

Trypanosome infections in naturally infected horses and donkeys of three active sleeping sickness foci in the south of Chad

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Research

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Abstract

Background Equine trypanosomiasis are complex infectious diseases with overlapping clinical signs defined by their mode of transmission. Despite their economic impacts, these diseases have been neglected by the scientific community, the veterinary authorities and regulatory organizations. To fill the observed knowledge gap, we undertook the identification of different trypanosome species and subspecies naturally infecting horses and donkeys within the Chadian sleeping sickness focus. The end objective, being to investigate the potential role of these domestic animals as reservoirs of the human infective *Trypanosoma brucei gambiense*. Blood samples were collected from 155 donkeys and 131 horses in three human African trypanosomiasis (HAT) foci of Chad. Rapid diagnostic test (RDT) and capillary tube centrifugation (CTC) test were used to search for trypanosome infections. DNA was extracted from each blood sample and different trypanosome species and subspecies were identified with molecular tools.

Results From 286 blood samples collected, 54 (18.9%) and 36 (12.6%) were respectively positive for RDT and CTC. PCR revealed 194 (67.8%) animals with trypanosome infections. The kappa Cohen coefficients used to evaluate the concordance between the diagnostic methods were low; ranging from 0.087 ± 0.04730 to 0.48 ± 0.06951 . Trypanosomes of the subgenus *Trypanozoon* were the most prevalent (29.4%), followed by *T. congolense forest* (11.5%), *T. congolense savannah* (4.9%) and *T. vivax* (4.5%). Two donkeys and one horse from the Maro HAT focus were found with *T. b. gambiense* infections. Between animal species and HAT foci, no significant differences were observed in the infection rates of different trypanosomes.

Conclusion This study revealed several trypanosome species and sub species in donkeys and horses, highlighting the existence of AAT in HAT foci of Chad. The identification of *T. b. gambiense* in donkeys and horses suggests considering these animals as potential reservoir for HAT in Chad. The presence of both human-infective and human non infective trypanosomes species highlights the need for developing joined control strategies for HAT and AAT.

Background

Trypanosomiasis are infectious diseases affecting both humans and animals. Human African Trypanosomiasis (HAT), also known as sleeping sickness, is an important public health disease caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. The former parasite causes the chronic form of HAT in west and central Africa while the latter induces an acute form which is found in eastern and southern Africa [1]. These human-infective parasites can be transmitted to livestock and wildlife, which can serve as reservoirs for HAT [2]. On the basis of the HAT-related mortality, HAT has been ranked ninth out of 25 human infectious and parasitic diseases in Africa [3, 4]. During the last decades, efforts undertaken in the fighting of HAT brought this disease under control and led to its inclusion in the WHO "roadmap for eradication, elimination and control of neglected tropical diseases", with a target set to eliminate HAT as a public health problem by 2020 [5]. Achieving these goals requires investigations on

animal reservoir that has been considered as one factor that could compromise the elimination and eradication of HAT. It is in this light that several trypanosome species and subspecies including *T. b. gambiense* were reported in various animal species of western and central African HAT foci. Although donkeys and horses are commonly used by inhabitants of some HAT foci for traction and transport, no data on trypanosome infections in these animals has been published in such settings. However, these animals are exposed to trypanosome infections and could alter the dynamics of HAT infection and thus jeopardize eradication efforts.

African animal trypanosomiasis (AAT) are responsible of major constraints to livestock production in affected countries. Its direct impact is linked to the reduction of livestock productivity while the indirect impacts are associated with a reduced efficiency of draught animals for crop production [6, 7]. Although several trypanosome species have been reported in domestic and wild animals residing within HAT foci in west and central Africa [8, 9, 10, 11, 12, 13], equines have not been addressed thus far (mules, donkeys and horses). Indeed, the equine population is estimated to more than 127 million with approximately 85% in low income countries [14]. The positive impact of equines has been widely acknowledged upon poverty reduction, gender equality and environmental stability [15, 16]. Equines maintain the health and welfare of 300 to 600 million people globally, often within the most vulnerable communities [17]. They play important role in transport and traction [18], contribute significantly to household income [19, 20, 21] and create opportunities for women and children [20, 22]. Viewed their importance, attempts have been refocused to tackle infectious diseases that could compromise the welfare and productivity of these animals [14, 15, 16, 17]. In this light, equine trypanosomiasis was reported as one of the infectious diseases that may have greatest impact upon working equines [7].

Caused by trypanosomes of the genus *Trypanosoma*, equine trypanosomiasis is a complex of infectious diseases called dourine, nagana and surra. These diseases are characterized by overlapping clinical features that can be defined by their mode of transmission [23]. They give rise to important economic losses in Africa, the Middle East, Asia and Latin America [24]. They can be considered as animal diseases that are seriously neglected, both by the scientific community as by veterinary authorities and regulatory organizations [24]. Nagana is due to *T. vivax*, *T. congolense* and/or *T. brucei* subspecies and is transmitted by tsetse flies; Surra is caused by *T. evansi* and is mechanically transmitted by biting flies while dourine is due to *T. equiperdum* and is sexually transmitted [23]. With these transmission modes, designing appropriate control measures requires a better understanding of the epidemiology of equine trypanosomiasis by identifying trypanosomes that naturally infect horses and donkeys. In HAT foci, such investigation could generate data for the improvement of epidemiological knowledge on AAT and animal reservoirs of HAT.

The present study was designed to identify trypanosome species in naturally infected horses and donkeys of three active sleeping sickness foci of Chad and to see if these animals can serve as reservoir hosts for *T. b. gambiense*.

