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Humoral immune responses to *Theileria parva* in cattle as measured by two-dimensional Western blotting

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Abstract Humoral immune responses to schizont antigens from six stocks of *Theileria parva* were compared by two-dimensional Western blotting using sera from cattle that had been infected with a *T. parva* stock or a clone. Isoelectric points of a polymorphic immunodominant molecule (PIM) of schizonts that induces strong antibody responses in cattle ranged from acidic to basic. Molecular masses (Mr) of the PIM of the respective *T. parva* stocks were as follows: *T. parva* Muguga, 86 kDa; Mariakani, 83 kDa; Marikebuni, 83 kDa; Uganda, 83 kDa; *T. parva* Boleni, 83 kDa; and *T. parva* 7014, 100 kDa. Among nine cattle infected with *T. parva* Muguga, four produced antibodies to a basic antigen having an Mr of 32 kDa. The PIM of *T. parva* Muguga, *T. parva* Boleni, and *T. parva* 7014 reacted strongly with serum obtained from an animal that had been infected with *T. parva* Muguga. Two-dimensional Western blotting using antischizont monoclonal antibodies enabled us to differentiate between stocks of *T. parva*.

Introduction

Theileria parva is a protozoan parasite transmitted by the tick *Rhipicephalus appendiculatus* that causes an acute and often fatal lymphoproliferative disease of cattle in eastern, central, and southern Africa, called East Coast fever (ECF). Three subspecies of *T. parva* have been described as causing disease in cattle: *T. p. parva*, transmitted between cattle and causing classic ECF; *T. p. lawrencei*, transmitted from buffalo to cattle and causing corridor disease; and *T. p. bovis*, occurring in the southern distribution of the disease during the period of adult *R. appendiculatus* activity and causing January disease.

These subspecies have been distinguished by the clinical signs of the diseases they cause and by their behavior (Uilenberg 1981; Dolan 1989b). The disease caused by *T. p. bovis* is generally milder than that caused by *T. p. parva* or *T. p. lawrencei*. *T. p. lawrencei* may be similar in virulence to *T. p. parva*, but the former produces fewer schizonts and piroplasms in cattle than does the latter. However, the subspecies are morphologically and serologically indistinguishable (Lawrence 1977; Uilenberg 1981). Furthermore, immunofluorescence tests using antischizont monoclonal antibodies (mAbs; Minami et al. 1983; Conrad et al. 1987b, 1989a), DNA hybridization (Conrad et al. 1987a, 1989b), and two-dimensional gel electrophoresis (Sugimoto et al. 1989) did not show a clear distinction between the buffalo- and cattle-derived parasites. It has since been agreed that this trinomial nomenclature should be dropped and that the term *T. parva* be used for all three parasites (Dolan 1989a).

Cattle can be immunized against ECF by the infection and treatment method involving simultaneous infection with sporozoites and treatment with long-acting tetracycline (Radley 1981). This method induces good protection against challenge with the same parasite stock or a limited number of heterologous stocks having an mAb profile similar to that of the stock used for initial immunization (Radley et al. 1975; Irvin et al. 1983; Minami et al. 1983). However, certain heterologous stocks may break through this protection. The antigenic diversity that exists among *T. parva* stocks presents a serious impediment to immunization, and a clear understanding of the heterogeneity in *T. parva* stocks is required for the development of an effective vaccination program against ECF.

In the present study, two-dimensional gel electrophoresis was combined with Western blotting to study the humoral immune responses to six stocks of *T. parva* in cattle.

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Materials and methods

Schizont-infected cell lines

Theileria parva-infected bovine cell lines were established in vitro by incubation of the cloned T-cell line 657.G6 (Brown and Grab 1985) or of bovine peripheral blood mononuclear cells with sporozoites obtained from tick salivary glands (Brown et al. 1973). The sporozoites were derived from stabilates of *T. parva* Muguga, 3087; Mariakani, 3029 and 3178; Marikebuni, 2245, 3014, and 3219; Uganda, 3066 (all cattle-derived, causing ECF, and isolated in Kenya except for the Uganda stock); *T. parva* Boleni, 3039 (cattle-derived, causing January disease, and isolated in Zimbabwe); and *T. parva* 7014 and 3081 (buffalo-derived and isolated in Kenya). The infected cells were maintained by serial passage in RPMI-1640 medium (Gibco, Paisley, Scotland) containing heat-inactivated fetal bovine serum (15%), L-glutamine (2 m*M*), gentamicin (50 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and HEPES buffer (20 m*M*). Cloned stabilates were prepared by the method of Morzaria et al. (1989). Uninfected T-cell line 657.G6, maintained in medium containing T-cell growth factor (Brown and Grab 1985), was used as the control.

Preparation of cell lysate for electrophoresis

T. parva-infected or uninfected lymphocytes were solubilized in a sample buffer (pH≥9.5) containing 9 *M* urea, 4% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo., USA), 2% 2-mercaptoethanol, and 2% Ampholine (pH 9–11; Pharmacia LKB biotechnology AB, Bromma, Sweden) at room temperature for 15 min (Anderson et al. 1985). The samples were centrifuged (Eppendorf 5413, Hamburg, Germany) at 8000 *g* for 1 min, and the supernatant was stored at –70° C prior to use. Protein concentrations of cell lysates were determined according to Lowry et al. (1951) using bovine serum albumin as the standard.

