 hours of observation after adaptation to continuous culture. Numbers represent mean number of parasitized cells/5000 red blood cells (numbers in brackets represent ranges of four replicates).

Time (hours)	Isolates			
	TS-2	TS-4	NA	KP
0	25 (23-37)	28 (26-29)	29 (27-32)	24 (22-25)
48	101 (98-107)	141 (138-146)	81 (76-84)	120 (115-127)
fold-increase	4	5	3.5	5

Table 2 Growth of Thai isolate TS-4 with different starting parasitaemias during 48 hours of observation. Each number represents mean of 4 replicates. Numbers in brackets represent percent parasitemias.

Time (hours)	Number of parasitized cells per 5000 red blood cells			
0	200 (4%)	100 (2%)	50 (1%)	25 (0.5%)
48	275 (5.5%)	202 (4.04%)	142 (2.84%)	130 (2.6%)
fold-increase	0.8	2	2.8	5

Sensitivity of B. bovis to quinuronium sulfate. The responses of the four isolates to quinuronium sulfate at varying concentrations are summarized in Figure 1. There was only a slight inhibitory effect on parasite growth at a drug concentration of 3.12×10^{-7} g/ml. At 6.25×10^{-7} g/ml, pronounced inhibition of parasite growth was observed in all isolates. Over 50% inhibition occurred in every isolate at a drug concentration of 2.5×10^{-6} g/ml, and 92-99% inhibition occurred at 5.0×10^{-6} g/ml. The IC_{50}s of all four isolates are summarized in Table 3. Our Thai isolates *B. bovis* are similarly responsive to quinuronium sulfate, having the same range of IC_{50} (i.e., $1.72\text{-}2.43 \times 10^{-6}$ g/ml).

Emergence of drug resistant B. bovis in continuous culture. In all four isolates, only a few parasites survived the drug treatment at a concentration of 5.0×10^{-6} g/ml. The morphology of the parasites appearing on the thin films was very abnormal. Parasites in schizont stages were enlarged, vacuolated and poorly stained, and their paired pear-shaped stages were recognized only as small dense dots without

cytoplasm. Interestingly, a very small number of healthy parasitized cells could still be detected from culture TS-2 which had been exposed to $2.5x10^{-6}$ g/ml of the drug on day 10 post treatment. These few

