Research Square

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Transmission of trypanosomiasis by tsetse flies in three active HAT foci of the Republic of Congo

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Research Article

Keywords: Transmission, stetse flies, HAT, Republic of Congo

Posted Date: September 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2010128/v1

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Abstract

Introduction: Human African Trypanosomiasis **(**HAT) is a neglected tropical disease still endemic in the Republic of Congo. Despite continuous detection of HAT cases in the country, there is still not enough data on trypanosome infections in tsetse flies, trypanosome species and tsetse flies species distribution in endemic foci. The present study is intended to fill this gap and improve understanding on trypanosome circulation in three active foci in the centre and south of Congo.

Methods: Pyramid traps were set in various places in villages to collect tsetse flies both during rainy and dry season. Once collected, tsetse flies were identified using morphological keys. DNA extracted from flies was processed by PCR for species identification and for detection of trypanosome. A second PCR was ran for different trypanosome species identification.

Results: A total of 1291 tsetse flies were collected. The average apparent density of fly per day was 0.043 in Mpouya, 0.73 in Ngabé and 2.79 in Loudima. *Glossina fuscipes quazensis* was the predominant tsetse fly found in Ngabé and Mpouya, while *Glossina palpalis palpalis* was the only tsetse fly found in Loudima. A total of 224 (17.7%) flies were detected infected by trypanosomes; 100 (7.91%) by *Trypanosoma congolense savannah*, 22 (1.74%) by *Trypanosoma congolense forest*, 15 (1.19%) by *Trypanosoma vivax*, 83 (6.56%) by *Trypanosoma brucei* (s.l.) and 2 (0.16%) undetermined species. A total of 57 co-infections between *T. brucei* (s.l.) and *T. congolense savannah* or *T. brucei* (s.l.) and *T. congolense forest* were found only in *G. p. palpalis*. Loudima recorded the highest number of infected tsetse flies.

Conclusion: The study provided updated information on the dynamics of tsetse fly populations as well as on the transmission of trypanosome species in the different active HAT foci in Congo. The data suggest high transmission of animal trypanosomes and possibly human trypanosomes in these foci. The study stress the need for active surveillance in these endemic foci.

Introduction

Trypanosomiases are parasitic diseases affecting humans and animals, caused by many species of trypanosomes and transmitted by tsetse flies of the genus Glossina [1]. Many species of trypanosomes infect cattle, including *T. brucei, T. congolense* and *T. vivax* [2]. These species cause African animal trypanosomiasis (AAT), resulting in livestock losses and reduced productivity. *T. brucei rhodensience* and *T. brucei gambiaense* are known to infect humans causing human African trypanosomiasis (HAT) which is a fatal disease. *T. b. gambiense* is common in Central and West Africa, while *T. brucei rhodensiense* is common in East Africa. They are known to cause chronic and acute forms of the disease respectively [3, 4, 5] .

HAT is one of the neglected tropical diseases that have been targeted by WHO for elimination as a public health problem by 2030. The disease occurs in more than 36 countries in sub-Saharan Africa and an estimated 65 million people are at risk of HAT transmission [6]. The populations most at risk from tsetse

fly bites are rural populations engaged in farming, fishing, herding or hunting. *Trypanosoma brucei gambiense* is the most prevalent species and accounts for 98% of all reported sleeping sickness cases [6]. In 2009, the number of reported cases fell below 10,000 for the first time in 50 years with 2,804 cases reported. However, the disease is heterogeneously distributed across the continent.

In the Republic of Congo, despite a reduction in cases, the disease remains endemic. Between 2014 and 2017, cross sectional surveys carried out in different active foci in the country, notably in the Bouenza, Plateau, Pool and Cuvette regions, resulted in the detection of 142 patients infected out of 36725 people examined [7]. Passive screening in health care centres in the city of Brazzaville and throughout the country identified 14 patients infected out of 1322 people examined in 2015 [8]. Despite efforts made by the government and partners, sleeping sickness persists in many foci.

To better understand the epidemiology of human and animal trypanosomiasis, it is important to generate data on the distribution and dynamics of tsetse flies populations [3, 9]. To date, there is a lack of data on the distribution and dynamic of tsetse fly populations as well as trypanosome parasite circulating in active HAT foci in Congo. Indeed, since the 1990s not much entomological studies have been carried out on HAT in Congo as a result there is not enough information on the dynamic of HAT and it vectors. The National Control Programme for sleeping sickness (PNLTHA) in Congo, due to lack of resources, mainly limit it activities to diagnostic campaigns and treatment of population at risk.