Table 1 Parasite stocks and animals used

Parasite stock	Animal numbers	Infection with
1. *Theileria parva* Muguga	F267, F313, G97 G191, G194	Bulk stabilate[a] 3087
	E18, E99	Sporozoites; challenge with infected ticks (both from infections with 3087)
	F118	Autologous cells infected with irradiated sporozoite bulk stabilate 3087
	F191	Cloned sporozoite stabilate[b] 3308
2. *T. parva* Mariakani	E220, E252	Cloned sporozoite stabilate 3178
3. *T. parva* Marikebuni	E15	Cloned cells[c] infected with stabilate 2245
	F18	Cloned stabilate[d] 3219
	F338	Cloned sporozoite stabilate 3262
4. *T. parva* Uganda	D810, F111, F270	Cloned cells infected with stabilate 3066
	F310	Ticks from animal F111
5. *T. parva* Boleni	D487	Bulk stabilate 3039 using infection and treatment
	F199	Cloned sporozoite stabilate 3230
	G2	Bulk stabilate 3039
6. *T. parva* 7014	D482	Bulk stabilate 3081 using infection and treatment
	E166	Bulk stabilate 3081
	E231, F172	Cloned cells infected with bulk stabilate 3081

[a] Stabilate – produced from an infection with an uncloned stock
[b] Cloned sporozoite stabilate – produced from an infection with a cloned parasite (Morzaria et al. 1989)
[c] Cloned cells – lymphocytes cloned by limiting dilution following infection in vitro
[d] Cloned stabilate – produced from an infection using cloned cells; parasite not necessarily cloned

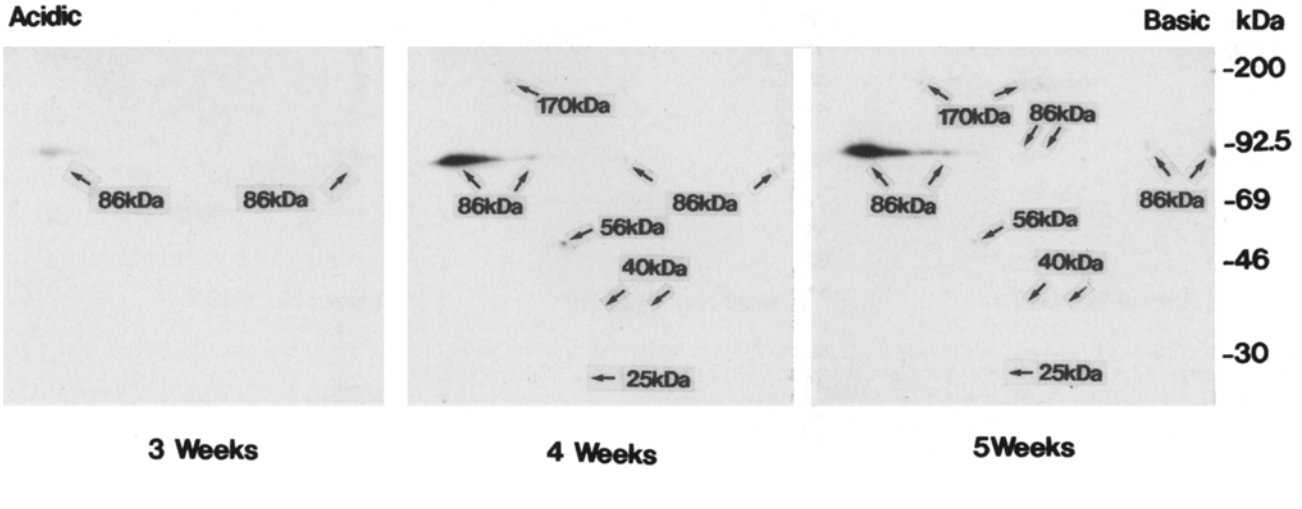


Fig. 1 Antibody response to *Theileria parva* Muguga at various intervals after infection. Sera from animal F313 infected with *T. parva* Muguga were obtained at 3, 4, and 5 weeks after infection. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

