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Human Africa Trypanosomiasis in Littoral Region of Cameroon: an Updated With First Evidence on the Circulation of *Trypanosoma Brucei Gambiense* in Manoka Island

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Abstract
Aims: A survey on Human African Trypanosomiasis (HAT) is essential for prevention and preparedness for epidemics. The objective of this study is to assess the circulation of human trypanosomes and their vectors across quiescent HAT foci of the Littoral Region of Cameroon.
Methodology: A descriptive study on the presence of *Trypanosoma brucei gambiense* and its potential vectors was carried out in Youpwe, Yabassi, Sodiko, Manoka island and Cape-Cameroon Island from February to April 2022.Tsetse flies collected from the five selected locations using pyramidal traps, were first classified by species according to their morphology, then by sub-species with Polymerase Chain Reaction Diagnostic (PCR-Diag). *Trypanosoma* species and sub-species were subsequently identified and genotyped using a Nested PCR.
Results: *Glossina palpalis palpalis* was the unique tsetse subspecies recorded across the five locations. The tsetse infection rate by *Trypanosoma* ssp. varied between 5.35% in Cape-island and 35.71% in Manoka island. Three *Trypanosoma* species were detected: *T. brucei* s.l. 32/500 (6.4%), *T. congolense* 15/500 (3.0%), and *T. vivax* 8/500 (1.6%). The sub-species *T. b. gambiense* responsible for HAT was detected in tsetse flies from Manoka (2/150: 1.33%), whereas *T. congolense* consisted of *T. congolense "forest"* and *T. congolense "savannah"* types.
Conclusion: The presence of *T. b. gambiense* and *T. congolense sub-species* in Manoka and nearby suggests residual circulation of human and animal trypanosomes in quiescent HAT foci of the littoral region of Cameroon.

Keywords: Human African Trypanosomiasis, *Trypanosoma brucei gambiense*, *Trypanosoma congolense*, *Glossina Palpalis Palpalis*, Littoral Region, Cameroon

1. Introduction

Trypanosomiasis is a parasitic disease caused by a protozoa belonging to the genus *Trypanosoma*, and transmitted to vertebrate hosts by a blood-feeding (tsetse) fly of the genus *Glossina* [1]. These diseases occur in sub-Saharan Africa and affects both humans and animals. The distribution of Trypanosomiasis throughout the continent depends on the distribution of their vectors, and covers about 10 million km^2 or about one third of the continent [2]. In 2017, more than 65 million people were still at risk of Human African Trypanosomiasis (HAT), and about 977 cases were notified each year in 37 countries [3]. An estimated number of 50 million of livestock was at risk of African Animal Trypanosomiasis (AAT), and in areas with

high potential livestock production, direct and indirect economic losses due to AAT are estimated around 5 billion euro per year [4]. HAT is currently considered a re-emerging disease and as one of parasitic neglected tropical diseases that is a major public health problem, especially in rural areas of sub-Saharan Africa [5]. Cameroon is classified as a meso-endemic country for HAT with less than 100 reported cases in the five still active HAT foci, namely Campo and Bipindi in the South Region, Doumé in the East Region, Fontem and Mamfé in the South-West Region, which benefit from recurrent studies. Due to epidemiological status of Trypanosomiasis, it is important to extent the disease surveillance to any foci or hotspot that could present favourable bio-ecological, socio-economical and anthropological conditions for the emergence of the disease. This will contribute to the achievement of the World Health Organization (WHO) goal of eliminating and interrupting trypanosomiasis transmission by 2030 [6]. Thus, gathering key information or data on the effective circulation of trypanosomes and their vectors might serve to assess the associated risks [7]. [8] The Wouri foci and surrounding potential hotspots for HAT in the Littoral Region of Cameroon have been neglected for a long time, and have no longer benefited from large-scale studies despite the presence of trypanosome-infected tsetse flies [9] [10] and the wild animal reservoir of the Douala-Edéa Wildlife Reserve [11]. As part of these favourable factors, we have to updated key indicators in order to drawn a map of public health risks associated with HAT and AAT circulation in these locations which included remote areas (Cape-Cameroon and Manoka islands). The present study intended to assess the circulation of human trypanosomes and their vectors in quiescent HAT foci and potential hotspots of the Littoral region of Cameroon. The objective of this research is to gather updated information on the composition of trypanosomes and tsetse vector hosts, as well as on the circulation of the causal agent of HAT (*T. b. gambiense*) across the foci and potential hotspots of the Littoral Region of Cameroon.

