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Molecular epidemiology of *Theileria parva* in Eastern Democratic Republic of Congo: implications to the introduction of the Muguga Cocktail vaccine for East Coast Fever

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Abstract

Background

East Coast Fever (ECF) is one of the most economically important tick-borne diseases of cattle in Eastern, Central, and Southern Africa, caused by the intracellular protozoan parasite *Theileria parva* (*T. parva*). This study investigated the seroprevalence and genetic diversity of *T. parva* populations in the eastern Democratic Republic of Congo (DRC) to inform immunization strategies against ECF. A cross-sectional study was conducted between September 2012 and January 2013 in three provinces of eastern DRC (North Kivu, South Kivu, and Ituri) to assess the seroprevalence and confirm the presence of *T. parva* in cattle. Blood samples from 31 villages across different agro-ecological zones were analyzed using the PIM ELISA, and ELISA-positive samples were further screened by *p104* PCR. An immunization and field challenge trial using the Muguga Cocktail vaccine was conducted with 100 seronegative cattle. Molecular characterization of samples collected from the sentinel, immunized, and control groups was performed by sequencing the *p67*, *Tp1*, and *Tp2* genes and conducting microsatellite genotyping.

Data were analyzed using MEGA, DnaSP, GenAIEx, FSTAT, and LIAN to determine genetic variation, population structure, and linkage equilibrium among *T. parva* isolates.

Results

Of the 756 serum samples analyzed, 27.5% tested positive for *T. parva* antibodies by ELISA, with Ituri showing the highest seroprevalence and North Kivu the lowest. PCR confirmed *T. parva*

DNA in 87.5% of ELISA-positive samples. Sentinel cattle exposed in Kabasha village developed severe ECF, confirming high transmission pressure, and were used to establish the immunization and challenge site. Vaccination with the Muguga Cocktail induced seroconversion in 94% of cattle, with 46.5% surviving field challenge. Sequence analysis of *p67*, *Tp1*, and *Tp2* genes revealed limited clustering with Muguga strains and high polymorphism, particularly in *Tp2* (13.2%). Microsatellite genotyping detected substantial allelic diversity and population sub-structuring, with unique alleles predominant across loci ($F_{st} = 0.118$).

Conclusion

This study revealed high and variable *T. parva* seroprevalence across eastern DRC, with genetic analyses indicating the coexistence of Muguga-like and distinct local parasite populations. The low survival rate in immunized cattle highlights limitations of the Muguga Cocktail against field strains. These findings highlight the presence of diverse *T. parva* populations and emphasize the need for continuous genetic monitoring and tailored vaccine strategies to enhance ECF control in the region.

Key words: *Theileria parva*; East Coast fever; seroprevalence; genetic diversity; Muguga cocktail; immunization; Eastern Democratic Republic of Congo; molecular characterization

Background

East Coast Fever (ECF) is one of the most economically significant tick-borne diseases affecting cattle in Eastern, Central, and Southern Africa, caused by the intracellular protozoan parasite *Theileria parva* (1,2). The transmission of this disease occurs through the three-host tick, *Rhipicephalus appendiculatus* (3), predominantly parasitizing cattle (4). In the Democratic

Republic of the Congo (DRC), ticks belonging to the *Rhipicephalus* genus are notably found to be most abundant in cattle (5). Research conducted in North Kivu, a province in the eastern part of DRC, focused on identifying tick species and their correlation with the seroprevalence of *T. parva* in cattle raised under an extensive farming system, revealed dominance of *R. appendiculatus* in the area, suggestive of a potential state of endemicity to *T. parva* infection in the area (6).

The life cycle of *T. parva* is intricate, involving obligatory developmental stages in both mammalian and vector hosts (7). Cattle become infected through the inoculation of sporozoite forms present in tick saliva. The disease significantly affects exotic and crossbred cattle, along with indigenous calves under six months old, resulting in elevated mortality rates. In endemic areas, chronic ECF manifests with less quantifiable effects such as reduced growth, diminished milk production, poor weight gain, low fertility rates, paralysis, and vulnerability to secondary attacks from other parasites (8). Consequently, ECF remains a primary driver of tick control strategies across several African countries.

Control of ECF involves the use of a combined system of acaricides, chemotherapy, livestock movement and Infection and Treatment Method (ITM). The utilization of acaricides remains a widespread method of tick control, albeit obstacles such as elevated expenses, emergence of tick resistance and insufficient dipping facilities. Despite these challenges, a considerable number of farmers resort to acaricides for ECF control even though the most effective preventive approach is the infection and treatment method (ITM). ITM involves the simultaneous inoculation of a live, potentially lethal dose of *T. parva* sporozoites and a long-acting oxytetracycline (9). While ITM is stock-specific, combinations of stocks, such as the widely used 'Muguga cocktail' (10) comprising *T. parva* Muguga, Kiambu 5, and Serengeti-transformed stocks, provide broad protection. Despite its efficacy, ITM adoption faces challenges due to difficulties with the cold

chain and concerns about vaccine strains establishing in resident tick populations and intermingling with local parasite genotypes (11).

The effectiveness of ITM depends on selecting an appropriate immunizing stock compatible with local *T. parva* genotypes, underscoring the importance of molecular characterization of local *T. parva* strains in eastern DRC. Despite the region's shared agro-ecological and epidemiological characteristics with neighboring endemic countries, molecular epidemiological data on *T. parva* remain scarce. Previous studies across East Africa have revealed considerable genetic and antigenic diversity among *T. parva* isolates, influenced by regional tick populations, host factors, and livestock management practices, which can have implications for vaccine efficacy (12). Characterizing local parasite populations is therefore crucial for maximizing cross-immunization potential and identifying suitable vaccine candidates. While there is no direct correlation between molecular profiling and immunogenicity, characterizing local strains helps narrow down the parasite stocks or isolates for cross-immunization trials, thereby reducing the cost of introducing immunization. For comparative purposes, different and well-characterized strains are often included in a single vaccine, exemplified by the success of the Muguga Cocktail in immunization trials.

The sequencing of *T. parva* CTL antigens has emerged as a valuable tool for assessing the diversity of *T. parva* in endemic areas (13–16). In addition, the identification of polymorphic minisatellites and microsatellite markers in the *T. parva* genome allows direct genotyping of isolates from blood samples using specific primers (12,17,18). These locus-specific markers, based on variable number tandem repeats (VNTRs) in non-coding regions, serve as valuable tools for studying population structure due to their technical simplicity in analysing repeat motif copy number variation. The utilization of minisatellites and microsatellites in *T. parva* genotyping has unveiled diverse

population structures (17–20). Numerous studies highlight substantial diversity among *T. parva* isolates, with observed genetic exchange when multiple stocks infect the tick vector (12,14,17–23). In addition, the p67 gene encodes a major sporozoite surface antigen of *T. parva* involved in host-cell invasion and is one of the most extensively studied antigens of *T. parva* (24)(25). It contains polymorphic regions that are useful markers for assessing genetic diversity and population structure in *T. parva* (25). The marker has been extensively used in epidemiological studies to differentiate parasite genotypes, distinguish cattle and buffalo-associated parasite populations, and investigate the distribution of *T. parva* strains of relevance to vaccine development and disease control (26)(27).

In the DRC, communities in the eastern regions heavily depend on livestock for their livelihoods, and the presence of theileriosis poses a significant impediment to the growth and enhancement of the livestock sector. Consequently, implementing effective control measures, particularly through immunization, is crucial, given its cost-effectiveness compared to chemotherapy. Thus, recognizing the importance of genetic exchange in *T. parva* populations, the aim of this study was to determine the seroprevalence and characterize the population genetics of *T. parva* isolates in eastern DRC in order to assess the suitability of the Muguga Cocktail for ECF control. To achieve this, the study employed sequence and microsatellite analyses, together with immunization and field challenge trials, to generate evidence supporting the potential introduction of the Muguga Cocktail vaccine in the region.

Methods

Study design and Timeline

The project was implemented in three distinct and sequential phases between 2012 and 2017 to enable the progression from epidemiological characterization of *T. parva* distribution, through assessment of transmission dynamics, to evaluation of immunization under field conditions.

A cross-sectional epidemiological survey was conducted between September 2012 and January 2013 to determine the seroprevalence and geographical distribution of *T. parva* infection among apparently healthy cattle in eastern Democratic Republic of Congo (DRC). Blood samples collected during the survey were shipped to the Centre for Ticks and Tick-Borne Diseases (CTTBD), Lilongwe, Malawi, for serological and molecular analyses. Laboratory analyses were performed between 2014 and 2015. Additional molecular characterization of selected samples was subsequently undertaken at the University of Zambia in 2015.

Using the epidemiological data generated in Phase I, Kabasha village was selected for a longitudinal field study. In November 2016, susceptible sentinel cattle were introduced into the study site to monitor natural *T. parva* transmission dynamics and tick vector challenge under field conditions. The sentinel cattle were monitored continuously for a period of two months, and the study concluded in January 2017.

