IMMUNOLOGICAL ANALYSIS OF *BABESIA EQUI* PIROPLASM PROTEINS AND DEVELOPMENT OF POLYMERASE CHAIN REACTION BASED ASSAY FOR THE DIAGNOSIS OF EQUINE PIROPLASMOSIS

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(*BABESIA EQUI* ピロプラズム蛋白質の免疫学的解析とDNA診断の開発)

by

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LIST OF ABBREVIATIONS

ABTS	Azino-di-[3-ethylbenzthiazoline]sulfonic acid
bp	Base pair
CF	Complement fixation
DAB	Diaminobenzidine tetrahydrochloride
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyne-linked immunosorbent assay
hr	Hour
IF	Immunofluorescence
IFAT	Immunofluorescence antibody test
kDa	Kilo Dalton
MAP	Multiple antigenic peptide
MoAbs	Monoclonal antibodies
Mr	Molecular mass
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS with Twee-20
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SM	Skim milk
TBS	Tris buffer saline
TE buffer	Tris EDTA
2D	Two dimensional

PREFACE

Babesia equi and *B. caballi* are obligate intracellular parasites of the phylum *Apicomplexa* and cause hemoparasitic diseases in equines. Both parasites are transmitted by ticks and are endemic in most tropical and subtropical areas of the world (Friedhoff *et al.* 1990). Fourteen species of ixodid ticks of three genera, *Dermacentor*, *Rhipicephalus* and *Hyalomma*, have been identified as vectors of either *B. equi* or *B. caballi*. *B. equi* is transmitted only transstadially by nine of these tick species, while *B. caballi* is transmitted both transstadially (four tick species) and transovarially (nine tick species) (Neitz 1956 ; Stiller and Frerichs 1979 ; Stiller *et al.* 1980 ; de Waal and Potgieter 1987 ; de Waal 1990). Due to the wide distribution of the various tick vectors, equine babesiosis presents a global problem for equestrian sports and trade. *B. caballi* and *B. equi* exist together in most regions of the world.

Clinical diagnosis can be made on the basis of typical clinical signs, evidence of exposure to infected ticks or history of blood transfusion. Clinical signs alone are often non-specific and the disease may be confused with a variety of others such as equine influenza, encephalosis virus infections, equine infectious anaemia and trypanosomiasis. It is also not possible to differentiate between *B. equi* and *B. caballi* infections on clinical signs alone (deWaal 1992). The equine piroplasma-parasitized inapparent carrier commonly presents a special problem of diagnosis since outward signs of the disease are not evident (Knowles 1988). Infected animals become carriers probably for life and potentially intrauterine infection can occur throughout the breeding life of the mare (Holbrook *et al.* 1973). Procedures employed to test for the presence of the hemoparasites in chronically infected but otherwise healthy animals include the thick blood smear technique to demonstrate *Babesia*-infected erythrocytes, immunodepression with corticosteroids, splenectomy, brain biopsy only for *B. bovis* and blood inoculation from a suspected carrier to a susceptible splenectomized recipient (Mahony 1962 ; Callow *et al.* 1986).

Definitive diagnosis of the disease is made by demonstration of the parasite in peripheral blood smears. The limits of detection of parasitaemia in stained blood smears are ~ l0-20 parasites / µl of blood (Lanar *et al.* 1989). However, after establishment of the carrier state, there may be a complete absence of circulating parasites in blood, making detection of parasites in blood smears very difficult. Thus the infected horse will be undetected and will remain the reservoir of infection for tick vectors (Todorovic and Carson 1981). Holman *et al.* (1993) reported that culture of horse blood for *B. caballi* identified four carrier horses among nine previously infected horses which were found negative by microscopic examination of blood smears before the start of *in vitro* culture. Currently, regulatory control of equine babesiosis in many countries relies on serological tests (Complement fixation and immunofluorescence tests) to confirm the diagnosis and to identify infected horses whoes movements should be restricted. However, the serological status of a horse may not accurately reflect the current status of a *Babesia* infection in the animal. Indeed, parasitaemias occur prior to the development of a serological response and antibody activity may be detectable long after the ability to transmit the infection has been lost (Enigk 1950). Moreover, serological methods, although easy to perform, are unable to distinguish between active and previous infections.

Each serological assay has some advantages or disadvantages depending on its level of sensitivity, specificity, simplicity, and cost effectiveness. At present, most workers propose the combination of at least two different assays to increase the diagnostic reliability of the serodiagnosis of *Babesia* infections (Tenter and Friedhoff 1986; Weiland 1986; Weiland and Reiter 1988). The complement fixation (CF) test, introduced by Hirato *et al.* (1945) remains the serologic test of choice for equine piroplasmosis. The CF test is not infallible and false positive and negative results do occur (Frerichs *et al.* 1969 ; Donnelly *et al.* 1980 ; Tenter and Friedhoff 1986). Böse and Peymann (1994) compared the CF test with the ELISA and Western blot for *B. caballi*. For sera taken from day 14 after experimental infection the sensitivity of the ELISA was 98.3 %, of the Western blot 94.9 %, of the immunofluorescence antibody test (IFAT) 96.6 %, and only

28.8 % for the CF test.

Recent advances in serodiagnosis designed for the detection of anti-*Babesia* antibodies were partly achieved by improving and standardizing the indirect immunofluorescence (IF) and CF tests. These tests provide no information about the molecules against which the immune response of horses to *B. equi* and *B. caballi* is directed. Consequently, efforts have been made to demonstrate parasite-specific antigens by the generation of monoclonal antibodies and by Western blotting (Knowles *et al*. 1991 a and b ; Böse and Hentrich 1994).

Conventional techniques including serology and microscopy do not always meet the requirements as low levels of parasitaemia can not be detected by blood smears and in serological assays only antibodies against the specific antigens are detected. Therefore, methods using DNA as target molecules have been tested as diagnostic tool. DNA probes for the detection of either *B. equi* or *B. caballi* in blood have been reported (Posnett and Ambrosio 1989 and 1991). The *B. equi* probe is able to detect parasitaemias of 0.0028%, and the *B. caballi* probe can detect parasitaemia of 0.0016%. Polymerase chain reaction (PCR), which was introduced in 1985 has been used to find and identify microorganisms in the environment, among others in samples of soil, sediments and waters. Selected segments of any DNA molecule can be amplified exponentially by PCR. As the polymerase chain reaction can amplify the target sequence many times, detection of even lower parasitaemias in carrier horses is possible. PCR can amplify DNA in a specific sample up to 200,000-fold and could result in even more sensitive diagnostic method. PCR for *Babesia* spp. have been developed which can detect parasite DNA in blood with a parasitaemia of 10^{-9} which is 100 times more sensitive than the limits of microscopic detection (Fahrimal *et al* 1992; Figueroa *et al* 1992; Persing *et al* 1992). Multiplex nonradioactive PCR tests have been developed which can detect *B. bigemina*, *B. bovis* and *Anaplasma marginale* in a single assay; however, the sensitivity is reduced to 10^{-6} for *Babesia* spp. (Figueroa *et al* 1993) .

After identification of immunodominant proteins of infecting organisms the next important step may be the use of these proteins for the prevention of the disease. The recent advances in immunology and biotechnology have stimulated much research on the control of parasitic diseases through vaccination. Some of the fundamental steps in the design of a parasitic vaccine are (1) to identify the protective antigens, (2) to produce them in useful quantities, and (3) to administer them in the most suitable manner. A vaccination program for tournament horses with either attenuated merozoites or inactivated antigens would be desirable (Schein 1988).

In the first part of this thesis, *B. equi* piroplasms were purified from erythrocytes of experimentally infected horse and the immunogenic proteins of piroplasms ranging from Mr 15 kDa to 96 kDa were identified using serum from experimentally infected horse. The N-terminal amino acid sequences of immunodominant surface proteins of p28 and p30 were determined and peptides were synthesised according to the N-terminal sequences of these proteins. Piroplasm antigens of *B. equi* and *B.* caballi were analysed using antisera produced against p28 and p30 synthetic peptides. In the second part of the thesis, monoclonal antibodies were produced against *B. equi* piroplasms and epitopes on 18 kDa surface protein were characterized using monoclonal antibodies. PCR was developed in the third part of the thesis which can amplify the gene encoding p37 of *B. equi* from *B. equi* DNA in horse blood with 0.001% parasitaemia.

PART I

PROTEIN CHARACTERIZATION OF *BABESIA EQUI* PIROPLASMS ISOLATED FROM INFECTED HORSE ERYTHROCYTES

INTRODUCTION

Babesia equi and *B. caballi* are intraerythrocytic protozoa which are tick-borne and give rise to diseases in Equidae generally called piroplasmosis. The diseases are characterized by fever, anemia, icterus, hepato- and splenomegaly. Most of the horse and donkey population of the world (approximately 120 million) is exposed to equine babesiosis and lives in endemic areas (Jasiorowski *et al.* 1985). The differential recognition of *B. equi* and *B. caballi* in mixed equine piroplasma infections is crucial. Various serological techniques have been used to identify infected animals. Serological differentiation between *B. equi* and *B. caballi* is possible by CF test and , to a certain extent, by indirect IF test during early infection, but not by ELISA. Strong cross-reactions are observed between *B. equi* and *B. caballi* when diagnosed by ELISA (Weiland 1986). Most researchers propose that a combination of at least two different assays should be used to increase the diagnostic reliability of the serodiagnosis of *Babesia* infections. Therefore, there is a need to identify new antigens to be used for the improvement of existing serodiagnostic assays. In this study, the *B. equi* piroplasms were purified by a combination of N_2 gas cavitation and Percoll density gradient centrifugation and piroplasm proteins were analyzed by one- and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The immunodominant proteins of piroplasms recognized by sera from infected horses were also identified by immunoblotting method.

N-terminal amino acid sequences of surface immunodominant 28 and 30 kDa *B. equi* proteins were determined and rabbit antisera were produced against peptides which had been synthesised according to the N-terminal sequences of p28 and p30 using a multiple antigenic peptide (MAP) system (Tam 1988). This system has been developed to induce effective immune responses against synthetic peptides. It uses a small peptidyl core matrix bearing radially branching synthetic peptides as dendritic arms and can be injected without conjugating to carrier proteins to produce anti-peptide antibodies. Piroplasm antigens of *B. equi* and *B. caballi* were analysed using antisera produced against p28 and p30 synthetic peptides.

MATERIALS AND METHODS

Purification of piroplasms

Nonsplenectomized horses were inoculated intravenously with 5 ml of *B. equi* infected blood which had been obtained from National Veterinary Services Laboratories, U.S. Department of Agriculture, Iowa, USA. Heparinized blood was collected from the horse when the parasitemia was > 15%, as determined by Giemsa staining. Erythrocytes were washed three times with phosphate-buffered saline (PBS) and were suspended in PBS to give a 50% (v/v) suspension. The suspension was passed through a cellulose column (CF11, Whatman, Kent, England) to remove leukocytes. The erythrocytes were disrupted by a nitrogen gas cavitation method as described by Shimizu *et al.* (1988) and the lysate was centrifuged at 1,400 xg for 20 min to remove unlysed erythrocytes. The supernatant was then centrifuged at 12,700 xg for 20 min. The pellet was applied on 60 % (v/v) Percoll solution prepared in Tris-buffered saline (TBS ; 10 mM Tris- H Cl, 150 mM Na Cl, pH 7.4) containing 5 mM EDTA and centrifuged at 3,600 xg for 20 min. The supernatant which contained piroplasms and erythrocyte membranes was applied on 20 % (v/v) Percoll and centrifuged at 3,600 xg for 20 min. The pellet containing piroplasms were stored at - 80 °C.

Piroplasms of *B . caballi* were also prepared as described above from erythrocytes of splenectomised horses but this fraction contained erythrocyte membranes as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The pellet containing piroplasms was stored at - 80 °C until use. Membranes of uninfected horse erythrocytes prepared by the method of Edwards *et al.* (1979) were used as control antigen.

Phase partition by Triton X-114

Piroplasm proteins were subjected to Triton X-114 phase partition according to the method of Bordier (1981). Purified piroplasms were treated with TBS containing 1% (v/v) Triton X-114 and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. The lysate was centrifuged for 1 hr at 100,000 xg and the pellet was discarded. The supernatant was carefully layered over an equal volume of a 6 % (w/v) sucrose cushion in TBS containing 1 mM PMSF. After incubation at 30 °C for 15 min, the sample was centrifuged at 1,000 xg for 15 min at room temperature . The top aqueous phase and bottom detergent phase were collected. Each fraction was precipitated with 4 volumes of acetone and centrifuged at 6000 xg for 10 min at 4 °C. The pellets were stored at - 80 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

One-dimensional SDS-PAGE was performed using a 10-20 % (w/v) gradient polyacrylamide gel according to the method described by Laemmli (1970). Samples were solubilised in Tris-HCl buffer (0.0625 M, pH 6.8) containing 2 % (w/v) SDS and 5 % (w/v) 2-mercaptoethanol (Laemmli 1970) at 100 °C for 2 min. Solubilized samples of erythrocyte membranes obtained from an uninfected horse by the method of Edwards *et al.* (1979) were also analyzed. After electrophoresis, gels were stained with Coomassie brilliant blue G-250 (Kodak, New York, USA) or with a silver staining kit (Dai-ichi Pure Chemicals Co., Tokyo, Japan). For two-dimensional (2D) SDS- PAGE (Anderson and Anderson 1978 a,b), samples were solubilized in the solubilization buffer composed of 9 M urea , 4 % (v / v) NP-40, 2 % (v / v) 2-mercaptoethanol and 2 % (v / v) ampholines. Isoelectric focusing was used as first dimensional gel electrophoresis. The ampholines used were a mixture of pH 4-6 and 3.5 - 10 ampholines (Pharmacia LKB

Biotechnology AB, Bromma, Sweden). A 10-20 % gradient polyacrylamide gel was used as second dimension. Gels were silver stained after electrophoresis as described above.

Immunoblotting

After separation by SDS-PAGE, the piroplasm proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) sheets (Immobilon transfer membranes; Millipore Bedford, Mass., USA) according to the immunoblotting technique of Dunn (1986). The sheets were blocked for 1 hr at room temperature in 0.01 M PBS (pH 7.2), 0.1% (v/v) Tween-20 (PBST) containing 5% skim milk (SM). These were washed three times with PBST and incubated for 1 hr at room temperature with sera collected from horses infected with *B. equi* (diluted 1:200 and 1:50) or *B. caballi* (diluted 1:50) in PBST with 1% SM. The sera had CF titres of 1:80 and 1:640, respectively. A preinoculation horse serum was used as a control. The sheets were washed and incubated for 1 hr at room temperature with rabbit anti-horse IgG peroxidase conjugate (Cappel Corp., West Chester, PA, USA) diluted 1:1000 in PBST with 1 % SM. After washing, the sheets were treated with a freshly prepared substrate solution containing 1.3 mM diaminobenzidine tetrahydrochloride (DAB; Nakarai Chemicals, Ltd, Kyoto, Japan), 1.3 mM $CoCl_3$ and 0.02% (v/v) hydrogen peroxide in PBS.

Determination of N-terminal sequences of membrane proteins

Proteins partitioned into the Triton X-114 phase were subjected to SDS-PAGE and transferred electrophoretically to PVDF membrane sheet (Immobilon SQ, Millipore Bedford, Mass., USA). Protein bands stained by Coomassie blue R-250 were cut out and applied to a pulsed liquid automated protein sequencer (476A, Applied Biosystems, foster city, CA, USA).

