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Molecular epidemiology of *Theileria parva* in the field

D. Geysen[1], R. Bishop[2], R. Skilton[2], T. T. Dolan[3] and S. Morzaria[2]

1 *Institute of Tropical Medicine, Antwerp, Belgium*
2 *ILRI, Nairobi, Kenya*
3 *Livestock Services, Nairobi, Kenya*

Summary Molecular tools based on seminested RFLP-PCR techniques to characterize field parasites in bloodspots dried on filter paper permitted investigation of the extent and the dynamics of diversity of *Theileria parva* populations in the field. Parallel molecular studies explored the long-term genome stability of various isolates by probing Southern blots of *Eco*RI digested total genomic DNA with four different reference nucleic acid probes. Three polymorphic single copy loci encoding for antigen genes were developed for seminested PCR detection in order to apply them for a multilocus approach in population genetic studies. Seven alleles were identified for the polymorphic immunodominant molecule (PIM) locus by using restriction enzymes, and 4 alleles each for the p150 and p104 loci. A simple DNA extraction method gave good results in amplifying these loci from carrier animals using samples of blood dried on filter papers. Results from probing Southern blots of cultures taken at sequential timepoints indicate relative genome stability in *T. parva* in comparison to other parasitic protozoa such as *Plasmodium*. Comparatively homogeneous profiles in sympatric isolates from Zambia were identified using all four probes and PCR amplified products which contrasted with the variety found amongst Kenyan stocks. Preliminary characterization of *T. parva* field samples from the Southern Province of Zambia strongly suggest clonal expansion of one of the components of a non-Zambian trivalent vaccine used on a limited scale in the Province from 1985 until 1992.

keywords *Theileria parva*, molecular epidemiology, DNA probes, PCR

correspondence D. Geysen, Institute of Tropical Medicine, 155 Nationalestraat, 2000 Antwerp, Belgium

Introduction

Theileria parva is a tick-borne protozoan parasite that infects T and B lymphocytes of cattle. The parasite develops within the lymphocyte into a schizont, transforming the target cell into a clonally proliferating lymphoblast. The parasite divides in synchrony with the host cells, and the parasitized cells invade tissues throughout the body, resulting in a severe and often fatal disease known as East Coast fever (ECF). In response to an unknown signal merogony follows schizogony, producing the piroplasm blood stages which are infective to engorging ticks. All parasite stages are haploid except for a short diploid zygote stage in the tick intestinal cells (Gauer *et al*. 1995). Vaccination against the disease is based on an infection and treatment method consisting of inoculating cattle with a defined dose of live sporozoites and a simultaneous injection of a long-lasting tetracycline to control the infection (Radley *et al*. 1975).

Elaborate and expensive cross-immunity trials are the basis for determinating the strain composition of vaccine stocks. Two different approaches are being followed: a 'local' strain approach, using a broadly protective local stock of *T. parva;* and a 'cocktail' approach, using a combination of three stocks to provide broad immunity over most of the ECF region. In Zambia, the local strain approach (using *T. parva* Katete) has been used on a large scale in Eastern Province, whereas immunization with an exotic trivalent cocktail vaccine (Muguga cocktail) on a limited scale was used in the Southern Province. We report on the application of newly developed molecular characterization tools, which enable better characterization of *T. parva* stocks and analysis of *T. parva* parasite populations in the field before and after immunization with local or exotic stocks.

Materials and methods

T. parva stocks

Eight *T. parva* tissue culture isolates from the prevaccination era in both the Eastern and Southern Provinces were isolated

to define the extent of immunologically different strains in both provinces. The three components of a trivalent vaccine used in Southern Province and a recent Zambian field isolate from this province (Mazabuka) after immunization were included in the study. All stocks were cloned by limiting dilution of infected cells and studied over a 2-year period to assess their genomic stability. These 12 parasites also formed the basis for the development of molecular characterization tools for analysis of parasite populations in the field.

