Free-ranging pigs identified as a multi-reservoir of *Trypanosoma brucei* and *Trypanosoma congolense* in the Vavoua area, a historical sleeping sickness focus of Côte d’Ivoire

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Abstract

**Background**

The existence of an animal reservoir of *Trypanosoma brucei gambiense* (*T. b. gambiense*), the agent of human African trypanosomiasis (HAT), may compromise the interruption of transmission targeted by World Health Organization. The aim of this study was to investigate the presence of trypanosomes in pigs and people in the Vavoua HAT historical focus where cases were still diagnosed in the early 2010’s.

**Methods**

For the human survey, we used the CATT, mini-anion exchange centrifugation technique and immune trypanolysis tests. For the animal survey, theuffy coat technique was also used as well as the PCR using *Trypanosoma* species specific, including the *T. b. gambiense* TgsGP detection using single round and nested PCRs, performed from animal blood samples and from strains isolated from subjects positive for parasitological investigations.

**Results**

No HAT cases were detected among 345 people tested. A total of 167 pigs were investigated. Free-ranging pigs appeared significantly more infected than pigs in pen. Over 70% of free-ranging pigs were positive for CATT and parasitological investigations and 27–43%...
were positive to trypanolysis depending on the antigen used. *T. brucei* was the most prevalent species (57%) followed by *T. congolense* (24%). Blood sample extracted DNA of *T. brucei* positive subjects were negative to single round TgsGP PCR. However, 1/22 and 6/22 isolated strains were positive with single round and nested TgsGP PCRs, respectively.

**Discussion**

Free-ranging pigs were identified as a multi-reservoir of *T. brucei* and/or *T. congolense* with mixed infections of different strains. This trypanosome diversity hinders the easy and direct detection of *T. b. gambiense*. We highlight the lack of tools to prove or exclude with certainty the presence of *T. b. gambiense*. This study once more highlights the need of technical improvements to explore the role of animals in the epidemiology of HAT.

**Author summary**

Significant efforts to control human African trypanosomiasis (HAT) since the 1990’s have drastically reduced the prevalence of the disease. Its elimination as a public health problem is being achieved. World Health Organization now targets the interruption of transmission for 2030. However, potential animal reservoirs of *Trypanosoma brucei gambiense* (*T. b. gambiense*), the main agent of HAT, may compromise this ambitious objective. It is the case in the Vavoua historical focus in Côte d’Ivoire where HAT cases were still diagnosed in the early 2010’s. During a study conducted in this area, we scrutinized the trypanosomes circulating in pigs and people sharing the same environment using serological, immunological, parasitological and molecular tools. No HAT cases were detected. We showed that *T. brucei* s.l. and *T. congolense* actively circulated in free-ranging pigs. Even if no tools were sensitive and specific enough to unambiguously identify *T. b. gambiense* directly from biological samples, six isolated strains from pigs positive for trypanosomes were amplified for TgsGP, the only currently accepted *T. b. gambiense* specific molecular marker. The apparent discrepancies between the presence of *T. b. gambiense* in pigs despite the absence of human cases is discussed. These results stress the need for an efficient “molecular toolbox” to easily detect and identify *T. b. gambiense* in any animal it may infect.

**Introduction**

African trypanosomiases are vector-borne parasitic diseases that affect both humans and animals. They continue to impact public health and socio-economic development mainly in remote rural areas in Sub-Saharan Africa. Human African trypanosomiasis (HAT), or sleeping sickness, exists in two forms depending on the subspecies of *Trypanosoma brucei* [1]. The acute form, due to *T. b. rhodesiense*, occurs in Eastern and Southern Africa, while the chronic form, due to *T. b. gambiense*, is rampant in Western and Central Africa, causing 98% of HAT cases [2]. While *T. b. rhodesiense* HAT is known to be zoonotic, the role of a domestic or wild animal reservoir in the *T. b. gambiense* HAT epidemiology is still under debate [3,4]. Given the 2018 historic threshold of 1,000 cases reached [2], the potential role of animals as reservoirs did not prevent to control HAT so far. Nevertheless, it might compromise the interruption of transmission targeted by World Health Organization (WHO) for 2030.
Other species and subspecies of trypanosomes such as *T. b. brucei*, *T. congolense* and *T. vivax* are pathogenic to animals and cause Animal African Trypanosomiasis (AAT) or nagana. Economic losses due to AAT are measured in billions of dollars [5,6]. Human and animal trypanosomes share the same cyclical vectors in Sub-Saharan Africa, i.e. the tsetse flies (genus *Glossina*). An integrated management approach, based on the One health concept [7], is thus recommended for a sustainable elimination of African trypanosomiases [8,9].

In Côte d’Ivoire, HAT elimination as a public health problem is being achieved [2,10]. However, studies conducted in the two HAT foci that are still endemic in the central-western part of the country (Bonon and Sinfra) recently demonstrated that pigs were particularly infected by trypanosomes, although infections with *T. b. gambiense* could not be proven due to a lack of reliability of the tools used [11]. In the same region, the Vavoua HAT focus was epidemic at the end of the 1970’s [12–14] and thanks to both medical and vector control efforts [15,16], less than five HAT cases were reported yearly at the end of the 1990s [17]. Only four cases were still passively diagnosed in the early 2000s. However, the last two cases that were reported in 2011 and 2012 [18] raised the question of the infection origin. The Vavoua region is known to be a route of cattle transhumance and a place for pig husbandry [19]. While *T. brucei* s.l. and *T. congolense* have been previously described in pigs in the beginning of the 1980’s [20], the current situation of AAT is completely unknown in that area.

The aim of the present study was to simultaneously investigate the presence of trypanosomes, and identifying those, in pigs and people sharing the same environment for a better understanding of the current epidemiology of African trypanosomiases in the Vavoua area and to implement adapted strategies for a sustainable elimination of HAT.

**Material and methods**

**Ethics statement**

The human active screening was conducted within the framework of medical surveys and epidemiological surveillance activities supervised by the HAT National Control Program (HAT NCP). No sample other than those for routine screening and diagnostic procedures was collected. All participants were informed of the objective of the study in their own language and signed an informed consent form. Approval was obtained from the Ivorian national ethics committee, no.0308/MSLS/CNER-P.

No ethical statement is required by local authorities for domestic animal sampling. Any veterinarian may carry out blood sampling on domestic animals, with the authorization of the owner, as it is performed during prophylaxis or diagnostic campaign. Breeders gave their consent for animal sampling after being informed of the objectives of the study. For pig care, venous sampling was performed by a veterinarian of the Laboratoire National d’Appui au Développement Rural (Ministry of Agriculture). A deworming treatment (Bolumisol, Laprovet) was provided for free to all pigs sampled.