Production of anti synthetic peptide sera

p28 peptide (Fig 7, D^{1}-T^{16}) and p30 peptide (Fig 7. E^{1}-L^{16}) for immunization were synthesized, based on the respective N-terminal sequences using the multiple antigenic peptide system (Tam 1988). An automated peptide synthesizer (PSSM-8, Shimazu, Kyoto, Japan) with F-moc chemistry was used. One milligramme of each peptide was injected intramuscularly to the rabbits with Freund's complete adjuvant. After 2 weeks, booster injections were given twice at an interval of 2 weeks with Freund's incomplete adjuvant and sera were collected one week after the third immunisation. The immunoblotting was carried out as described above.

RESULTS

Piroplasm purification and protein analysis

After disruption of infected equine erythrocytes, piroplasms were purified by differential centrifugation and density gradient centrifugation in Percoll solutions. The piroplasm preparation was analyzed by one- and two-dimensional SDS-PAGE to estimate contamination with erythrocyte proteins (Figs. 1 and 2). In one dimensional SDS-PAGE analysis, proteins ranging from 18 to 96 kDa were detected in piroplasm preparations. However, 18 kDa band of piroplasm protein seemed to comigrate with a 18 kDa erythrocyte protein (Fig. 1). By 2D SDS-PAGE (Fig. 2 A), most of the major protein spots of the piroplasm appeared in the neutral to basic area of the gel. An 18 kDa spot was identified in the piroplasm preparation, but the erythrocyte preparation did not have any protein in the same area (Fig. 2B). The 18 kDa protein of equine erythrocytes (Fig. 1) was located on basic side of the 2D gel (Fig. 2B). Major erythrocyte membrane proteins, including bands 1 and 2 (spectrin), band 3 (Mr 96 kDa), band 5 (actin, Mr 43 kDa) were not detected in the piroplasm preparations (Fig. 2 B).

Triton X-114 phase partition

To identify membrane-bound proteins, Triton X-114 phase partition were performed. As shown in Fig. 3, proteins of Mr 28, 30 and 32 kDa were partitioned into Triton X-114 phase, and predominated in this fraction. Several other minor protein bands including 18 kDa protein were also detected.

Detection of proteins recognized by sera from infected horses

A serum from an experimentally infected horse was reacted with 15,18, 28, and 30 kDa proteins and several other high molecular weight purified *B. equi* proteins (Fig. 4, lane C). Strong reactions with 18 and 28 kDa membrane-bound proteins were detected

(Fig. 4, lane B). A weak reaction with immunogenic protein of 30 kDa was also identified in Triton X-114 phase (Fig. 4, lane B). In immunoblotting of proteins separated by 2D SDS-PAGE, two immunodominant proteins of Mr 18 and 28 kDa were localized in the neutral and basic areas in the gel, respectively (Fig. 5).

Immunogenecities of piroplasm proteins of *B. equi* and *B. caballi* were analysed by immunoblotting using sera from horses infected with *B. equi* or *B. caballi* (Fig. 6). A serum from a *B. equi* -infected horse reacted with 18, 28, 30, 33 and 37 kDa proteins and cross-reacted with *B. caballi* proteins of 28, 30 and 46 kDa (Fig 6. panel A). The serum from a *B. caballi* infected horse strongly reacted with 23, 25, 29, 31, 36, 40, 44, 52, 56, 62 and 70 kDa proteins and cross-reacted with 24, 44, and 78 kDa proteins of *B. equi* (Fig 6. panel B).

Determination of N-terminal sequences of membrane proteins

The membrane bound *B. equi* proteins of 28 and 30 kDa were enriched by the Triton X-114 phase partioning method before determination of their N-terminal amino acid sequences. The N-terminal amino acid sequences of p30 and p28 are shown in Figure 7. Both sequences contained a stretch rich in charged amino acids, lysine (K) and glutamic acid (E), which is a characteristic sequence found at N-terminals of immunodominant piroplasm surface proteins of the bovine *Theileria* species, *T. sergenti* and *T. buffeli.* As aligned in Figure 7, p32 of *T. sergenti* and p33 of *T. buffeli* contained several conserved amino acids with the two *B. equi* proteins. The reactivities of *B. equi* and *B. caballi*-infected horse sera were tested against *T. sergenti* piroplasm proteins in order to investigate antigenic relationships among these parasites (Fig. 6). Both of the sera reacted with the 44 kDa and several other proteins of *T sergenti*, but not with p32 of *T. sergenti*.

Detection of proteins recognized by anti synthetic peptide sera

Based on the N-terminal amino acid sequences of p28 and p30, peptides were synthesised using the MAP system and used for immunization of rabbits. Reactions of rabbit sera against p30 and p28 synthetic peptides were determined by immunoblot analysis (Fig. 8). The peptide sera reacted with their corresponding proteins, p30 and p28 of *B. equi* piroplasms, respectively. No cross reactions between p28 and p30 were detected by the sera although the two peptides contained a common sequence, PK-SGAVV. The serum against p28 peptide also reacted weakly with 94, 64 and 39 proteins. The serum against p30 peptide reacted with 68 and 62 kDa bands of *B. equi*, *B. caballi*, *T. sergenti*, and uninfected horse erythrocyte membrane proteins.

DISCUSSION

The comparison of the protein spot patterns of *B. equi* piroplasms and membrane from uninfected equine erythrocytes separated in 2D SDS-PAGE demonstrated very little contamination from equine erythrocyte membranes in the piroplasm preparation. Separation of a morphologically intact parasite with minimum contamination of host erythrocyte is the first step in the antigenic analysis of intraerythrocytic protozoan parasites. A variety of methods used for this purpose have been reviewed by Mahoney (1972) and Mahoney and Goodger (1981). The quality of liberated parasites can be evaluated in terms of purity with regard to contamination by the host cell and the morphological integrity, viability and antigenic activity of the parasite. Shimizu *et al.* (1988) purified *Theileria sergenti* piroplasms from infected erythrocytes by the nitrogen gas cavitation method and showed by light and electron microscopy that the morphological integrity of piroplasms was maintained. The piroplasms obtained were found to be a suitable antigen for the development of ELISA for the diagnosis of infection. In the present study, *B. equi* piroplasms released by the nitrogen gas cavitation method were further purified by centrifugation using Percoll.

The purification method for *B. equi* piroplasms developed in the present study gives the scope to improve the sensitivity of the present serological tests including the CF test, indirect IF test and ELISA. Böse and Daemen (1992) suggested the use of Western blotting for the diagnosis of *B.* caballi infections to avoid the contradicting results of CF and IIF tests. Recently, piroplasm proteins of *B. equi* and *B. caballi* were reported with Mr ranging from 210 to 25 kDa and 141 to 30 kDa, respectively (Knowles *et al.* 1991a ; Böse and Daemen 1992). Methods for differential diagnosis of *B. equi* and *B. caballi* can be improved by using the purified *B. equi* piroplasms antigen. By a combination of Triton X-114 phase partition method and immunoblotting several immunodominant *B. equi* piroplasm proteins were identified in this study. Three of them, of Mr 18, 28

and 30 kDa proteins are revealed to be membrane-associated. It is necessary to determine whether these proteins are involved in reactions in various serological tests for equine babesiosis including CF test, ELISA and indirect IF test. Further characterization of these immunodominant proteins should be carried out to improve these serological tests in terms of sensitivity and specificity.

Knowles *et al.* (1991a) immunoprecipitated 44, 36, 34 and 28 kDa proteins metabolically labelled with ^{3}H using a monoclonal antibody (36/133.97) against live *B. equi* merozoites. The 28 kDa protein was identified as immunodominant because of its ability to induce high titred antibody response in both naturally and experimentally infected horse. They did not detect serological responses to the 18 kDa protein, which was identified as one of the immunodominant proteins in the present study. This disagreement between this study and their results may be attributed to the difference in the methodology used. If the 18 kDa protein is poorly synthesized or radiolabeled *in vitro*, this cannot be detected by the immunoprecipitation.

Anti-peptide sera were produced against p28 and p30, immunodominant piroplasm proteins of *B. equi* based on the sequence of N-terminal amino acids. The sera reacted with their corresponding proteins in immunoblot analysis. Anti-p28 peptide serum reacted specifically with *B. equi* antigen; it is useful for serological differentiation of the two *Babesia* species of horses. This serum reacted with other piroplasm proteins, which indicates that they share B cell epitope(s). Alternatively, they are proteolytically produced from one higher molecular weight precursor, as known for the surface proteins of *Plasmodium falciparum* (Kemp *et al.* 1990). Anti-p30 peptide serum reacted with *B. caballi*, *T. sergenti* and uninfected horse erythrocyte membrane preparations. This result indicates that N-terminal sequence of p30 may contain similar sequences to those of equine and bovine erythrocyte membrane proteins.

N-terminal amino acid sequence analysis of p30 and p28 of *B. equi* piroplasm proteins also revealed their relationships with the surface proteins of other piroplasms in cattle, p 32 of *T sergenti* and p34 of *T. buffeli* (Kawazu *et al.* 1992 ; Matsuba *et al.* 1993),

which provides evidence for a close relationship of *B. equi* and *Theileria* species. *B. equi* is often considered to be excluded from the genus *Babesia* because of the presence of *Theileria*-like intralymphocytic schizonts (Mehlhorn and Schein 1984). Structural similarity of these piroplasm surface molecules indicates that they may have similar functions and play important roles in their interaction with host cells including growth within erythrocytes and invasion of uninfected erythrocytes. A surface protein of the malaria parasite at its intraerythrocyte stage, MSP-1, interacts with erythrocyte spectrins, and this interaction is important for the parasite development from trophozoites into mature schizonts (Herrera *et al.* 1993). The interaction may be mediated by tripeptide motifs, KEL or LEK in the molecule. Similar motifs have been identified in p32 of *T. sergenti*.

There are several practical problems in differential diagnosis between *B. equi* and *B. caballi* by current serological tests, including CF test and ELISA. These may be due to several cross-reacting proteins of the two parasite species and to the production of antibodies against their own erythrocyte proteins in infected horses. MoAbs have been produced against *B. equi* piroplasm surface molecules (Knowles *et al.* 1991 a) and used for competitive ELISA tests (Knowles *et al.* 1991 b). Their MoAb immunoprecipitated three polypeptides of 28, 34 and 36 kDa in similar amounts, and the 44 kDa polypeptide in a smaller quantity. The anti-p28 peptide serum produced in this experiment reacted predominantly with p28, with low reactivities against other piroplasm proteins. As the nature of the antigen involved is better chemically defined by antipeptide sera, they can be used as immunological reagents for the development of more specific and sensitive diagnostic tests.

Progress has been made towards the development of a vaccine against bovine babesiosis using merozoite-derived antigens (Wright *et al.* 1985 ; Goodger *et al.* 1987) . A practical problem with the use of merozoites is their purification and large scale production. Recently emphasis has been shifted from crude antigen preparations towards purified polypeptides produced by biochemical techniques. Subunit vaccines

can be produced in large amounts as the desired protein can be synthesized in bacteria, yeast, or animal cells using recombinant DNA technology. Further characterization of 18, 28 and 30 kDa proteins is important in the search of a better immunogenic reagents for the diagnosis and vaccination of *B. equi*.

ABSTRACT

Proteins of *Babesia equi* piroplasms were characterized. The piroplasms of *B. equi* were purified by lysis of infected horse erythrocytes with N_2 gas cavitation followed by separation in Percoll density gradient centrifugation. Molecular weights (Mr) of major proteins separated by two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis were 18, 28, 30, 41, 43, 54, 66.5 and 96 kDa. Immunoblot analysis using serum from an experimentally infected horse revealed 6 immunodominant proteins of 15, 18, 28, 30, 41 and 96 kDa. Three immunodominant proteins of Mr 18, 28 and 30 kDa were membrane- bound proteins as revealed by Triton X-114 phase partitioning.

N-terminal amino acid sequences of two membrane proteins of *Babesia equi* piroplasms were determined. The sequence of p28 contained a region rich in charged amino acids (lysine and glutamic acid), which is a characteristic primary structure found in N-terminal region of major piroplasm surface proteins of the bovine *Theileria* parasites, *T. sergenti* and *T. buffeli*. Peptides were synthesized according to the N-terminal sequences of p28 and p30, and antisera were produced. The anti-peptide sera reacted with the corresponding proteins of piroplasms. As these sera showed no cross-reactions with *B. caballi* piroplasm antigens, they could be used for serological differentiation of the two *Babesia* species in horses.

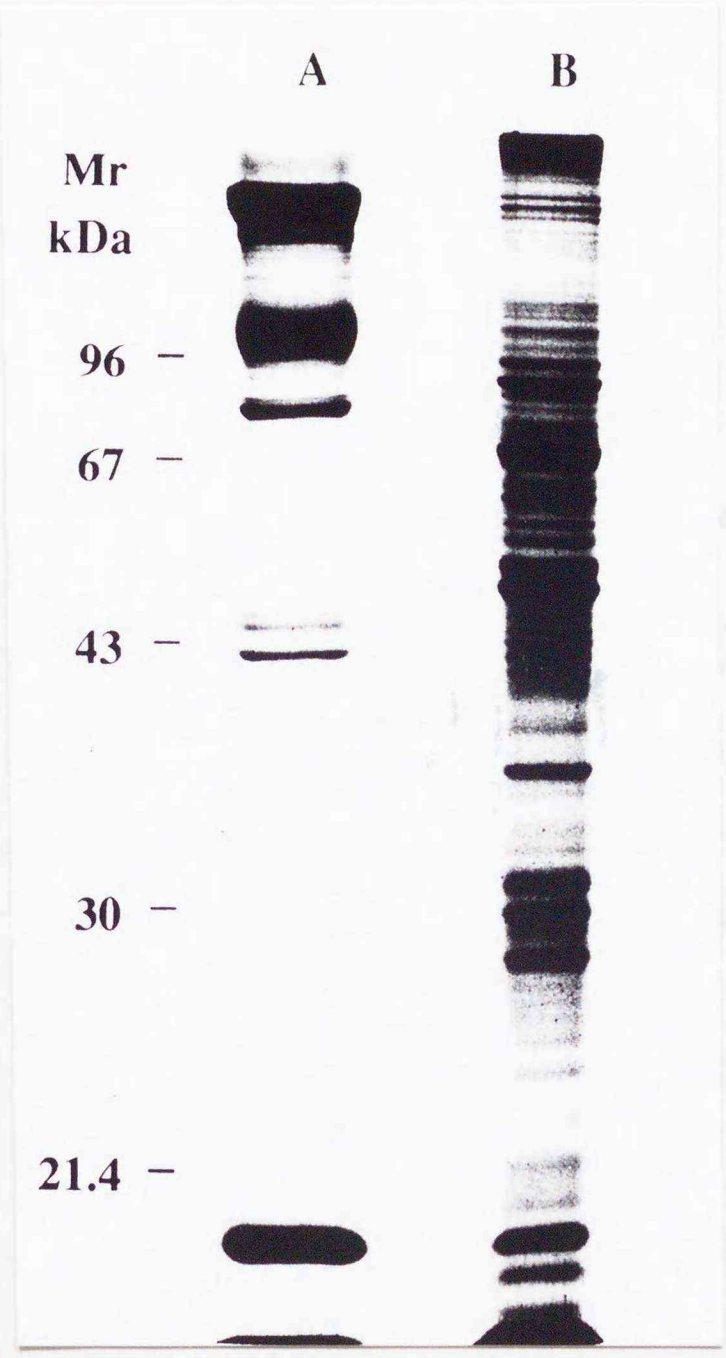

Fig. 1. Protein analysis of *B. equi* piroplasms by one-dimensional SDS-PAGE.

