The transmission of African Animal Trypanosomiasis (nagana) in two districts in the forest zone of Ghana

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Abstract

Animal African Trypanosomiasis also known as 'nagana' is a debilitating vector-borne disease caused by trypanosome parasites and transmitted to human and animals by tsetse fly of the Glossina genus within sub-Saharan Africa. The parasite lives extracellularly and multiplies in the blood and fluid tissues of the mammalian host, leading to morbidity and mortality of the infected animal if untreated. As no known vaccine is currently available to combat the disease, prevention is hinged mainly on vector control and the use of trypanocides. We therefore, carried out a cross-sectional survey to investigate the composition of vectors, parasite diversity and transmission intensity. A total of 229 tsetse flies, 65 pigs and 20 cattle blood samples were collected from two districts in the Eastern region. We performed Cytochrome c (*cox1*) and internal transcribed space (ITS1) dependent PCR assays to detect and identify tsetse fly species and trypanosome parasites circulating in the study area. More than thrice as many female flies 225 (98%) as male flies 74 (32.3%) were collected using biconical traps. The overall vector density of 4.3 flies/trap/day was observed. Sequence analysis revealed that all collected tsetse flies belonged to *Glossina palpalis palpalis* and PCR results revealed trypanosome prevalence of 58.9%, 46.2% and 0% in tsetse flies, pigs and cattle respectively. *Trypanosoma congolense* was the predominant parasite species with prevalence of 80.7% in flies and 60% in pigs. There was evidence of multiple trypanosome infection in both tsetse flies and pigs with *T. congolense*/*T. simiae* occurring highest with prevalence of 37.03%. The parasite prevalence in the flies and pigs within the communities was high with significant difference associated ($\chi 2$ = 16.42, P= 0.0367; $\chi 2$ = 28.06, P=0.0009). Blood meal analysis revealed feeding on both domestic *Sus scrofa domesticus* (pig) and *Phacochoerus africanus* (warthog) as the source of tsetse blood meal. Tsetse flies remain highly infected with trypanosomes and may continue to transmit the parasites to livestock and human populations in the communities.

Keywords: Trypanosomiasis, *T. congolense, T. brucei,* tsetse flies, nagana, pigs, Ghana.

Introduction

Trypanosomiasis, is a vector-borne infectious disease of huge public health and economic importance in sub-Saharan Africa [1–3] . It is a vector-borne parasitic disease caused by a flagellate protozoans belonging to the genus Trypanosoma. These parasites circulate in the blood and Central nervous system of their host (humans and livestock) and are transmitted by a bloodsucking dipteran of the genus Glossina [4]. Several parasite species cause infection in humans and animals. *Trypanosoma brucei gambiense* [4]determines the human's chronic disease form in West and Central Africa while *Trypanosoma brucei rhodesiense* [5]. circulating in East and Southern Africa determines the acute form of the disease. The World Health Organization (WHO) estimates suggest 65 million people being at risk of trypanosomiasis with a high proportion going without any medical monitoring (OMS). The disease in animals though is known as the African Animal Trypanosomiasis, otherwise known as 'nagana', a Zulu-originated word which means 'powerless' or 'useless' [6]. These parasites include *Trypanosoma congolense, Trypanosoma vivax, Trypanosoma b. brucei, T. simae* and several other subspecies. Unlike in domestic animals, where severe infection usually leads to death if untreated the disease has a relatively mild effect on wild animals [7–9]. At least 17,500 new cases are reported every year in meat production areas generating loss of nearly $ 4.5 billion each year [10,5]

The disease is mostly restricted to rural forested communities that rely heavily on agriculture [5][6]. As reported by W.H.O in 2010, the African continent is known to account for 90% of total number of reported cases for Human African Trypanosomiasis (HAT) in addition to huge losses in animal production[13, 14]. As a result, Africa loses up to US$1.5 billion annually [15]. Information on the current situation of HAT in Ghana remains scanty as reported by W.H.O [16]; however, 'nagana', which is mostly transmitted by *Glossina palpalis palpalis* and is perhaps the most widely distributed tsetse fly species in Africa, remains highly endemic in Ghana[16–18].

The presence of tsetse flies is a threat to livestock production [20] in the Suhum municipality as many pigs have suffered death in the past (personal communication with Mr. Gbeve, Veterinary Officer). This study was therefore carried out to determine the prevalence of trypanosome parasites in the pigs and cattle blood as well as the invertebrate host (vector)

using molecular techniques to identify disease risk areas in the Suhum municipality for future studies.

Methodology

Study area

This study was conducted in the selected communities in the Ayensuano and Kraboa districts located 6° 2′ 3.84″ N, 0° 27′ 8.64″ W in the Eastern region of Ghana. The region is positioned in the semi-deciduous forest zone with an annual rainfall of 1500 mm. Thus, most of the communities at the outskirts of the Suhum municipality have more than half of their population engaged in agriculture. The type of soil in the region is conducive for the cultivation of cash and food crops. As a result, crop farming and rearing of livestock are the main agricultural activities in the district. Farmers in remote areas have satellite homes with a small domestic farming as supporting income or the main economic livelihood for the family. Two different areas within the municipality were visited in February 2018 and February 2019. Previous studies indicated no variation in transmission across the climatic seasons (https://www.entomoljournal.com/archives/2020/vol8issue5/PartT/8-2-351-455.pdf)

Study design

A cross-sectional community-based survey was employed where all pigs, cattle and human participants were sampled within the study period.

Fly trapping and dissection

The flies were captured using un-baited biconical traps[8,21–23] that were mounted close to the pigsties but under a shade to minimise fly mortality. The coordinates of each trap was recorded with a Global Positioning System. After fly collection, an index of apparent abundance (IAA) of tsetse was calculated as the number of flies found per trap per trapping day[24]. The flies were grouped into males and females based on the presence or absence of the hypopygium (a round structure on the last segment of the abdomen)[25]. Each fly was immobilized at 4°C prior to dissection, and the wings, legs, proboscis, and midgut placed into 2ml micro centrifuge tubes for preservation. However, only the midgut was investigated for trypanosomes in this study.

Ethical consideration

Ethical clearance was obtained from the Committee on Human Research, Publications and Ethics KNUST and the Komfo Anokye Teaching Hospital. Informed consent was obtained from the farmers prior to sampling.

Blood sample collection

Blood samples were collected from, 20 cattle and 65 pigs. Each cow was bled for 6mL of blood from the jugular vein into a heparinized vacutainer and same volume of blood was drawn from the marginal ear vein of each pig into an EDTA tube. After sampling, the vacutainers were kept in a refrigerator till further processing.

DNA Extraction

The tsetse fly midgut was homogenized in a solution of RNAlater and Tris-buffer (50Mm pH 9.0) using a motor-driven homogenizer and micro pestles (Kimble® Kontes disposable pellet pestles, Sigma-Aldrich), and 100µL of each homogenate was transferred into 1.5mL Eppendorf tube. DNA was extracted from the midgut with DNeasy® Blood and Tissue Kit according to the manufacturer's instructions and 60µL of each was eluted and stored at -20 °C.