Theileria parva Zam2, Zam3 (*T. parva* Chitongo), Zam5, Zam22 (*T. parva* Mandali), Zam23 are Southern Province stocks isolated by F. Musisi. The first three were isolated in the cold seasons of 1982–83 and the latter two in the cold season of 1985. *Theileria parva* Mazabuka was isolated in 1993 by T. Katuda and A. Nambota in the Southern Province. *Theileria parva* Katete, Genda and Lundazi are Eastern Provine stocks isolated in 1983 by D. Geysen. *T. parva* Katete was developed as a local vaccine stock. The *T. parva* Muguga (Kenya, stabilate 73), Kiambu5 (Kenya, stabilate 68) and Serengeti-transformed (Tanzania, stabilate 69) are the components of the trivalent vaccine and their origins have been described elsewhere (Radley *et al.* 1975). Blood samples from clinical cases were collected in Southern Province, Zambia during May 1996 and from healthy but recovered animals related with suspected ECF disease outbreaks over the whole Province during 1997 (February, May and July).

Characterization methods

For Southern blotting on RFLP-DNA, 4 radiolabeled probes, the Tpr probe (Sohanpal *et al.* 1995), the telomere probe (Allsopp & Allsopp 1988), the LA6 probe (Bishop *et al.* 1993a) and the minisatellite probe (Bishop *et al.* 1999) were used on *Eco*RI digested genomic DNA from tissue culture parasites. These probes provide a good overall picture of the parasite genome (Bishop *et al.* 1993b). Common extraction procedures based on phenol, isoamyl alcohol and chloroform were used for *in vitro* parasite cultures (Maniatis *et al.* 1989). RFLP profiles of cultured parasites taken at the start of the work were compared with those after one and two years in culture.

RFLP-PCR assays for three polymorphic antigen loci were developed for use on field samples (PIM locus characterized by Toye *et al.* (1991), p104 locus characterized by Iams *et al.* (1990) and the p150 locus characterized in 1998 by Skilton *et al.* 1998). An extraction method based on boiling and Chelex resin complexing (Wooden *et al.* 1993) was used on the filter paper blood samples from the field. PCR products were digested with various restriction enzymes, run on a 10% polyacrylamide gel for two hours at 200 V and stained with silver.

Results

Southern blot on RFLP-DNA

Minor differences were found among various cloned cultures of the same parent stock when using the Tpr and telomere probes (Figures 1–4). Analysis of the profiles of the different probes indicate that the Zambian isolate profiles were similar to each other, suggesting a relative homogeneity (except isolate Zam5) among the stocks from Zambia. The cocktail component profiles were distinctly different from the local Zambian stocks, the Muguga and Serengeti components of the trivalent vaccine had similar but not identical profiles. The recent Mazabuka field isolate was identical to these two cocktail components using the two most informative probes (LA6 and Minisat). The molecular profiles remained stable over a two year period except for the telomere probe, for which the largest differences on passage were exhibited by the Mazabuka stock.

RFLP-PCR

The p150 and p104 antigen loci (Figure 5) exhibited moderate polymorphism with four alleles identified among the isolates studied. The PIM locus was the most polymorphic with 10 alleles besides marked size polymorphism in both DNA and protein length. Clear differentiation of the isolates coming

