ウシ及びウマ・バベシア原虫 in vitro 長期培養法の確立とその応用に関する研究

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研究成果報告書

はしがき

赤血球内寄生原虫であるバベシアは牛、馬などに感染し世界的に甚大な被害を与えている。日本では *Babesia ovata* による牛バベシア症が認められており、牧野衛生上の大きな問題となっている。また、馬ピロプラズマ病はバベシア原虫*B. equi* 及び *B. caballi*の感染によって引き起こされ、家畜法定伝染病に指定されている。幸いに、現在のところ日本には存在しないと考えられているが、近年わが国において海外からの競走馬、乗馬および肉用馬の輸入が急増し、日本への侵入の可能性は否定できない現状にある。さらに、本伝染病はダニによって伝播され、日本国内においてもこれらの病原体を媒介する種類のダニが見つかっていることから、仮に侵入すると流行する危険性が十分にあり、ウマバベシア原虫の日本への侵入は絶対に阻止しなければならず、輸入管理防圧体制の強化が益々重要視されている。しかしながら、これらの原虫の培養法が未だ確立されていないため、研究材料の確保が難しく、特異性や感度の高い診断法、および抗バベシア剤やワクチンの開発についての検討が遅れている。

本研究では、ウシ及びウマのバベシア原虫 の in vitro 長期連続培養法について検討を行い、ウシバベシア原虫*Babesia ovata* 、ウマバベシア原虫*B. equi* 及び *B. caballi*の連続培養法を確立した。また、これら培養で得られた原虫を抗原として用いた間接蛍光抗体法による血清中の抗体調査を行ったところ、モンゴルでは８０％以上の馬が感染している事が明らかとなり、日本のウマ血清においても抗体の存在が疑われる例が認められた。また、特異的抗原の精製を目的として*B. equi* 及び *B. caballi* に対するモノクローナル抗体を作製した。今後の、これらの抗体の認識する抗原を用いた新しい診断法の開発が必要である。

研究組織

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研究成果の概要

１。ウシバベシア原虫 *Babesia ovata* の *in vitro* 連続培養法の検討

凍結保存されたウシバベシア原虫*B. ovata* 感染赤血球を予めウシ赤血球を移入したSCIDマウスに接種して増殖させ、in vitro 長期培養に用いた。その結果、１９９培養液（４０％ウシ血清添加、pH 7.0）、赤血球容積が１０％、低酸素の気相（５％酸素、５％炭酸ガス、９０％窒素）の培養条件下で*B. ovata* の増殖が認められた。２代以後は３－４日間の継代培養間隔で赤血球感染率が６－１０％に達し長期連続培養が可能となった。また、数代継代後は５％ CO2のガス条件下でも培養可能であることが判明した。更に、培養した原虫を凍害防止剤（ポリビニールピロリドン）を加えたVega y Martinez リン酸緩衝液からなる保存液で凍結後、液体窒素で保存し、再び培養した結果でも原虫の増殖が認められた。また、培養に用いられる赤血球の保存は従来他のウシバベシア原虫では１週間以内とされていたが、本研究では２ヶ月間保存可能であることが明かになった。

２。ウマバベシア原虫のin vitro培養法の検討

実験的に*B. equi* 感染させたウマより感染赤血球を準備し、培養法につき検討した。その結果、１９９培養液（ハイポキサンチン、４０％ウマ血清添加、pH 7.0）、赤血球容積が５～１０％、低酸素のガス（２％酸素５％炭酸ガス９３％窒素）の培養条件下で*B. equi* の増殖が認められた。２代以後は５－７日間の継代培養間隔で赤血球感染率が２－３％に達し連続培養が可能となった。また同様に、*B. caballi* ではRPMI1640培養液（L-glutamin、４０％ウマ血清添加、pH 7.0）、赤血球容積が５～１０％、低酸素のガス（２％酸素５％炭酸ガス９３％窒素）の培養条件下で*B. caballi* の増殖が認められた。２代以後は５－７日間の継代培養間隔で赤血球感染率が２－３％に達し連続培養が可能となった。培養に適合した*B. equi* と*B. caballi* は低酸素ガス濃度から５％炭酸ガス９５％空気の気相に移して培養する事により、それぞれ１５～２０％及び８～１０％の血球感染率を得ることが可能になった。また、*Babesia equi*の培養ではアルブミン、α2-マクログログロブリン、β-リポプロテインを添加することにより血清濃度を４０％から２５％に減少させることが可能となった。

３。間接蛍光抗体法による血清抗体調査

１）間接蛍光抗体法によるウマの*Babesia equi* 原虫

現在まで日本においてはウマ*Babesia equi* 原虫によるヒトおよびウマの感染報告はない。しかし、肉用ウマなどの輸入が盛んな現在、人畜共通感染原虫としての*B.equi*に対

する基礎研究の第一歩として南アフリOnderstepoort獣医学研究所の標準株を用いて我が国のウマ血清を調査した。用いた血清は、１９９２年ー１９９３年に北海道および九州地域で採取した４９４例（内国産馬４３９例、外国産馬５５例）である。間接蛍光抗体法によりOnderstepoort 研究所の判定基準（ウマ血清希釈８０倍を陽性）に準じて検索した。その結果は陰性４７５例、偽陽性１６例、陽性に近い偽陽性３例の成績を得た。そこで、オーストラリア*B.equi* 株およびドイツBerlin大学株を用いてOnderstepoort株による偽陽性血清の抗体価を比較した。異なる３か国由来の*B. equi*株に対する血清抗体価は必ずしも同様ではなく、３株すべてに偽陽性を示した例は認められなかった。

２）間接蛍光抗体法によるモンゴルウマ血清中のバベシア抗体調査

培養で得られた*Babesia equi*及び*B.caballi* を抗原とした間接蛍光抗体法について検討した。赤血球感染率３～４%が原虫抗原として適当であり、８０倍以上を陽性とした。日本のウマ血清１５例は陰性であった。また、モンゴルのウマ血清１１０例について抗体調査を行ったところ、８１.８％が両原虫に対する抗体を保有していることが判明した。

４。*Babesia equi* に対するモノクローナル抗体の作製

３で述べたように、培養で得られた虫体を抗原として用いた間接蛍光抗体法は血清診断に使用可能であるが、培養虫体より調整された粗抗原を用いたELISAでは、赤血球成分の混入によると考えられる非特異的反応が多く実用的に用いることはできなっかた。そこで、赤血球成分の混入のない抗原の精製を目的として*Babesia equi*に対するモノクロナール抗体(mAb)の作製を試みた。その結果、虫体とのみ反応するハイブリドーマが９クローンが得られた。その中から３回クローニングを行って得られたハイブリドーマ1.H11.B2.G3とした。1.H11.B2.G3 の産生するmAbは*Babesia equi* とのみ反応し、*B.caballi*及び馬赤血球に反応しないことが確認された。このmAbのサブクラスはIgG1であり、ウエスタンブロット法により、19kDa の抗原を認識することが判明した。また、500 μg/ml 濃度のmAb 添加により、対照に比べ原虫の増殖が50%抑制された。今後、アフニティクロマトグラフィーによりこのmAb が認識する抗原を精製し、ELISA用の抗原として至適であるか否かついて検討する予定である。

以上の結果、５％ CO2のガス条件下でウシバベシア原虫*Babesia ovata* 、ウマバベシア原虫*B. equi* 及び *B.caballi*の連続培養が可能となった。また、培養で得られた原虫は間接蛍光抗体法の抗原として有用である事が明らかとなった。今後、培養で得られた原虫を用いることにより、更に特異性・感度の高い診断法の開発や及び遺伝子解析などに大いに役立つことが期待される。

研 究 成 果

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Continuous in vitro Cultivation of *Babesia ovata*

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ABSTRACT

Basic method for in vitro cultivation of *Babesia ovata* was examined using a method which was developed by Vega et al. for cultivation of *B. bigemina*, a closely related organism. The parasites obtained from an infected SCID mouse were initiated their multiplication within adult bovine RBC in Medium 199 supplemented with 40% adult bovine serum under a low oxygen atmosphere, 5% O_2, 5% CO_2 and 90% N_2. Although no proliferation of *B. ovata* maintained in 5% CO_2 in air was seen during the initial 5 days, the parasites passaged three times under the low oxygen atmosphere were readily cultured in 5% CO_2 in air, as well as under the low oxygen atmosphere. The parasites were propagated in the RBC stored in Vega y Martinez solution at 4°C for up to 2 months. *Babesia ovata*-infected RBC from cultures were successfully cryopreserved in 10% polyvinyl-pyrrolidone in Vega y Martinez solution and used to initiate new culture not only under the low oxygen atmosphere but also in 5% CO_2 in air.