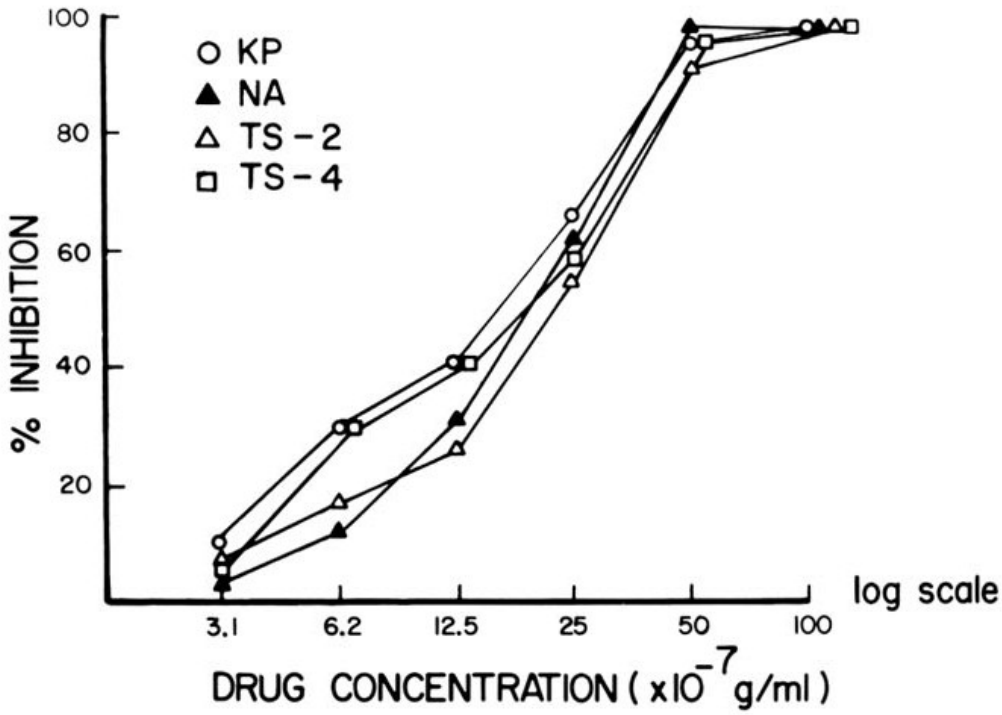

Figure 1. Response of *Babesia bovis* Thai isolates to quinuronium sulfate: KP (o), NA (▲), TS-2 (Δ) and TS-4 (□). Each point represents the mean percent inhibition at the concentration indicated (Ranges are not shown as they are less than 3%).

Table 3 The calculation of linear regression equations and calculated IC_{50}s value for quinuronium sulfate on *B. bovis* growth in vitro.

B. bovis Culture line	Equations	Inhibitory concentrations (g/ml) (IC_{50}s)
KP	Y = 13.3+2.1X	$1.72x10^{-6}$
NA	Y = 1.19+2.0X	$2.35x10^{-6}$
TS-2	Y = 4.67+1.8X	$2.43x10^{-6}$
TS-4	Y = 11.9+1.7X	$2.14x10^{-6}$
TS-2R	Y = 17.5+18.9X	$1.7x10^{-5}$

parasites had servived and recovered, and was propagated as a new isolate, redesignated as TS-2R. This line was maintained for an additional 3 weeks before it was re-exposed to the drug at a concentration of $2.5x10^{-6}$ g/ml. Surprisingly, this drug level did not exert any inhibitory effect on its growth. In a follow-up study, its response to quinuronium sulfate was evaluated in comparison with that of the original isolate. TS-2R was clearly more resistant to quinuronium sulfate than parent culture TS-2 (Figure 2). To achieve 50% inhibition of growth of TS-2R, it required a drug concentration of $1.7x10^{-5}$ g/ml, 7 times higher than required by the original TS-2 (Table 3). Only a few parasites were seen at a drug concentration of $5.0x10^{-5}$ g/ml.

