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A short-term *in vitro* cultivation of Thai isolate *Theileria* sp.

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ABSTRACT The short-term *in vitro* cultivation of intraerythrocytic stages of Thai isolates of the *Theileria* parasite was successfully established in this study. Of the four types of culture media used in the experiment, M199 supplemented with 40% fetal bovine serum (FBS) proved to be the best medium. The mean percentage of living parasites cultured in this medium was significantly higher than that in the others, ie, M199+fetal calf serum (FCS), RPMI+FBS and RPMI+FCS ($p<0.05$). In addition, many developmental stages, ie, match form, ring form, and 2-piroplasm, 3-piroplasm and 4-piroplasm forms, were observed at significant numbers in M199+FBS medium after 48-72 h of cultivation. It was also demonstrated in this study that the blood samples with a high percentage of parasitemia, ie, >4.5%, should not be cultured at cell suspension concentrations higher than 12.5%, whereas those with low percentage of parasitemia, ie, <4.5%, could be cultured at any percentage of cell suspension. On the other hand, our results revealed that culturing at approximately 12.5% cell suspension provided the highest percentage of both multiplied form and living parasites. Production of an optimal cultivation system that would lead to the successful continuous *in vitro* cultivation of intraerythrocytic stages of *Theileria* parasite remains to be investigated.

KEYWORDS: benign *Theileria*, Thai isolate, *in vitro* cultivation.

INTRODUCTION

Bovine theileriosis is an intraerythrocytic protozoan parasite transmitted to grazing cattle by ticks. One group of these parasites is benign *Theileria*, which includes many species of veterinary importance. Benign *Theileria* parasites include *T. sergenti, T. buffeli* and *T. orientalis,* which are distributed in Japan, Australia and elsewhere. The parasite causes a disease characterized by mild anemia and hyperthermia, but severity and fatality are not rare under conditions in the field. Frequently, anemia due to the presence of intraerythrocytic piroplasm can induce abortion and mortality in the infected cattle. Though the first report of *Theileria* parasite in Thai native cattle from the Southern part of Thailand was recorded in 1971[1], the *Theileria* species and their vectors in Thailand have not been identified. Moreover, the life cycle of the Thai *Theileria* parasite needs to be studied before any conclusions could be made. Nevertheless, it has been proposed that benign *Theileria* groups are abundant throughout the country.[2] Recent studies using the major piroplasm surface protein (MPSP) and small subunit ribosomal RNA (ssrRNA) gene indicated that Thai isolates of *Theileria* are more closely related to the *T. sergenti/buffeli/orientalis* group parasites than to malignant species of *Theileria.*[2-4] Since the pathogenesis of the benign *Theileria* species is found to be related to the proliferation of the parasites in the host's red blood cells, chemotherapy of the benign theileriosis has mainly been focused on destroying the intraerythrocytic stages. Although some drugs are currently available for the treatment of bovine theileriosis, they are not necessarily satisfactory because of their adverse effects and the emergence of parasite with increased drug resistance. Thus, there is a great demand for developing better anti-theilerial drugs. Under the current situation, however, drug development is very difficult because of the lack of a suitable *in vitro* system to screen effica-

cious compounds. This is also true for the situation of theileriosis in Thailand. Though the *in vitro* culture system for *T. sergenti*, a benign *Theileria*, has been recently developed[5], no such system has been achieved for Thai isolates. Chemotherapy and prophylaxis is one means of controlling method of this disease; however, there is no data about the sensitivity of Thai *Theileria* sp. to any common anti-theilerials used in the country. Due to a significant impact of benign theileriosis to livestock productivity in Thailand, an *in vitro* culture of Thai isolates of *Theileria* sp. was established in this study. Successful *in vitro* cultivation not only makes the study of the drug sensitivity of Thai *Theileria* possible, but may also contribute to the investigation of its life cycle, determination of the species of its tick vector, and screening for new potent anti-theilerial compounds.

