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RESEARCH ARTICLE

REVISED Detection of blood pathogens in camels and their associated ectoparasitic camel biting keds, *Hippobosca camelina*: the potential application of keds in xenodiagnosis of camel haemopathogens [version 2; peer review: 2 approved]

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Abstract

Background: Major constraints to camel production include pests and diseases. In northern Kenya, little information is available about blood-borne pathogens circulating in one-humped camels (*Camelus dromedarius*) or their possible transmission by the camel haematophagous ectoparasite, *Hippobosca camelina*, commonly known as camel ked or camel fly. This study aimed to: (i) identify the presence of potentially insect-vectored pathogens in camels and camel keds, and (ii) assess the potential utility of keds for xenodiagnosis of camel pathogens that they may not vector.

Methods: In Laisamis, northern Kenya, camel blood samples (n = 249) and camel keds (n = 117) were randomly collected from camels. All samples were screened for trypanosomal and camelpox DNA by PCR, and for *Anaplasma, Ehrlichia, Brucella, Coxiella, Theileria*, and *Babesia* by PCR coupled with high-resolution melting (PCR-HRM) analysis.

Results: In camels, we detected *Trypanosoma vivax* (41%), *Trypanosoma evansi* (1.2%), and " *Candidatus* Anaplasma camelii" (68.67%). In camel keds, we also detected *T. vivax* (45.3%), *T. evansi* (2.56%), *Trypanosoma melophagium* (1/117) (0.4%), and " *Candidatus* Anaplasma camelii" (16.24 %). Piroplasms (*Theileria* spp. and *Babesia* spp.), *Coxiella burnetii*, *Brucella* spp., *Ehrlichia* spp., and camel pox were not detected in any samples.

Conclusions: This study reveals the presence of epizootic pathogens in camels from northern Kenya. Furthermore, the presence of the same pathogens in camels and in keds collected from sampled camels suggests the potential use of these flies in xenodiagnosis of haemopathogens circulating in camels.

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Camelus dromedarius, Hippobosca camelina, haemopathogens, xenodiagnosis, high-resolution melting analysis

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REVISED **Amendments from Version 1**

We have addressed comments from the reviewers and made the following changes

The type of sampling is specified as opportunistic sampling and was adopted for convenience by sampling camels from diverse geographical locations as they converge at specific water drinking points. Otherwise it will be challenging to conduct daily sampling of camels considering that camel owners are nomadic pastoralists with busy lifestyles characterized by long distance movements together with their animals and other belongings.

Other minor changes include;

Throughout the article, "disease pathogens" was changed to read to "pathogens",

Absolute fractions in the results section of the abstract were removed,

Edits such as rephrasing some statements was done, e.g. "unpredictable rainfalls" was changed to "unpredictable rainfall"; "short and long rains" to "short and long wet seasons",

Table 3 (Biting flies were trapped…) is edited as follows; new column inserted showing the number days sampled; column heading "Fly density per trap/day" changed to "Daily fly captures",

New addition; Figure 5: Pairwise alignment of 16S rRNA sequences of "*Candidatus* Anaplasma Camelii" amplified from camel blood sample 60D with GenBank-retrieved nucleotide sequence (KF843824) showed 100% identity. N is ambiguous nucleotide.

The following citations have been added to the current version; Moore & Messina, 2010; Nelson, 1963; Rahola *et al.*, 2011; Truc *et al.,* 2013; Vanhollebeke *et al.*, 2006

Any further responses from the reviewers can be found at the end of the article

Introduction

Camels are the most valuable livestock for pastoralist farmers living in arid and semi-arid lands (ASALs) in Kenya (Mochabo *et al.*, 2005). Among other benefits, they provide milk, meat, transport, and income through sale of animal products (Faye, 2014; Oryan *et al.*, 2008). There are no other livestock species that have such versatile uses to pastoralists living in ASALs (Faye, 2014). Over three million one-humped camels are estimated to be in northern Kenya (FAOSTAT, 2015; KNBS, 2010), which represents the third largest camel population in Africa after Somalia and Sudan (Lamuka *et al.*, 2017). Camels are resilient to harsh conditions of ASAL regions characterized by long periods of drought, scarcity of vegetation and water, and unpredictable rainfall. However, camel pests and diseases are the major constraints to camel production (Higgins, 1985; Kassa *et al.*, 2011; Mochabo *et al.*, 2005). Additionally, the constant association between camels and humans, co-herding of livestock species, and communal watering of animals, as well as sharing of water troughs by the domestic and wild animals, exacerbate the spread of zoonotic diseases, which poses a great risk to public health among livestock and humans in Kenya's north (Bengis *et al.*, 2002; Kazoora *et al.*, 2014; Lamuka *et al.*, 2017; Younan & Abdurahman, 2014). Thus, there is a need for constant surveillance of infectious agents circulating within the camel herds in order to guide control and treatment of these diseases.