INTRODUCTION

The success of continuous cultivation of *Babesia bovis* with microaerophilous stationary phase system (Levy and Ristic 1980) stimulated the cultivations of other *Babesia* parasites such as *B. divergens* (Vaayrynen and Tuomi 1985), *B. canis* (Molinar et al. 1982), *B. gibsoni* (Onishi et al. 1993), *B. caballi* (Holman et al. 1993) and *B. equi* (Holman et al. 1994), and these success of in vitro cultivation contributed to the morphological and biochemical study, drug sensitivity and supply of antigen or immunogens for the prevention of disease (Kellermann et al. 1985).

Babesia ovata is a relatively large *Babesia* parasite and widespread in Japan. It is not highly pathogenic for cattle, but splenectomized cattle show high parasitemias accompanied by anemia, hemoglobinuria, and in some cases, death (Ishihara and Minami 1977; Minami and Ishihara 1980). Morphologically, the intraerythrocytic forms of *B. ovata* are closely resembled *B. bigemina*. Vega et al. (1985a) reported that *B. bigemina* was cultured continuously under in vitro labo-

ratory conditions, and they also established a cryopreservation method of cultured *B. bigemina* (Vega et al. 1985b). We applied these methods to the cultivation of *B. ovata* by taking advantage of morphological similarity of two parasites. The present study reports establishment of in vitro culture of *B. ovata* and of cryopreservation of the parasite.

MATERIAL AND METHODS

Parasites: *Babesia ovata* (Miyake strain) was provided from the Kyushu Branch of National Institute of Animal Health (Kagoshima, Japan). A frozen stabilate of *B. ovata* was inoculated into, instead of cattle, a splenectomized SCID mouse which was transfused with bovine RBC, according to the method of Tsuji et al. (1992). When parasitemia reached at 2.3%, infected RBC were collected from the SCID mouse by a cardiac puncture with a syringe containing glass beads, and the defibrinated blood was then washed twice with Vega y Martinez (VYM) phosphate-buffered saline solution (Vega et al. 1985a) and used for in vitro cultivation.

Culture media: Three culture media, RPMI 1640, Medium 199 (M 199) and Minimum Essential Medium (MEM), were purchased from Flow Laboratories (ICN Biomedical Inc., CA, USA). The culture media contained $NaHCO_3$ (2.2g/l) or 25mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and pH of all the media was adjusted to 6.70-6.90 and supplemented with bovine serum. Antibiotics were not added to any culture media used in the experiment.

Bovine erythrocytes and sera: Normal venous blood from adult Holstein cows of the University farm was aseptically collected and was immediately defibrinated by shaking with glass beads. The defibrinated blood was centrifuged at 800 *g* for 10 min at 4°C and serum was separated and distributed into sterile 15 ml plastic tubes in 4.0 ml aliquots, and stored at -80°C until use. Remaining RBC were subsequently washed twice in VYM solution at 800 *g* for 10 min at 4°C to remove buffy coat cells, and resuspended in VYM solution to a final 30-50% concentration for storage at 4°C until use. Fetal bovine and calf sera were purchased from ICN Biomedical Japan Co. Ltd. (Osaka, Japan).

Culture conditions: An infected RBC suspension was prepared at 10% concentration (v/v) by mixing one part of packed RBC with 9 parts of serum-supplemented medium. Duplicate RBC suspensions were placed in wells of sterile, disposable 24-well multiple plates (one ml/well) (Corning Laboratory Sciences Company, U. S. A.) and incubated under a low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) or in 5% CO_2 in air. Culture medium was changed daily with fresh medium.

Cryopreservation of parasites: Stock solution of 20% polyvinylpyrrolidone (PVP) was made in VYM solution according to Vega et al. (1985b) and used as a cryoprotectant solution. When parasitemia was higher than 5%, packed infected RBC were mixed with an equal volume of 20% PVP and suspended in 1.5 ml cryotubes. The PVP concentration of the final mixture was 10%. The vials were rapped with tissue papers and frozen at -80°C overnight. The vials were then stored in liquid nitrogen following day.

The frozen vials were taken from liquid nitrogen container and rapidly thawed in a 37°C water bath. The thawed samples were immediately diluted in 10 ml of VYM and centrifuged at 800 *g* at 4°C for 10 min. The resulting pellets were resuspended in fresh culture medium containing 10% normal bovine RBC and one ml of RBC suspension was dispensed in wells of 24-well microplates. The

plates then were incubated under the low oxygen atmosphere or in 5% CO_2 in air. Culture medium was changed daily and the growth of parasite was monitored in Giemsa-stained blood smears by means of light microscopy.

RESULTS

Infected blood was obtained from the splenectomized SCID mouse infected with *B. ovata* at the time of initiation of culture. The defibrinated and washed blood was suspended in a mixture of M 199 supplemented with 40% adult bovine serum containing 10% normal RBC (v/v). One ml of suspension was transferred to a well of two separate 24-well microplates. The plates were incubated in humidified atmosphere of 5% CO_2 in air or under the low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) at 37°C. No growth of parasites was observed in culture in 5% CO_2 in air. In contrast, growth of *B. ovata* was observed in the cultures maintained under the low oxygen atmosphere (Fig. 1). Although parasitemia was very low (less than 0.3%) until day 3, thereafter parasitemia steadily increased on days 4 and 5 (0.6% and 1.2%, respectively). Subculture was made on day 5 by diluting 1:2 with a 10% normal RBC suspension. The second subculture was carried out on day 9 by diluting 1:10 with 10% normal RBC suspension when parasitemia reached at 4.2%. The parasites were then subcultured every 3 to 5 days depending on the growth of parasites, and maintained continuously in vitro for more than 1 year. Parasitemias in excess of 6% were frequently observed. After three passages of parasite under the low oxygen atmosphere, growth of parasite was observed in 5% CO_2 in air, as well as under the low oxygen atmosphere (Fig. 2).

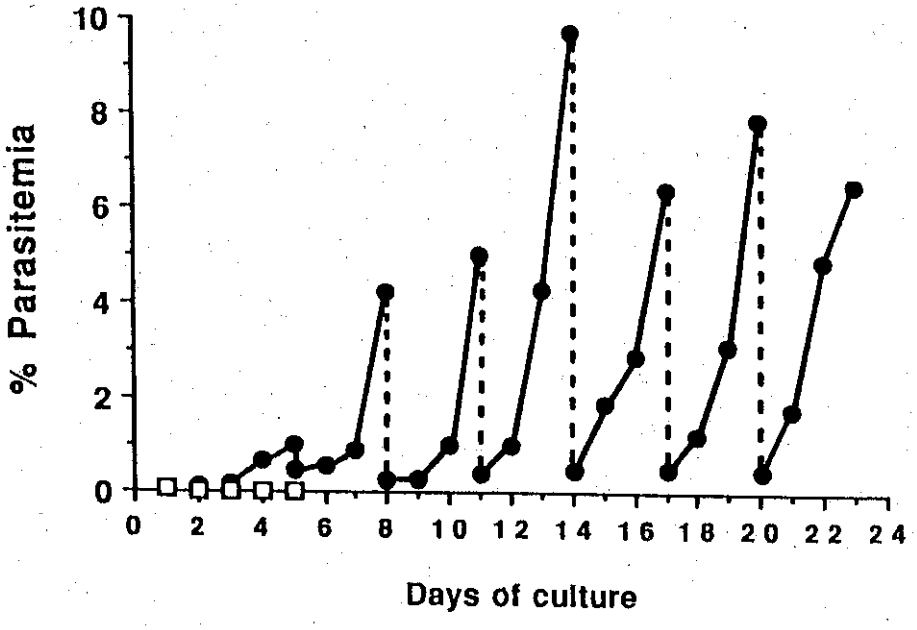

Fig. 1. Growth of *B. ovata* cultured from the blood of an infected SCID mouse in different atmospheres. Days at subcultures are indicated with dotted lines. Symboles; low oxygen, 5% O_2, 5% CO_2 and 90% N_2 (●) and 5% CO_2 in air (□).

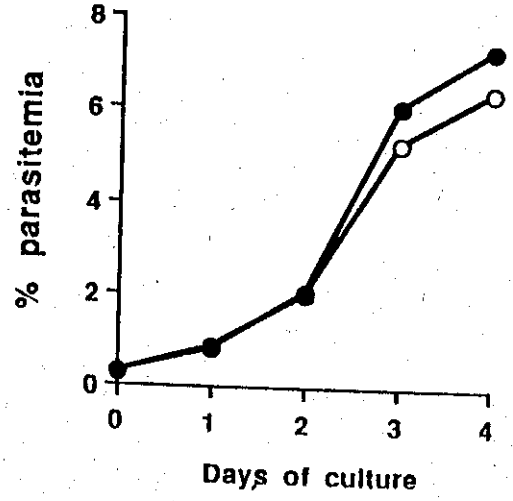

Fig. 2. Growth of *B. ovata* in different atmospheres after three passages. Symboles; low oxygen (○) and 5% CO_2 in air (●).

