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Antigenic differences between Japanese *Theileria sergenti* and other benign *Theileria* species of cattle from Australia (*T. buffeli*) and Britain (*T. orientalis*)

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Abstract. Serological comparisons among piroplasm antigens of the benign *Theileria* species of cattle from Japan, Australia and Britain, which are frequently referred to as *T. sergenti*, *T. buffeli* and *T. orientalis*, were carried out. The results obtained from comparative enzyme-linked immunosorbent assay (ELISA) using sera from infected cattle suggest that *T. sergenti* could be differentiated from both *T. buffeli* and *T. orientalis* by their serological dissimilarities. Western blotting combined with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) revealed that both the 33-kDa piroplasm protein of *T. sergenti* and the similar 34-kDa protein of *T. buffeli* and *T. orientalis* corresponded to immunodominant antigens against cattle. The other 32-kDa proteins of *T. buffeli* and *T. orientalis* also represented immunodominant antigens. Cross-reactivities of the 32- and 34-kDa proteins were observed between *T. buffeli* and *T. orientalis*, whereas the 33-kDa protein of *T. sergenti* could be differentiated from the similar 34-kDa proteins of *T. buffeli/orientalis*. The present study suggests that *T. sergenti* should be separated from *T. buffeli* and *T. orientalis* on the basis of their serological dissimilarities.

The classification of benign species of *Theileria* that occur outside Africa is very confused. Based on serological and morphological identities, Uilenberg et al. (1985) concluded that the stocks of benign *Theileria* species from Japan, Australia, Britain, Iran and the United States were identical with a more pathogenic stock from Korea. Because the latter parasite is considered to correspond to *T. sergenti* as described in the Russian literature (Yakimoff and Dekhtereff 1930), Uilenberg et al. (1985) reported that all belonged to one species and proposed the name *T. orientalis*. However, Stewart et al. (1987b) suggested the use of the name *T. buffeli* according to Callow (1984) for Australian *Theileria* species as based on the species of the tick vector.

Recently, we proposed that the benign *Theileria* species from Japan, Australia and Britain, which are frequently referred to as the *T. sergenti*, *T. buffeli* and *T. orientalis* group parasites, should be classified into two groups, i.e. *T. sergenti* and *T. buffeli/orientalis*, according to the results of transmission experiments using various species of tick vectors (Fujisaki et al. 1991a, b) and the protein analysis of piroplasms by two-dimensional gel electrophoresis (Sugimoto et al. 1991b).

This paper presents the results of serological comparisons of *T. sergenti*, *T. buffeli* and *T. orientalis* using a comparative enzyme-linked immunosorbent assay (ELISA) and Western blotting in combination with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Materials and methods

Theilerial stocks

Theileria sergenti (Ikeda stock; Fujisaki et al. 1985) maintained at our laboratory in ticks that were fed on infected cattle were used in this study. *T. buffeli* (Warwick stock; Stewart et al. 1987a) was provided by Dr. N.P. Stewart in infected ticks. *T. orientalis* (Essex stock; Morzaria et al. 1974) was provided in infected blood by Dr. G. Uilenberg.

Infection of calves with theilerial stocks

Holstein calves between 4 and 6 months of age were screened for antibodies against *T. sergenti* by ELISA. Six animals that were negative for *T. sergenti* were splenectomised at approximately 1 month prior to infection and were kept in tick-free individual pens. Two calves were infected with *T. sergenti* by subcutaneous injection of a tick-derived sporozoite suspension or by allowing infected ticks to feed on them. Infection with *T. buffeli* or *T. orientalis* was induced in two calves each by syringal passage of infected blood. Following infection, calves were tested at intervals of 1–2 days to determine the sequential course of parasitaemia by exam-

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ining Giemsa-stained blood smears. The antisera used for ELISA and Western blotting were obtained from the calves after the parasitaemia had progressed markedly.

