Parasitol Res (1998) 84: 408–416

ORIGINAL PAPER

Joseph Katende · Subhash Morzaria · Philip Toye
Robert Skilton · Vish Nene · Catherine Nkonge
Antony Musoke

An enzyme-linked immunosorbent assay for detection of *Theileria parva* antibodies in cattle using a recombinant polymorphic immunodominant molecule

Received: 19 October 1997 / Accepted: 19 November 1997

Abstract Field and experimental bovine infection sera were used in immunoblots of sporozoite and schizont lysates of *Theileria parva* to identify candidate diagnostic antigens. Four parasite antigens of Mr 67,000 (p67), 85,000 (the polymorphic immunodominant molecule, PIM), 104,000 (p104), and 150,000 (p150) were selected for a more detailed analysis. The p67 and p104 antigens were present only in the sporozoite lysates, whereas PIM and p150 were found in both sporozoite and schizont lysates. The four antigens were expressed as recombinant fusion proteins and were compared with each other in an enzyme-linked immunosorbent assay (ELISA) and in the whole-schizont-based indirect fluorescent antibody test (IFAT) in terms of their ability to detect antibodies in sera of experimentally infected cattle. The PIM-based ELISA provided a higher degree of sensitivity and specificity than did the ELISA using the other three recombinant antigens or the IFAT. Further evaluation of the PIM-ELISA using experimental sera derived from cattle infected with different hemoparasites and field sera from endemic and nonendemic *T. parva* areas showed that the assay had a sensitivity of $> 99\%$ and a specificity of between 94% and 98%.

Introduction

Theileria parva is a tick-transmitted protozoan parasite of cattle and African buffalo. It causes a disease in cattle that is variously called East Coast fever, January disease, or corridor disease (reviewed in Irvin and Morrison 1987). The parasite is transmitted by the tick *Rhipicephalus appendiculatus* and has two stages in the mammalian host: the schizont, which is found in lymphocytes, and the piroplasm, which occurs in erythrocytes. In the tick vector the infective sporozoite stage is found in the salivary glands and is introduced during feeding. The disease causes high morbidity and mortality in susceptible cattle and is a major impediment to improvement of cattle productivity in eastern, central, and southern Africa. There are approximately 25 million cattle at risk, and in 1989 alone, losses due to the disease were estimated at U.S. $ 168 million (Mukhebi et al. 1991).

Currently the diagnosis of *T. parva* infection is based on the demonstration of parasites in Giemsa-stained blood and lymph-node smears and the detection of serum antibodies to schizont antigens using the indirect immunofluorescent antibody test (IFAT; Burridge and Kimber 1972; Goddeeris et al. 1982). The parasitological method has major limitations in that *T. parva* schizonts and piroplasms are difficult to differentiate from those of other *Theileria* species, namely, *T. taurotragi*, *T. mutans*, *T. buffeli*, and *T. velifera*. The IFAT has many disadvantages, which include cross-reactivity due to the use of crude antigens, difficulty in standardization, subjectivity in interpretation of the results, and the impracticability of processing large numbers of samples. It would therefore be desirable to develop a serology test more sensitive and specific than the currently available assays to provide definitive screening for antibodies following *T. parva* infection. In this paper we report on the development of a highly sensitive and specific-enzyme linked immunosorbent assay (ELISA), which is based on a defined recombinant *T. parva* antigen.

J. Katende · S. Morzaria (✉) · P. Toye
R. Skilton · V. Nene · C. Nkonge · A. Musoke
International Laboratory for Research on Animal Diseases,
P.O. Box 30709, Nairobi, Kenya
Fax: 0254-2-631499
E-mail: S.Morzaria@cgnet.com

Materials and methods

Animals

All experimental cattle were Boran (*Bos indicus*) steers that were aged 4–8 months and were raised in a tick-free environment. Before use they were screened by IFAT for antibodies against *Theileria parva* (Goddeeris et al. 1982), *T. mutans*, *Babesia bigemina* (Burridge 1971), *Trypanosoma vivax*, *T. brucei*, and *T. congolese* (Katende et al. 1987) and by ELISA for antibodies against *Anaplasma*

marginale (Voller 1977). Only those found to be negative in all the tests were used in this study.

Parasites

The details of the parasites used to infect experimental animals in this study are summarized in Table 1.

Infection of experimental cattle with various hemoparasites and collection of sera

Cattle were experimentally infected with various hemoparasites for the production of antisera. *T. parva* infections were produced in five groups of cattle. The first group of four calves aged 6 months received ticks infected with *T. parva* Muguga stabilate 3087. The second group of two calves was inoculated subcutaneously with 1 ml of sporozoites of *T. parva* Marikebuni stabilate 3014. The third, fourth, and fifth groups, consisting of two cattle each, were similarly inoculated with 1 ml of sporozoites of *T. parva* Muguga stabilate 3087, Uganda stabilate 3066, *T. parva* Boleni stabilate 3039, and *T. parva* buffalo-derived stabilate 3081, respectively. A large group of 112 cattle was infected at different times with either *T. parva* Muguga stabilate 3087 or Marikebuni stabilate 3014. The cattle were treated with Parvaquone (Clexon, Pitman Moore, UK) 2 days after they showed schizont parasitosis and pyrexia.

For the production of antisera against other theilerial species, ticks infected with the Intona strain of *T. mutans* were fed on four calves. Another group of two cattle were infected by intravenous inoculation of 5 ml of *T. buffeli* Marula blood stabilate 3890, and a further two cattle were infected similarly with 5 ml of *T. taurotragi* blood stabilate 3211.

Antisera against *B. bigemina*, *B.bovis*, and *A. marginale* were prepared as follows. Four calves were individually inoculated via the jugular vein with 1 ml of sporozoites of *B. bigemina* stock B-2 sporozoite stabilate 3899, whereas two calves were similarly inoculated with 1 ml of *B. bovis* blood stabilate 3818 and one calf was inoculated with 1 ml of *B. bovis* blood stabilate 3119. Two animals were individually inoculated intravenously with 1 ml of *A. marginale* stabilate BK 131.