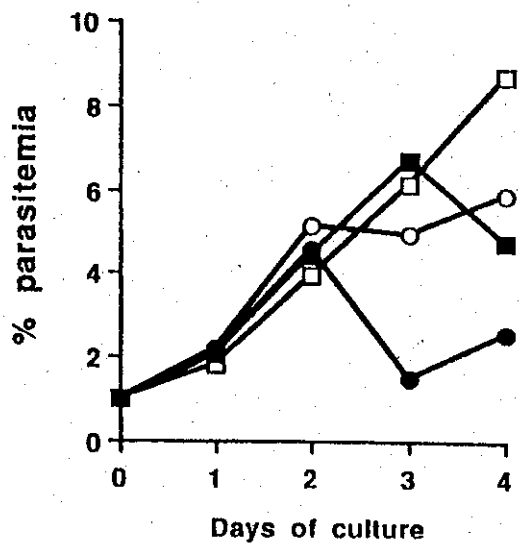

Fig. 3. Growth of *B. ovata* using various cultu media. Symboles; $NaHCO_3$-buffered M 199 ([HEPES-bufferedM 199 (■), $NaHCO_3$-buffered MEM (○) and $NaHCO_3$-buffered RPMI 1640 ((

Three culture media, M 199, RPMI 1640 and MEM buffered with $NaHCO_3$, and one medium, M 199 buffered with HEPES, were examined on the growth of parasites. All the media contained 40% adult bovine serum. No significant difference in parasitemia was found among the 4 media on day 2. However, M 199 buffered with $NaHCO_3$ supported higher parasitemia than other three media on day 4 (Fig. 3). $NaHCO_3$ showed superior effect to support the parasite growth than HEPES when it was added to M 199. Therefore M 199 buffered with $NaHCO_3$ was selected for further experiments.

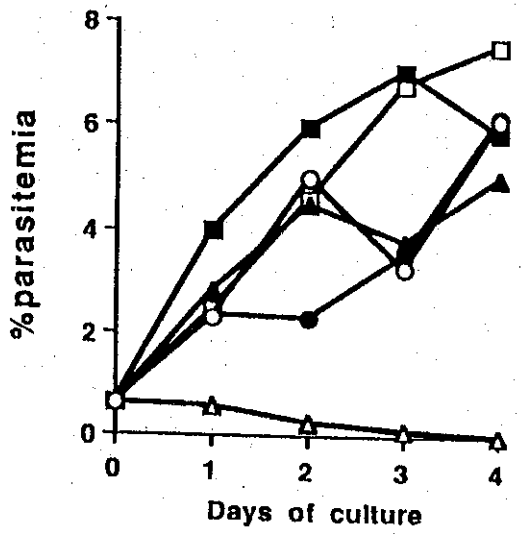

Fig. 4. Growth of *B. ovata* using adult bovine serum at different concentrations with M 199. Symboles; 0% (△), 20% (●), 40% (□), 60% (■), 80% (▲) and 100% (○).

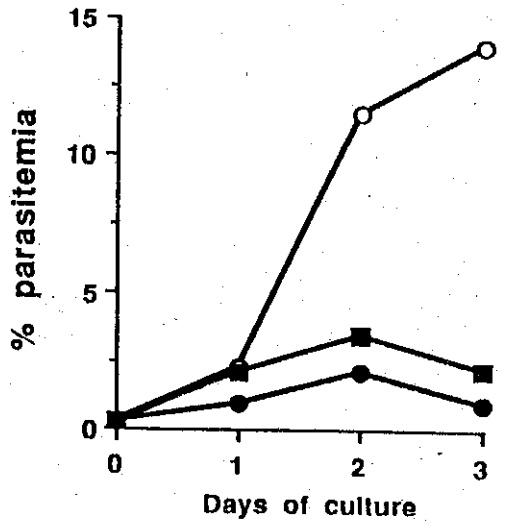

Fig. 5. Growth of *B. ovata* using different sera with M 199. Symboles; adult bovine serum (○), calf serum (●) and fetal bovine serum (■).

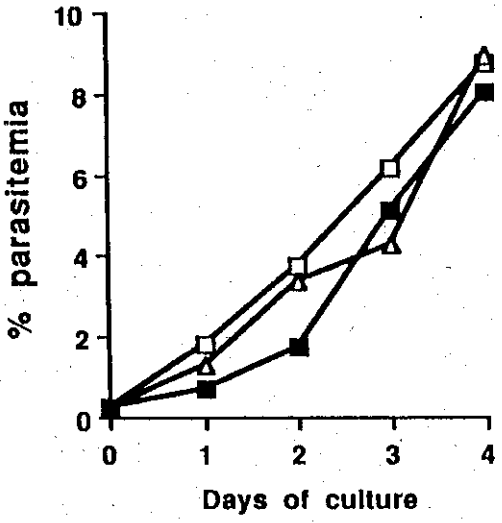

Fig. 6. Growth of *B. ovata* in normal adult bovine RBC stored at 4 °C for different periods. Symboles; freshly collected (□), stored for 37 days (△) and stored for 57 days (■).

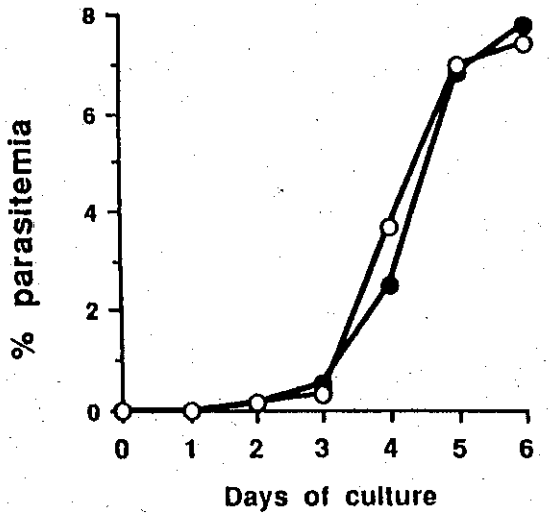

Fig. 7. Growth of *B. ovata* in different atmospheres after cryopresrvation. Symboles; low oxygen atmosphere (○) and 5% CO_2 in air (●).

After $NaHCO_3$-buffered M 199 was selected in the previous experiment, the effect of different concentrations of adult bovine serum on the growth of parasites was examined. Although 20% or higher concentrations seemed to support the growth of parasites, the highest parasitemia was observed at the 40% of serum concentration (Fig. 4). However, supplement with 40% of fetal bovine serum or calf serum did not support the growth of parasite (Fig. 5). Heat-inactivation of serum (at 56°C for 30 min) did not affect the growth of parasites (data not shown).

The effect of storage of adult bovine RBC on the growth of parasites was examined for practical consideration. Normal RBC were collected on day of experiment or collected from the same donor and had been stored for 37 and 57 days in VYM solution at 4°C, respectively. No difference of the parasite growth was seen in RBC stored for different periods of time (Fig. 6). As the result of this finding, normal RBC were stored for up to 2 months in VYM solution at 4°C.

Cultured parasites from the sixth passage in the RBC were cryopreserved using 20% PVP in VYM buffer as a cryoprotectant. Cultures were initiated again from the cryopreserved parasites propagated under the low oxygen atmosphere. Low numbers of *B. ovata* within RBC were first observed on the day 2 after the initiation of culture. On days 4 and 6, parasitemias of culture were 0. 3% and 3.6%, respectively. On day 7, at the time when the parasitemia was 4.9%, first subculture was made. The parasitemia in the subculture was reached at 7.7% three days after the initial subcultivation, thereafter successful subcultures were made every 3-5 days. In a separate experiment, the effect of culture atmosphere on the cryopreserved *B. ovata* was examined by initiating cultures again from the cryopreserved materials either under the low oxygen atmosphere or in 5% CO_2 in air. The atmosphere of 5% CO_2 in air, as well as the low oxygen atmosphere, supported the growth of parasite (Fig. 7).