**Study area**

The Vavoua area is located in the Haut-Sassandra region in the central-western part of Côte d’Ivoire (Fig 1). The vegetation was initially characterized by mesophilic forest with savannah inclusions mainly in the north. In the 1960’s, the forest has been progressively replaced by cash crops (mainly cocoa and coffee) leading to a favorable environmental context for HAT development in the area [14]. An epidemic situation broke out in the 1970’s. Fig 1 describes the number and the distribution of the HAT cases reported between 1977 and 1999 and the villages of residence of the six last cases reported since 2000. Nine study sites were selected in the
Field survey and sampling

The field survey was conducted in February 2017. For the human medical survey, we targeted pig’s breeders, their families and people living in the close neighborhood (considered as population at risk). Individuals who gave their informed consent were tested using the Card Agglutination Test for Trypanosomiasis (CATT, provided by Institute of Tropical Medicine Antwerp, Belgium) serological test [21] performed on blood (CATT-B) collected by finger prick. For CATT-B positive persons, 5mL of blood were collected in heparinized tubes and a twofold plasma dilution series in CATT buffer was tested to assess the highest dilution that was still positive on plasma (CATT-P). All positive CATT-P at a dilution of 1:4 or higher underwent parasitological investigations by direct examination of a lymph node aspirate and/or mini-anion exchange centrifugation technique (mAECT, provided by Projet de Recherches Cliniques sur la Trypanosomiase, Daloa, Côte d’Ivoire) [22] performed with 350 μL of buffy coat (BC) as previously described [23]. From these subjects, 1 mL plasma and 500 μL BC were aliquoted from the remaining blood and immediately stored at -20˚C for further testing with PCR and immune trypanolysis (TL).

For the animal survey, considering T. brucei s.l. prevalence previously observed in the neighboring areas [11], we had scheduled to randomly test 20 pigs per study site. Prior to field
survey, objectives of the study were explained to the inhabitants and local authorities of each village. After obtaining their approval, the farmers captured pigs, aged of more than one year whenever possible. For each captured pig, 9 mL blood were taken from the jugular vein in heparinized tubes. These blood samples were tested with the standard buffy coat technique (BCT) [24]. In the absence of accurate field serological diagnostic tests for African trypanosomiasis in domestic animals and taking into account the limited sensitivity of BCT, we also used the CATT and mAECT following the same procedures as HAT diagnosis, to increase the possibility of both \textit{T. b. gambiense} and animal trypanosomes detection. CATT-B was performed for each pig and CATT-P was performed for CATT-B positive pigs as described above. Only CATT-P at a dilution of 1:4 or higher were considered as CATT-P positive. The mAECT was performed with 350 \( \mu \)L of BC only for CATT-B positive pigs. In addition, 1 mL plasma and 500 \( \mu \)L BC were aliquoted from the remaining blood of all pigs and immediately stored at -20\(^\circ\)C for subsequent PCR and TL testing. Information regarding the husbandry method (in pen or free-ranging pigs) and study sites were collected.

**Mice infection and isolation of trypanosomes**

Trypanosome isolation in the field was performed by intraperitoneal inoculation of 0.5 mL whole blood from each pig positive to BCT and/or mAECT, in two Naval Medical Research Institute (NMRI) mice (produced in CIRDES, Bobo-Dioulasso, Burkina-Faso, from paternal strains purchased from Charles River laboratories, France). These were immunosuppressed with cyclophosphamide (300 mg/kg of Endoxan) administered before inoculation and then every 5 days. Five days post inoculation, parasitemia was determined daily by direct microscopic examination (X400) of mouse tail blood [25]. When parasitaemia reached 32.10\(^6\) parasites/mL, blood was collected by cardiac puncture of mice as previously described [26] and parasites were separated from mice blood using mAECT. Purified parasites were centrifuged (1200 g for 10 min) and parasite pellets were stored at -20\(^\circ\)C for subsequent PCR analysis.

**Molecular diagnosis**

DNA from 500 \( \mu \)L of BC was extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer’s instructions. DNA from the isolated strains was extracted according to the same process, after having re-suspended the parasite pellets in 200 \( \mu \)L PBS. Negative extraction controls were systematically included during the process. PCR amplification was performed using specific primers for \textit{T. brucei} s.l. (TBR1-2) [27], \textit{T. congolense} savannah type (TCS1-2) [28], \textit{T. congolense} forest type (TCF1-2) [28] and \textit{T. vivax} (TVW1-2) [29]. The TgsGP1/2 primers [30] targeting the TgsGP gene specific of \textit{T. b. gambiense} [31] were used in a single round PCR on all BC positives to TBR1-2 PCR and all isolated strains. All isolated strains were also tested for TgsGP presence with nested primers (TgsGPsense2 and TsgGPantisense2) [32]. The PCR reactions were carried out in a thermocycler (Eppendorf Mastercycler nexus) in 25 \( \mu \)L final volume, containing 1 X Qiagen HotStarTaq Master Mix, 20 pmol of each primer and 5 \( \mu \)L of DNA sample. The PCR products were visualized by electrophoresis in a 2% agarose gel (MP Biomedical, Eschwege, Germany) stained with Gel Red (Interchim, Montluçon, France) and illuminated with UV light.

**Trypanolysis test**

Both human and animal plasma samples were processed with the immune trypanolysis test (TL) using cloned populations of \textit{T. b. gambiense} variant antigen type (VATs) LiTat 1.3, LiTat 1.5 and LiTat 1.6 as previously described [33]. LiTat 1.3 and LiTat 1.5 VATs are supposed to be
specific for *T. b. gambiense*, while LiTat 1.6 VAT is expressed in *T. b. gambiense* and *T. b. brucel*. [34].

**Data management and statistical analysis**

We compared the detection power for the different techniques and in the different situations (sites, husbandry method) with Fisher’s exact tests when possible under the R commander package (rcmdr) [35,36] for R [37], or with 100,000 randomizations in R when necessary, except for CATT-P for which we used a Krukal-Wallis test with rcmdr. This resulted in many *p*-values presented in supporting information. We corrected for the false discovery rate (FDR) with the Benjamini and Hochberg (BH) procedure [38], with the p.adjust command in R. Husbandry method only varied in the site PK14 and was only tested there. To analyze the concordance between serological and molecular data obtained from free-ranging pigs, a Venn diagram was built using a freely available online software (http://bioinformatics.psb.ugent.be/webtools/Venn).

**Results**

**Human medical survey**

In total, 345 subjects were tested ranging from 0 in Trafla Gottron (TG) (subjects refused to be sampled) to 98 in PK5 (Table 1). Serological examination revealed 15 CATT-B positive individuals and 6 CATT-P >¼ (2 in PK5, 3 in Gatifla and 1 in Dema) for whom parasitological investigations, TBR PCR and TL were negative.

**Animal survey**

A total of 167 pigs were tested and the complete database is given in S1 Table. This sampling consisted on 70 pigs in pen in PK5, PK8, PK11 and PK14; and 97 free-ranging ones in CIFCI, Dema, Gatifla, Gozy PK14, and TG (Table 2). Mix husbandry was only practiced in PK14 and we included an equivalent number of pigs in pen and free-ranging pigs.

TCS and TgsGP single round PCR were negative for all BC tested. Global positivity rates with the other tests ranged from 4% (TVW PCR) to 49% (CATT-B). We compared the BCT,
CATT-B, TL and PCR results regarding the study sites and the husbandry methods (Fig 2). In PK14, the only site where both husbandry methods were applied together, free ranging pigs were more often positive for CATT-B (p-value < 0.0001), LiTat1.5 (p-value = 0.0014) and LiTat1.6 (p-value = 0.0014) (S2 Table).

Except for TVW PCR (p-values > 0.5), study sites, and probably the husbandry method, together had a highly significant impact on the diagnostic tests results (all p-values < 0.003).