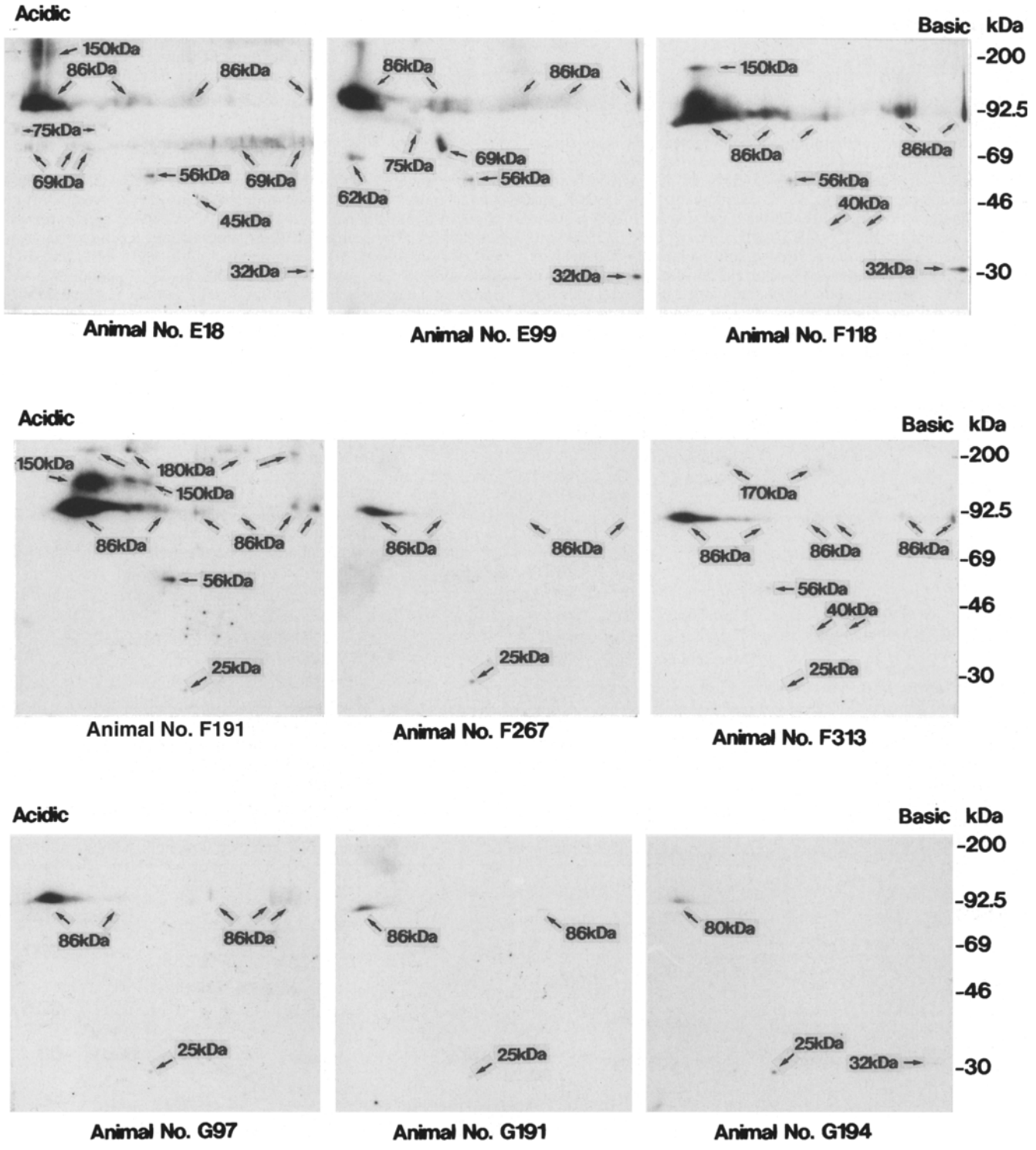


Fig. 2 comparison of antibody responses to *T. parva* Muguga in different cattle. Sera were obtained from animals E18, E99, F118, F191, F267, F313, G97, G191, and G194. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

Immune bovine sera

Bovine antisera were obtained from cattle (*Bos indicus*) that had been infected with each *T. parva* stock or clone as described in Table 1. Antibody titers of sera to *T. parva*-infected lymphocytes, measured by an immunofluorescence test (Goddeeris et al. 1982), were 1:1000 or 1:5000.

Monoclonal antibodies

MAbs 5 (originally BT5/2.2.5) and 7 (BT5/35.3.5) were raised against cells infected with *T. parva* Muguga (Pinder and Hewett 1980), and mAb 21 (IL-S27.12) was raised against a cell line isolated from steer U792, which had been infected with a buffalo-derived *Theileria* parasite (Conrad et al. 1989a). Ascitic fluid from mice carrying the hybridoma cells was used as antibody.

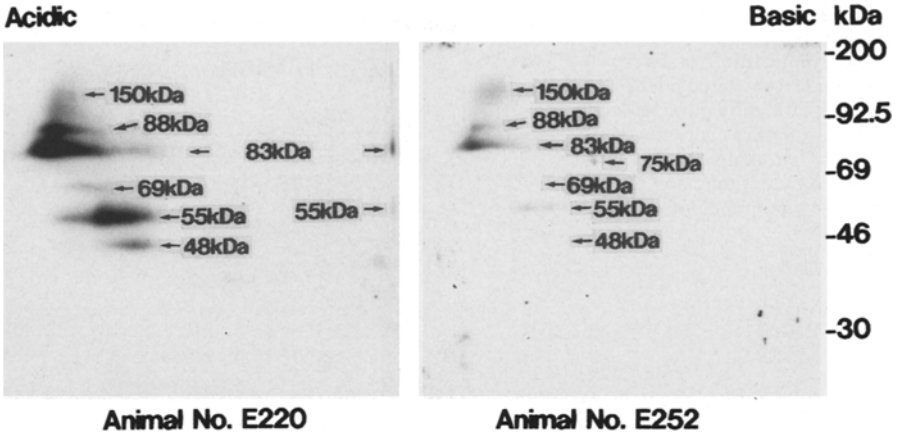


Fig. 3 Comparison of antibody responses to *T. parva* Mariakani in different cattle. Sera were obtained from animals E220 and E252. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