Blood samples for serum acquisition were collected from all the cattle prior to infection and twice weekly for a period of 250 days post-infection. Sera were prepared and stored at −60 °C.

Sera prepared from three cattle infected with tsetse-transmitted *T. brucei*, *T. congolense*, or *T. vivax* were obtained from Dr. R. Masake at ILRI. In addition, sera collected from 15 animals that had been experimentally infected with *Schistosoma bovis* were provided by Dr. Khitma Hassan Elmaliki, Department of Preventive Medicine, Veterinary School, University of Khartoum, Sudan. Two serum samples were also obtained from cattle infected with *Fasciola hepatica* during the chronic stage of infection.

Field sera were obtained from cattle from different parts of the world (Table 2). Positive standard sera were collected from animals BJ 410 and C9. Animal BJ 410 was infected with *T. parva* (Muguga), and serum was obtained when the antibody response exceeded 1:3,200 in an ELISA using crude schizont antigen (Katende, unpublished data). Animal C9 was immunized against *T. parva* (Muguga) by infection and treatment and was rechallenged by application of infected ticks. The positive reference control serum was collected from animal C9 when the antibody titer exceeded 1:5,000 as determined by the IFAT. Negative control serum was obtained from an animal raised under tick-free conditions. It tested negatively in the IFAT for antibodies against *T. parva*, *T. mutans*, *T. buffeli*, *T. taurotragi*, *A. marginale*, *B. bigemina*, *T. brucei*, *T. congolense*, and *T. vivax*.

Preparation of schizont antigen and antibovine conjugates

The *T. parva* schizont antigen used in the IFAT was prepared by fixation of *T. parva* Muguga-infected lymphocytes in suspension with acetone and formaldehyde (Goddeeris et al. 1982). The antibovine immunoglobulin (Igs) conjugates were prepared by conjugation of fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP) to goat antibovine Ig as described by Katende et al. (1990).

Immunoblotting

Immunoblotting was carried out by standard procedures. The lysates of sporozoites and schizonts were separated by electrophoresis through a 7.5 to 17.5%-gradient sodium dodecyl sulfate-polyacrylamide gel (Laemmli 1970), and the separated antigens were transferred onto nitrocellulose membranes (Towbin et al. 1979). The membranes were probed with sera, at a dilution of 1:20, from naturally and experimentally infected cattle that had previously been shown to contain antibodies to *T. parva* by IFAT. The blots were developed with ^{125}I anti-bovine Ig followed by autoradiography.

Preparation of the recombinant antigens

The cDNA encoding antigen p67 was isolated by immunoscreening of a *lambda* gt11 library with immune sera (Iams et al. 1990; Nene

Table 1 Tick-borne pathogens and other protozoan parasites used to infect experimental animals

Parasite species	Stock	Location	Country	Stabilate number	Reference
Theileria Parva	Muguga	Kikuyu	Kenya	3087	Brocklesby and Barnett 1961
T. parva	Marikebuni	Coast Province	Kenya	3014	Irvin et al. 1983
T. parva	Uganda	Kigungu	Uganda	3066	Minami et al. 1983
T. parva	Boleni	Boleni	Zimbabwe	3039	Lawrence and Mackenzie 1980
T. parva	Buffalo 7014	Laikipia	Kenya	3081	Morzaria et al. 1990
T. mutans	Intona	Transmara	Kenya	3289	Mutugi 1987
T. buffeli	Marula	Naivasha	Kenya	3890	S. Williamson[a]
T. taurotragi	Eland	Laikipia	Kenya	3211	Stagg et al. 1983
Babesia bigemina	B-2	Naivasha	Kenya	3899	S. Williamson[a]
B. bovis	K 1614(a)	Brisbane	Australia	3818	W. Jorgensen[b]
B. bovis	Zanzibar	Zanzibar	Tanzania	3119	S. Morzaria
Anaplasma marginale	ILRI	Kapiti	Kenya	BK 131	J. Katende
Trypanosoma brucei	427	Tororo	Uganda	ILB42	Cunningham and Vickerman 1962
T. congolense	212	Serengeti	Tanzania	IL1180	Geigy and Kauffmann 1973
T. vivax	Y486	Yakawada	Nigeria	IL306	Leeflang et al. 1976

[a] Kenya Agricultural Research Institute (KARI), Muguga
[b] Tick Research Center, Wacol, Australia

Table 2 Field sera collected from cattle in East Coast fever-endemic and free zones in different parts of the world

Country	Location	Number of sera	Important tick-borne pathogens and other protozoan parasites present
Kenya	Kaloleni	161	*T. parva*, *T. mutans*, *A. marginale*, *B. bigemina*, *Cowdria ruminantium*
Uganda	Kampala and Jinja	321	*T. parva*, *T. mutans*, *A. marginale*, *B. bigemina*, *C. ruminantium*
Uganda	Nabiswera	128	*T. parva*, *T. mutans*, *A. marginale*, *B. bigemina* *Trypanosoma* species
Zaire	Kivu	77	*T. parva*, *T. mutans*, *A. marginale*, *B. bigemina*
Kenya	Kapiti	721	*T. taurotragi*, *A. marginale*, *B. bigemina*
UK	Edinburgh	179	None
Burkina Faso	Bobo-Dioulasso	110	*T. mutans*, *A. marginale*, *B. bigemina*, *Trypanosoma* species
Ivory Coast	Kohrogo	162	*T. mutans*, *A. marginale*, *B. bigemina*, *Trypanosoma* species
Sudan	Shanbati	61	*T. annulata*, *T. mutans*, *B. bigemina*