Materials and Methods

Infected blood and non-infected blood samples

Two naturally *Theileria* sp. infected calves, aged between 4 and 7 months, from Rattanapum District, Songkla Province, in the South of Thailand were used as sources of infected blood samples in this study. The percentage of parasitemia was found to be 0.01 and 0.05%. To achieve higher parasitemia, they were splenectomized at National Institute of Animal Health, Department of Livestock Development. After 5 days of splenectomy, daily parasitemia was monitored by making thin blood smears from jugular's vein, fixing in absolute methanol, staining with Field 's stain and examining microscopically under a 100x objective lens. Once parasitemia had reached 4-18%, the infected blood was collected for the study. Non-infected blood used for adjusting the percentage of parasitemia in one experiment was collected from a healthy non-splenectomized calf without any *Theileria* infection experience.

Culture media

Two types of culture media were used in this study. The first one was RPMI1640 (GIBCO, Life Technologies, Grand Island, NY) supplemented with HEPES (25 mM), L-glutamine (0.3 g/L), sodium bicarbonate (1.5 g/L), reduced glutathione (1 mg/L), gentamicin sulfate (50 mg/L), and hypoxanthine (2 μM). The second was Medium M199 with Hanks' salts, 25mM HEPES buffer and L-glutamine (GIBCO). RPMI was purchased, as powder while M199 was in a liquid formula. Before being used for culture, both media were supplemented with 40%(v/v) of either heat-inactivated (HI) fetal calf serum (FCS) or HI-fetal bovine serum (FBS). These working media were referred to as RPMI+FCS, RPMI+FBS, M199+FCS and M199+FBS in this study.

Experiment 1: Establishment of cultures

The method used for cultivation of *Theileria* Thai isolated in this study was based on those used for *Babesia* sp. with slight modification.[6-7] Briefly, 50 ml of blood sample showing approximately 4% parasitemia was aseptically collected from jugular venipuncture of infected calf (calf no. 1, on day 7 post-splenectomy) into a sterile flask and defibrinated by shaking with glass beads. White blood cells were removed from the blood sample by passing through a Whatman CF11 cellulose column. Then, the blood samples were washed once with 1x Vym's solution by centrifugation at 1,000 g for 10 min at 15°C. The packed red cells were resuspended to make a 50% (v/v) cell suspension in 4 different types of working media as mentioned above. The percentage of cell suspension of each blood sample was then diluted from 50% to 10% with each type of corresponding working medium. Each parasite inoculum sample was finally dispensed in 1 ml aliquots into each of duplicate wells in the 8 inner wells of 24 well-culture plates (Nunc). Sterile water was added to the outer wells to provide humidity. The culture plates were placed at 37°C in 5% CO_2 in a humidified incubator for 72 h. The culture medium overlaying the settled layer of erythrocytes was replaced daily with the corresponding working medium. Duplicate thin blood smears were made from each well after removing the medium. The number of infected cells with various forms of parasites was counted against 5,000 erythrocytes observed. The mean numbers calculated from four slides made from duplicate wells of each test were expressed as a percentage. The numbers of erythrocytes containing living and dead parasites were also differentially counted, expressed as percentage and statistically analyzed by the Firedman test. The dead or abnormal forms of parasites were characterized by the presence of pyknotic nuclei and little or no cytoplasm.

Experiment 2: Determination of the optimal percentage of initial parasitemia and cell suspension

This experiment was performed in order to find the appropriate percentages of initial parasitemia and cell suspension for cultivation of *Theileria* parasites. A blood sample harboring approximately 18% parasitemia collected from one infected calf (calf no. 2, on day 14 post splenectomy) was prepared and

adjusted to 50% cell suspension in M199+FBS using the same procedure as described in the Experiment 1. The 50% cell suspension of 18% parasitemia blood sample was 2-fold diluted with 50% non- infected cell suspension to obtain varying percentages of parasitemia ie, 18%, 9%, 4.5% and 2.2% respectively. Blood samples with varying parasitmia were subsequently adjusted to 2-fold varying percentage of cell suspensions ie, 50%, 25%. 12.5%, 6.3% and 3.2%, in M199+FBS medium. The parasite inoculum was cultured in 24 well-culture plates and incubated at 37° C for 24 h in 5% CO_2 in a humidified incubator. The numbers of infected cells containing living and dead parasites, single and multiplied forms were counted against 5,000 erythrocytes and expressed as percentages. The results were statistically analysed by using the independent sample-T-test.