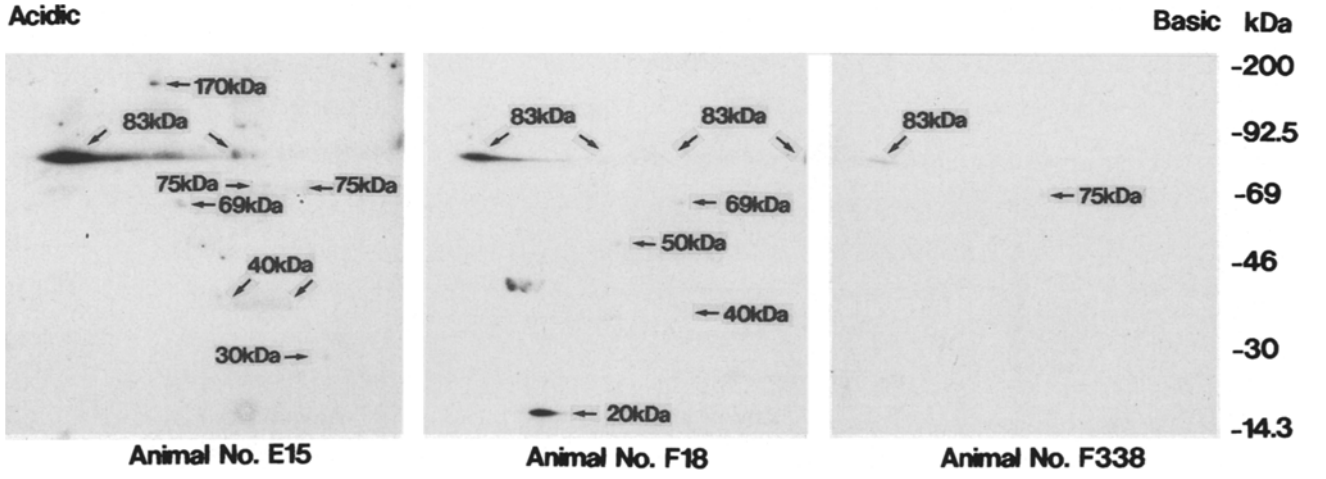


Fig. 4 Comparison of antibody reponses to *T. parva* Marikebuni in different cattle. Sera were obtained from animals E15, F18, and F338. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

Two-dimensional gel electrophoresis and Western-blot analysis

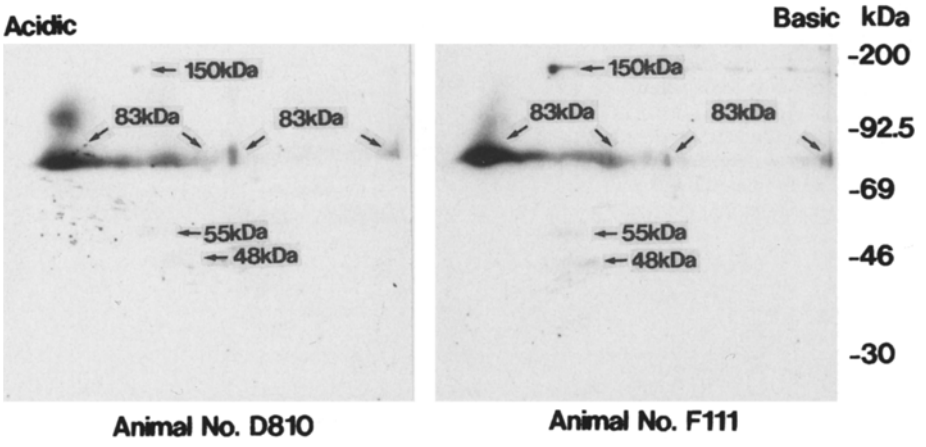
Separation of *T. parva* antigens was carried out by two-dimensional polyacrylamide gel electrophoresis according to the ISO-DALT system (Anderson and Anderson 1978a, b). Solubilized cell samples were thawed and centrifuged at 8000 *g* for 1 min immediately before use to remove insoluble aggregates. In the first dimension, isoelectric-focusing tube gels containing a 1:2 mixture of pH 4–6 and pH 3.5–10 Ampholine (Pharmacia LKB Biotechnology AB) were used. After prefocusing, cell lysate (100–200 µg in 20 µl buffer) was applied on the isoelectric focusing gel and run for 20 h at 600 V. In the second dimension, polyacrylamide gradient gels (7.5%–16.5%) were used. A ^{14}C-methylated protein mixture (14.3–200 kDa; Amersham International, Buckinghamshire, UK) was used as a molecular-mass marker.

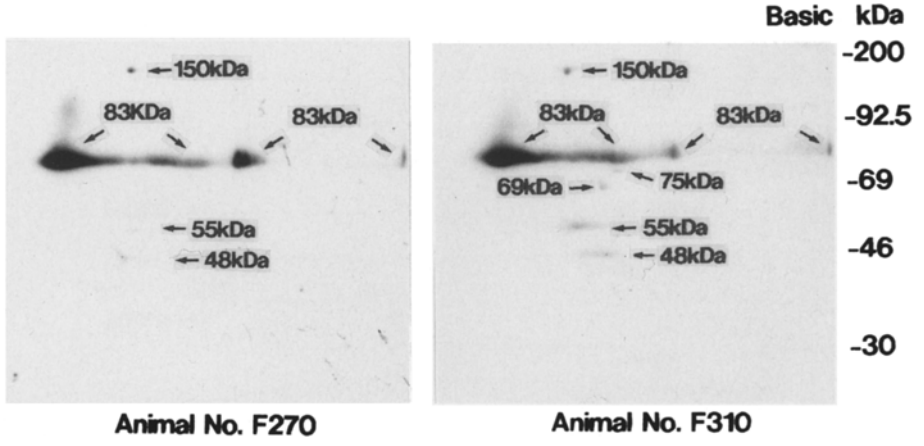
Following electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham International) electrophoretically using a transfer system (Hoeffer Scientific Instruments, San Francisco, Calif., USA). The transfer was performed in a buffer containing 25 m*M* TRIS, 192 m*M* glycine, 20% methanol, and 0.1% sodium dodecyl sulfate with a constant voltage of 80 V for 2 h. Thereafter, free protein-binding sites on the nitrocellulose membranes were blocked with 10% skimmed milk in TRIS-buffered saline (TRIS-NaCl; 20 m*M* TRIS, 150 m*M* NaCl, pH 8.1) for 1 h or overnight. After a washing with 5% skimmed milk in TRIS-NaCl (TRIS-NaCl-Milk) containing 0.1% Tween 20, the nitrocellulose membranes were reacted with immune bovine sera diluted 1:50 in TRIS-NaCl-Milk at room temperature for 2 h. After a washing with TRIS-NaCl-Milk containing 0.1% Tween 20, the nitrocellulose membranes were incubated with ^{125}I-labeled protein G (10 µCi/50 ml; Amersham International) in TRIS-NaCl-Milk at room temperature for 1 h. For detection of antigens with mAbs, the membranes were incubated with ^{125}I-labeled anti-mouse Ig (10 µCi/50 ml; Amersham International) after the reaction with mAbs. The membranes were washed, dried, and exposed to X-ray film (Fuji Photo Film Co., Ltd., Kanagawa, Japan) for autoradiography.