Phase III involved an immunization and field challenge study conducted at Kabasha farm, North Kivu Province, DRC. The study was designed to evaluate the efficacy of the Muguga Cocktail vaccine against ECF under field conditions in Eastern DRC. The primary outcome was the ECF clinical disease reactions in vaccinated and non-vaccinated cattle over the study period. Preparatory activities commenced in April 2017 and included identification and procurement of suitable experimental cattle. Blood samples were collected from candidate animals and shipped to CTTBD in Lilongwe for serological and molecular screening to identify cattle free of *T. parva*.

infection. The animal trial commenced in September 2017 and was completed in November 2017, during which the efficacy of immunization was evaluated under natural field challenge conditions. Further molecular characterization of selected samples from the sentinel, immunized, and control groups was subsequently conducted at the University of Zambia in 2015.

Study sites

A cross-sectional study was carried out in three provinces of eastern DRC: North Kivu, South Kivu, and Ituri, each comprising three agro-ecological zones—low altitude (< 1200m), medium altitude (1200 to 1800m), and high altitude (> 1800m) (Fig.1). The study included a total of 31 villages (North Kivu = 12, South Kivu = 8, and Ituri = 11) distributed across the provinces (Fig.1). Territories within each province were carefully selected based on their concentration of the most significant cattle population and their suitability as an agro-ecological zone for the *R. appendiculatus* tick. Specifically, two territories were chosen in North Kivu (Beni and Lubero), three in South Kivu (Kabare, Walungu, and Uvira), and two in Ituri (Irumu and Mambasa). Due to wartime occupation during the sampling period, some villages in Ituri experienced limited dispersion in terms of latitude and longitude. Consequently, certain territories such as Walikale and Rutshuru in North Kivu (with indigenous cattle populations) and Mwenga in South Kivu (with endangered wildlife species) were excluded.

Kabasha village in North Kivu was selected as a sentinel-animal study site mainly because of its proximity to The Graben Catholic University, the study's host institution, and because farm owners there gave consent to participate. Security, logistics, and access constraints prevented establishment of sentinel cattle in other provinces, so we were limited to one sentinel-animal site. We conducted a preliminary study in November 2016 on two farms in Kabasha village, chosen

because they are lowland sites where tick burdens exceed 150 ticks per animal in the heavy rainy season. A total of 90 cattle were sampled: 60 animals from Kabasha Farm to estimate *T. parva* prevalence there, and 30 animals from Joachim Farm (near Kabasha Farm), where no clinical ECF cases had been reported, to assess whether Joachim Farm could provide *T. parva* free animals for use as sentinels. Serology at Kabasha Farm showed a *T. parva* seroprevalence of 95% (95% CI: 85.6–98.4). All 30 animals from Joachim Farm tested negative for *T. parva*; five of these were purchased and placed as sentinel cattle in a paddock at Kabasha Farm to estimate the level of field challenge.

The immunization and field challenge study was carried out at Kabasha farm, North Kivu.

Source of animals

The five sentinel cattle were Friesian crossbreeds purchased from farmers in Beni Territory who practiced strict and consistent tick control using acaricides. The animals were only purchased after testing negative for *T. parva* antibodies on PIM ELISA. Cattle for the immunization and field challenge study were sourced from smallholder farmers in the highland areas of Lubero and Beni territories, North Kivu Province. The animals were predominantly Friesian crossbreeds. To ensure naïve status, only cattle that tested seronegative by PIM ELISA for antibodies against *Theileria parva*, *Theileria mutans*, *Anaplasma marginale*, and *Babesia bigemina* were selected for inclusion in the study.

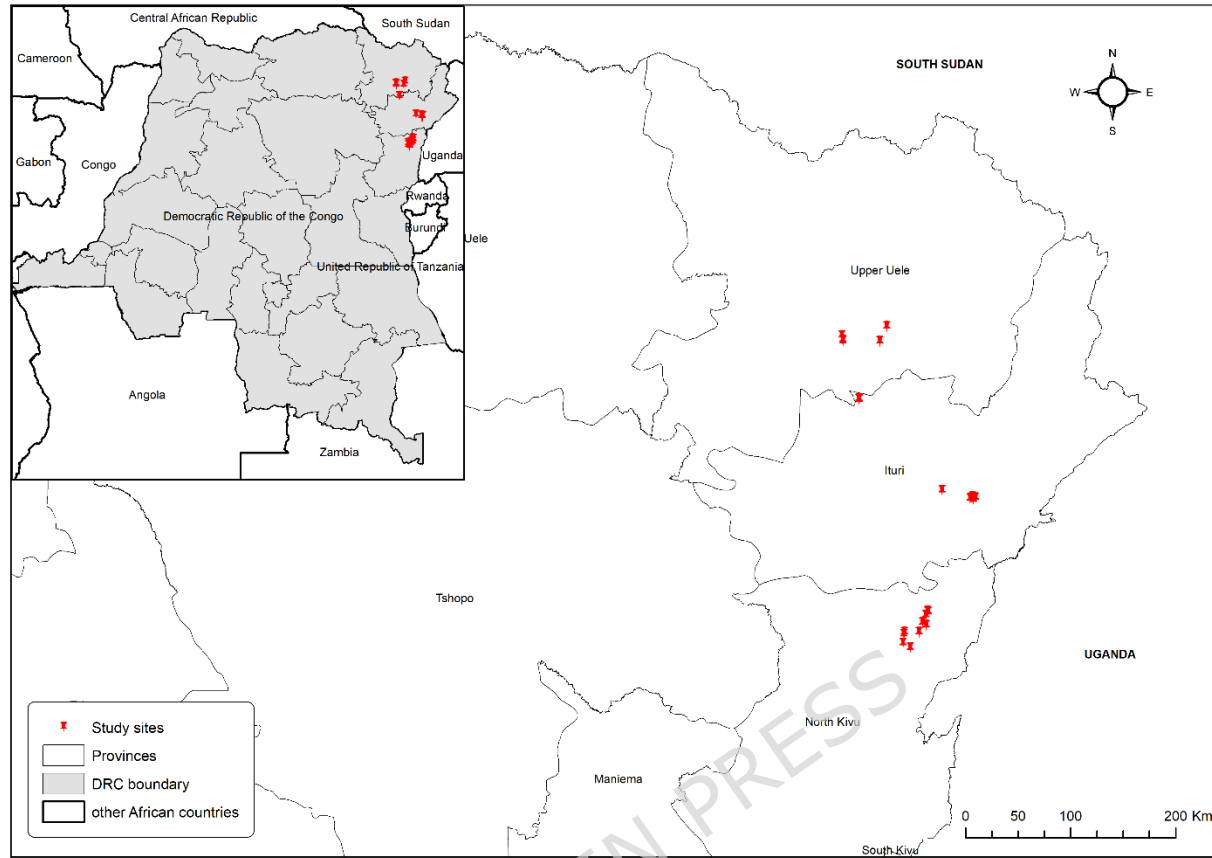


Fig.1: Map of DRC showing study sites in the eastern region

Sample size determination

The sample size was calculated according to a formula by (28) using an expected *T. parva* seroprevalence of 43% based on a previous study in North Kivu (29), a 95% confidence level, and a 5% precision. This yielded a minimum of 377 animals. After adjusting for a design effect of 2 and a 5% non-response rate, the final target sample size was 792. A total of 756 samples were collected and analyzed, providing adequate precision for estimating *T. parva* seroprevalence across the three study provinces.

For the immunization and challenge study, the sample size was conveniently determined due to logistical limitations, security constraints, and the operational challenges of conducting

longitudinal livestock studies in Eastern DRC. A total of 100 cattle were enrolled, comprising 50 animals in the vaccinated group and 50 in the non-vaccinated control group.

Sample collection

Blood samples were collected from cattle via jugular venipuncture using EDTA-coated vacutainer tubes, with aliquots spotted onto FTA filter papers (Whatman Bio-Science) for molecular analysis and into plain tubes for ELISA testing. The collected whole blood and serum were stored at -20°C at the Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, Catholic University of Graben, Butembo, DRC. Subsequently, the samples were transported to the Center for Ticks and Tick-borne Diseases (CTTBD) in Lilongwe, Malawi, for *T. parva* ELISA and PCR screening. For further characterization, DNA was sent to the School of Veterinary Medicine, University of Zambia (UNZA), Lusaka, Zambia.

Serological analysis

The *PIM* ELISA was used for *T. parva* screening of serum samples collected according to the protocol described by (30). All animals used in the immunisation and field challenge study were those that tested negative on ELISA for antibodies against *Theileria parva*, *Theileria mutans*, *Anaplasma marginale* and *Babesia bigemina*.

DNA extraction and PCR

DNA was extracted and purified from cattle blood samples and FTA filter papers (Whatman Bio-Science) following the method as previously described (12). Field samples were screened for *T. parva* DNA using *T. parva* specific *p104* gene primers as described previously (31). Due to

logistical constraints, only samples that tested positive by ELISA were subjected to PCR amplification of the *p104* gene.

Immunization and field challenge trial

One hundred (n=100) *T. parva* negative cattle were purchased, confined at UCG/Butembo farm, and underwent strict tick control using SUPONA EXTRA (1/2000) to prevent premature parasite challenge. After acclimatization, the animals were divided into two groups, A (n=50) and B (n=50). Cattle were randomly allocated to either the vaccinated or control group in a 1:1 ratio. Randomization was conducted at the individual animal level using a computer-generated random allocation sequence prepared prior to enrolment. To reduce selection bias, allocation was implemented by a study team member not involved in clinical assessment or outcome evaluation.