DISCUSSION

The present study demonstrated that *B. ovata* could be cultivated continuously in vitro using a method which was developed for in vitro cultivation of *B. bigemina*, a closely related organism. A low oxygen atmosphere (5% O_2, 5% CO_2 and 90% N_2) was necessary to initiate growth of *B. ovata* in bovine RBC. The reduced oxygen requirement for in vitro culture is also reported in *B. bigemina* (Vega et al. 1985a), *B. caballi* (Holman et al. 1993) and *B. equi* (Holman et al. 1994). These results suggest that oxygen gas tension seems to be a key factor for initiation of in vitro culture of *Babesia* parasites. After three passages, however, *B. ovata* could be maintained in bovine RBC culture under 5% CO_2 in air (high-O_2) as observed in *Plasmodium falciparum* (Waki et al. 1984) and *B. bigemina* (Vega et al. 1985b). The oxygen requirement of *B. bovis* was varied according to initial parasitemia of subculture (Rodriguez et al. 1982). *Babesia bovis* grew readily in cultures in 5% CO_2 in air when an initial parasitemia was 0.5% or above, but the parasites did not when the initial parasitemia was 0.1% or less. If this similar effect occurred with *B. ovata*, threshold of the growth of *B. ovata* in 5% CO_2 in air might be lower than of *B. bovis*.

Serum supplement to medium is essential to in vitro growth of *B. ovata*. Cultures without serum did not support even limited parasite multiplication, but cultures containing greater than 20% serum supported the growth. Cultures with 40% of serum concentration showed the highest multiplication. Furthermore, effects of fetal bovine and calf sera on the the growth of parasites were compared with those of adult bovine serum, since young animals are known to be more resistant to *Babesia* infection than adults (Levy and Ristic 1981). Commercially obtained fetal bovine and calf sera did not support the growth of *B. ovata*. One possible explanation may relate to the presence of an inhibitory factor(s) to parasite growth in young animals as suggested in *B. bovis* (Levy et al. 1982), or lacking a supporting component(s) for growth of *B. ovata*. Further study of effect of RBC and leukocytes from young animals will need to determine the role of blood components in the phenomenon of age resistance.

The storage of RBC for longer period is very important for practical consideration of this culture method. The present study demonstrated that storage of RBC in VYM buffer for up to 2 months at 4°C did not affect parasite growth and during the period severe hemolysis was not observed. This finding is contrast to the cultivation of *B. bovis* and *B. bigemina*, where that RBC can be stored at most for one week only (Vega et al. 1985a; Levy and Ristic. 1980). De Verdier et al. (1981) reported the favorable effect of citrate-phosphate-dextrose supplemented with adenine-guasonine on the stability of human blood stored at 4°C. High concentration of glucose in VYM solution supplies energy source for RBC, and adenine and guasonine enhance ATP and 2,3-DPG production, and ATP is closely linked to preservation of biconcave shape of RBC. An addition of adenine to human blood preservation media extended the storage period of RBC from 21 to 28 days or 35 days (Sasakawa 1983). These favorable effects may occur with respect to the storage of bovine RBC for longer periods in the case of *B. ovata* cultivation.

The reestablishment of in vitro culture, using frozen *Babesia* parasites, would eliminate the need for maintaining infected animals. The successful cryopreservation of *B. bovis* for initiation of in vitro cultures was reported by Palmer et al. (1982) using 10% PVP in Pack's saline G solution supplemented with extra glucose. However, this cryoprotectant solution did not work with *B. bigemina*.

Vega et al. (1985b) developed a modified procedure for cryopreservation of *B. bigemina* with 10% PVP in VYM solution instead of Pack's saline G. Application of this procedure to *B. ovata* was successful in the initiation of culture of cryopreserved parasites and animal inoculation (data not shown). Growth of *B. ovata* was observed in cultures under a low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) as observed in *B. bigemina* (Vega et al. 1985b). Furthermore, *B. ovata* could be also cultured in 5% CO_2 in air. This result may indicate that high infectivity of cryopreserved *B. ovata* was retained during cryopreservation of parasites, since relatively high oxygen atmosphere of 10% O_2, 5% CO_2, and 85% N_2 could support the initiation of growth of *B. bigemina*, if a high ratio of infectivity was used (Vega et al. 1985b). These results suggest that 10% PVP in VYM solution is highly suitable to cryopreserve *B. ovata*.

The present study suggests that *B. ovata* can be cultured readily using a conventional CO_2 incubator after establishment of in vitro culture or cryopreservation of parasites from cultured materials. By establishment of in vitro cultivation, animal infection for maintaining parasites and the risk of spread to susceptible animals could be avoided. In addition, in vitro cultivation can be used to produce specific antigen for serology, and to study the life cycle and biochemistry of parasites and to develop effective and safe vaccines.

ACKNOWLEDGEMENTS

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寄生虫病学
短報

Improved *in vitro* cultivation of *Babesia caballi*

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IN VITRO CULTURE OF BABESIA CABALLI

ABSTRACT. *Babesia caballi* infected erythrocytes were collected from the blood of an experimentally infected horse and could be continuously cultivated in vitro with parasitemia ranging from 2-4% in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20 mM HEPES and 40% adult horse serum in a low oxygen atmosphere (2%O_2, 5%CO_2 and 93% N_2). All attempts to increase parasitemmia failed using other culture media, serum concentrations and culture vessels. However, parasite growth was enhanced by transfer of cultures from a low oxygen to 5% CO_2 in air, with parasitemia ranging from 8-10 %.

Key words: *Babesia caballi*, *in vitro* cultivation,

Equine piroplasmosis, caused by *Babesia caballi* and *Babesia equi*, is considered to be the most important tick-borne disease of horses in tropical and subtropical regions of the world [10]. Japan is free from the disease, however, a number of imported horses from abroad including endemic areas has increased and the existence of tick vectors, *Dermacentor reticulus* and *Rhipicephalus sanguineus* has been also reported [13]. If infected animals are introduced in Japan, there is a possibility that these parasites can readily be spread. Therefore, the detection of carrier animals is very important to prevent the introduction of infected animals to the country. Horses to be imported to Japan have to be tested for babesiosis by serological tests. In addition to relatively low sensitivity and specificity of serological tests, antigen supply for serological tests is not sufficient in Japan, because equine babesiosis is designated as an exotic disease for which P3-level facility is required.

The development of a continuous microaerophilous stationary phase culture system for a related parasite, *Babesia bovis* [7], represented a major breakthrough in *Babesia* research. Culture-derived *B. bovis* antigens were, thus, made available replacing the requirement for experimentally infected animals in antigen production. Several researchers have cultured *B. caballi in vitro*, however, the yields of parasites from *B. caballi* cultures is very low [1, 2], which hampers the progress of development of diagnostic methods or biological studies. The present study was designed to define the optimal continuous culture conditions for *B. caballi* to improve the yields of parasite antigen.

Blood samples for cultures were collected from a horse experimentally infected with a frozen stabilate of *B. caballi* (USDA strain) at P3-level facility of the Epizootic

Research Station, Equine Research Institute, The Japan Racing Association. Blood from the jugular vein, containing approximately 1.0 % parasitemia, placed in tubes containing EDTA (ethylenediamine tetra-acetic acid) as anti-coagulant were sent by air packed in ice to The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine. Upon arrival, the sample was washed three times by centrifugation with RPMI 1640 medium buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) to remove the buffy coat. After the final wash, the supernatant was removed and 50 μl packed infected erythrocytes were transferred to a 24-well culture plate containing 1 ml of buffered RPMI 1640 with 40% horse serum and supplemented with 2 mM L-glutamine and 50 μl packed normal horse erythrocytes. Cultures were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 in air or in reduced oxygen atmosphere containing 5% CO_2, 2% O_2 and 93% N_2.

Whole blood from an adult horse was collected in sterile Ehrlenmeyer flasks with glass beads and immediately defibrinated by regular shaking. Defibrinated blood was centrifuged at 800 *g* for 10 min at 4 ℃ Serum was separated and distributed into sterile 15 ml plastic tubes in 4 ml aliquots and stored at -30 ℃ ntil use. Remaining erythrocytes were also washed three times by centrifugation with RPMI 1640 medium buffered with 20 mM HEPES, for the removal of buffy coat. The final pellet was resuspended in an equal volume of RPMI 1640 medium and stored at 4 ℃ for up to two months.

The medium was changed daily by using 1 ml of fresh medium and percentage of parasitemia was monitored by microscopic examination of Giemsa-stained blood smears. During the first five days, the percentage of parasitemia was very low

(<0.1%) both in a low oxygen atmosphere and in 5% CO_2 in air. Parasites in 5% CO_2 in air were no longer detected at days 6 and 7 and thereafter the experiment was discontinued. Increased parasitemia was observed in a low oxygen atmosphere on culture days 6 and 7. Subcultures were made on days 7 and 12 in a low oxygen atmosphere, and parasitemia increased to a maximum of 3% (Fig. 1). After the third passage, continuous cultures were successfully carried with parasitemia between 2-4% and subcultures were made every 5 to 7 days, depending on the growth. Two hundred ml of infected erythrocyte suspension was transferred to a well containing 900 μl of RPMI 1640 medium described above and 100 μl of packed donor erythrocytes.