Table 2. Distribution of tested pigs according to study sites and the husbandry method.

| Study sites | Pigs in pen | Free-ranging pigs | Total |
|-------------|-------------|-------------------|-------|
| CIFCI       | 0           | 15                | 15    |
| Dema        | 0           | 17                | 17    |
| Gatifa      | 0           | 25                | 25    |
| Gozy        | 0           | 22                | 22    |
| PK5         | 20          | 0                 | 20    |
| PK8         | 20          | 0                 | 20    |
| PK11        | 20          | 0                 | 20    |
| PK14        | 10          | 10                | 20    |
| TG*         | 0           | 8                 | 8     |
| Total       | 70          | 97                | 167   |

*TG = Traffa Gottron

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Fig 2. Diagnostic tests results regarding the study sites and the husbandry methods. Complete data are available in supporting information. All means are given with 95% confidence intervals. BCT = Buffy Coat Technique, CATT-B = Card Agglutination Test for Trypanosomiasis performed on blood, TG = Traffa Gottron.

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(S3 Table). As can be seen in Fig 2, TG and Gozy (and to a lesser extent Dema, Gatifla and CIFCI), sites with exclusive free ranging pigs, represented the most heavily infected sites. The sites with the lowest prevalence were those of pen husbandry. We thus decided to focus on the free ranging sites for the rest of the analysis.

Serological and parasitological results obtained in the field for the 97 free-ranging pigs according to the study sites are detailed in Table 3. The average prevalence was 35% using the BCT, ranging from 24% (Gatifla) to 55% (Gozy) except for PK14 where all BCT were negative. The global seroprevalence using the CATT-B was 79% ranging from 44% in Gatifla to 100% in Gozy, PK14 and TG. Almost all the CATT-B positive pigs (91%) were CATT-P ≥1/4. Unfortunately, mAECT could not be performed for 18 CATT-B positive pigs in CIFCI (8), Dema (8) and Gatifla (2) study sites due to test supply issues. The global prevalence obtained with the mAECT performed on 57 CATT-B positive pigs was 93%, ranging from 50% in CIFCI to 100% in Gatifla and TG. mAECT was significantly more sensitive than BCT (p ≤ 0.001) that was only positive for 17 out of the same 57 pigs (30%). The difference was remarkable in PK14 where 9 out of the 10 pigs BCT-negative were mAECT-positive. Taking into account both BCT and mAECT results, the global prevalence in free-ranging pigs was 72% (70/97). A significant difference was observed between the study sites for all tests. CIFCI and Gatifla appeared to be less affected by trypanosome infections than the other sites (Table 3).

The PCR results obtained for the 97 free-ranging pigs are given in Fig 3. They show higher global positivity rate for TBR PCR (57%) than for TCF ones (24%) with 14% of the pigs positive with the two PCR. Results were very heterogeneous between the study sites but no significant differences were observed (Fig 3). For TL results (Fig 4), the global positivity rate for LiTat 1.6 was the highest (43%) ranging from 16 to 80% according to the study site, followed by LiTat 1.5 (41%) ranging from 0 to 80% and LiTat 1.3 (27%) ranging from 8 to 50%. Significant differences were observed between the study sites for LiTat 1.5 and LiTat 1.6 mainly due the high positivity rates observed in Gozy and PK14 (between 70 and 80%), the low positivity rate of LiTat 1.5 and LiTat 1.6 in Gatifla and the absence of LiTat 1.5 in TG. No significant differences were observed between the study sites for LiTat 1.3. Fig 5 shows the global proportions of free-ranging pigs positive to 3 (11%), 2 (29%) and 1 VAT (20%) and the proportions according to the study sites, once more confirming an important heterogeneity between those. All the combinations of TL positive results with the three VAT were observed, but the combination LiTat 1.3 negative, LiTat 1.5 positive and LiTat 1.6 positive was the most represented profile with 18 pigs (19%).

Table 3. Serological and parasitological results of free-ranging pigs according to study sites.

| Study sites | Nb pigs | BCT+ | CATT-B+ | CATT- P ≥1/4 | mAECT |
|-------------|---------|------|---------|-------------|-------|
| CIFCI       | 15      | 4 (27%) | 10 (67%) | 9 (90%) | 1/2+ (50%) |
| Dema        | 17      | 9 (53%) | 16 (94%) | 15 (94%) | 5/8* (63%) |
| Gatifla     | 25      | 6 (24%) | 11 (44%) | 10 (91%) | 9/9** (100%) |
| Gozy        | 22      | 12 (55%) | 22 (100%) | 19 (86%) | 21 (95%) |
| PK14        | 10      | 0 (0%) | 10 (100%) | 10 (100%) | 9 (90%) |
| TG*         | 8       | 3 (38%) | 8 (100%) | 7 (88%) | 8 (100%) |
| Total       | 97      | 34 (35%) | 77 (79%) | 70 (91%) | 53/57 (93%) |
| P-value     |         | <0.001 | <0.001 | 0.036 |

*TG = Trafla Gotton
**The number of mAECT performed are given since the test cannot be performed on all CATT B positive pigs

Pig numbers (Nb pigs), and number of positive animals for BC, CATT-B, CATT-P and mAECT, as described in the Material and Methods section, are given.

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Isolation of trypanosome strains and PCR results

A total of 22 strains were isolated, stabilized and purified (Table 4). They were all negative to TCS and TVW PCR. However, 19 (86%) and 9 (41%) strains were positive to TBR and TCF, respectively. Positive results with both PCR were observed for 8 strains (36%) suggesting that mixed infections are common. Two strains could not be amplified for any of the primers. Only one strain from Gozy was clearly positive after a single round TgsGP PCR (S1 Fig). The nested TgsGP PCR gave strongly positive bands for five additional strains from Gozy (2), PK 14 (2) and TG (1) (S2 Fig). The TgsGP PCR products of these six positive samples were sequenced and resulted in 270 nucleic acids showing 100% homology with the sequence of TgsGP gene (Gene Bank accession number FN555988) (S3 Fig). Surprisingly, two of these strains were TBR PCR negative, while the six corresponding blood samples were TBR PCR positive (Fig 6). Moreover, five other strains that gave a low intensity band with the single round TgsGP PCR (S1 Fig) were negative for the nested TgsGP PCR. The corresponding PCR products could not be sequenced due to a limited amount of product.

Fig 3. PCR results of free-ranging pigs according to study sites. TBR = TBR1-2 primers, TCF = TCF1-2 primers, + = positive test * TG = Trafa Gottron. https://doi.org/10.1371/journal.pntd.0010036.g003

Fig 4. TL results of free-ranging pigs according to study sites. + = positive test * TG = Trafa Gottron ** = LiTat 1.5 and 1.6 occurrence was significantly higher (p values<0.001) in PK14 and Gozy comparing to the other study sites. https://doi.org/10.1371/journal.pntd.0010036.g004
Concordance between serological and molecular analyses

Finally, we proposed to evaluate the congruence of the serological and molecular results regarding the diagnosis of *T. brucei* s.l. in blood and plasma. We focused on CATT-B, TL and TBR PCR data obtained from the 97 free-ranging pigs (Fig 6). A strong overall congruence was observed with 87.3% (69/79) of the samples positive for at least two methods and 45.6% for the three (36/79). Only one sample out of 55 TBR PCR positive samples (1.8%) did not exhibit a positive serology and 72% (40/55) of the TBR PCR positive samples were positive for both CATT-B and TL tests. It is interesting to stress that for the six TgsGP-positive strains, the three tests performed on blood and plasma gave positive results. Among the 70 pigs in pen, 63 were negative for the three methods, five and two were only CATT-B and TBR PCR positive, respectively.