2. Material And Methods

2.1 Study locations

The Littoral region (20,239 km^2) belongs to the coastal area of Cameroon and the main city is Douala. The Region has a population of approximately 3,900,000 [12] and has four divisions, each named after a river (the Wouri, Nkam, Moungo, and Sanaga Maritine). The climate is tropical with a dry season from November to February and a rainy season from March to October. However, it rains throughout the year and the average rainfall is 3600 mm per year [13] . The climate is typically hot and humid with an average annual temperature of 27°C and relative humidity of 85% [13]. The most remarkable plant formation is the mangrove characterized by the presence of *Rhizophora racemosa* and *Rhizophora harrisonii* trees, covering the islands of the estuary, the banks of the Wouri and its tributaries. There are also old and degraded secondary forests in the depression zones, which are privileged areas for vertebrate hosts and the movement of tsetse flies seeking for blood sources. Five locations belonging to old Wouri foci (Wouri division) and its surrounding towns in Nkam division were selected for the study (Figure 1): i Yabassi (04°27'N; 09°57'E), the capital of the Nkam division, ii. Sodiko-Village (4°08’N, 09°40’E) located near Bonaberi on the edges of Wouri river, in the subdivision of Douala IV, iii. Youpwe district (04°00'N; 09°42'E), a fishing port in the subdivision of Douala II, iv. the Manoka Island (03°47'N; 09°39'E) in the south the Wouri estuary, the largest (88 km^2) of the 24 islands of the subdivision of Douala VI and v. the Cap-Cameroon Island (03°54'N; 09°28'E) in atlantic ocean at about 50 km distant to the Douala port.