Results

Before culturing (h 0) most parasites appeared predominantly in the match form (Fig 1A) and with only a few in 2-piroplasm form (Fig 1B) as shown in Fig 2. The distribution of the developmental stages of the parasites during 72 h of cultivation in 4 types of culture media is expressed as histograms of percentage parasitemia in Fig 2. Differentiation of match form into ring form (Fig 1C) and 2-piroplasm form was observed in all types of media

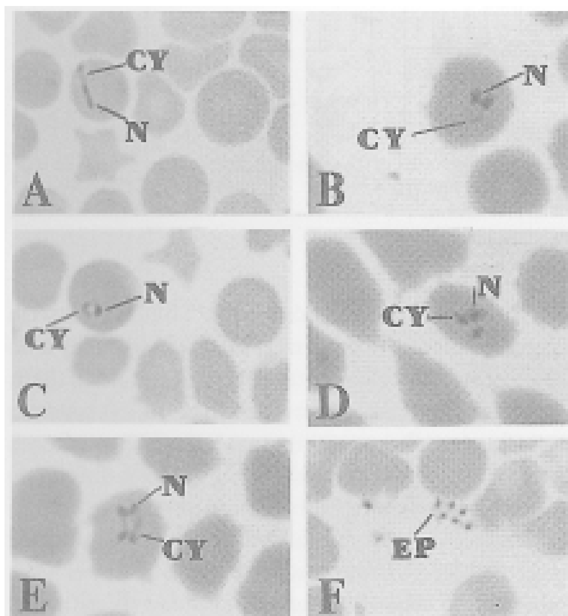

Fig 1. Intraerythrocytic forms (piroplasms) of Thai isolate *Theileria* sp. from the culture. Match form (A); 2-piroplasm form (B); Ring (C); 3-piroplasm form (D); 4-piroplasm form (E); extracellular piroplasm (F) (N = nucleus; CY = cytoplasm; EP = extracellular piroplasm)

after 24 h of culture. However, 3-piroplasm form (Fig 1D) and 4-piroplasm form (quadruplet or tetrad form) (Fig 1E) were found only in M199 medium (Fig 2A, 2B). The result clearly demonstrated that development of parasites in M199+FCS (Fig 2A) was not as good as that in M199+FBS (Fig 2B). The continual change from single to multiplied forms (2-, 3- and 4-piroplasms) was obviously seen in M199+FBS throughout 72 h of cultivation (Fig 2B). Though the percentage of parasitemia in M199+FBS did not increase during 72 h of observation, all forms of parasites were apparently healthy and no dead parasites were observed (Fig 3B), unlike in the other media (Fig 3A, 3C, 3D). However, it is interesting to note that a significant number of extracellular parasites was found with this type of medium after 48 h; most of which were aggregated, forming clusters of 4 parasites or more (Fig 1F). The development of parasites in RPMI (Fig 2C, 2D) appeared to progress to a much lesser extent than in M199 (Fig 2A, 2B). Though the parasite could develop from a match form to 2-piroplasm form after 48 h, no 3- and 4-piroplasm forms were found. A rapid decrease in parasitemia was recognized after only 24 h and no living parasites appeared in the culture after 48-72 h of cultivation. The percentages of dead parasites found in RPMI +FCS and RPMI+FBS media (Fig 3C, 3D) were significantly higher than those observed in M199+FCS and M199+FBS (Fig 3A, 3B) ($p<0.05$). Compared with M199 medium, RPMI was shown to be less suitable for culturing the Thai isolates of *Theileria* parasite by considering the percentage of both multiplied forms and living parasites. Our study provided the evidence that, among the 4 culture media used in this study, M199+FBS was the most appropriate medium for the short-term *in vitro* culture of the Thai *Theileria* parasite isolates.