et al. 1992). The gene encoding full-length antigen p67 was expressed in the pMG1 plasmid vector system, where the expressed product is a fusion protein in which the first 85 amino acids are derived from NS1, a nonstructural protein of influenza virus A (Young et al. 1983; Musoke et al. 1992). Biochemically purified NSI-p67 was provided by SmithKline Beecham (Animal Health, USA). The cDNA encoding full-length PIM was isolated using eukaryotic expression cloning technology (Toye et al. 1995) and then subcloned into pGEX3X (Smith and Johnson 1988) for expression. Genes encoding antigens p104 (Iams et al. 1990) and p150 (M. Macklin, unpublished results) were isolated by screening of genomic expression libraries, constructed in *lambda* gt11, with immune sera. The complete p104 gene encodes a protein of 924 amino acids (Iams et al. 1990). A *Bam*HI-*Mun*I DNA fragment encoding antigenic determinant(s) encoded by C-terminal amino acids 721–924 was cloned into pGEX-1N (Smith and Johnson 1988). The p150 gene encodes a protein of 1,453 amino acids (R. Skilton, unpublished results). An *Eco*RI fragment of DNA encoding the C-terminal amino acids 1,181–1,453 was cloned into pGEX-1N.

The PIM, p104, and p150 recombinant antigens, as glutathione S-transferase (GST) fusion proteins, were expressed by (isopropyl-beta-D-thiogalactopyranoside) (IPTG) induction of *Escherichia coli* cultures transformed with recombinant pGEX. Fusion proteins were purified by affinity chromatography on glutathione-agarose as described previously (Smith and Johnson 1988).

Enzyme-linked immunosorbent assays

Polysorb micro-ELISA plates (Polysorb, Nunc, Denmark) were coated with recombinant antigens at a concentration of 50 ng/well at 37 °C for 90 min. Excess antigen solution was discarded, and the uncoated sites on the walls of the microtiter plate and the non-specific sites on the antigen were blocked by the addition of 0.25% casein followed by incubation at 37 °C for 1 h. Test sera, diluted 1:200 in Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) containing 0.1% Tween 20 and 2% skimmed milk, were added to the wells of the micro-ELISA plate in two replicates (150 µl/well). The control sera (strong positive, weak positive, and negative control) were diluted as described for the test sera. These and the conjugate control (DPBS alone) were added to the plate in four replicates. The antibodies were allowed to bind to the antigen by incubation of the plate for 25 min at room temperature (RT) under continuous gentle agitation on a micro-agitator (Heidolph, France). The unbound antibodies were removed by extensive washing of the plate before the addion to each well of 150 µl of anti-bovine Ig HRP conjugate diluted 1:5,000 in DPBS containing 0.1% Tween 20 and 2% skimmed milk.

The plate was incubated and washed as described above. The color reaction was developed by the addition of 150 µl of sodium citrate buffer (pH 4.0) containing 1% hydrogen peroxide as the substrate and of 40 m*M* 2,2′-azino-*bis*(3-ethylbenz-thiazoline-6-sulfuric acid), diammonium salt (ABTS), as the chromogen followed by incubation for 30 min in a dark environment. During incubation the micro-ELISA plate was shaken for 5 min every 15 min to ensure maximal color development. The optical density (OD) was determined in a Titertek Multiscan Mcc340 spectrophotometer. The OD values were expressed as percentages of positivity (PP), which were calculated as follows: (*OD of test*/*OD of strong positive*) × *100* (Wright et al. 1993).

Results

Selection of candidate antigens

For identification of antigens of potential use in an ELISA, schizont and sporozoite lysates were examined by immunoblot analysis using sera from naturally infected animals. This analysis demonstrated that most of the 160 field sera recognized 4 major proteins of Mr 67,000 (p67), 85,000 (PIM), 104,000 (p104), and 150,000 (p150; Fig. 1). Antigens p67 and p104 were detected only in the sporozoite preparations, whereas the other two proteins were present in both sporozoite and schizont lysates (Fig. 1). In all, 92% of the field sera contained antibodies to PIM, whereas antibodies to antigens p104, p67, and p150 were found in 83%, 80%, and 78% of the sera, respectively.

To compare these antigens further, we examined their reactivity in an ELISA with sera from experimentally infected cattle. As it is difficult and impractical to purify large quantities of these antigens by biochemistry or immunopurification techniques, the genes encoding the respective antigens were cloned and expressed as recombinant fusion proteins. The four recombinant antigens were evaluated in an ELISA using sequential sera collected from four cattle infected by tick application.

All four recombinant antigens detected antibodies on day 17 after infection (Fig. 2), but the antibody PP values recorded for PIM were greater than those noted for the other antigens. The antibodies to PIM remained detectable for up to 215 days after infection as compared with 120 days for p104, 90 days for p150, and 60 days

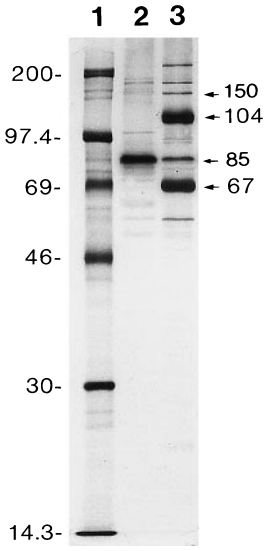

Fig. 1 Western-blot gel showing *1* molecular-weight markers, *2* *Theileria parva* Muguga schizont lysate, *3* *T. parva* Muguga sporozoite lysate. The blot was developed with sera from naturally infected cattle as the first antibody.

for p67 in the ELISA. These results indicated that the PIM-ELISA had a higher degree of sensitivity than did the ELISA using the other three antigens for the detection of *Theileria parva* antibodies. Similarly, the PIM-ELISA was shown to be more sensitive than the schizont-IFAT (results not shown).