Cattle and pig blood. DNA was extracted from, pig and cattle whole blood using DNeasy blood and tissue kit in accordance with the manufacturer's protocol. The extracted DNA was quantified using Nanodrop® ND-1000 spectrophotometer at 260 nm.

PCR Amplification of the ITS1 gene for the Identification of Trypanosome spp.

The ITS1 was amplified by nested PCR to identify trypanosomes in tsetse midgut, cattle and pigs using the primers [26,27] listed in Table 1. Each PCR reaction was carried out in a total volume of 25µL mixture each containing 2µM of each primer, 0.2mM of mix dNTPs, 1X of Buffer (Dream Taq), 0.1U of Taq polymerase and molecular-graded water. The cycling conditions were the same for both nested reactions with denaturation at 94 °C for 1 minute, annealing at 54 ^{0}C for 30 seconds, extension at 72 °C for 30 seconds. The reaction mixture was cycled for 30 times in each PCR. However, the mixture was incubated at 95 °C for 3 minutes prior to the cycling and there was final extension at 72 °C for 5 minutes after the cycling. All PCR reactions were carried out in a Mastercycler Personal. The resulting PCR

products were electrophoresed on a 1.5% agarose gel run in Tris-borate-EDTA buffer at 100V for 1 hour.

T. congolense-specific PCR

Samples which showed a band-size approximately 700bp with generic primers were further run with *T. congolense* specific primers to confirm the presence of *T. congolense.* The species-specific primers (Table 1) used in a nested PCR also targeted the ITS region of the parasites DNA. Sample preparation and PCR reactions were identical to ITS1 nested PCR as already described in this paper.

Amplification of the cox 1 gene for the identification of tsetse fly species

Primer	Direction of the sequence (5' → 3')	Target	Tm	Expected band size

To identify the tsetse fly species in the study area, a part of the *cox1* gene [28] was amplified in a one-step PCR following the protocol as described by Ngomtcho and others [27]

Blood meal Analysis

The extracted DNA from tsetse midgut were screened for the mitochondrial cytochrome oxidase b subunit 1 (COI) gene for all vertebrates in a one-step conventional PCR as described by Muturi and others [29].

Table 1. Primers, annealing temperature and target as used in this study

Abbreviations: GAPDH, *Glyceraldehyde 3-phosphate Dehydrogenase; T.con*, *Trypanosoma congolense*; TGR, *Trypanosoma grayi*; *cox1*, cytochrome c oxidase 1; Out, outer primer; In, inner primer; F, forward; R, reverse; Tm, annealing temperature

ITS OutF ITS OutR ITS1 InF ITS1 InR	TGC AAT TAT TGG TCG CGC CTT TGC TGC GTT CTT TAG AGG AAG CAA AAG AAG CCA AGT CAT CCA TCG	All Trypanosome sp.	54°C	variable
TcITS1-OutF TcITS1-OutR TcITS1-InF TcITS1-InR	TGCAATTATTGGTCGCGC TGTTGGTCGACACTGAGA TCGCGTGTCTCACGT TCAAAGATTGGGCAATGT	*T. congolense*	54°C/56°C	681(kilifi) / 781 (forest)
Cox 1- F Cox 1- R	TTGATTTTTTGGTCATCCAGAAGT TGAAGCTTAAATTCATTGCACTAATC	Generic Glossina	55°C	900

Sequencing of *cox 1* fragments

Cox1 fragments were sequenced for the identification of tsetse fly species in the study area. The resulting gel product from *cox1* PCR were purified using GeneJET Gel Extraction Kit (Cat. no. K0701, Thermo Scientific) according to the manufacture's protocol. The gel containing the DNA fragments were excised with a clean scalpel into pre-weighed 1.5ml Eppendorf tubes. The cut gels were purified according to the manufacturer's protocol and 50µL of each sample was collected in the elution buffer. The DNA was quantified with a Nanodrop® ND-1000 spectrophotometer at 260nm. 12µL each amplicon containing 18ng/100basepairs was prepared for sequencing at Seqlab GmbH. The sequences obtained were evaluated using the Genius software (version 5.5.9.0) and for the purpose of comparison, the sequences were compared with NCBI Nucleotide Blast database at (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and TriTryp at (https://tritrypdb.org/tritrypdb/).

Statistical analysis

We processed our data using Microsoft Excel software and transported it to Graph Pad Prism for the statistical analysis. The trypanosome distribution and infection rate in tsetse flies and pigs within the communities were compared using Chi-squared. The differences of infection rate were checked between male and female tsetse flies, age groups of pigs, and amongst communities. The comparisons were considered as significantly different at P-value <0.05.

