

Evaluation of the sensitivity of a rapid diagnostic test (RDT) detecting anti-*Trypanosoma brucei gambiense* antibodies to the polymerase chain reaction (PCR) test in animals from outbreaks of human African trypanosomiasis in Chad



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ABSTRACT

Introduction: Human African trypanosomiasis (HAT) has been targeted for interruption of transmission by 2030. This study was undertaken to assess the sensitivity of a RDT test detecting anti-*T. b. gambiense* antibodies to PCR testing in domestic animals from HAT foci in Chad.

Method: Blood samples were taken from 443 goats, 339 sheep, 228 dogs, 98 pigs, 1,466 cattle, 155 donkeys and 151 horses. The rapid diagnostic test (RDT) was used to detect trypanosomes. DNA was extracted from buffy coats and *T. b. gambiense* was identified by PCR.

Results: Of the 2,860 domestic animals sampled, 8.98% were positive for anti-*T. b. gambiense* antibodies. RDT targeting anti-*T. b. gambiense* antibodies and PCR were simultaneously positive and negative for 257 (9%) and 21 (0.73%) respectively. The RDT was positive in 236 animals (8.25%) that were PCR-negative. For RDT targeting anti-*T. b. gambiense* antibodies and PCR targeting *T. b. gambiense*, 549 samples (82.1%) showed concordant results, while discordant results were reported for 120 samples (17.9%). Of the 549 samples with concordant results, 14 (2.6%) and 535 (97.4%) were positive and negative, respectively. Sensitivity and specificity were 66.67% and 91.44% respectively. Comparing the sensitivity of TDR according to the species analyzed, it was found that this value varied from 0% in cattle, 37.50% in sheep, 60% in goats and 100% in other animals (sheep, pigs, donkeys and horses). RDT specificity ranged from 79.74% in Asians to 97% in Cattle.

Conclusion: Results comparing RDT and PCR targeting *T. b. gambiense* in each animal species were poor. RDT does not appear to be suitable for the reliable identification of *T. b. gambiense* in domestic animals.

Keywords: Evaluation, RDT, PCR, detecting, *T. b. gambiense*, Domestic animals.

1. INTRODUCTION

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease that mainly affects rural populations in sub-Saharan African countries [1]. Transmitted by an arthropod vector of the genus *Glossina*, this infectious disease is caused by protozoan parasites belonging to the *Trypanosoma brucei* species, and includes *Trypanosoma brucei brucei*, and *Trypanosoma brucei rhodesiense* [1]. *T. b. brucei* infects domestic and wild animals and induces African animal trypanosomiasis.

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The other two species are infectious to humans and cause HAT. *T. b. gambiense* is responsible for the chronic form of the disease in humans and occurs in Central and West Africa, while *T. b. rhodesiense* is responsible for the acute form of the disease and occurs in East Africa. Thanks to the efforts of the international community and individual countries, HAT has now been greatly reduced, and is still present in only twenty or so countries [2]. Chad, RCA and RDC are among the most affected countries [3]. In Chad, the disease is particularly prevalent in the south of the country, where the most active outbreaks can be found [4]. Recent studies report a prevalence of over 8% in the Mandoul, Maro and Moissala foci [5]. Control of the disease relies on diagnosis, treatment and vector control. Until very recently, widely used diagnostic techniques for disease detection included Capillary Tube Centrifugation (CTC) and Card Agglutination Trypanosomiasis Test (CATT) [6, 7]. The recent development of rapid diagnostic tests (RDTs) now enables easier detection of HAT in humans [8]. The RDT has shown good efficacy in the diagnosis of HAT in different regions of Africa [9]. Although effective, this tool has so far not been used to detect TBG in animals, even though animals represent a major TBG reservoir [10,11]. The use of rapid diagnostic tests (RDTs) can help in the epidemiological surveillance of HAT, particularly in areas where animals are transhumant, as is the case in southern Chad. In this context, can the use of RDTs enable better diagnosis of TBG in animals? Furthermore, we don't know whether the sensitivity and specificity of this test would be affected by cases

of co-infection by different species of trypanosomes? And whether different animal species play a similar role as TBG reservoirs?

In this study, we propose to: compare the performance of diagnostic tools for the detection of *Trypanosoma brucei gambiense* in animals in Chad.