Due to the polymorphism of PIM it was necessary to assess whether the Muguga recombinant PIM would detect antibodies in sera from cattle infected with different *T. parva* stocks. For this purpose, five pairs of cattle, each infected with one of the five stocks of *T. parva* (Muguga, Uganda, Marikebuni, Boleni, and the buffalo-derived stock 7014) exhibiting polymorphic PIM (Toye et al. 1991), were tested. All cattle developed antibodies that were detectable in the PIM-ELISA at days 13–28 after infection and remained positive until day 150, when the experiment was terminated (see Fig. 6). In addition, analysis of the 21 post-infection sera obtained from 111 cattle, immunized by the infection and treatment method against *T. parva* Muguga (99 animals) and Marikebuni (12 animals), showed that all animals were positive, with PP values lying above the cutoff value of 18.

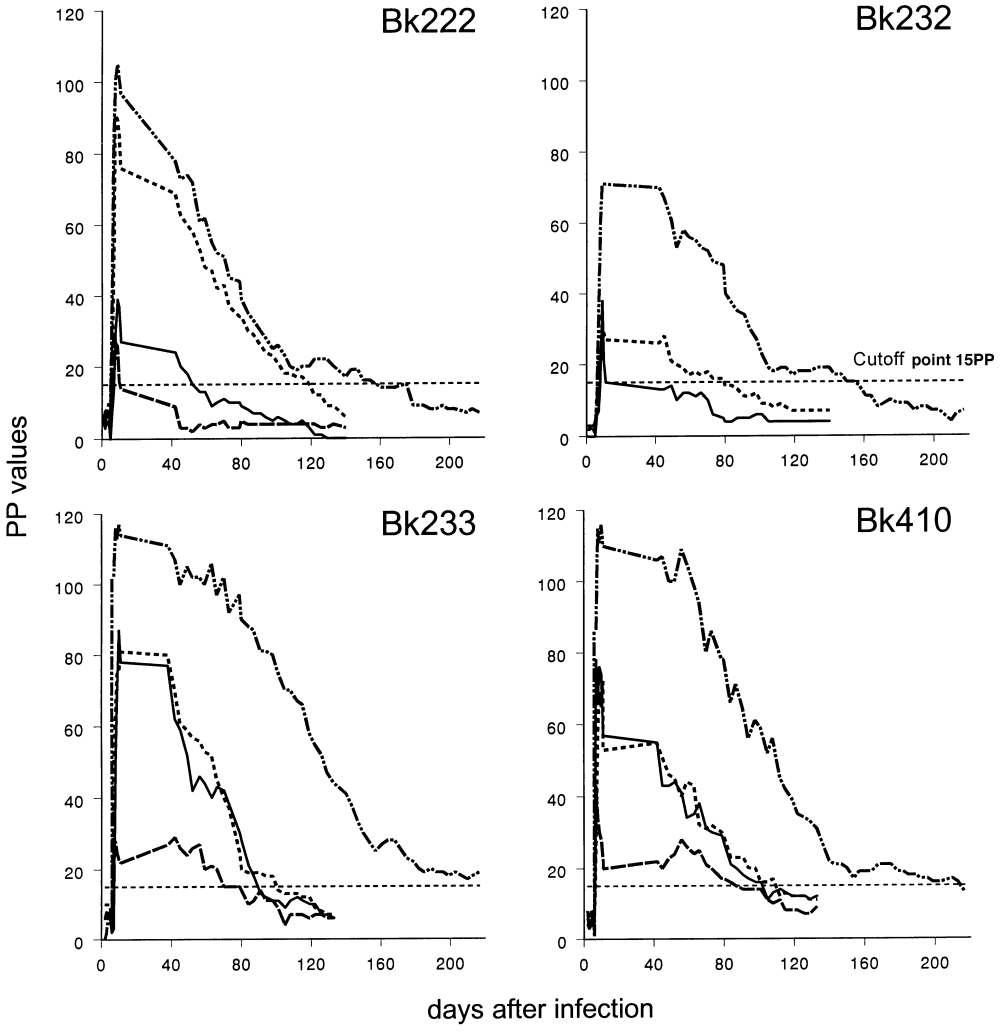

Fig. 2 Percentage of positivity recorded for antibodies against p67(––), PIM(–··–), p104 (——), and p150 (- - -) in the ELISA in four animals (BK222, BK232, BK233, and BK410) that had been experimentally infected with *T. parva* Muguga

Evaluation of the sensitivity of the PIM-based ELISA with field sera

The test was evaluated using sera collected from cattle in East Coast fever-endemic areas. In all, 77 serum samples collected from adult Ankole cattle from North Kivu in western Zaire (Makunyaviri and Habimana 1993) and another 321 obtained from East African zebu cattle from small-scale farms located within a radius of 50 km from Kampala, Uganda, were examined. The sera from Zaire had antibody prevalence rates of 88% in the PIM-ELISA and 61% in the IFAT. Similarly, the Uganda sera had prevalence rates of 83% (Fig. 3) and 77% as determined by ELISA and IFAT, respectively. Further comparison of the results revealed that 75% of the ELISA-positive Zaire sera and 95% of the ELISA-positive Uganda sera were also positive in the IFAT. In both sets the sera that were positive in the IFAT were also positive in the ELISA.