Group A received 1ml of the Muguga cocktail vaccine and long-acting Oxytetracycline simultaneously, while Group B received 1ml of the diluent and served as non-immunized controls. All animals were closely monitored for 35 days under strict tick control. All animals were bled on Days 0, 21, 28, and 35 post-immunisation. The sera were stored at -20°C at the Faculty of Veterinary Medicine at UCG/Butembo, DRC, before shipping to ILRI/Kenya for PIM ELISA analysis (30).

The study was conducted as a single-blinded trial. Field personnel responsible for clinical monitoring, diagnosis, and outcome assessment were blinded to the vaccination status of the animals. Vaccination records were maintained separately and were accessible only to designated personnel responsible for treatment administration. To maintain blinding, animals were identified using unique identification numbers without indication of group allocation. Data analysts were

also blinded to group assignment during the initial stages of data analysis, with group codes only revealed after completion of primary analyses.

Before exposing both groups to tick/parasite challenge, animals underwent thorough daily washing for three consecutive days (Days 33, 34, and 35 post-immunization) to eliminate residual acaricide, facilitating easy and high tick attachment during exposure. On Day 35 post-immunization, all animals were field-exposed in Kabasha village, grazing freely. Throughout the exposure period, animals received no tick control or ECF treatment but were treated for other diseases. Daily temperature monitoring occurred, and if an animal exhibited a high temperature ($> 39.5^{\circ}\text{C}$) for two consecutive days, a blood smear was checked for haemoparasites. Animals with enlarged lymph nodes underwent biopsy smears stained with Giemsa. Blood samples were further collected from animals that succumbed to ECF during the exposure period and sent to the University of Zambia, School of Veterinary Medicine, for molecular characterization.

***p67*, *Tp1* and *Tp2* genes PCR and cycle sequencing**

The *T. parva* *p67*, *Tp1*, and *Tp2* genes were amplified using the Amplitaq Gold PCR kit (Invitrogen, CA) with primers designed by (27)(32). Briefly, in a 20 μL reaction, consisting of 10 ng template DNA, 10 μL 2x Amplitaq Gold master mix, 0.5 μM of each primer, and distilled water, the amplification cycles were as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles at 96°C for 30 seconds, 55°C (*p67*) 50°C (*Tp1*) and 53°C (*Tp2*) for 45 seconds, and 68°C for 60 seconds, with a final extension step at 72°C for 5 minutes. PCR products were visualized on 1.5% ethidium bromide-coated agarose gels, with expected product sizes of 906 bp, 432 bp, and 525 bp for *p67*, *Tp1*, and *Tp2*, respectively.

The obtained PCR products underwent purification using the Monofas purification kit (GL Sciences, Japan), following the manufacturer's instructions. Subsequently, cycle sequencing was

carried out using the Big Dye Terminator v3.1 cycle sequencing kit (Life Technologies, Applied

Marker	Chromosome number	size (bp)	Reference
MS39	4	263	(33)
MS25	3	325	(33)
MS7	1	372	(33)
ms9	3	230	(33)
MS33	4	214	(12)
MS19	2	307	(33)

Biosystems) following the manufacturer's instructions. The resulting cycle sequencing products were purified to remove excess labeled dNTPs, buffers, and enzymes through the ethanol precipitation method, followed by denaturation. Finally, capillary electrophoresis was performed on the ABI 3500 genetic analyzer (Life Technologies).

Microsatellite and minisatellite genotyping

The genotyping markers used for field samples are listed in Table 1. Fluorescent dye-labeled forward primers and previously specified annealing temperatures (14) were used. PCR mix and reaction conditions followed established protocols (14). After amplification, PCR products were visualized on a 1.5% agarose gel coated with ethidium bromide. Successful PCR products were denatured and electrophoresed using the ABI SeqStudio genetic analyzer (Life Technologies). DNA fragment sizes from control, immunized, sentinel and vaccine strains (Muguga, Kiambu, Serengeti, and Chitongo vaccine isolates) were determined using GeneMapper software ver. 5 (Applied Biosystem, Waltham, Massachusetts, USA). The software scored peaks with the highest area as the most dominant allele, and based on this, a Multi-locus genotype (MLG) was constructed to represent the most dominant genotype within each sample.

Table 1. Satellite markers used to genotype samples from Eastern DR Congo**Data analysis*****ELISA, Immunisation and challenge analysis***

For serological analysis the OD values were expressed as percent positivity (PP) relative to a reference strong-positive control serum. Any test serum with a PP value of 20 or above was considered positive.

For comparing responses between immunized and non-immunized animals, disease reactions to East Coast Fever were interpreted based on a combination and extent of measurements of pyrexia, parasitosis, and haematological parameters, together with other clinical observations. Responses to infection were subsequently categorized into four outcome groups: no reaction (NR; non-reactor), mild reaction (MR; mild reactor), moderate reaction (MOD; moderate reactor), and severe reaction (SR; severe reactor), in accordance with established criteria used in experimental and field studies of ECF (10).

Sequence analysis

For nucleotide sequence verification, Blast analysis on the NCBI website was used. The sequences obtained were assembled and edited using Genetyx version 12. Alignment with Muguga, Kiambu 5, Serengeti transformed, and Chitongo vaccine strain reference sequences, along with other East African references, was performed using Clustal W1.6. A fasta file of this alignment was generated and converted to a MEGA file format for constructing phylogenetic trees with MEGA version 6 (34). Phylogenetic trees were constructed using 1000 bootstrap replicates. Multiple sequence alignments of amino acid sequences, translated from nucleotide alignments, were conducted to

analyze CTL epitopes on both *Tp1* and *Tp2*. DNA polymorphisms for each gene were calculated using DnaSP version 5 (35). The single likelihood ancestor counting method, using the F81 model and a confidence level of 0.05 on the Data Monkey website (<http://www.datamonkey.org>), was employed to calculate the mean ratio of non-synonymous substitutions to synonymous substitutions (dN/dS) per site (36,37). The distribution of genetic variation and population differentiation among sequences was investigated through AMOVA performed using GenAIEx6 (38).

Network version 10 was utilized to assess haplotype similarities between *Tp1* and *Tp2* nucleotide sequences of the vaccine stocks and field samples (<http://fluxus-engineering.com/>). All nucleotide sequences obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ) with designated accession numbers LC863893-LC863940.

Microsatellite analysis

Initially, microsatellite analysis was employed using the microsatellite toolkit (<http://animalgenomics.ucd.ie/sdeparck/ms-toolkit/>) to assess the level of similarity within the MLG. The results were visualized through allele frequency distribution and Principal Component Analysis (PCA) created using GenAIEx6 (31). Population sub-structuring was quantified using the FSTAT computer package version 2.9.3.2 (<https://www2.unil.ch/popgen/softwares/fstat.htm>). To test the null hypothesis of panmixia and linkage equilibrium, LIAN (Haubold and Hudson, 2000) was employed. This involved calculating the standardized index of association, the variance of pairwise differences (VD), the variance of differences required for panmixia (VE), and L, representing the 95% confidence interval for VD. Negative or close-to-zero values of the index of association suggest panmixia (random mating), while positive values significantly greater than zero indicate non-panmixia (nonrandom mating). The rejection of the null hypothesis of panmixia

is indicated when VD is greater than the L value, signifying linkage disequilibrium (LD). Conversely, acceptance of the null hypothesis of panmixia occurs when the calculated VD is less than the L value, indicating linkage equilibrium (LE).

Results

Characteristics of cattle in the study area

Three variables were taken into account for sampling: (i) age group, (ii) sex, and (iii) agro-ecological zone. A minimum of 250 animals were sampled from each province, resulting in a total of 756 animals. The majority of the sampled animals were adult females (> 24 months) (Table 2), reflecting the common practice of slaughtering males before they reach 12 months of age. The distribution of animals was consistent across all agro-ecological zones (AEZ).

Table 2: Distribution of the characteristics of cattle in the study area

Variables	Levels	Frequency	Percentage
Age group (Month)	< 6	3	0.4
	6 to 12	106	14.02
	13 to 24	115	15.21
	> 24	532	70.37
Sex	Males	194	25.66
	Females	562	74.34
Agro-ecological zone	Low altitude (<1200m)	355	46.96
	Medium altitude (1200 to 1800m)	270	37.71
	High altitude (> 1800m)	131	17.33

Serological screening for T. parva

Out of the 756 serum samples analyzed, 208 (27.5%) tested positive for *T. parva*, and 24 yielded inconclusive results on ELISA (Table 3). Ituri province showed the highest seroprevalence, followed closely by South Kivu, while North Kivu had the lowest seroprevalence.

Table 3: Summary of cattle *T. parva* antibodies detected using PIM ELISA

Province	Number of cattle sampled	ELISA positive (%)	Confidence interval at 95%	ELISA inconclusive	ELISA negative
Ituri	250	101 (40.4)	34.5-46.6	9	140
North Kivu	255	18 (7)	4.5-10.9	5	232
South Kivu	251	89 (35.5)	29.8-41.6	10	152
Total	756	208 (27.5)	24.4-30.8	24	524

PCR screening for *T. parva*

Out of the 208 ELISA-positive samples, 182 (87.5%) tested positive for the *T. parva p104* gene (Table 4). The highest positivity was observed in North Kivu, followed by South Kivu and Ituri provinces.