Method

Study sites

This cross-sectional study was conducted in three active HAT foci located in the extreme southern part of Chad. These HAT foci include the Maro, Mandoul and Moissalla (figure 1).

- The HAT focus of Mandoul (8° 6'57"N; 17°06' 58"E) was previously called the Bodo HAT focus [25]. It is located at about 50 km from Doba, the capital of "Logone Oriental" region. It has 45 villages and belongs to areas showing low risk for HAT [26-27]. Its temperature varies between 22 and 38° C and the average annual rainfall is 1000 mm [28]. The landscape is mainly dominated by forest galleries and wooded savannah that provided favorable conditions for tsetse flies. The inhabitants of this focus practice peasant farming around the forest galleries where they build their huts or houses. The main agricultural activities are cotton, millet and sesame cultivation. Inhabitants also practice extensive animal breeding (cattle, sheep, goats, pigs and some equines). During the dry season, the Mandoul River offers a meadow to many Bororos herders in transhumance.

- The Mara HAT focus (8°28'33"N; 18°46'10"E) is located at 55 km from Sarh, the capital of the "Moyen Chari" region. It contains 33 villages and belongs to foci showing moderate risk for HAT [27]. Most of these villages are located near the great Sido River. Its temperature varies between 25 to 38° C and the precipitation varies between 800 mm to 1300 mm. The vegetation is made up of savannah and clear forests with dotted trees. This vegetation offers favorable environmental conditions for the reproduction and survival of tsetse flies. Inhabitants of this HAT focus practice peasant agriculture with millet and cassava cultivation being the most predominant agricultural activities. They also practice fishing, gathering, hunting and animal breeding (cattle, sheep, goats, pigs and horses). The presence of nomadic pastoralists such as Bororo and Arabs leads to a very large cross-border movement of populations between Chad and the Central African Republic.

- The Moissala HAT focus (8°20'25"N; 17°45'58"E) is part of the great historical HAT focus of Middle Chari [25]. It extends on the left and the right sites by Nana-Barya, along the complicated network of Moula, Dou and Bahr Kô, between Bahr Sara (Ouham) and Chari. It is located in the South of Koumra, the Capital of the Mandoul region, at about 400 km of the Central Africa Republic border. It has 25 villages and belongs to foci showing moderate risk for HAT [27]. The temperature varies between 24 and 38° C and the average annual rainfall is about 1100 mm. The vegetation is formed by forest galleries which offer favorable conditions for the reproduction and development of tsetse flies. Inhabitants of this HAT focus practice peasant farming cotton, millet and sesame cultivation. They also practice extensive animal breeding (cattle, sheep, goats, pigs and some equines).

In the three HAT foci, the majority of inhabitants were traditional small farm holders practicing small scale animal husbandry. Sheep and goats are usually reared together with cattle. Donkeys and horses are commonly used for transportation and traction. The grazing system is essentially free grazing.

Ethical considerations

The protocol of this study was approved by the Bioethics Committee according to the decree: N° 462/PR/PM/MESRI/SG/CNBT/2017. Subsequently, the review board of the molecular parasitology and entomology unit of the Department of Biochemistry of the Faculty of Science of the University of Dschang gave its approval. Two weeks before the sampling, a sensitization mission was performed in each HAT focus. During each mission, the local administration, the religious and traditional authorities of each HAT focus were informed and the objectives of the study were explained in detail. These authorities gave their approval before all samples collection. Verbal consent was obtained from all farmers whose animals were included in the study after a detailed explanation of the study and its objectives.

Sample collection, immunologic and parasitological analyses

Donkeys and horses were sampled during field surveys in three active HAT foci of Chad. The first survey was performed from April to May 2018 and the second one in 2019 during the same period. Before each survey, the objective of the study was re-explained to inhabitants and local authorities of the villages. One day before the sampling, the inhabitants were asked to restrain and/or keep their animals. In each village, all donkeys and horses that had spent at least 3 months in the study zone were selected. From each animal, about 5 ml of blood were collected into EDTA coated tubes. The collection was performed from the jugular vein in horses and donkeys. The tubes were labelled and carefully packed to avoid crossed contamination. All horses sampled in this study were of “Poney du Logone” or “Poney Musey” breed while donkeys were local breed [29].

Capillary tube centrifugation test, as described by Woo [30], was performed on each blood sample to search for trypanosomes. To identify animals that had been in contact with *T. b. gambiense*, the gHAT rapid diagnostic test (RDT) was performed in parallel as described by Matovu et al. [31]. The RDT named SD BIOLINE HAT was used in this study. It was developed using native VSGs (Nat-LiTat 1.3 and Nat-LiTat 1.5) obtained from the Institute of Tropical Medicine (ITM) in Antwerp, Belgium [31]. It detects anti-VSG LiTat 1.3 and anti-VSG LiTat 1.5 antibodies [32, 33, 34].

At the end of immunological and parasitological tests, the remaining blood samples were centrifuged at 13,000 rpm for 5 minutes. The buffy coat was transferred into 1.5 mL micro-tubes and stored in an electric cooler. The buffy coat samples were transported, in the electric cooler, to the molecular parasitology and entomology unit of the Department of Biochemistry of the Faculty of Science of the University of Dschang in Cameroon. They were stored at -20° until DNA extraction for molecular analyses.

During sampling collection, each animal was examined by a veterinarian and its clinical status recorded.