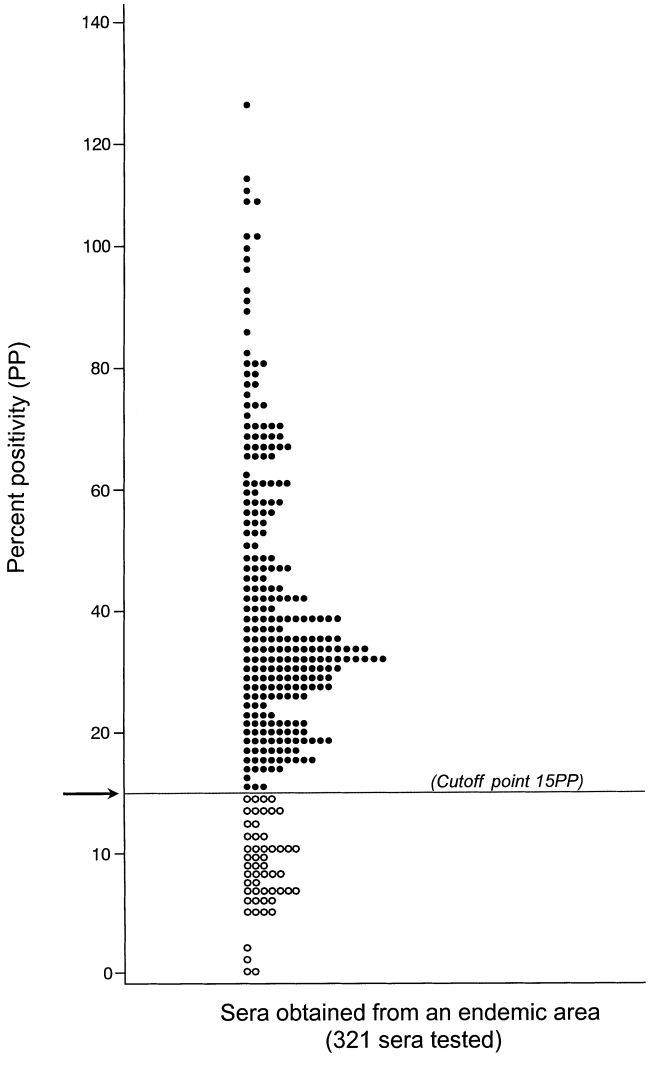

Fig. 3 Percentage of positive (*PP*) calculations, illustrating the sensitivity of the PIM-based ELISA for *T. parva*. A total of 321 sera obtained from animals from an endemic area were tested

Sequential sera collected for a period of 9 months from 128 cattle kept on a farm in Nabiswera, western Uganda, where there was epidemiological and clinical evidence of *T. parva* (Okelo Onen, personal communication), were tested by ELISA. The results indicated that once animals had seroconverted, the antibodies to PIM persisted throughout the period of observation (results not shown).

Selection of strong and weak positive standards and establishment of the positive/negative threshold

Standard sera are necessary to ensure that the sensitivity of an ELISA is consistent between assays (Wright et al. 1993). The standards consist of a strong positive and a weak positive serum as well as a negative control serum. The ratio of the OD or PP value obtained with the strong positive serum to that obtained with the weak positive serum should not vary considerably between assays. The negative serum control monitors the background reaction; it is also necessary for the establishment of a positive/negative threshold value.

For PIM-ELISA the strong and weak positive sera and the positive/negative threshold were established by titration of a reference antiserum from 1:50 to 1:12,800. This reference serum was obtained from animal C9, which had been infected twice with sporozoites of *T. parva* (Muguga). A standard curve was produced by plotting of the OD against the reciprocal of dilutions of antiserum C9 (Fig. 4). The strong positive control serum was obtained from animal BJ 410 at 30 days after infection. When it was tested at a routine dilution of 1:200 an OD reading of 0.841 was obtained. When this OD was located on the established standard curve generated for C9 antiserum it corresponded to a dilution that lay on the linear portion of the curve just below the plateau (Fig. 4, C++ representing 100 PP). The positive control serum was therefore selected as antiserum BJ 410 diluted 1:200. When the BJ 410 was tested at a dilution of 1:1,500 an OD value of 0.420 was obtained, which was equivalent to 50 PP (Fig. 4, C+ 50 PP). The BJ 410 antiserum diluted 1:1,500 in negative serum was therefore selected as the weak positive control serum.

The positive/negative threshold was taken as the lowest point on the linear portion of the curve generated for C9 antiserum. This point corresponded to an OD value of 0.131, which represents a PP of 15 relative to the OD of 0.841 recorded for the strong positive serum. Therefore, a PP of 15 was taken as the positive/negative threshold for this ELISA. This value was confirmed by testing of 960 serum samples from Holland and of another 721 from Kapiti Plains, Kenya, where *T. parva* does not exist. When 25D were added to the mean PP values recorded for the 960 and 720 serum samples, values of 16.1 and 16.4, respectively, were obtained. These values were not significantly different from that

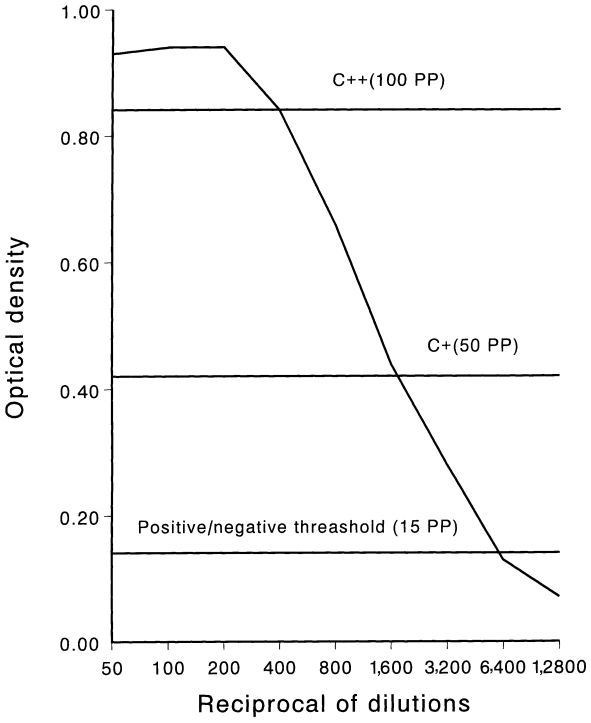

Fig. 4 Indirect ELISA titration curve of hyperimmune serum prepared from the blood of an animal infected with *T. parva* Muguga. *C++(100 PP)* represents the optical density recorded at a dilution of 1:200 of the strong standard positive serum. *C+(50 PP)* represents the optical density noted at a dilution of 1:1,500 of the strong standard (in negative reference serum) that was equivalent to 50 *PP* and was used as the weak positive standard. The positive/negative threshold (15 *PP*) represents the lowest optical density on the linear scale of the curve, which is the lowest antibody activity this assay can detect

obtained using a single sample of highly positive antiserum C9.