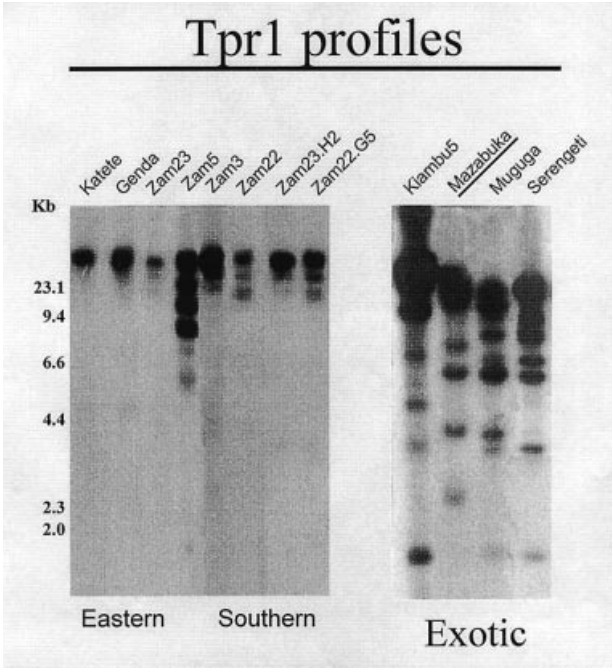

Figure 1 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with Tpr1. DNA's are from Eastern and Southern Zambian isolates and exotic isolates.

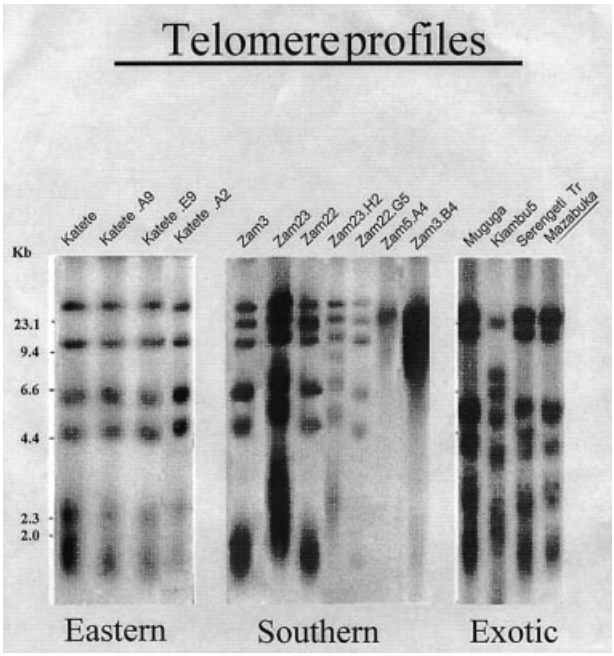

Figure 2 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with telomere. DNAs are from Eastern and Southern Zambian isolates and exotic isolates.

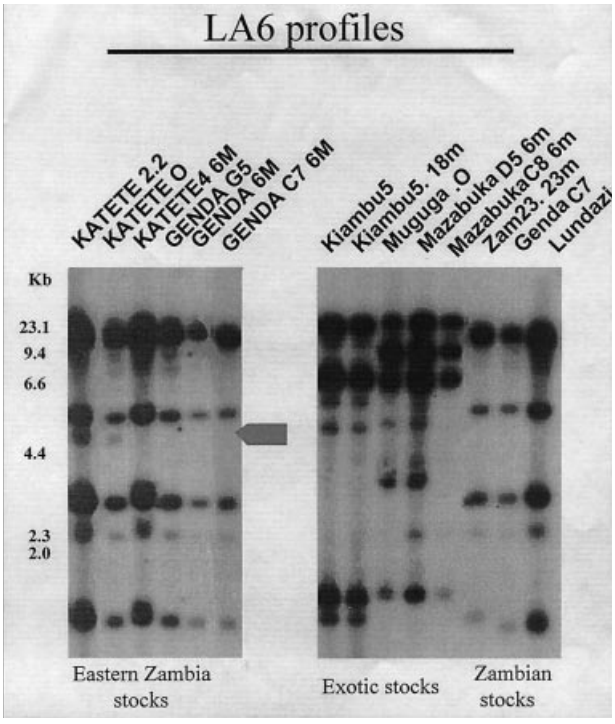

Figure 3 LA6 profiles of Eastern, Southern and exotic isolates with the marker pointing at the extra 5.5 Kb fragments in the Katete stock.

