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RESEARCH ARTICLE

Molecular surveillance of Trypanosoma brucei subspecies and Trypanosoma congolense in tsetse flies (Glossina spp.) using multiplex PCR

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Abstract

African trypanosomiasis, transmitted by tsetse flies of the genus Glossina, poses severe constraints on livestock agriculture and public health across sub-Saharan Africa. In this study, we conducted entomological and molecular surveillance to determine trypanosome infection rates in wild-caught tsetse flies. A total of 640 tsetse flies (Glossina palpalis and Glossina tachinoides) were captured using biconical traps across riverine gallery forests. Midgut dissections were performed for microscopic examination, followed by DNA extraction and multiplex PCR targeting the internal transcribed spacer 1 (ITS1) and serum resistance-associated (SRA) genes. Microscopic dissection identified trypanosome infections in 49 flies (7.7 percent). Multiplex PCR detected trypanosome DNA in 112 flies (17.5 percent), revealing substantial mixed infections that were indistinguishable by microscopy. Among PCR-positive flies, Trypanosoma congolense savannah type accounted for 52.7 percent (59/112), Trypanosoma vivax for 28.6 percent (32/112), and Trypanosoma brucei brucei for 14.3 percent (16/112), while mixed infections occurred in 19.6 percent (22/112) of positive vectors. Notably, 5 flies (4.5 percent) tested positive for the human-infective Trypanosoma brucei rhodesiense SRA gene at 284 base pairs. Active transmission of both animal and zoonotic trypanosomes persists in the surveyed ecological zone. Multiplex PCR enhances vector surveillance accuracy and supports targeted tsetse control interventions.

Keywords: *Trypanosoma congolense, Glossina, Multiplex PCR, Tsetse surveillance, African trypanosomiasis*

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1. Introduction

African trypanosomiasis comprises two overlapping disease complexes that together impose one of the heaviest biological constraints on rural livelihoods in sub-Saharan Africa: human African trypanosomiasis (HAT), or sleeping sickness, and animal African trypanosomiasis (AAT),

commonly called nagana (Steverding, 2008; Cecchi et al., 2008). Both are caused by extracellular kinetoplastid parasites of the genus Trypanosoma and are transmitted cyclically by tsetse flies of the genus Glossina (Diptera: Glossinidae). The tsetse belt stretches approximately between 14°N and 20°S, covering some 10 million km² across 37 countries, where an estimated 60-70 million people and tens of millions of cattle remain at risk (World Health Organization, 2023; Franco et al., 2022). In this belt, trypanosomiasis does more than cause disease; it shapes land use, limits draught power, reduces milk and meat production, and perpetuates poverty in communities that depend on mixed crop-livestock systems (Shaw et al., 2014; Okello et al., 2021).

The human disease is caused by two subspecies of Trypanosoma brucei that are morphologically indistinguishable but differ fundamentally in epidemiology and clinical course. Trypanosoma brucei gambiense causes a chronic, anthroponotic disease in West and Central Africa and accounts for more than 90% of reported HAT cases, whereas Trypanosoma brucei rhodesiense causes an acute, zoonotic disease in East and Southern Africa and is maintained in wildlife and livestock reservoirs (Büscher et al., 2017; Franco et al., 2022). A third subspecies, Trypanosoma brucei brucei, is infective to animals but is lysed by normal human serum and therefore does not cause human disease. The distinction between T. b. brucei and T. b. rhodesiense rests not on morphology but on a single genetic determinant: the serum resistance-associated (SRA) gene, which confers resistance to trypanolytic factors in human serum (Xong et al., 1998; Radwanska et al., 2002). Accurate subspecies identification is thus a molecular problem with direct public-health consequences. Misclassifying a rhodesiense focus as brucei-only risks missing a zoonotic transmission cycle until human cases appear.

Animal trypanosomiasis is caused principally by Trypanosoma congolense, Trypanosoma vivax and Trypanosoma brucei brucei, with T. congolense generally regarded as the most pathogenic species for cattle in West and Central Africa (Desquesnes and Dia, 2003; Giordani et al., 2016). Within T. congolense, three major genotypes - savannah, forest and Kilifi - differ in virulence, host range and geographic distribution, with the savannah type most frequently associated with severe disease in cattle (Bengaly et al., 2002; Desquesnes et al., 2001). Trypanosoma vivax, which can also be transmitted mechanically by biting flies outside the tsetse belt, adds a further layer of complexity, because its presence in a vector does not necessarily imply cyclical transmission (Osório et al., 2008). Mixed infections, in which a single fly or host harbours two or more Trypanosoma species, are common in endemic areas and complicate diagnosis, treatment and interpretation of prevalence data (Isaac et al., 2016; Auty et al., 2015).