The results of Experiment 2 conducted with M199+FBS medium using varying percentages of initial parasitemia and cell suspension are summarized in Fig 4 and 5. Fig 4 shows the percentage of single and multiplied parasites after 24 h of cultivation in M199+FBS, while Fig 5 shows the percentage of living and dead parasites observed during the same period. The results revealed that regardless of percentage of parasitemia, the highest percentage of living parasites and multiplied forms were achieved at 12.5% cell suspension ($p<0.05$). The results also demonstrated that parasite inoculum with low percentages of initial parasitemia could be cultured at a wide range of cell suspension concentrations (Fig 4A, Fig 5A). The percentage of living parasites and multiplied forms were found to be more or less

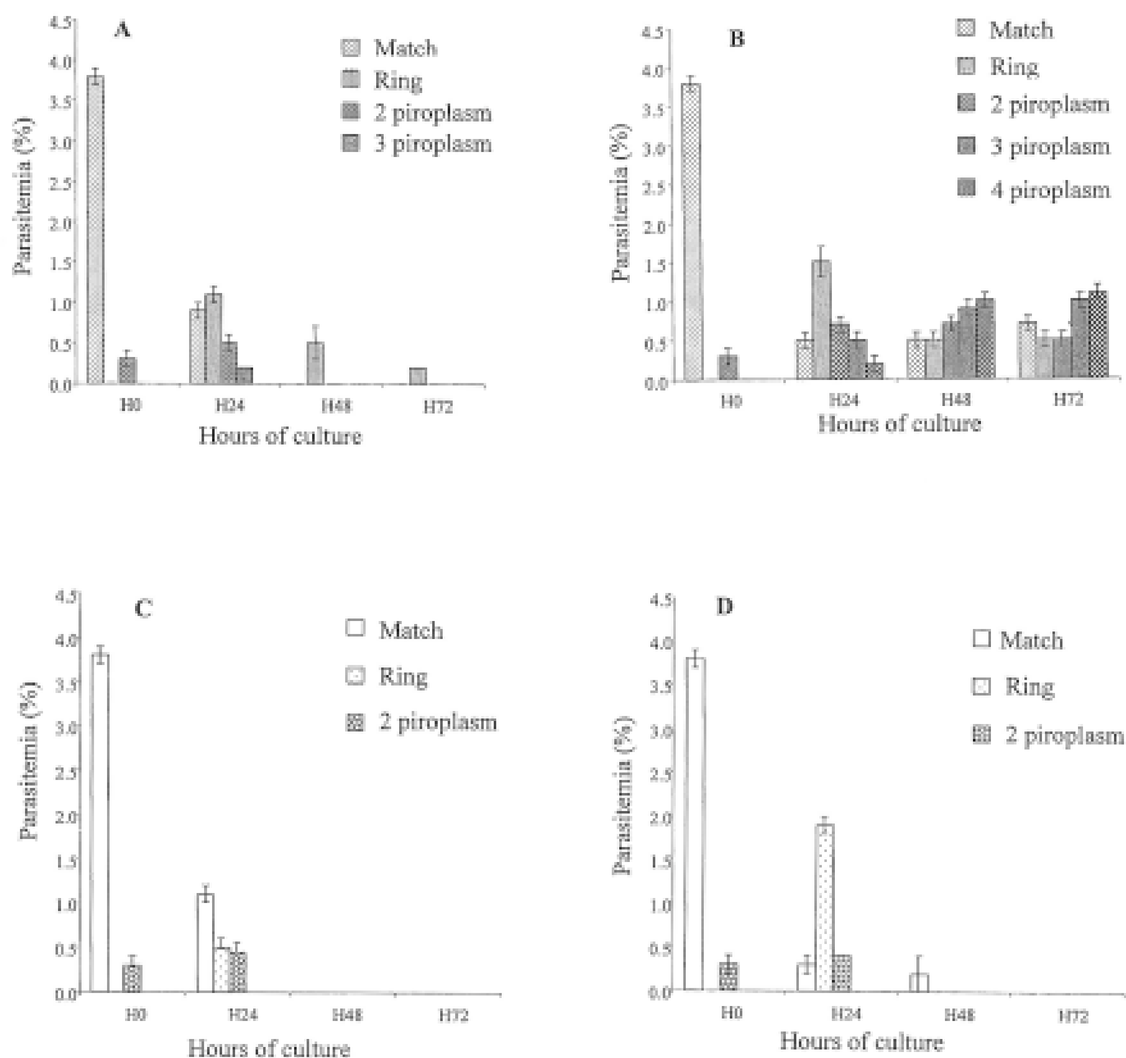

Fig 2. Histograms of the mean percentages of parasitized cells containing various forms of Thai *Theileria* sp. isolates during 72 h of cultivation in M199+FCS (A), M199+FBS (B), RPMI+FCS (C) and RPMI+FBS (D).