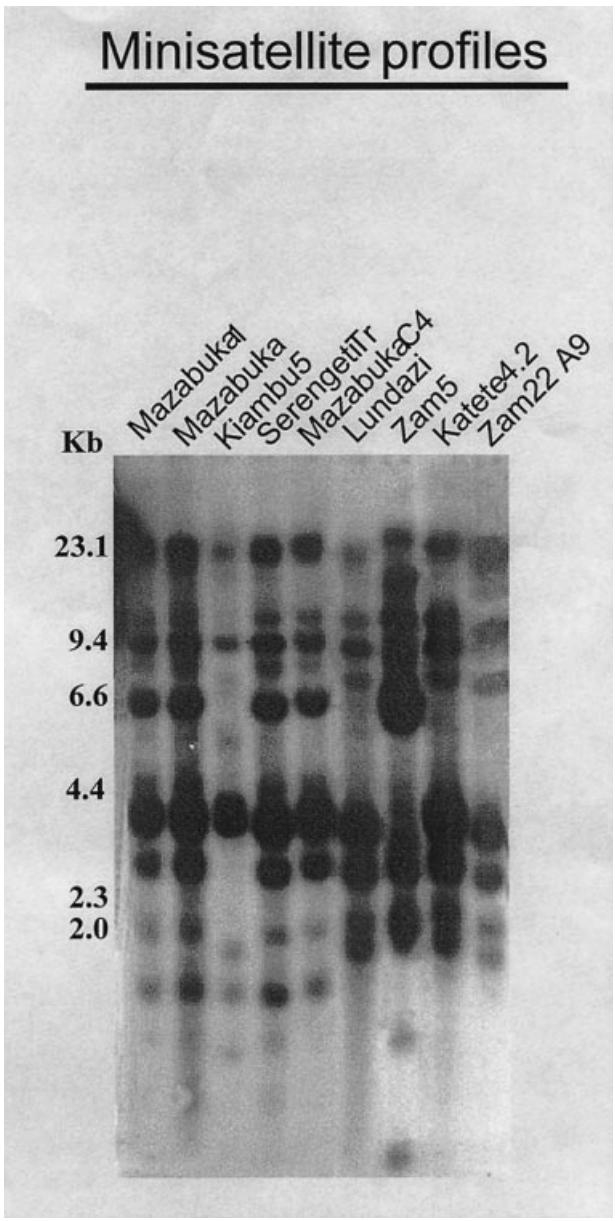

Figure 4 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with minisatellite. DNAs are from Eastern and Southern Zambian isolates and exotic isolates.

from the two geographical areas in Zambia (Katete, Genda and Lundazi against Zam2, Zam3, Zam5, Zam22 and Zam23) and within each Province was possible (Eastern Province with differences between Katete and Genda stocks; Southern Province with differences between Zam5 and the other Zams). The PIM polymorphisms alone can characterize subdivisions in the *T. parva* populations in the field. No

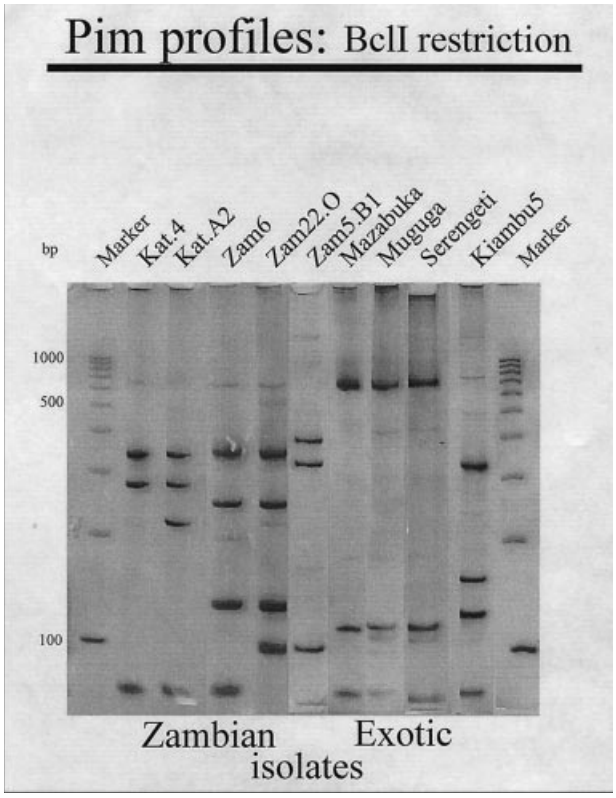

Figure 5 Restricting profiles of amplified PIM loci from Zambian and exotic *Theileria parva* isolates, using BC1 I restriction enzymes.