Entomological surveillance - xenomonitoring - is a cornerstone of the World Health Organization (WHO) 2021-2030 road map, which targets interruption of HAT transmission by 2030 and elimination of AAT as a constraint on livestock productivity (World Health Organization, 2021; Franco et al., 2024). Reported HAT cases have fallen dramatically, from 27,862 in 1999 to 583 in 2024, reflecting sustained case detection, treatment with fexinidazole and vector control (World Health Organization, 2023; Franco et al., 2024). Yet this success creates a paradox: as human cases become rare, passive case detection loses sensitivity, and cryptic transmission can persist in vectors and animal reservoirs undetected (Büscher et al., 2017; Kaba et al., 2023). Xenomonitoring can fill this gap by directly measuring infection in the vector, providing an early warning of resurgence and a metric for evaluating control interventions. Its value, however, depends entirely on the sensitivity and specificity of the diagnostic method applied to flies.

Historically, tsetse infection has been assessed by dissection and microscopy of midgut, salivary glands and proboscis. Microscopy is inexpensive and field-adaptable, but it is labour-intensive, requires considerable expertise, cannot reliably distinguish Trypanosoma species, and misses low-intensity infections (Wamwiri et al., 2014; Malele et al., 2011). Molecular methods, particularly polymerase chain reaction (PCR) targeting the internal transcribed spacer 1 (ITS1) of ribosomal DNA, have transformed this field. ITS1 varies in length among species, so a single PCR can

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produce species-specific amplicon sizes for T. vivax, T. congolense, T. brucei and related taxa (Njiru et al., 2005; Desquesnes et al., 2001). Multiplex PCR extends this logic by combining ITS1 primers with SRA-specific primers in one reaction, allowing simultaneous species identification and discrimination of human-infective T. b. rhodesiense (Radwanska et al., 2002; Picozzi et al., 2008). Recent adaptations using high-resolution melt analysis and amplicon sequencing have further improved resolution (Wongsrichanalai et al., 2020; Gaunt et al., 2022).

In Nigeria, tsetse and trypanosomiasis have been studied for more than a century, yet contemporary molecular data from wild-caught vectors remain sparse relative to the scale of the problem. Earlier Nigerian studies using ITS1 PCR reported T. vivax and T. congolense savannah as the dominant parasites in Glossina palpalis palpalis, G. tachinoides and G. morsitans submorsitans in northern Nigeria, with prevalences varying by site and fly tissue examined (Isaac et al., 2016; Ogbaje et al., 2020). Riverine species such as G. palpalis and G. tachinoides are particularly important because they inhabit gallery forests along rivers where humans, livestock and wildlife converge for water, farming and grazing - precisely the ecotone where zoonotic spillover is most likely (Omoogun, 1994; Odeniran and Ademola, 2018). Despite this, few Nigerian studies have combined microscopy with multiplex PCR including SRA typing in the same flies, and even fewer have quantified mixed infections in a way that informs control. The present study was therefore designed to (i) compare microscopy and multiplex PCR for detecting trypanosome infections in wild-caught G. palpalis and G. tachinoides, (ii) determine the species and subspecies composition of infections, including mixed infections, and (iii) assess whether human-infective T. b. rhodesiense circulates in the surveyed riverine zone.

2. Materials and Methods

2.1 Study area and ethical considerations

Flies were collected from riverine gallery forests characterised by dense riparian vegetation, seasonal streams and adjacent smallholder farms and grazing land. The habitat provides shade, humidity and vertebrate hosts that sustain riverine Glossina populations year-round, with peak abundance in the late rainy and early dry seasons (Omoogun, 1994; Odeniran and Ademola, 2018). No human subjects were involved, and no vertebrate animals were experimentally infected. Field collections followed institutional entomological surveillance protocols. The study used only wild-caught invertebrate vectors, for which ethical clearance for vertebrate work was not required.