Purification of piroplasms

For the preparation of antigens for ELISA, *Theileria* piroplasms were isolated from infected erythrocytes by the nitrogen cavitation method (Shimizu et al. 1988) followed by discontinuous density-gradient centrifugation in Percoll. After parasitaemia had reached levels of >20%, blood was collected in a sodium citrate solution. The erythrocytes were depleted of leucocytes by several washes in phosphate-buffered saline (PBS; 0.01 M, pH 7.2) and were then resuspended in PBS to give a 20% (v/v) suspension. This suspension was exposed to a pressure of 56 kg/cm^2 nitrogen for 1 min and then decompressed rapidly in a cell disruption bomb (Parr Instrument Co., USA). To remove remaining intact erythrocytes, the lysate was centrifuged twice at 750 *g* for 30 min at 4° C. The supernatant containing piroplasms was centrifuged at 4800*g* for 30 min at 4° C. Resultant sediments were suspended in an appropriate volume of PBS, placed on top of a 40% and 60% (v/v) Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) discontinuous density gradient and ultracentrifuged at 20000 rpm for 20 min in a SW 27 rotor (Beckman Instruments Inc., USA). Piroplasms were collected from the band formed at the interface of the 40% and 60% Percoll solution, washed twice with PBS and stored at −80° C until use.

For the preparation of samples for 2D-PAGE, theilerial piroplasms were purified from infected erythrocytes using the method described previously by Sugimoto et al. (1991 a). Blood was collected from the calves after the level of parasitised erythrocytes had reached >10% and was washed three times with 10 mM TRIS-HCl and 150 mM NaCl (pH 7.4, TRIS-saline). The erythrocytes were resuspended in TRIS-saline at a concentration of 50% (v/v) and were applied to a column packed with cellulose powder (Type B, Advantec; Tokyo, Japan) for the removal of contaminated leucocytes. After one washing, erythrocytes were resuspended in TRIS-saline and then incubated with 300 hemolytic units *Aeromonas hydrophila* hemolysin/ml for 10 min at 37° C. The hemolysin was purified from the *A. hydrophila* Ah-1 strain using the method of Asao et al. (1984). The erythrocyte lysate was placed on top of a 40% and 60% (v/v) Percoll discontinuous density gradient and ultracentrifuged as described above. Piroplasms were collected from the band formed at the interface of the 40% and 60% Percoll solution, washed twice with TRIS-saline containing 5 mM ethylenediaminetetraacetic acid (EDTA, pH 7.4) and treated with solubilising solution for electrophoresis.

ELISA procedure

The ELISA reaction was performed as previously described by Shimizu et al. (1988) except that azinobenzthiazoline (ABTS) was used as a substrate. The piroplasm suspension prepared by the nitrogen cavitation method was incubated with an equal volume of a 4% solution of Triton X-100 in PBS for 3 h at 4° C and used as ELISA antigens. Optimal dilution of reagents was obtained by checkerboard titrations. The horseradish peroxidase (HRP)-conjugated IgG fraction of rabbit anti-bovine IgG (Cappel Lab. Inc., USA) was diluted to 1:7500. ELISA antigens prepared from *T. sergenti*, *T. buffeli* and *T. orientalis* piroplasm suspensions were diluted to 1:32000, 1:16000 and 1:32000, respectively. Sera collected from calves that had been infected with each stock were diluted to 1:100–1:800.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed as described elsewhere (Sugimoto et al. 1991 b). Non-equilibrium pH gradient gel electrophoresis (NEPHGE, O'Farrell et al. 1977) was used as the first step. Piroplasm samples were solubilised in buffer composed of 9 M urea, 4% (v/v) Nonidet P-40, 2% 2-mercaptoethanol and 2% (v/v) ampholines (pH 3.5–10; LKB, Uppsala, Sweden); 7.5 µl of each sample was loaded on the first-dimensional tube gel and electrophoresed for 1 h at 400 V. In the second step, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% (w/v) polyacrylamide gel according to the method described by Laemmli (1970). After electrophoresis, the gels were silver-stained or processed for Western blotting.