After continuous *in vitro* culture of *B. caballi* has been established, various conditions were investigated to enhance parasite growth. Three cuture media, RPMI 1640 (Dainippon Pharmaceutical Co., Ltd., Osaka), Iscove`s modified Dulbecco's medium Hybri-Max and Iscove's modified Dulbecco's medium (Sigma, St. Louis), three different serum concentrations (20,40, 60 %), and three diffrent size of culture vessels (24-well plate, 6-well plate and Petri dish) were compared as to their effect on *in vitro* propagation of *B. caballi* parasites in a low oxygen atmosphere. All media with 40% horse serum supported parasite growth with no significant difference as observed on day 5. *B. caballi* reached the highest observed parasitemia in RPMI 1640 with 40% serum and grew least in 20% serum. The size of culture vessels did not enhance parasite growth.

After eight passages in a low oxygen atmosphere, parasite growth was reexamined under conditions of a low oxygen atmosphere or 5% CO_2 in air. On

the fourth culture day, the parasitemia had doubled in 5% CO_2 in air reaching as high as 6.3% on day 7, while the parasitemia in low oxygen was only 3.3% on day 7 (Fig. 2). Henceforth, routine maintenance of *B. caballi* was done under 5 % CO_2 in air and parasitemias usually ranged from 8-10% but parasitemias in excess of 15% were sometimes observed (Fig. 3).

Establishment of *in vitro* culture of equine *Babesia* is particularly important for antigen production without animal infection in a non-endemic country like Japan. The present study demonstrated that *B. caballi* can be continuously cultivated *in vitro* and confirmed the previous report that a low oxygen atmosphere is necessary for the initiation of *B. caballi* cultures [4]. The requirement of reduced oxygen tension for the establishment of *Babesia* cultivation was reported in other *Babesia* parasites such as *B. bigemina*, *B. ovata* and *B. equi* [5,6,11]. Low oxygen tension is also required when starting a culture with low *B. bovis* parasitemia [9] or cryopreserved samples of *B. bigemina* [12].

The levels of parasitemia remained low in *in vitro* culture with a low oxygen atmosphere. Attempts to improve the parasitemia using different culture media, serum concentrations, or vessel volumes were in vain. The parasitemia was doubled or even greater when parasites were transferred to 5% CO_2 in air. Observed parasitemia was between 8-10% and sometimes over 15% which is the highest ever reported for *B. caballi*. Similar improvement of parasitemia was observed in *B. equi* [14] and *B. divergens* [3, 8]. These results may indicate that actively dividing parasites require normal oxygen levels. The improvement of parasite growth in an ordinary 5 %CO_2 incubator can avoid the use of animal infection and will greatly enhance further studies on biochemistry, metabolic pathways, growth requirements and drug sensitivity of this important equine

pathogen.

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Figure legends

Fig. 1. Growth of *Babesia caballi* cultured from the blood of an experimentally infected horse from initiation of culture through 2 passages. Mean percent parasitemia of a low oxygen atmosphere (○) and 5% CO_2 in air (□). Closed circles indicate the points of subcultures.

Fig. 2. Mean percent parasitemia of cultute-adapted *Babesia caballi* in a low oxygen atmosphere (○) and in 5% CO_2 in air (□).

Fig. 3. Giemsa-stained thin smear of cultured *Babaesia caballi* from 70th passage showing parasitemia of 16.4 %. Paired pyriforms and several different single forms are shown. x890.

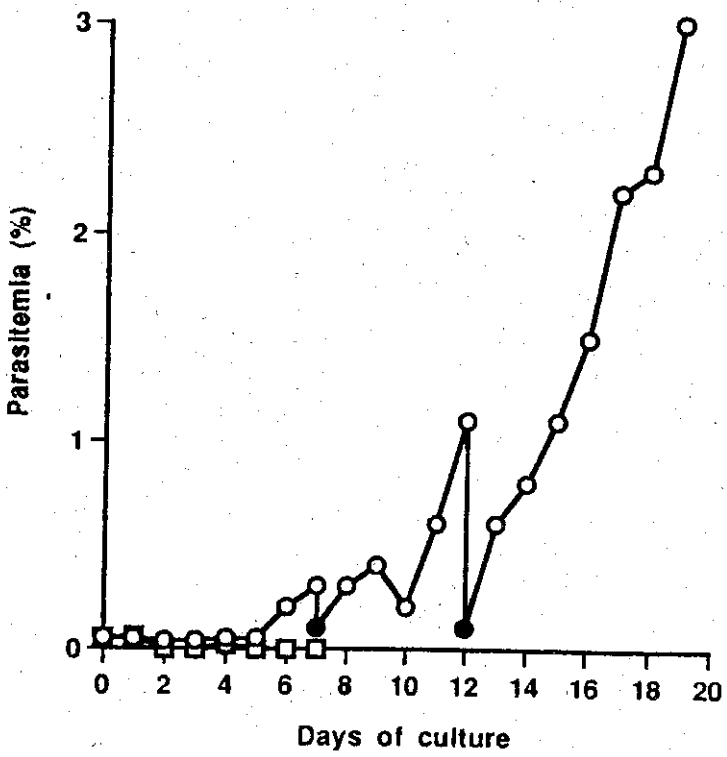

Fig.1

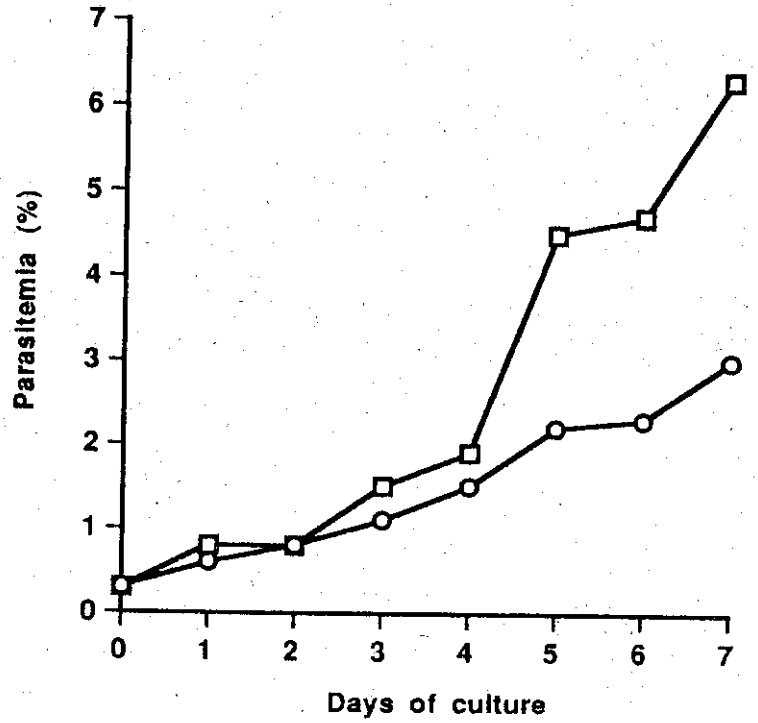

Fig.2

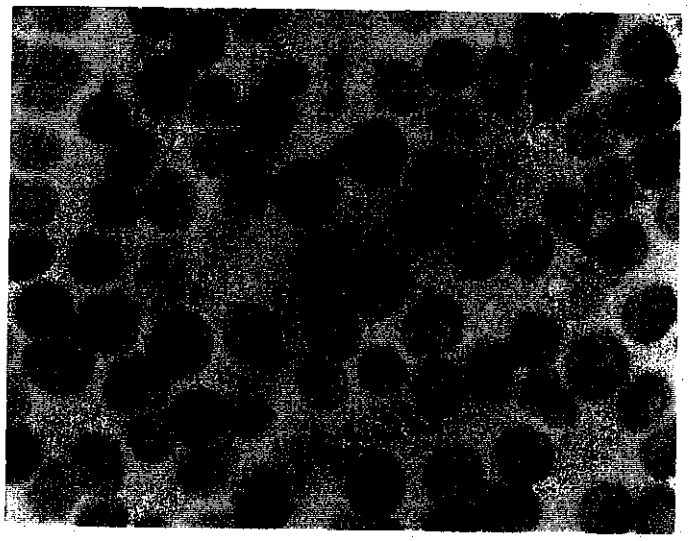

Fig.3

Babesia caballi in vitro培養法の改良（短報）

Avazed Avgaandorjiin ・五十嵐郁男・兼丸卓美[1)]・蛭海和子・蛭海啓行・小俣吉孝[2)]・斎藤篤志[2)]・小山田隆[3)]・長澤秀行 ・豊田裕 ・鈴木直義[3)]（帯広畜産大学原虫病分子免疫研究センター、[1)]日本中央競馬会競走馬総合研究所栃木支所、[2)]帯広畜産大学家畜生理学教室、[3)]北里大学畜産学部獣医寄生虫学教室）