Establishment of the sensitivity and specificity of the assay

For establishment of the sensitivity of this ELISA, 12 animals that had been experimentally infected with different isolates of *T. parva* were used as a source of positive sera. The 12 animals sero-converted between days 13 and 28 after infection as measured by the IFAT and PIM-ELISA. The sensitivity of the test was calculated according to Martin et al. (1989) as indicated in Table 3. Analysis of the data showed that the sensitivity of the assay was $> 99\%$.

To establish the specificity of the assay we used a total of 721 serum samples from Kapiti Plains, Kenya, derived from a herd with no epidemiological, clinical, or serological evidence of *T. parva* infection. However, many of these samples were serologically positive for *Anaplasma marginale*, *T. taurotragi*, and *Babesia bigemina* (J. Katende, unpublished observation).Animals were selected and arranged into groups of four according to age as follows: 4 days to 3 months, 4–12 months, 13–24 months, and 3–4 years. On screening of the sera for antibodies to PIM, 709 sera gave PP values below the positive/negative threshold value of 15, whereas 12 sera gave a value exceeding 15 (Fig. 5).

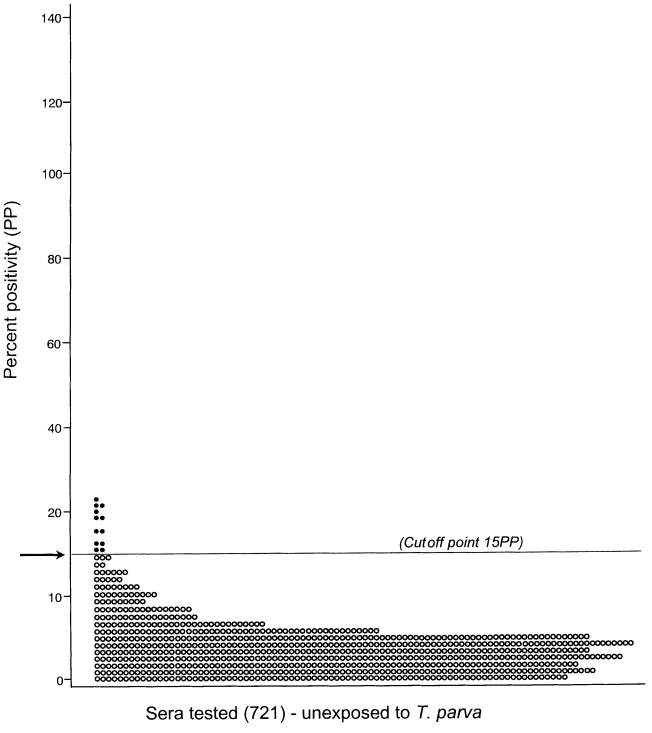

Fig. 5 Percentage of positivity (*PP*) calculations, illustrating the specificity of the PIM-based ELISA for *T. parva*. The total of 721 sera from a population unexposed to *T. parva* were tested

Table 3 Establishment of the sensitivity and specificity of the improved ELISA for *T. parva* based on PIM[a] (*A* True-positive finding, *B* false-positive finding, *C* false-negative finding, *D* true-negative finding, + ve positive, − negative)

	Infected	Healthy	Total
Test + ve	A (12)	B (12)	A + B
Test −ve	C (0)	D (709)	C + D
Total	A + C (12)	B + D (721)	(A + B) + (C + D)

[a] The *numbers in parentheses* indicate the actual numbers that were either positive or negative in the experiments. Sensitivity is the proportion of animals that test positively among the infected population and is calculated as $[A/(A+C)] \times 100$, whereas specificity is the proportion of those that tested negatively among the known healthy population is calculated as equals $[D/(B+D)] \times 100$

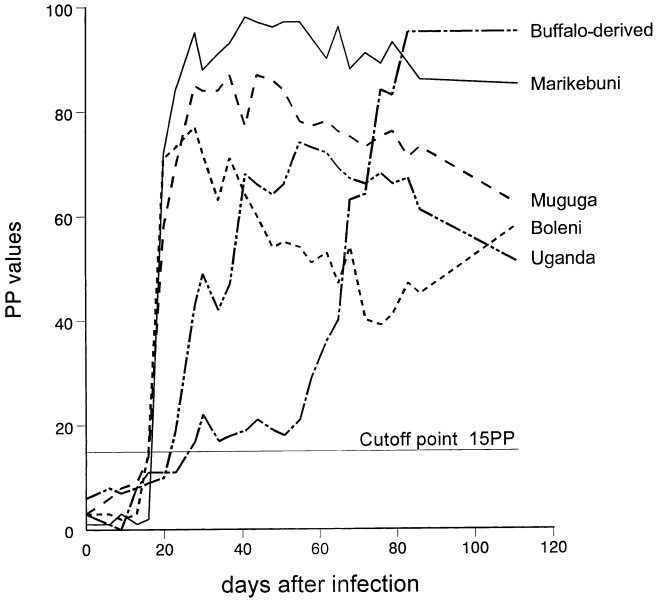

Fig. 6 Antibody responses (in PP values) to the PIM antigen as detected in four animals experimentally infected with sporozoite stabilates of five *T. parva* isolates

The analysis of the data gave specificities of 99%, 97%, 100%, and 97% for the groups aged 14 days to 3 months, 4–12 months, 13–24 months, and 3–4 years, respectively. The overall specificity was 98%.

Cross-reactivity with other parasite antigens

The PIM antigen was expressed as a fusion protein with the enzyme glutathione S-transferase (GST). Analogues of GST are present in *Schistosoma bovis* and *Fasciola hepatica* (Hillyer et al. 1992). Since infections with these parasites occur within East Coast fever (ECF)-endemic areas, it was necessary that we rule out the possibility of false-positive findings due to GST by testing sera obtained from cattle that had been experimentally infected with *S. bovis* and *F. hepatica* during the acute and chronic stages of infection. All 15 sera from *S. bovis*-infected animals tested negatively (PP < 15) in the PIM-ELISA assay, as did the 2 sera collected from cattle infected with *F. hepatica* (PP 15).