Western blotting

After separation by 2D-PAGE, piroplasm proteins were electrophoretically transferred to polyvinylidene difluoride sheets (Immobilon transfer membranes; Millipore, USA) according to the immunoblotting technique of Dunn (1986). The sheets were incubated overnight for blocking in 0.01 M PBS (pH 7.2) and 0.1% (v/v) Tween-20 (TPBS) containing 3% (v/v) fish gelatin (Sigma, USA) and were placed in TPBS containing 1% (v/v) fish gelatin, and serum was collected from calves that had been experimentally infected with either *T. sergenti*, *T. buffeli* or *T. orientalis* at dilutions of 1:800, 1:400 or 1:400, respectively. After 1 h the sheets were washed four times in TPBS and then incubated for 1 h with HRP conjugate diluted at 1:2500 with TPBS containing 1% (v/v) fish gelatin. After four further washes in TPBS, bound HRP conjugate was detected using a freshly prepared substrate solution containing 1.3 mM diaminobenzidine tetrahydrochloride (DAB; Nakarai Chemical, LTD., Kyoto, Japan), 1.3 mM cobalt chloride and 0.02% (v/v) hydrogen peroxide in TPBS.

Biotin labelling and detection of surface proteins

Surface proteins on piroplasms were labelled with biotin as described by Hurley et al. (1985). Piroplasms obtained by the Ah-1 hemolysin method were washed twice with PBS and resuspended in 0.5 ml PBS. Then, 100 µg biotinylation reagent (Sulfo-NHS-Biotin; Pierce, USA; 50 mg/ml in dimethylsulfoxide) was added to the suspension and the latter was incubated for 10 min at room temperature. Labelled piroplasms were washed twice with PBS and treated with solubilising solution for electrophoresis. The proteins were separated by 2D-PAGE and electrophoretically transferred to an Immobilon sheet. After overnight incubation with TPBS containing 3% (v/v) fish gelatin for blocking, biotinylated proteins were detected using an avidin-HRP conjugate (Cappel Lab. Inc.) and the substrate solution as described above.

Results

Serological comparisons of piroplasm antigens by ELISA

The cross-reactivity of the piroplasm antigens of *Theileria sergenti*, *T. buffeli* and *T. orientalis* with sera collected from calves that had been infected with either of these parasite species was tested by ELISA as shown in Table 1. The sera collected from calves that had been infected with *T. orientalis* showed higher reactivity with *T. buffeli* as well as *T. orientalis* antigen, but their reactivity against *T. sergenti* antigen was lower. The sera collected from calves that had been infected with *T. buffeli* also displayed intense reactions with the homologous antigen and intermediate reactions with *T. orientalis* antigen. The reactivity of these four sera with *T. sergenti* antigen was always lower than that with *T. buffeli* and

Table 1. Comparative ELISA among piroplasm antigens of *Theileria sergenti*, *T. buffeli* and *T. orientalis*

Antiserum	Animal number	Antigen		
		T. sergenti	*T. buffeli*	*T. orientalis*
T. sergenti	29	1[a]	0.6	0.55
	52	1	0.47	0.39
T. buffeli	82	0.22	1	0.68
	90	0.58	1	0.71
T. orientalis	80	0.54	1.08	1
	83	0.85	1.34	1

[a] An optical density of 415 was measured and the value obtained for a heterologous combination of antigen and antisera was standardised to that found for a homologous combination

T. orientalis antigens. The sera collected from calves that had been infected with *T. sergenti* showed relatively low reactivity with both *T. buffeli* and *T. orientalis* antigens.