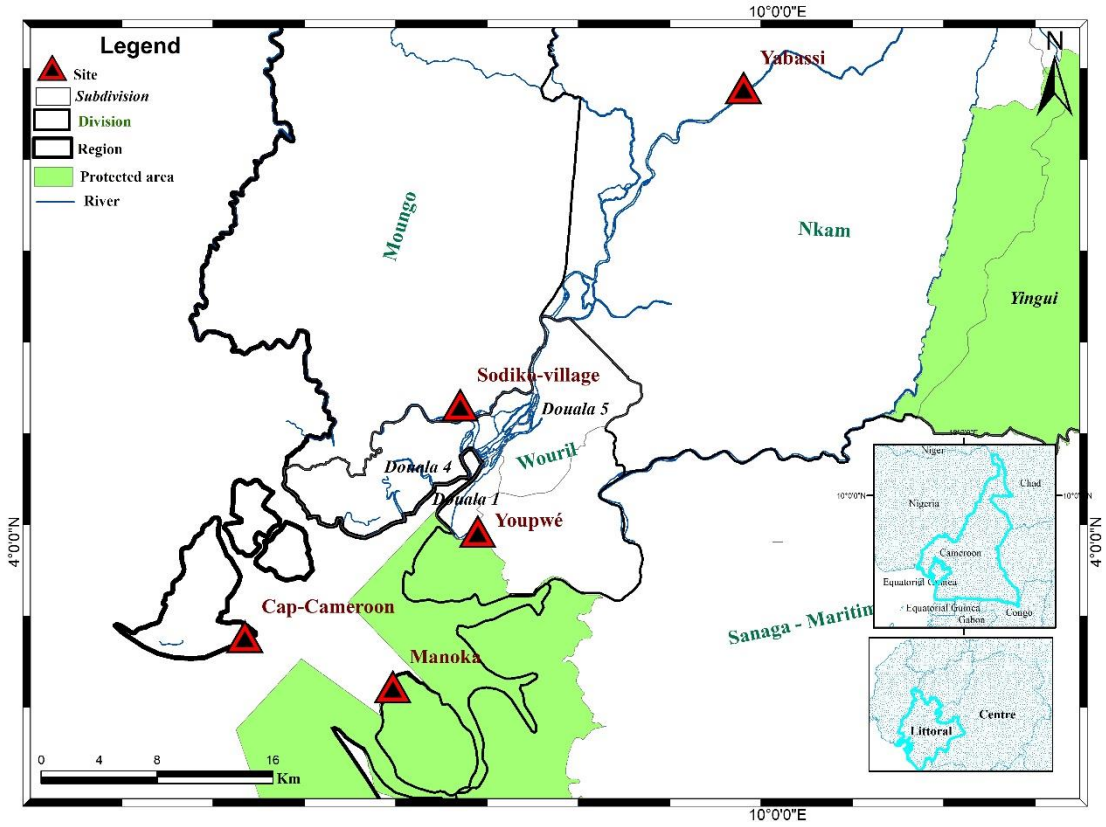

Figure1: Map of the Littoral Region of Cameroon showing subdivisions and sampling sites

2.2 Tsetse collections

We collected tsetse flies in the five selected sites, between February and April 2022. Pyramidal traps developed by Gouteux and Lancien [14] were set up in different geo-referenced facies known to attractive for tsetse (forest, mangrove, beach/forest edge, village edge). Each trap was visited twice a day (at 9:00 AM and 2:00 PM) during 4 successive days to collect trapped flies. Tsetse flies captured were morphologically separated at species and sex stages following identification keys [15] and software [16]. Non teneral tsetse specimens were preserved in individual tubes containing 70% alcohol for further molecular analysis in laboratory.

2.3. Molecular identification of tsetse species/subspecies

DNA was extracted from whole tsetse body using the cethyl trimethyl ammonium bromide (CTAB) method [17] and tsetse species were identified by PCR-Diag, based on the polymorphism of the nuclear marker Internal Transcribed Spacer 1 (ITS1) and according to protocol described by Dyer *et al.* [18]. Amplification was performed with the primers Diag forward (5'-TGGA CTTCGGATTAAGTACAACA-3') and Diag reverse (5'TCATTATCGCGCTATTAAGGTAAGC-3'), in a final volume of 15 µl contained 1.5 µl of 10X PCR buffer, 1.5 mM MgCl2, 10 pmol of each primer, 150 mM of each dNTP, 0.3 U of Taq DNA polymerase and 3µl of DNA extract diluted tenfold. The amplification program was as follows: a denaturation step at 94 °C for 5 min followed by 35 amplification cycles; each of these cycles included a denaturation step at 94 °C for 30 s, an annealing step at 56 °C for 30 s and an extension step at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. The amplified products were separated by electrophoresis into discriminant band sizes on 2% agarose gel. The stained gels with species/subspecies patterns were then visualized and photographed under ultraviolet light.

2.4. Molecular identification of trypanosome species

Trypanosome DNA was extracted from tsetse material and the detection of trypanosome species and subspecies was performed following the PCR protocol of Desquesnes et *al.* [19]. The amplification of the ITS1 region of the trypanosome ribosomal DNA included two PCR rounds and two specific primers: TRYP18.2C (5'-GCAAATTGCCCAATGTCG-3' TRYP4R 5'-GCTGCGTTCTTCAACGAA-3') for the first PCR and IRFCC (5'-CCTGCAGCTGGATCAT-3' TRYP5RCG 5'-ATCGCGACACCTTGTG-3') for the