Results

Antibody response to *Theileria parva* Muguga at various intervals after infection

Sera were obtained from animal F313, infected with *T. parva* Muguga, at 3, 4, and 5 weeks after infection and antibody responses to schizont antigens were analyzed by two-dimensional Western blotting. Antibody responses to antigens in the acidic area and basic end having a molecular mass (Mr) of 86 kDa were detected at 3 weeks after infection (Fig. 1). As infection progressed, antibody responses to 86-kDa antigens having various isoelectric points ranging from acidic to basic and antigens having various Mr (25, 40, 56, and 170 kDa) were





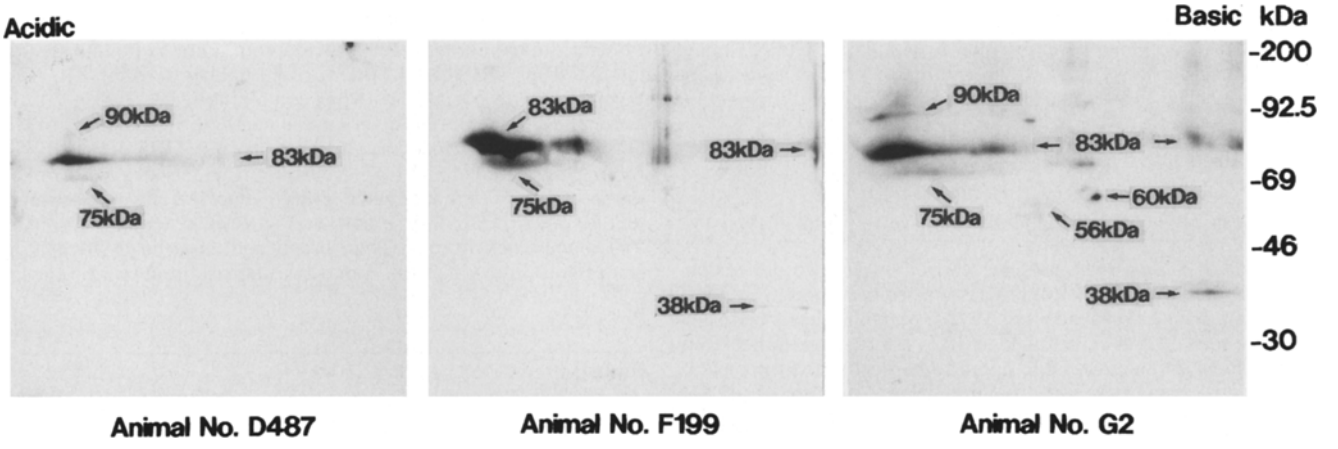


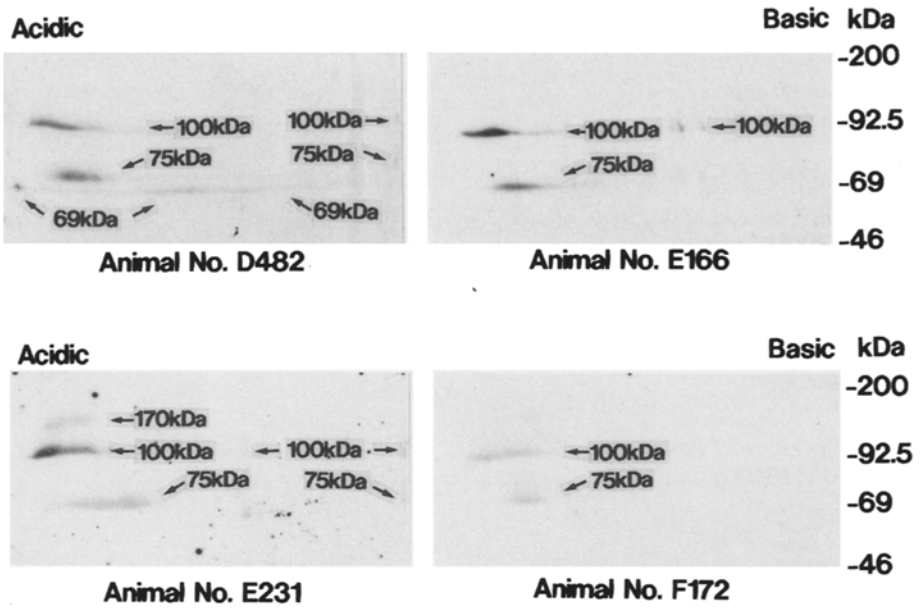
Fig. 5 Comparison of antibody responses to *T. parva* Uganda in different cattle. Sera were obtained from animals D810, F111, F270, and F310. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

Fig. 6 Comparison of antibody responses to *T. parva* Boleni in different cattle. Sera were obtained from animals D487, F199, and G2. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

detected (Fig. 1). The sera from animal F313 did not react with the lysate of uninfected 657.G6 control cells (data not shown). Furthermore, sera obtained from an uninfected control animal did not react with the lysate of *T. parva* Muguga-infected 657.G6 cells (data not shown).