*Babesia caballi*感染馬の血液を40%ウマ血清加RPMI1640培養液を用いて初代培養を行ったところ、２%O_2 ５%CO_2 93%N_2の気相条件下で虫体の増殖が認められ、２代以後は、5-7日間隔の継代で赤血球感染率が2-4%に達し、連続培養が可能となった。培養液、血清濃度、培養容器の検討では、感染率の増加は認められなかった。しかし8代継代後、５%$CO_2$95%空気の気相条件下で培養することにより赤血球感染率が８－１０%に上昇した。

Prevalence of Equine Piroplasmosis in Mongolia

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INTRODUCTION

Equine piroplasmosis is caused by *Babesia equi* and *Babesia caballi* and is endemic in equines throughout most of the tropical and subtropical regions of the world (Friedhoff, Tenter and Mueller, 1990). *B. caballi*, a typical large *Babesia* only infects and multiplies in erythrocytes and is transmitted transovarially and transstadially by its tick vectors (Friedhoff, 1988). In the case of *B. equi,* however, it has been reported that the parasite apparently first multiplies in lymphocytes (Schein,Rehbein, Voigt & Zweygarth 1981; Rehben, Zweygarth, Voigt & Schein 1982), before invading erythrocytes where merozoites can frequently be seen arranged in a typical Maltese cross formation.

Babesia parasites are usually only detected in blood smears during the acute stage of the infection and animals that recover from the disease remain carriers of these parasites. It has been reported that horses can remain carriers of *B. caballi* parasites for up to 4 years, while they probably remain carriers for their life after infection with *B. equi* (Friedhoff, 1982). Identification of these carriers and previously exposed horses can most easily be accomplished by demonstrating specific antibodies in the serum with a serological tests (Weiland and Reiter, 1982). The indirect fluorescent antibody test (IFAT) is the most widely used serological technique to detect specific antibodies in protozoan infections. Ristic and Sibinovic (1964) first reported about the use of the IFA test to detect *B. caballi* antibodies in horses. Maddan and Holbrook (1968) successfully applied this test to differentiate between *B. equi* and *B. caballi* infection in horses and did

not report any cross reaction. Antibodies detectable by the IFAT start to rise shortly after the first parasites appear in blood smears, and persist for long periods (Donnelly, Joyner, & Frank, 1980; Weiland 1986; De Waal. 1995).

In Mongolia, Dash (1957 and 1959, cited by Dash, 1967), using microscopic examination of thin blood smears, reported that both *B. equi* and *B. caballi* occur wide spread in horses. The tick vectors in this region is also unknown, but *D. nuttalli* (Olenev, 1929, cited by Dash, 1986), *D. salvarum* (Shulth, 1933, cited by Dash, 1986) and *H. dromedari* (Svirskaya, 1961, cited by Dash, 1986) frequently infect horses and all have been reported to be vectors of *B. caballi*, elsewhere. However, no serological survey has been conducted to determine the prevalence of these two parasites in horses. In this study *in vitro* propagated parasites were used as antigen in the IFAT to determine the prevalence of *B. equi* and *B. caballi* antibodies in field horses from Central Mongolia.

MATERIALS AND METHODS

Strains of Babesia species

B. equi and *B. caballi,* both USDA strains, were used in this study and were obtained from experimentally infected horse at Tochigi Research Station of Equine Research Institute, Japan Racing Association.

Serum samples

Serum from experimentally infected horses with *B. equi,* OP-isolate, (horse No 460-9) and *B. caballi,* SWA isolate, (horse No 042-9) were obtained from Onderstepoort Veterinary Institute (OVI), South Africa and used as positive control serum. Negative serum was obtained from a horse raised under tick-free conditions at OV I. Sera were collected from 15 Babesia-free horses in Japan and from 110 horses of the Mongolian native breed, originating from two different herds in Central Mongolia. Blood was allowed to clot at room temperature overnight before serum was removed and stored at -20°C. At the same time as collecting blood, thin blood smears were also prepared, and stained with Giemsa solution.

Antigen preparation for IFAT

Antigen was prepared from *in vitro* propagated *B. equi* or *B. caballi* infected erythrocytes (Avarzed. A, Igarashi. I, Kanemaru.T, Omata. Y, Saito. A, Nagasawa. H, Toyoda. Y and Suzuki.N. 1995). *In vitro* propagated *Babesia* parasites were transferred from 1 well of a 6-well culture plate in to a 15 ml centrifuge tube and centrifuged for 15 min at 700 g at 4°C. After the first centrifugation, the erythrocyte pellet was washed 5 times by centrifugation for 5 min at 350 g and reconstituted with 10 ml PBS(-)[147mM NaCl, 5mM NaH_2PO_4, 5mM Na_2HPO_4, pH 7.2] each time. Any visible white blood cell layer was removed after each wash. After the fifth wash, 1 part packed red blood cells was reconstituted with 2 parts of 4% bovine serum albumin in PBS(-)(1:2, v/v). The blood mixture was dispensed onto previously cleaned glass slides using a 1 ml syringe and 27G needle. Fifteen antigen spots, 5 mm in diameter (3 rows of 5 spots) were prepared per slide. The slides were dried for 30 minutes at 45°C in a drying oven (Forced convection oven FC-610, Toyo Seisaku, Co., Ltd) and thereafter for a further 30 min at room temperature. The slides were then wrapped in tissue paper and covered with aluminum foil and stored at -20°C.

IFAT technique

Antigen slides were removed from storage, at -20°C, and placed in an drying oven at 45°C for 1 h. The antigen spots on the slides were encircled with a pap pen (Kantoukagaku, Japan) and fixed in a large volume of cold acetone (-20°C) for 5 minutes. Two fold serum dilutions were prepared in PBS(-) starting at 1/10 to 1/5120 dilution. Every test included *B. equi* and *B. caballi* positive and negative sera as well as PBS control. Ten microliters of each serum dilution were then placed on the antigen spots. Slides were incubated in a humid chamber at 37°C for 30 min. The slides were then rinsed in fresh PBS(-) and washed once for 10 minutes in PBS(-) followed by a further washing with deionized-distilled water for 5 min. Goat anti-horse IgG FITC conjugate (Bethyl Laboratories, Inc., USA.) was diluted 1/80 in 0.01% Evans blue-PBS(-) to reduce nonspecific fluorescence and 10 ml of diluted conjugate was pipetted onto each spot. The slides were incubated, rinsed and washed as described before. After the last wash slides were allowed to air dry slightly before being overlaid with glycerin-PBS(-) (1:1) and covered with a 24x60 mm cover slip. The slides were examined under a fluorescent microscope using a 40x objective lense (Microphot EPI-FL, Nikon, Japan).Fluorescence was interpreted as positive (+), trace(±) or negative (-). The highest serum dilution showing positive (+) fluorescence was taken as the titer of the sample.

RESULTS

Validation of IFA with parasites from in vitro culture.

In vitro propagated *B. equi* and *B. caballi* were investigated as antigen sourse in the IFAT. When the test was performed with *B. equi* or *B. caballi* antigen and homologous positive control sera, strong specific fluorescence of the parasites within erythrocytes was observed (Fig. 1). Some cross-reactions were observed between the positive reference serum and the reciprocal antigen at low serum dilutions <1/40 (data not shown) but no specific fluorescence was observed at 1/80 or higher dilutions. Sera, collected from 15 horses born and reared in Japan, and regarded as *Babesia*-free showed some non-specific fluorescence at low serum dilutions (≤1/40) on both *B. equi* and *B. caballi* antigens (Fig. 2). Therefore, a titer of≥80 with in vitro cultured antigens in vitro culture was considered as being positive for the infections.

Screening of IFA titer with Mongolian sera.

In vitro propagated *B. equi* and *B. caballi* were also used to investigate the prevalence of antibodies to *B. equi* and *B. caballi* in horses from Central Mongolia. Although no blood parasites were detectable in the Giemsa-stained blood smears from any of these horses, a wide range of end titers was found in the 110 Mongolian horses 1/20 to 1/2560 (Fig. 3). Based on criterion for positivity as described above, the prevalence of *B. equi* and *B. caballi* infection in Mongolia was 88.15% and 84.5%, respectively (Table 1). The ages of the Mongolian horses varied from 1 to 19 years and *B. equi* and *B. caballi* antibodies were detected in all age groups with ninety horses (81.8%) being seropositive to both infections (Table 2).