second PCR [20]. Both primer pairs hybridize in the conserved 18S and 5.8S region of the DNA of the different trypanosomes, allowing the amplification of a specific DNA fragment for each trypanosome species. PCR reactions were performed in a final volume of 15 µl and contained 1.5 µl of 10X PCR buffer, 1.5 mM MgCl2, 10 picomoles of each primer, 150mM of each dNTP and 0.3 unit of Taq DNA polymerase 3µl of DNA extract. For the second PCR reaction, the first amplified products were diluted 10-fold and 3µl of each dilution was used as template. The amplification sequence was: i. a denaturation step at 94°C for 3min followed by 30 amplification cycles; each cycle containing a denaturation step at 94°C for 30s, ii. an annealing step at 58°C for 1min, and iii. an extension step at 72°C for 1 min followed by a final extension at 72°C for 5min. After the second round of PCR, 8 µl of amplified products were resolved on 2% agarose gel which was subsequently stained with ethidium bromide prior the visualization and photography under ultraviolet light. The DNA fragments amplified around 650 bp for *T. congolense*, 400 bp for *T. brucei* and 150 bp for *T. vivax*.

2.5. Trypanosome genotyping

The trypanosome genotyping consisted of the identification of subspecies or molecular forms belonging to *T. congolense* and *T. brucei* complexes or groups of species. Therefore, some of positive samples for *T. congolense* were proceeded for PCR methods described by Masiga *et al*. [21] and Moser *et al*. [22], using the following specific primers: for *T. congolense "forest"*, TCF1 (5'-GGACACGCCAGAAGTACTT-3') and TCF2 (5'-GTTCTCGCACCAAATCCAAC-3'), for *T.congolense "savannah"*, TCN1 (5'-TCGAGCGAGAACGGGCACTTTGCGA-3') and TCN2 (5'-ACAATTAGGGACAAACAAATCCCGC-3'). Both PCR reactions were performed in a final volume of 15 µl contained 1.5 µl of 10X PCR buffer, 1.5 mM MgCl2, 10 pmol of each primer, 150 mM of each dNTP, 0.3 U of Taq DNA polymerase and 3µL of DNA extract diluted tenfold. The amplification program consisted of: a denaturation step at 94 °C for 5 min followed by 40 amplification cycles; each of these cycles included a denaturation step at 94 °C for 30 s, an annealing step at 60 °C for 30 s and an extension step at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. The amplified products were separated by 2% agarose gel electrophoresis. The stained gels were visualized and photographed under ultraviolet light. Two amplification patterns were expected: at 341 bp for *T. congolense "forest*" infections and at 350 bp for *T. congolense "savannah"* infections. *Trypanosoma brucei* s.l. positive samples were subjected to nested PCR for the distinction of the sub-species *T. b. gambiense* from other sub-species of the complex, targeting the *T. gambiense* specific glycoprotein gene (TgsGP). This PCR showed two amplification rounds using the following specific primers: TsGP1 (5'-GCTGCTGTGTTCGGAGAGC-3'), and TsGP2 (5'-GCCATCGTGCTTGCCGCTC-3') as described by Radwanska *et al* [23] and TgsGPs (5 - TCAGACAGGGCTGTAATAGCAAGC-3) and TgsGPas (5 - GGGCTCCTGCCTCAATTGC TGCA-3) [24]. Each PCR was performed in a final volume of 25 µl contained, 2.5 µl of 10X PCR buffer, 1.5 mM MgCl2, 10 picomoles of each primer, 200mM of each dNTP and 0.3 unit of Taq DNA polymerase 3µl of DNA extract. For the second PCR reaction the first amplified products were diluted 10-fold and 3µl of each dilution was used as template. The amplification program was: a denaturation step at 94°C for 3min followed by 40 amplification cycles for the first round and 25 amplification cycles for the second round; each cycle containing a denaturation step at 94°C for 30s, an annealing step at 58°C for 1min, and an extension step at 72°C for 1 min followed by a final extension at 72°C for 5min. All samples that showed an amplification band at 270 bp were considered to carry *T. b. gambiense* infections. Other profiles that needed further analyses were not included in this study.