Extraction of genomic DNA

Genomic DNA was extracted from each buffy coat sample using the Cethyl Trimethyl Ammonium Bromide (CTAB) method. Briefly, 500 µL of buffy coat and 1 mL of nuclease free water were mixed in a 2 mL micro-tube. The mixture was vigorously homogenized and then centrifuged at 11,000 rpm for 15 minutes. The supernatant was removed and 600 µL of CTAB buffer (CTAB at 5%; 1 M Tris, pH 8; 0.5 M

EDTA, pH 8; 5 M NaCl) was added to the resulting pellet. This latter was re-suspended and incubated in a water bath at 60°C for 30 min. To the content of each micro-tube, 600µL of chloroform/isoamyl alcohol (24/1) mixture were added. Each micro-tube was slowly homogenized for 15 min and the upper aqueous phase was removed and transferred to a new 1.5 mL micro-tube. DNA was precipitated by adding 600 µL of isopropanol. The mixture was gently homogenized for 5 min and then incubated overnight at -20° C. After this incubation, each micro-tube was centrifuged at 13,000 rpm for 15 min. DNA pellet was then washed twice with cold 70% ethanol and dried overnight at room temperature. The resulting DNA pellet was re-suspended in 50 µL of sterile nuclease-free water and then stored at -20° C until use.

Molecular identification of different trypanosome species

Trypanosome identification was achieved by amplifying the internal transcribed spacer 1 (ITS1) of ribosomal DNA of different trypanosome species as described by Ravel et al. [35]. For this identification, two PCR rounds were performed; the first round was carried out in a final volume of 25 µL containing 1X PCR buffer [10 mM Tris - HCl (pH 9.0), 50 mM KCl], 2 mM MgCl₂, 1 µL (10 picomoles) of each primer (5'-CAAATTGCCAATGTCG-3'/5'-GCTGCGTTCTTCAACGAA-3'), 0.5 µL (200 mM) of dNTPs, 1 µL (one unit) of Taq DNA polymerase (New England Biolab 5 U/µL), 5 µL of DNA extract and 14 µL of nuclease free water. The amplification program began with a denaturation step at 94° C for 3 minutes and 30 seconds followed by 30 amplification cycles; each of these cycles contained a denaturation step at 94° C for 30 seconds, an annealing step at 58° C for one minute, and an extension step at 72° C for one minute followed by a final extension at 72° C for 5 minutes.

The amplified products of the first PCR round were diluted 10 fold and 3 µL of each dilution was used as template for the second PCR round. This latter was performed with two other primers (5'-CCTGCAGCTGGATCAT-3'/5'-ATCGCGACACGTTGTG-3'). The amplification program was similar to that of the first PCR round. After the nested PCR, amplicons were separated by electrophoresis on 2% agarose gel that was subsequently stained with ethidium bromide and visualized under UV light.

Different trypanosome species were identified on the basis of length polymorphism of their ITS1 fragments. For instance, *T. congolense* strains generate DNA fragments of around 650bp (630bp for *T. congolense* forest and 610bp for savannah) while fragments of about 150bp and 400bp were respectively expected for *T. vivax* and all trypanosomes belonging to the sub genus *Trypanozoon* (*T. brucei* s.l., *T. evansi*, *T. equiperdum*).

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

Following the amplification of ITS1 sequences, all samples that showed DNA fragment between 600 to 650 bp, corresponding to the expected size of *T. congolense* species, were subjected to another PCR where specific primers were used to identify *T. congolense* forest "type" or *T. congolense* savannah "type". These specific identifications were done as described by Simo et al. [8] using TCF₁ (5'-GGACACACGCCAGAAGGTACTT-3') and TCF₂ (5'-GTTCTCTCGCACCAAATCCAAC-3') primers for *T. congolense* forest "type" [36] and TCS₁ (5'-CGAGCGAGAACGGGCAC-3') and TCS₂ (5'-

GGGACAAACAAATCCCGC-3') primers for *T. congolense* savannah "type" [37]. PCR reactions were carried out in a final volume of 25 µL containing 1x PCR buffer [10 mM Tris - HCl (pH 9.0), 50 mM KCl], 3 mM MgCl₂, 1 µL (15 picomoles) of each primer, 0.5 µL (200mM) of dNTPs, 1 µL (one unit) of Taq DNA polymerase, 3 µL of DNA extracts and 16 µL of sterile water. The amplification program was comprised of a denaturation step at 94° C for 3 min 30 s, followed by 40 amplification cycles comprising, for each cycle, a denaturation step at 94° C for 30 seconds, a hybridization step at 60° C for one minute and elongation step at 72° C for 1 minute. A final elongation was done at 72° C for 5 minutes.

The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide (0.3 µg / ml). The DNA bands were visualized under ultraviolet (UV) light and then photographed.

Search for *Trypanosoma brucei gambiense*

This was done only on samples that showed a DNA fragment of about 400 bp corresponding to the expected size of trypanosomes belonging to the subgenus *Trypanozoon* (*T. b. brucei*, *T. evansi*, *T. b. gambiense*, *T. b. rhodesiense*). On these samples, *T. b. gambiense* was identified as described by Cordon-Obras et al. [12]. This was done using a nested PCR with two pairs of primers specific to *T. b. gambiense*. The primer pairs TgSGP1 (5'GCT GCT GTG TTC GGA GAG C-3' and TgSGP2- (5'-GCC ATC GTG CTT GCC GCT C-3') described by Radwanska et al. [38] (2002) and, TgsGPs (5'-TCA GAC AGG GCT GTA ATA GCA AGC-3') and TgsGPas (5'-GGG CTC CTG CCT CAA TTG CTG CA-3') designed by Morrison et al. [39] were used.