differences were found between cloned and parental populations *in vitro*. The *T. parva* Muguga and Serengeti-transformed components of the trivalent vaccine were identical on all allelic markers used in this study (Table 1). Field samples from 131 cases from Southern Province were characterized (Figure 6) and 126 (96%) found with a *T. parva* Muguga/Serengeti profile. Three of these showed a mixed profile: two samples contained profiles of Muguga/Serengeti and a local stock and one contained profiles of Kiambu 5 and a local stock. Only two samples were showing a local Zam3 profile.

Discussion

DNA analysis using different probe and RFLP-PCR marker assays gave consistent and matching results, with the allelic markers giving a higher resolution in differentiation among the various parasite populations. The isolates showed good homogeneity, which contrasts with the wide heterogeneity seen in the Kenyan stocks (Conrad *et al.* 1987; Allsopp & Allsopp 1988; Baylis *et al.* 1991; Bishop *et al.* 1997). This is in agreement with published results from the highveld in Zimbabwe (Bishop *et al.* 1994). Minor differences in Tpr restricting profiles of Muguga, Serengeti-transformed and Mazabuka were found between parental bulk and clonal profiles derived from these. This is in agreement with the known dynamism of this locus (Bishop *et al.* 1997). The discriminative power of the Tpr repetitive probe, which detects a multicopy gene family locus in the middle of chromosome 3, has been reported previously (Morzaria *et al.* 1995) but its biological relevance is still unknown. These variable results with the Tpr probe suggest that results from its use as a strain characterization marker should be interpreted with caution. The other probe profiles did not change between the parental stock and their clones, between clones from the same parent stock nor over time in culture, indicating an overall stability of the genome. The only exception was the Mazabuka stock which showed prominent differences between profiles over time which were not reflected in the clonal populations of this isolate.

The origin of the Southern Province *T. parva* stocks has never been documented, as the disease suddenly appeared in this province during the late 1970s. This study clearly shows that the preimmunization isolates from Eastern and Southern Provinces of Zambia show similar profiles but different to those from Zimbabwe, indicating that Eastern and Southern Province stocks had a common Eastern African origin. The Zam5 isolate is prominently different from all the other Zambian or exotic stocks. Unfortunately, several attempts to infect experimental animals with cell culture-derived material failed so that its cross immunity profile could not be determined.

The allelic marker approach correlated well with the probe results and would be the method of choice using bloodspots on filter paper as no cell culture isolation techniques are needed for this approach. Because PIM also presents marked size polymorphism, it offers an additional advantage in screening for different alleles.

The field data strongly suggest that one of the stocks (Muguga or Serengeti-transformed) introduced with the trivalent vaccine in Southern Province is widely disseminated in the field populations in the province. Live vaccination was deployed from December 1990–92 around Monze involving about 6000 animals, although field trials (1986–87) involving about 1000 animals were also organized near Choma. An alternative hypothesis is that a parasite with a genotype similar to one of the trivalent components was introduced from elsewhere in the region and although this is unlikely, it cannot be formally excluded.