Lane A : membrane from uninfected horse erythrocytes. Lane B : purified piroplasm. Gel was stained with Coomassie brilliant blue G250.

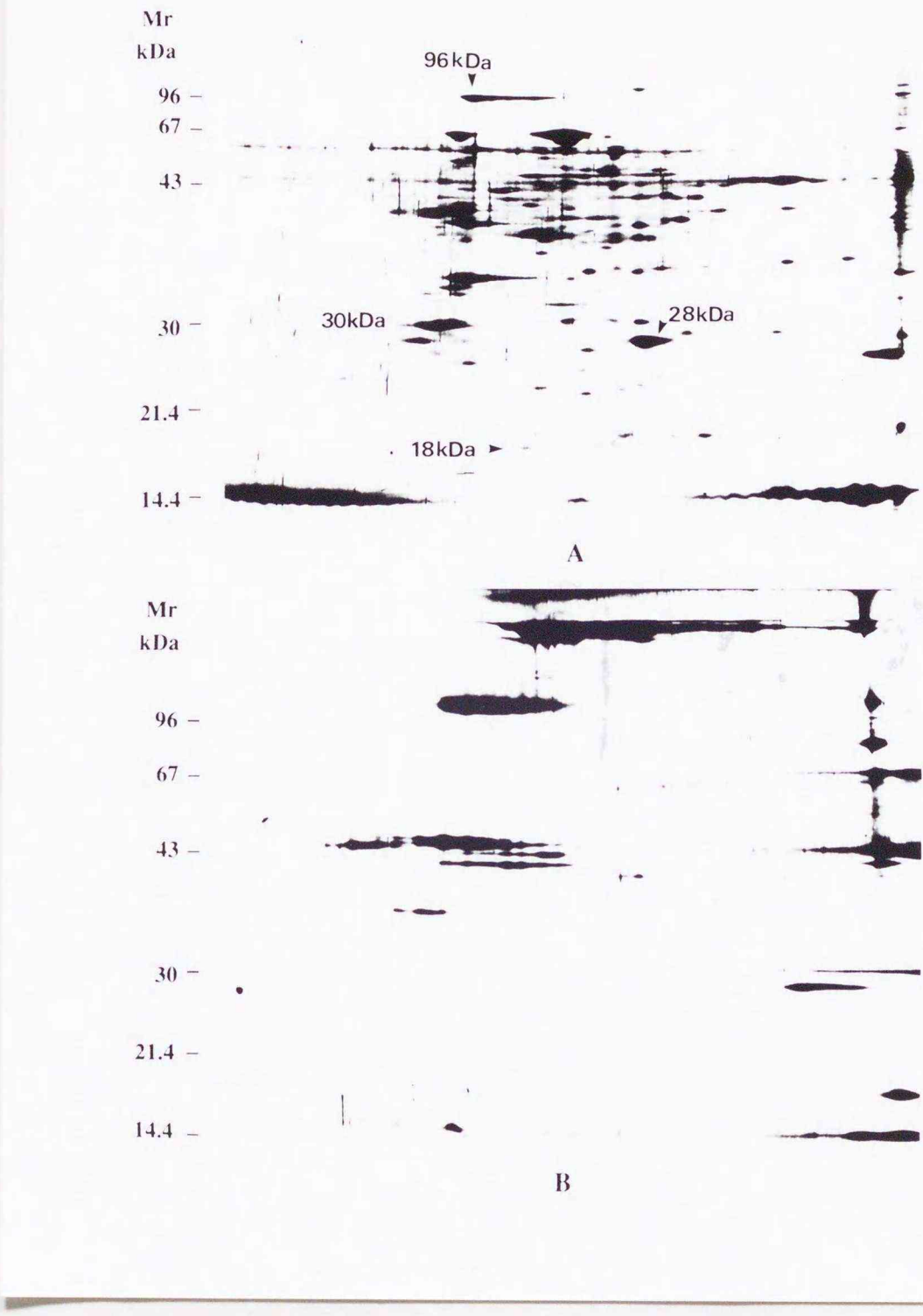

Fig. 2. Protein analysis of *B. equi* piroplasms by two-dimensional SDS-PAGE.

A : purified piroplasm. B : membrane from uninfected horse erythrocytes. Gels are oriented with basic end to the right and acidic end to the left. Gels were silver stained after electrophoresis. Spots marked by arrow heads (Mr 96,30,28 and 18 kDa) correspond to the spots detected by 2D-immunoblot (Fig. 5A).

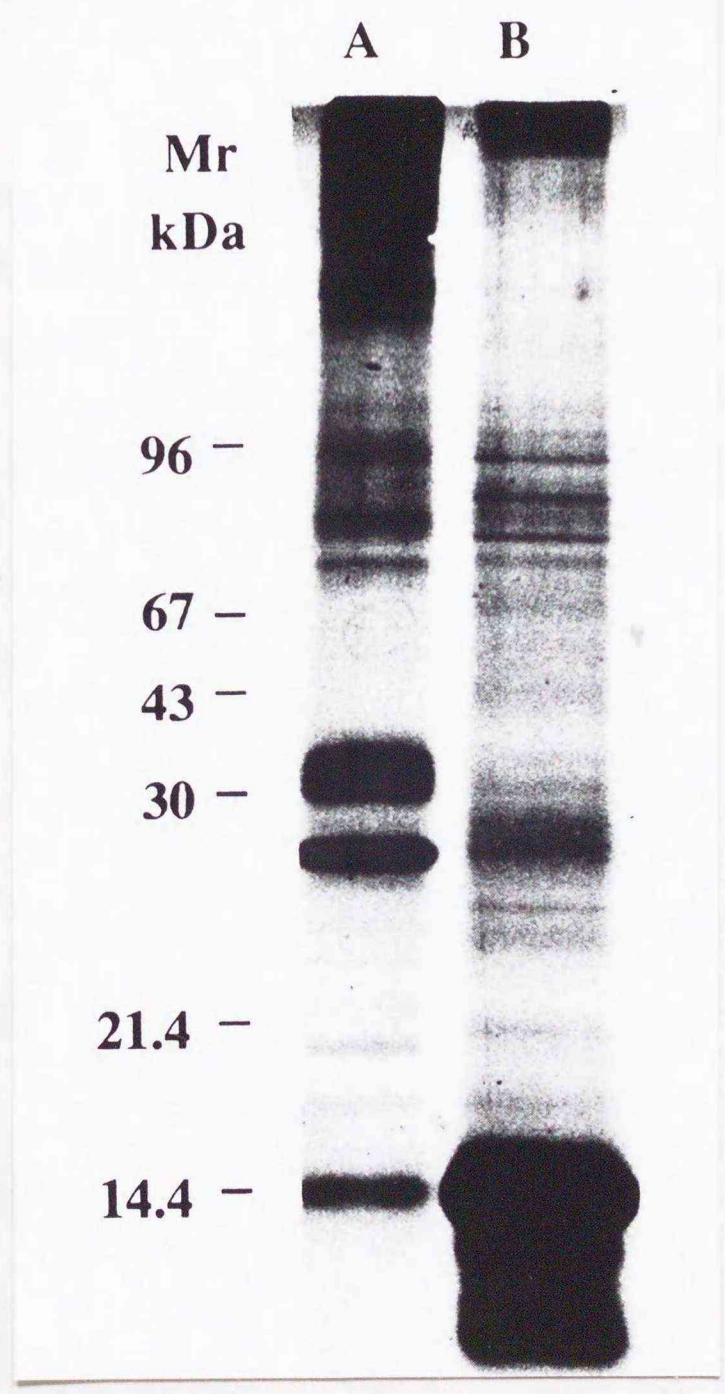

Fig. 3. Triton X-114 phase partition of *B. equi* piroplasm proteins.

Lane A : detergent phase and Lane B : water phase proteins . Gels were silver stained after electrophoresis.

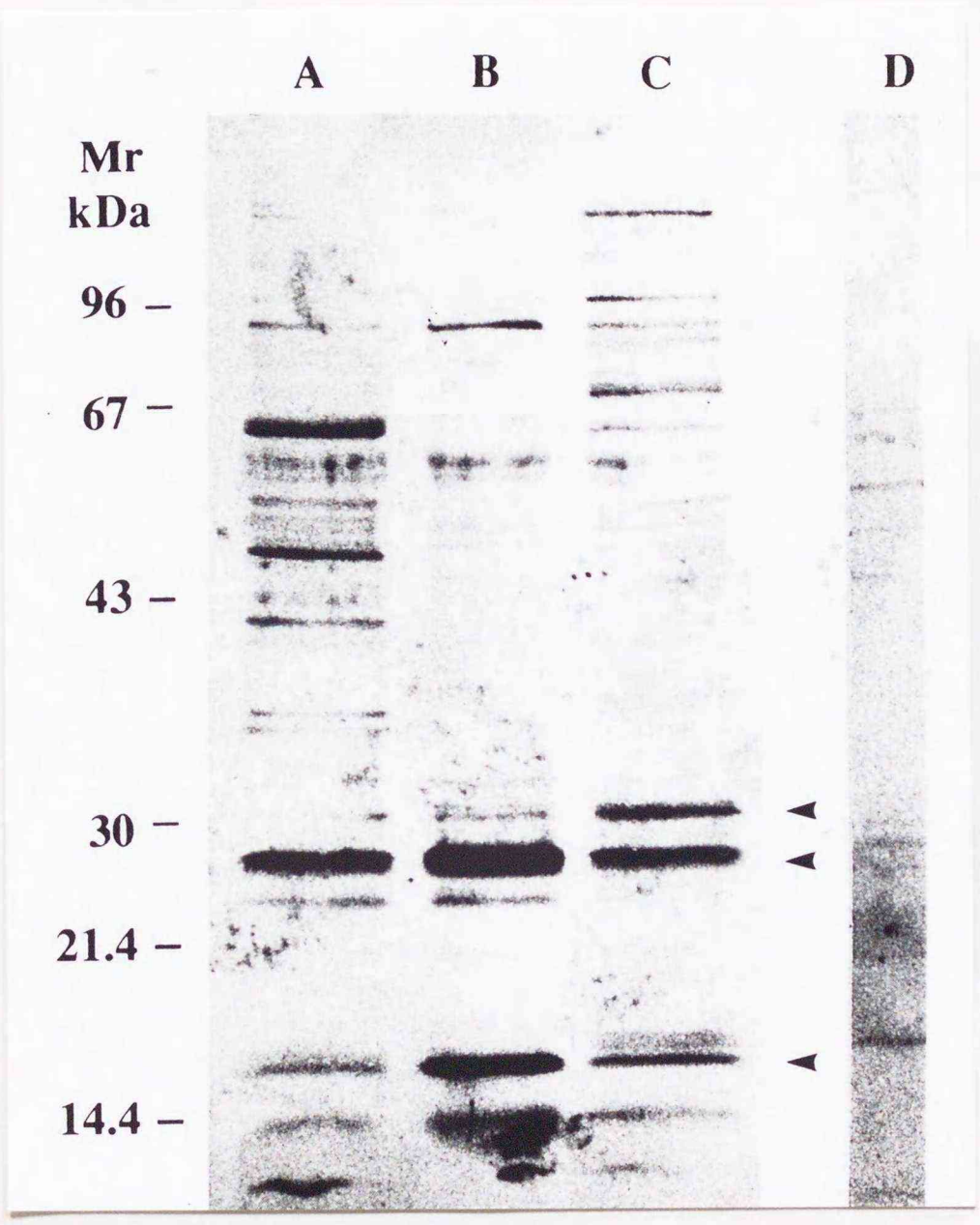

Fig. 4. Immunoblotting of *B. equi* piroplasms proteins partitioned by Triton X-114.

Lane A : water phase. Lane B: detergent phase and Lanes C and D : purified *B. equi* piroplasms . Lanes A, B and C were probed with a serum from experimentally infected horse. Lane D was probed with a preinoculation horse serum. Positions of Mr 18, 28 and 30 kDa proteins are marked by arrow heads.

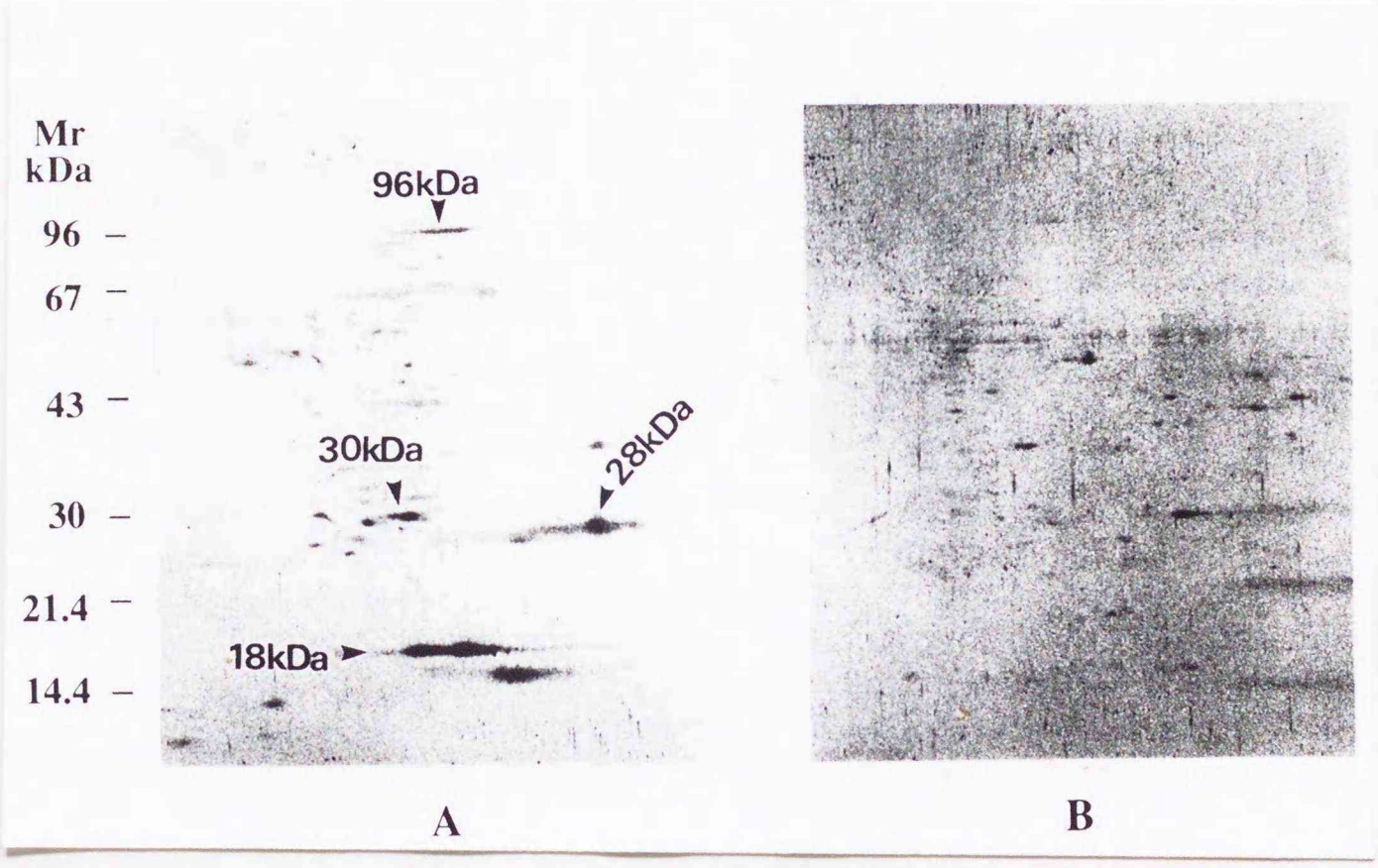

Fig. 5. Identification of immunodominant proteins of *B. equi* piroplasms by 2D SDS-PAGE and immunoblotting.