The first PCR round was carried out in a total volume of 25 µl containing 2.5 µL of 10X PCR buffer [Tris - 10 mM HCl (pH 9.0), 50 mM KCl, 3 mM MgCl₂], 1 µL (15 picomoles) of each of primers (TgSGP1/2), 0.5 µL (100 mM) of dNTPs, 1 µL (one unit) of Taq DNA polymerase, 5 µL of DNA extract and 14 µL of sterile water. The amplification program contained an initial denaturation at 95° C for 3 minutes followed by 45 cycles of 95° C for 30 seconds, 63° C for 1 minute and 72° C for 1 minute. A final elongation was done at 72° C for 5 minutes. Amplified products of the first PCR were diluted 10 times and 5 µL of each dilution were used as DNA template for the second PCR round. For this latter, primers TgsGPs and TgsGPas were used and only 25 amplification cycles were performed in the same conditions as for the first PCR round.

The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide (0.3µg/ml). DNA bands were visualized under ultraviolet (UV) light and then photographed.

Data analyses

Statistical analyses were performed to compare the trypanosome infection rates between animal species and HAT foci. This was done using the XLSTAT 2016 software. The Chi² test (χ^2) was used to compare, between animal species and different HAT foci, the infection rates of different trypanosomes. The threshold for significance was set at below 5%. To estimate the concordance between results generated

by the tests used to identify different trypanosome infections, the kappa coefficient was determined according to Cohen [40], and interpreted as described by Altman [41].

Results

Results of parasitological (CTC) and immunological (RDT) tests

For this study, 286 animals including 155 (54.19%) donkeys and 131 (45.80%) horses were sampled in the three HAT foci of Chad. Forty seven (16.43%) animals including 30 (10.48%) donkeys and 17 (5.94%) horses were from the Mandoul HAT focus, 180 (62.93%) including 84 (29.37%) donkeys and 96 (33.56%) horses from the Maro HAT focus and 59 (20.62%) animals including 41 (14.33%) donkeys and 17 (5.94%) horses from the Moissala HAT foci (Table 1). Comparing results of RDT between the three HAT foci, slight variations were observed without any significant difference ($X^2 = 0.19$; $P = 0.91$) (Table 1). Similarly, no statistically significant difference was observed in the trypanosome infection rates despite some little variations between HAT foci for the parasitological tests (CTC) ($X^2 = 0.04$; $P = 0.98$) (Table 1).

Of the 286 equines, 54 (18.9%) were positive for RDT: 32 (20.6%) donkeys and 22 (16.8%) horses (Table 2). Between the number horses and donkeys positive to RDTs, no significant difference was observed ($X^2 = 0.69$; $P = 0.41$) was observed (Table 1). The parasitological test (CTC) enabled to revealed trypanosomes in 36 (12.6%) animals: 22 (14.2%) donkeys and 14 (10.7%) horses. Between these animals, no significant difference ($X^2 = 0.79$; $P = 0.37$) was observed in their trypanosome infection rates (Table 2).

Molecular detection of different trypanosomes

In this study, a variety of trypanosome species and subspecies including *T. vivax*, *T. congolense* forest and savannah, and trypanosomes belonging to the subgenus *Trypanozoon* were identified. From 286 animals that were examined in this study, trypanosome DNA was found in 194 of them (Tables 1 and 2). This gives an overall prevalence of 67.8% (194/286); 71.6% (111/155) in donkeys and 63.4% (83/131) in horses (Table 2). At the species level, *T. vivax* had the lowest infection rate (4.5%) followed by *T. congolense* (16.4%). Amongst the 47 animals found with *T. congolense* infections, 33 (11.5%) were due to *T. congolense* forest and 14 (4.9%) to *T. congolense* savannah. Between horses and donkeys, no significant difference was found in the trypanosome infection rates neither for *T. vivax* nor for different *T. congolense* subspecies.

Trypanosomes belonging to the sub genus *Trypanozoon* (*T. evansi*, *T. equiperdum* and *T. brucei*) were found with the highest infection rate of 29.4%. Between horses and donkeys, no significant difference ($X^2 = 0.82$; $P = 0.37$) was found in the infection rates of trypanosomes of the subgenus *Trypanozoon* (Table 2). The primers used to identify trypanosomes were not able to differentiate trypanosomes of the subgenus *Trypanozoon*. Therefore, the 84 animals found with DNA fragment of the molecular size of

trypanosomes of the subgenus *Trypanozoon* could be infected by *T. brucei* s.l., *T. evansi*, *T. equiperdum* or a mixture of two or three of these subspecies.

Between HAT foci, the trypanosome infection rates varied slightly without any significant difference (Table 1). The highest trypanosome infection rate of 76.6% (36/47) was observed in the Mandoul HAT focus (Table 1). No significant difference was found in the infection rates of different trypanosome species and subspecies despite some slight variations observed between HAT foci (Table 1).

Mixed infections involving double and triple infections were found in 25.2% (72/286) of animals: 23.9% (37/155) of donkeys and 26.7% (35/131) of horses. The double infections include *T. congolense* with trypanosomes of the subgenus *Trypanozoon*, *T. vivax* with trypanosomes of the subgenus *Trypanozoon*, and *T. congolense* with *T. vivax*. Double infections of *T. congolense* with trypanosomes of the subgenus *Trypanozoon* were higher in donkeys (17.41%) compared to horses (15.26). Triple infections were found in 1.5% (2/131) of donkeys and 3.2% (5/155) of horses. They include *T. congolense*, *T. vivax* and trypanosomes of the subgenus *Trypanozoon*. Such mixed infections were underestimated given that mixed infections could occur amongst trypanosomes of the subgenus *Trypanozoon*.