within the same range by using 2.2% parasitemia and 3.2% to 50% cell suspension of the parasite inoculum. However, the parasite inoculum having higher percentage of parasitemia eg >4.5% (Fig 4B, 4C, 4D and Fig 5B, 5C, 5D) should be cultured at 12.5% cell suspension or lower in order to obtain a good development of the parasites. This observation might be attributed to competition for nutrients and toxicity of the accumulated waste products in those cultures with high parasitemia. Experiments 1 and 2 were repeatedly carried out with an alternate Thai isolate of *Theileria* parasite from calf no. 1 and no. 2 and similar results were obtained (unpublished data).

Discussion

Though the species of the Thai isolates of *Theileria* parasite has not been elucidated, the establishment of a short-term *in vitro* culture of their intraerythrocytic stages was obtained from this study. By culturing the parasites in M199+FBS medium, they could undergo development from single to multiplied forms including 4-piroplasm form after 24 h of culture. A remarkable increase in the number of multiplied forms was seen after 48-72 h of cultivation. This finding correlated well with the previous studies on the virulent species ie, *T. annulata, T. parva*.[8-9] The development of *T. parva* in M199+FBS from single to multiplied forms was observed on day 1 through day 8 and the percentage

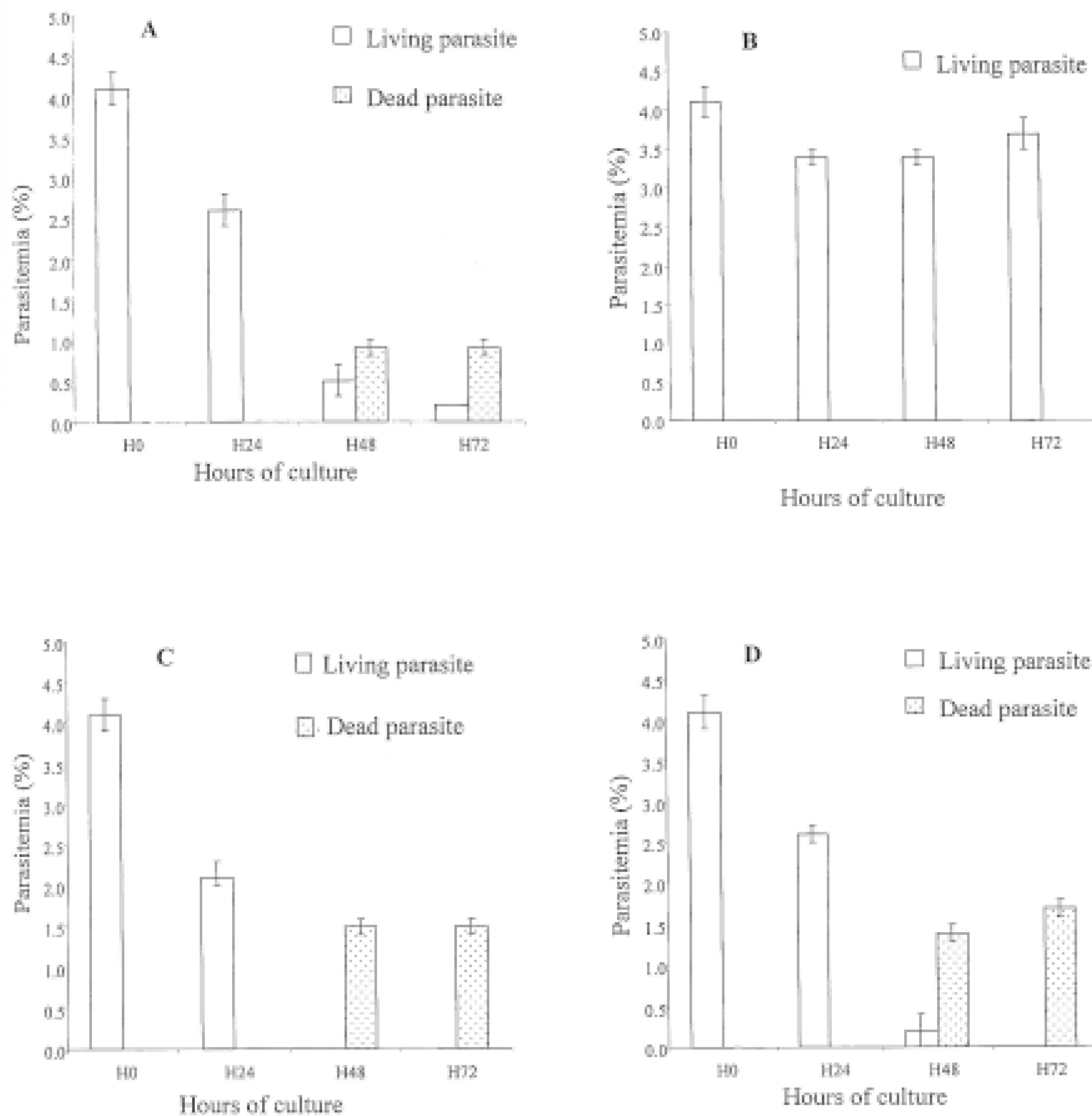

Fig 3. Histograms of the mean percentages of parasitized cells with living and dead parasites during 72 h of cultivation in M199+FCS (A), M199+FBS (B), RPMI+FCS (C) and RPMI+FBS (D).