Serological cross-reactivity as determined by Western blotting

Piroplasm proteins separated using 2D-PAGE were transferred to Immobilon sheets and probed with serum collected from calves that had been infected with either *T. sergenti*, *T. buffeli* or *T. orientalis* as shown in Figs. 1–3. The protein spots marked as 33K in Fig. 1 and 34K in Figs. 2 and 3 were those that were characteristic for *T. sergenti* and for other two parasites, respectively (Sugimoto et al. 1991b). The serum from the *T. sergenti*-infected calf reacted intensely with the 33-kDa protein of *T. sergenti* (Fig. 1B; spot 33K) and weakly with several other proteins including the 32-kDa protein (Fig. 1B, spot 1). The reactivity of serum collected from the *T. buffeli*- or the *T. orientalis*-infected calf with the 33-kDa protein of *T. sergenti* was weaker than that observed in the homologous combination (Fig. 1B–D).

Intense reactions with the 32- and 34-kDa proteins were observed between the homologous combination of *T. buffeli* antigen and the antiserum (Fig. 2C, spots 34K and 2). The serum collected from the calf that had been infected with *T. orientalis* also reacted intensely with the same proteins of *T. buffeli* (Fig. 2D). The reactivity of the serum collected from the calf that had been infected with *T. sergenti* with these proteins was weak (Fig. 2B).

Intense reactions with the 32- and 34-kDa proteins were also observed between the homologous combination of *T. orientalis* antigen and the antiserum (Fig. 3D, spots 34K and 1). The serum collected from the calf that had been infected with *T. buffeli* reacted intensely with the same proteins of *T. orientalis* (Fig. 3C). In contrast, the reactivity of the serum collected from the calf that had been infected with *T. sergenti* with these *T. orientalis* proteins was weak (Fig. 3B).

Analysis of biotinylated surface proteins of T. sergenti *and* T. buffeli *piroplasms*

Immunodominant proteins of piroplasms, the 33-kDa protein of *T. sergenti* and the 34-kDa protein of *T. buffe-*