More specifically, we will:

- determine the sensitivity and specificity of rapid diagnostic tests (RDT) compared with PCR for the detection of *T. b. gambiense* in animals;
- assess the sensitivity and specificity of co-infection with different trypanosomes;
- determine the prevalence of *T. b. gambiense* in different animal species.

2. METHODS

2.1. Study sites

This cross-sectional study was carried out from February 2021 to June 2022 in three HAT foci in the extreme south of Chad.

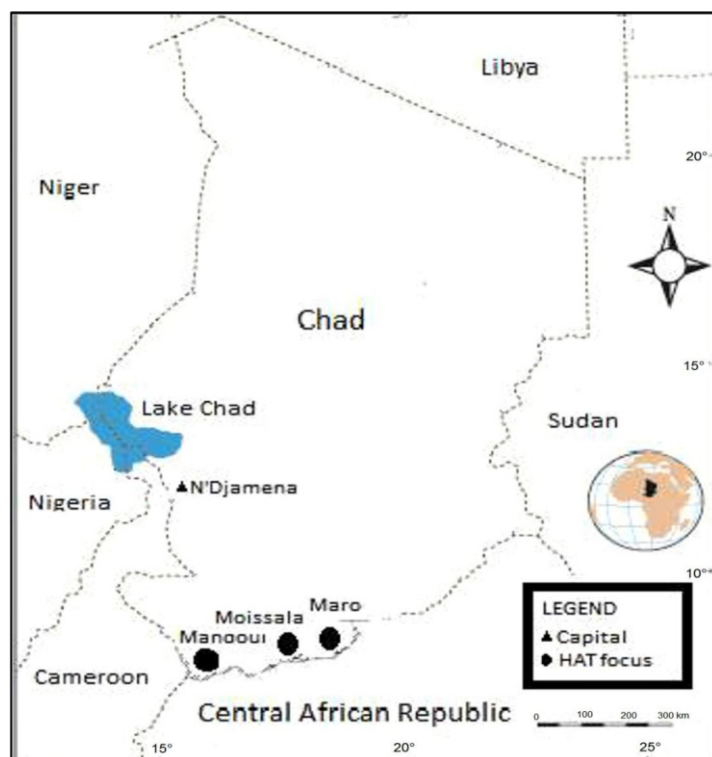


Fig. 1 Map showing sleeping sickness foci where donkeys and horses were sampled in the south of Chad

- The Mandoul HAT outbreak (8605700 N; 170605800 E) is located 50 km from Doba, the capital of the Logone Oriental region near Chad's border with Cameroon and the Central African Republic. One of the most active foci for *T. b. gambiense* transmission [12, 4], it covers 41 villages with an estimated population of 13.799. The temperature in this region ranges from 22 to 38 °C and the average annual rainfall is 1.000 mm [13]. The landscape is dominated by gallery forest and wooded savannah. The domestic animals examined came from the 41 villages in the household
- The Maro THA focus (82803300 N; 184601000 E) is located 55 km from Sarh, the capital of the Moyen-Chari region. It lies on the border with the Central African Republic, and comprises 33 villages with an estimated total population of 8.526. It is one of the HAT foci presenting a moderate risk of *T. b. gambiense* infection [2]. Temperatures range from 25 to 38 °C, and rainfall from 800 mm to 1300 mm.

Vegetation consists of savannah and open forest dotted with trees. For this study, animals were sampled in 31 villages.

- The Moissala THA focus (82002500 N; 174505800 E) is part of the large historical THA focus of the Moyen Chari. It stretches along the network dominated by the Nana-Barya River between the Bahr Sara (Ouham) and the Chari rivers. Located south of Koumra, the capital of the Mandoul region, around 400 km from the border with the Central African Republic, the Moissala focus is one of the HAT foci at moderate risk of infection by *T. b. gambiense* [2]. Its population is estimated at 12.234, spread over 41 villages. In this HAT outbreak, the temperature varies between 24 and 38 °C with an average annual rainfall of 1100 mm. Vegetation is dominated by gallery forests. The domestic animals examined came from 42 villages.