Comparison of antibody responses to *T. parva* Muguga in different cattle

Antibody responses in nine cattle infected with *T. parva* Muguga were compared (Fig. 2). Major antibody responses were detected to antigens having an Mr of 86 kDa in all cattle. Four cattle (E18, E99, F118, and G194) produced antibodies to a basic antigen having an Mr of 32 kDa, and six cattle (F191, F267, F313, G97, G191, and G194) produced antibodies to a neutral antigen having an Mr of 25 kDa.



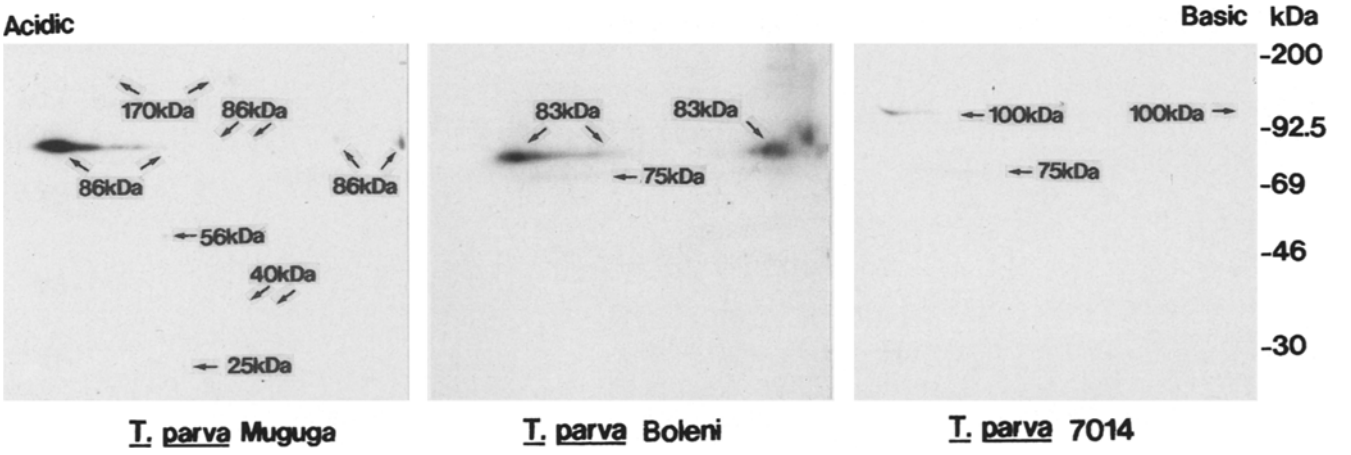


Fig. 7 Comparison of antibody responses to *T. parva* 7014 in different cattle. Sera were obtained from animals D482, E166, E231, and F172. Molecular-mass markers are indicated on the *right*. Antigens detected by the sera are indicated by *arrows with molecular mass*

Fig. 8 Cross-reactivity between different *T. parva* stocks. Antigens of each *T. parva* stock were probed with serum from animal F313, which had been infected with *T. parva* Muguga. Molecular-mass markers are indicated on the *right*. Antigens detected by the serum are indicated by *arrows with molecular mass*

Antibody responses to other cattle-derived stocks of *T. parva*

In *T. parva* Mariakani-infected cattle (E220 and E252), major responses were detected against 83-kDa antigens (Fig. 3). Responses were also detected against neutral and basic antigens having Mr of 48, 55, 69, 75 (animal E252), 88, and 150 kDa (Fig. 3). In *T. parva* Marikebuni-infected cattle (E15, F18, and F338), major responses were detected against 83-kDa antigens (Fig. 4). Responses were also detected against 20- (neutral, animal F18), 30- (basic, animal E15), 40- (neutral, animals E15 and F18), 50- (neutral, animal F18), 69- (neutral, animals E15 and F18), and 75-kDa (neutral, animals E15 and F338) antigens (Fig. 4). In *T. parva* Uganda-infected cattle, major antibody responses were detected against 83-kDa antigens (Fig. 5). Responses were also detected against neutral antigens having Mr of 48, 55, and 150 kDa in all cattle (Fig. 5). Western-blot patterns in *T. parva* Uganda-infected cattle were very similar to those in *T. parva* Mariakani-infected cattle (Figs. 3, 5).