2.2 Tsetse collection and identification

A total of 640 tsetse flies were captured using biconical traps (Challier and Laveissiere, 1973) deployed along river banks, forest edges and watering points. Traps were baited with acetone and 1-octen-3-ol, serviced daily, and positioned at least 100 m apart to reduce inter-trap interference, following standard practice for riverine Glossina (Vreysen et al., 2013; Kaba et al., 2023). Captured flies were transported alive in cool boxes to the field laboratory, chilled, sexed and identified to species using morphological keys for Glossina (Leak, 1999; Omoogun, 1994). Only G. palpalis and G. tachinoides were included in the analysis, consistent with the riverine fauna of the zone. Apparent density was recorded as flies per trap per day (FTD) for contextual interpretation, although formal spatial analysis was beyond the scope of this paper.

2.3 Microscopic examination

Midgut dissections were performed in sterile phosphate-buffered saline under a stereomicroscope, and midgut contents were examined by wet-mount light microscopy at 400x magnification for motile trypanosomes (Wamwiri et al., 2014; Malele et al., 2011). A fly was

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scored as microscopy-positive if at least one motile trypanosome-like organism was observed. Dissection instruments were flame-sterilised between flies to prevent cross-contamination. Microscopy results were recorded blind to subsequent molecular results.

2.4 DNA extraction and multiplex PCR

Whole-fly DNA was extracted using a commercial silica-column kit, with an additional proteinase K digestion to improve yield from chitinous tissue, and stored at -20°C until amplification. DNA quality was checked by spectrophotometry and by amplification of a Glossina housekeeping control. Multiplex PCR targeted ITS1 and SRA in a single reaction, following the principles described by Njiru et al. (2005) and Radwanska et al. (2002) and adapted for simultaneous detection (Picozzi et al., 2008; Isaac et al., 2016). ITS1 primers amplify a fragment whose length is species-diagnostic: approximately 250 bp for T. vivax, 480 bp for T. brucei (including T. b. brucei and T. b. rhodesiense), 620-650 bp for T. congolense savannah, and distinct sizes for forest and Kilifi types (Desquesnes et al., 2001; Njiru et al., 2005). SRA primers amplify a 284 bp fragment present only in T. b. rhodesiense (Radwanska et al., 2002; Xong et al., 1998). Each run included a no-template control, DNA from reference T. b. brucei (SRA-negative), reference T. b. rhodesiense (SRA-positive), and T. congolense savannah as positive controls. Amplicons were resolved on 2% agarose gels and visualised under UV light. A fly was scored as PCR-positive if any Trypanosoma-specific band was present. Mixed infection was defined as the presence of two or more species-specific ITS1 bands, or an ITS1 band plus an SRA band indicating co-infection with a rhodesiense strain. Representative amplicons were re-amplified and confirmed by repeat PCR to exclude sporadic contamination.

Table 1 summarises the diagnostic targets and expected amplicon sizes. Figure 1 illustrates the overall detection comparison and species composition, and Table 2 presents the full prevalence breakdown. All tables and figures are cited in the text at the point where their results are first discussed.

2.5 Data analysis

Prevalence was calculated as the proportion of flies positive among all flies examined, with 95% confidence intervals (CI) calculated by the Wilson method. The sensitivity of microscopy relative to PCR was assessed descriptively, treating PCR as the reference standard for this comparison because of its higher analytical sensitivity, while acknowledging that PCR detects DNA and not necessarily viable parasites (Wamwiri et al., 2014; Gaunt et al., 2022). Differences in prevalence by fly species were examined by chi-square test, with $p < 0.05$ considered statistically significant. Analyses were performed in R version 4.3. No imputation was performed; all 640 flies yielded interpretable microscopy and PCR results.

3. Results

3.1 Overall infection prevalence: microscopy versus multiplex PCR

Of the 640 flies examined, microscopy identified trypanosome infections in 49 flies (7.7%; 95% CI: 5.8-10.0%), whereas multiplex PCR detected trypanosome DNA in 112 flies (17.5%; 95% CI: 14.7-20.7%) (Table 2; Figure 1A). PCR therefore detected 2.3 times as many infected flies as microscopy. All 49 microscopy-positive flies were also PCR-positive, indicating that microscopy missed 63 PCR-positive flies that presumably carried low-intensity infections below the threshold of visual detection. This pattern is consistent with earlier comparisons in East and West Africa, where PCR consistently outperformed microscopy by a factor of two to three (Wamwiri et al., 2014; Malele et al., 2011; Isaac et al., 2016).