The objective of the present study is to generate entomological and parasitological data on tsetse flies species and trypanosome infections in tsetse flies caught in three endemic foci in the Republic of Congo.

Methodology

Study sites

The study was carried out in three active foci situated in the south and centre of the Republic of Congo.

- Mpouya (2°36'57"S, 16°12'43"E): this foci is located in the Plateau administrative division about 300 km north of Brazzaville. The foci is compose of several villages bordering the Congo river. Its vegetation is essentially made of savannah with sparse vegetation with few high trees. The population mainly practice fishing and farming for living.

- Ngabé (3°12'52"S, 16°10'1"E) is situated about 200 km north of Brazzaville in the Pool region. This foci is also compose of several villages bordering river Congo. The vegetation is made of savannah and forest gallery along the edges of rivers. The population mainly practice farming, hunting and fishing for living.

- Loudima (4°6'45"S, 13°3'30"E) is located in the Bouenza division about 300km south of Brazzaville. The vegetation is dominated by grassfield. Main activities practiced by the population is farming.

In each foci tsetse flies sampling was conducted in four to five villages.

(Figure 1)

Tsetse flies sampling

Entomological surveys were conducted in November 2019, November 2020 (rainy season) and June 2021 (dry season). During each survey, pyramid traps**[10]**were set up in five villages in Ngabe, four villages in Mpouya and Loudima for at least three days in different places (close to the river or swamps, shaded areas and in the village). A total of 251 traps were set, including 106 in November 2019, 94 in November 2020 and 51 in june 2021. For each trap, the geographical coordinates were recorded using a GARMIN GPS (eTrex® 10). The minimum distance between traps was 100m. The traps were visited twice daily (10 A.M. and 4 P.M.). Once the tsetse flies were captured, they were identified morphologically to determine the sex and species**[11]** and then preserved in microtubes containing alcohol. In the field, these microtubes were kept in a refrigerated cooler (at +4°C) and in the laboratory at -20°C until DNA extraction.

DNA extraction

DNA was extracted from whole tsetse fly by the cethyl trimethyl ammonium bromide (CTAB) method**[12]**. In the laboratory, the alcohol was evaporated at room temperature and 600 µl of CTAB buffer (5% CTAB; 1 M Tris, pH 8; 0.5 M EDTA, pH 8; 5 M NaCl) was added, the tsetse fly was ground with a pestle and then incubated in a water bath at 60 °C for 30 min. Then, 600 µl of chloroform/isoamyl alcohol (24/1) was added to each tube. This mixture was slowly homogenised for 15 min and then centrifuged at 13 000 rpm for 15 min. The upper aqueous phase was removed and transferred to another 1.5 mL microtube. The DNA was precipitated by the addition of 600 µl isopropanol. After gentle homogenisation of each microtube for 5 min and incubation overnight at -20 °C, each microtube was centrifuged at 13,000 rpm for 15 min. The DNA pellet was then washed twice with cold 70% ethanol and dried overnight at room temperature. The resulting DNA pellet was resuspended in 50 µL of sterile nuclease-free water and stored at -20 °C or used directly for PCR.

Molecular identification of tsetse fly species

The principle of this PCR is based on the amplification of the ITS1 region ("internal transcribed spacer 1") of the tsetse genome, using the primer pair "Diag forward" (5'-TGGACTTCGGATTAAGTACAACA-3') and "Diag reverse" (5'-TCATTATGCGCTATTAAGGTAAGC-3')**[13]**. For this identification, only one PCR cycle was performed; the cycle was performed in a final volume of 25 µl containing 1× PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl), 3 mM MgCl2, 1 µl (15 pmol) of each primer, 0.5 µl (200 mM) of dNTPs, 0.1 µl (one unit) of Taq DNA polymerase, 2.5 µl of DNA and 17.4 µl of sterile water. The amplification programme began with a denaturation step at 95 °C for 3 min and 30 s, followed by 30 cycles of amplification; each cycle contained a denaturation step at 95 °C for 30 s, a hybridisation step at 56 °C for 1 min and an extension step at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min.

The amplification products were run on a 2% agarose gel to enable species identification.