of 4-piroplasm or quadruplet form reached 10-20% on day 8.[9] Though our cultures were not kept longer than 72 h, we have found that the Thai *Theileria* parasite isolate could survive in the culture up to day 6 (unpublished data). At present, an *in vitro* cultivation system is still not available for the intraerythrocytic stages of the *Theileria* parasite that provides increasing parasitemia. In most systems that have been described, the parasites could develop to the dividing stages but no increase in parasitemia was seen after cultivation. The result observed with RPMI medium was found to be similar to the previous study of *T. sergenti*, a benign *Theileria* (Japanese strain).[5] *T. sergenti* cultured in RPMI+FBS was shown to possess active metabolism during the first 18 h of cultivation, demonstrated by its ability to incorporate [^{3}H] hypoxanthine. This allowed the drug sensitivity of *T. sergenti* to anti-hemosporozoal agents to be evaluated. However, the developmental stages of the parasites were not examined. Our results clearly showed that the Thai *Theileria* isolate underwent development from single form to 2-piroplasm form after 24 h. No more advanced stages beyond this were found with RPMI medium during 72 h of observation. A rapid decrease of living parasites was recognized in both RPMI+FCS and RPMI+FBS media after 24 h and none were observed after 48 h. It was presumably that *T. sergenti* in the previous study