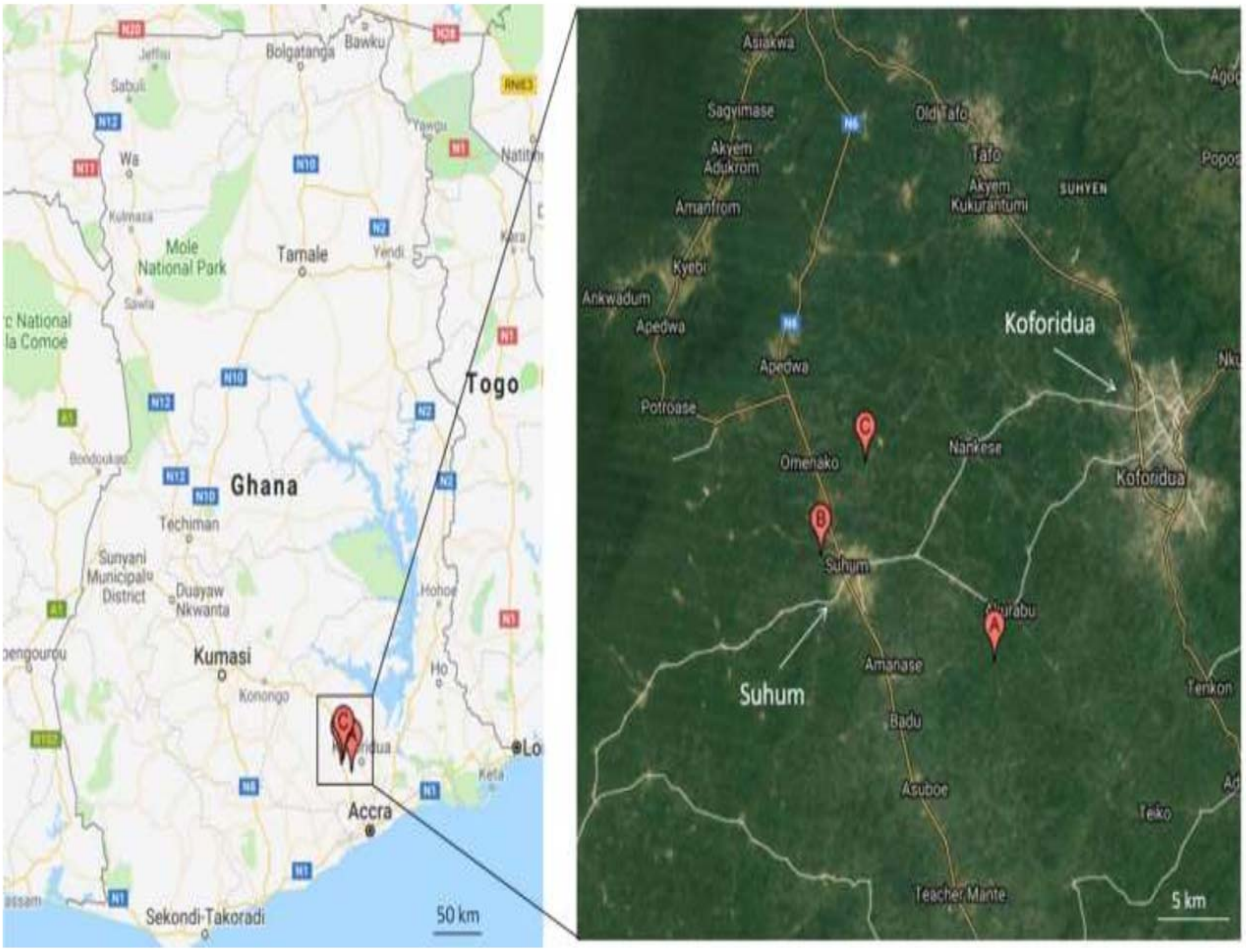
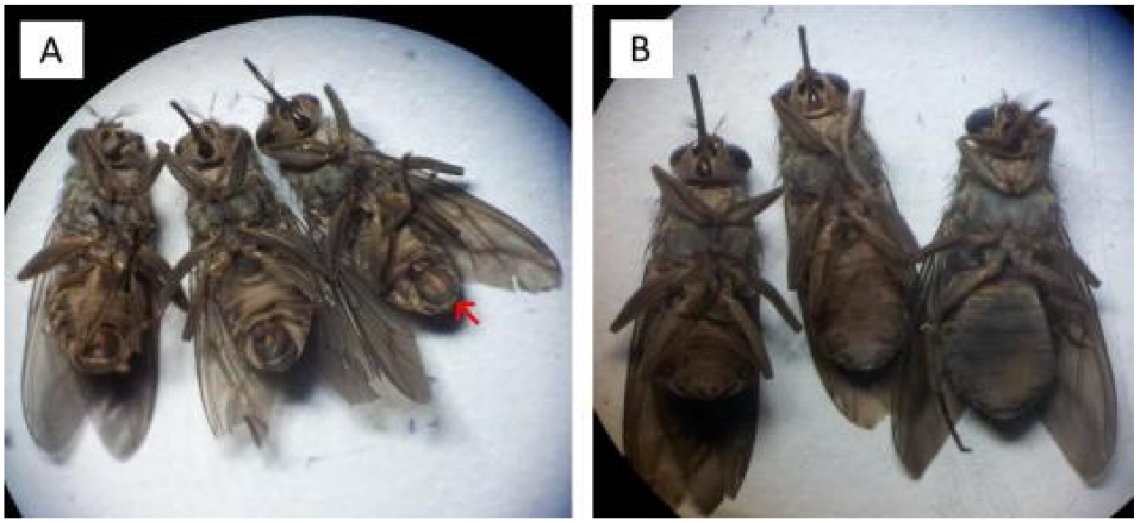


Figure 1. *Tsetse flies were collected in some selected communities in the Suhum/Kraboa/ Coaltar district. Two trap was set up at Santramor (A), two at Pinto (B) and two at Adenta (C), one at Nkatekwan, and one at Ayekokooso.*

Results

The overall average apparent density of tsetse flies during the survey period was 4.3 flies/trap/day. We trapped 229 tsetse flies, comprising 74 males and 225 females respectively (Fig 2). Amongst the communities we visited, tsetse fly infestation was low at Whanabenya with only 4 flies captured, while Nkatekwan recorded the highest of the fly numbers (Table 2).



(Insert figure 2)

Figure 2: A: *Male tsetse flies trapped in the field were identified by the presence of a hypopygium (at the external genitalia indicated by the red arrow);* ***B****. Female tsetse flies were identified by the absence of a hypopygium on the last segment of the abdomen*[30]

Molecular detection of trypanosomes in the tsetse midgut and pig whole blood

Nested PCR was performed with DNA extracted from tsetse fly gut tissue and pig whole blood using generic primers to amplify the 5.8S and 18S genes of *Trypanosomatidae.* Interestingly, trypanosomal DNA was detected in a large number of flies from all but one community, Ayekokoo so. None of the cattle was found positive for trypanosomal DNA whereas many of the pigs encountered harboured trypanosomal DNA (Table 2). There was simultaneous infection of trypanosomes in the tsetse midgut[31].

Table 2. Number of trypanosomal DNA detected in the tsetse flies and pigs collected in the

FLIES	Total Number Examined	PCR Results (+)	Prevalence	X^2	P-value
Adenta	71	45	63.38		
Ayekokoso	8	0	0.00		
Magazine	10	4	40.00		
Nkatenkwan	49	31	63.27		
Santramor	57	32	56.14	16.42	0.0367
Teacher	5	3	60.00		
Whanabenya	4	2	50.00		
Pinto	19	13	68.48		
Zorh	6	5	83.33		
PIGS					
Magazine	7	1	14.29		
Nkatenkwan	10	10	100.00		
Santramor	4	4	100.00		
Teacher	7	2	28.57		
Whanabenya	7	1	14.29		
Zorh	5	3	100.00	28.06	0.0009
Bonkua No.1	7	2	28.57		
Budu	7	1	14.29		
Nankese	2	2	100.00		
Mensakrom	7	2	28.57		
CATTLE					