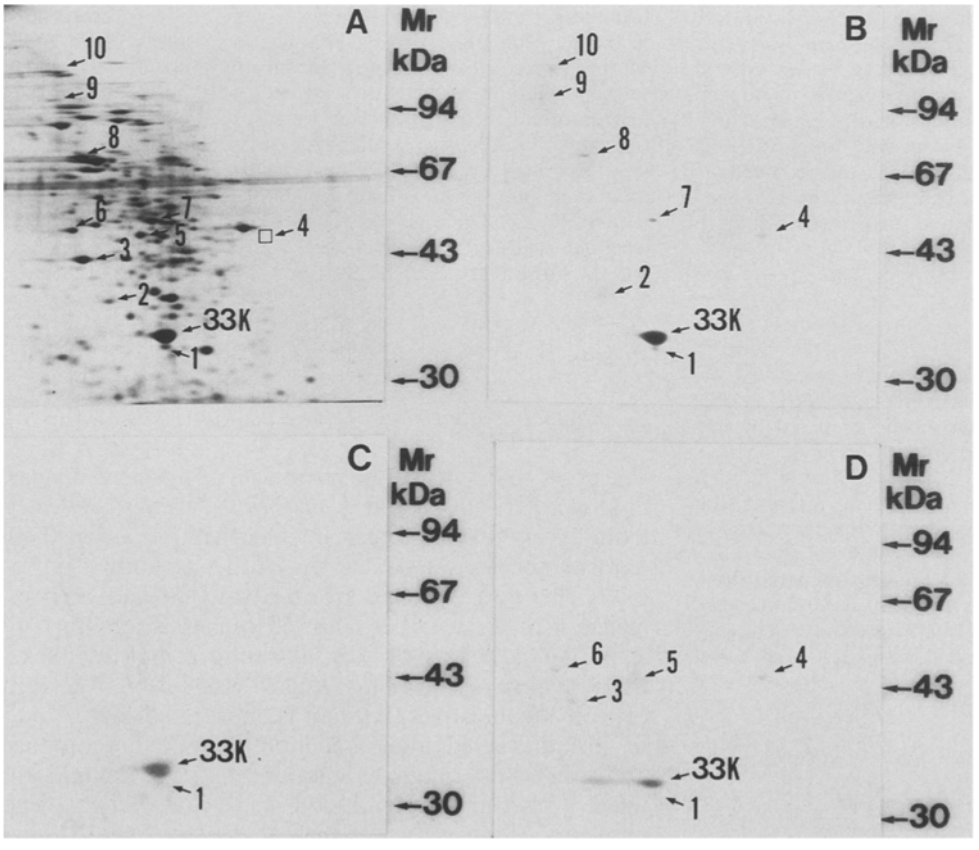

Fig. 1A–D. Western blot analysis of *Theileria sergenti* (Ikeda stock) piroplasm antigens using sera collected from calves that had been experimentally infected with *T. sergenti*, *T. buffeli* and *T. orientalis*. **A** Silver-stained 2D-PAGE of *T. sergenti* piroplasm proteins. **B, C, D** Profiles of *T. sergenti* piroplasm antigens probed with **B** serum (diluted 1:800) from a calf that had been infected with *T. sergenti*, **C** serum (diluted 1:400) from a calf that had been infected with *T. buffeli* or **D** serum (diluted 1:400) from a calf that had been infected with *T. orientalis*. The gel and corresponding Immobilon sheets were orientated with the acidic end to the *left* and the basic end to the *right*. Molecular weight markers (in kDa) are indicated on the *right*. *33K* indicates a protein spot of 33 kDa. Other protein spots detected by the sera are indicated by *arrows* and numbered *1–10*