Molecular identification of different trypanosome species

The identification of trypanosomes was performed by amplification of the internal transcribed spacer 1 (ITS1) of the ribosomal DNA of different trypanosome species, as described by **Ravel et al [14]**. For this identification, two PCR cycles were performed; the first cycle was performed in a final volume of 25 µl containing 1× PCR buffer (10 mM Tris-HCl (pH 9. 0), 50 mM KCl), 2 mM MgCl2, 1 µl (10 pmol) of each primer (5'-CAA ATT GCC CAA TGT CG-3' and 5'-GCT GCG TTC TTC AAC GAA-3'), 0. 5 µl (200 mM) of dNTPs, 1 µl (one unit) of Taq DNA polymerase (5 U/µl), 5 µl of DNA and 14 µl of nuclease-free water. The amplification programme began with a denaturation step at 94 °C for 3 min and 30 s, followed by 30 cycles of amplification; each cycle contained a denaturation step at 94 °C for 30 s, a hybridisation step at 58 °C for 1 min and an extension step at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min.

The amplified products from the first round of PCR were diluted 10-fold and 3 µl of each dilution was used as template for the second round of PCR. The second round of PCR was performed with two different primers (5'-CCT GCA GCT GGA TCA T-3' and 5'-ATC GCG ACA CGT TGT G-3'). The amplification program was identical to that of the first round of PCR. After nested PCR, the amplicons were separated by electrophoresis on a 2% agarose gel which was then stained with midori green advance and visualised under UV light.

Trypanosomes species were identified on the basis of the length of ITS1 fragments. For example, T. congolense strains generate DNA fragments of about 650 bp (630 bp for *T. congolense* forest and 610 bp for *T. congolense* savannah) whereas fragments of about 150 bp and 400 bp, respectively, are expected for *T. vivax* and all trypanosomes belonging to the Trypanozoon subgenus (*T. brucei* (s.l.), *T. evansi* and *T. equiperdum*).

Identification of *Trypanosoma congolense* forest and *Trypanosoma congolense* savannah

After amplification of the ITS1 sequences, all samples that had a DNA fragment between 600-650 bp, corresponding to the expected size of *T. congolense*, were subjected to a further PCR where specific primers were used to separate *T. congolense* forest to *T. congolense* savannah. These specific identifications were performed as described by **Simo et al. [15]**using primers TCF1 (5'-GGA CAC ACG CCA GAA GGT ACT T-3') and TCF2 (5'-GTT CTC TCG CAC CAA ATC CAA C-3') for T. congolense forest "type" **[16]**, and TCS1 (5'-CGA GCG AGA ACG GGC AC-3') and TCS2 (5'-GGG ACA AAC AAA TCC CGC-3') for *T. congolense* savannah "type"**[17]**. PCR reactions were performed in a final volume of 25 µl containing 1× PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl), 3 mM MgCl2, 1 µl (15 pmol) of each primer, 0.5 µl (200 mM) of dNTPs, 1 µl (one unit) of Taq DNA polymerase, 3 µl of DNA and 16 µl of sterile water. The amplification program consisted of a denaturation step at 94°C for 3 min 30 s, followed by 40 cycles of amplification including a denaturation step at 94°C for 30 s, a hybridisation step at 60°C for 1 min and an elongation step at 72°C for 1 min, followed by a final elongation step at 72°C for 5 min.

The amplified products were separated by electrophoresis on a 2% agarose gel containing midori green advance. The DNA bands were visualised under ultraviolet (UV) light and a picture of the gel was taken and stored.

Data analysis

The apparent density of tsetse flies was calculated using the following formula ADT = C/(T*D) where C represents the number of flies collected, T the number of traps used and D the number of days of trapping. The Anova was used to compare the ADT between foci. Statistical analysis were conducted using the software MedCalc**[18]**. The infection rate of tsetse flies between sites was compared using the pearson chi-square test. The threshold for significance was $P < 0.05$.

Results

Entomological surveys

During the three entomological surveys 251 pyramid traps were set up and captured 1291 tsetse flies (190 in 2019, 462 in 2020 and 639 in 2021) (Table 1). Tsetse flies collected belonged to three species including *Glossina palpalis palpalis* (*G. p. palpalis*), *Glossina fusca congolensis* (*G. f. congolensis*) and *Glossina fuscipes quazensis* (*G. f. quazensis*). *G.f. quazensis* was the most abundant fly in Ngabé and Mpouya, while *G. p. palpalis* was the only tsetse fly found in Loudima (picture 1). The highest ADT was recorded in the site of Loudima whereas the lowest was recorded in Mpouya. A significant difference was observed when comparing the ADT between sites (P 0.05).