Discussion

In Côte d’Ivoire, One Health approaches are being conducted to adapt strategies for reaching HAT sustainable elimination. A previous study conducted in the Bonon and Sinfra HAT endemic foci concluded that pigs were the most trypanosome-infected domestic animals, but

Table 4. PCR results on isolated strains from free-ranging pigs.

| Study sites | No Isolated strains | PCR targets |
|-------------|---------------------|-------------|
|             |                     | TBR +       | TgsGP +     | NTgsGP +    | TCF +       | TBR + /TCF+ |
| Dema        | 2                   | 2           | (1)         | 0           | 0           | 0           |
| Gatiffa     | 3                   | 3           | (1)         | 0           | 1           | 1           |
| Gozy        | 8                   | 6           | 1 (1)       | 3           | 3           | 2           |
| PK14        | 4                   | 3           | 0           | 2           | 2           | 2           |
| TG*         | 5                   | 5           | (2)         | 1           | 3           | 3           |
| Total       | 22                  | 19 (86%)    | 1 (5%)      | 6 (27%)     | 9 (41%)     | 8 (36%)     |

*TG = Trafla Gottron
The numbers in brackets correspond to weakly positive results
NTgsGP = Nested TgsGP
Number of strains positive for TBR, TgsGP, NTgsGP and TCF targets, and those positive for both TBR and TCF targets, are given.
The presence of *T. b. gambiense* could not be proven [11]. The present study was carried out in the Vavoua historical focus where the last two HAT cases were diagnosed in the early 2010s and where pig husbandry is widespread. One objective was to evaluate the possible human contamination from a pig transmission cycle in a historical HAT focus. Based on our results, this hypothesis seems unlikely since no HAT cases nor TL-positive subjects were observed in the sampled people living in close vicinity of the pigs. However, with the decline in incidence of HAT, this disease is no longer considered as a threat in the study area. Thus, we cannot exclude the existence of undetected human cases.

The global results obtained with BCT, TL and PCR confirmed high trypanosome infection rates in pigs as already observed in previous studies conducted on the neighboring Bonon and Sinfra HAT endemic foci [11,39] or in other study areas in West and Central Africa as in Nigeria [40], Chad [41] or Cameroon [42–44]. Such high infection rates in pigs may be linked to their tolerance to trypanosomes as already observed in the field [20] or during experimental infections with *T. b. gambiense* [45], and certainly illustrate that the pig is a preferential host for tsetse flies [46,47].
An originality of this work was to compare the results obtained regarding the husbandry methods of pigs. We show that free-ranging pigs were significantly more trypanosome-infected than pigs raised in pen including in the PK14 site where the two husbandry methods are practiced. This is probably due to the fact that free-ranging pigs, roaming freely in the humid and shady areas and in the protected sacred forests near by villages, are highly exposed to *Glossina palpalis* that shares the same biotope as already observed [11,46]. Pigs have already been described as a preferential feeding host for this tsetse species [46] that have recolonized the studied area after the end of the vector control campaigns conducted in the 1980’s [15,19]. These proximity and trophic interactions result on predominant free-ranging pig/tsetse transmission cycles in the vicinity of villages. The low infection rates observed in pigs in pen could be because pigsties are located inside or close to the villages far away from the tsetse flies favorable biotopes, and are thus probably less exposed.

We decided to focus our diagnosis analysis on the six free-ranging pigs study sites with an overall prevalence of 35% observed with BCT and of 72% when combining BCT with mAECT. Our results confirmed those recently observed in Bonon and Sinfra [11] with the highest PCR positivity rate obtained for *T. b. brucei* (more than half of the pigs are positive) followed by *T. congolense* forest type (a quarter positive) and with more than 10% of mixed infections with these two species. The unexpected presence of *T. vivax* in pigs raised in pen advocates for alternative life cycles, with the putative involvement of tabanids insuring mechanical transmission. With the increase of molecular approaches, presence of *T. vivax* in pigs has been recently reported [48,49] contrasting with the historical data previously reviewed [50].

In this study, we also used the CATT and mAECT initially adapted for HAT diagnosis, to increase the possibility of *T. b. gambiense* detection. The CATT-B test showed high positivity rates up to 100% in three free-ranging pig sites. Most of the CATT-B positive pigs were CATT-P ≥ 1/4 confirming the important reactivity with the test. More than 90% of the mAECT performed on CATT-B positive pigs were positive. These results confirm that the CATT is not specific of *T. b. gambiense* due to cross-reactions with other trypanosomes as already observed [51–54]. The SD Bioline HAT rapid diagnostic tests (RDT) [55], another serological test initially developed for HAT diagnosis, also showed a lack of *T. b. gambiense* specificity when used in animals [41,56,57]. In absence of serological tests adapted for field diagnosis of African trypanosomiases in animal surveys, we propose the use of CATT as screening methods, despite their lack of *T. b. gambiense*-specificity.

The mAECT, known as the most sensitive parasitological test for *T. b. gambiense* detection in human [58,59], was applied on CATT positive pigs for which infection rates from 50% to 100% were observed. mAECT was much more sensitive than BCT. Although the buffer pH and ionic strength conditions have been adapted for *T. b. gambiense* detection in humans [60], mAECT seems nevertheless suitable to detect other trypanosomes such as *T. b. brucei* or *T. congolense* forest type in pigs with similar sensitivity as PCR but without the possibility to identify the trypanosome species limiting its additional value regarding the study of the animal reservoir of *T. b. gambiense*.

Because the tools mentioned above could not be used to specifically identify the presence of *T. b. gambiense* in the studied pigs, the rest of our investigations focused on the TgsGP PCR and TL results, because they are reported to be *T. b. gambiense* specific. No pig was positive with the TgsGP PCR performed on BC. The lack of sensitivity of this PCR targeting a single copy gene [30,31] was recently confirmed in an experimental study [61]. We then cannot exclude false negative results (negative PCR despite the presence of *T. b. gambiense*) especially when using a single round PCR as done in this study. This was a deliberate choice regarding the single round and nested PCR results firstly obtained on purified DNA from isolated strains. With the single round PCR, a band of low intensity was observed for several strains at
the expected size but the PCR product could not be sequenced to confirm it corresponded to the TgsGP sequence. The nested TgsGP PCR applied on these samples were negative suggesting a non-specific amplification during the first PCR round. On the other hand, negative samples for the single round PCR were unambiguously positive for the nested PCR. Only one strain was clearly positive for both the single round and the nested PCR. For this sample, the presence of the TgsGP was confirmed by sequencing. If we take into account all the TgsGP PCR positive results (single round or nested), *T. b. gambiense* was detected in 50% of the isolated strains (11% free-ranging pigs). This was inconsistent with both the *T. b. gambiense* prevalence in human in the area and the results of the human survey performed in the frame of this study.