DISCUSSION

Until recently most researchers have performed the IFAT for equine piroplasmosis on infected erythrocyte antigen prepared from experimentally infected horses (Ristic & Sibinovicl. 1964, Madden & Holbrook.1968, Tenter & Friedhoff. 1986, Weiland, 1986 and De Waal, 1995). With the successful establishment of *in vitro* cultures of *Babesia*, it is now possible to employ culture derived antigens in this test. In vitro propagated parasites, used as a diagnostic antigen for the IFAT in the present study, can avoid the use of experimentally infected animals as an antigen sources. Although Boese (1994) reported the use of micro-aerophilous stationary phase (MASP) cultures as antigen source of *B. caballi* IFA, they did not comment on antigen preparation procedure. We experienced many difficulties in an established IFAT procedure when the MASP cultures of *B. equi* or *B. caballi* were used as antigens for IFAT in comparison with the antigens obtained from infected horses (unpublished observations). Consequently various modification had to be implemented to allow repeatable and consistent results. In this study, cultures with 3-4% parasitized erythrocytes were found to be most suitable. Cross reactions of control sera with heterologous antigen were observed at serum dilutions of up to 1/40. Similar results were reported by other workers using antigen from experimentally infected horses (Ristic & Sibinovic 1964, Madden & Holbrook 1968, Weiland 1986, Tenter &Friedhoff 1986, De Waal 1995). Although we were unable to completely eliminate the non-specific binding of conjugate to *B. caballi* antigen, it was distinctly different from the positive control and did not interfere with the interpretation of the test.

The Mongolian horse, famous for its riding qualities animal, is also used for racing, draught, milk and meat production. Horse husbandry in Mongolia has still remained a pastoral nomadic system in Mongolia, which includes seasonal migrations and rotations of migrations routes. This seems to be a possible source of re- and mixed infections of horses with *Babesia* parasites. The present study confirmed that both babesial infections occur in horses in Central Mongolia. As shown in Fig 3, ninety horses had high titers (1/160-1/2560) for B. *equi* and eighty two horses for *B. caballi*. The results from this limited survey may suggest that equine piroplasmosis is probably endemic in Central Mongolia. Very few clinical cases of equine piroplasmosis are reported from horses in Central Mongolia probably due to most horses being infected early in life and become as carriers for (as 93.7% of 1 year old horses sera positive to both parasites) when they are protected by colostral immunity and other non-specific factors (De Waal, 1995). The tick vectors of these parasites in Mongolia is still unknown, but *Dermacentor nuttalli* (OleSnev, 1929, cited by Dash, 1967), *D. salvarum* (Shulth,1933, cited by Dash, 1967) and *Hyalonma dromedari* (Svirskaya, 1961, cited by Dash, 1967) ticks frequently infect horses and all have been reported to be vectors of *B. caballi* in Europe and Russia (Friedhoff, 1988; Markob, 1935, cited by Dash, 1967). Further surveys and studies on vectors of equi piroplasmosis are necessary to to evaluate the epidemiological status of equine piroplasmosis in Mongolia.

ACKNOWLEDGEMENT

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Fig 1. Immunofluorescent reactions; A. *Babesia caballi* antigen with *B. caballi* positive serum, B. *B. equi* antigen with *B. equi* positive serum.

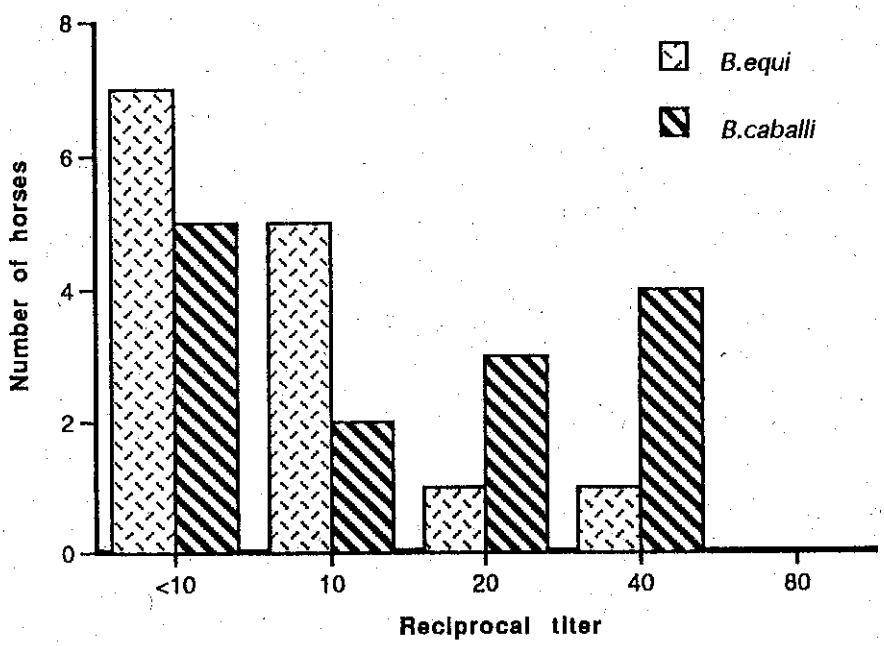

Fig 2. Distribution of IFAT end titers for Babesia equi and *Babesia caballi* in *Babesia*- free horses from Japan.

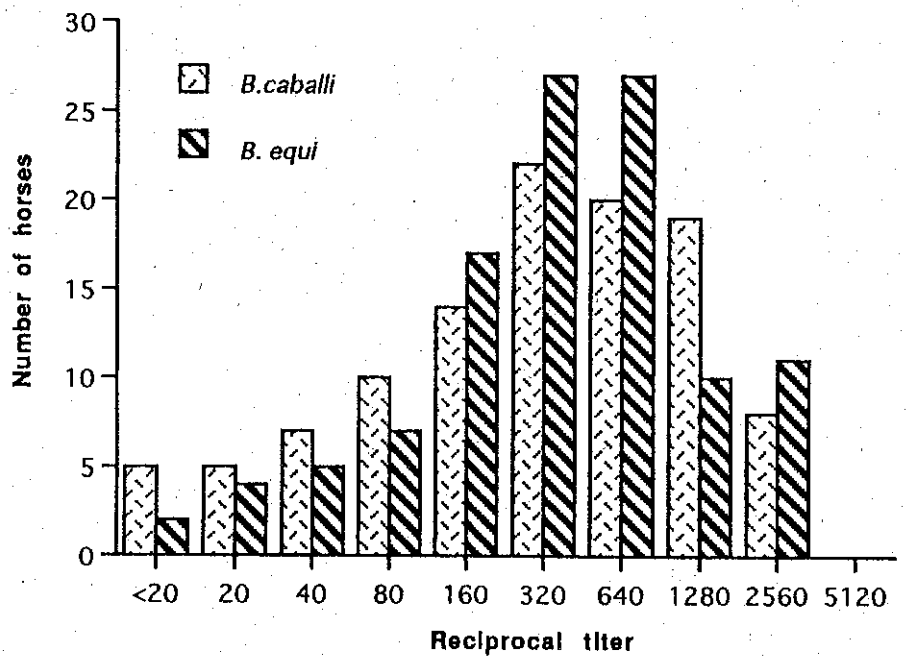

Fig 3. Distribution of IFAT end titers for *Babesia equi* and *Babesia caballi* in Mongolian horses.

Table 1. Summary of IFAT results of Mongolian horse sera.

	number of horses	
	seronegative[1]	seropositive[2]
Babesia equi	13 (11.9%)	97 (88.1%)
Babesia caballi	17 (15.5%)	93 (84.5%)

[1.2]Reciprocal titer of <80 or >80, respectively.

Table 2. Prevalence of *Babesia equi* and *B. caballi* in different age groups of Mongolian horses.

	Seronegative		Seropositive	
Age	*B. equi*	*B. caballi*	*B. equi*	*B. caballi*
1	-	1	16	15
2	1	3	21	19
3	2	3	12	11
4	-	3	13	10
5	-	-	11	11
6-10	6	3	23	26
11-19	4	4	1	1

Preliminary Survey on Horse Serum Indirect Fluorescence Antibody Titers in Japan Against Protozoan Parasites *Babesia equi* and *Babesia caballi* (Onderstepoort strain) Originating from South Africa

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Key words: *Babesia caballi, Babesia equi*, sera diagnosis, IFAT, Survey

ABSTRACT

No clinical surveys concerning equine babesioses in Japan have been reported. A total of 494 horse serum samples (439 domestic horses and 55 imported) were collected from Kyushu and Hokkaido in Japan. In the serum samples tested by the IFAT, none showed a positive reaction of a 1:80 serum dilution. However, 3 of the 19 trace or false-positive (±) cases were observed as pseudo-positive (± −+) in a weak and sharp fluorescence around the parasite of *B. equi* and/or *B. caballi* by the IFAT. These results might be required to further studies until the accurate diagnostic method in *in vitro* culture system can be established in Japan.