Table 1
Tsetse fly densities per study sites

Foci	No. Tsetse fly	No of teneral flies (%)	No. Trap	ADT	No of males (%)	No of females (%)	Sex ratio
Mpouya	5	0 (0)	38	0.043	0 (0)	5 (100)	0
Ngabé	179	5 (0.03)	81	0.73	58 (32.40)	121 (67.60)	0.5
Loudima	1107	11 (0.01)	132	2.79	462 (41.73)	645 (58.27)	0.7
P value				0.001			

(Table 1)

Tsetse fly identification was done morphologically in the field and molecularly in the laboratory (picture 1)

(Table 2)

Table 2
Distribution of different tsetse species from the three sleeping sickness foci of the Republic of Congo

HAT foci	Tsetse fly species			Total
	G.p. palpalis	*G.f. quazensis*	*G.f. congolensis*	
Mpouya	-	5	-	5
Ngabé	-	177	1	179
Loudima	1107	-	-	1107
Total	1107	182	1	1291

G.p. palpalis : Glossina palpalis palpalis ; G.f. quazensis : Glossina fuscipes quazensis ; G.f. congolensis : Glossina fusca congolensis

Molecular identification of trypanosomes

Of the 1291 tsetse flies captured 1265 were screened, molecular examination revealed that 224 (17.7%) tsetse flies were infected by at least one trypanosome species. Different species of trypanosomes were detected: *T. c. savannah, T. c. forest, T. vivax* and *T. brucei* sl. *Trypanosoma brucei* sl and *T. C. savannah* were the main trypanosome species found in tsetse flies. Loudima recorded the highest number of infected fly followed by Ngabé. Some flies (N = 57) were detected with coinfections *T.c.savannah* + *T. brucei* and *T.c. forest* + *T. brucei* (Table 2). All co-infections were detected in Loudima.

(Table 3)

Table 3
Molecular identification of trypanosomes in tsetse flies

Foci	No. tested	*T. c. s.* (%)	*T. c. f. (%)*	*T. vivax (%)*	*T. b.* (s.l.) (%)	*Coinfection T.b + T.c.s*	*Coinfection T.b + T.c.f.*	unknown species
Loudima	1103	95 (8.61)	21 (1.9)	11 (1)	82 (7.43)	49 (85.96)	8 (14.04)	4 (0.36)
Mpouya	5	0	0	0	1 (20)	0	0	0
Ngabé	157	5 (3.18)	1 (0.64)	4 (2.55)	0	0	0	0
Total	**1265**	100 (7.91)	22 (1.74)	15 (1.19)	83 (6.56)	49 (3.87)	8 (0.63)	4 (0.16)

No: Number; *T.c.s. : Trypanosoma congolense savannah ; T.c.f. : Trypanosoma congolense forest ; T.b* (s.l.) : *Trypanosoma brucei* (senso lato).

Discussion

Although active detection of sleeping sickness cases are almost regularly conducted in sleeping sickness foci in the Republic of Congo [7, 8] updated data on tsetse flies distribution and trypanosome species circulating in the country is not available for most foci. The present study was conducted to fill this gap in order to better understand the epidemiological situation of HAT in different active foci in Congo. Tsetse flies were more abundant in Loudima compared to Ngabé and Mpouya. *G. p. palpalis* was the only species caught in Loudima while *G. f. quazensis* was the main species found in Ngabé and Mpouya. *G. f. quazensis* is a species commonly found in gallery forests along rivers in humid savannah areas, whereas *G. p. palpalis* is found in both forest and savannah environments [11]. These species distribution ranges are similar to previous findings [19]. However slight changes in species abundance and composition were recorded between foci and previous findings [20, 21] and support the possible influence of environmental or anthropic changes (deforestation, extension of human settlements, increase in agricultural land surfaces) on tsetse flies distribution as reported elsewhere [22, 23].

Tsetse flies were more abundant during dry season as compared to the rainy season. Frequent overflowing of rivers and inundation of large areas during the rainy season, could have kill larvae developing in the soil and limit the emergence of flies from larvae to the adult stage. River banks or swamps or places with high humidity have always constituted ideal breeding ground for tsetse larvae [24].