Molecular identification of *T. b. gambiense*

The two set of primers used to search for *T. b. gambiense* infections enabled to amplify a DNA fragment of 270bp which is specific to this *T. brucei* subspecies (Figure 2). Of the 84 samples found with trypanosomes of the subgenus *Trypanozoon*, three animals were revealed with infections due to *T. b. gambiense*; 2 donkeys and one horse (Table 2). This gives an overall infection rate of 1.0% (3/286) for *T. b. gambiense*: 1.3% (2/155) in donkeys and 0.8% (1/131) in horses. No significant difference ($\chi^2 = 0.19$; $P = 0.66$) was found between *T. b. gambiense* infections in donkeys and horses. Remarkably, *T. b. gambiense* infections were found only in three animals of the Maro HAT focus; giving thus an overall infection rate of 1.7% (3/180). This *T. brucei* subspecies was not found in animals sampled in the Mandoul and Moissala HAT foci (Table 1).

Concordance between RDT, CTC and PCR for the detection of *T. b. gambiense*

From the 286 animals examined in this study, 54 were positive for RDT, 36 for CTC and 194 for PCR. Concordant results between RDT and CTC were reported for 256 (89.5%) samples: 25 (8.7%) and 260 (90.9%) samples were respectively positive and negative for both tests. Between RDT and CTC, the value of concordance index expressed here as kappa Cohen coefficient was 0.48 ± 0.06951 (95%CI: 0.34337 - 0.6158), indicating a moderate strength of agreement.

Regarding RDT and PCR, these tests were concordant for 240 (86.9%) samples: 38 (13.3%) and 202 (70.6%) samples were respectively positive and negative for both tests. The value of kappa Cohen coefficient was 0.42 ± 0.05991 (95%CI: 0.29921 - 0.53406); indicating also a moderate strength of agreement for RDT and PCR.

Between RDT and PCR detecting *T. b. gambiense*, these tests were concordant for 235 (82.2%) samples: 3 (1.04%) and 232 (81.1%) samples were simultaneously positive and negative for both tests. The value of kappa Cohen was 0.087 ± 0.04730 (95%CI: -0.00559 - 0.17983). These results indicate poor strength of agreement between these tests.

Discussion

To address the problem linked to African trypanosomiases, considerable efforts have been undertaken to identify trypanosomes in tsetse and various animals of HAT foci in west and central Africa. Despite data generated on trypanosome infections in animals from these foci, equine trypanosomiasis has not been addressed. It is to fill this knowledge gap that the presence of different trypanosome species was investigated in horses and donkeys from three HAT foci of Chad. This first study on equine trypanosomiasis in central African HAT foci revealed several trypanosome species and subspecies including *T. congolense*, *T. vivax* and trypanosomes of the subgenus *Trypanozoon* in donkeys and horses. These results are in agreement with those reported elsewhere [42, 43, 44].

The high infection rate of 67.8% revealed by PCR-based methods compared to 12.6% obtained with CTC suggests that most animals were infected by trypanosomes with low parasitaemia that was below the detection threshold of the CTC. Although our overall infection rate of 67.8% is lower than 91% reported by Pinchbeck et al. [44] in West Africa, this value is considerably higher than any of the previously published figures, which range from 7% [45] to 61% [43]. The differences reported by these studies could be explained by the methods used to identify trypanosomes, the sampling sites and the population of investigated animals. Previously published data are largely based on microscopy which, from this study and previous ones [42, 44], exhibits a much lower sensitivity compared to 1–20 trypanosomes/ml for PCR-based method [46]. In studies reporting higher prevalence of above 90%, the majority of animals were clinical unhealthy and consequently, were more likely to be infected by trypanosomes [43, 44].

The infection rate reported here is high than 27.08 % and 18.7% respectively reported in domestic and wild animals of other Central African HAT foci [9, 11]. These differences may result from the animal species and the transmission patterns in each setting. Horses and donkeys were investigated here while pigs, sheep, goats, primates, rodents, carnivores and pangolins were analyzed in other studies [9, 11]. Moreover, the environmental conditions in HAT foci of Chad are different from those of HAT foci of the forest regions. In such contexts, the trypanosomes' transmission will vary in response to the diversity of tsetse fauna.

The identification of *T. congolense* forest and savannah, *T. vivax* and trypanosomes of the sub genus *Trypanozoon* indicates the presence of AAT in HAT foci of Chad. The high infection rate reported in donkeys (71.6%) than horses (63.4%) contradicts results obtained elsewhere [44, 47]. In general, horses are considered more susceptible to trypanosome infections than donkeys [44]. Although the reasons explaining the high susceptibility of donkeys are still unknown, we can speculate on; i) the nutritional behavior of vector populations, ii) the density of biting flies that are responsible for the mechanical and

cyclical transmission of trypanosomes and, iii) the behavior of animal species in each epidemiological setting. These factors could interfere with trypanosomes' transmission and consequently, on the infection rates. The absence of significant difference in the prevalence of any trypanosome species suggests similar transmission pattern of trypanosomes in horses and donkeys, and also in different HAT foci. This could be explained by the fact that horses and donkeys are used for the same purposes and hence, are exposed to the similar levels of trypanosomes' transmission. Entomological investigations on tsetse (blood meal analysis, dynamics of tsetse populations) and other biting arthropods could enable to determine their nutritional behavior in relationship to horses and donkeys and consequently, the probability for each animal to acquire trypanosome infections.