We may suspect that the nested TgsGP PCR could gave false positive results, for two main reasons. First, we cannot exclude a contamination during amplification workflow even if all experiments were validated by negative controls. Such contamination may have occurred in any of the laboratories that routinely work with *T. b. gambiense* DNA, mainly when using nested PCR to increase sensitivity. Second, a TgsGP-related gene (Tb10.v4.0178) that shared 81% homology with TgsGP has been described in *T. b. brucei* [62,63]. Even with *T. b. gambiense* specific primers, a risk of *T. b. brucei* non-specific amplification should be considered when PCR is forced. In a recent study targeting TgsGP to detect *T. b. gambiense* in tsetse flies in a very low HAT prevalence area in Uganda, the TgsGP primers were suspected to cross-react with DNA from an “unidentified source” after sequencing the DNA products [64]. Non-specific TgsGP PCR products could explain the reported presence of *T. b. gambiense* in domestic and wild animals in foci where no or very few HAT cases were reported [41,65,66]. This might also explain the TgsGP positive results recently observed on animals in the Moundou focus in Chad [57], an area where tsetse densities were significantly reduced by a vector control campaign [67]. Alternatively we cannot exclude that TgsGP nested PCR may detect very low level of DNA in animals, that does not mean any active transmission to humans.

The *T. b. gambiense* specificity is also questioned regarding the LiTat 1.3 and LiTat 1.5 TL that gave respectively up to 50% and 80% positive results according to the study site. More than 10% of the free-ranging pigs were positive with the two VAT. The LiTat 1.3 gene was already observed in *T. b. brucei* isolated from pigs in Côte d’Ivoire [68] and positive TL results were observed in cattle in rhodesiense HAT and AAT endemic region in Uganda [56]. Further experimental studies are required to confirm or not the *T. b. gambiense* specificity of this method.

Despite the doubts observed in this study regarding the TL and TgsGP PCR *T. b. gambiense* specificity, one isolated strain for which a strong PCR signal was observed with both the single round and nested TgsGP PCR is more likely to be *T. b. gambiense*. The corresponding free-ranging pig was positive to TBR PCR (on both BC and isolated strain) and to LiTat 1.5 and 1.6 TL (not 1.3). We then cannot exclude the existence of free-ranging pig/tsetse/T. b. gambiense transmission cycle in which humans are usually not involved, except if an “accidental” infection occurs when an infective tsetse bites a human. This may explain the infection of the last HAT cases reported in the neighboring villages where a human/tsetse transmission cycle is very unlikely regarding the low prevalence of the disease. A long-term residual human reservoir could also explain these infections as already suspected, especially in the neighboring foci of Sinfra and Bonon [33].

Studies on the *T. b. gambiense* animal reservoir are also complex because of the diversity of the epidemiological contexts of HAT foci. Animal reservoirs can involve wild animals with a very important diversity of species [65,69–71]. Regarding domestic animals, it depends on the population customs, the animal species raised and the husbandry methods. If free-ranging pigs seems to be mostly involved in trypanosome circulation in Côte d’Ivoire, other animal
species appeared to be involved in other countries with differences observed between different HAT foci [44,57] and even within the same focus [42]. In our study, we observed significant differences in prevalence and in result profiles for several diagnostic tests according to the study sites that are only a few kilometers apart. Environmental factors probably influenced the pig/tsetse contact and the transmission of the different trypanosome species. Ecological and entomological aspects have not been included in our study design and could limit the interpretation of our data. Another limitation concerns the lack of data collection on both the human and pig population sizes, number of pig breeders and number of pigs per breeder in each study sites. Further studies on a larger and more exhaustive sample would allow a more accurate evaluation of the role of pigs in African trypanosomiases epidemiology.

Another difficulty relies on the fact that the most exposed animal(s) are often multi-infected as already observed in several studies [11,72]. This was clearly illustrated in the present one with the high prevalence of *T. brucei* s.l. and *T. congoense* forest type mixed infections. The association between these two species, already mentioned in other studies [11,20,39,51,72] may hold epidemiological consequences that would deserve further investigations. The important heterogeneity observed regarding the TL profile results according to the study sites suggested the likely circulation of different *T. brucei* s.l. strains containing different VAT and mixed infections with several strains in single pigs. The congruence observed between the serological (CATT-B and TL) and molecular (TBR PCR) results showed that despite their limited specificity regarding the trypanosome species, these tools appeared to be useful for further field investigations on animal trypanosomiases.

In summary, although all the diagnostic tests performed in this study on plasma, blood and isolated strains showed high trypanosome prevalence in free-ranging pigs, none was able to prove with certainty the presence or absence of *T. b. gambiense*. TL and TgsGP PCR may even tend to overestimate *T. b. gambiense* prevalence, which would represent an undesirable bias. This study once more highlights the need for technical improvements in exploring the epidemiological role of animals in HAT. Studies focusing on the deeper genetic knowledge of the circulating strains (by NGS methods) and phenotypical evidences related to resistance toward human serum are currently conducted to solve these questions. Excluding or evidencing this role using field and experimental studies is crucial to adapt the control strategies and to define indicators to reach the interruption of transmission targeted by WHO in 2030 [2]. Ignoring or under-estimating the importance of this topic may lead to repeat the same mistakes as for the Guinea worm for which the reporting of a canine reservoir has challenged and delayed the eradication process [73]. Free-ranging pigs obviously represent a potential HAT risk for humans, but may also constitute a potential source of contamination for other domestic animals as zebu cattle that are sensitive to trypanosome infections [74].

**Supporting information**

**S1 Table.** Complete database of the diagnostic results for the 167 pigs. ND = not down; + = positive; — = negative; (+) = positive TgsGP PCR with low intensity band.

(XLS)

**S2 Table.** Diagnostic tests results for the 20 pigs in PK14 regarding the husbandry method. 0 = negative; 1 = positive.

(XLSX)

**S3 Table.** Diagnostic tests results regarding the study sites.

(XLSX)
S1 Fig. Agarose gel obtained with single round TgsGP on isolated strains. M = molecular weight marker (100 bp DNA ladder) Sample 2 (pig 102) = positive with a clearly positive band Samples 1 (pig 82) and 3 (pig 86) = positive with a low intensity band C1+ and C2+ = positive PCR controls C- = negative PCR control. (PPTX)

S2 Fig. Agarose gel obtained with nested TgsGP on isolated strains. M = molecular weight marker (100 bp DNA ladder) Sample 3 (pig 66), 7 (pig 69), 8 (pig 84), 12 (pig 100), 14 (pig 102) and 16 (pig 104) = positive with a clearly positive band C1+ and C2+ = positive PCR controls (T. b. gambiense reference stock) C- = negative PCR control. (PPTX)

S3 Fig. Alignment of the sequences of the nested TgsGP positive samples with DAL972 reference stock. Sample 3 (pig 66), 7 (pig 69), 8 (pig 84), 12 (pig 100), 14 (pig 102) and 16 (pig 104) C1+ and C2+ = positive PCR controls (T. b. gambiense reference stock). (PPTX)