Camels are vertebrate hosts of various haematophagous arthropods including *Hippobosca* spp. (also known as keds or hippoboscids), horse flies, stable flies, *Lyperosia* spp., and ticks (Higgins, 1985). In addition to the direct effects such as blood loss, annoyance, and painful feeding bites, these biting pests can be vectors of infectious pathogens (Baldacchino *et al.*, 2013; Higgins, 1985; Young *et al.*, 1993). Biting flies such as tabanids and *Stomoxys* have been implicated in the transmission of viruses (including bluetongue and Rift Valley fever viruses), rickettsiae (e.g. *Anaplasma, Coxiella*), *Bacillus anthracis*, and protozoa (*Besnoitia besnoiti, Haemoproteus metchnikovii, Trypanosoma theileri, Trypanosoma evansi, Trypanosoma equiperdum, Trypanosoma vivax, Trypanosoma congolense, Trypanosoma simiae, Trypanosoma brucei*) in their specific vertebrate hosts (reviewed by Baldacchino *et al.*, 2013).

Hippoboscids (keds) are obligate haematophagous ectoparasites of mammals and birds. They belong to the family Hippoboscidae within the superfamily Hippoboscoidae (Petersen *et al.*, 2007; Rahola *et al.*, 2011). This family of haematophagous dipterans is divided into three subfamilies, *Lipopteninae, Ornithomyinae*, and *Hippoboscinae* (Rani *et al.*, 2011). *Hippoboscidae* and *Glossinidae* (tsetse; i.e. the definitive vector of African trypanosomes) belong to the same superfamily *Hippoboscoidae*, which is characterized by adenotrophic viviparity (Petersen *et al.*, 2007). Members of *Hippoboscidae* act as vectors of several infectious agents including protozoa, bacteria, helminths, and viruses (Rahola *et al.*, 2011). *Hippobosca camelina* is the predominant ectoparasite of camels in northern Kenya. This haematophagous fly acquires blood meals mainly from camels for its nourishment and reproduction. The role of keds in disease transmission is not well established. Furthermore, as primarily long-term camel blood-feeders, they may have potential in xenosurveillance of pathogens within camel herds that they may not transmit. Therefore, this study was undertaken to (i) detect the presence of infectious viruses, bacteria, protozoa, and rickettsial pathogens, particularly those responsible for zoonoses, in camels and hippoboscids associated with them, and (ii) study the potential utility of hippoboscids in xenodiagnosis.

Methods
Study area
The study was carried out in Laisamis (1° 36' 0" N 37° 48' 0" E, 579 m above sea level) located in Marsabit County, northern Kenya (Figure 1). The County of Marsabit in Kenya has a total area of 70,961km² and occupies the extreme part of northern Kenya (Source: County Commissioner's Office, Marsabit, 2013). Area of the Laisamis sub-County that consists of four County Assembly Wards is 20,290 km² with a population of 84,056 people consisting of about 41,240 males and 42,871 females (KNBS, 2013). Laisamis electoral ward, one of the four County Assembly Wards of Laisamis sub-County in Marsabit County, has an area of 3,885 km². A total population of 203,320 camels was reported in Marsabit County, where our present study was conducted (SSFR, 2017).

Weather conditions
The average temperature in Laisamis is 26.5°C (19°C – 30°C; March is the warmest month, whereas July is the coldest month
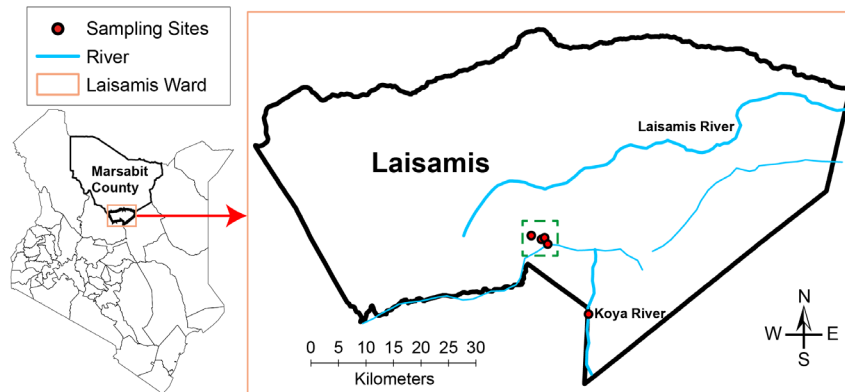
Figure 1. A map of Kenya showing the study sites in Laisamis, Marsabit County, Kenya. Camel blood samples were collected from camel herds during the day, shortly after drinking water from the wells dug along Koya semi-permanent river (circle filled in red), whereas camel keds were collected from the same herds later at night when these camels returned to their temporary settlements shown on the map by circles (filled in red) inside a dotted green square.