A : probed with a serum from experimentally infected horse. B : probed with a preinoculation horse serum. Spots marked by arrow heads (Mr 96,30,28 and 18 kDa) correspond to the spots in silver-stained 2D-PAGE gel (Fig. 2A).

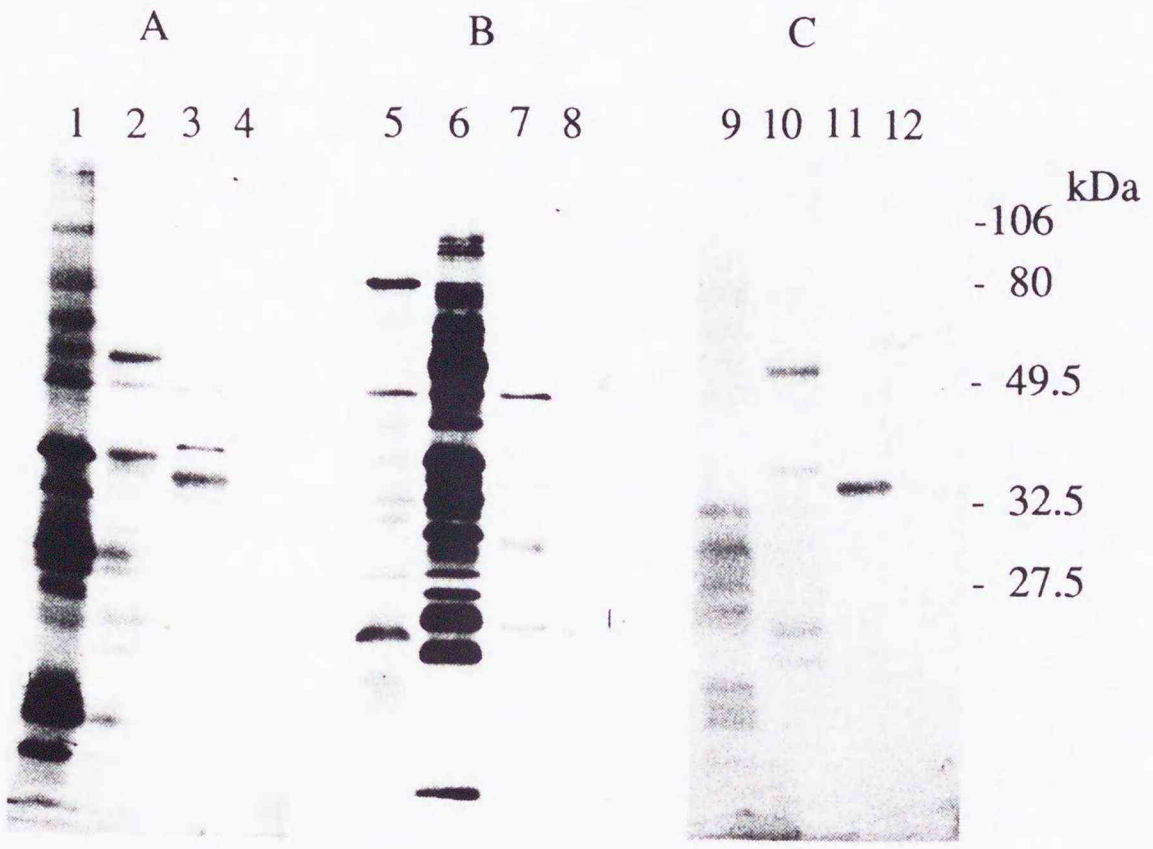

Fig 6. Analysis of piroplasm antigens of *B. equi*, *B. caballi* and *Theileria sergenti* by immunoblotting with experimentally infected horse sera.

Proteins of *B. equi* (lanes 1,5,9) *B. caballi* (lanes 2 ,6,10) , *T. sergenti* (lanes 3 ,7,11) and uninfected equine erythrocyte membrane (lanes 4 ,8,12) were separated in 12.5 % polyacrylamide gels and probed with sera of *B. equi*-infected (panel A) , *B. caballi*-infected (panel B) or uninfected (panel C) horses.

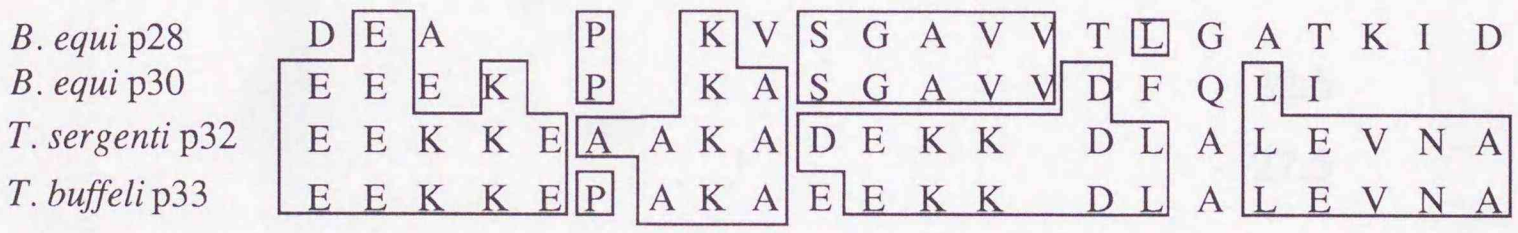

Fig 7. N-terminal amino acid sequences of *B. equi* p28 and p30 and alignment with the deduced sequence of *T. sergenti* and *T. buffeli* piroplasm surface proteins.

Identical residues are boxed. GenBank accession numbers of *T. sergenti* and *T. buffeli* proteins are D12691 and D11047, respectively.

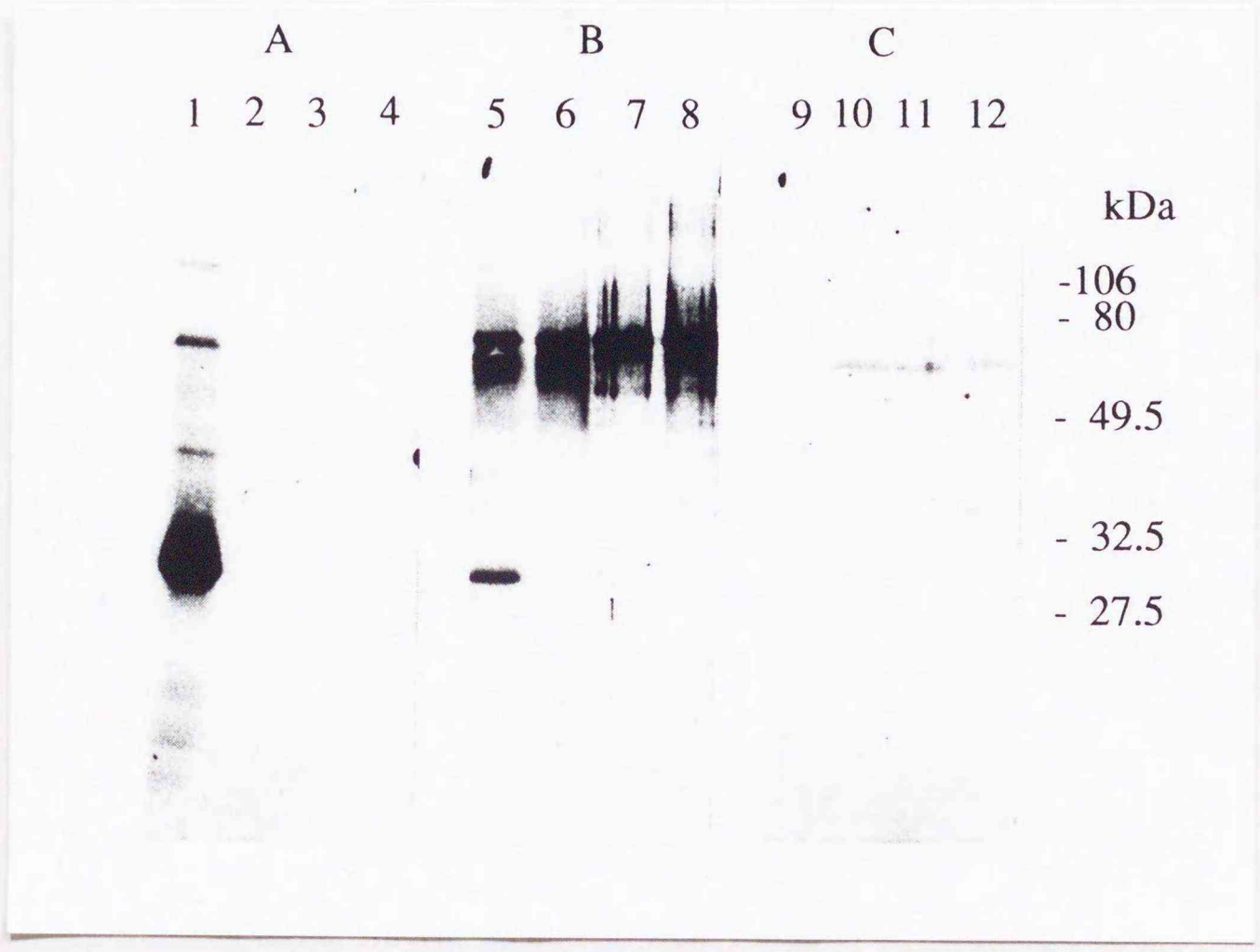

Fig 8. Analysis of piroplasm antigens of *B. equi*, *B. caballi* and *T. sergenti* by immunoblotting with anti-p28 and p30 peptide rabbit sera.

Proteins of *B. equi* (lanes 1 ,5,9.), *B. caballi* (lanes 2,6,10) , *T. sergenti* (lanes 3,7,11) and uninfected equine erythrocyte membrane (lanes 4,8,12) were separated in 12.5 % polyacrylamide gels and probed with anti-p28 (panel A), p30 peptide (panel B) and normal (panel C) rabbit sera.

PART II

CHARACTERIZATION OF EPITOPES ON AN 18 KDA PIROPLASM SURFACE PROTEIN OF *BABESIA EQUI*

INTRODUCTION

Equine babesiosis is one of the major problems in horse trade especially due to the danger of disease transmission during the movement of race horses from country to country. Efforts have been made to demonstrate parasite-specific antigens by the generation of monoclonal antibodies (MoAbs) against *B.equi* proteins and Western blot analysis using immune sera against the *B. equi.* A competitive-inhibition enzyme-linked immunosorbent assay (ELISA) using a MoAb recognizing a geographically conserved epitope has been described (Knowles *et al.* 1991 a and b). None of the antigens diagnostic for European isolates of *B equi* were recognized by sera from field-infected horses with *B.caballi* from Brazil (Böse and Hentrich 1994). Previously six immunodominant *B. equi* piroplasm proteins were identified (Ali *et al.* 1993- first part of this thesis). Out of these, Mr 18, 28 and 30 were found to be membrane bound proteins. It is necessary to determine whether these proteins are involved in reactions in various serological tests for equine babesiosis. In this experiment MoAbs against *B. equi* piroplasms were produced. These MoAbs were used in competitive inhibition and two-site ELISA to characterize epitopes on the 18 kDa surface protein of *B. equi* piroplasms.

MATERIALS AND METHODS

Parasite and antigen preparations

Nonsplenectomized horses were inoculated with *Babesia equi* -infected blood that had been obtained from National Veterinary Services Laboratories (United States Department of Agriculture, Iowa, USA). Piroplasms of *B. equi* were purified from infected horse erythrocytes as described before (Ali *et al.* 1993 - first part of this thesis). For preparation of ELISA antigen, purified piroplasms were treated with 2% Nonidet P-40 in phosphate-buffered saline (PBS, pH 7.2) at 4 °C for 3 hr. After centrifugation at 2,000xg for 10 min, the supernatant was used for ELISA according to the method of Shimizu *et al.* (1988).

South African *B. equi* antigen used in the Western blot analysis was kindly supplied by Dr. E. Zweygarth, Onderstepoort Veterinary Institute, Onderstepoort, South Africa. Dr. V.T. Zablotsky of All Russia Institute of Experimental Veterinary Medicine (VIEV), Moscow, Russia has kindly provided the Russian *B. equi* antigen used in this experiment.

Preparation of monoclonal antibodies

Six weeks old BALB/c mice were subcutaneously immunized with 100 µg protein / 0.1 ml of *B.equi* piroplasms emulsified with the same volume of Freund's complete adjuvant. They were boosted with the same amount of the antigen two weeks after the first injection. The spleen was removed 3 days after a second booster. Spleen cells ($1x10^8$ cells) were fused with $1x10^7$ myeloma cells (P3U1) in the presence of ployethylene glycol (PEG 1500, Boehringer Mannheim, GmbH, Mannheim, Germany). Hybrid cells were selected by HAT medium (hypoxanthine-aminopterine-thymidine). Hybridoma supernatants were screened by immunoblotting. The antibody-producing

hybridomas were cloned twice by the limiting dilution method in a 96 well plastic microplate.

To determine the class, subclass and light chain type of the MoAbs, ammonium sulphate precipitated and protein G affinity purified MoAbs were examined by a MoAb isotyping kit (Amersham International plc., Amersham, UK).

Purification and biotinylation of MoAbs

MoAbs were purified from hybridomas grown in serum-free medium and from ascitic fluids by precipitation with 50% ammonium sulphate and protein G affinity chromatography using MAb Trap G II kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). For biotinylation of MoAbs, sulfosuccinimidobiotin (Rockford, Illinois, USA) was used to covalently bind biotin to purified MoAbs. One ml of the purified MoAbs (1 mg/ml) was mixed at a ratio of 250 µg of ester / mg of antibody and incubated at room temperature for 4 hr. Incubation reaction was stopped by the addition of 20 µl of 1 M NH_4Cl per 250 µg of ester. The mixture was dialyzed against PBS and 0.1% thimersal was added as preservative. Labelled MoAbs were stored at 4 °C till use.

The specificity and optimal concentration of the biotinylated MoAbs were determined by a standard ELISA, as described below, using *B. equi* piroplasm antigen and avidin-conjugated peroxidase (Zymed Laboratories, San Francisco, CA, USA).

Phase partition by Triton X-114

Piroplasm proteins were subjected to Triton X-114 phase partition according to the method of Bordier (1981) as explained in the Part I of this thesis under the same heading.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Parasite proteins were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunobloted on PVDF membrane as described in the first part of this thesis (Ali *et al.* 1993) The PVDF sheets were incubated for 1 hr at room temperature with MoAbs. The sheets were washed and incubated for 1 hr at room temperature with goat anti-mouse IgG peroxidase conjugate (Jackson Immunoresearch Inc., West Grove, PA, USA) diluted 1:1000 in PBST with 1 % SM. After washing, the sheets were treated with a freshly prepared substrate solution containing 1.3 mM DAB, 1.3 mM $CoCl_3$ and 0.02% (v/v) hydrogen peroxide in PBST.