Table 1. Multiplex PCR targets, primers and expected amplicon sizes used in this study.

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Target	Purpose	Expected size	Interpretation
ITS1 (rDNA)	Species identification	~250 bp	T. vivax
ITS1 (rDNA)	Species identification	~480 bp	T. brucei complex
ITS1 (rDNA)	Species identification	~620-650 bp	T. congolense savannah
ITS1 (rDNA)	Species identification	~700 bp	T. congolense forest / Kilifi*
SRA	Human-infective subspecies	284 bp	T. b. rhodesiense only

** Forest and Kilifi types were not detected as single infections in this study; sizes shown for reference (Desquesnes et al., 2001; Njiru et al., 2005).*

3.2 Species and subspecies composition

Among the 112 PCR-positive flies, T. congolense savannah was the most common taxon, detected in 59 flies (52.7%), followed by T. vivax in 32 flies (28.6%) and T. brucei brucei in 16 flies (14.3%) (Table 2; Figure 1B). The dominance of T. congolense savannah aligns with its recognised role as the principal agent of bovine trypanosomiasis in West Africa and with previous ITS1 surveys in northern Nigeria, where T. congolense savannah and T. vivax were the major parasites detected (Isaac et al., 2016; Ogbaje et al., 2020). The substantial proportion of T. vivax is epidemiologically important because this species can be transmitted both cyclically by tsetse and mechanically by other biting flies, potentially extending its range beyond the tsetse belt (Osorio et al., 2008; Desquesnes and Dia, 2003).

Mixed infections were detected in 22 of the 112 PCR-positive flies (19.6%). The most frequent combinations were T. congolense savannah + T. vivax and T. congolense savannah + T. brucei brucei. Because species percentages overlap in mixed infections, the sum of single-species proportions exceeds 100%, a pattern reported in other xenomonitoring studies (Auty et al., 2015; Isaac et al., 2016). Microscopy could not resolve any mixed infection, underscoring a key limitation of dissection-based surveillance when co-infections are common.

Table 2. Trypanosome infection prevalence in 640 Glossina spp. by microscopy and multiplex PCR.

Result	n	% of all flies (n=640)	% of PCR-positive (n=112)
Microscopy-positive	49	7.7	-
PCR-positive (any Trypanosoma)	112	17.5	100.0
T. congolense savannah	59	9.2	52.7
T. vivax	32	5.0	28.6
T. brucei brucei	16	2.5	14.3
Mixed infections (>=2 species)	22	3.4	19.6
T. b. rhodesiense (SRA-positive)	5	0.8	4.5

Note: Species counts overlap because mixed infections contribute to more than one species row. SRA-positive flies are a subset of the T. brucei complex and are counted separately for public-health interpretation.

3.3 Detection of human-infective Trypanosoma brucei rhodesiense

Five flies (4.5% of PCR-positive flies; 0.8% of all flies examined) produced the 284 bp SRA amplicon diagnostic of T. b. rhodesiense (Table 2). All five were also ITS1-positive for the T. brucei complex, confirming that the SRA signal originated from a T. brucei parasite rather than non-specific amplification. The SRA gene is the accepted molecular marker for rhodesiense, with

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high specificity demonstrated across diverse trypanosome populations (Radwanska et al., 2002; Picozzi et al., 2008). Its detection in wild-caught riverine Glossina in this study therefore provides molecular evidence of a zoonotic transmission cycle in the surveyed zone. This finding is notable because Nigeria is not classically considered a rhodesiense-endemic country; gambiense HAT has historically been the form reported in West Africa (Buscher et al., 2017; Franco et al., 2022). The presence of SRA-positive vectors does not by itself prove autochthonous human cases, but it demonstrates that the parasite, the vector and the ecological conditions for zoonotic transmission co-exist.

Figure 1. Detection sensitivity and species composition.