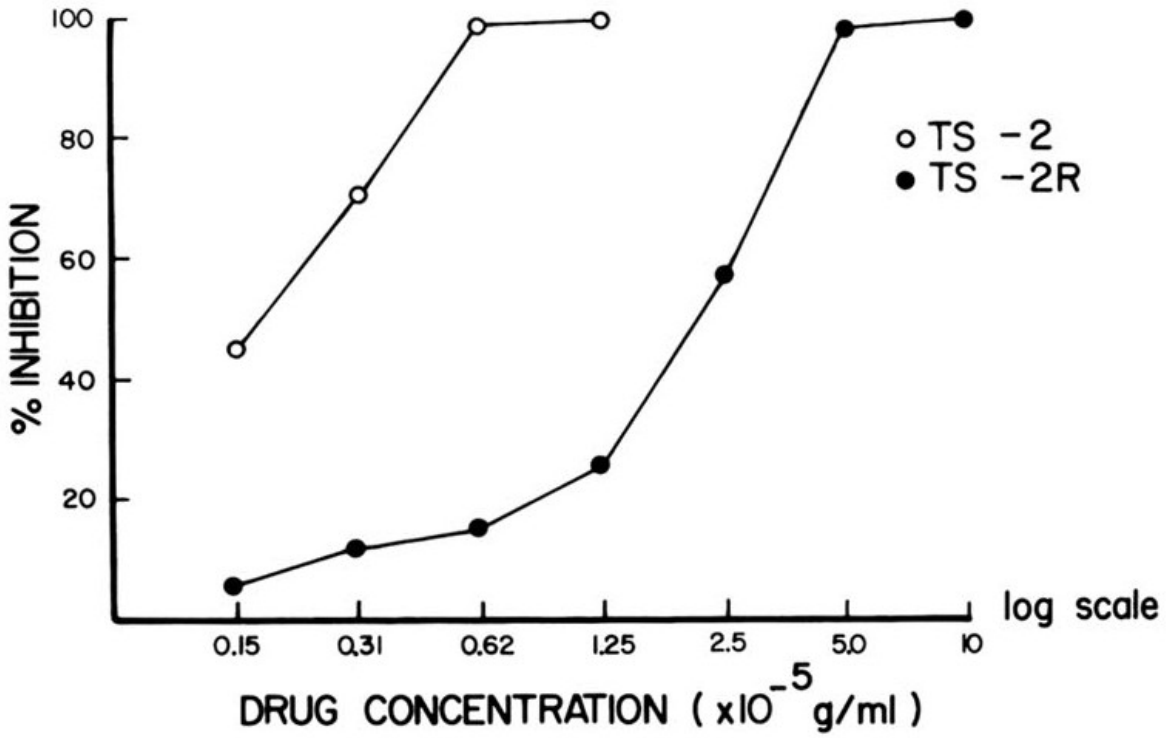

Figure 2. Responses of isolate TS-2 and line TS-2R of *Babesia bovis* to quinuronium sulfate. Each point represents mean percent inhibition at the concentration indicated (Ranges of are not shown as they are less than 3%).

DISCUSSION

During our preliminary study, an attempt was made to establish continuous cultures of Thai isolates *B. bovis* using the method of Vega et al (1985) in which the blood samples were washed three times and white blood cells were removed after each centrifugation. However, it was found that some white blood cells were still present resulting in later disintegration of the cells and loss of cultures after 1 to 2 weeks. Thus, we removed the white blood cells by passing the blood samples through the CF11 column and the washing was carried out only once. This procedure seemed to be suitable for an initiation and establishment of continuous cultures derived from samples which had been transported for approximately 24 hours.

Our study shows that growth of *Babesia* parasites is influenced by the initial percent parasitaemia (Table 2). The IC_{50} evaluated from cultures with initial parasitaemia exceeding 1% varied considerably from experiment to experiment. Experiments started with 0.4-0.6% parasitaemia resulted in better reproducibility of IC_{50} values (our unpublished data). The in vitro microtest assay of drug sensitivity of *Babesia* sp. should therefore be carried out using a low starting parasitaemia.

Our results from drug sensitivity tests of Thai isolates collected from different geographical areas of the country revealed a similarity of response to quinuronium sulfate, with IC_{50} values ranging from 1.72 to $2.43x10^{-6}$ g/ml. Previously we reported that the IC_{50} of a Mexican strain for quinuronium sulfate was $4x10^{-7}$ g/ml. It seems likely that the Thai strains of *B. bovis* were less sensitive to this drug than the Mexican strain. However, it would be premature to draw a definite conclusion since there are other factors which should be considered carefully. Firstly, Thai *Babesia* used in this study were fresh isolates whereas the Mexican *Babesia* used in the previous study had been cultured for four years. Culture conditions might have some effect on the drug response patterns of *Babesia* sp. Both genotypic and phenotypic changes (including drug sensitivity) have been found to occur in malarial parasites in long-term cultures (Brockelman et al 1988). Secondly, Thai *Babesia* used in this study had not been cloned. Isolates of *Babesia* may consist of mixed populations with varying drug sensitivities, as has been reported for malarial parasites (Thaithong et al 1984). Thai *Babesia* should therefore be cloned before the drug responsiveness of the isolates is compared. The emergence of resistant *Babesia*, strain TS-2R, from the treated culture was probably a result of drug selection pressure. This phenomenon was previously observed in *Plasmodium falciparum* in which a pyrimethamine resistant strain could be selected from certain uncloned culture, but not from the cloned culture, after a single exposure to a high dose of the drug (Tan-ariya et al 1984).

As the reason for failure of quinuronium sulfate treatment against bovine babesiosis in Thailand is not yet understood, we cannot predict when *B. bovis* resistance to other drugs available in the market will emerge. It is urgent to study this matter further.

Responses of *Babesia* sp. to common babesiacidal drugs need to be evaluated, as well as drug sensitivities of cloned parasites. Cross resistance to commonly used drugs among resistant parasites should also be studied. All of these problems can be investigated in vitro.

Needless to say, an in vitro assessment of *B. bovis* resistance to drug can only be extrapolated to in vivo situation in the field with caution. However, the in vitro system provides a practical and available mean for monitoring shift of parasitic resistance to drugs and can render itself, with further refinement, for controlled studies of this and other biological changes of the parasites.

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