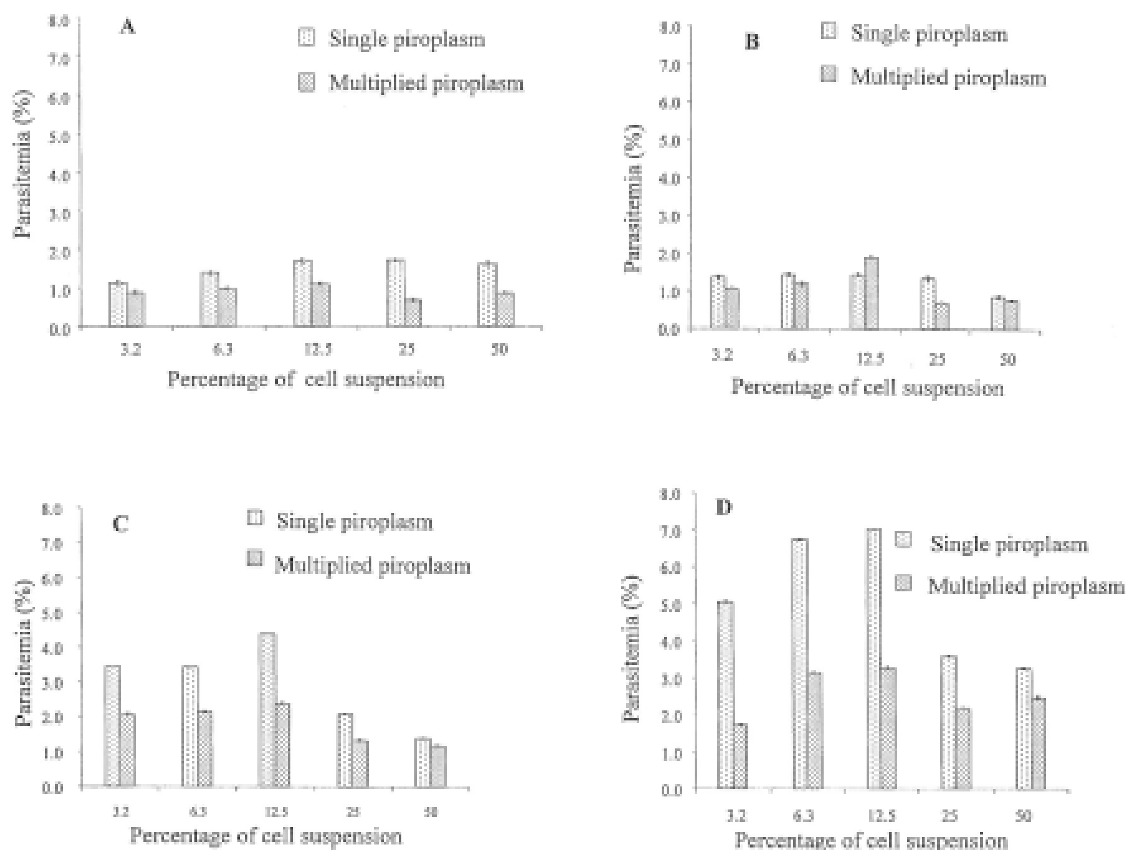

Fig 4. Histograms of the mean percentages of parasitized cells with single and multiplied piroplasma forms of Thai isolates of *Theileria* sp. after 24 h of cultivation with 2.2% (A), 4.5% (B), 9% (C) and 18% (D) parasitemia at varying percentages of cell suspensions.

could develop from single form to only 2-piroplasm form as observed in our study. The present study has provided enough evidence to conclude that M199+FBS is the most appropriate medium tested for cultivating the Thai *Theileria* parasite isolate. The present study also demonstrated that FBS was superior to FCS in supporting growth and development of the Thai isolates of *Theileria* parasite, which confirmed the previous report. FBS is presently one of the most universally applicable cell culture additives for stimulation of cellular proliferation and biological production.[10] The failure of FCS to support growth of the parasite may be due to toxicity caused by high protein, high phospholipid and total cholesterol contents as previously described.[11]

Though we did not examine the ultrastructure of the parasites in this study, it is speculated that the 4-piroplasm form predominantly found after 48-72 h was the schizogonic stage of the parasite as previously shown by electron microscopy by other investigators.[8-9, 12] In addition, the 2-piroplasm form should be the parasites in the process of binary fission[12] according to their Y-shaped-morphological appearance. The 3-piroplasm form frequently found in the culture was presumably the immature schizont which would eventually reach the maturity with 4 merozoites later on. The presence of dividing forms, both 2- and 4-piroplasm in our culture system strongly suggested that the Thai *Theileria* isolate parasites could undergo their development in the host erythrocytes, which means that they could be kept metabolically active for at least 72 h in culture. The success of short-term in vitro cultivation of the Thai *Theileria* sp. isolates and the defined appropriate percentage of initial parasitemia and cell suspension for cultivation were reported for the first time in this study. Therefore, screening of drug sensitivity, studying of drug action and drug resistance of the Thai *Theileria* sp. now appeared to be feasible. Investigation of the life cycle and species of tick vector of the Thai *Theileria* isolate parasite is also assisted by use of the intraerythrocytic forms of parasite obtained from the culture. Observation of extracellular parasites in the culture likely indicated that schizogony could be completed, and the merozoites were capable of leaving the host cells.