Although molecular tests enabled, with relatively high sensitivity and excellent specificity, to identify *T. congolense*, *T. vivax* and *Trypanozoon* taxa, no single test is able to differentiate unequivocally trypanosomes of the sub genus *Trypanozoon* [24]. The presence of tsetse flies in HAT foci of Chad indicates that some trypanosomes belonging to the subgenus *Trypanozoon* may be due to *T. brucei s.l.* This hypothesis is strengthened by the identification of *T. b. gambiense* in animals of one HAT focus. The geographical localization of these HAT foci does not exclude the possibility of having, in addition to *T. vivax*, *T. evansi* infections that can be mechanical transmitted by some biting flies [48]. This hypothesis is more plausible with previous identification of mechanical vectors such as *Stomoxys* and *Tabanidae* in these HAT foci [26]. Investigations on the nutritional behavior of biting flies could enable to better understand trypanosomes' transmission in each HAT focus. The slightly high prevalence of trypanosomes of the subgenus *Trypanozoon* in donkeys (31.6%) compared to horses (26.7%) is in agreement with results obtained in the Gambia [44]. In addition, the slightly low prevalence of *T. vivax* infections in donkeys (3.9%) than horses (5.3%) corroborates also results of Pinchbeck et al. [44]. Understanding these differences requires subsequent investigations on the nutritional behavior of different biting flies.

Compared with *T. congolense* prevalence of 64% reported by Dhollander *et al.* [43], our low prevalence of 16.4% could be explained by the fact that the majority of animals previously investigated were anaemic and consequently, were more likely to carry trypanosome infections. The co-existence of *T. congolense* forest and savannah indicates that the geographical limit (*T. congolense* savannah and forest in the savannah and forest zones, respectively) tends to change with time. The high infection rate of *T. congolense* forest (12.9%) compared to *T. congolense* savannah (4.9%) could be explained by the geographical localization of most HAT foci in the forest galleries.

In addition to simple infections, the present study showed that approximately 25.2% of animals (23.9% of donkeys and 26.7% of horses) carried mixed infections comprising different trypanosome species and subspecies. These results are in agreement with previous observations highlighting that mixed infections may be more frequent where several species co-exist [42, 44]. It is important to point out that these mixed infections are probably underestimated because some mixed infections could exist between trypanosomes (*T. evansi*, *T. equiperdum* and *T. brucei s.l.*) of the subgenus *Trypanozoon*. With the high

number of double and triple infections reported in this study, there is a need to understand their evolution and their potential impacts on animal health, and the transmission dynamics of trypanosomes.

This study revealed for the first time *T. b. gambiense* in animals of HAT foci of Chad, especially in horses and donkeys. These results suggest horses and donkeys as potential reservoirs of *T. b. gambiense* in HAT foci of Chad. Interestingly, no animal from the Mandoul and Moissala HAT foci was found with infection due to *T. b. gambiense*. The three infections found in horses and donkeys were reported in the HAT focus of Maro. These results could be explained by vector control initiated since 2013 in some HAT foci [49] through the deployment of tiny targets for the reduction of tsetse density and consequently, the trypanosomes' transmission [50]. Compared to other HAT foci of Chad, the Maro focus reports the highest number of *T. b. gambiense* infections in humans. Putting together results of *T. b. gambiense* infections in human and animals, it appears that the human-infective trypanosome was found in animals when the disease prevalence was high in humans. These observations contradict those of other HAT foci where *T. b. gambiense* was detected in animals of HAT foci showing low to very low disease prevalence [8, 12, 13]. The discrepancies between these results could be linked to animal species as well as the epidemiological patterns in each focus. In our study, horses and donkeys are regularly in close contact with human and therefore, could be more subjected to tsetse flies that have fed on humans infected by trypanosomes. In such context, these animals are more likely to be involved in the transmission cycle involving human (human-tsetse-horses/donkey). In other studies, the animals identified as potential reservoir for *T. b. gambiense* are more likely involved in animal transmission cycle [12, 13, 51, 52, 53]. Investigations on tsetse blood meals from different HAT foci could improve the understanding of the contact frequency between tsetse and such animals. The epidemiology implications of these animals may vary according to the epidemiological patterns of each HAT focus.

The RDT used here is an immune-chromatographic test for the screening of HAT and it was expected to be positive only if the host has been in contact with *T. b. gambiense* [54]. The sero-prevalence of 18.9% revealed here is too high since only 3 (1.04%) animals were found with *T. b. gambiense* infections. The low specificity of RDT is in line with observations by Matovu et al. [31] reporting similar specificity in animals from AAT endemic region. It may results from the fact that the antigens used in RDTs could cross-react with epitopes of other trypanosome species, but are not probably predominant in these species [31]. This is not surprising if we considered the high similarity reported at the genomic level between different trypanosome species [55, 56]. As already reported in cattle [31], the RDT used for specific identification of *T. b. gambiense* seems not convenient for horses and donkeys. This hypothesis is strengthened by the low Kappa cohen coefficient; thus indicating the low strength of agreement between RDT and the PCR used to identify *T. b. gambiense* in animals. The Kappa cohen coefficients remain low between RDT and CTC as well as RDT and PCR, indicating also low or moderate agreement between these tests. All the results plaid for the fact that the antigens used in RDT may cross-react with other antigens not yet identified.