2.4. Data Analysis

The apparent tsetse fly density per trap per day (ADT) was estimated using the following formula: ADT = C/TD; where C is the number of tsetse flies captured, T is the number of traps deployed, and D is the number of days trapped. The chi-square test was used to compare ADTs between biotopes/environments, locations and sex ratio as well as the infection rates of tsetse flies according trypanosome species and locations. The difference was considered significant for a p-value less than 0.05.

3. Results

3.1 Tsetse density and distribution

A total of 1008 tsetse flies were captured in all sites (Table 1).

Table 1: Total number of tsetse flies and daily Apparent Density per Trap in study locations

Sampling locations	species	Individual samples			ADT
		female	male	total	
Manoka island	*Glossina palpalis*	215	145	360	3.75
Youpwe	*Glossina palpalis*	150	82	232	2.41
Sodiko-vllage	*Glossina palpalis*	140	57	197	2.05
Cap Island	*Glossina palpalis*	77	110	187	1.94
Yabassi	*Glossina palpalis*	23	9	32	0.33
Total		605	403	1008	10.48

ADT=Apparent Density per day per trap

All belonged to *Glossina palpalis palpalis* Rosbineau-Desvoidy 1949 sub-species. The overall apparent tsetse density was 0.42F/T/D, and showed insignificant variations by study locations (p=0.22). The highest APD was recorded in Manoka (3.75F/T/D) and the lowest in Yabassi (0.33F/T/D).
In general, tsetse fly was more frequent in the mangrove 46.82% (472/1008) and less frequent in the thickets 7.24% (73/1008) (Figure 2). The number of female flies (605: 60%) was slightly greater than males (403: 40%) (p= 0.157), and the sex ratio M/F was 0.6.