study area

Ayekokooso	10	0	0.00		
Akorabo	10	0	0.00		

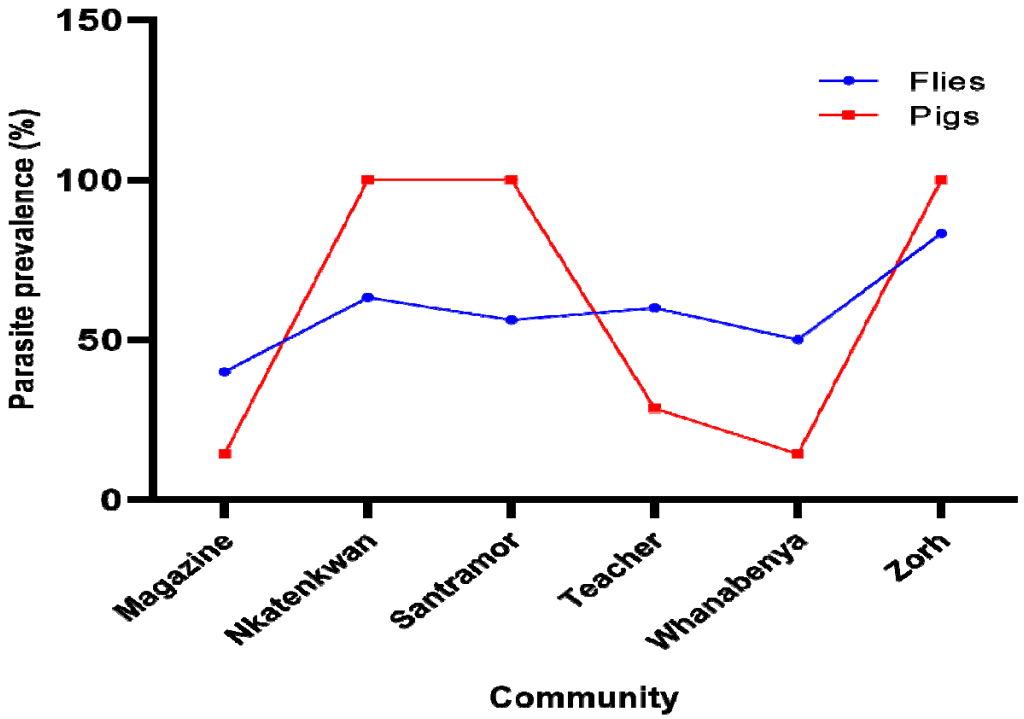
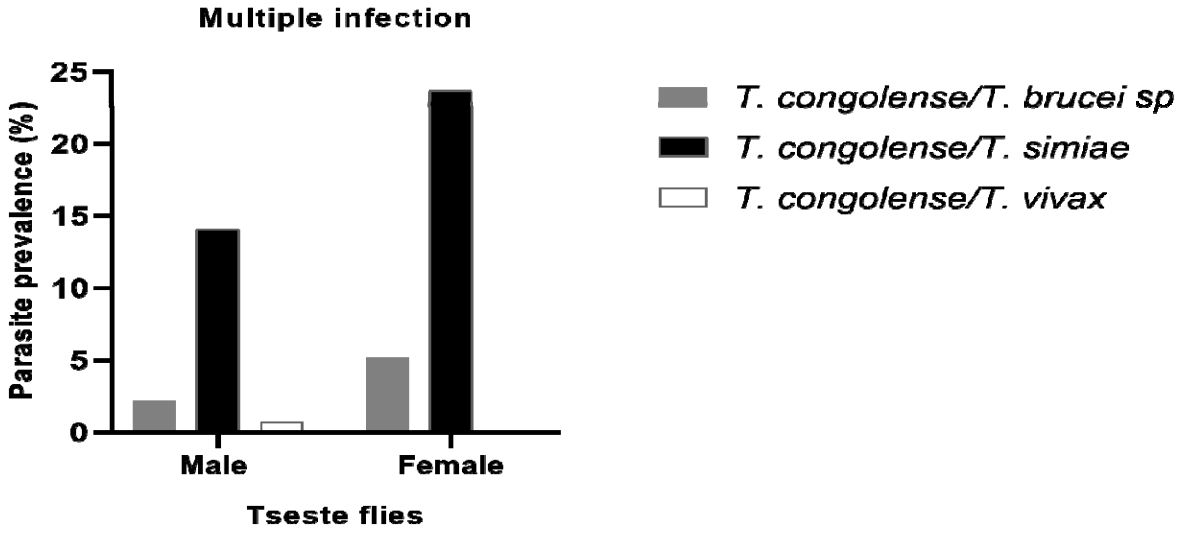


Figure 3. Relationship of the parasite prevalence between tsetse flies and pigs collected from the same communities



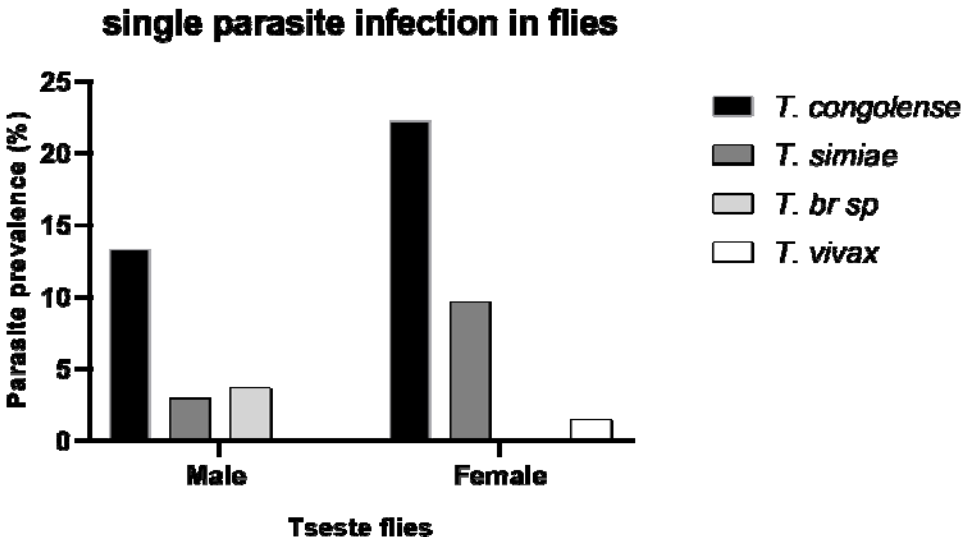


Figure 4. Single and multiple trypanosome infection in collected tsetse flies in relation to fly sex