Conclusion

This study revealed high natural trypanosome infection rate and several trypanosome species and sub species in donkeys and horses of HAT foci of Chad. The absence of significant difference in the infection rate of different trypanosome species or subspecies suggests similar transmission patterns of trypanosomes in these HAT foci. The identification of *T. b. gambiense* in donkeys and horses suggests that these animals are potential reservoirs of human-infective trypanosomes in Chad. These animals must be taken into account for refining of control strategies aiming to eliminate and interrupt HAT transmission. The identification of several animal trypanosomes as well as human-infective trypanosome highlights the need of developing control strategies to fight HAT and AAT with the overarching goal of improving animal and human health.

Declarations

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Authors' contributions

JV contributed to study design, sample collection and molecular identification of trypanosomes. TZAA helped in the molecular identification of trypanosomes and the drafting of the manuscript. MM participated in the conception of the study. GS participated in the conception and the drafting of the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this article.

Ethics approval and consent to participate

This study was approved by the Bioethics Committee according to the decree: N° 462/PR/PM/MESRI/SG/CNBT/2017. Subsequently, the review board of the molecular parasitology and entomology unit of the Department of Biochemistry of the Faculty of Science of the University of Dschang gave its approval.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: Trypanosome infections according to HAT foci

HAT foci	NE	RDT+	T+	PCR results						
				TB	TC+	TCS+	TCF+	TV+	TBG+	Total
Mandoul	47	8 (17.0%)	6 (12.8%)	17 (36.2%)	8 (17.0%)	1 (2.1%)	7 (14.9%)	3 (6.8%)	0	36 (76.6%)
Maro	180	34 (18.9%)	23 (12.8%)	49 (27.2%)	31 (17.2%)	9 (5.0%)	22 (12.2%)	5 (2.8%)	3 (1.7%)	119 (66.1%)
Moissala	59	12 (20.3%)	7 (11.9%)	18 (30.5) %	8 (1.4%)	4 (6.8)	8 (13.5%)	5 (8.5%)	0	39 (66.1%)
Total	286	54 (18.9%)	36 (12.6%)	84 (29.4%)	47 (16.4%)	14 (4.9%)	37 (12.9%)	13 (4.5%)	3 (1.04%)	194 (67.8%)
χ^2	-	0.19	0.04	1.48	0.45	4.17	1.12	3.76	1.79	
p-value	-	0.91	0.98	0.48	0.80	0.12	0.57	0.15	0.41	

NE: Number of animals examined; RDT: Rapid diagnosis test; CTC: T+: trypanosome infections revealed by Capillary tube centrifugation; TB: trypanosomes belonging to the subgenus *Trypanozoon*; TC: *Trypanosoma congolense*; TCS: *Trypanosoma congolense* savannah type; TCF: *Trypanosoma congolense* forest type; TV: *Trypanosoma vivax*; TBG: *Trypanosoma brucei gambiense*.

Table 2: Trypanosome infections according to animal species

Animal species	NE	RDT+	T+	PCR results						
				TB	TC+	TCS+	TCF+	TV+	TBG+	Total
Donkeys	155	32 (20.6%)	22 (14.2%)	49 (31.6%)	27 (17.4%)	8 (5.2%)	19 (12.3%)	6 (3.9%)	2 (1.3%)	111 (71.6%)
Horses	131	22 (16.8%)	14 (10.7%)	35 (26.7%)	20 (15.3%)	6 (4.6%)	14 (10.7%)	7 (5.3%)	1 (0.8%)	83 (63.4%)
Total	286	54 (18.9%)	36 (12.6%)	84 (29.4%)	47 (16.4%)	14 (4.9%)	33 (11.5%)	13 (4.5%)	3 (1.0%)	194 (67.8%)
χ^2	-	0.69	0.79	0.82	0.24	0.70	0.14	0.35	0.19	
p-value	-	0.41	0.37	0.37	0.62	0.40	0.71	0.55	0.66	

NE: Number of animals examined; RDT: Rapid diagnosis test; CTC: T+: trypanosome infections revealed by Capillary tube centrifugation; TB: trypanosomes belonging to the subgenus *Trypanozoon*; TC: *Trypanosoma congolense*; TCS:

Trypanosoma congolense savannah type; TCF: *Trypanosoma congolense* forest type; TV: *Trypanosoma vivax*; TBG: *Trypanosoma brucei gambiense*.