Table 4: PCR detection of *T. parva* using *p104* gene

Province	Number of samples	PCR positive (%)	Confidence interval at 95%	PCR negative
Ituri	101	86 (85.1)	76.8-90.8	15
North Kivu	18	18 (100)	-	0

South Kivu	89	78 (87.6)	79-93	11
Total	208	182 (87.5)	82.3-91.3	26

ECF Reactions of sentinel cattle

Over the 20-day exposure period, the sentinel cattle placed at Kabasha village succumbed to a severe form of ECF, resulting in the euthanasia of four animals. Postmortem examinations revealed significant pulmonary edema and hemorrhages in the abomasum and small intestines. Schizonts were also identified in impression smears of the spleen, liver, and lymph nodes. The fifth animal experienced moderate to severe reactions but ultimately recovered (Table 5). Based on these findings, Kabasha village was selected as a suitable location for the immunization and field challenge trial.

Table 5: ECF Reactions of the 5 Sentinel Cattle placed at Kabasha Village

Animal ID Number	Days to Temperature	Duration of Temp	Day to Schizonts	Duration of Schizonts	Day to Piroplasms	Duration of Piroplasms	Time to euthanasia	Classification of Reactions*
UC-16-8	23	17	23	11	20	14	34	SR
UC-18-24	13	12	15	9	14	11	24	SR
UC-20-19	15	9	16	7	15	8	23	SR
UC-21-12	-	-	30	13	35	ND	-	MOD
JO-9-11	23	7	25	4	21	8	29	SR

NR: Non-Reactor, **MR:** Mild Reactor, **MOD:** Moderate Reactor, **SR:** Severe Reactor.

Immunization and Tick parasite challenge trial

Serological analysis of the 50 cattle immunized with the Muguga cocktail stabilate revealed a commendable success rate, with 94% (47 animals) demonstrating seroconversion. In the non-immunized control group, 14% (7 out of 50 animals) showed cross-reactivity with PIM indicating a low baseline exposure to similar parasites. Because 86% of the controls remained below the diagnostic cut-off level, it proves that the high antibody levels in the vaccinated group were strictly due to the immunization itself, rather than random background environmental exposure, signifying successful immunization. Unfortunately, seven of the initially immunized cattle succumbed to intercurrent infections before detectable ECF emerged, necessitating their exclusion from the analysis.

Among the remaining 43 in the immunized group, 23 cattle succumbed to severe ECF, while 20 successfully withstood the field challenge (46.5%). Nine fell into the non-reactor category, five in the mild category, and six in the moderate category (Table 6). In the control group, all animals had signs of severe ECF and were euthanized. Postmortem lesions were pathognomonic for ECF.

Table 6: ECF reactions of both immunized and unimmunized (control) animals

Experimental Group	Number of Animals	NR	MR	MOD	SR	Number Surviving	Percent Protection
Control	49	0	0	0	49	0	0
Immunized	43	9	5	6	27	20	46.5

NR: Non-reactor, MR: Mild reactor, MOD: Moderate reactor, SR: Severe reactor.

Sequence diversity and phylogenetic analysis of the *p67*, *Tp1*, and *Tp2* genes

p67 locus

A fragment of the *Theileria parva p67* gene, spanning coordinates 2946 to 3767 bp and encompassing a characteristic exon-intron-exon structural architecture, was successfully

sequenced across the control, immunized, and sentinel cohorts. Historically, four distinct *p67* alleles have been defined based on length and sequence polymorphisms: Allele 1, characterized by a diagnostic 129 bp deletion; Allele 2, which lacks this deletion (36); Allele 3, characterized by a larger 179 bp deletion; and Allele 4, which lacks the 179 bp deletion but maintains high sequence identity with Allele 3 (37).

In this study, sequence alignment revealed that a consistent 129 bp deletion was strictly fixed across all isolates analyzed. Subsequent phylogenetic reconstruction demonstrated that all sequences from the field trial formed a single, highly uniform clade that clustered exclusively with reference sequences belonging to Allele 1. There was an absolute absence of sequences branch-clustering with, or showing structural homology to the remaining reference lineages (Alleles 2, 3, or 4) (Figure 2). This uniform distribution confirms that the parasite population under field challenge in this region of the Democratic Republic of the Congo consists solely of the Allele 1 structural archetype.

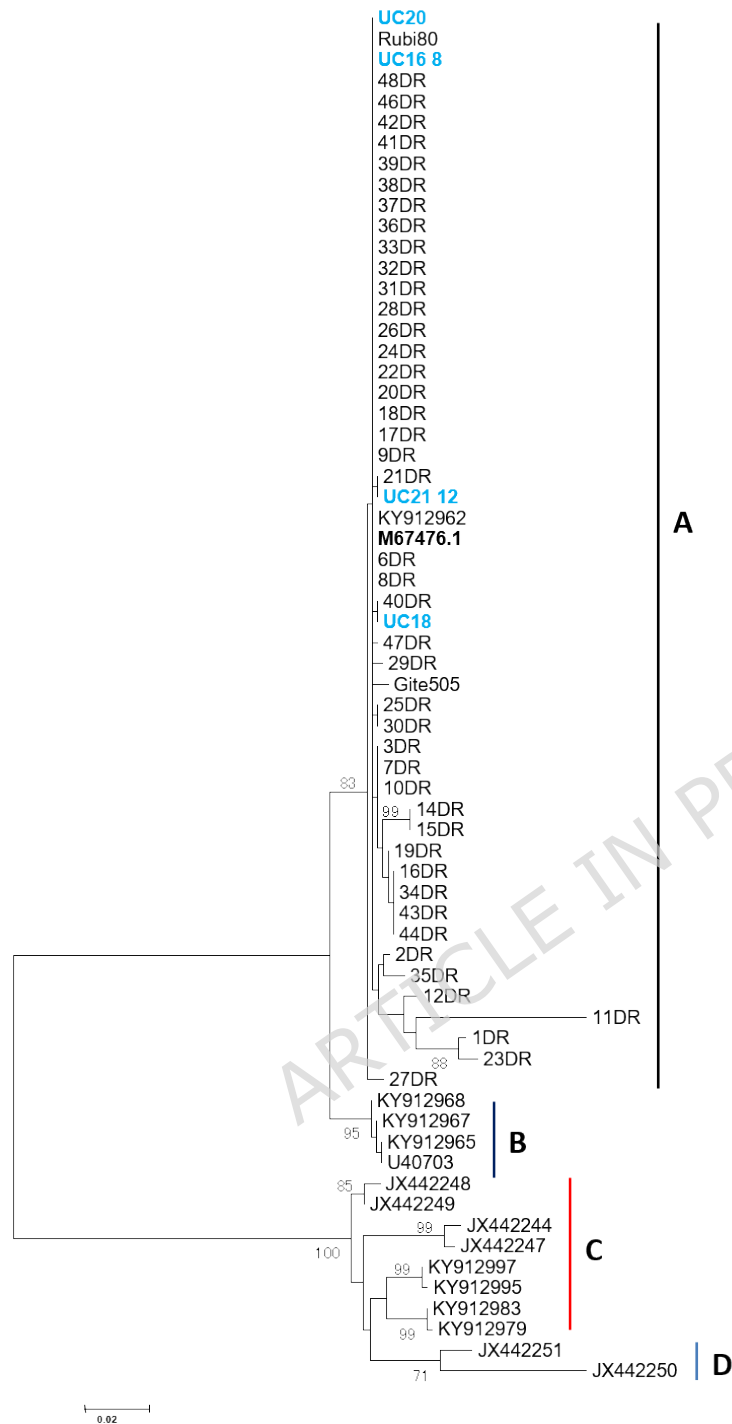


Figure 2: *p67* phylogenetic tree based on 821 bp nucleotide sequences from sentinel and field challenge sequences from the Democratic Republic of the Congo along with reference sequences from East and Southern Africa. The tree was constructed using MEGA version 6 with 1000

bootstrap replicates as a measure of confidence. Cluster A are sequences in Allele 1, Clusters B, C, and D are reference sequences in Alleles 2, 3, and 4 respectively.

***Tp1* locus**

The *Tp1* CTL gene (432 bp) of *T. parva* was sequenced from the p104 gene-positive samples. A total of 33 samples exhibited 100% amino acid sequence homology with the MC epitope. Among these, two samples (UC16, UC18) were from the sentinel group, while the remaining 31 were from the immunized (n=13) and control (n=18) groups. Three variant epitopes were observed: VGYPKVEEEML (n=1, immunized), VGYPKVKEEMI (n=4, all immunized), and VGYPKVKEEII (n=9, 5 immunized, 3 controls, and 1 sentinel). The identified epitopes in the control, immunized, and sentinel groups were largely similar to those found in the Muguga cocktail. Collectively, the samples produced a DNA polymorphism of 0.6% and a mean ratio of dN/dS of 2.15, with one positive selection site indicating positive selection.