Females flies were always more abundant than males and represented 60.42% and 58.27% of *G. f. quazensis* and *G. p. palpalis* respectively. These findings are similar to data recorded by **Gouteux et al** [25]. The sampling of both males and females flies in collection places suggest that these areas are both suitable breeding and hunting sites for the flies.

G. f. quazensis and *G. p. palpalis* were detected infected by trypanosomes. These species are major vectors of HAT in both forest and savannah areas [23, 26]. Five trypanosome species including: *T. c. savannah, T. c. forest, T. vivax, T. brucei* (s.l.) and an unknown species were detected. The majority of trypanosome species recorded are responsible for african animal trypanosomiasis (AAT) or nagana. The frequent circulation of trypanosomes infecting animals is probably a serious threat for livestock in these foci. The proportion of tsetse flies infected by trypanosome was high and similar to findings recorded elsewhere [23, 27]. Due to frequent record of sleeping sickness cases in human, it is possible that trypanosomes infecting human are also present in the area. Indeed a high number *Trypanosoma brucei* s.l. infections were recorded. Further examination of these samples is requested to confirm the presence of *T. b. gambiense*. The high prevalence of *T. brucei* s.l. infections support the presence in the area of a high reservoir of this pathogen. High infections due to Trypanosoma congolense savannah was recorded and was similar to data recorded elsewhere [26, 28, 29].

Over 29% of flies collected were infected by at least one trypanosome. It is possible that the true infection rate could have been overestimated due to the fact that the whole mosquito was processed by PCR to detect infections. Studies conducted so far indicated different infection rate when screening the midgut, the proboscis, salivary glands or the whole body [23, 30]. Moreover because detection of trypanosome

DNA was used as a proof of active infection this could also have overestimated the infection rate since dead trypanosomes DNA could have been amplified this alongside other factors could have induced overestimation of the infection rate [31]. Less than 8% of the flies processed were blood fed this significantly reduced bias associated with the presence of trypanosomes in the blood meal. However, it is likely that infections detected during this study are both immature and mature infections since no dissection was conducted.

Female tsetse flies were found to be more infected compare to male. This difference could result from the fact that, female live longer than males [4, 32]. Similar findings have been registered in Chad [30].

Mixed infections were detected during this study this pattern is in line with studies conducted so far across Africa [28, 33]. The presence of mixed infections could be associated to a high prevalence of these pathogens in mammals host and probably frequent contact between tsetse flies and infected animals or humans. Yet the presence of mixed infections was found to vary according to foci, time period or countries [26, 28]. Factors influencing trypanosome abundance and diversity in a given foci are still not well understood and deserve further investigations. The high infection rate of flies by different trypanosomes species are a proof of high vectorial competency of tsetse flies to different trypanosome species [30].

Human African trypanosomiasis is still a public health concern in most countries in sub-Saharan Africa and the emergence and spread of the disease could impede development efforts. It is important in this light that immediate action be taken to achieve elimination goals the fastest possible [34]. Studies on tsetse flies distribution and trypanosome species circulating in both animal and humans are key to understand the epidemiological situation of African animal and human African trypanosomiasis and for the implementation of efficient control strategies aiming at eliminating sleeping sickness in endemic foci. The circulation of animal trypanosomes could have damaging effect on the economy. By affecting animal health, trypanosomes particularly *T. congolense* reduce livestock production (quality and quantity of meat) and limit cattle rearing activities [35]. Because anthropisation processes such as deforestation, dams construction or the practices of intensive agriculture have reduced Tsetse flies distribution range, the mapping of affected areas could be a key component of vector control activities on the field [36]. Targeted control activities could lead to the reduction of control costs but also to improve the efficacy of control actions on the field [37].

In active foci, the use of insecticide impregnated traps or screens could be promoted for improve control. The regular treatment of infected livestock with trypanocides could also be undertaken alongside these measures.

Conclusion

The study provided updated information on the risk of human and animal trypanosomiasis in 3 active endemic foci in Congo. The study suggested high circulation of animal trypanosomes in these foci. It is likely that trypanosome infecting human such *T. b. gambiense* could be present since all *T. brucei* s.l.

samples were not identified to the species level. These findings call for urgent actions to control these foci in order to stop the expansion of HAT to the rest of the country. HAT in Congo have significantly decrease due to concerted efforts and this momentum need to be maintain in order to achieve elimination goals by 2030.