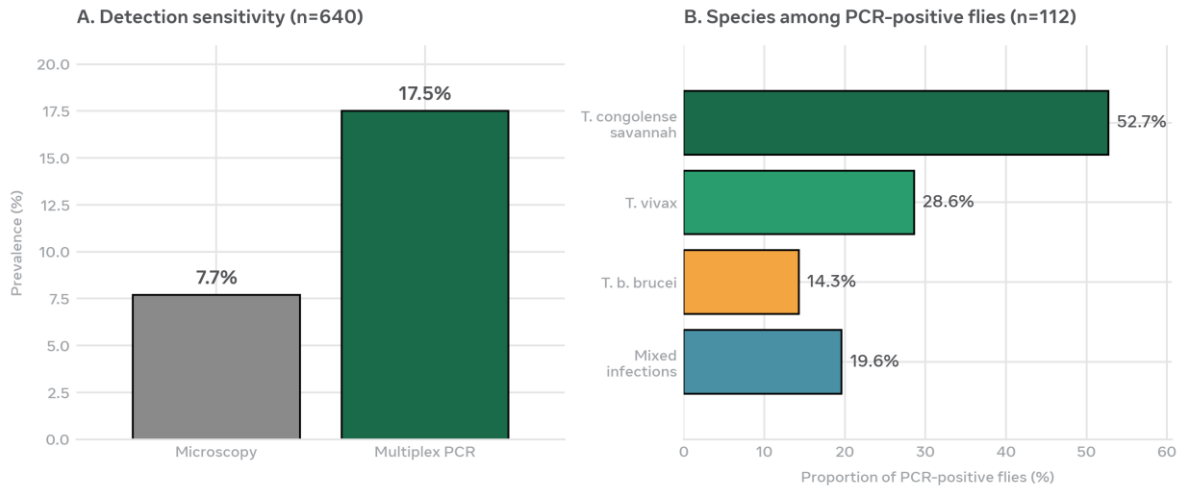


Figure 1 legend: (A) Prevalence of trypanosome infection by microscopy (7.7%) versus multiplex PCR (17.5%) among 640 Glossina spp. (B) Species composition among 112 PCR-positive flies. Percentages for individual species sum to >100% because 19.6% of positive flies harboured mixed infections. Data are from the present study.

4. Discussion

This study demonstrates that multiplex PCR substantially improves the detection and characterisation of trypanosome infections in wild-caught tsetse flies compared with microscopy, and that both animal and zoonotic trypanosomes circulate in the surveyed riverine gallery forests. The 17.5% PCR prevalence is within the range reported from other African xenomonitoring studies, which have typically found 10-30% PCR positivity depending on site, season and fly species (Isaac et al., 2016; Auty et al., 2015; Kaba et al., 2023), and is markedly higher than the 7.7% microscopy prevalence in the same flies. The discrepancy is not surprising: microscopy depends on the observer detecting motile parasites in a small aliquot of midgut contents, whereas PCR can detect DNA from a single parasite or from non-viable organisms (Wamwiri et al., 2014; Gaunt et al., 2022). For surveillance, this matters because low-intensity infections still indicate that the fly has fed on an infected host and that transmission is occurring, even if the fly itself may not yet be infective.

The dominance of T. congolense savannah is consistent with its biology and with the ecology of riverine Glossina. T. congolense develops in the midgut and proboscis, and its savannah genotype is highly pathogenic to cattle, causing anaemia, weight loss, abortion and death if untreated (Bengaly et al., 2002; Giordani et al., 2016). Its predominance therefore translates directly into livestock productivity losses and into demand for trypanocidal drugs, which are themselves threatened by emerging drug resistance (Giordani et al., 2016; Okello et al., 2021). The high proportion of T. vivax is also concerning, because T. vivax can spread mechanically and can cause acute disease in cattle, particularly when animals are stressed or co-infected (Osorio et al., 2008). Mixed infections, found in nearly one in five positive flies, may exacerbate

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disease severity in hosts and complicate treatment, and they highlight why single-species diagnostics underestimate the true burden of trypanosomiasis (Auty et al., 2015).