Figures

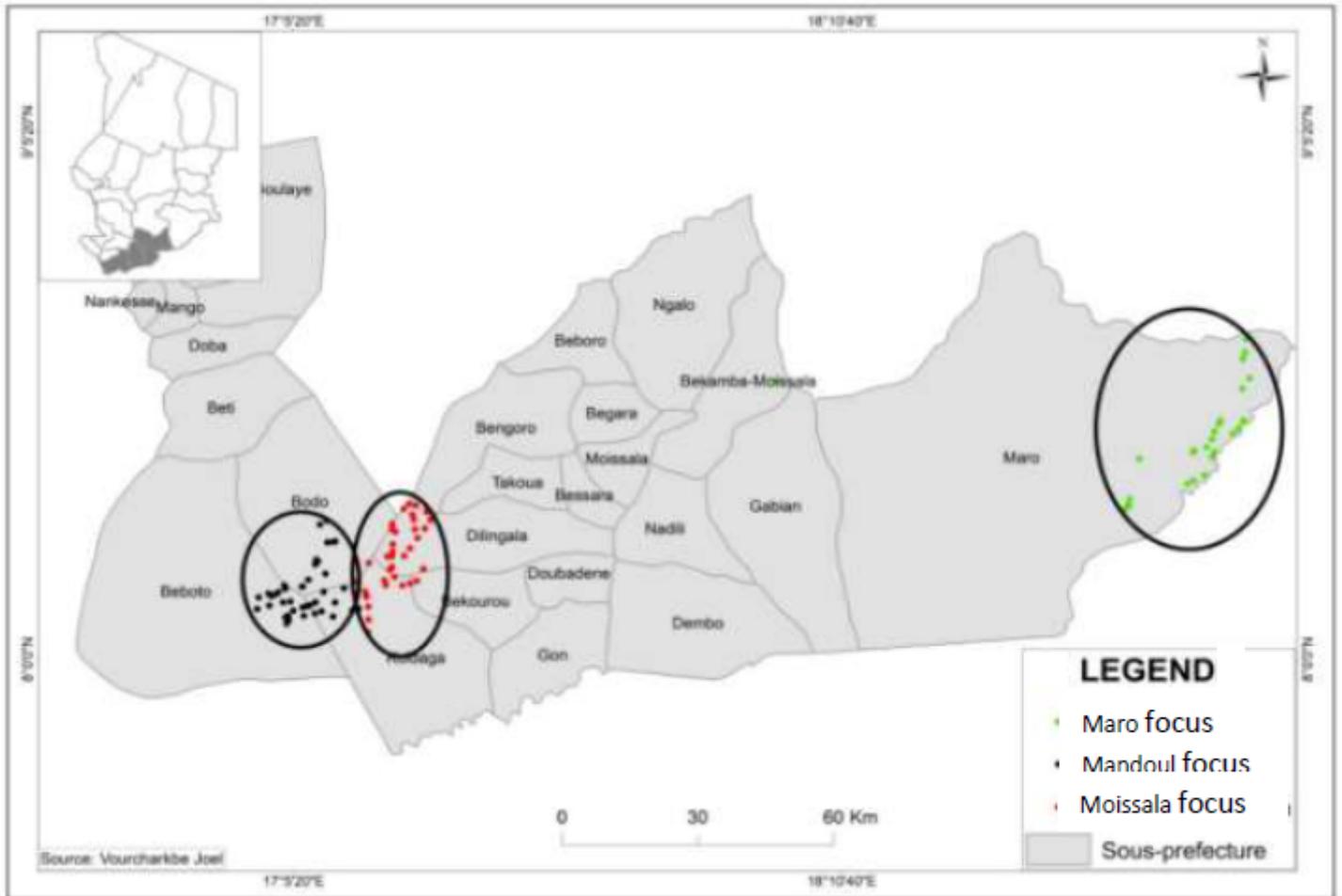


Figure 1

Map showing locations where donkeys and horses were sampled in the three active sleeping sickness foci of Chad

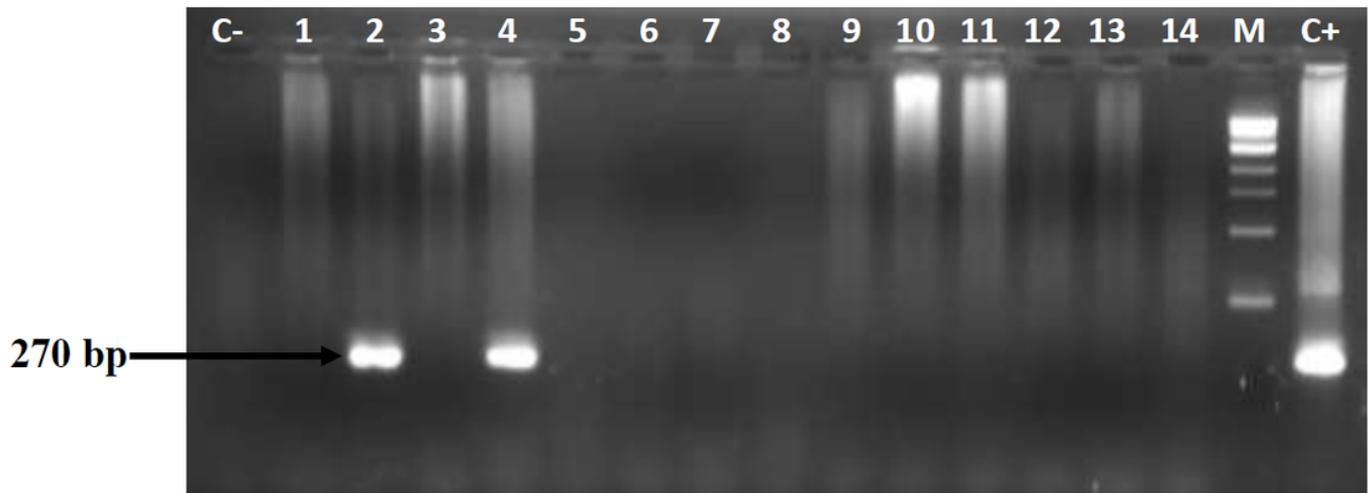


Figure 2

Electrophoretic profiles illustrating the amplification of specific DNA fragment of *T. b. gambiense* from donkey and horse samples. C-: Negative control, C+: Positive control (purified DNA from human *T. b. gambiense* isolate); M: Molecular marker (1 kb ladder); 2 and 4: samples with infections due to *T. b. gambiense*; 1, 3, 6, 7, 8, 9, 10, 11, 12, 13 and 14: samples harboring trypanosomes of the subgenus *Trypanozoon*, but without infection due to *T. b. gambiense*.

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