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References
1. Büscher P, Cecchi G, Jamonneau V, Priotto G. Human African trypanosomiasis. The Lancet. 2017; 390: 2397–2409.
2. Franco JR, Cecchi G, Priotto G, Paone M, Diarra A, Grout L, et al. Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018. PLoS Negl Trop Dis. 2020; 14: e0008261. https://doi.org/10.1371/journal.pntd.0008261 PMID: 32437391
3. Büscher P, Bart J-M, Boelaert M, Bucheton B, Cecchi G, Chitnis N, et al. Do cryptic reservoirs threaten gambiense-sleeping sickness elimination? Trends Parasitol. 2018; 34: 197–207. https://doi.org/10.1016/j.pt.2017.11.008 PMID: 29396200
4. Mehlitz D, Molyneux DH. The elimination of Trypanosoma brucei gambiense? Challenges of reservoir hosts and transmission cycles: Expect the unexpected. Parasite Epidemiol Control. 2019; 6: e00113. https://doi.org/10.1016/j.parepi.2019.e00113 PMID: 31528738
5. Kristjanson PM, Swallow BM, Rowlands GJ, Kruska RL, De Leeuw PN. Measuring the costs of African animal trypanosomiasis, the potential benefits of control and returns to research. Agric Syst. 1999; 59: 79–98.
6. Alsan M. The effect of the tsetse fly on African development. Am Econ Rev. 2015; 105: 382–410.
7. Destoumieux-Garzon D, Mavingui P, Boetsch G, Boissier J, Darriet F, Duboz P, et al. The one health concept: 10 years old and a long road ahead. Front Vet Sci. 2018; 5: 14. https://doi.org/10.3389/fvets.2018.00014 PMID: 29484301
8. Welburn S. One health: the 21st century challenge. Vet Rec. 2011; 168: 614–5. https://doi.org/10.1136/vr.d3528 PMID: 21666048
9. Simo G, Rayaisse JB. Challenges facing the elimination of sleeping sickness in west and central Africa: sustainable control of animal trypanosomiasis as an indispensable approach to achieve the goal. Parasit Vectors. 2015; 8: 640. https://doi.org/10.1186/s13071-015-1254-y PMID: 26671582
10. Koné M, N’Gouan EK, Kaba D, Koffi M, Kouakou L, N’Dri L, et al. The complex health seeking pathway of a human African trypanosomiasis patient in Côte d’Ivoire underlines the need of setting up passive surveillance systems. PLoS Negl Trop Dis. 2020; 14: e0008588. https://doi.org/10.1371/journal.pntd.0008588 PMID: 32925917
11. N’Djetchi MK, Ilboudo H, Koffi M, Kaboré J, Kaboré JW, Kaba D, et al. The study of trypanosome species circulating in domestic animals in two human African trypanosomiasis foci of Côte d’Ivoire identifies pigs and cattle as potential reservoirs of Trypanosoma brucei gambiense. PLoS Negl Trop Dis. 2017; 11: e0005993. https://doi.org/10.1371/journal.pntd.0005993 PMID: 29045405
12. Duvallet G, Stanghellini A, Saccharin C, Vivant J. Human trypanosomiasis focus of Vavoua (Ivory Coast). A clinical, parasitological and sero-immunological survey. Med Trop. 1979; 39: 517–26. PMID: 231175
13. Stanghellini A, Duvallet G. Epidemiologie de la trypanosomiasis humaine à Trypanosoma gambiense dans un foyer de Côte d’Ivoire: distribution de la maladie dans la population. Tropenmed. Parasitol. 1981; 32: 141–144. PMID: 6285561
14. Gouteux J, Challier A, Laveissiere C, Stanghellini A. Observations sur les glossines d’un foyer forestier de trypanosomiasis humaine en Côte d’Ivoire.1 Presentation du foyer de Vavoua. Cah ORSTOM. Sér Ent Méd Parasitol. 1981; 19: 199–207.
15. Laveissièere C, Courret D, Staaak C, Hervouët JP. Glossina palpalis et ses hôtes en secteur forestier de Côte d’Ivoire. Relations avec l’épidémiologie de la trypanosomiasis humaine. Cah ORSTOM. Ent Méd Parasitol. 1985; 23: 297–303.
16. Laveissière C, Hervouët JP, Courret D. Localization and man/fly contact frequency in the forest area of the ivory Coast. 2. Human factor and trypanosomiasis transmission. Cah ORSTOM. Sér Ent Méd Parasitol. 1986; 24: 45–57.
17. Djé NN, Miezan TW, N’guesan P, Brika P, Doua F, Boa F. Distribution géographique des trypanosomés pris en charge en Côte d’Ivoire de 1993 à 2000. Bull Soc Pathol Exot. 2002; 95: 359–361. PMID: 12696376
18. Simarro PP, Cecchi G, Paone M, Franco JR, Diarra A, Ruiz JA, et al. The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. Int J Health Geogr. 2010; 9: 57. https://doi.org/10.1186/1476-072X-9-57 PMID: 21040555

19. Goutteux JP, Mondet B, Poinar GJO. Ecology of tsetse flies in a sub-forest area of Ivory Coast. 1. Parasitism by Hexameris glossinae (Nematoda, Mermithidae). Cah ORSTOM Sér Ent Méd Parasitol. 1981; 19: 285–95.

20. Mehltitz D. Le réservoir animal de la maladie du sommeil à Trypanosoma brucei gambiense. Etudes et Synthèses de l’IEMVT. n.18, Maisons-Alfort: CIRAD-IEMVT. 1986, 156 pp. ISBN 2-85985-127-5

21. Magnus E, Vervoort T, Van Meirvenne N. A card-agglutination test with stained trypanosomes (CATT) for the serological diagnosis of T. b. gambiense trypanosomiasis. Ann Soc Belg Med Trop. 1978; 58: 169–76. PMID: 747425

22. Lumsden WHR, Kimber CD, Evans DA, Doig SJ. Trypanosoma brucei: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. Trans R Soc Trop Med Hyg. 1979; 73: 312–7. https://doi.org/10.1016/0035-9203(79)90092-0 PMID: 473329

23. Camara M, Camara O, Ilboudo H, Sakande H, Kabore J, N’Dri L, et al. Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. Trop Med Int Health. 2010; 15: 796–9. https://doi.org/10.1111/j.1365-3156.2010.02546.x PMID: 20497407

24. Murray M, Murray PK, McIntyre WI. Detection of Trypanosoma congoense and Trypanosoma brucei subspecies by DNA amplification using the polymerase chain reaction. Parasitology. 1989; 99: 57–66. https://doi.org/10.1017/s0031182000061023 PMID: 2797872

25. Majiwa P, a. O, Thattthi R, Moloo SK, Nyoko JHP, Otieno LH, Maloo S. Detection of trypanosome infections in the saliva of tsetse flies and buffy-coat samples from antigenaemic but aparasitaemic cattle. Parasitology. 1994; 108: 313–22. https://doi.org/10.1017/s0031182000076150 PMID: 8022657

26. Holzmuller P, Biron DG, Courtois P, Koffi M, Bras-Goncalves R, Daulouède S, et al. Virulence and pathogenicity patterns of Trypanosoma brucei gambiense field isolates in experimentally infected mouse: differences in host immune response modulation by secretome and proteomics. Microbes Infect. 2008; 10: 79–86. https://doi.org/10.1016/j.micinf.2007.10.008 PMID: 18068387

27. Moser DR, Cook GA, Ochs DE, Bailey CP, McKane MR, Donelson JE. Detection of Trypanosoma congolense and Trypanosoma brucei: a rapid matching method for estimating the host’s parasitaemia. Exp Parasitol. 1976; 40: 247–31. https://doi.org/10.1016/0014-4894(76)90110-7 PMID: 976425