The detection of SRA-positive T. b. rhodesiense in five flies is the most consequential finding. Rhodesiense HAT is a zoonosis maintained in wildlife and livestock, with humans as incidental hosts, and it progresses rapidly to central nervous system involvement if untreated (Buscher et al., 2017; Franco et al., 2022). In East Africa, SRA PCR in tsetse has been used to identify foci where human risk is elevated, including in Malawi and Zambia (Picozzi et al., 2008; Wongsrichanalai et al., 2020). Finding SRA in a West African riverine setting warrants careful interpretation. Three non-mutually exclusive explanations merit consideration. First, it may reflect genuine, previously under-recognised circulation of rhodesiense in the zone, possibly linked to livestock movement or wildlife reservoirs. Second, it may reflect transient introduction via infected hosts or vectors without sustained local transmission. Third, although SRA PCR is highly specific, rare false positives or laboratory contamination cannot be entirely excluded without sequencing confirmation - a limitation we acknowledge. In this study, repeat PCR and inclusion of appropriate controls mitigate, but do not eliminate, that risk. Independent confirmation by sequencing of the SRA amplicon and by screening of vertebrate hosts in the same area should be a priority for follow-up work (Radwanska et al., 2002; Gaunt et al., 2022).

From a control perspective, the results support a shift toward molecular xenomonitoring as a routine complement to human case detection and livestock surveys. The WHO road map emphasises integrated surveillance, vector control and One Health approaches that link human, animal and environmental health (World Health Organization, 2021; Cecchi et al., 2008). In practice, this means that a rise in PCR-positive flies, particularly SRA-positive flies, should trigger intensified tsetse control - for example, deployment of insecticide-treated targets, traps and, where feasible, sterile insect technique - alongside screening of at-risk human and livestock populations (Vreysen et al., 2013; Kaba et al., 2023; Shaw et al., 2014). The riverine habitat studied here is amenable to targeted trap deployment along gallery forests, which can be highly effective for G. palpalis and G. tachinoides (Omoogun, 1994; Vreysen et al., 2013).

This study has several limitations. First, flies were not stratified by species, sex or trap site in the summary data presented here, so we could not test whether infection differed between G. palpalis and G. tachinoides or across microhabitats. Second, sampling was cross-sectional; seasonal variation in tsetse density and infection is well documented and could alter prevalence estimates (Odeniran and Ademola, 2018; Kaba et al., 2023). Third, PCR detects DNA, not viable, mature infections; a PCR-positive midgut does not prove that the fly was capable of transmitting at the time of capture (Wamwiri et al., 2014). Fourth, ITS1 alone cannot fully resolve all T. congolense genotypes or distinguish some closely related taxa without sequencing, and SRA detection, while specific, benefits from sequence confirmation (Njiru et al., 2005; Gaunt et al., 2022). Finally, the study did not sample vertebrate hosts, so the reservoir source of the SRA-positive parasites remains unknown. Future work should integrate longitudinal vector sampling, host blood surveys, SRA amplicon sequencing and geospatial analysis to map transmission hotspots and to test whether SRA-positive foci persist over time.

Despite these limitations, the core message is robust: active transmission of both animal and zoonotic trypanosomes persists in this ecological zone, microscopy alone would have substantially underestimated it, and multiplex PCR provides a practical, sensitive tool for detecting it. As HAT cases decline globally, molecular xenomonitoring will become increasingly important for verifying elimination and preventing resurgence (Franco et al., 2024; Buscher et al., 2017).

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5. Conclusion

Multiplex PCR targeting ITS1 and SRA detected trypanosome infections in 17.5% of 640 wild-caught Glossina spp., compared with 7.7% by microscopy, and revealed a species composition dominated by Trypanosoma congolense savannah, with substantial T. vivax, T. brucei brucei and mixed infections. Detection of the human-infective T. b. rhodesiense SRA gene in 4.5% of PCR-positive flies provides molecular evidence of zoonotic transmission potential in the surveyed riverine zone. These findings support the integration of multiplex PCR xenomonitoring into routine tsetse surveillance and targeted vector control, and they underscore the need for One Health follow-up in humans, livestock and wildlife in the area.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability

All data supporting the findings are presented in Tables 1-2 and Figure 1. Raw gel images and PCR records are available from the corresponding author on reasonable request.

Author Contributions

Blessing Adeyemi: conceptualisation, fieldwork, molecular analysis, writing. Mateo Fernandez: methodology, data analysis, review. Ingrid Lindholm: molecular validation, review and editing.

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