The genetic data from the field samples indicate a homogeneous, clonal population structure, probably of an epidemic nature, such as is found after introduction or generation of a parasite strain with a selective advantage (Maynard Smith *et al.* 1993). Cross-immunity trials demonstrated excellent cross-protection between a local

Table 1 Probe allelic marker and mAbs results from various *T. parva* isolates. Genotypes are defined by letters. Identical letters define identical geotypes, similarity among genotype is expressed by using the same letter but different numbers

	Allelic markers				RFLP probes					
Isolate	p104	p150	PIM	L1,2	Telo	Tpr	M13	LA6	mAbs	TpR
Exotics										
1 KEN kiambu	A	E	P	R	A	G	Q	M	A	A
1 KEN Muguga	B	E	I	T	B	H2	R	N	B	B
1 TANSerengeti	B	E	I	T	B	H2	R	N	B	B
Zimbabwe										
ZIM Boleni	C	F	U	S	C	I		O	C	
Zambian isolates										
3 ZAMKatete	A	G	L	R	D1	J1	S	P1	D	C
3 Zam K4	A	G	O	R	D1	J1	S	P1		
3 Zam langa	A	G	L	R	D1	J1	S	P1	D	C1
3 ZAM Genda	A	G	O	R	D1	J1	S	P1	D	C
3 ZAM Lundazi	A	G	O	R	D1	J1	S	P1	D	C
2 ZAM 2	A	G	J	R	D3	J2	T	P1	D	D
2 ZAM 3 Chitongo	A	G	J	R	D3	J2	T	P1	D	C
2 ZAM 22 Mandali	A	G	J	R	D3	J2	T	P1	D	C1
2 ZAM 23	A	G	J	R	D3	J2	T	P1	D	C1
2* ZAM 5	D	H	M	R	E	K	U	P2	D	E
Recent isolate										
4 Mazabuka*	B	E	I	T	B	H1	R	N	B	B
Numbers of alleles	4	4	7	3			5	4	4	

*Recent field isolate.

isolate from Southern Province (Zam3 stock) and the trivalent cocktail vaccine. Partial (60–70%) or full cross-protection is the rule among *T. parva* infections, especially among stocks derived from cattle. However, there is a marked difference in virulence between the Southern Province stocks, which are relatively mild (resembling Zimbabwean cattle stocks), and the stocks originating from the Northern and Eastern Province.

A plausible hypothesis for the dominance of one of the newly introduced stocks in the field would be vaccination under epidemic conditions in which there are very suitable conditions for tick survival and multiplication and an abundance of naive hosts. These conditions might provide the opportunities for the new parasite to become established. If there was a good vectorial relationship between the new parasite and the local tick population and a lack of a specific host immune response to the introduced parasite, the strain could rapidly outnumber the local strain(s).

These studies might also provide important information on the strain structure of *Theileria* populations in the field. It has been suggested by Gupta *et al*. (1996) that immune responses to immunodominant antigens are the main driving force in subdividing field populations of infectious agents into distinct strains. These will be determined by the dominant polymorphic epitopes initiating the strongest immune responses. Molecular epidemiological results reveal that the PIM locus of *T. parva* on its own could be used to discriminate among the *T. parva* isolates identified so far in Zambia and a considerable body of other data suggests that it is an important immunodominant antigen. Future studies will focus on this antigen.

The development of good molecular characterization tools for *T. parva* parasites are especially useful in determining the various parasite population structures present in the field over the whole ECF region. Different epidemiological regions are known to exhibit different parasite population dynamics and degrees of diversity. Determining appropriate live parasite immunization methods must be based on the immunological diversity found in an area. Other than cross-immunity studies that may be impractical or impossible, molecular characterization offers the best indication of the relatedness of parasites. In a homogeneous situation, as shown for parts of Zambia and Zimbabwe, the introduction of an exotic, multivalent vaccine could create major problems arizing from recombination possibilities that were not present before.