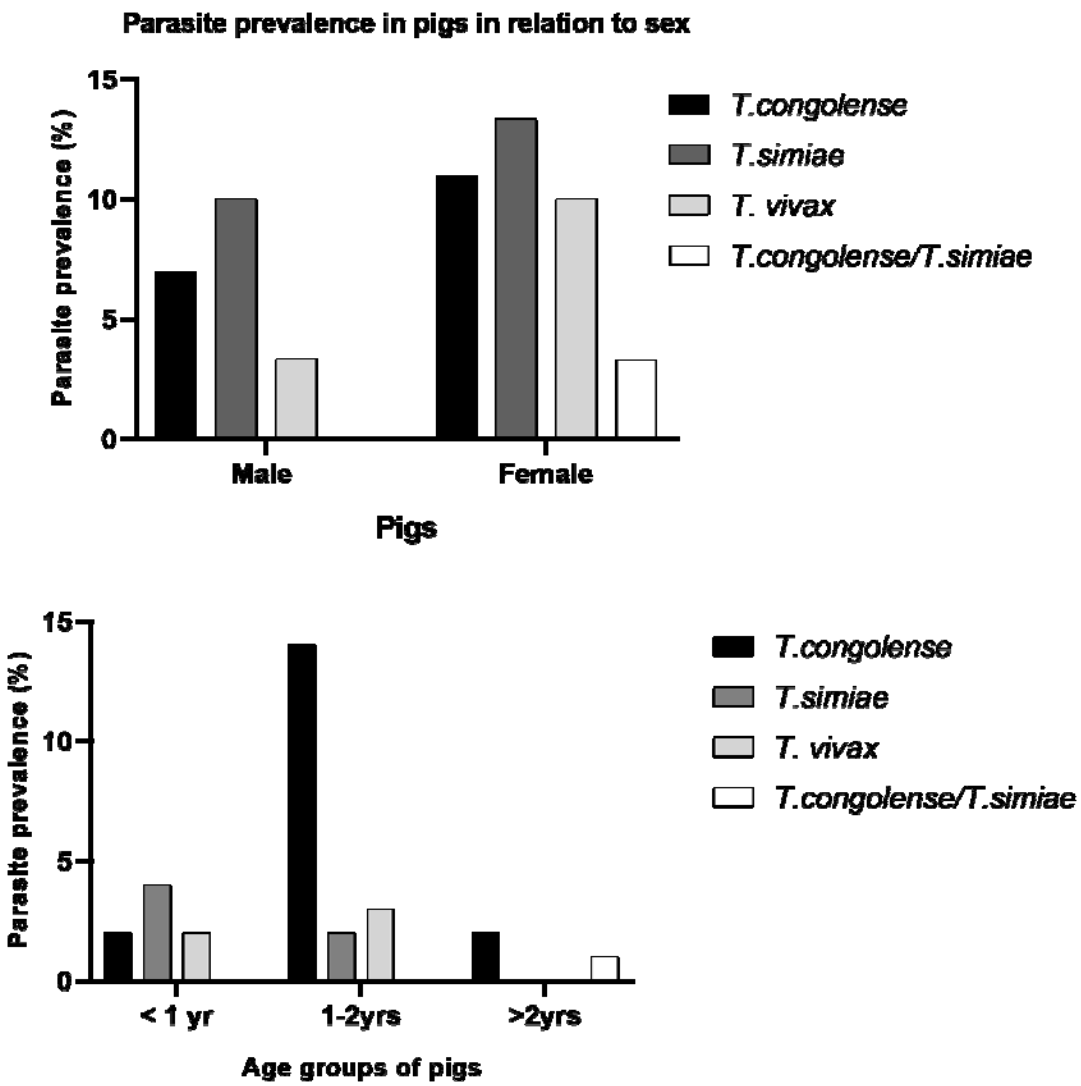


Figure 5. Trypanosome infection within sex and age groups of collected pigs.

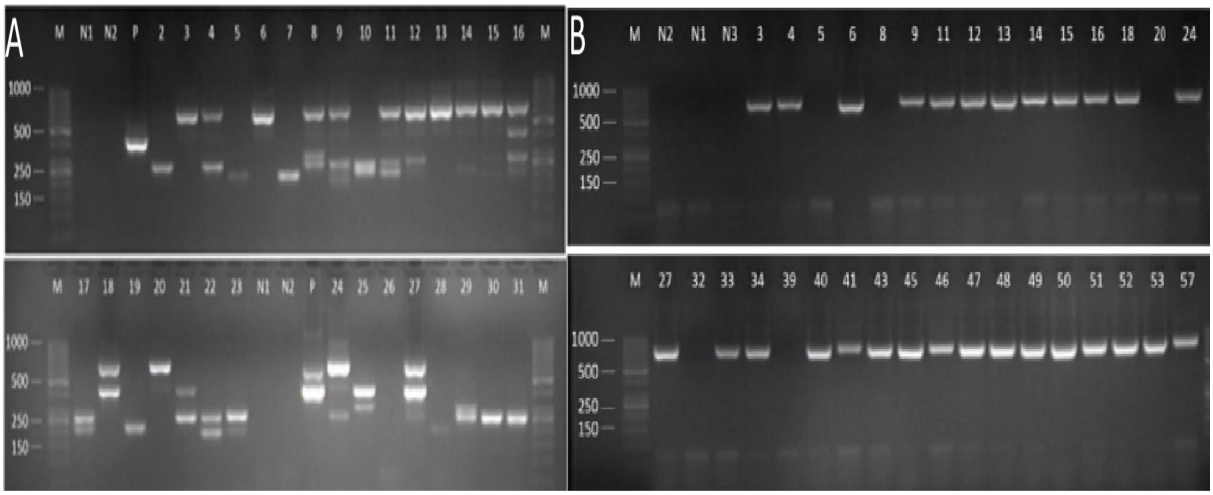


Figure 6. G-stained 2% agarose gel showing amplification of PCR products from tsetse midgut infected with trypanosomes. **A**: Lane (M) molecular marker GeneRuler 50bp Ladder (Thermo Scientific). (N1 and n2): negative controls (from both the first and second reactions), P: positive control (gDNA of T. brucei). Amplicons with sizes of ~700 indicate T. congolense, 420-500bp derive from T. brucei-like species defined by their similar ITS1 length in this study, the base pairs around 370-400 corresponds to T. simiae. Amplicons size of 200-320bp is T. vivax. **B**: shows amplification of T. congolense (~700bp fragment size) from T. congolense-specific PCR. Lane (M) molecular marker GeneRuler 50bp Ladder (Thermo Scientific). (N1, N2 and N3 are all negative controls: lane 3 to 18 were all T. congolense positive samples.**)**

T. congolense-specific nested PCR

All tsetse fly and pig samples that showed PCR positive were further subjected to species-specific nested PCR to confirm the presence of *T. congolense* and also determine the specific subspecies of *T. congolense* responsible for the infection (i.e. the savannah, the forest or the riverine type). For *T. congolense* forest and savannah, amplicons in the range of 760-780bp were expected, for *T. congolense* kilifi a size of 680bp was expected.

Molecular detection and identification of trypanosome in cattle and pigs

A total of 20 cattle and 65 pigs from the selected communities were screened in nested PCR. All 20 cattle were negative for trypanosomal DNA while 30(46.2%) out of 65 pigs sampled were positive for different trypanosome species. The predominant trypanosome species circulating in pigs was *T. congolense* (forest type) and *T. simiae*. Majority of the pigs that were found positive for trypanosomal DNA were recorded at Nkatekwan (12.3%) followed by Santramor (6.1%). There was no significant difference of trypanosome prevalence amongst communities($P>0.05$). The pigs were grouped into three age categories; (i.e. < 1 year, 1-2years and >2years). Only two pigs were found older than two years and they all

showed positive for trypanosome parasite. 46% out of those aged between 1 and 2 years harboured different species of the parasite while 7(21%) out of 33 young pigs (i.e.<1year old) were found positive for the parasite.

Molecular identification of tsetse fly blood meal source

DNA from tsetse blood meal were amplified positive signal for *cytochrome b gene* in 10 samples was observed. Five (5) out of ten (10) samples were sequenced while the other 5 failed to produce good sequences. Three (3) of the flies that showed positive signal were collected from Santramor and 2 were collected from Nkatekwan.

Table 3. Blood meal source determined by comparison with sequences of species in the NCBI GeneBank database.