Phylogenetic reconstruction of the *Tp1* locus demonstrated that all field isolates sequenced in this study segregated within a single, dominant lineage designated as Major Cluster A (Figure 3). High-resolution analysis of this lineage revealed sub-structuring into distinct sub-clusters, exposing variations in relatedness to the vaccine strains. Specifically, isolates UC16 and UC18, obtained from the sentinel cohort, formed a distinct minor cluster that grouped directly with the Muguga Cocktail reference strains within sub-cluster AIIa. Conversely, sentinel isolate UC20 diverged from this group, positioning within sub-cluster AIa. Field sequences derived from both the non-immunized control and immunized breakthrough cohorts were distributed across two distinct minor sub-clusters, designated AIa and AIIb. Sub-cluster AIIb exhibited high sequence homology and a close phylogenetic relationship with the Muguga Cocktail vaccine strains situated in sub-cluster AIIa. In contrast, sub-cluster AIa formed a phylogenetically divergent lineage, displaying a marked evolutionary distance from the vaccine reference stocks in clusters B and C (Figure 3).

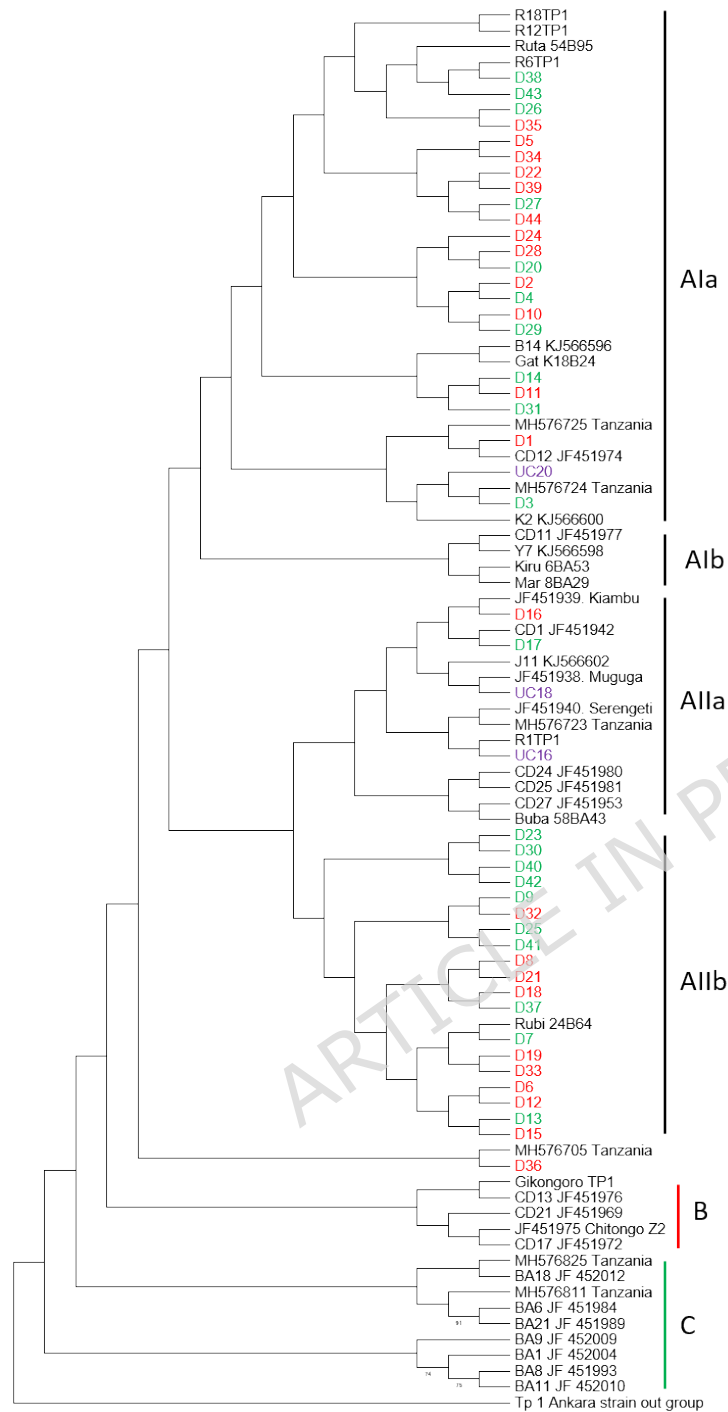


Figure 3: *T. parva* Tp1 phylogenetic tree. The tree was constructed from 432 bp nucleotide sequences using MEGA version 6 with 1000 bootstrap replicates for confidence. Sentinel group samples are shown in purple, immunized group samples in green, and control group samples in red.

Analysis of *TpI* haplotypes using network 10 showed a star-like pattern with nine haplotypes (H11, H16, H15, H22, H18, H8, H23, H9, H8) radiating from haplotype H7 (Figure 4), indicating the possibility of population expansion. H7, mainly comprising samples from the control group, was the most abundant haplotype (n=15). H1, despite being one of the minor haplotypes, included sequences from the sentinel, control, and immunized groups. All other haplotypes either comprised one group (e.g., H22 with control group samples) or included samples from both the control and immunized groups. The MC haplotypes were least represented overall, suggesting that the majority of samples from both the control and immunized groups differed from the MC (Figure 4).

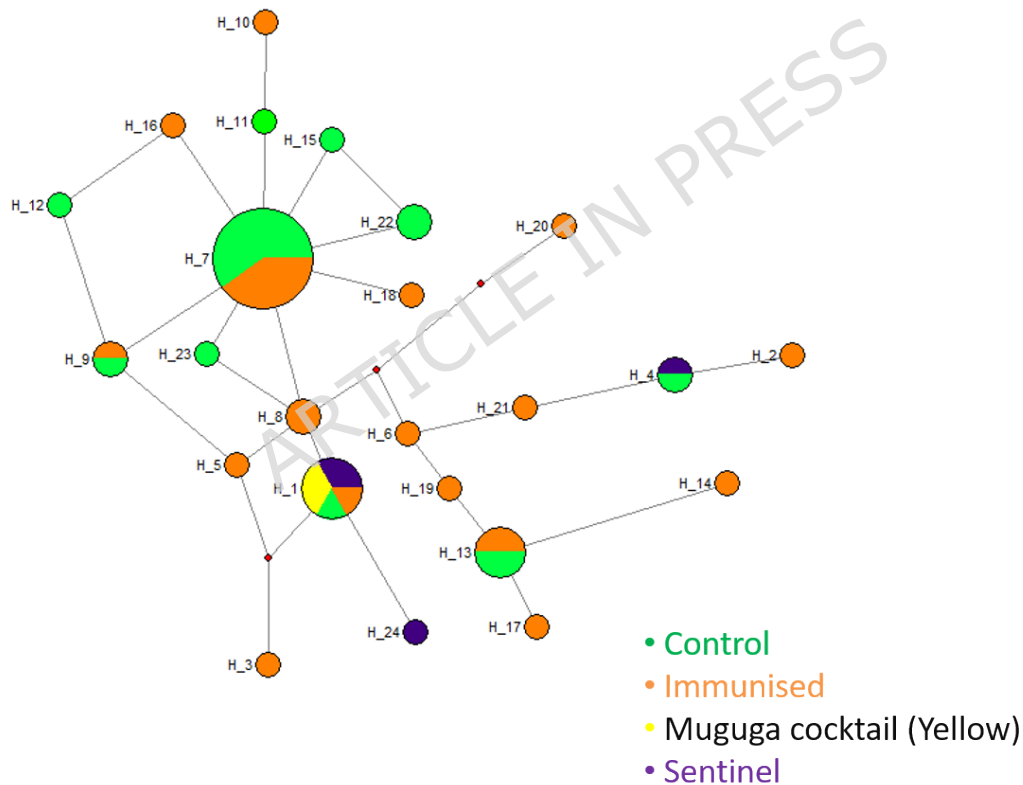


Figure 4: Median-joining network of the *T. parva* *TpI* gene, constructed using Network 5 and based on the polymorphic sites of *TpI*. The network displays a star-like radiation pattern, with circle sizes corresponding to haplotype frequency and color codes indicating the origin of the samples.

***Tp2* Locus**

The *Tp2* gene encodes a 174-amino acid protein that consists of six epitopes: CTL 1 (SHEELKKLGML), CTL 2 (DGFDRDALF), CTL 3 (KSSHGMGKVGK), CTL 4 (FAQSLVCVL), CTL 5 (QSLVCVLMK), and CTL 6 (KTSIPNPCKW). The complete *Tp2* gene (531 bp) of *T. parva* was sequenced from samples in the sentinel, immunized, and control groups. Regarding CTL 1, no MC epitopes were identified in both control and immunized samples; instead, ten (10) variants were identified (Table 7). MC epitopes were only identified on CTL 4, 5, and 6 in 6, 6, and 11 post-immunization samples, respectively. The observed MC variants were 10, 10, 7, 9, 10, and 7 on epitopes 1 to 6, respectively. Out of a total of 258 epitopes identified across all loci, only 23 were similar to MC epitopes, while the majority (n=235) were variants of the MC epitope (Table 6). Additionally, all epitopes identified from sentinel sequences were variants of the MC epitope. Overall, the MC epitope was poorly represented across all six epitopes of the *Tp2* protein (Table 7). A DNA polymorphism score of 13.2%, indicating high nucleotide polymorphism, was calculated from the sentinel, control, and immunized samples. The mean ratio of dN/dS was 0.659 with 15 purifying selection sites.