INTRODUCTION

Babesia equi and *Babesia caballi* are horse intraerythrocytic protozoan parasites transmitted by ticks almost worldwide(Tenter and Freidhoff, 1986). Up to now, there have been no clinical reports concerning equine babesioses in Japan, and Japan is still free from equine babesiosis. Possible vectors of horse piroplasma, however, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* exist in Japan (Yamaguti et al. 1971). As a standard test of horse piroplasmoses in Japan, the complement fixation test (CFT) is used for protection of disease control and safety importation of horses from foreign countries. Consequently, we surveyed horse babesioses using the indirect fluorescence antibody test (IFAT) as a preliminary survey in Japan.

MATERIALS AND METHODS

Strains of Babesia equi and Babesia caballi: Slide smears of *B. equi* and *B. caballi* for IFAT and the standard positive and negative sera were transported at -20C from Onderstepoort Veterinary Institute, South Africa. The antigen spots on the slides were encircled with a pap pen and fixed in cold acetone. Slides were wrapped in tissue papers covered with aluminum foil, and stored at -20C until use.

Serum samples collected: Serum samples collected from Kyushu, Hokkaido were kindly supplied by various veterinary practitioners in 1992-1993. The total of 494 was from 439 domestic horses bred in Japan and from 55 horses imported from foreign countries.

Ten samples of equine babesia positive sera by CFT were kindly provided by Dr. N. Komatsu, Yokohama Animal Quarantine Service, the Ministry of Agriculture and Fishery. They were kept at -20C until use.

IFAT technique: Eighty fold serum dilutions in each sample were prepared in PBS(-). Every test included *B. equi* and *B. caballi* standard positive and negative sera provided by Onderstepoort Veterinary Institute as well as PBS control. Ten microliters of each serum dilution was then placed on the antigen spots and slides were incubated in a humid chamber at 37C for 30 min. The slides were then rinsed in fresh PBS(-) and washed once for 10 min in PBS(-) followed by a further washing with deionized-distilled water for 5 min. Goat anti-horse IgG FITC conjugate (Bethyl Lab., Inc., USA) was diluted 1:80 in 0.01% Evans blue-PBS(-) to reduce nonspecific fluorescence and 10 ml of diluted conjugate was pipetted onto each spot. The slides were incubated, rinsed and washed as described before. After the last wash slides were allowed to air dry slightly before being overlaid with glycerin-PBS(-) (1:1) and covered with a 24 x 60 mm cover slip. The slides were examined under a fluorescence microscope using a 40x objective lens (Microphot EPI-FL, Nikon Co., Japan). Fluorescence was interpreted as positive (++), pseudo-positive (+ - ±), trace (false-positive)(±), or negative (-).

RESULTS

Determination of equine sera by IFAT: 494 samples at a serum dilution of 1:80 were tested by IFAT either in *B. equi* or *B. caballi* spotted blood smears, together with the positive and the negative standard sera. As shown in Table 1, 19 cases of 494 were trace (false-positive)(±) in the *B. equi* IFAT. Three of the 19 cases gave a strong and sharp trace showing a weak and clear fluorescence around parasites. We marked the three as pseudo-positive (+−±), showing both *B. equi* and *B. caballi* . The other 475 samples were all negative. In the three pseudo-positive sera of *B. equi* and *B. caballi* in the IFAT, two cases were positive in 1:5 dilution in the *B. caballi* CFT(tested by Dr. T. Kamio, NIAH, Tsukuba, personal communication).

Comparison of the CFT positive sera with IFAT: Ten equine babesia CFT positive sera which detected babesia parasites inside erythrocytes by the National Animal Quarantine Service, Yokohama, were tested by the IFAT. As shown in Table 2, in 7/10 cases (70%) identical

results were obtained with IFAT. Test samples of No. 5, 6 and 7 in *B. equi* were lower than 1:5 to 1:5 by the CFT, and they reacted in the IFAT showing a titer of 1:80 to 1:320. The sample No. 7 with a titer lower than 1:5 by the CFT was positive in the IFAT showing a titer of 1:80.

Cross reactions between B. equi and B. caballi: In the IFAT as shown in Table 3, *B. equi* antigen reacted to 1:320 of anti-*B. equi* serum, and to 1:40 of anti-*B. caballi* serum as the positive titre, respectively. *Babesia caballi* antigen reacted also to 1:320 of anti-*B. caballi* serum and to 1:20 of anti-*B. equi* serum (trace(±) in 1:40) as the positive titre, respectively.

DISCUSSION

Currently, regulatory control of equine babesiosis in the United States and other countries (Brazil, Canada, Japan, and Australia, for example) relies on serological testing (Holman et al., 1993). Although the CFT is the official test of the U.S. Department of Agriculture, it has been shown to yield both false-positive and false-negative results for *B. caballi* (Enigk, K., 1950; Tenter & Friedhoff, 1986). At present, the ELISA for *Babesia* infections is the test with the highest sensitivity. Western blot and IFAT provide comparable results, while the CFT must be regarded as obsolete due to low sensitivity which does not meet the requirements for export testing or epidemiological studies (Boese and Peymann, 1994). For the final confirmation of *Babesia* infection in horses, the parasites should be identified from the horse, and consequently, the ability to expand the parasite population to detectable levels through in vitro culturing represents a final diagnostic procedure that might be used to confirm current methods (Holbrook et al, 1968). IFAT is laborious and provides a somewhat subjective method, however, with the reason that we could see every slide directly to observe the *Babesia*, we carried out a survey on the horses in some regions of Japan to see whether they are completely free from the IFAT *Babesia* tests. Three cases of the 494 horse sera were not completely negative from the test in 1:80 serum dilution by IFAT, showing a weak and clear fluorescence around the parasites. Two of the three cases were positive at 1:5 serum dilution by the CFT examined by Dr. T. Kamio, National Institute of Animal Health (personal communication). We collected sera at random from 3 different regions in Japan, consequently, we could not try to test these three cases again. Thus, further studies for equine babesiosis in Japan are required until the accurate diagnostic methods and

the in vitro culture system for the improved tests can be established.

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Table 1.
Determination of equine sera by the IFAT *Babesia equi* (Onderstepoort strain)

Serum tested	Negative (−)	Trace (±)	Pseudo-positive (± - +)	Positive (+ - ++)	Total
1. Onderstepoort					
Positive serum				1	1
Negative serum	1				1
No. of control samples	1			1	2
2. Japan					
A. Hokkaido					
A stock farm	327	7	1	0	335
B stock farm	100	1	0	0	101
B. Kyushu					
C stock farm	48	8	2	0	58
No. of samples	475	16	3	0	494

Note: All samples at serum dilution of 1:80 were tested by IFAT.

Table 2.

Validation of the CFT for *Babesia equi* and/or *Babesia caballi* given by the Yokohama Animal Quarantine Servoce ,and comparison with our IFAT

Case No.	Sera from horses infected with *B. equi* and/or *B. caballi**											
	CFT						IFAT					
	B. equi			*B. caballi*			*B. equi*			*B. caballi*		
No. of Exam.	1	2	(S**)	1	2	(S**)	1	2	(S**)	1	2	(S**)
1(EI-2)	5-10	5	(+)	<5	<5	(-)	80	80	(+)	<80	<80	(-)
2(EI-5)	< 5	<5	(-)	<5	<5	(-)	<80	<80	(-)	<80	<80	(-)
3(EI-13)	5	10	(+)	20	20	(+)	80	80	(+)	160	80	(+)
4(EI-17)	<5-5	<5-5	(±)	40	40	(+)	<80	<80	(-)	320	80	(+)
5(EI-24)	< 5	<5	(-)	20	20	(+)	320	320	(+)	80	80	(+)
6(EI-25)	<5-5	<5-5	(±)	5	5	(+)	160	160	(+)	80	80	(+)
7(EI-32)	< 5-5	<5-5	(±)	<5	<5	(-)	80	80	(+)	80	<80	(±)
8(EI-39)	40	40	(+)	10	5-10	(+)	320	160	(+)	160	160	(+)
9(EI-46)	< 5	<5	(-)	<5	<5	(-)	<80	<80	(-)	<80	<80	(-)
10(EI-49)	5-10	5-10	(+)	5-10	10	(+)	80	160	(+)	80	160	(+)

*Nos. 1-10 horses examined contained intraerythrocytic parasites in each blood smear slide. Results of the CFT for *B. equi* and *B. caballi* were tested by Dr. N. Komatsu at the Yokohama Animal Quarantine Service.

All sera tested by the IFAT were diluted more than 1:80 as a standard positive result.

S** means the score mark, serum positive(+), trace or false positive(±), or nagative (-) result.