of the year). About 413 mm of precipitation falls annually, the average rainfall amounts and rain days differ between years. Long wet seasons occur mostly during April - June, while short wet seasons are experienced in October - December. On the other hand, short dry seasons occur between January and March, whereas long dry spells are experienced between July and September (SSFR, 2017). However, unpredictable and irregular climatic patterns are becoming more common, with no rainfall in some years leading to frequent droughts in the arid and semi-arid regions of northern Kenya.

Study design and sample collection

This field study was cross-sectional in design and involved opportunistic sampling of camels from diverse geographical locations as they converge at specific water drinking points. Daily sampling of camels found along the river was convenient strategy considering that camel owners are nomadic pastoralists with busy lifestyles characterized by long distance movements together with their animals and other belongings.

Due lack of historical data on camel diseases in Laisamis sub-County, there was no basis for calculation of the sample sizes, thus we collected as many samples as possible during the sampling duration.

We did not have data on the total number of camel herds kept by the pastoralist community whose main occupation at 87% is livestock herding (SSFR, 2017). We defined camel herd as a group of camels that spend significant amount of time together by living, feeding, or migrating together. Camels in each herd ranged from 8 – 90 camels.

Camel blood samples

In September 2017, 249 clinically healthy dromedary camels of both sexes (203 females and 46 males) were sampled in Laisamis sub-County, along Koya River (01° 23' 11" N, 37° 57' 11.7" E). Koya River was selected as sampling site as it contains

permanent watering points. Sampling was preferred in dry season of September when the camel ked densities are highest in contrast to the wet season. We sampled all camels in each and every herd at water drinking points for five consecutive days.

About 5 mL of camel blood was drawn from jugular vein into a heparinised vacutainer and immediately preserved in liquid nitrogen at -196°C for transportation to molecular biology laboratories at the International Centre of Insect Physiology and Ecology (*icipe*, Nairobi) for analysis.

Collection of camel keds, *H. camelina*

Camel keds closely associate and move with their host as they firmly attach to the hairs on camel's skin using tarsal claws. These blood feeders are mainly observed on the underbelly (Figure 2), although they can be found on other parts of the body such as the neck and hump. Since we observed that keds are best collected under the cover of darkness at night, we collected blood samples at the water drinking point, then later in the evening followed the same camel herds for fly collection. Flies were collected off camels from four sites (Sarai – 01° 30' 33.2" N, 037° 52' 34.4" E; Sarai Maririwa/Kilakir – 01° 35' 20.5" N, 037° 48' 39.7" E; Lapikutuk Lelembirikany' – 01° 30' 42.9" N, 037° 52' 53.5" E; Noldirikany' – 01° 30' 04.2" N, 037° 54' 50.7" E) by handpicking using spotlights that were briefly switched on and off in order to locate flies on the camels. Camel keds were randomly collected from 21 sampled camel herds in 5 days and we aimed to collect all camel flies found on the camel's body in all sampled herds. Freshly collected camel keds were preserved in absolute isopropanol and transported to *icipe* for molecular screening of infectious agents. Morphological identification of camel keds was done through comparison with known hippoboscid collections at the Zoology museum of the University of Cambridge (UK), and the Natural History Museum in London. DNA barcoding of COI gene to resolve species of keds was unsuccessful possibly because these flies are little studied and have poor representation in the databases.
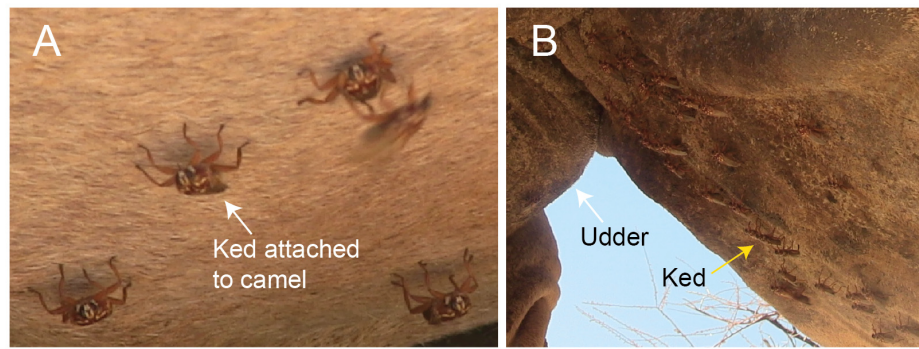
Figure 2. *Hippobosca camelina* flies infesting camels. These ectoparasites are mostly found on the belly of the host as shown in **A** & **B**. Over 30 flies were found concentrated on a small section of underbelly of the camel next to the udder. These flies mostly infest the underbelly and occasionally on the other parts of the camel's body where they are not prone to disturbance by the host.