Table 7: *Tp2* epitope variants identified in samples from eastern DRC.

Number	CTL 1	CTL 2	CTL 3	CTL 4	CTL 5	CTL 6
1	SDNELDTLGLL (13)	EGFDRDALF (10)	KSSHGMGKIGK (10)	FAQSLMCVL (5)	QSLMCVLMK (5)	VNDIPNPCKW (1)
2	SDELKKMGMV (6)	PDLKKNRLF (1)	KSSHGMGKIGR (3)	FAQSIVCVI (1)	QSIVCVIMK (1)	VSDIPNPCSG (1)
3	SDELNNLGMV (1)	PDFDKNRLF (19)	LSSHGMGKIGR (21)	LAQSIVCVV (1)	QSIVCVVSK (1)	KPSIPNPCEW (1)
4	SDELNKLGM (5)	EGFDKEKLF (7)	KSSHGMGIVGR (6)	LAQSIKCVL (1)	QSIKCVLHK (1)	KPSVPNPCEW (20)
5	SDELDTLGLL (9)	PDFDRNTLF (1)	LSSHGMGKIGK (1)	LAASI KCVS (19)	ASIKCVSHH (12)	VNDIPNPCKW (7)
6	SDELNKMGM (5)	PDFDKNTLF (1)	LTSHGMGKIGR (1)	FVQSIMCVI (7)	ASIKCVSQY (7)	KTSVPNPCEW (1)
7	SDELKKMGML (1)	EGFDKDTLF (1)	KSSKSMGIVGR (1)	FAPSIKCVS (1)	QSIMCVINK (7)	KPSVPNPCEW (1)

8	SEAELRKMGMI (1)	PDFDRDALF (1)		FAQSLMCVS (1)	PSIKCVSQY (1)	
9	TEEELKKMGMV (1)	GNFDRELLF (1)		LAPSIKCVS (1)	QSLMCVSQN (1)	
10	SDDELDNLGLL (1)	PGFDKNTLF (1)			PSIKCVSHH (1)	
Total	43	43	43	37	37	32

Phylogenetic analysis of the polymorphic *Tp2* gene resolved into three primary lineages, with the field isolates from this study partitioning exclusively within Clusters D and E (Figure 5). Cluster D was further stratified into two distinct sub-clusters: sub-cluster DI, which displayed a close phylogenetic association with the reference Muguga Cocktail vaccine strains, and sub-cluster DII, which grouped with the Chitongo reference strain (Figure 5). The distribution of the field isolates across these lineages was characterized as follows: Sub-cluster DI (vaccine associated), encompassed 11 sequences, comprising 6 isolates from the immunized cohort and 5 from the non-immunized control cohort, Sub-cluster DII (Chitongo associated) comprised the largest proportion of isolates (n = 23), grouping 12 immunized, 10 control, and 1 sentinel sequence and cluster E (divergent field lineage) contained 9 sequences, consisting of 5 immunized, 2 control and 2 sentinel isolates. The topology of the *Tp2* phylogenetic tree demonstrated that the vast majority of the field isolates analyzed in this study (n = 32, 74.4%) segregated away from the Muguga Cocktail vaccine strains, aligning instead with alternative reference lineages or forming independent field clades (Figure 5).

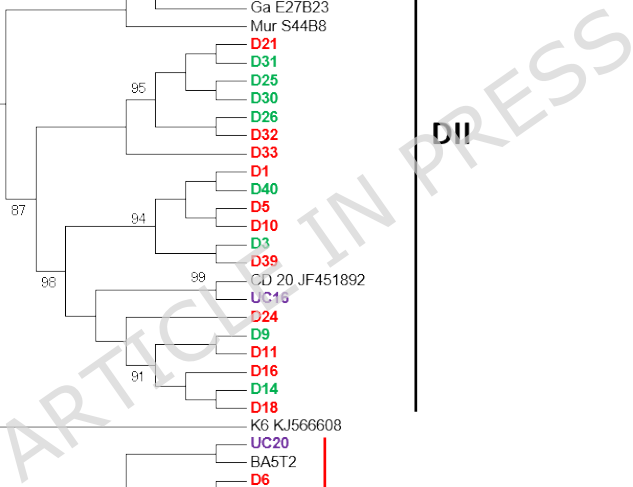


Figure 5: *T. parva* *Tp1* phylogenetic tree. The tree was constructed from 531 bp nucleotide sequences using MEGA version 6 with 1000 bootstrap replicates as a measure of confidence. Sentinel, immunised and control sequences are given in bold and purple, green and red, respectively.

The analysis of haplotype diversity revealed distinctive and non-shared haplotypes in the MC and sentinel samples, which were not shared among the immunized or control groups (Figure 6). Specifically, Muguga cocktail haplotypes H1 and H2 exhibited a direct relation only to H8, encompassing both immunized and control group samples. Within the sentinel haplotypes, only haplotype H17 showed a direct connection to H13, involving both immunized and control group sequences. The remaining sentinel haplotypes were indirectly linked to other haplotypes, such as H15, through median vectors. In summary, network analysis demonstrated that sentinel haplotypes were not connected to the MC haplotypes, nor were they associated with the haplotypes found in both the immunized and control groups.

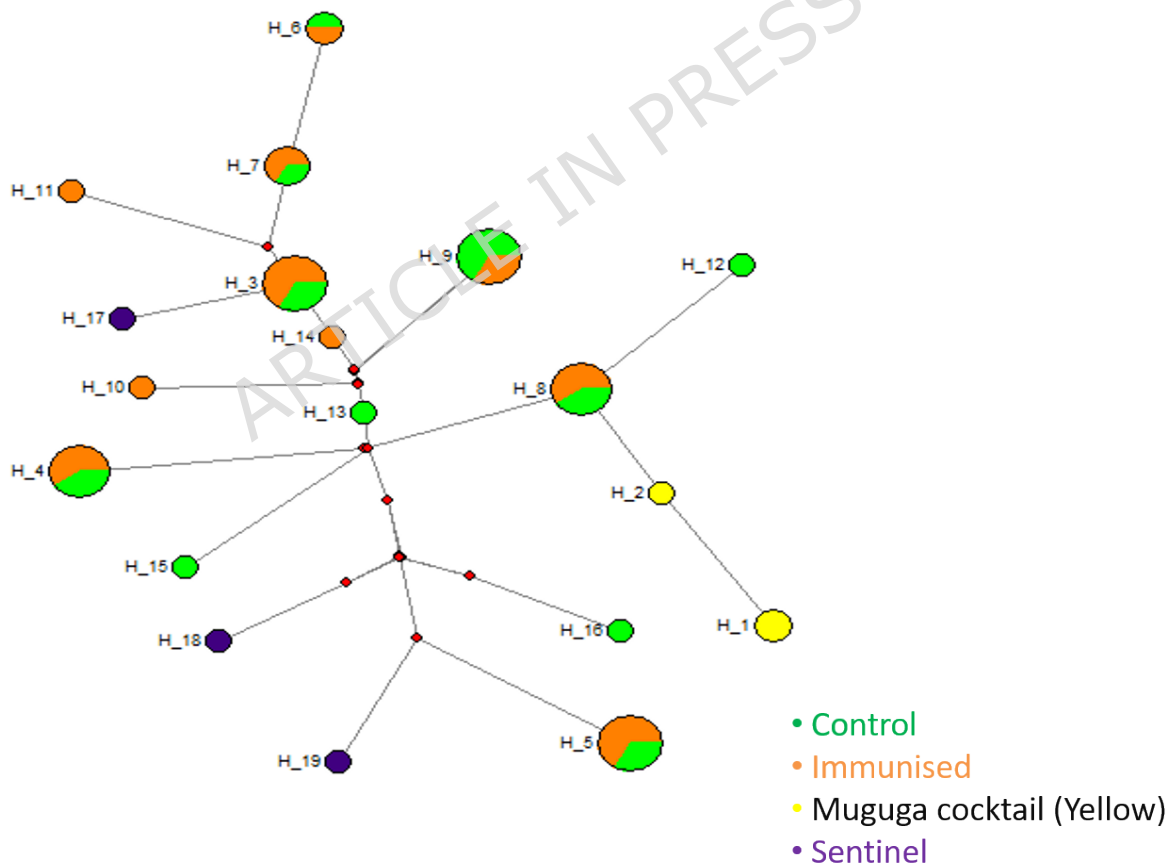


Figure 6: Median-joining network of the *T. parva* *Tp2* gene constructed using Network 5 and based on the polymorphic sites of *Tp2*. The size of the circle corresponds to haplotype frequency and the color codes indicate the origin of the samples.

Marker diversity and allelic variation

Samples from control, immunized, and sentinel cattle from the DRC were genotyped using a panel of five microsatellites (MS39, MS25, MS7, MS33, and MS19) and one mini-satellite marker (ms9), covering the four chromosomes of *T. parva*. Among the markers, MS39 was the most polymorphic, producing 25 alleles, while MS19 was the least polymorphic, identifying only 13 alleles (Table 8). High genetic diversities were also observed in sentinel, control and immunized populations (Table 8).