Declarations

Acknowledgements

We gratefully acknowledge the financial support of the “Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC)”, the “Communauté Economique des Etats de l’Afrique Centrale (CEMAC)”, the German Federal Ministry for Economic Cooperation and Development (BMZ) through the German Development Bank (KfW).

We are also grateful to the inhabitants and administrative authorities of the villages of Mpouya, Ngabe and Loudima

Funding

This work received financial support from the OCEAC MTN KFW program. The funding body did not have any role in the design, collection of data, analysis and interpretation of data and in drafting of the manuscript.

Data Availability

The datasets supporting the findings of this paper are all included in this paper.

Competing interests

The authors declare that they have no conflict of interest

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Figures

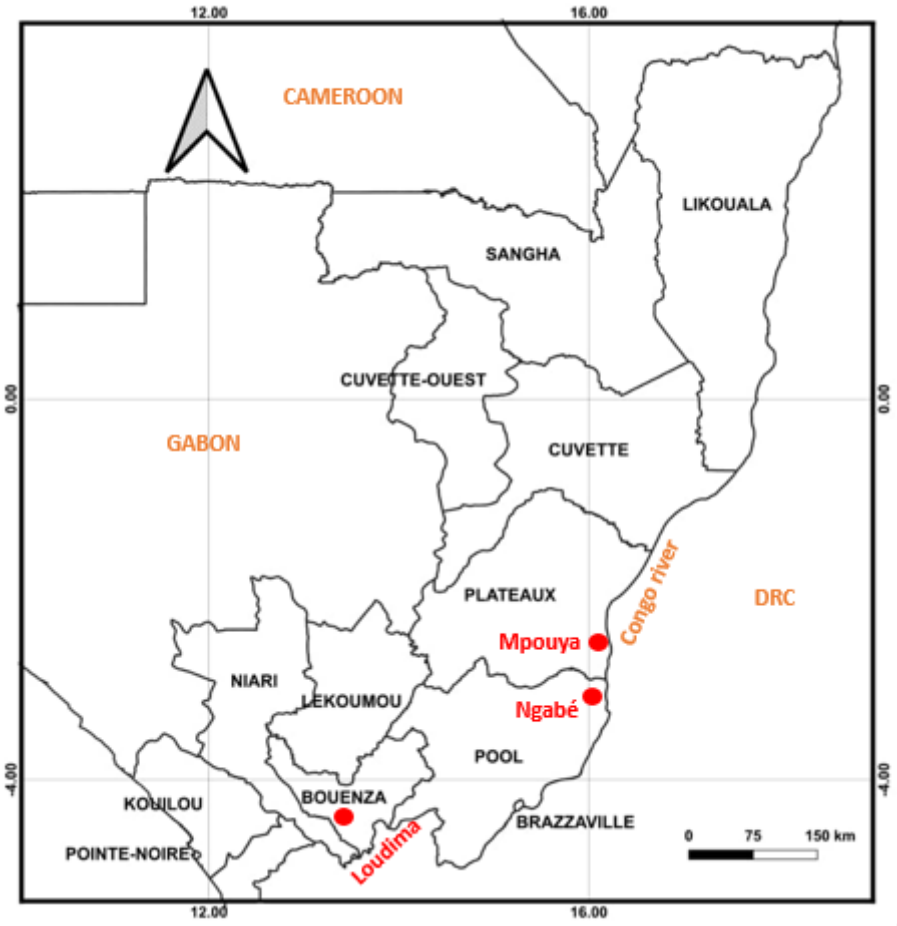


Figure 1

Map of the Congo Republic showing the situation of study sites.

(Source: map made with QGIS software).

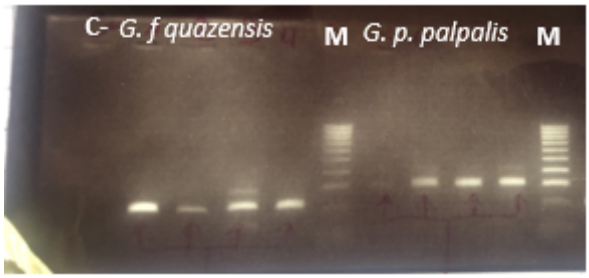


Figure 2

Detection of *G. f. quazensis* and *G. p. palpalis*