Collection of other biting flies

In order to determine occurrence of tsetse flies and other species of haematophagous biting flies found in Laisamis sub-County and Koya, we deployed monoconical traps, using cow urine and acetone as attractants. Three traps were deployed per site on daily basis from 09:00 - 18:00 next to livestock pens and near watering points along Laisamis River. The inter-trap distance was at least 100 meters. Daily trap collections were pooled, fly species sorted, counted, and then the flies were preserved in 50 mL Falcon tubes half-filled with absolute ethanol for later morphological identification.

Ethical approval

This study was undertaken in strict adherence to experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee at *icipe* (REF: IACUC/ICIPE/003/2018). All efforts were made to minimize pain and discomfort during sampling. For instance, camel keepers, with whom camels were familiar, were allowed to restrain their camels for sample collection. Samples were collected after receiving informed verbal consent from camel keepers. All camel keepers were neither able to read nor write, thus verbal rather than the written consent was adopted as the pragmatic approach.

DNA extraction

Each *H. camelina* fly was surface-sterilized with 70% ethanol and allowed to air dry for 10 min on a paper towel on top a clean bench. Individual flies were placed into a clean 1.5 mL centrifuge tubes containing sterile 250 mg of zirconia beads with 2.0 mm diameter (Stratech, UK) and ground in liquid nitrogen in a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA) for 3 min. Genomic DNA was extracted from camel keds and camel blood samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Detection of pathogen DNA

Detection of *Coxiella burnetii*, *Anaplasma* spp., *Ehrlichia* spp., *Brucella* spp., and piroplasms belonging to *Theileria* and *Babesia* genera employed PCR followed by DNA fragment analysis based on high-resolution melting (HRM) analysis (Šimenc & Potočnik, 2011) in a Rotor-Gene Q thermocycler

(Qiagen, German). *Coxiella burnetii* DNA was screened for using primers (Table 1) targeting the IS1111 gene (Tokarz *et al.*, 2009). *Anaplasma* and *Ehrlichia* species were detected by PCR amplification using genus-specific primers (Table 1). In detection of *Anaplasma* and *Ehrlichia* species, the PCR-HRM target for 16S rRNA lies within the longer 16S rRNA region, thus the 1000 bp barcode region (amplified by conventional PCR) instead of 16S rRNA HRM products were sequenced. *Babesia* and *Theileria* spp. DNAs were amplified using primers RLB-F1 and RLB-R1 (Table 1) targeting the hypervariable V4 region of 18S rRNA genes (Georges *et al.*, 2001). The PCRs were carried out in 10 μL reaction volumes, containing 2.0 μL of 5× HOT FIREPol EvaGreen HRM mix (no ROX) (Solis BioDyne, Estonia), 0.5 μL of 10 pmol of each primer, 6.0 μL PCR water and 1.0 μL of template DNA. For *Brucella* spp., the reactions were carried out in 10 μL reaction volumes, containing 2.0 μL of 5× HOT FIREPol EvaGreen HRM mix (no ROX) (Solis BioDyne, Estonia), and 0.5 μL of 10 pmol of each primer of three primers; *Brucella arbutus* forward primer, *B. melitensis* forward primer, and *Brucella* spp. universal reverse primer targeting the IS711 gene (Probert *et al.*, 2004), 5.5 μL PCR water and 1.0 μL of template DNA. PCR amplification was preceded by an initial enzyme activation at 95°C for 15 min, followed by 10 cycles at 94°C for 20 sec, step-down annealing from 63.5°C with decrements of 1°C after each cycle for 25 sec, and primer extension step at 72°C for 30 sec; then 25 cycles of denaturation at 94°C for 25 sec, annealing at 50.5°C for 20 sec, and extension at 72°C for 30 sec followed by a final elongation at 72°C for 7 min. Immediately after PCR, HRM profiles of amplicons were obtained by increasing temperature gradually from 75 to 90°C at 0.1°C/2 sec increments. Changes in fluorescence with time (dF/dT) were plotted against changes in temperature (°C).