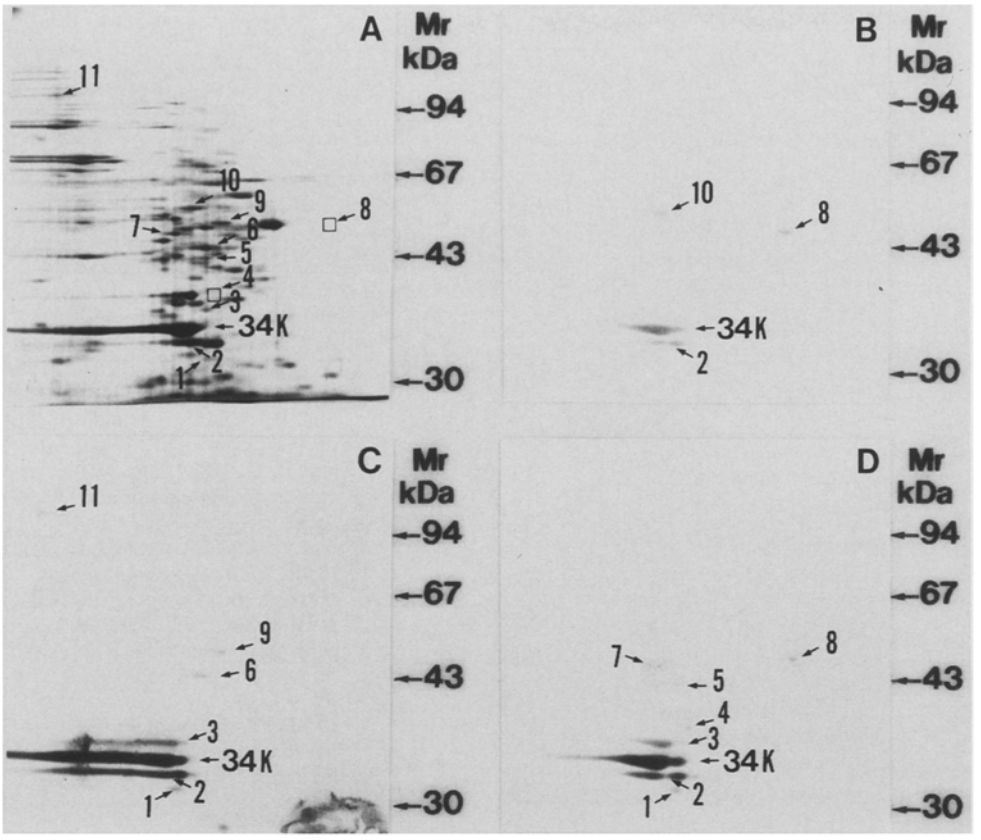

Fig. 2A–D. Western blot analysis of *Theileria buffeli* (Warwick stock) piroplasm antigens using sera collected from calves that had been experimentally infected with *T. sergenti*, *T. buffeli* and *T. orientalis*. **A** Silver-stained 2D-PAGE of *T. buffeli* piroplasm proteins. **B, C, D** Profiles of *T. buffeli* piroplasm antigens probed with **B** serum (diluted 1:800) from a calf that had been infected with *T. sergenti*, **C** serum (diluted 1:400) from a calf that had been infected with *T. buffeli* or **D** serum (diluted 1:400) from a calf that had been infected with *T. orientalis*. The gel and corresponding Immobilon sheets were orientated with the acidic end to the *left* and the basic end to the *right*. Molecular weight markers (in kDa) are indicated on the *right*. *34K* indicates protein spots of 34 kDa. Other protein spots detected by the sera are indicated by *arrows* and numbered *1–11*