To evaluate further the specificity of this assay, we used sequential sera collected from animals that had been experimentally infected with other hemoparasites, namely, *T. mutans*, *T. taurotragi*, *T. buffeli*, *B. bigemina*, *B. bovis*, *Trypanosoma brucei*, *T. vivax*, and *T. congolense*. All values obtained were below PP 15, indicating that the epitopes on the recombinant PIM were not recognized by antibodies to these parasites in cattle.

The test was also evaluated using 61 sera from northern Sudan, where *T. annulata* is endemic. Of the 61 sera, 59 tested positively for *T. annulata* in the IFAT, with titers ranging between 1:200 and $> 1:5,000$. When these sera were tested in the PIM-ELISA, one sample had a PP of 15 and the values recorded for the rest were below the positive/negative threshold.

Discussion

An ELISA based on the recombinant PIM antigen from the Muguga stock was developed for the detection of *Theileria parva* antibodies in cattle. This assay is superior both to other ELISAs based on *T. parva*-specific antigens p104, p150, and p67 and to the IFAT. The results obtained in the PIM-ELISA show that the assay detects antibodies for as long as 215 days after a single infection with *T. parva*. This period is significantly longer than that observed for the IFAT test, which detects antibodies for approximately 110 days.

The size and epitope polymorphism exhibited by PIM (Shapiro et al. 1987; Toye et al. 1991) did not affect the detection of antibodies in sera from cattle infected with four polymorphic stocks of *T. parva*. This may have been due to the presence of conserved epitopes across many *T. parva* stocks that are recognized by the bovine immune system (Toye et al. 1996). Sequence data obtained from different PIM antigens suggest that the molecule consists of conserved termini flanking a central variable region (Toye et al. 1996). The size polymorphism of the antigens is considered to be due to variations in the length of the central region. Preliminary results indicate that the PIM antigen expressed by parasites from the *T. parva* (Boleni) stock is the least homologous with the Muguga PIM antigen. The ability of serum from an animal infected with the Boleni stock of the parasite to recognize the recombinant version of the Muguga PIM in the experiments described herein suggests that the polymorphism of the antigen will not interfere with the use of the PIM-based ELISA for detection of infected animals in a field situation.

To monitor the consistency among test runs we included the strong and weak positive controls and the negative controls in four replicates on each ELISA plate. These controls were treated in the same way as any test sample with respect to the dilution and test procedure. In addition to the three controls, four replicates of a conjugate control were included on every plate for monitoring of background activity in the system. Two replicates of a test sample were considered adequate for interpretation of the data. For the results to be accepted, both replicate values recorded for the test samples had to be either above or below the positive/negative threshold point. If the replicate values were on both sides of this point the sample was retested. The highest OD values obtained during repeated routine testing with the strong positive and weak positive controls were taken as the upper control limits (UCLs) for the two controls. Similarly, the lowest OD values obtained during repeated routine testing with the strong and weak positive controls were taken as the lowest control limits (LCLs) for the two standards. This set of internal quality controls, included in every run, determined whether the assay

operated within accepted limits and also monitored the variability within and between assays.

All the OD values recorded for test samples were expressed as a percentage of the strong positive control standard. Percentage of positivity relative to a strong standard, although semiquantitative, has a number of distinct advantages: it requires a single dilution; it is expressed on a continuous scale of 0–100; and, if more quantitative data are required, a standard curve can be established by titration of a positive reference control, which can be used to convert OD values to units that are directly proportional to antibody activity (Wright et al. 1993).

The PIM-based ELISA in its present form will be amenable for use in the screening of large numbers of bovine sera for antibodies against *T. parva* at national or provincial laboratories. The high degree of sensitivity and specificity exhibited by the PIM-based ELISA indicates that the antigen could be used for the development of rapid pen-side tests using existing technologies such as latex particle agglutination or red blood cell agglutination based on bifunctional antibody (Wilson et al. 1991).

Acknowledgements We are grateful to Dr. W. Thorpe of ILRI; Dr. C.P. Otim of the Uganda Animal Health Center, Entebbe; Dr. G. Duvallet of CRTA; and Bobo-Dioulasso for the assistance they provided in the collection of sera from Kenya, Uganda, Burkina Faso, and Ivory Coast, respectively. We are indebted to Drs. S. Williamson, A.M. Makumyaviri, and H. Suliman for the provision of sera from Scotland, Kivu, Zaire, and Northern Sudan, respectively, as well as to Dr. A. Young for the *R. appendiculatus* ticks infected with *T. parva* Muguga. We also thank Mrs. A.N. Mungai, M. Omenya, A. Sore, S. Njuguna, and J.Nyanjui for the excellent technical help and Mrs. R. Okonji for typing the manuscript. This is ILRI publication 98006.

References

Brocklesby DW, Barnett SF (1961) Morbidity and mortality rates in East Coast fever (*Theileria parva* infection) and their application to drug screening procedures. Br Vet J 17: 529–531

Burridge MJ (1971) Application of the indirect fluorescent antibody test in experimental East Coast fever (*Theileria parva* infection in cattle). Res Vet Sci 12: 338–341

Burridge MJ, Kimber CD (1972) The indirect fluorescent antibody test for experimental East Coast fever (*Theileria parva* infection in cattle). Evaluation of cell culture schizont antigen. Res Vet Sci 13: 451–455.