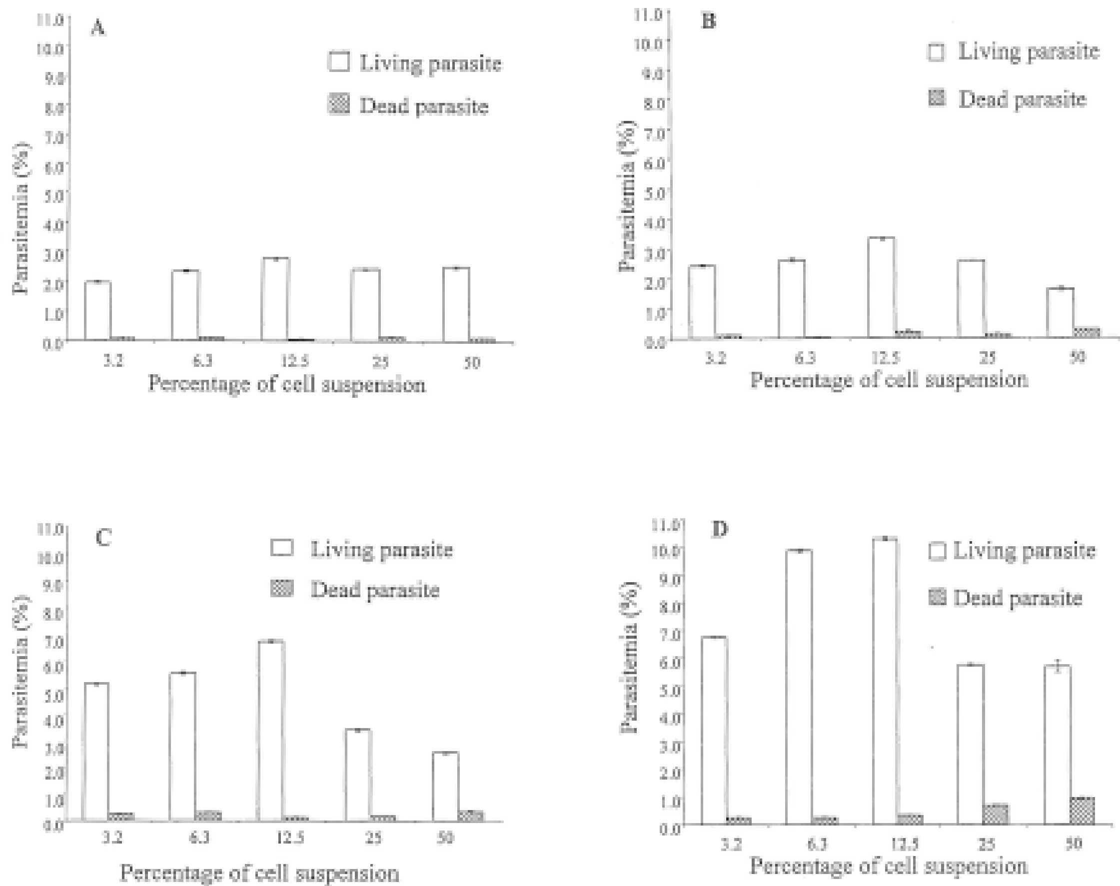

Fig 5. Histograms of mean percentages of parasitized cells with living and dead parasites of Thai isolates of *Theileria* sp. after 24 h of cultivation with 2.2% (A), 4.5% (B), 9% (C) and 18% (D) parasitemia at varying percentages of cell suspensions

Since it has been suggested that only merozoites can penetrate the red cells, further study on the optimal cultivation system that could sustain the viability of the extracellular form (merozoite) should be conducted to ensure the successful reinvasion of merozoites, which is necessary for the continuous cultivation of the *Theileria* parasite.

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