Mammalian host species	Cyt b detection rate	NCBI (Genebank accession number)
Phacochoerus africanus (Common warthog)	1/28	KJ192881
Sus scrofa breed Fengjing mitochondrion (Pig)	4/28	MH603005.1

Discussion

The study discovered the presence of *Glossina palpalis palpalis* in the area and that corroborate the report by Ebhodaghe and others [32], in a study conducted to investigate the distribution of tsetse fly in the same region. Tsestse species have varying ecological requirement in order to thrive in a particular environment [27,28]. For instance, *Glossina palpalis* prefers forest in which desired environmental factors are invariable [29,30]. However, other species such as *G. mositant* and *G. tachnoides* that have been previousely reported in the northern Ghana were absent in this study. According to the tsetse distribution models, those species usually thrive in a vegetation that is partially altered by human activities [28,29]. Thus the absence of morsitant and tachninoides in the southern part of the country as reported in this study and others could be an indication of a predisposition towards a northerly retreat of these species. The existence of the *palpalis* group in the study area indicates a health challenge due to their ability to transmit the sleeping sickness. Though all tsetse species can transmit trypanosomes but the *palpalis* group is known as the most

significant transmitting vector for trypanosomiasis [39]. They are capable of transmitting *Trypanosoma gambiense*, which causes sleeping sickness in both western and central Africa [40]. They are also efficient in transmitting animal pathogenic trypanosomes including *T. vivax* and *T. congolense*. Thus, their presence in the area poses a challenge to the public health since both the people and livestock are exposed to the bite of the tsetse fly[18]. Apparent densities did not differ significantly amongst the tsetse biotopes as the factors that determine tsetse density including the vegetation cover and the availability of hosts were almost the same for all sites. The overall apparent density recorded was 4.3 flies/trap/day and that was slightly higher than that reported in the new-Juaben district in the same region [8]. The possible reasons could be the low density of livestock population, and/or the use of unbaited traps for the collection of the flies.

Trypanosomes infection in tsetse fly

This study indicates high infection rate of trypanosome infection in the tsetse midgut. This is somewhat intriguing because the maturation of trypanosome in the tsetse midgut is influenced by several factors[23,25]. For instance, the maturation of T. congolense is facilitated by a secretion in the midgut known as lectin. Thus, in the event that the lectin production is inhibited, the trypanosome parasite remains in the immature procyclic forms in the midgut [43]. Also, whenever tsetse fly takes up infected blood meal, several trypanosome parasites are mostly cleared away from the midgut by the tsetse immune system. Thus very few trypanosomes are able to escape the defence mechanism of the tsetse immunity. For these reasons, many trypanosomes are unable to mature and migrate from the midgut to the salivary gland. This could probably be the reason for the higher percentage of trypanosomal DNA in the tsetse midgut. It is good to note that DNA is relatively stable and can circulate in body tissues over a period of time and PCR is relatively more sensitive molecular tool to detect and identify even residual traces of trypanosome DNA in varying hosts[44]. Therefore, the presence of DNA alone in a host may not be enough justification for the presence of the active trypanosome parasite. As mentioned earlier, more female tsetse flies collected in this study were found positive for trypanosomal DNA with higher prevalence 125 (58.4%) than 63 (29.4 %) in the male flies (Fig 3). The lower prevalence of trypanosome in the male tsetse flies does not agree with the literature as few studies have assessed the influence of sex on susceptibility of trypanosome. It appeared the male tsetse was more susceptible than female tsetse in the following studies [36,37]. The reason could be due to the uneven sex ratios of tsetse flies captured in this study which probably skewed the statistical tendency towards the

female flies as female tsetse revealed 71%, 37% of *T. congolense* and *T. brucei* respectively compared with the prevalence 40%, 16% of *T. congolense* and *T. brucei* in males. *T. congolense* was the dominant trypanosome species in the study area with the prevalence of 45.8%. This was higher than that reported in a study carried out in the same region [17]. Ngomtcho and others found a much lower infection rate (24.8%) of *T. congolense* in the same vector species *G. palpalis palpalis* in northern Cameroon. There was an evidence of concurrent colonisation of mixed trypanosome infection in the tsetse midgut. This might come from tsetse blood meal infected with different trypanosome species or successive blood meal uptake from different pigs with different trypanosomes species.

Trypanosoma species in pigs and cattle

This study reveals trypanosome infection rate of 46.1%, and 0% in pigs and cattle respectively and this was higher than that reported by Baure et al., in a study carried out in the same region [19]. The trypanosome infection in livestock as revealed in the current study is in line with report by other authors [38–43] and this suggests that pigs are potential reservoirs of sleeping sickness as reported in Cameroon [44–46]. This also implies that other domesticated animals such as dogs and goats might be harbouring the parasites in the area as reported in other parts of Africa [54,55,42]. In this study, three trypanosome species were identified with *T. congolense* as the predominant spcies in 60% of the pigs and this corroborate the findings from [47–48]while about 20% and 16% were found carrying *T. simiae* and *T. vivax* respectively. This is consistent with the study by Penchenier and others[58]where species–specific PCR was performed to reveal high levels of *T. vivax* infection in pigs. Interestingly, we observed a strong correlation between the pigs and tsetse flies from the same site as high trypanosome prevalence in flies corresponds to increase in prevalence in the pigs (Fig 3).

We screened multiple cattle breeds including ‘Zebu’, and ‘Ndama’ that are generally known to be trypanotolerant and one susceptible breed, Sanga [59]. Interestingly, no DNA was found in any of the cattle. This could be attributed to the regular use of trypanocides in even the susceptible breed. This is consistent with the report in a study conducted to investigate trypanosomes in cattle in southern Ghana [60]. We observed low tsetse densities in some of the sampling areas including the cattle ranch site although the frequent administration of trypanocides cannot be excluded. The findings in this study could be attributed to the control practices ensured by the cattle farmers as they asserted that occasionally veterinary officers are invited to administer trypanocidal medication to the cattle. Also, the farmers occasionally

spray the cattle against all biting flies which might have reduced the tsetse fly-cattle contact on the farms.

Blood meal analysis

Sequence analysis of the cytochrome oxidase b gene revealed two vertebrates*; Sus scrofa domesticus* (domestic pig) and *Phacochoerus africanus* (warthog) as host species (Table.3). The availability of blood meal source is one of the most important factors for tsetse fly to thrive in a habitat[61].Thus, the presence of vertebrate host including pigs are key indicator of the presence of tsetse fly in an area. For that reason, the sampling strategy was skewed towards blood meal source (pigs). Nevertheless, tsetse flies are known to take blood meal from a wide range of vertebrate host and can even adopt to feeding from new host in the absence of the usual host [62]. Subsequently, all but one fly had fed from domestic pigs. The only fly that revealed the DNA of *Phacochoerus africanus* (warthog) was collected from Nkatekwan. This community was surrounded by secondary forest that could serves as habitat for both the tsetse flies and the *Phacochoerus africanus*

The outcome of the current study revealed that *Glossina palpalis palpalis* is the only tsetse fly species in the Suhum/Kraboa/Coaltar district responsible for the transmission of trypanosomes in pigs. ITS1 PCR technique shows that *T. congolense, T. brucei,* and *T. simiae* infection were present in the tsetse fly with *Trypanosoma congolense* being the predominant trypanosome species in the study area. Therefore, scheming an environmentally-friendly control approaches to reduce both the reservoir host and the tsetse fly vector is needed to reduce the prevalence of nagana in the Suhum/Kraboa/Coaltar district. Also, further studies involving a wide range of domestic animals should be carried out to determine prevalence and risk factors of nagana in different seasons to establish seasonal variation in the animal trypanosomiasis transmission.