Table 8: Alleles identified and genetic diversity in sentinel, immunized and control groups

	Population	N	MS39	MS25	MS7	ms9	MS33	MS19
Number of Alleles	Control	20	15	10	11	14	13	9
	Immunized	22	12	10	10	14	16	8
	Sentinel	5	4	4	3	5	3	4
	Muguga Cocktail	3	3	3	3	3	3	3
	Total		25	20	16	24	24	13
Gene Diversity	Control	20	0.968	0.863	0.895	0.958	0.958	0.858
	Immunized	22	0.887	0.879	0.866	0.944	0.961	0.853
	Sentinel	25	0.900	0.900	0.800	1.0	0.700	0.900

	Muguga	3	1.0	1.0	1.0	1.0	1.0	1.0
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Across the six loci, there were both shared and unique alleles. The MC exhibited a total of 18 alleles, with 14 being unique to MC and 4 shared with the control, immunized, and sentinel populations. The control and immunized populations each possessed a total of 35 and 30 unique alleles, respectively. When considering all populations together, the total number of unique alleles across the six loci was 91, while the number of shared alleles was 38. The greater proportion of unique alleles indicated genetic sub-structuring among the populations (Figure 7).

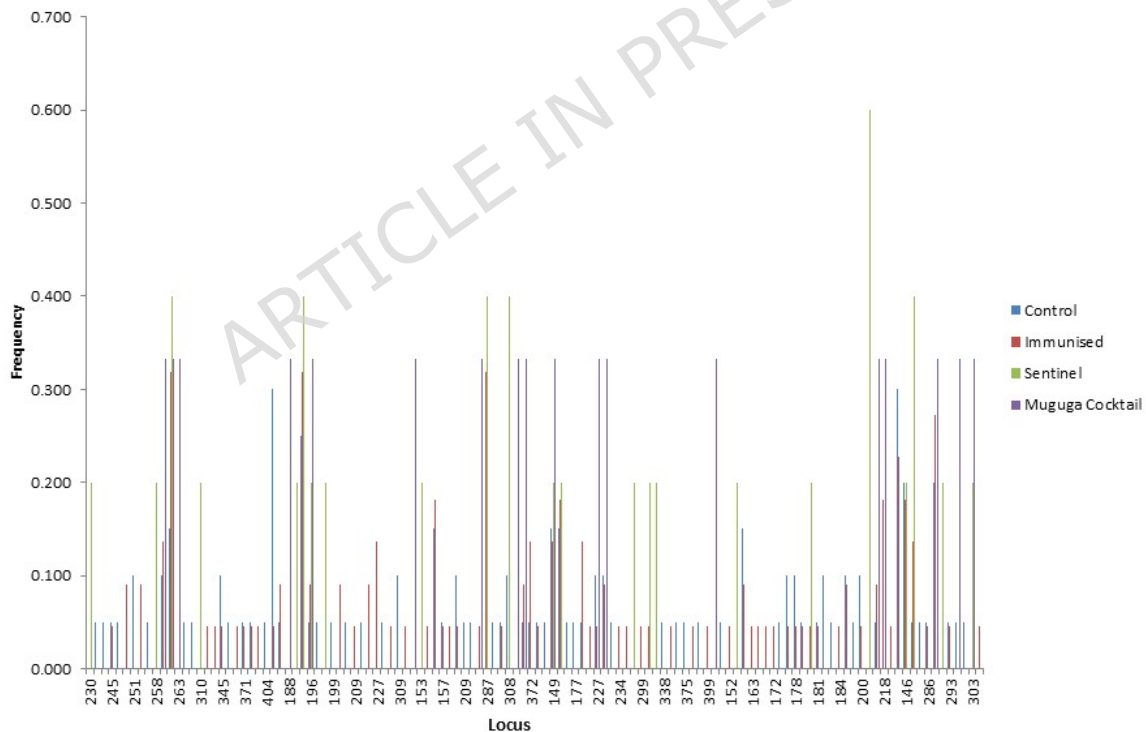


Figure 7: Overall frequency of alleles from sentinel, immunised, control and vaccine groups. The dataset presents shared and unique alleles determined by calculating the proportions of the

predominant alleles relative to the total of each satellite marker. Histograms were generated using the multi-locus genotype data.

Similarity analysis, population differentiation, and diversity

To evaluate the observed genetic sub-structuring depicted in Figure 7, Principal Component Analysis (PCA) was employed. The PCA results revealed that samples from the Democratic Republic of the Congo (DRC) occupied all four quadrants, whereas the Muguga cocktail (MC) samples were confined to a single quadrant (Figure 8). Notably, thirty-six control (n=13), immunized (n=18) and sentinel (n=5) samples closely clustered with the MC in cluster A, while the remaining eleven (11) were divided between cluster B (n=6) and C (n=5), comprising of both control and immunized samples. This cluster pattern indicates a degree of sub-structuring. The degree of sub-structuring, as illustrated in Figures 7 and 8 was further assessed using Wright's F index. Fst values of 0.119 and 0.096 were obtained when cluster population A, (MC, control, sentinel and immunized samples), was compared to cluster populations B and C, respectively. When all populations were treated as one, an Fst value of 0.118 was obtained.

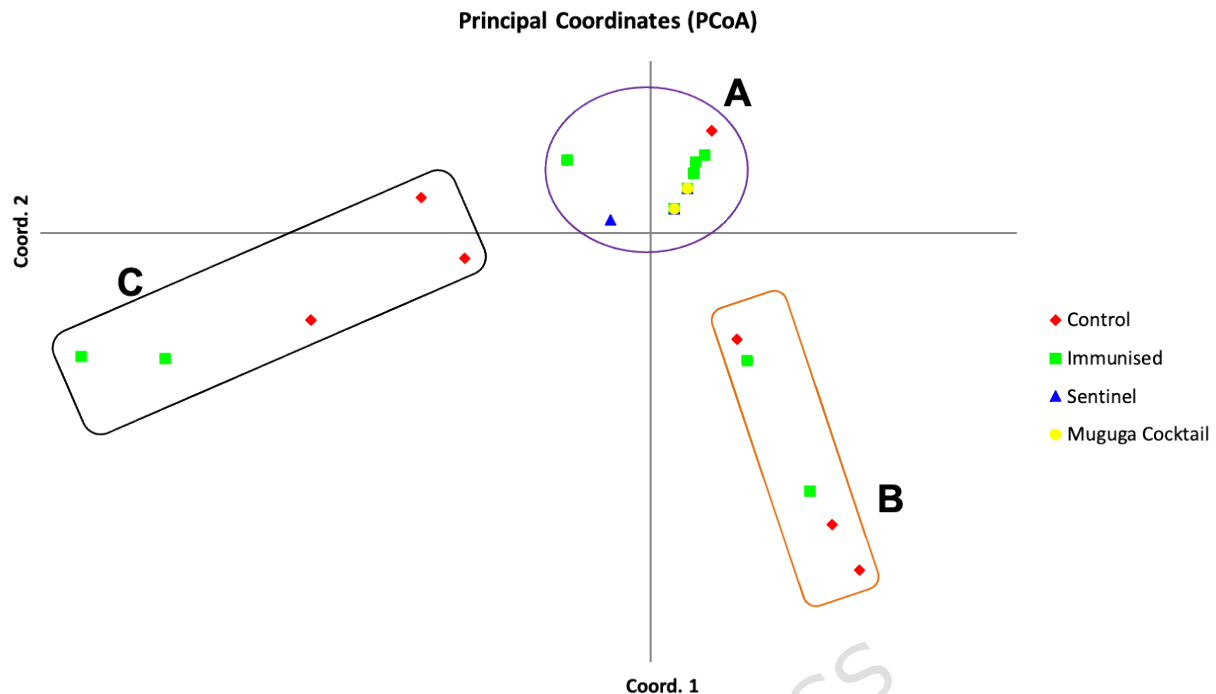


Figure 8: Principal Component Analysis (PCA) for sentinel, immunised, control and muguga cocktail vaccine samples revealing sub-structuring among three populations: Population A includes muguga cocktail, immunised, control and sentinel samples; Population B and C comprises of immunised and control samples. The numbers on each axis indicate the proportion of variance explained by the population dataset.