Antibody responses to other stocks of *T. parva* derived from buffalo or from cattle infected with the January-disease parasite

In *T. parva* Boleni-infected cattle, major responses were detected against 83-kDa antigens (Fig. 6). Responses were also detected against 38- (basic, animals F199 and G2), 56- (neutral, animal G2), 60- (neutral, animal G2), 75- (acidic, all animals), and 90-kDa (acidic, animals D487 and G2) antigens (Fig. 6). In *T. parva* 7014-infected cattle, major responses were detected against 100-kDa antigens (Fig. 7). These 100-kDa antigens were related to the 86-/83-kDa antigens recognized following infection with the other parasite stocks, as they reacted strongly with antischizont mAb 5 (Fig. 9C). Responses

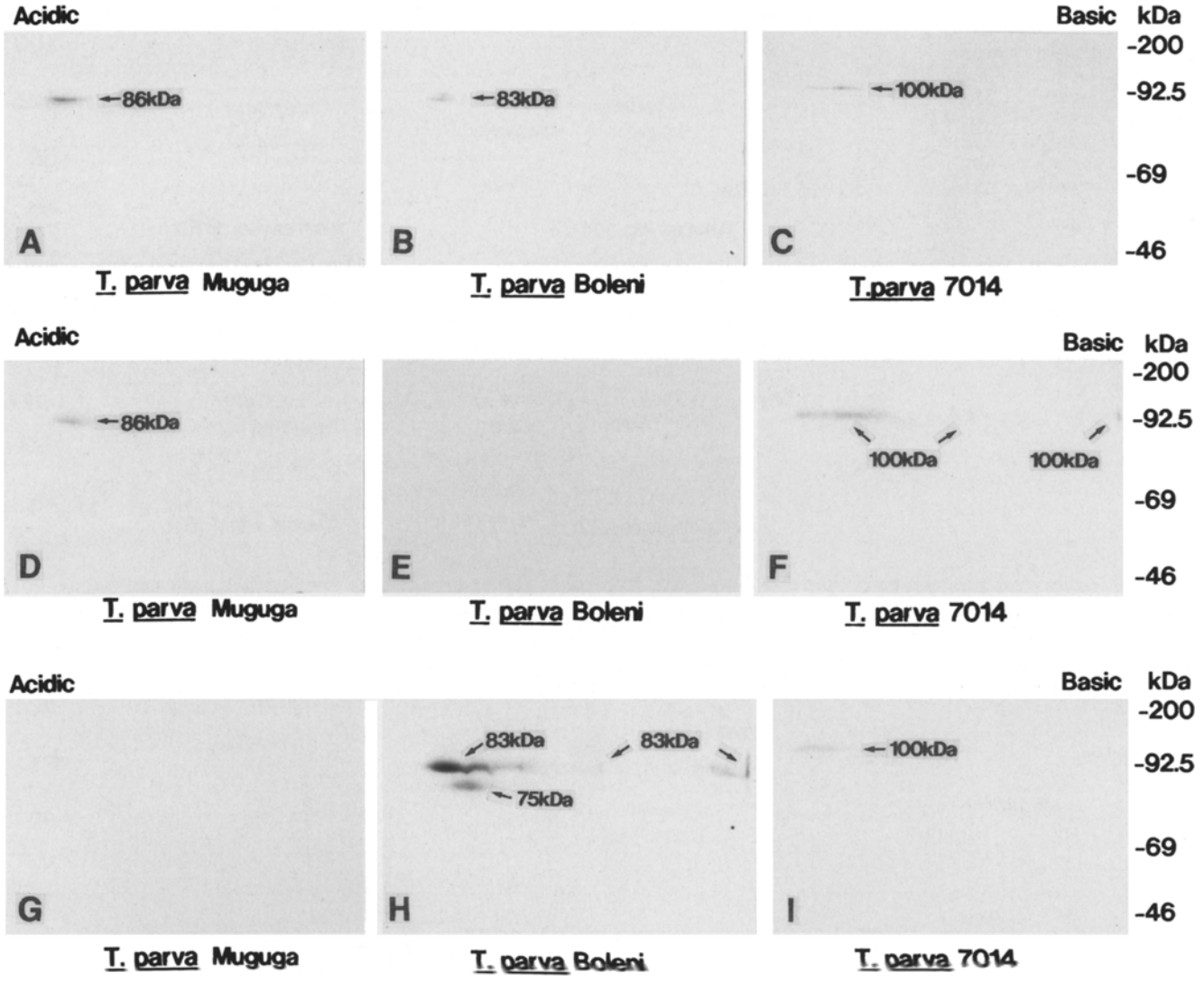


Fig. 9A–I Identification of schizont antigens by monoclonal antibodies. Schizont antigens of *T. parva* Muguga, *T. parva* Boleni, and *T. parva* 7014 were analyzed using monoclonal antibodies 5 (**A–C**), 7 (**D–F**), and 21 (**G–I**). Molecular-mass markers are indicated on the *right*. Antigens detected by the monoclonal antibodies are indicated by *arrows with molecular mass*

were also detected against 69- (animal D482), 75- (basic, all animals), and 170-kDa (basic, animal E231) antigens (Fig. 7).

Cross-reactivity between different stocks of *T. parva*

The patterns of reactivity of serum from a *T. parva* Muguga-infected animal with antigens of the other *T. parva* stocks were compared. Major schizont antigens of *T. parva* Boleni (83 kDa, acidic and basic) and *T. parva* 7014 (100 kDa, acidic and basic) reacted strongly with a serum from *T. parva* Muguga-infected animal F313 (Fig. 8). These results suggest that these antigens are cross-reactive between the stocks of *T. parva*.