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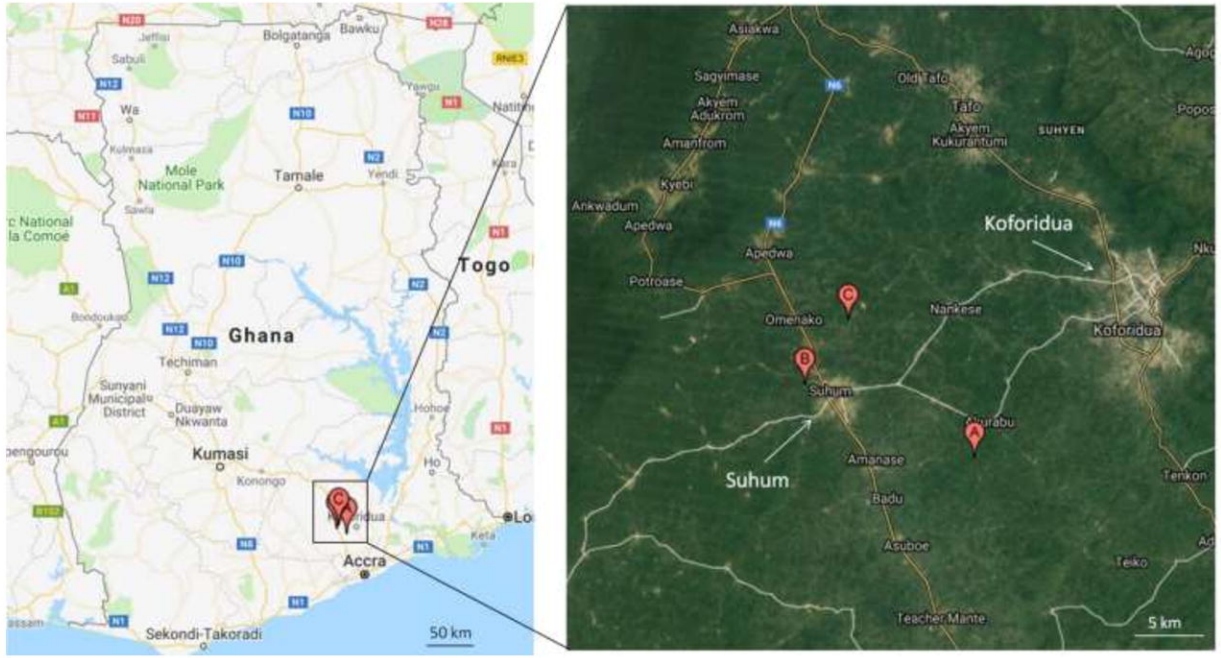
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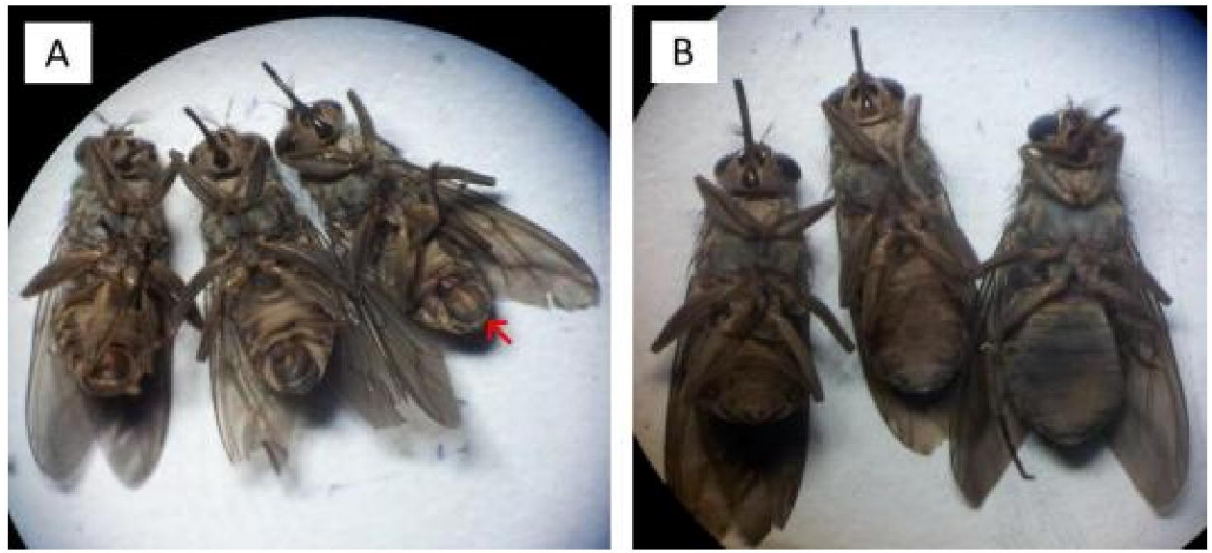
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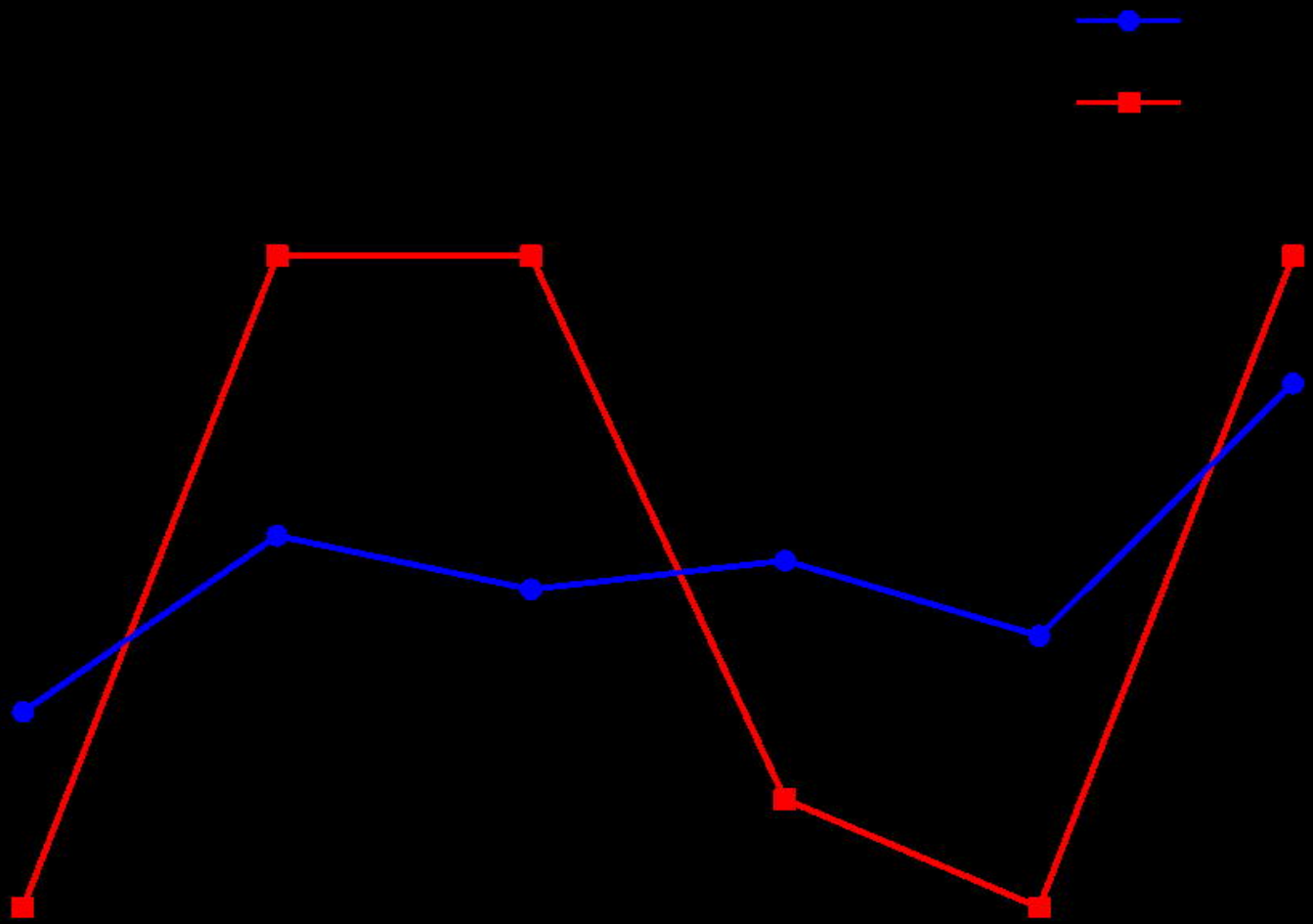