2.2. Sample collection

Domestic animals, including pigs, dogs, sheep and goats, were sampled during three field surveys carried out in villages in each of the three HAT foci in Chad. Sampling was carried out in all villages where at least one case of HAT had been reported in the last decade. These villages were selected based on (i) their proximity to villages where a case of HAT had been reported, (ii) the presence of different trypanosome species; and (iii) the presence of biotopes favorable to tsetse fly and trypanosome transmission. The first survey was carried out from March 7 to 27, 2021 in the Maro household, the second from April 2 to May 12, 2021 in the de Mandoul household, and the last survey from May 25 to June 14, 2022 in the Moissala household. Before each survey, the aim of the study was explained for the second time to the inhabitants of each THA household. Before each sampling, the inhabitants of each village were invited to tie their animals around their houses. In each village, only animals that had spent at least 3 months in the study area were selected. With the cooperation of the owners, around 5 ml of blood was collected from each pet and introduced into EDTA tubes. Blood was collected from the jugular vein in goats and sheep, while in pigs and dogs it was collected from the subclavicular and cephalic vein respectively. In some dogs, blood samples were not collected due to their aggressiveness. Each blood tube was labelled and carefully packaged.

2.3. Immunological and parasitological tests

The immunological test or gambiense-HAT rapid diagnostic test (RDT) was used to identify animals thought to have been in contact with *T. b. gambiense*. The RDT named SD BIOLINE THA was the test used in this study. Developed using native variable surface glycoproteins (VSGs) (Nat-LiTaT 1.3 and Nat-LiTaT 1.5) obtained from the Institute of Tropical Medicine (ITM) in Belgium, this test detects anti-VSG LiTaT 1.3 and anti-VSG LiTaT 1.5 antibodies [14, 15]. This TDR test was performed as described by Matovu [16]. Blood samples remaining in EDTA tubes were centrifuged at 13,000 rpm for 5 minutes. After centrifugation, the buffy coat was removed from each tube and transferred to 1.5 ml microtubes, which were stored in an electric cooler and transported to the laboratory. They were then stored at -20°C until DNA extraction for molecular analysis.

2.4. Extraction of genomic DNA

DNA was extracted from each buffy coat using the cetyltrimethyl ammonium bromide (CTAB) method described by Navajas [17]. Briefly, in each 2 ml micro-tube, 500 µl of buffy coat was mixed with 1 ml of water. The mixture was centrifuged at 11.000 rpm for 15 minutes.

The supernatant was discarded, each microtube was vigorously homogenized and 600 µl of CTAB buffer (5% CTAB; 1 M Tris, pH 8; 0.5 M EDTA, pH 8; 5 M NaCl) was added to the pellet. This mixture was resuspended and incubated in a water bath at 60°C for 30 minutes. Next, 600 µl of chloroform/isoamyl alcohol mixture (24 parts chloroform/1part isoamyl alcohol) was added to the contents of each microtube. This new mixture was then slowly homogenized for 15 minutes and centrifuged at 11,000 rpm for 15 minutes. The upper aqueous phase containing the nucleic acids was removed and transferred to another 1.5 ml microtube. DNA was precipitated by the addition of 600 µl isopropanol. After gently homogenizing each microtube for 5 minutes, the tubes were incubated at -20°C for 24 hours to facilitate nucleic acid precipitation. The mixture was then centrifuged at 13,000 rpm for 15 minutes. The alcohol was completely removed and the tubes were left open on the bench overnight to eliminate residual alcohol. The DNA pellet was washed twice with cold 70% ethanol and dried overnight at room temperature. The resulting DNA pellet was resuspended in 50 µl sterile water and stored at -20°C until use. This DNA extract was used for molecular identification of the various trypanosome species.

2.5. Molecular identification of *Trypanosoma brucei gambiense*

T. b. gambiense was detected in all samples showing a DNA fragment of around 177 bp corresponding to the size expected for trypanosomes of the *Trypanozoon* subgenus (*T. b. brucei*, *T. evansi*, *T. b. gambiense*, *T. b. rhodesiense* or *T. equiperdum*). Molecular identification of *T. b. gambiense* was carried out using two PCR cycles as described by Cordon-Obr [18]. During identification, two pairs of primers specific for *T. b. gambiense* were used:

TgSGP-1/2 (TgSGP-1: 50-GCT GCT GTG TTC GGA GAGC-30; TgSGP-2: 50-GCC ATC GTG CTT GCC GCT C-30) described by Radwanska [19].

and TgsGP-a/as (TgsGP: 5-TCA GAC AGG GCT GTA ATA GCA AGC-3; TgsGP-as: 5-GGG CTC CTG CCT CAA TTG CTG CA-3) designed by Morrison [20].