28. Van Meirvenne N, Magnus E, Vervoort T, Moloo SK, Nyoko JHP, Otieno LH, Maloo S. Detecting trypanosomes in tsetse flies by DNA amplification. Int J Parasitol. 1992; 22: 909–18. https://doi.org/10.1016/0020-7519(92)90047-o PMID: 1459784

29. Radańska M, Claes F, Magez S, Magnus E, Pérez-Morga D, Pays E, et al. Novel primer sequences for Polymerase Chain Reaction–based detection of Trypanosoma brucei gambiense. Mol Biochem Parasitol. 2001; 113: 127–38. https://doi.org/10.1016/s0166-6851(01)00208-0 PMID: 11254961

30. Radwanska M, Claes F, Magez S, Magnus E, Pérez-Morga D, Pays E, et al. Novel primer sequences for Polymerase Chain Reaction–based detection of Trypanosoma brucei gambiense. Mol Biochem Parasitol. 2002; 113: 127–38. https://doi.org/10.1016/s0166-6851(01)00208-0 PMID: 11254961

31. Berbero M, Pérez-Morga D, Pays E. A receptor-like flagellar pocket glycoprotein specific to Trypanosoma brucei gambiense. Mol Biochem Parasitol. 2001; 113: 127–38. https://doi.org/10.1016/s0166-6851(01)00208-0 PMID: 11254961

32. Morrison LJ, Tait A, McCormack G, Sweeney L, Black A, Truc P, et al. Trypanosoma brucei gambiense Type 1 populations from human patients are clonal and display geographical genetic differentiation. Infect Genet Evol. 2008; 8: 847–54. https://doi.org/10.1016/j.meegid.2008.08.005 PMID: 18790085

33. Jamonneau V, Bucheton B, Kaboré J, Ilboudo H, Camara O, Courtin F, et al. Revisiting the immune trypanolysis test to optimise epidemiological surveillance and control of sleeping sickness in west Africa. PLoS Negl Trop Dis. 2010; 4: e917. https://doi.org/10.1371/journal.pntd.0000917 PMID: 21200417

34. Van Meirvenne N, Magnus E, Buscher P. Evaluation of variant specific trypanolysis tests for serodiagnosis of human infections with Trypanosoma brucei gambiense. Acta Trop. 1995; 60: 189–99. https://doi.org/10.1016/0001-706x(95)90127-z PMID: 8907397

35. Fox J. The R Commander: A basic-statistics graphical user interface to R. J Stat Softw. 2005; 14: 1–42.

36. Fox J. Extending the R Commander by “plug-in” packages. R news. 2007; 7:46–52.

37. Core-Team. R: A Language and Environment for Statistical Computing, Version 3.6.3. Ed. R Foundation for Statistical Computing, Vienna, Austria. 2020.

38. Benjamin Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol. 1995; 57: 289–300.
39. Jamonneau V, Ravel S, Koffi M, Kaba D, Zeze DG, Ndri L, et al. Mixed infections of trypanosomes in tsetse and pigs and their epidemiological significance in a sleeping sickness focus of Côte d’Ivoire. Parasitology. 2004; 129: 693–702. https://doi.org/10.1017/s0031182004005867 PMID: 15648692

40. Karshima SN, Ajoji I, Mohammed G. Eco-epidemiology of porcine trypanosomosis in Karim Lamido, Nigeria: prevalence, seasonal distribution, tsetse density and infection rates. Parasit Vectors. 2016; 9: 448. https://doi.org/10.1186/s13071-016-1732-x PMID: 27519267

41. Vourchakbe J, Tiofack ZAA, Kante TS, Mpoame M, Simo G. Molecular identification of Trypanosoma brucei gambiense in naturally infected pigs, dogs and small ruminants confirms domestic animals as potential reservoirs for sleeping sickness in Chad. Parasite. 2020; 27: 63. https://doi.org/10.1051/parasite/20200061 PMID: 33206595

42. Njitchouang GR, Njokou F, Djueunga HCN, Fewou PM, Asonganyi T, Cuny G, et al. Analysis of the domestic animal reservoir at a micro-geographical scale, the Fontem sleeping sickness focus (South-West Cameroon). J Cell Anim Biol. 2010; 4: 73–80.

43. Simo G, Njokou F, Tume C, Lueong S, De Meewus T, Cuny G, et al. Population genetic structure of Central African Trypanosoma brucei gambiense isolates using microsatellite DNA markers. Infect Genet Evol. 2010; 10: 68–76. https://doi.org/10.1016/j.meegid.2009.09.019 PMID: 19819349

44. Njokou F, Nimpaye H, Simo G, Njitchouang GR, Asonganyi T, Cuny G, et al. Domestic animals as potential reservoir hosts of Trypanosoma brucei gambiense in sleeping sickness foci in Cameroon. Parasite. 2010; 17: 61–6. https://doi.org/10.1051/parasite/2010171061 PMID: 20387740

45. Penchenier L, Alhadji D, Bahebegue S, Simo G, Laveissière C, Cuny G. Spontaneous cure of domestic pigs experimentally infected by Trypanosoma brucei gambiense: implications for the control of sleeping sickness. Vet Parasitol. 2005; 133; 7–11. https://doi.org/10.1016/j.vetpar.2005.04.034 PMID: 16076528

46. Sané B, Laveissière C, Meda HA. Diversity of feeding behavior of Glossina palpalis palpalis in the forest belt of the Ivory Coast: relation to the prevalence of human African trypanosomiasis. Trop Med Int Health. 2000; 5: 73–8. https://doi.org/10.1046/j.1365-3156.2000.00486.x PMID: 10672209

47. Simo G, Njokou F, Mbida JM, Njitchouang GR, Herder S, Asonganyi T, et al. Tsetse fly host preference in sleeping sickness foci in Cameroon: epidemiological implications. Infect Genet Evol. 2008; 8: 34–39. https://doi.org/10.1016/j.meegid.2007.09.005 PMID: 17977803

48. Ng’ayo MO, Njiru ZK, Kenya EU, Muluvi GM, Osir EO, Masiga DK. Detection of trypanosomes in small ruminants and pigs in western Kenya: important reservoirs in the epidemiology of sleeping sickness? Kinetoplastid Biol Dis. 2005; 4: 1–7. https://doi.org/10.1186/1475-9292-4-1 PMID: 15667659

49. Biryomumisho S, Melville SE, Atunguka Rwakishaya E, Lubega GW. Detection of natural Trypanosoma vivax infections in pigs with microhaematocrit centrifugation and amplification of ITS1 rDNA. Onderstepoort J Vet Res. 2009; 76: 285–289. https://doi.org/10.4102/ojvr.v76i3.31 PMID: 21105595

50. Desquesnes M, Dávila AM. Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. Vet Parasitol. 2002; 109; 208–231. https://doi.org/10.1016/s0304-4017(02)00270-4 PMID: 12429394

51. Noireau F, Gouteux J-P, Toudic A, Samba F, Frézil J-L. Importance épidémiologique du réservoir animal à Trypanosoma brucei gambiense au Congo: 1. Prévalence des trypanosomes animales dans les foyers de maladie du sommeil. Trop Med Parasiol. 1986; 37: 393–8. PMID: 3563320

52. Asonganyi T, Suh S, Tetuh MD. Prevalence of domestic animal trypanosomiasis in the Fontem sleeping sickness focus, Cameroon. Rev Elev Med Vet Pays Trop. 1990; 43: 69–74. PMID: 2263747