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed according to the method described previously (Shimizu *et al.* 1988) with little modification. Briefly, optimal concentrations of antigen, antibody and enzyme conjugate were determined by checker board titration. Serial two fold dilutions of 36 µg per ml piroplasm proteins / 0.1 ml in 0.05 M carbonate / bicarbonate buffer (pH 9.6) per well, were tested through ELISA using serial two-fold dilutions of enzyme conjugate (from 1:500 to 1:2000), standard positive and negative (Ali *et al.* 1993) horse sera (from 1:20 to 1:1280) and unlabelled MoAbs (from 1.25 µg / ml to 50 µg / ml).

For color development, 0.1 ml of substrate (0.2 mM azino-di-[3-ethylbenzthiazoline] sulfonic acid; ABTS) was used. The plates were read at 405 nm in a spectrophotometer (Corona Electric Co., LTD. Tokyo, Japan). The end point of ELISA was estimated from the dose-response curve and the antibody titer was expressed as the reciprocal of the highest dilution of MoAbs showing an absorbance over 0.1.

Competitive binding assay between MoAbs

The assay was performed according to the method described previously (Zavala *et al.* 1983). The wells of microplate were coated with 0.1 ml of *B.equi* piroplasm lysate (protein concentration 18 µg / ml) in 0.05 M carbonate-bicarbonate buffer (pH: 9.6) and kept overnight at 4 °C. Non-specific sites were blocked with 0.5% SM in PBST at room temperature for 1 hr.

As an antibody competitor, 0.1 ml of serial 2-fold dilutions of unlabelled purified monoclonal antibodies (0-20 µg) were added. After incubation at room temperature for 1 hr, the plates were washed with PBST and 0.1 ml of biotin-labelled MoAbs (20 µg IgG / ml) were added. Following incubation at room temperature for 1hr and washing with PBST, 0.1 ml of avidin-peroxidase (Zymed Laboratories, San Francisco, CA, USA) diluted 1:1,000 in 0.2% SM-PBST was added and the plates were incubated at room temperature for 1 hr. The wells without *B. equi* antigen followed by biotin-labelled antibody were used as negative controls. For color development, 0.1 ml of 0.2 mM ABTS was used. The absorbance of the wells was read at 405 nm in a spectrophotometer (Corona Electric Co., Tokyo, Japan).

Two site-ELISA

Two-site ELISA was performed by the method described previously by Zavala *et al.* (1983) and Burkot *et al.* (1984). The microplate was coated with 0.1 ml of purified unlabelled MoAb C2, C5 and D3 (20 µg/ml) and left overnight at 4 °C. After washing with PBST, non-specific binding sites were blocked as described above. Plates were washed and 0.1 ml of serial 2-fold dilutions of 18 µg / ml solubilized piroplasm antigen were added. After incubation at room temperature for 1 hr, 0.1 ml of

biotin-labelled homologous MoAb (20 µg / ml) was added. Plates were incubated at room temperature for 1 hr, and processed as described above. Antibody-coated wells incubated without antigen followed by biotin-labelled antibody were used as negative controls.

RESULTS

Characterization of the monoclonal antibodies (MoAbs)

Immunoblotting was carried out for the determination of the specificity of MoAbs and for the identification of the molecules recognized by these MoAbs. A total of 54 clones secreting antibodies against *B.equi* piroplasms were obtained in 5 fusion experiments. Supernatants of three (i.e. C2, C5 and D3) out of 54 hybridomas, that reacted with the 18 kDa protein (p18) of *B. equi* in immunoblotting, were selected for further studies. None of the three MoAbs reacted either with *B. caballi* or equine erythrocyte proteines in Westernblot analysis (Fig. 9 ; Lanes 3 to 5). These MoAbs also recognized an 18 kDa protein in the Westernblot of a *B. equi* strain collected from Russia (Fig. 10 : Lanes 1 to 3). Other dominant proteins of the Russian strain were Mr 40.5, 28 and 16.5 kDa. However, these MoAbs did not recognize p18 in Westernblot of *B. equi* strain collected from South Africa (Fig.10 ; Lane 4). Determination of immunoglobulin isotype showed that all of the three MoAbs were IgG1 (κ).

Triton X-114 phase partition of piroplasm antigen

The Triton X-114 phase partition method was used to determine whether 18 kDa antigen is an integral membrane protein. When the extracted materials were electroblotted and reacted with MoAbs, all of which recognize a 18 kDa molecule of the *B. equi* piroplasm, this molecule was partitioned exclusively into the detergent phase (Fig. 1: Lane 2). This immunoblot is probed with MoAb C5 and is representative of all the three MoAbs used in this study.

Sensitivity of biotin-labelled MoAbs

Checker board titration showed that the 0.1 ml of *B. equi* piroplasm antigen (18

μg / ml) per well could produce a optimum reaction in ELISA. Therefore, this amount of the antigen was used for the determination of reactivity of the biotin-labelled MoAbs. From the results (shown in Fig. 11), 20 μg / ml biotin-labeled MoAb was used in the following test, since this amount of each MoAb could give the optimum reaction. The specificity of the biotin-labelled MoAbs was demonstrated by the lack of reactivity in negative control wells.

Competitive ELISA between MoAbs

Competitive ELISA was performed to determine whether any of the MoAbs recognized the same epitopes on the 18 kDa protein. MoAbs at different concentration (0-20 μg) were used in competition with the heterologous biotin-labelled MoAb (20 μg/ml). Results were expressed as percentage of inhibition of binding of each MoAb by the others. As shown in Fig. 12, each MoAb inhibited binding of homologous as well as heterologous biotinylated MoAb. MoAbs C5 and D3 produced a reduction of 83.3% and 84.6% in the binding of biotin labelled C2 at 20 μg/ml concentration. The reciprocal figures were 32.6% and 37.8% for biotynilated C5 and D3, respectively. The percentage of inhibition of biotin labelled D3 by C5 was 55.7% at 20 μg/ml level. The figure in reciprocal assay was 69.0%. The inhibitory effects in competition binding of homologous antibodies were from 65 to 75%.

Two-site ELISA

Two-site ELISA was performed to determine the presence of repetitive epitopes in the 18 kDa protein of *B. equi*. MoAbs immobilized on microplate wells were incubated with *B. equi* merozoite lysate and then homologous biotin-labelled MoAb was added to the plate. As shown in Figure 13, biotin-labelled MoAbs C2,C5 and D3 did not show any positive reactions.

DISCUSSION

Exposed epitopes on the sporozoite and merozoite membranes or on infected erythrocyte membranes are likely to be the targets for a protective immune response in *Babesia* infections (McElwain *et al.* 1987). The contamination of babesial proteins with large amounts of host cell products is a problem for the isolation of pure babesial antigens. The identification of such antigens is required if their efficacy as immunogen has to be investigated. Generation of MoAbs that bind to merozoite surface proteins is a way which leads to the identification of protective immunogen.

The aims of the present study were to develop MoAbs against the previously identified immunodominat proteins of *B. equi* (Ali *et al.* 1993) and to use these MoAbs in different diagnostic assays for equine piroplasmosis. As a first step, a panel of MoAbs was developed against *B. equi* piroplasms. Three MoAbs reacting with an 18 kDa surface membrane protein (p18) of *B. equi* in immunoblot analysis were used to characterize the epitopes on p18.

The results of competitive ELISA indicate that MoAbs recognized the same epitope on the p18 molecule as each MoAb inhibited the binding of homologous as well as heterologous biotinylated MoAb. Comparatively higher reduction in binding of biotin-labelled C2 in competition with C5 and D3 in inhibition assay may be due to the differences in their affinity for the epitope on the p18.

In this experiment, Triton X-114 phase partitioning of *B. equi* piroplasms proteins confirmed that 18 kDa antigen is an integral membrane protein of *B. equi* piroplasms. None of the three MoAbs mentioned in this study reacted either with *B. caballi* or with equine erythrocyte proteins. These MoAbs recognized the p18 in the Russian strain of *B. equi* but not in the strain obtained from South Africa. This suggests that there are antigenic differences between the *B.equi* strains isolated from different parts of the world. Böse and Hentrich (1994), demonstrated that none of the antigens diagnostic for European

isolates of *B equi* were recognized by sera from field-infected horses from Brazil. Single protein identification and absence of crossreaction with *B. caballi* or equine erythrocyte proteins recommend the use of these MoAbs in differential diagnosis of mixed equine piroplasma infections.

ABSTRACT

Monoclonal antibodies (MoAbs) were produced against *B. equi* piroplasms. Three MoAbs reacting with an 18 kDa surface membrane protein (p18) of *B. equi* in immunoblot analysis, were used to characterize the epitopes on p18. All the three MoAbs recognized the same epitope on p18 as indicated by competitive Enzyme linked immunosorbent assay (ELISA). Negative results in two-site ELISA suggest absence of repetitive epitopes on p18. Triton X-114 phase partitioning confirmed that 18 kDa antigen is an integral membrane protein of *B. equi* piroplasms. As these MoAbs identified a single protein and showed no crossreaction with *B. caballi* or equine erythrocyte proteins, these can be a candidate to be used in the differential diagnosis of mixed equine piroplasma infections.

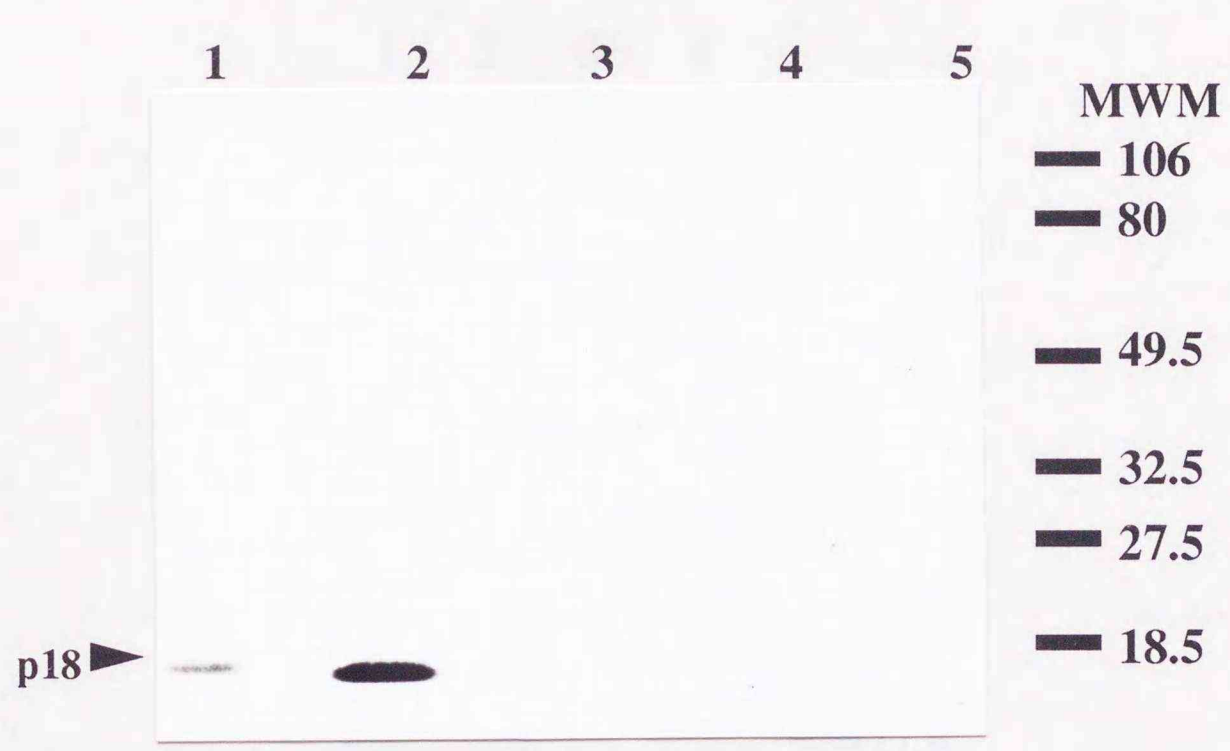

Fig. 9 Recognition of 18 kDa *B. equi* piroplasm surface protein by MoAbs.

B. equi and *B.caballi* piroplasm proteins were subjected to Triton X-114 phase partitioning, separated by electrophoresis on 12.5 % SDS-polyacrylamide and transferred to PVDF membrane sheet. The blot was probed with MoAb C5 and is representative of all the three MoAbs used in this study. Lanes: 1. *B. equi* whole piroplasm antigen ; 2. *B. equi* detergent phase; 3. *B. caballi* whole piroplasm antigen; 4. *B. caballi* detergent phase ; 5. Equine erythrocyte proteins. Position of 18 kDa protein is shown on the left (MW Marker $x10^3$).

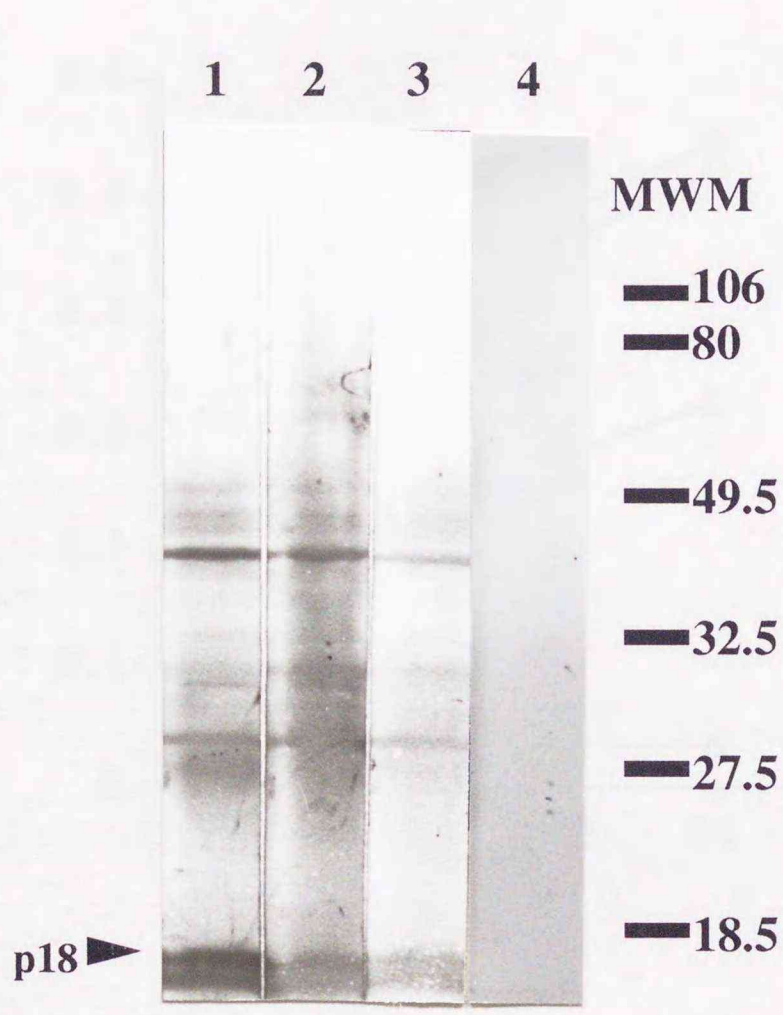

Fig. 10 Reaction of MoAbs with *B. equi* whole piroplasm antigens obtained from South Africa and Russia.