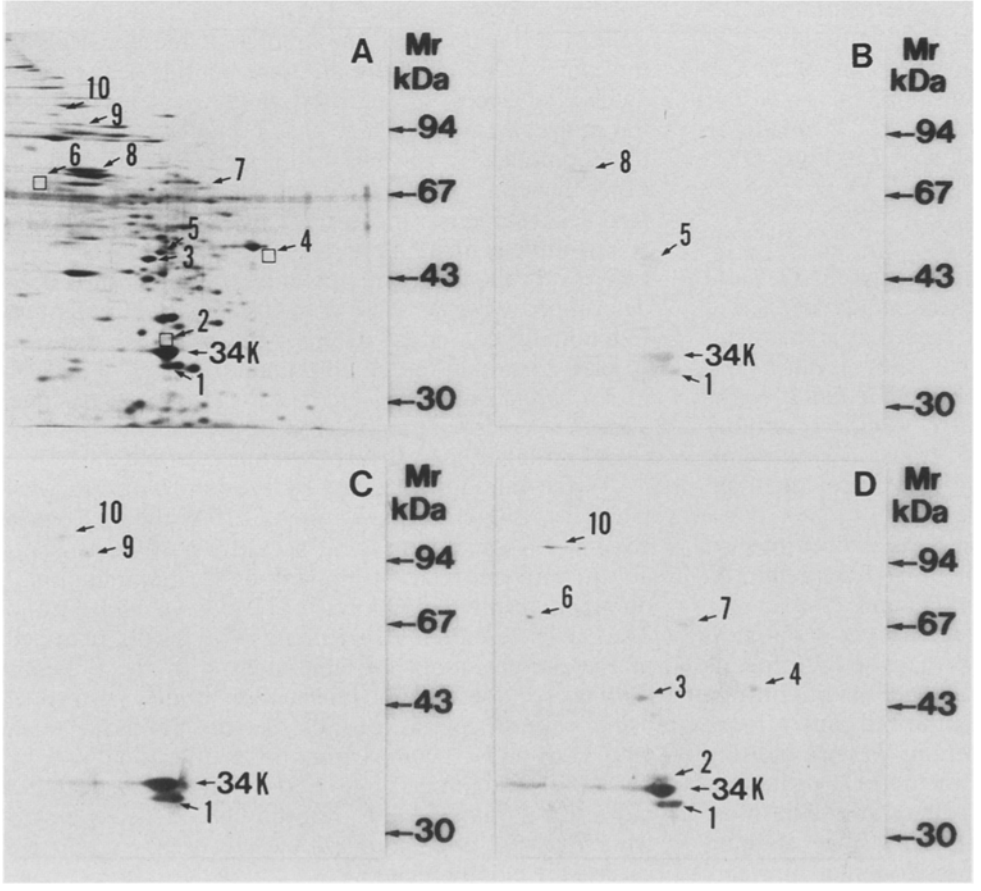

Fig. 3A–D. Western blot analysis of *Theileria orientalis* (Essex stock) piroplasm antigens using sera collected from calves that had been experimentally infected with *T. sergenti*, *T. buffeli* and *T. orientalis*. **A** Silver-stained 2D-PAGE of *T. orientalis* piroplasm proteins. **B, C, D** Profiles of *T. orientalis* piroplasm antigens probed with **B** serum (diluted 1:800) from a calf that had been infected with *T. sergenti*, **C** serum (diluted 1:400) from a calf that had been infected with *T. buffeli* or **D** serum (diluted 1:400) from a calf that had been infected with *T. orientalis*. The gel and corresponding Immobilon sheets were orientated with the acidic end to the *left* and the basic end of the *right*. Molecular weight markers (in kDa) are indicated on the *right*. *34K* indicates protein spots of 34 kDa. Other protein spots detected by the sera are indicated by *arrows* and numbered *1–10*