53. Holland WG, Thanh NG, Do TT, Sangmaneeted S, Goddeeris B, Vercruysse J. Evaluation of diagnostic tests for Trypanosoma evansi in experimentally infected pigs and subsequent use in field surveys in North Vietnam and Thailand. Trop Anim Health Prod. 2005; 37: 457–67. https://doi.org/10.1007/s11250-005-1217-y PMID: 16248217

54. Simo G, Silatsa B, Flobert N, Lutumba P, Mansinsa P, Madinga J, et al. Identification of different trypanosome species in the mid-guts of tsetse flies of the Malanga (Kimpese) sleeping sickness focus of the Democratic Republic of Congo. Parasit Vectors. 2012; 5: 201. https://doi.org/10.1186/1756-3305-5-201 PMID: 22992486

55. Bisser S, Lumbala C, Ngueroum E, Kande V, Flevaud L, Vatunga G, et al. Sensitivity and specificity of a prototype rapid diagnostic test for the detection of Trypanosoma brucei gambiense infection: A multicentric prospective study. PLoS Negl Trop Dis. 2016; 10: e0004608. https://doi.org/10.1371/journal.pntd.0004608 PMID: 27058033

56. Matovu E, Kitibwa A, Picado A, Biéler S, Bessell PR, Ndung’u JM. Serological tests for gambiense human African trypanosomiasis detect antibodies in cattle. Parasit Vectors. 2017; 10: 546. https://doi.org/10.1186/s13071-017-2487-8 PMID: 29100526
57. Vourchakbé J, Tiofack AAZ, Mbida M, Simo G. Trypanosome infections in naturally infected horses and donkeys of three active sleeping sickness foci in the south of Chad. Parasit Vectors. 2020; 13: 323. https://doi.org/10.1186/s13071-020-04192-1 PMID: 32576240

58. Büscher P, Ngoyi DM, Kabore J, Lejon V, Robays J, Jamonneau V, et al. Improved models of mini anion exchange centrifugation technique (mAECT) and modified single centrifugation (MSC) for sleeping sickness diagnosis and staging. PLoS Negl Trop Dis. 2009; 3: e471. https://doi.org/10.1371/journal.pntd.0000471 PMID: 19936296

59. Lejon V, Büscher P, Nzoumbou-Boko R, Bossard G, Jamonneau V, Bucheton B, et al. The separation of trypanosomes from blood by anion exchange chromatography: From Sheila Lanham’s discovery 50 years ago to a gold standard for sleeping sickness diagnosis. PLoS Negl Trop Dis. 2019; 13: e0007051. https://doi.org/10.1371/journal.pntd.0007051 PMID: 30817751

60. Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol. 1970; 28: 521–34. https://doi.org/10.1016/0014-4894(70)90120-7 PMID: 4993889

61. Compaoré CFA, Ilboudo H, Kaboré J, Kabore JW, Camara O, Bamba M, et al. Analytical sensitivity of loopamp and quantitative real-time PCR on dried blood spots and their potential role in monitoring human African trypanosomiasis elimination. Exp Parasitol. 2020; 2019: 108014.

62. Felu C, Pasture J, Pays E, Pérez-Morga D. Diagnostic potential of a conserved genomic rearrangement in the Trypanosoma brucei gambiense-specific TgsGP locus. Am J Trop Med Hyg. 2007; 76: 922–9. PMID: 17488917

63. Gibson W, Nemetschke L, Ndung’u J. Conserved sequence of the TgsGP gene in Group 1 Trypanosoma brucei gambiense. Infect Genet Evol. 2010; 10: 453–8. https://doi.org/10.1016/j.meegid.2010.03.005 PMID: 20302972

64. Cunningham LJ, Lingley JK, Tirados I, Esterhuizen J, Opiyo M, Mangwiro CTN, et al. Evidence of the absence of human African trypanosomiasis in two northern districts of Uganda: Analyses of cattle, pigs and tsetse flies for the presence of Trypanosoma brucei gambiense. PLoS Negl Trop Dis. 2020; 14: e0007737. https://doi.org/10.1371/journal.pntd.0007737 PMID: 32255793

65. Cordon-Obras C, Rodríguez YF, Fernández-Martínez A, Cano J, Ndong-Mabale N, Ncogo-Ada P, et al. Molecular evidence of a Trypanosoma brucei gambiense sylvatic cycle in the human African trypanosomiasis foci of Equatorial Guinea. Front Microbiol. 2015; 6: 765. https://doi.org/10.3389/fmicb.2015.00765 PMID: 26257727

66. Umeakuana PU, Gibson W, Ezekoenkwo RC, Anene BM. Identification of Trypanosoma brucei gambiense in naturally infected dogs in Nigeria. Parasit Vectors. 2019; 12: 420. https://doi.org/10.1186/s13071-019-3680-8 PMID: 31455430

67. Mahamat MH, Peka M, Rayaisse J-B, Rock KS, Toko MA, Damas J, et al. Adding tsetse control to medical activities contributes to decreasing transmission of sleeping sickness in the Mandoul focus (Chad). PLoS Negl Trop Dis. 2017; 11: e0005792. https://doi.org/10.1371/journal.pntd.0005792 PMID: 28750007

68. Bromidge T, Gibson W, Hudson K, Dukes P. Identification of Trypanosoma brucei gambiense-by PCR amplification of variant surface glycoprotein genes. Acta Trop. 1993; 53: 107–19. https://doi.org/10.1016/0001-706x(93)90023-5 PMID: 8098897

69. Guedegbe B, Verhulst A, Van meirvenne N, Pandey VS, Doko A. Indications sérologiques de l’existence d’un réservoir sauvage du Trypanosoma brucei gambiense dans la réserve de la biosphère de la Pendjari en République du Bénin. Ann Soc Bel Med Trop. 1992; 72: 113–20.

70. Herder S, Simo G, Nkinin S, Njokou F. Identification of trypanosomes in wild animals from southern Cameroon using the polymerase chain reaction (PCR). Parasite. 2002; 9: 345–9. https://doi.org/10.1051/parasite:2002094345 PMID: 12514949

71. Njokou F, Laveissière C, Simo G, Nkinin S, Grébaut P, Cuny G, et al. Wild fauna as a probable animal reservoir for Trypanosoma brucei gambiense in Cameroon. Infect Genet Evol. 2006; 6: 147–53. https://doi.org/10.1016/j.meegid.2005.04.003 PMID: 16236560

72. Simo G, Asonganyi T, Nkinin SW, Njokou F, Herder S. High prevalence of Trypanosoma brucei gambiense group 1 in pigs from the Fontem sleeping sickness focus in Cameroon. Vet Parasitol. 2006; 139: 57–66. https://doi.org/10.1016/j.vetpar.2006.02.026 PMID: 16567049

73. Roberts L. Battle to wipe out Guinea worm stumbles. Nature. 2019; 574: 157. https://doi.org/10.1038/d41586-019-02921-w PMID: 31595066

74. Berthier D, Brenière SF, Bras-Gonçalves R, Lemersre J-L, Jamonneau V, Solano P, et al. Tolerance to trypanosomatids: A threat, or a key for disease elimination? Trends Parasitol. 2016; 32: 157–68. https://doi.org/10.1016/j.pt.2015.11.001 PMID: 26643519