Piroplasm proteins were separated by electrophoresis on 12.5 % SDS-polyacrylamide gel and were transferred to PVDF membrane sheet. Russian *B. equi* antigen probed with MoAbs C2 (Lane 1), C5 (Lane 2) and D3 (lane3); Lane 4. South African antigen probed with C2. Position of 18 kDa protein is shown on the left (MW Marker $x10^3$).

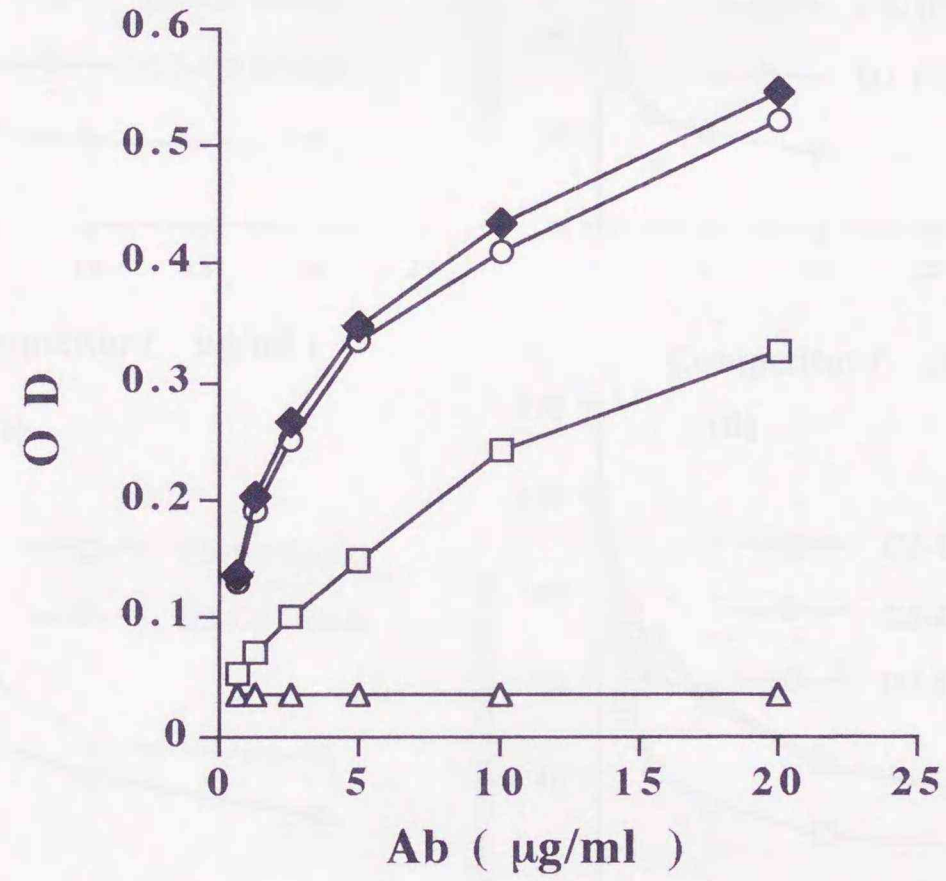

Fig. 11 Determination of sensitivity of biotin-labelled MoAbs.

Serial 2-fold dilutions of biotin-labelled MoAbs (20 μg/ml) were incubated in microplate wells coated with solubilized *B.equi* piroplasm antigen. Antibodies bound to the antigen-absorbed well were detected by ELISA. Wells without antigen were used as negative controls. All values are the mean of triplicated samples. —□—C2; —◆—C5;—O—D3; control —△—(without antigen)

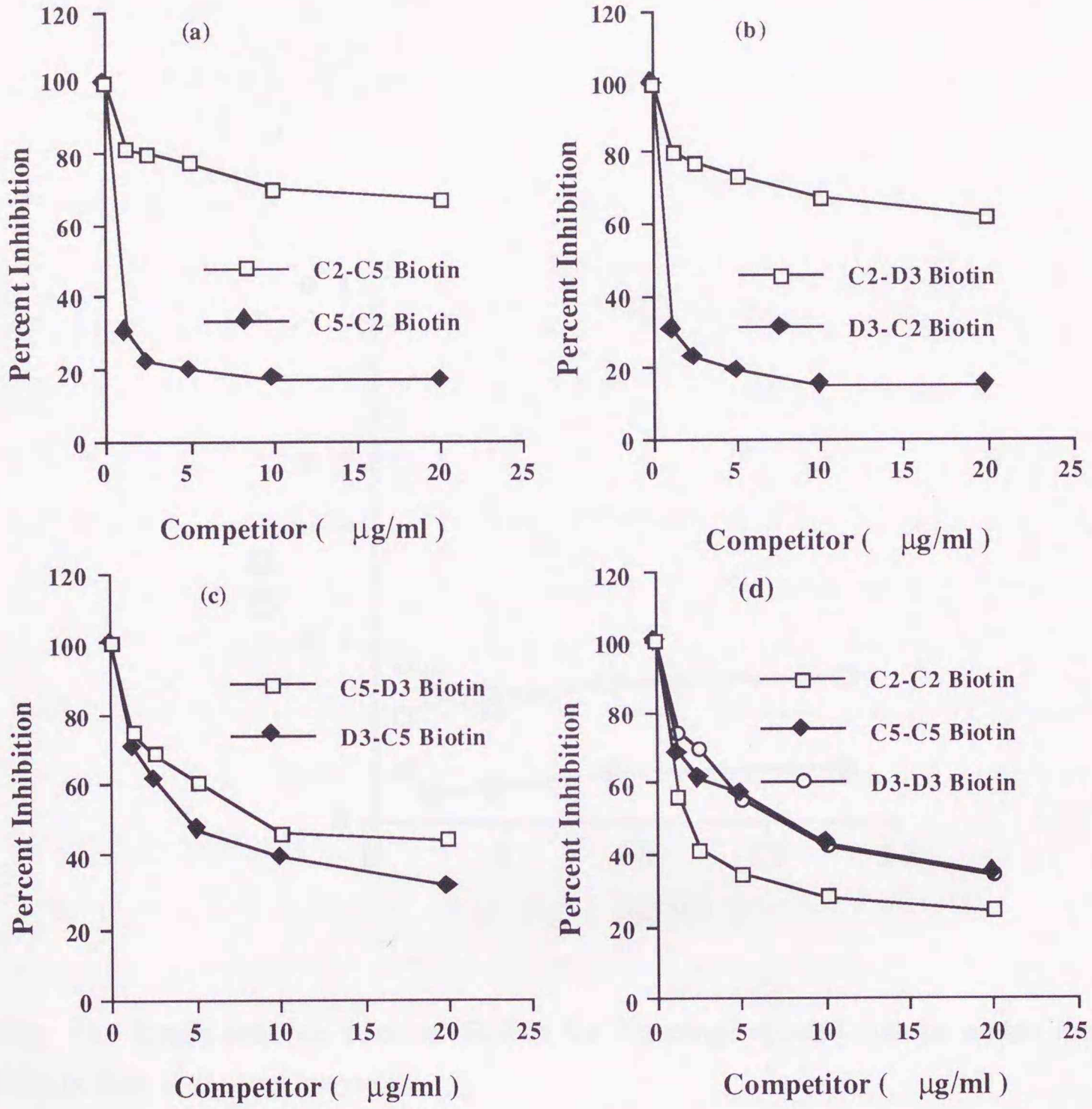

Fig. 12 Competitive ELISA assay between MoAbs.

Unlabeled MoAbs as competitors (0-20 μg) were incubated in microplate wells coated with 0.1 ml of the piroplasms antigen (18 μg/ml). After washing, the wells were incubated with biotin-labeled MoAbs (20 μg/ml). (a) MoAb C2 to compete with biotin-labeled MoAb C5 and their reciprocal assay. (b) MoAb C2 to compete with biotin-labeled MoAb D3 and their reciprocal assay. (c) MoAb C5 to compete with biotin-labeled D3 and their reciprocal assay. (d) Homologous unlabeled MoAb as a competitor of biotin-labeled MoAb. All values given are the mean of triplicated samples.

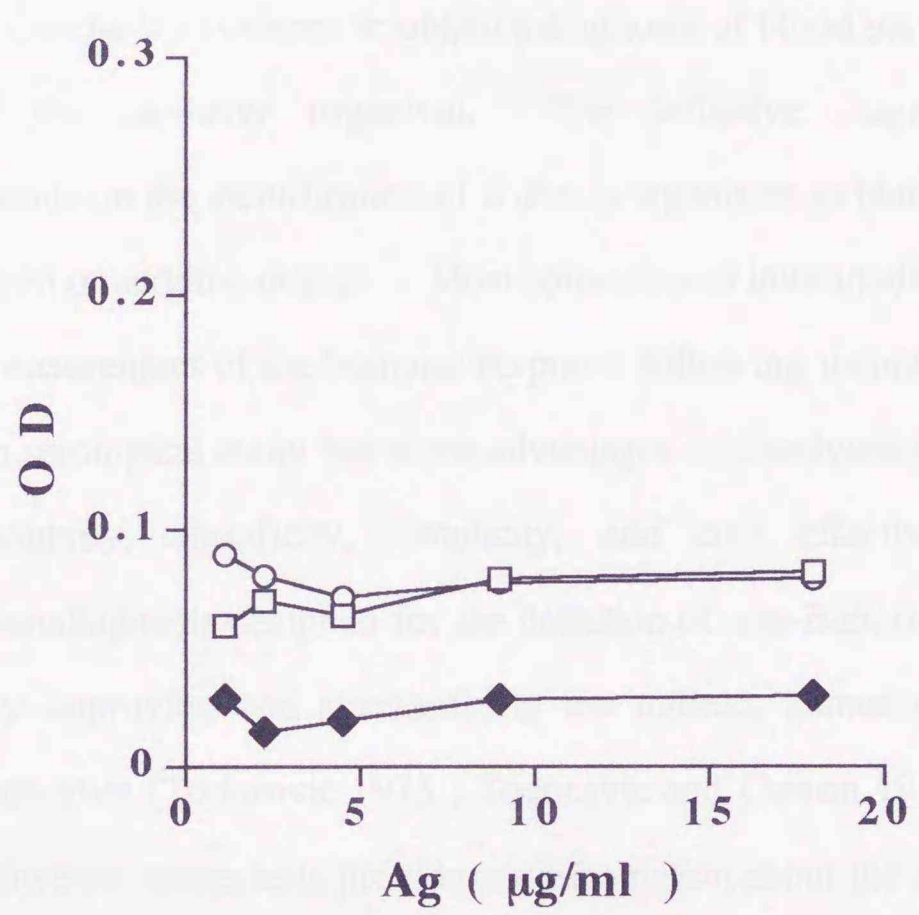

Fig. 13 Single-antibody two-site ELISA for detecting repeated epitope within the 18 kDa protein of *B. equi* merozoite.

Piroplasm solubilized antigens were incubated in microplate wells coated with one of the MoAbs (C2, C5, D3) specific to 18 kDa protein. After washing, homologous biotin-labeled MoAb was used to detect the antigen captured on the microplate surface. All values given are the mean of triplicated samples. −□−C2; −◆−C5; −O−D3

PART III

POLYMERASE CHAIN REACTION-BASED ASSAY FOR THE DETECTION OF *BABESIA EQUI* IN HORSE BLOOD

INTRODUCTION

The only conclusive evidence to obtain a diagnosis of blood parasite infections is demonstration of the causative organism. The definitive diagnosis of equine piroplasmosis depends on the identification of *Babesia* organisms in blood smears stained with Giemsa solution or acridine orange. Most conventional immunodiagnostic methods are used for the measurement of the humoral response following natural or experimental babesiosis. Each serological assay has some advantages or disadvantages depending on its level of sensitivity, specificity, simplicity, and cost effectiveness. Recent developments in serodiagnosis designed for the detection of anti-*Babesia* antibodies were partly achieved by improving and standardizing the indirect immunofluorescence and complement fixation tests (Todorovic 1975 ; Todorovic and Carson 1981 ; Weiland and Reiter 1988). However, these tests provide no information about the molecules against which the immune response of horses to *Babesia equi* and *B. caballi* is directed. One common theme has been the identification of diagnostic antigens through various processes. Consequently, efforts have been made to identify parasite-specific antigens by the generation of monoclonal antibodies and by Western blotting. Characterization of immunodominant piroplasm surface proteins of *B. equi* is described in the previous chapters of this thesis (Ali *et al.* 1993 ; Sugimoto *et al.* 1994 ; Ali *et al.* 1995).

Procedures employed to test for the presence of the hemoparasites in chronically or persistently infected but otherwise healthy animals include the thick blood smear technique to demonstrate *Babesia*-infected erythrocytes, immunodepression with corticosteroids, splenectomy, brain biopsy (*Babesia bovis* only) and blood subinoculation from a suspected carrier to a susceptible splenectomized recipient (Mahony 1962 ; Callow

et al. 1986). Polymerase chain reaction (PCR), which was introduced in 1985 has been used successfully for detection of various infectious organisms and is applicable both in diagnosis and in epidemiology. As this technique provides a powerful tool to detect and identify minimal numbers of microorganisms, it can be helpful in correct diagnosis of equine piroplasmosis in chronically infected carrier animals, allowing its more efficient control. This will be a very useful technique especially where horses are destined for the export market as parasite DNA is detected and not, as is the case of serological tests, only antibodies to the parasites. In this part of the study, the use of PCR to specifically amplify the gene of the 37 kDa protein of *B. equi* is described.

MATERIALS AND METHODS

Parasite stocks and DNA

DNAs of the Ikeda stock of *Theileria sergenti*, *B. bovis*, *B. bigemina* and *B. ovata* were kindly supplied by Dr. K. Fujisaki of Institute of Animal Health, Ibaraki, Japan. Chitose stock of *T.sergenti* was maintained in our laboratory. DNAs of *T.parva* and *T. annulata* were received from Dr. A. Nambota of University of Zambia, Zambia and Dr. E. Pipano of Kimron Veterinary Institute, Israel, respectively. *B. equi* and *B. caballi* (Department of Agriculture, Iowa, USA) were obtained from Equine Research Station, Japan Racing Association, Japan. South African *B. equi* stock was provided by Dr. E. Zweygarth, Onderstepoort Veterinary Institute, Onderstepoort, South Africa. Dr. V.T. Zablotsky of All Russia Institute of Experimental Veterinary Medicine, Moscow, Russia has kindly provided the Russian *B. equi* strain used in this experiment. DNAs from infected erythrocytes of horse imported from China were supplied by Dr. N. Komatsu, Animal Quarantine Office, Yokohama, Japan.