The first PCR run was performed in a total volume of 25 l containing 1 PCR buffer (Tris - 10 mM HCl (Ph 9.0), 50 mM KCl, 3 mM MgCl₂), 15 picomoles of each primer (TgSGP-1/2), 100 mM of each dNTP, one unit of Taq DNA polymerase, 5 l of DNA extract and 14 l of sterile water. The amplification program included an initial denaturation step at 95° C for 3 minutes. This was followed by 45 cycles comprising a denaturation step at 95° C for 30 s, a hybridization step at 63° C for 1 min and an elongation step at 72° C for 1 min. Final elongation was performed at 72° C for 5 min. Amplicons from the first PCR were diluted 10-fold and 5 µl of each dilution was then used as DNA template for the second PCR in which TgsGPs/as primers were used. In this nested PCR, only 25 cycles of amplification were performed under the same conditions as for the first PCR.

Amplicons from the second PCR were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide (0.3 µg/ml). The DNA bands were then visualized and photographed under ultraviolet (UV) light. All samples in which a DNA fragment of around 270 bp was revealed after PCR and electrophoresis were considered to carry *T. b. gambiense* infections.

2.6. Data analyses

The infection rates of trypanosomes revealed by RDT and those of *T. b. gambiense* were compared with PCR results, and the

sensitivity and specificity of RDTs were determined in different situations. These comparisons were carried out using XLSTAT 2016 software. The chi-square test (χ^2) was used to compare infection rates between animal species. The difference was considered significant if the p-value was less than 0.05.

3. RESULTS

Detection of *Trypanosoma brucei gambiense* using RDTs

Of the 2,860 domestic animals sampled, 8.98% were positive for anti-*Trypanosoma brucei gambiense* antibodies. The highest seroprevalence was observed in asians (20.64%), followed by equines (16.79%) and canines (15.78%). Seroprevalence of anti-*T. b. gambiense* antibodies was significantly different ($\chi^2 = 137.30$; $p < 0.0001$) between animal taxa (Table 1). By excluding cattle from the analysis, the prevalence of *Trypanosoma brucei gambiense* in other animals was no longer significantly different ($\chi^2 = 1.02$; $p = 0.96$), indicating that cattle do not have a similar *T. b. gambiense* prevalence rate to other animals.

Table 1: Seroprevalence of anti-*Trypanosoma brucei gambiense* antibodies by animal taxon in the Mandoul, Maro and Moissala outbreaks in southern Chad.

Animal taxa	Anti- <i>T. b. gambiense</i> antibody tests			
	Numbers examined	Positive RDT	Percentage (%)	95% Confidence Interval (%)
Goats	443	64	14.45	(11.13 - 18.45)
Sheep	339	46	13.57	(9.93 - 18.1)
Canines	228	36	15.79	(11.06 - 21.86)
Pigs	98	12	12.24	(6.33 - 21.39)
Cattle	1466	45	3.07	(2.23 - 4.10)
Asins	155	32	20.65	(14.12 - 29.14)
Equines	131	22	16.79	(0.52 - 25.43)
Total	2860	257	8.98	(7.92 - 10.154)
χ^2		137.3		
P value		< 0.0001		

3.1. PCR detection of *Trypanosoma brucei gambiense*

Using the PCR technique, except for cattle, all the other animal taxa examined in this study had infections due to *T. b. gambiense* (Table 2). Of the 21 animals infected with *T. b. gambiense*, 1.80% were goats, 1.49% sheep, 1.75% dogs, 1.02% pigs, 1.29% donkeys and 0.76% horses. The highest prevalence (1.80%) was found in goats, followed by canines (1.75%). Sheep (1.49%), pigs (1.02%) and asians (1.29%) showed similar prevalences of *T. b. gambiense* infection. In contrast, the lowest prevalence of infection (0.76%) was observed in equines. Comparing *T. b. gambiense* prevalences by species, a significant difference ($\chi^2 = 23.99$; $p = 0.0005$) was observed (Table 3). Excluding cattle, the difference was no longer significant ($\chi^2 = 1.02$; $p = 0.96$).