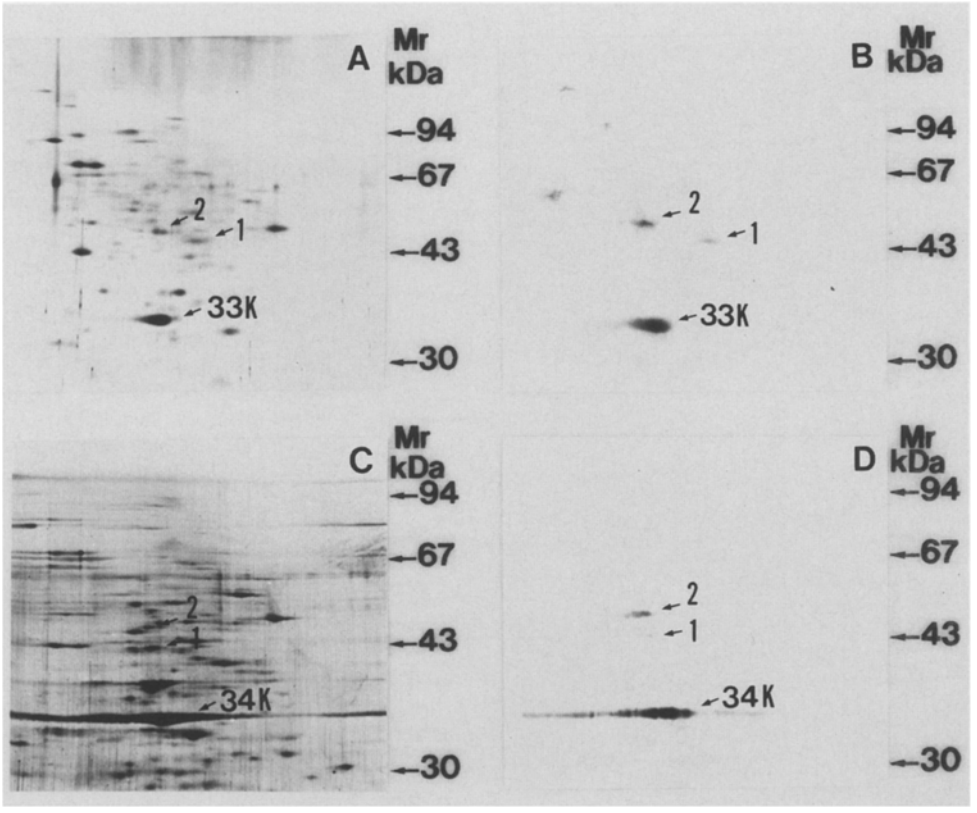

Fig. 4A–D. Identification of surface proteins on piroplasms of *Theileria sergenti* (Ikeda stock) and *T. buffeli* (Warwick stock). **A, C** Silver-stained 2D-PAGE of **A** *T. sergenti* and **C** *T. buffeli* piroplasm proteins after biotin labelling. **B, D** Surface protein profiles on piroplasms of **B** *T. sergenti* and **D** *T. buffeli*. The gels and corresponding Immobilon sheets were orientated with the acidic end to the *left* and the basic end to the *right*. Molecular weight markers (in kDa) are indicated on the *right*. *33K* indicates *T. sergenti* piroplasm protein spots of 33 kDa and *34K* indicates *T. buffeli* piroplasm protein spots of 34 kDa. Other protein spots labelled as surface components are indicated by *arrows* and numbered (*1, 2*)

li were labelled with biotin (Fig. 4A, B, spot 33K; Fig. 4C, D, spot 34K). The other immunodominant 32-kDa protein of *T. buffeli* was not biotin-accessible (Fig. 4C, D). Other minor surface proteins of 52 kDa (Fig. 4, spot 2 in *T. sergenti* and spot 2 in *T. buffeli*) were also detected by the serum from the *T. orientalis*-infected animal (spot 5 in Fig. 1D, spot 7 in Fig. 2D).

Discussion

The results obtained from the comparative ELISA and Western blot analysis of *Theileria sergenti*, *T. buffeli* and *T. orientalis* antigens using sera from cattle that had been infected with either of these parasites revealed close serological relationships between *T. buffeli* and *T. orientalis*. The results also indicated that *T. sergenti* is serologically dissimilar to the other two *Theileria* species, although several cross-reactive proteins were identified among those three theilerial species.

The most immunodominant proteins against infected cattle were the 33-kDa basic protein of *T. sergenti* and the 34-kDa basic proteins of *T. buffeli* and *T. orientalis*, which were serologically cross-reactive. The serological cross-reactivity observed between the 34-kDa proteins of *T. buffeli* and *T. orientalis* was evident. In contrast the 33-kDa protein of *T. sergenti* showed only a weak reaction with both heterologous sera in Western blotting and could be differentiated from the 34-kDa protein of *T. buffeli*/*T. orientalis*. These proteins are also most abundant in piroplasms as revealed by silver staining of 2D-PAGE gels. As these immunodominant proteins are biotin-accessible, they are considered to be exposed on the piroplasm surface. These 33-/34-kDa major piroplasm surface proteins may represent the homologous molecule with a partially different peptide sequence or deferential glycosylation. Interestingly, proteins in a similar molecular weight range have been detected as a major component of piroplasm or merozoite proteins in other theilerial species, including the 30-kDa merozoite/piroplasm-stage-specific surface protein of *T. annulata* (Glascodine et al. 1990) and the 32-kDa piroplasm proteins of *T. mutans* (Katende et al. 1990). It should be determined whether these proteins are structurally and functionally conserved over a wide range of theilerial species. The other immunodominant proteins of 32 kDa in *T. buffeli* and *T. orientalis* were also cross-reactive. These molecules are considered to correspond to an internal protein due to their biotin inaccessibility.

As the findings obtained by Western blottings were parallel to those obtained using ELISA, the serological cross-reactivity among these parasites may be attributable to antigenic relationships among the major immunodominant proteins: 33 kDa in *T. sergenti* and 32 and 34 kDa in *T. buffeli*/*T. orientalis*. The results obtained in the present study are consistent with the previous findings we obtained in transmission studies performed using several species of tick vectors (Fujisaki et al. 1991a, b) and in comparisons of profiles analysed by 2D-PAGE (Sugimoto et al. 1991b). All of these studies indicate that Japanese *T. sergenti* should be separated from *T. buffeli* and *T. orientalis*, and that the latter two species are closely related.

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