Preparation of DNA

Parasite DNA was prepared from purified piroplasms and parasitized equine or bovine erythrocytes. Infected and uninfected erythrocytes were washed in phosphate-buffered saline (PBS; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM K_2HPO_4) by centrifugation at 3,000 xg for 10 min. The buffy coat was removed and the genomic DNA was extracted either directly from erythrocyte pellet by Sepa Gene (Sanko Juntaku Co, Tokyo, Japan) or by the method described previously (Tanaka *et al.* 1992). Briefly, the erythrocytes were lysed either by *Aeromonas hydrophila* hemolysin (Sugimoto *et al.* 1991) or by nitrogen gas cavitation (Ali *et al.* 1993-first part of this thesis), and the parasite-enriched fraction was digested with proteinase K (0.3 mg/ml) and 1% sodium dodecyl sulfate (SDS). The lysates were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA in the aqueous phase was precipitated

with 2 volumes of cold 99.5% ethanol, then centrifuged at 10,000 xg for 10 min, dried, dissolved in sterile deionized water and stored at 4 °C till further use.

Alternatively, DNA was purified by Sepa Gene kit (Sanko Junyaku Co, Tokyo, Japan), according to the manufacturers instructions. Briefly, the erythrocyte pellet was hemolized by suspending it in 0.2% NaCl and centrifuged it at 1,000 xg for 5 min. Supernatant was discarded and the process was repeated till the supernatant was clear. Then 100 µl of solution I (Tris buffer) was added to the pellet and left it at room temperature for 10 min. Thereafter, 100 µl of solution II (guanidine thiocyanate) was added and pellet was suspended gently using a micropipette. Seven hundred microliter of Solution III (Phenol Chloroform) and 400 µl of solution IV (sodium acetate) were added and the suspension was mixed by vigorous handshaking. After centrifuging at 14000 rpm for 10 min, supernatant was collected in a new tube. Then the solution V (acetate buffer) was added (amount equal to the one tenth volume of the supernatant). After mixing by gentle inversion of tube equal volume of 2-propanol were added and tubes were cenrifuged at 14,000 rpm for 10 min. Supernatant was discarded and pellet containing DNA was washed with 70% ethanol. The DNA pellet was dissolved in sterile deionized water and stored at 4 °C till further use. DNA prepared from uninfected erythrocytes was used as control.

Polymerase chain reaction (PCR) amplification

PCR amplification was performed in a 50 µl reaction mixture containing 20 mM Tris-HCl, pH 8.4 / 50 mM KCl / 2.0 mM $MgCl_2$ / 200 µM each of the four dNTPs / 1.0 µM each of the oligonucleotide primers / 2.5 units of Taq (*Thermus aquaticus*) DNA polymerase (GIBCO BRL, Life Science Technologies Inc., Gaithersburg, MD, U.S.A.). Sequence of primers were derived from the sequence of p37 gene of *B. equi* (Kubota *et al.*

unpublished data). Fifty nanograms of genomic DNA was added to the reactions as template. The reaction mixture was overlaid with paraffin oil and subjected to 35 cycles of amplification in a programmable heating block (PTC™ Programmable Thermal Controller, MJ Research Inc., Watertown, Mass., USA). Each cycle consisted of 1 min of denaturation at 94 °C (4 min for the first cycle), 1 min of annealing at 57 °C, and 2 min of polymerization at 73 °C, with an additional 4 min at 73 °C after the last cycle .

The PCR-amplified products were analyzed by agarose gel electrophoresis. Ten microliters of PCR-amplified DNA was separated on a 1.5% agarose gel in Tris-acetate-EDTA (0.04 M Tris-acetate and 0.002 M EDTA [pH 8.1]) buffer. The gel was then stained with ethidium bromide (0.5 µg/ml) for 30 min and photographed.

Sensitivity of PCR

In order to determine the minimum possible amount of parasite DNA that can be amplified by PCR-based assay , serial 10-fold dilutions of purified *B.equi* piroplasm DNA from USA stock were amplified by PCR and the products were analyzed by electrophoresis as given above.

Serial 10-fold dilutions of *B.equi* infected erythrocytes from an experimentally infected horse were prepared to determine the minimum possible parasitaemia detection by PCR. Normal equine erythrocytes were mixed with *B. equi* infected erythrocytes to give parasitaemia from 10 % to 0. 00001 %. DNA extracted by Sepa Gene (Sanko Junyaku Co, Tokyo, Japan) from these dilutions of infected erythrocytes and were subjected to PCR amplification as described above.

RESULTS

Polymerase chain reaction (PCR) amplification of *B. equi* p37 gene

Two PCR primers of 20-residue oligonucleotides were synthesized to amplify the p37 gene of *B. equi* . The sequence of forward primer is ; 5' primer AAAGGCCCGTGTATCGTATT and of reverse primer is ; 3' primer TCTTCTTCCTCCTCTTCTGG. When genomic DNA of *B. equi* strains obtained from USA, South Africa and Russia were used (Fig. 14 ; Lanes 1 to 3), a single fragment of the expected size of approximately 935 bp was amplified. There was no amplification of DNA observed when *B. caballi* DNA was used in the PCR reaction (Fig. 14 ; Lane 4). However, these primers also amplified a single fragment of 1353 bp from genomic DNA template of *T. sergenti* obtained Chitose and Ikeda districts of Hokkaido, Japan (Fig. 14 ; Lane 5 and 6). No detectable amplifications from other *Babesia* or *Theileria* DNA took place under the same conditions (Fig. 14 ; Lanes 7 to 13)

When 10 genomic DNA templates of *B. equi* obtained from naturally infected horses from China were subjected to PCR, the same 935 bp fragment was amplified except in sample no. 2 (Fig. 15 ; Lane 2) having complement fixation (CF) titre < 5. CF titres of positive Chinese samples were from 5 to ≤ 40. However, Chinese sample no. 9 having CF titre < 5 was poorly amplified (Fig. 15 ; Lane 9).

Sensitivity of PCR

In order to determine the sensitivity of the PCR based assay, 10-fold serial dilutions of purified *B.equi* piroplasm DNA from USA stock were amplified by PCR and the products were analyzed by electrophoresis (Fig. 16). PCR was sensitive enough to detect 10 pg *B.equi* purified DNA used as template DNA in PCR reaction.

DNA extracted from serially diluted erythrocytes of an experimentally infected horse were also subjected to PCR. PCR could amplify *B. equi* DNA in horse blood with 0.001% parasitaemia (Fig. 17).

DISCUSSION

B. equi infection can be diagnosed routinely by direct microscopic examination of Giemsa-stained blood smears, which is time-consuming and requires expertise. This method may not always detect *B. equi*-infected carrier horses when parasitemia is at very low level. Advances in molecular biology have led to the development of diagnostic assays that use nucleic acid probes to detect pathogens directly, specifically, and sensitively. Repetitive DNA probes for *Babesia equi* have been reported by Posnett and Ambrosio (1989). These were sensitive enough to detect from 0.5% to 0.0025% of infected erythrocytes in the blood. However, to apply this hybridization method to clinical diagnosis, there is a serious problem to be solved. As contamination by proteins in DNA preparation may significantly reduce the sensitivity of the method, phenol-chloroform extraction should be repeated several times. On the other hand, the presence of contaminated protein in template DNA for PCR amplification does not cause problems in direct amplification of DNA in lysates from field samples. Thus, the DNA purification steps could be simplified.

The primers designed from the sequence of p37 of *B. equi* gene also amplified a 1,353 bp fragment when genomic DNA from bovine *T. sergenti* (Chitose and Ikeda stocks) were used as template DNA in the PCR. This suggests another link in the chain of observations made by different researchers in previous years regarding the relationship of *B. equi* with *Theileria* species (Moltmann et al. 1983 ; Mehlhorn and Schein 1984 ; Schein 19889). Some considerable differences in the development and reproduction of *B. equi* have been found e.g. existence of exoerythrocytic schizogony in lymphoid cells of experimentally infected horses (Schein *et al.* 1981) and occurrence of macro- and microschizonts in lymphocytes of infected horses *in vivo* and *in vitro* by electron microscopy studies (Moltmann *et al.* 1983). Comparisons of ribosomal DNA sequences of babesia parasites which exhibit the schizogony in lymphocytes (Ellis *et al.* 1992) and relationship of extrachromosomal nucleic acid element in several apicmoplexan parasites

e.g., *Toxoplasma gondii, Eimeria tenella, Theileria parva, B. microti, B. equi ,B. ovis* and *T. annulata* (Joseph *et al.* 1989 ; Schelp *et al.* 1995), favours the erection of a third genus with in the subclass *Piroplasmia.* In the first part of this thesis, N-terminal amino acid sequence analysis of p30 and p28 of *B. equi* piroplasm proteins also provides evidence for a close relationship of *B. equi* p30 and p28 with the surface proteins of other *Theileria* species of cattle, p 32 of *T sergenti* and p33 of *T. buffeli* (Kawazu *et al.* 1992 ; Matsuba *et al.* 1993).

The primers designed in this study appears to be highly specific for *B. equi* since there was no detectable amplification of DNA from equine erythrocytes and other hemoparasites including *Babesia* (*B. caballi, B. bovis, B. bigemina, B. ovata*) and *Theileria* (*T. parva, T. annulata, T. buffeli*) species. The amplification of a 1,353 bp DNA fragment by the primers designed in the present study will not effect the specificity of the test as *T. sergenti* is a cattle parasite and horses are not infected with this parasite. Moreover, the size of the DNA fragment (1,353 bp) amplified by these primers from *T. sergenti* DNA is different from the size of DNA fragment (935 bp) amplified from *B. equi* DNA used as template in this test. PCR based assay is sensitive enough to detect 10 pg of purified *B. equi* DNA used as template DNA. Amplification of parasite DNA by PCR has advantages over other methods of detecting horses persistently infected with *B. equi*, because parasites are difficult to detect by conventional diagnostic methods. The primers used in this study could amplify *B. equi* DNA in the infected horse blood with 10^{-3} parasitaemia. Used in conjuction with serological assays PCR test can facilitate more accurate diagnosis of equine piroplasmosis in carrier animals.

ABSTRACT

A polymerase chain reaction (PCR) based assay was developed to detect DNA of *Babesia equi* in naturally and experimentally infected horse blood. The PCR was used to amplify the gene of 37 kDa protein from piroplasm DNA. A pair of synthetic oligonucleotide primers designed from the gene encoding p37 of *B. equi* amplified a single fragment of approximately 935 bp when the genomic DNAs from USA, South African, Russian and Chinese *B. equi* stocks were used as template DNAs in PCR. These primers also amplified a single fragment of 1,353 bp from genomic DNA template of *Theileria sergenti* Chitose and Ikeda stocks. However, no detectable amplifications from DNAs of other *Babesia* or *Theileria* species took place under the same conditions. PCR was sensitive enough to detect 10 pg *B.equi* purified DNA used as template DNA in PCR reaction and could also amplify *B. equi* DNA in horse blood with 0.001% parasitaemia.

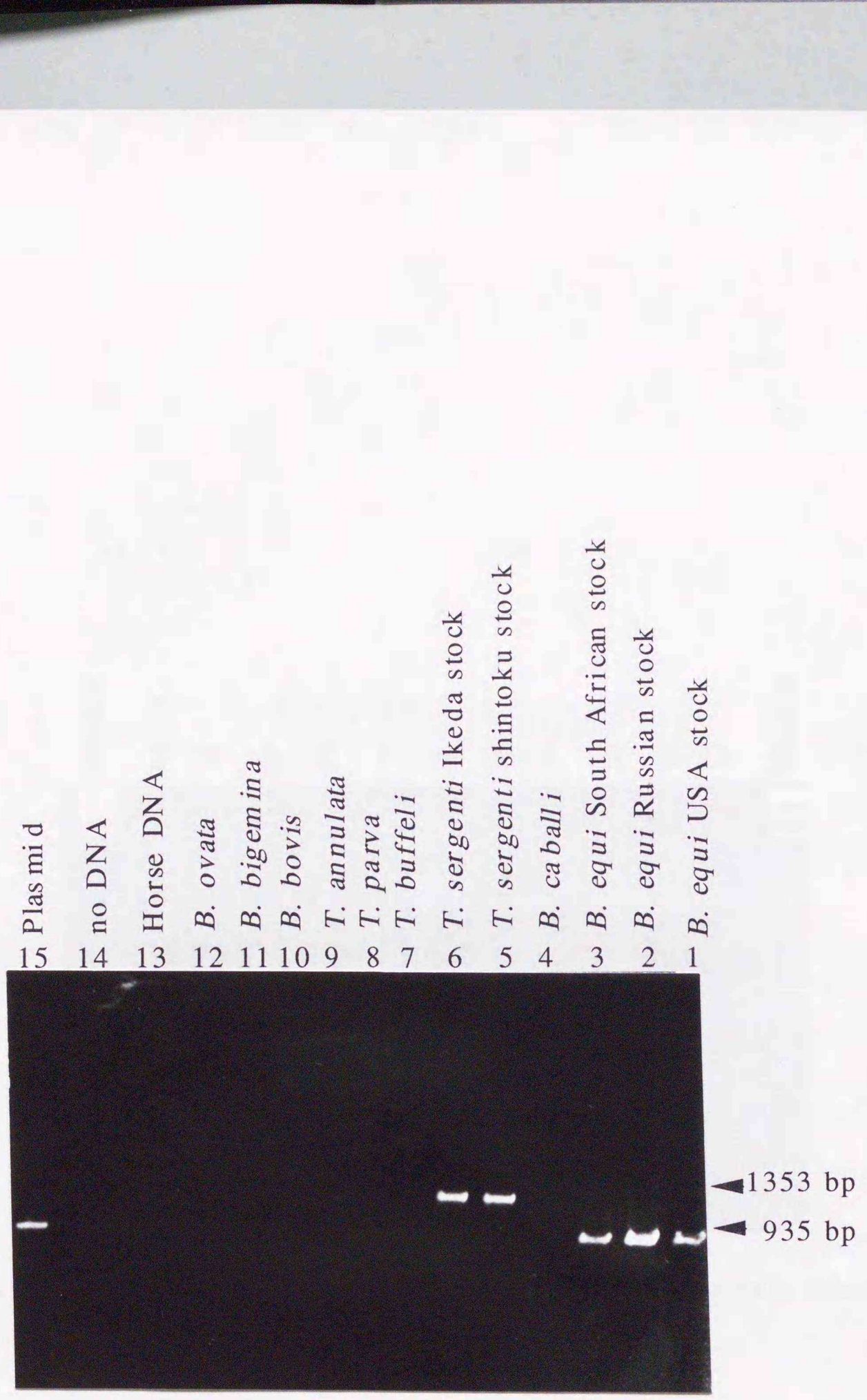

Fig. 14 PCR amplification of *B. equi* DNA from different geographic locations and of other hemoparasite DNAs.

Fifty nanograms of genomic DNA from different hemoparasites were amplified by PCR and 10 μl of PCR product from each sample were electrophoresed on a 1.5 % agarose gel and DNA were visualized by ethidium bromide.