Table 2: *T. b. gambiense* infection rates as a function of animal taxa in the Mandoul, Maro and Moissala outbreaks in southern Chad

Animal taxa	Numbers examined	TBG-Positive to PCR	Percentage (%)	95% confidence Interval
Goats	443	8	1.80	(0.78 - 3.55)
Sheep	339	5	1.47	(0.47 - 3.44)
Canines	228	4	1.75	(0.47 - 4.49)
Pigs	98	1	1.02	(0.02 - 5.68)
Cattle	1466	0	0	0%
Asins	155	2	1.29	(0.15 - 4.66)
Equines	131	1	0.76	(0.01 - 4.25)
Total	2860	21	0.73	(0.45 - 1)
χ^2		23.99		
P value		< 0.0005		

3.2. Concordance between RDT and PCR

RDT targeting anti-*T. b. gambiense* antibodies and PCR were simultaneously positive and negative in 257 (9%) and 21 (0.73%) animals respectively. The RDT was positive in 236 animals (8.25%) that were PCR-negative. For RDT targeting anti-*T. b. gambiense* antibodies and PCR targeting *T. b. gambiense*, 549 samples (82.1%) showed concordant results, while discordant results were reported for 120 samples (17.9%). Of the 549 samples with concordant results, 14 (2.6%) and 535 (97.4%) were positive and negative, respectively (Table 3). Sensitivity and specificity were 66.67% and 91.44%

respectively. Comparing the sensitivity of RDT according to the species analyzed, this value was found to vary from 0% in cattle, 37.50% in sheep, 60% in goats and 100% in other animals (sheep, pigs, donkeys and horses). RDT specificity ranged from 79.74% in Asians to 97% in Cattle.

Table 3: Sensitivity and specificity of RDT test detecting *T. b. gambiense*

Animal	PCR+	PCR-
RDT+	14	243
RDT-	7	2596
Total	21	2839
Sensitivity	66.67%	
	(43.03% - 85.41%)	
Specificity	91.44%	
	(90.35% - 92.44%)	
Positive Predictive value (*)	0.04%	
	(0.03% - 0.05%)	
Negative Predictive Value (*)	100%	
	(100% - 100%)	
Kappa concordance index	0.08±0.02	95% CI (0.04 to 0.13)
Animals Taxa	Sensitivity	Specificity
Goats	37.50%	85.98%
Sheep	60%	87.13%
Canines	100%	85.13%
Pigs	100%	88.66%
Cattle	0%	97%
Asins	100%	79.74%
Equines	100%	83.85%

4. Discussion

RDT tests were originally developed for the detection of *Trypanoma brucei gambiense* sleeping sickness in humans. These tests have recently shown that, under certain conditions of use, their indications can be extended to other *Trypanozoon*: *Trypanoma brucei evansi* and *Trypanoma brucei brucei* [21]. The present study highlights the sensitivity of the RDT test to *Trypanosoma brucei gambiense*. The rapid diagnostic test (RDT) revealed a seroprevalence of anti-*Trypanosoma brucei gambiense* antibodies of 8.98%. The RDT is an immuno-chromatographic test developed for the detection of human African trypanosomiasis (HAT). As it should only be positive when the host has been in contact with *T. b. gambiense* [3, 22], its 8.98% seroprevalence seems too high, as only 0.73% of animals were infected with *T. b. gambiense*. These results are in line with those reported by Matovu [16] in East Africa; highlighting the low specificity of RDT for the detection of *T. b. gambiense* in domestic animals.

The seroprevalence results could be explained by the fact that a positive RDT could indicate current or past infections, while a positive PCR can be interpreted as an active infection. We cannot exclude the fact that some positive RDTs may be the result of cross-reactions with epitopes of other trypanosome species [16]. In fact, sera from animals infected with either *T. congolense*, *T. b. brucei* or *T. evansi* cross-react with antigens used in the RDT and card agglutination test for the detection of human African trypanosomiasis [23, 24, 21]. Furthermore, of from patients with *T. b. gambiense* and *T. b. rhodesiense* induced cross-reactions with many of the trypanosome antigens, including VSG LiTat 1.3 and VSG LiTat 1.5, which reacted with sera from patients with *T. b. gambiense* and *T. b. rhodesiense* [25], demonstrating cross-reactivity between the antigens of different trypanosome subspecies. The high similarity reported at the genomic level between the different trypanosome species is a further argument in favor of this cross-reactivity [26, 27]. Our results are in line with published findings on the use of RDT on domestic animals [28]. It confirms the low specificity of RDT for the identification of *T. b. gambiense* in animals. The test is therefore unsuitable for screening domestic animals for *T. b. gambiense*.