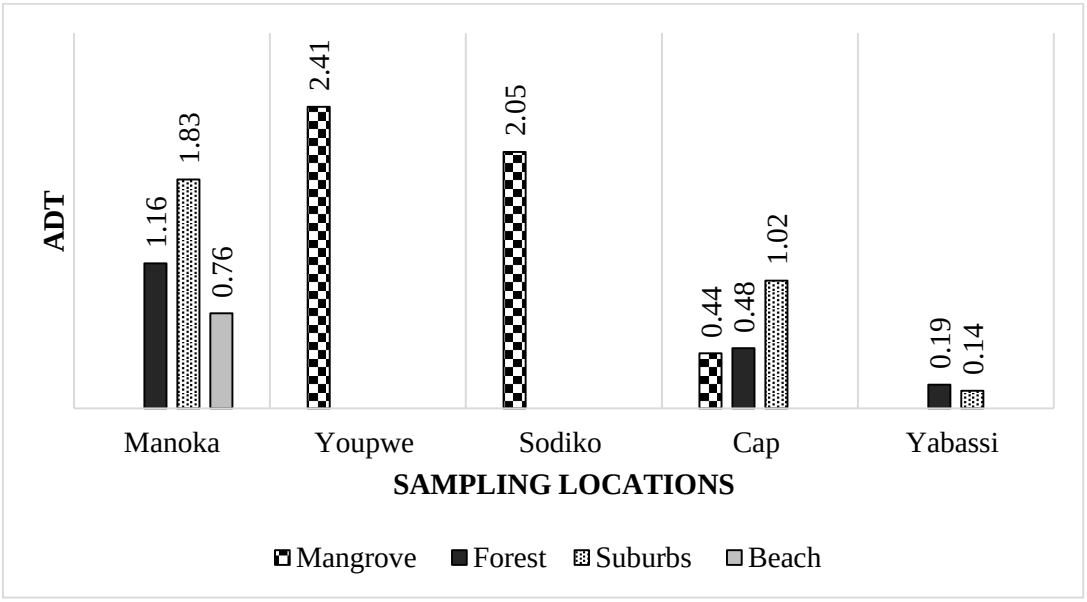

Figure 2: Daily tsetse Apparent density per trap (ADT) by sampling environments

3.2 *Trypanosoma* infections in tsetse flies

Of a total of 500 tsetse flies tested by PCR, 56 were positive for trypanosome infections, suggesting an overall prevalence of 11.2% (Table 2). The highest infection rate was recorded in Manoka 35.71% (20/56) followed by Youpwe 30.35% (17/56), Cape-Cameroun Town 16.07% (9/56) Sodiko-village 12.5% (7/56) and Yabassi 5.35% (3/56). These infections were caused by three trypanosome species, *T. brucei s.l.* in 6.4% (32/500), *T. congolense* in 3% (15/500) and *T. vivax* in 1.6% (8/500) of all samples. Single infections were frequent 98% (55/56) and only one *T. congolense*/*T. brucei* co-infection was recorded (Table 2).

Table 2: Frequency of *Trypanomosa* infections in tsetse flies (in percentage ± 95%SD) according to study locations

Locality	Total tested	T. vivax	*T. brucei*	*T. congolense*	*T. vivax / T. brucei*	Overall/per location
Manoka island	154	3 (1.95±1.11 %)	10 (6.49±1.99%)	6 (3.90±1.58%)	1 (0.65±0.63%)	20 (12.99±2.76%)
Youpwe	100	3 (3.00±1.73 %)	11 (11.00±3.21%)	3 (3.00±1.73%)	0 -	17 (17.00±3.83%)
Sodiko	100	0 -	6 (6.00±2.40%)	1 (1.00 ±1.02%)	0 -	7 (7.00±2.60%)
Cap island	110	2 (1.82±1.28 %)	3 (2.73±1.58%)	4 (3.64±1.84 %)	0 -	9 (8.18±2.65%)
Yabassi	32	0 -	2 (6.25±4.44%)	1 (3.12±3.21%)	0 -	3 (9.37±5.36%)
Total	500	8 (1.60±0.56 %)	32 (6.40±1.12%)	15 (3.00±0.77 %)	1 (0.20±0.20%)	56 (11.20±1.43%)

SD: Standard Deviation; T.: *Trypanosoma*

3.3 Sub-species polymorphism in *T. congolense* and *T. brucei*

Of the 15 *T. congolense* positive samples, ten (66.7%) showed *T. congolense "forest*" infections, two (13.3%) *T. congolense "savannah"* and one (6.7%) was a mixed "forest/savannah" co-infection (Table 3).

Table 3: Frequency of *Trypanosoma brucei* and *Trypanosoma congolense* sub-species in study locations

Locations	*T. brucei samples*		*T. congolense* samples			
	Total tested	*T. brucei gambiense*	Total tested	*"forest"* sub-species	*"savannah"* sub-species	*"forest/savannah"* co-infection
Manoka island	10	2	6	3	2	1
Youpwe	11	0	3	2	0	0
Sodiko	6	0	1	1	0	0
Cap island	3	0	4	3	0	0

Yabassi	2	0	1	1	0	0
Total (% ± 95%SD)	32	2 (6.25 ± 4.44 %)	15	10 (66.67 ± 12.07 %)	2 (13.33 ± 7.3 %)	1 (6.67 ± 6.39 %)

SD: Standard Deviation; T.: *Trypanosoma*

The remaining two *T. congolense* samples showed invalid profiles. Among the 32 *T. brucei* infections, two samples isolated from tsetse flies captured in the forest and at the edge of the Manoka village revealed positive for *Trypanosoma brucei gambiense*.