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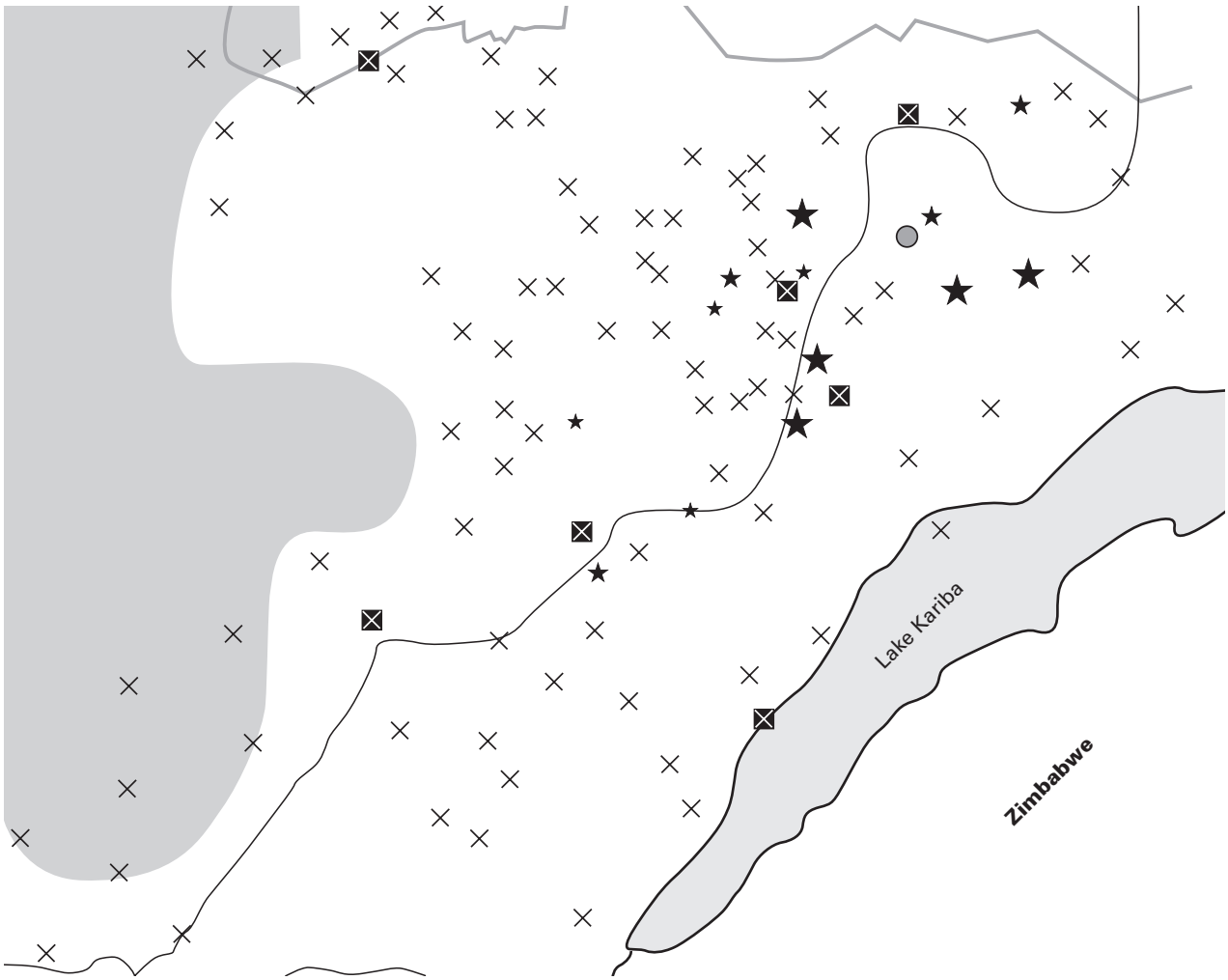

Figure 6 Distribution of *T. parva* isolates detected in filter paper blood samples from Southern Province of Zambia. ⊠ District headquarters; × Crush pens; ★ Muguga type; ● Southern type.

of the Zambian project at Mazabuka for collecting the samples, Dr F. Musisi for isolation of the early Southern Zambian stocks and Dr P. Spooner (ILRI, Nairobi) for the mAb characterization and the revitalization of Zambian tissue culture isolates. This study was carried out with the highly appreciated technical assistance of Mr F.Ceulemans and Mrs. R.Beudeker at ITM, Antwerp.

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