Discussion

The results of this study provide critical insights into the epidemiology and genetic diversity of *Theileria parva* in eastern DRC, an area where data on local parasite populations have been limited. The observed seroprevalence and molecular characterization of *T. parva* isolates highlight the presence of diverse parasite genotypes circulating in the region, consistent with reports from other East African endemic areas. Molecular techniques, such as PCR and DNA sequencing, have revolutionized the detection of previously unidentified pathogens in regions believed to be pathogen-free. By integrating serological, microsatellite, and sequence-based analyses, this study offers a comprehensive understanding of local parasite diversity, which is essential for guiding the

rational introduction of immunization strategies such as the Muguga Cocktail. To establish successful immunization programs and ensure optimal protection of the local cattle population, it is imperative to identify suitably matched vaccine strains of *T. parva* for use in new areas. Molecular-based studies on prevailing *T. parva* populations are essential for this purpose, as the infection and treatment method only confer strain-specific immunity (39,40). Positive outcomes from similar studies in Rwanda (31) and Burundi (14) emphasize the potential success of this approach. To assess the distribution of *T. parva* and to evaluate similarities between *T. parva* populations in Kabasha village, eastern DRC, and the Muguga cocktail vaccine stocks, this study employed an approach that included analyses of; seroprevalence, CTL antigen sequences, and minisatellite and microsatellites. The study also included a rigorous immunization and field challenge trial aimed at informing and developing effective immunization strategies against ECF in the eastern DRC region. Notably, to the best of our knowledge, this is the first study to utilize the sequence diversity of *T. parva* CTL antigens *Tp1* and *Tp2*, along with mini- and microsatellite analysis, and an immunization and field challenge trial, to characterize and compare field samples with known vaccine isolates in the DRC.

For the cross-sectional study, the majority of sampled cattle were adult females, aligning with common slaughtering practices involving male calves, which agrees with a similar study in North Kivu (29). The overall seroprevalence of *T. parva* was found to be 27.5%, with notable variations observed across the three surveyed provinces. This finding diverges significantly from previous epidemiological reports in the region; specifically, earlier investigations recorded *T. parva* seroprevalence rates of 43% in North Kivu (29) and 69.33% in South Kivu (41). Our findings are in agreement with related studies in neighboring Rwanda, Uganda, and South Sudan (42–44) that have reported differing seroprevalences of *T. parva*, emphasizing the endemic nature of the

parasite within the region and suggesting a shared regional epidemiological pattern of East Coast Fever. However, a key limitation of this study is that sampling was conducted between September 2012 and January 2013. Given the dynamic nature of tick-borne diseases, these data may not fully reflect the current epidemiological status or present distribution of *T. parva* in Eastern DRC. Over the past decade, shifts in cattle movement patterns, changes in climate, and evolving livestock management strategies may have altered transmission dynamics. Consequently, further up to date cross sectional studies are warranted to assess the present-day burden of the disease and to guide effective regional control strategies. The presence of the *p104* gene in the majority of ELISA-positive samples emphasizes its role as a useful and confirmatory diagnostic marker for *T. parva*. The sentinel cattle succumbed to ECF in Kabasha village indicating that the area was heavily infested with *T. parva* infected *R. appendiculatus* ticks which was then selected as a site of choice for the natural field challenge. A previous study in North Kivu where Kabasha village is located found *R. appendiculatus* as the most abundant tick species (29).

To identify the strains of prevailing *T. parva* populations and evaluate the effectiveness of the available Muguga cocktail vaccine, an immunization and field challenge study was undertaken. All the control animals and more than half of the vaccinated animals succumbed to ECF, resulting in an immunization success rate of only 46.5%. This indicated a failure of the vaccine to provide adequate protection against ECF and likely that the available stocks may not offer cross protection against the Kabasha village strain of *T. parva*. Previous studies in other countries using the Muguga cocktail have reported higher immunization success rates (45–48) suggesting that the Muguga cocktail doesn't cross-protect against the *T. parva* circulating in Kabasha village.

To further understand the characteristics of the *T. parva* population in Kabasha village and how it compares with the Muguga cocktail stocks, molecular analyses were conducted. Phylogenetic

analysis of the *p67* gene revealed the exclusive presence of cattle-derived *T. parva* strains, all possessing allele type 1, as observed in other studies (14). Analysis of the CTL *Tp1* and *Tp2* antigens showed three clusters, contrasting with findings from other studies (13–16,31). The sentinel samples (2/3) were closely related to the Muguga cocktail based on *Tp1* and exhibited distance relatedness to Muguga cocktail on *Tp2*. On both *Tp1* and *Tp2* phylogenetic analyses, immunized and control samples separated into two clusters. Within these clusters, both immunized and control samples were present, implying the presence of similar strains in both groups. On *Tp2*, two sentinel and control samples and five immunized samples formed a separate cluster, suggesting they could have originated from a different *T. parva* population. The majority of samples from the sentinel, immunized, and control groups didn't share the same antigenic profile with the Muguga cocktail, especially considering *Tp2* epitopes. This was evident from the high proportion of epitope variants on *Tp2*, despite the majority of epitopes on *Tp1* being similar to Muguga cocktail. This discrepancy may be attributed to differences in the conservation nature of both genes. Ultimately, the implication is that most *T. parva* field populations, while appearing Muguga cocktail-like, might not share the same immunological response and could constitute a different population from the Muguga cocktail. This is further supported by the few haplotypes that were shared between the sentinel, immunized, control populations, and the Muguga cocktail vaccine stocks. This observation aligns with previous studies in different regions (14,31,49). Additionally, evidence of expanding populations with similar haplotypes across the region suggests the possibility of open grazing, coupled with free trade in animals or movement among local farmers, driving the spread of *T. parva* infection. This practice hampers the implementation of adequate control strategies, such as frequent dipping and livestock movement bans, crucial in mitigating the effects of theileriosis. It is important to note that at the time of sampling, part of this

region was experiencing armed conflict; therefore, the samples used for molecular analysis were obtained from the same place (Kabasha village). Samples that appear different from the Muguga cocktail may represent breakaway populations that do not succumb to ITM using Muguga cocktail. Nevertheless, sequence analysis alone is insufficient to determine the most appropriate vaccine strain for field challenge trials with respect to *T. parva*. Due to the highly conserved nature of *Tp1* and diversity of *Tp2* genes, determining the similarity of proposed vaccine isolates to field populations is challenging. To enhance result resolution, population genetic analysis involving six (6) sites on the *T. parva* genome was conducted. Population genetic analysis revealed high gene diversities in all populations across all loci, indicating the presence of a diverse population. Despite the lack of close clustering with MC vaccine isolates on phylogenetic and network analysis, sentinel, immunized, and control samples also did not share the majority of alleles on microsatellite analysis. In contrast, PCA showed close clustering of samples comprising sentinel, immunized, and control populations, with a few samples clustering independently. This could be attributed to detecting Muguga cocktail vaccine strains, along with control samples infected with Muguga cocktail-like parasites. Furthermore, based on sequence diversity and population genetic data, it is likely that more than one population similar to the Muguga cocktail but genetically sub-structured from it is present at the study site. This is evidenced by the breakaway populations observed in clusters B and C on the PCA and the F_{st} values of 0.119 and 0.096 for comparisons of A vs. B and A vs. C, respectively. An F_{st} value of 0.118 when all populations are treated as a single population indicated moderate genetic differentiation among the populations.

Conclusions

In conclusion, this study revealed a high seroprevalence of *T. parva* that varied across the three provinces in eastern DRC. While some *T. parva* populations appeared Muguga cocktail-like,

significant differences in antigenic profiles were observed, indicating the presence of distinct populations. Despite the armed conflict in the region during sampling, the study provided valuable insights into the genetic diversity of *T. parva* populations, suggesting the presence of diverse populations. The immunization and field challenge trial indicated a low survival rate, underscoring the limitations of the current vaccine against field strains in eastern DRC. The study concludes that more than one population similar to the Muguga cocktail may be present in the study site, as observed in clusters B and C on PCA. Nevertheless, this study contributes significantly to the understanding of *T. parva* dynamics in the region, emphasizing the complexities of vaccine strain selection and the importance of continuous monitoring and adaptation of control strategies in the face of evolving parasite populations. Future studies incorporating deep sequencing or clone-based sequencing approaches would provide a more comprehensive understanding of *T. parva* population diversity and its implications for vaccine deployment.

List of abbreviations

ECF: East Coast Fever

DRC: Democratic Republic of Congo

PCA: Principal Component Analysis

MC: Muguga Cocktail

ITM: Infection and Treatment Method

Declarations

Ethical approval and Consent to participate

Administrative clearance and ethical permission to collect samples and conduct the field trial were officially granted by the Department of Agriculture, Livestock, and Fisheries (AGRIPEL) in the Democratic Republic of Congo. The regulatory and welfare oversight fell under the jurisdiction of AGRIPEL veterinary authorities. Informed consent for sampling was obtained from all livestock owners before specimen collection. The purpose and procedures of the study were clearly explained, and participation was entirely voluntary. It is essential to note that the animals underwent humane handling procedures throughout the sample collection and the immunization/field challenge study. Ethical clearance for the recombinant DNA experiments conducted in this study received approval from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) under REF. 233-2019.

Consent for publication

Not Applicable.

Availability of data and materials

All the nucleotide sequences obtained and used in this study have been deposited in the DNA database of Japan with accession numbers LC863893-LC863940. All other material is contained within the manuscript. Other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

WM and DKA contributed equally to this work and either could have been the first author, while other authors declare no competing interests.

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Author contributions

Experimental design, data analysis, writing manuscript draft, W.M. and D.K.A.; field sample collection, V.M., K.M.K., K.M.H.; immunisation and field challenge study, A.J.M., D.K.A. and W.M.; laboratory work, S.M., W.M. and D.K.A.; drafting and editing manuscript, V.M., S.M. and J.S.; Experimental design and manuscript draft editing, B.N., and A.J.M. All authors have read and approved the final version of the manuscript.

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