4. Discussion

The aim of this study was to gather updated information on the current epidemiological situation regarding the seasonal circulation of tsetse vectors and trypanosomes species in the old Wouri HAT foci and surrounding locations of the Littoral Region of Cameroon. From these preliminary data on the composition of tsetse vector and trypanosome species recorded from the selected study locations at the early rainy season, it seems very important to recommend active surveillance of the HAT/AAT indicators across active and quiescent foci in Cameroon. In fact, our study has confirmed the spread of *Glossina palpalis palpalis* as the unique tsetse species captured. This main observation is not in accordance with what recorded in previous studies [25]; [9]. According to these authors, the species *Glossina caliginea* was usually captured together with *Glossina palpalis* in these areas even if he has become rare over the years. Recent entomological study confirmed his presence in forest and mangrove of Manoka island [10]. This tsetse fly (*Glossina caliginea*) has a relative low dispersal capacity this allows it to remain under vegetation cover and inside mangroves compared with *G. palpalis*. Moreover, the presence of *Glossina Palpalis* in all locations may be due to its ubiquity, its dietary eclecticism and its strong capacity to adapt to all environments including anthropized environments. The highest tsetse densities were recorded in Manoka with an average ADT of 3.75F/T/D, similarly to average recorded by Atangana *et al* [10] in the same location (3.80 F/T/D) and by Mbang et al [26] in the National Park of Ivindo in Gabon (3.0 F/T/D). Factors that enhance the circulation of tsetse flies may be associated with low level of human activities and the mangrove coverage areas in Manoka and coastal locations (Youpwe and Sodiko-village) and the availability of alternative vertebrate hosts compared to continental locations such Yabassi (0.33 F/T/D) where human activities are more pronounced. The density of tsetse flies was high in the mangrove as observed by Eouzan and Ferrara [25]; Genevoix *et al*. [9] and Atangana *et al* [10]; Indeed, the mangrove swamp which covers an important part of the study area is very humid, and constitutes a privileged area for the movement of tsetse flies which find wild animals to feed on, and human who usually cross these areas for fishing or hunting activities.

This study also highlights the circulation of trypanosomes belonging to the species *T. brucei s.l., T. congolense*, and *T. vivax* which have been identified in tsetse flies at a rate of 6.4%, 3.0%, and 1.6% respectively. These trypanosomes also circulate in other foci in Cameroon [27] [7] [28], and elsewhere in Africa [29]. The presence of trypanosomes in tsetse flies suggests the existence of an active transmission of these parasites in our study localities [30]; [7]; [8]; [31] [32]. Furthermore, the presence of *T. brucei s.l.* in the majority of samples was not confirmed by the findings obtained by Eouzan and Ferrara [25] and Atangana *et al*; [10] where only *T. congolense*. and *T. vivax* species were identified, notably on the island of Manoka. The presence of wild animals in the Douala-Edéa Wildlife Reserve that are known to be the reservoir of these parasites and the sources of new infestations for the vectors, as well as the molecular tools we used in our analyses could explain this difference. The high frequency of *T. brucei* in tsetse flies suggests residual transmission of this parasite in the locality of Manoka. Thus, the detection of *T. b. gambiense* sub-species in one of main HAT vector species in central Africa region (*Glossina palpalis palpalis*) is indicative for a possible Human African Trypanosomiasis transmission in this locality. Another key finding of our study is the contribution of mangroves and forests to the circulation of potential tsetse vectors of HAT/AAT. These environments are favourable for tsetse proliferation due to the availability of alternative reservoirs such as

wild animals. Concerning AAT, the presence of *T. congolense*, especially the subspecies *T. congolense savannah*, although in the minority compared to *T. congolense forest*, is a real threat for the wild fauna of the Douala-Edea reserve and for the livestock activities in this area. The AAT caused by *T. congolense* sub-species is associated with high parasitaemia in a short period of time, resulting to a rapid death of the animal [33], whereas *T. vivax* shows low pathogenicity and is better controlled by animals [34] [35]. These findings underline the necessity to urgently improve current strategies for the control and surveillance of trypanosomiasis in these historic HAT foci.

5. Conclusion

This study allowed us to identify the species of tsetse fly and trypanosomes present in the quiescent Wouri HAT foci and suggest a possible transmission risk of human and animal trypanosomiasis. It will be therefore difficult to achieve the objective of elimination of HAT expected by 2030 and prevent epidemics without extending innovative vector control and case management approaches/strategies to all potential HAT hotspots.

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