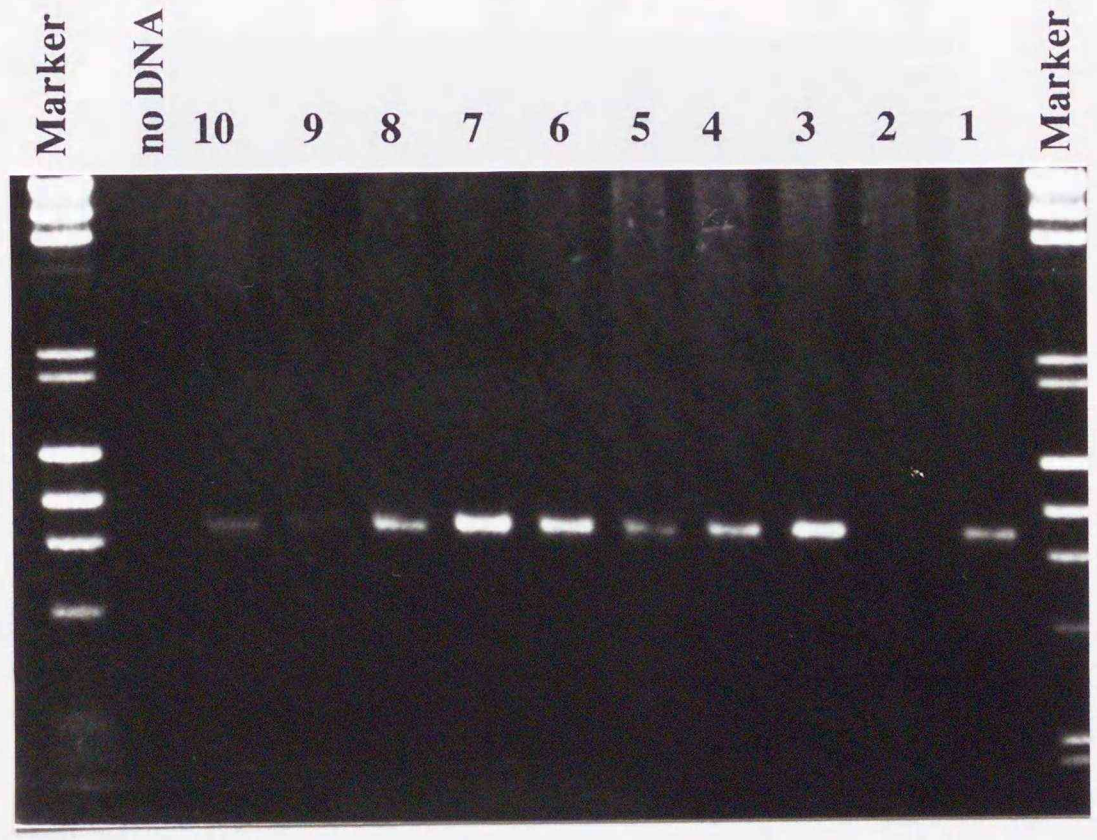

Fig. 15 PCR amplification of *B. equi* DNA extracted from naturally infected horse blood obtained from China.

Fifty nanograms of genomic DNA were amplified by PCR and 10 μl PCR product from each sample were electrophoresed on a 1.5 % agarose gel and DNA were visualized by ethidium bromide. Molecular mass markers are 23.13, 9.42, 6.56, 4.36, 2.32, 2.02, 1.353, 1.078, 8.72 and 6.03 kbp.

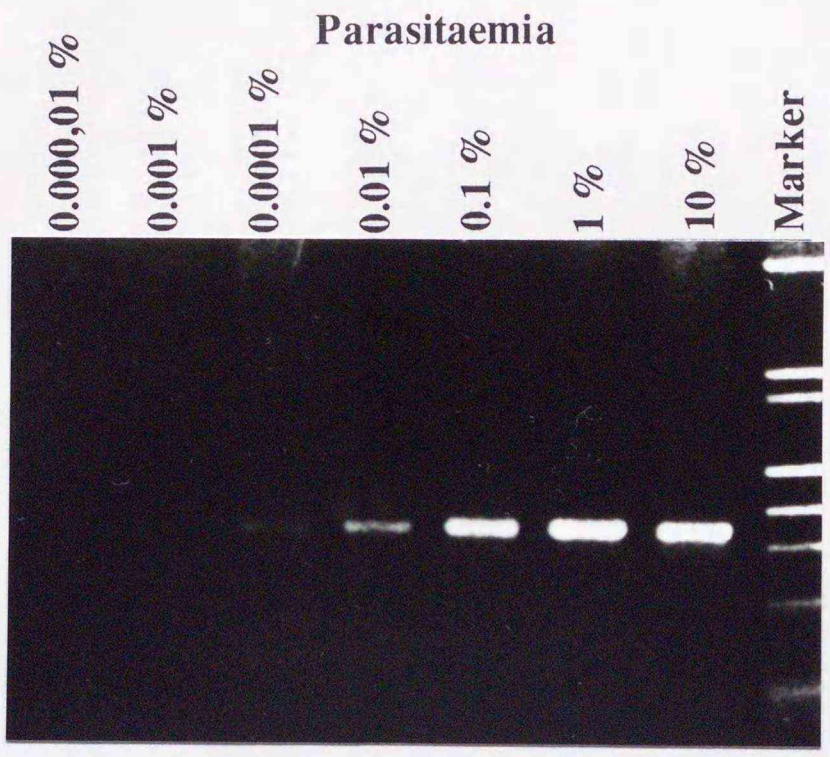

Fig. 16 Detection of PCR amplified *B. equi* DNA from experimentally infected horse blood.

Blood infected with 10 % parasitaemia was washed and 50 µl of packed infected erythrocytes were mixed with 450 µl of washed packed normal horse erythrocytes. This suspension was ten-fold serially diluted with washed packed normal horse erythrocytes to obtain blood samples with parsitaemia from 10 % to 10^{-5} %. DNA was extracted from each dilution and dissolved in 50 µl of sterile deionized water. Ten microliter DNA from each dilution was subjected to PCR and PCR products (10 µl from each sample) were electrophoresed on a 1.5 % agarose gel and DNA were visualized by ethidium bromide. Molecular mass markers are 6.56, 4.36, 2.32, 2.02, 1.353, 1.078, 8.72 and 6.03 kbp.

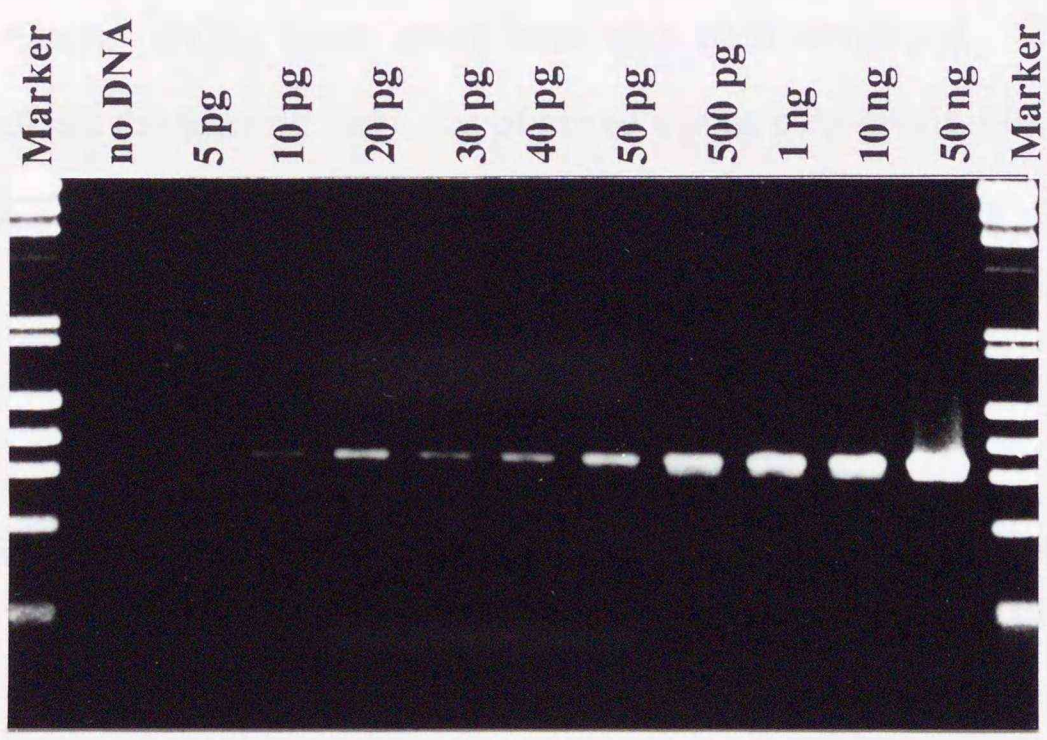

Fig. 17 Detection of PCR amplified DNA from purified *B. equi* piroplasm DNA.

Purified *B. equi* piroplasm DNA was tenfold serially diluted with sterile deionized water and subjected to PCR. PCR products (10 µl from each dilution) were electrophoresed on a 1.5 % agarose gel and DNA were visualized by ethidium bromide. Molecular mass markers are 23.13, 9.42, 6.56, 4.36, 2.32, 2.02, 1.353, 1.078, 8.72 and 6.03 kbp.

CONCLUSIONS

In this study immunodominant piroplasm surface proteins of *B. equi* and *B. caballi* have been characterized by one and two dimensional (2D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Triton X-114 partitioning, N-terminal aminoacid sequencing and development of monoclonal antibodies against *B. equi* piroplasms. Westernblot using antisynthetic peptide immune sera, monoclonal antibodies (MoAbs) based Enzyme linked immunosorbent assay (ELISA) and Polymerase chain amplification (PCR) based assay have also been developed. These diagnostic tools can facilitate the accurate diagnosis of mixed equine piroplasma infections in infected horse blood as they can specifically identify the *B. equi* piroplasm surface proteins and DNA both in acute or carrier state of the disease. The summary of salient results obtained is as below:

1. Molecular weights (Mr) of major *B. equi* piroplasm proteins separated by 2D SDS-PAGE were 18, 28, 30, 41, 43, 54, 66.5 and 96 kDa.

2. Immunoblot analysis revealed 6 immunodominant *B. equi* piroplasm proteins of 15, 18, 28, 30, 41 and 96 kDa as identified by an exprimentally infected horse immune serum.

3. Immunodominant *B. equi* piroplasm proteins of Mr 18 and 28 and 30 kDa were membrane- bound proteins as revealed by Triton X-114 phase partitioning.

4. Similarities in N-terminal aminoacid sequences of p28, p30 (*B. equi*) and p32 of (*T. sergenti*) suggests relationship of *Babesia* and *Theileria* species.

5. Anti-synthetic peptide sera produced against p28 and p30 of *B. equi* can be used for differential diagnosis of the two *Babesia* species (*B. equi* and *B. caballi*) in horses.

6. All the three MoAbs produced against the *B. equi* piroplasms recognized the same epitope on p18 of *B. equi* piroplasms as indicated by competitive inhibition ELISA.

7. Negative results in two-site ELISA (using MoAbs) suggest absence of repetitive epitopes on p18.

8. As the MoAbs produced identified a single protein and showed no crossreaction with *B. caballi* or equine erythrocyte proteins, these can be used in the differential diagnosis of mixed equine piroplasma infections.

9. A pair of synthetic oligonucleotide primers designed from the gene encoding p37 of *B. equi* amplified a single fragment of approximately 935 bp when the genomic DNA from USA, South African, Russian and Chinese *B. equi* stocks were used as template DNA in PCR based assay .

10. The amplification of a single fragment of 1,353 bp from genomic DNA template of *T. sergenti* from Chitose and Ikeda stocks suggesting a genetic similarity among *B. equi* and these stocks.

11. No detectable amplifications from other *Babesia* (*B. bovis*, *B. bigemina* and *B. ovata*) or *Theileria* (*T. parva*, *T. annulata* and *T. buffeli*)species, especially *B. caballi*, in PCR suggets that primers are specific for *B. equi*.

12. PCR was sensitive enough to detect 10 pg *B.equi* purified DNA used as template DNA in PCR reaction.

13. In conjuction with serological assays PCR test can be used for the accurate diagnosis of equine piroplasmosis in carrier animals as the test could amplify *B. equi* DNA in horse blood with 0.001% parasitaemia.

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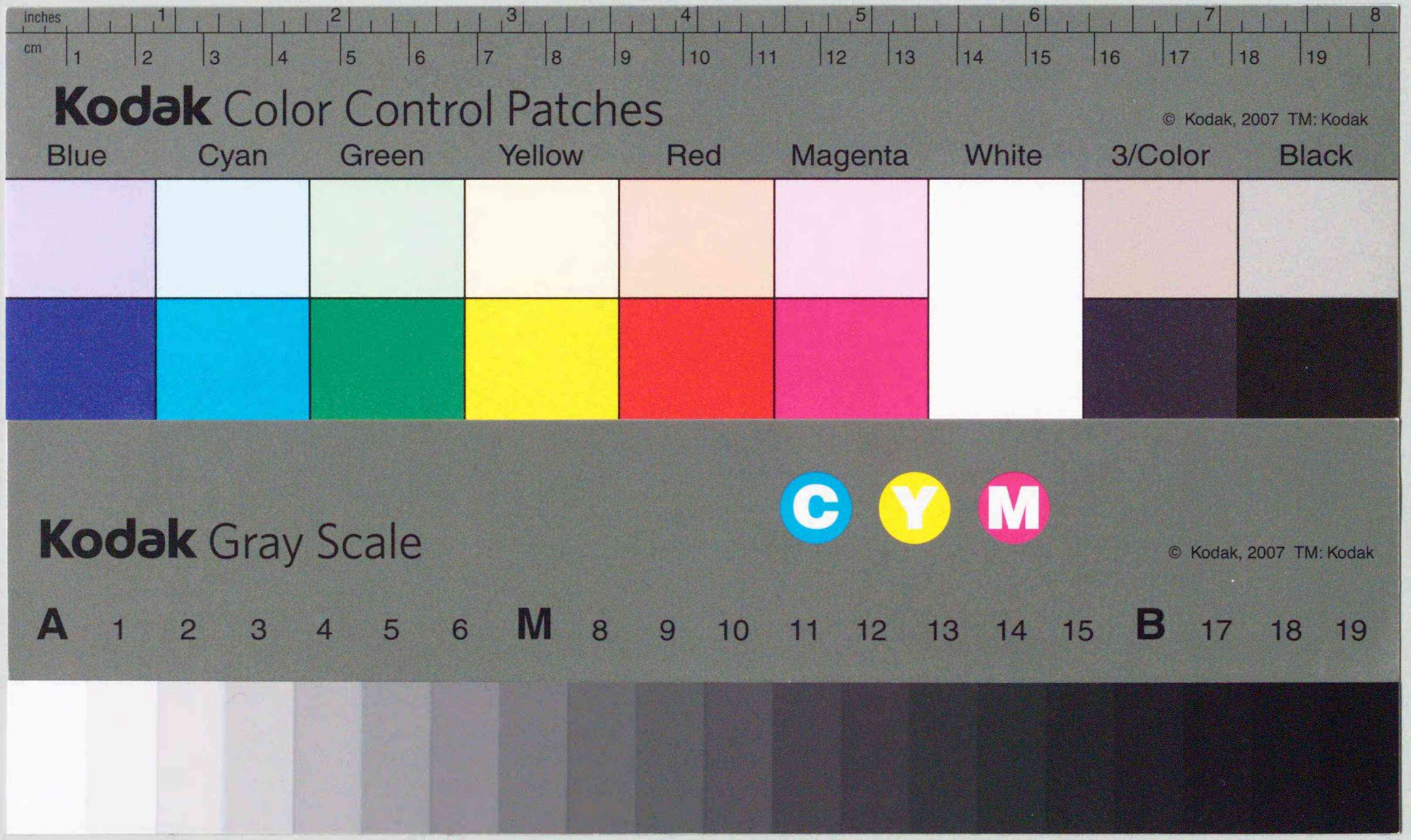
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