The sensitivity of RDTs was high in Sheep compared with other animals (Cattle, Dogs, Pigs, Asians and Equines), while specificity was higher in Asians. These results show that Sheep have preferential traits that attract glossiness compared to Equines. The low coefficients resulting from comparisons of RDT and PCR targeting *T. b. gambiense*, either for overall results or for each animal species, indicate a low strength of agreement between these two tests. The RDT does not appear to be suitable for reliable identification of *T. b. gambiense* in the domestic animals we studied. In comparisons between the RDT and PCR targeting trypanosomes of the *Trypanozoon* subgenus, certain variations were observed between animal species. Despite these variations, the coefficients between these tests remain low. Agreement strengths also remained low between animal species, confirming the low specificity of the RDT for the identification of trypanosomes in animals. Although the specificity of an RDT for HAT manufactured using recombinant antigens is very good when applied to animals in a region free of trypanosomiasis, it decreases considerably in a region endemic for African animal trypanosomiasis, limiting its potential as a diagnostic test. The specificity of an RDT made from native parasite antigens decreases with the age of the cattle, probably due to cross-reactions resulting from exposure to other pathogens. These cross-reactions make the tests less useful for identifying animal reservoirs of HAT [24, 23]. The seroprevalence of anti-trypanosome antibodies and the prevalence of trypanosome infections were higher in all three foci. These results could be explained not only by the favorable environmental conditions for the development of tsetse flies, in particular watercourses and gallery forests, but also by the large movements of animals between Chad and the Central African Republic.

Some animals carrying trypanosomes of the *Trypanozoon* subgenus are thought to be infected with trypanosomes belonging to species of the *T. brucei* complex. This hypothesis is confirmed by the detection of *T. b. gambiense* in the 21 animals from the three HAT outbreaks. This detection of *T. b. gambiense* infections in pigs, sheep and goats confirms previous findings in some HAT outbreaks in West and Central Africa [29, 18, 11, 30]. For this study, *T. b. gambiense* infection was detected in goats, sheep, canines, pigs, asians and horses. The presence of *T. b. gambiense* in these animals suggests that they are potential reservoirs of human-infectious trypanosomes in Chad. Living and moving together in similar environmental conditions, all the animal species studied in the present study may be subject to the same level of tsetse fly bites. The circulation of animals in areas where tsetse fly density is higher could result in an intensified risk of infection. Consequently, the most active and abundant animal species, such as goats, could be more exposed to tsetse fly bites. The absence of *T. b. gambiense* in cattle can be explained by the fact that, in January, transhumant pastoralists descend into the outbreaks in search of pasture, only to re-emerge from the outbreaks in May.

5. CONCLUSION

This study shows that the sensitivity of the RDT test is lower than its specificity for identifying *Trypanosoma brucei gambiense* in animals. The rapid diagnostic test (RDT) revealed a seroprevalence of anti-*T. b. gambiense* antibodies of 8.98%, which seems too high, as only 0.73% of animals were infected with *T. b. gambiense*. They confirm the low specificity of the RDT for identifying *Trypanosoma brucei gambiense* in animals. The test is therefore not suitable for screening domestic animals for *T. b. gambiense*.

Abbreviations

HAT: human African trypanosomiasis, WHO: World Health Organization; PCR: polymerase chain reaction; RDT: rapid diagnostic test CTAB: cethyl trimethyl ammonium bromide; ITS: internal transcribed spacer; UV: ultraviolet.

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Authors' contributions

VJ participated in the molecular identification of trypanosomes and the design and drafting of the manuscript. OIAD participated in the design of the study and DKI participated in the design and collection of samples in the field.

Availability of data and materials

All data generated and/or analyzed during this study are included in the article and its additional files.

Ethics approval and consent to participate

The protocol for this study was approved by the Bioethics Committee in accordance with Decree: No. 462/PR/PM/MESRI/SG/CNBT/2019. Two weeks before sampling, an outreach mission was conducted to each household in THA. During each mission, the local administration, religious and traditional authorities of each HAT household were informed and the objectives of the study were explained in detail. These authorities gave their consent before any samples were collected. Verbal consent was obtained from all farmers whose animals were included in the study after a detailed explanation of the study and its objectives.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Declaration of data availability

The data cannot be shared publicly for reasons of confidentiality. The participants have not permitted us to publicly disclose their identity or the recordings of the interviews and workshop.

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