Identification of schizont antigens by mAbs

Schizont antigens of the different *T. parva* stocks were analyzed by two-dimensional Western blotting using antischizont mAbs 5, 7, and 21. MAb 5 reacted with an acidic antigen having Mr of 86 kDa in *T. parva* Muguga, of 83 kDa in *T. parva* Boleni, and of 100 kDa in *T. parva* 7014 (Fig. 9A–C). MAb 7 reacted with an acidic antigen in *T. parva* Muguga having an Mr of 86 kDa and with antigens in *T. parva* 7014 having an Mr of 100 kDa (acidic, neutral, basic; Fig. 9D, F). MAb 7 did not react with schizont antigens in *T. parva* Boleni (Fig. 9E). MAb 21 reacted with antigens in *T. parva* Boleni having Mr of 83 (acidic, neutral, basic) and 75 kDa (acidic) and with an antigen in *T. parva* 7014 having an Mr of 100 kDa (acidic; Fig. 9H, I). MAb 21 did not react with antigens in *T. parva* Muguga (Fig. 9G).

Discussion

The results of this study using two-dimensional gel electrophoresis and Western blotting indicate that sera from

immune cattle reacted predominantly with *Theileria parva* antigens varying in Mr from 83 to 100 kDa. This result agrees with those obtained by Toye et al. (1991) and Sugimoto et al. (1992) and confirms this antigen as the polymorphic immunodominant molecule (PIM) of *T. parva*.

Heterogeneity among stocks of *T. parva* has been demonstrated by several methods. Minami et al. (1983) examined ten stocks of *T. parva* using an indirect immunofluroescence test with antischizont mAbs and demonstrated that these stocks fell into three groups on the basis of their reaction profiles. Conrad et al. (1987b) demonstrated that there was marked antigenic diversity among isolates from buffalo using the indirect immunofluorescence test, and many of the isolates consisted of mixed parasite populations. In addition, genomic diversity among stocks of *T. parva* was demonstrated by DNA hybridization (Conrad et al. 1987a). Later, Conrad et al. (1989a) reported that theilerial parasites that had similar reactions with a panel of antischizont mAbs were often genotypically different. They could not identify any specific phenotypic or genotypic characteristics of buffalo-derived theilerial parasites that would distinguish them from cattle-derived parasites (Conrad et al. 1989a). Sugimoto et al. (1989) analyzed schizont proteins of *T. parva* stocks by two-dimensional gel electrophoresis to identify specific proteins. *T. parva* stocks from cattle differed by only one protein spot; however, buffalo-derived *T. parva* showed several protein spots that were different from those of two cattle-derived *T. parva* stocks (Sugimoto et al. 1989). Differences in protein-spot patterns of schizonts from the Muguga, Marikani 3029, and Uganda stocks of *T. parva* were minimal and the patterns of the Mariakani 3029 and Uganda stocks were identical (Sugimoto et al. 1989).

In the present study, diversity in two-dimensional Western-blot patterns of schizont antigens from different *T. parva* stocks was detected using immune bovine sera. The Mr of the PIM in *T. parva* Muguga was 86 kDA; that in *T. parva* Mariakani, Marikebuni, Uganda, and Boleni was 83 kDa; and that in *T. parva* 7014 was 100 kDa, similar to the results obtained by Toye et al. (1991) and Sugimoto et al. (1992). In addition to the variation in Mr of the PIM, the isoelectric points of the PIM ranged from acidic to basic in the same stock of *T. parva*.

Sugimoto et al. (1992) reported that a basic antigen having an Mr of 32 kDa was detected by sera from two cattle (E18 and E99; also used in the present study) that had been infected with *T. parva* Muguga. Among nine cattle in the present study that had been infected with *T. parva* Muguga, four (E18, E99, F118, and G194) produced antibodies against the 32-kDa antigen and six (F191, F267, F313, G97, G191, and G194) produced antibodies to a neutral antigen having an Mr of 25 kDa. These findings indicate that individual differences exist in the antibody response to *T. parva* antigens. However, the use of different infection regimes and of cloned and uncloned parasites may have contributed to some of these differences.

The PIM of *T. parva* has been analyzed by Western blotting using antischizont mAbs (Shapiro et al. 1987; Toye et al. 1991; Sugimoto et al. 1992). Shapiro et al. (1987) demonstrated that four of the antischizont mAbs (4, 5, 6, and 12) reacted with the same antigen of schizont-infected cells. Toye et al. (1991) reported that the PIM was present in all *T. parva* stocks examined and that PIM was the only antigen that had been shown to react with antischizont mAbs on Western blotting. Sugimoto et al. (1992) demonstrated heterogeneity in both the Mr and the charge of PIM by two-dimensional Western blotting. In the present study, two-dimensional Western blotting using mAbs 5, 7, and 21 enabled us to differentiate among stocks of *T. parva* from different regions and derived from cattle or buffalo. Although individual variation in two-dimensional Western-blot patterns of schizont antigens was observed, fundamental patterns were unique in each *T. parva* stock.

Most, if not all, of the stocks used in this study appear to be mixtures of parasite types. The Marikebuni stock has been shown to consist of at least four parasite types with PIMs of different Mr (Toye et al. 1991). However, there appears to be a predominant component in this stock, as there probably is in the other stocks. The major antibody responses in cattle sera seem to be directed against the PIM of the predominant component of the stocks. Therefore, the two-dimensional Western-blotting procedure described herein was capable of detecting patterns that were unique to each stock.

Further studies should be made on the unique schizont antigens and unique mAbs that may predict the protective capacity and might be used in the selection of immunizing stocks for particular locations or in the search for candidate antigens for subunit vaccines.

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