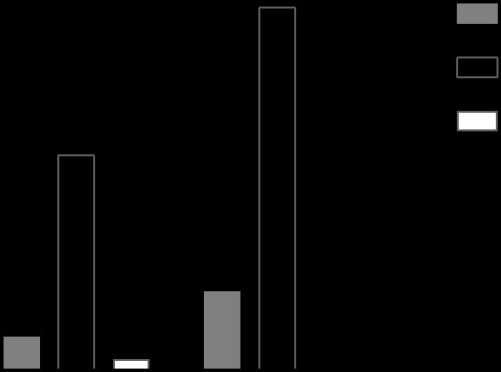

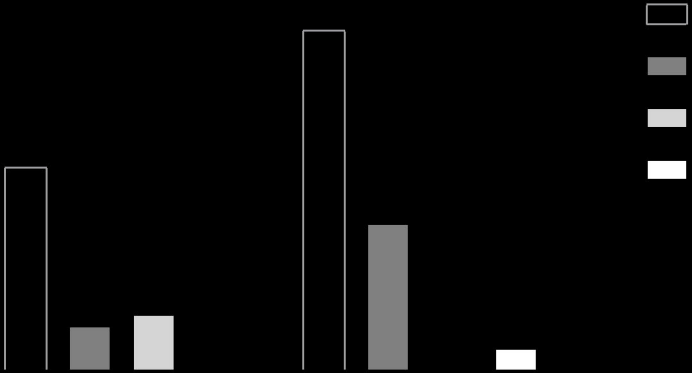

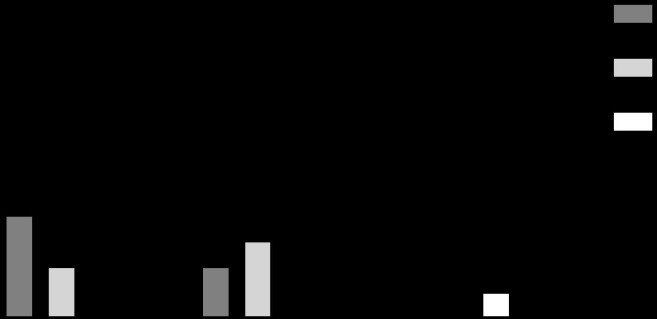

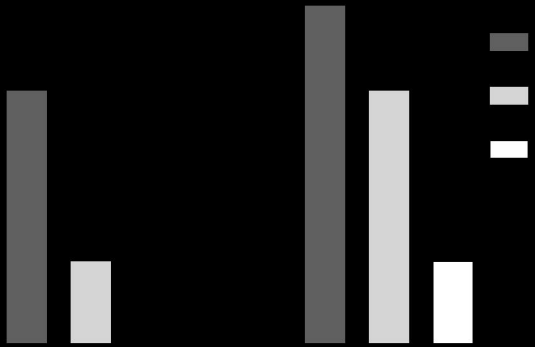

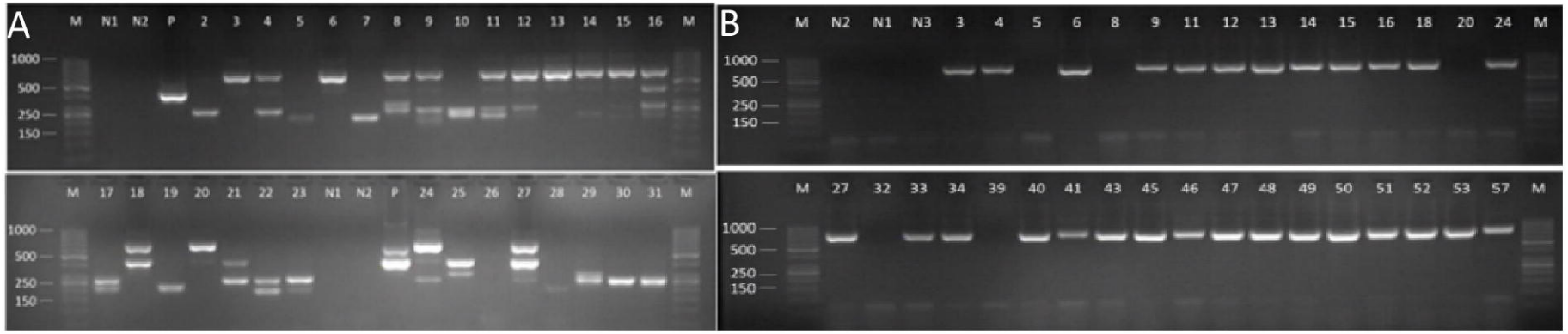
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