Cunningham MP, Vickerman K (1962) Antigenic analysis of the *Trypanosoma brucei* group using the agglutination reaction. Trans R Soc Trop Med Hyg 5: 48–59

Geigy R, Kauffmann M (1973) Sleeping sickness survey in the Serengeti area (Tanzania), 1971. 1. Examination of large mammals for trypanosomes. Acta Trop (Basel) 30: 12–23

Goddeeris BM, Katende JM, Irvin AD, Chumo RSC (1982) The indirect fluorescent antibody test for experimental and epizootiological studies of East Coast fever (*Theileria parva* infection in cattle). Evaluation of a cell culture schizont antigen fixed and stored in suspension. Res Vet Sci 33: 360–365

Hillyer GV, Soler De Galanes M, Battisti G (1992) *Fasciola hepatica*: host responders and nonresponders to parasite glutathione S-transferase. Exp Parasitol 75: 176–186

Iams KP, Young JR, Nene V, Desai J, Webster P, MoiYoi O ole, Musoke AJ (1990) Characterisation of the gene encoding a 104 kiloDalton microneme-rhoptry protein of *Theileria parva*. Mol Biochem Parasitol 39: 47–60

Irvin AD, Morrison WI (1987) Immunopathology, immunology and immunoprophylaxis of *Theileria parva* infection. In: Soulsby EJL (ed) Immune responses in parasitic infections: immunology, immunopathology and immunoprophylaxis. CRC, Boca Raton, Florida, pp 223–224

Irvin AD, Dobbelaere DAE, Mwamachi DM, Minami M, Spooner PR, Ocama JGR (1983) Immunisation against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* and cross-immunity in vivo. Res Vet Sci 35: 341–346

Katende JM, Musoke AJ, Nantulya VM, Goddeeris BM (1987) A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. Trop Med Parasitol 38: 41–44

Katende JM, Goddeeris BM, Morzaria SP, Nkonge CG, Musoke AJ (1990) Identification of a *Theileria mutans*-specific antigen for use in an antibody and antigen detection ELISA. Parasite Immun 12: 419–433

Laemmli VK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lawrence JA, Mackenzie PKI (1980) Isolation of a non-pathogenic *Theileria* of cattle transmitted by *Rhipicephalus appendiculatus*. Vet J 11: 27–35

Leeflang P, Buys J, Blotkamp C (1976) Studies on *Trypanosoma vivax*: infectivity and serial maintenance of natural bovine isolates in mice. Int J Parasitol 6: 413–417

Makunyaviri AM, Habimana M (1993) Prevalence de la theileriose bovine a *Theileria parva*. Surlesplateaucal' itombive ent centre-est Zaire. Rev Med Vet 144: 415–418

Martin WS, Meek AH, Willerberg P (1987) Measurement of disease frequency and production. In: Martin WS, Meek AH, Willerberg P (eds). Veterinary epidemiology: principles and methods. Iowa State University, Ames, Iowa, pp 48–76

Minami T, Spooner PR, Irvin AD, Ocama JGR, Dobberlaere DAE, Fujinaga T (1983) Characterisation of stocks of *Theileria parva* by monoclonal antibody profiles. Res Vet Sci 35: 334–340

Morzaria SP, Spooner SP, Bishop RP, Musoke AJ, Young JR (1990) *Sfi*I and *Not*I polymorphisms in *Theileria* stocks detected by pulsed field gel electrophoresis. Mol Biochem Parasitol 40: 203–212

Mukhebi AW, Perry BD, Kruska R (1991) Estimating economic losses caused by theileriosis and the economies of its control in Africa. Prev Vet Med 12: 73–85

Musoke AJ, Morzaria SP, Nkonge SP, Jones E, Nene V (1992) A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. Proc Natl Acad Sci USA 89: 514–518

Mutugi JJ (1987) Transmission studies of a pathogenic strain of *Theileria mutans*. In: Sutherst RW (ed) ACIAR proceedings, No 17, ACIAR, Canberra, pp 95–96

Nene V, Iams KP, Gobright E, Musoke AJ (1992) Characterisation of the gene encoding a candidate vaccine antigen of *Theileria parva* sporozoites. Mol Biochem Parasitol 51: 17–28

Shapiro SZ, Fujisaki K, Morzaria SP, Webster P, Fujinaga T, Spooner PR, Irvin AD (1987) A life-cycle stage specific antigen of *Theileria parva* recognised by anti-schizont monoclonal antibodies. Parasitology 94: 29–37

Smith DB, Johnson KS (1988) Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67: 29–37

Stagg DA, Young AS, Leitch BL, Grootenhuis JG, Dolan TT (1983) Infection of mammalian cells with *Theileria* species. Parasitology 86: 243–254

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 86: 4639–4643

Toye PG, Goddeeris BM, Iams KP, Musoke AJ, Morrison WI (1991) Characterisation of a polymorphic immunodominant molecule in sporozoites and schizonts of *Theileria parva*. Parasite Immunol 13: 49–62

Toye PG, Metzelaar MJ, Wijngaard PJL, Nene V, Iams K, Roose J, Nyanjui JK, Gobright E, Musoke AJ, Clevers HC (1995) Characterisation of the gene encoding the polymorphic immunodominant molecule, a neutralizing antigen of *Theileria parva*. J Immunol 155: 1370–1381

Toye P, Nyanjui J, Goddeeris B, Musoke AJ (1996) Identification of neutralization and diagnostic epitopes on PIM, the polymorphic immunodominant molecule of *Theileria parva*. Infect Immun 64: 1832–1838

Voller A (1977) Serology of African trypanosomiasis. Ann Soc Belg Med Trop 57: 273–280

Wilson KM, Gerometta M, Rylatt DB, Bundesen PR, McPhee DA, Hillyard CJ, Kemp BE (1991) Rapid whole blood assay for HIV-1 seropositivity using an Fab-peptide conjugate. J Immunol Methods 138: 111–119

Wright PF, Nilsson EAM, Vanrooij M, Lelenta Jeggo MH (1993) Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev Sci Tech Off Int Epiz 12: 435–450

Young JF, Dessehberger V, Palene P, Fergusson B, Shatzman AR (1983) Efficient expression of influenza virus NS1 non-structural proteins in *Escherichia coli*. Proc Natl Acad Sci USA 80: 6105–6109