Screening of pathogenic animal African trypanosomes and camel-pox virus was done by PCR in a ProFlex thermocycler (Applied Biosystems). Trypanosome DNA was amplified by targeting trypanosomal internal transcribed spacer region using the following universal primer sets described by Njiru *et al.* (2005); ITS1_CF and ITS1_BR (Table 1) in 10 μL PCR volumes containing 0.1 units of Phusion DNA polymerase (Finnzymes, Espoo, Finland), 2 μL of 5× HF buffer, 0.2 μL of 10 mM dNTPs,

Table 1. Primers for amplification of target genes for detection of disease-causing camel pathogens. Primers sequences were sent to inqaba biotec™ (Muckleneuk, Pretoria, South Africa) for synthesis.

Primer name	5'to 3' sequence	Target organism	Target gene	Product size (bp)	References for primers
IS1111F IS1111F	GTA ATA TCC TTG GGC GTT GAC G ATC TAC GCA TTT CAC CGC TAC AC	*C. burnetii*	*Coxiella* 16S rRNA	242	(Doosti *et al.,* 2014)
*Anaplasma*JVF *Anaplasma*JVR	CGGTGGAGCATGTGGTTTAATTC CGRCGTTGCAACCTATTGTAGTC	*Anaplasma* spp.	*Anaplasma* 16S rRNA	300	(Mwamuye *et al.,* 2017)
*Ehrlichia*JV F *Ehrlichia*JV R	GCAACCCTCATCCTTAGTTACCA TGTTACGACTTCACCCTAGTCAC	*Ehrlichia* spp.	*Ehrlichia* 16S rRNA	300	(Mwamuye *et al.,* 2017)
EHR16SD 1492R	GGTACCYACAGAAGAAGTCC GGTTACCTTGTTACGACTT	*Ehrlichia &* *Anaplasma* spp.	16S rRNA	1000	(Parola *et al.,* 2000; Reysenbach *et al.,* 1992)
RLB F RLB R	GAGGTAGTGACAAGAAATAACAATA TCTTCGATCCCCTAACTTTC	*Theileria &* *Babesia* spp.	*Theileria &* *Babesia* 18S rRNA	450	
CMLVC18LF CMLVC18LR	GCGTTAACGCGACGTCGTG GATCGGAGATATCATACTTTACTTTAG	Camel pox virus	C18L gene	243	(Balamurugan *et al.,* 2009)
B. arbutus F *B. melitensis* F *Brucella* IS711T R	GCGCTCAGGCTGCCGACGCAA GCGGCTTTTCTATCACGGTATTC GGGTAAAGCGTCGCCAGAAG	*Brucella* spp	IS711		(Probert *et al.,* 2004)
ITS1_CF ITS1_BR	CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAAC- GAA	*Trypanosoma* spp.	ITS1	250-720	(Njiru *et al.,* 2005)
ILO 7957F ILO 8091R	GCCACCACGGCGAAAGAC TAATCAGTGTGGTGTGC	*T. evansi*	RoTat 1.2	488	(Urakawa *et al.,* 2001)

0.2 μL of 10 mM of each primer and 6.3 μL of nuclease free water. The PCR conditions were as follows: 98°C for 1 min, 40 cycles of 98°C for 30 sec, 61°C for 30 sec, and 72°C for 45 sec, with a final elongation step of 7 min at 72°C. Camelpox virus C18L gene was amplified using CMLV C18LF and CMLV C18LR primers described by Balamurugan *et al.* (2009). The PCRs were carried out in a 10-μL reaction mixtures containing 5.0 μL of DreamTaq Green PCR master mix (2×) (Thermo Scientific), 0.5 μL of 10 mM of each primer, 1.0 μL of DNA template, and 3.0 μL of nuclease-free water. The PCR thermocycling conditions included; initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec followed with a final elongation of 72°C for 5 min. The PCR amplicons were electrophoresed on 1.5% ethidium bromide-stained agarose gel and visualized under ultraviolet light.

DNA purification and sequencing

Representative positive samples producing distinct amplicons with expected band sizes relative to the known positive DNA controls were selected for amplification in larger PCR reaction volumes (30-μL). The PCR amplicons were separated by electrophoresis in ethidium bromide-stained 1.5% agarose gels and visualized under ultraviolet light. The target bands were excised and gel purified using QIAquick PCR purification kit (Qiagen, Germany) according to manufacturer's instructions. The purified amplicons were sent to Macrogen Inc. (Netherlands) for Sanger sequencing.

Since it is not possible to resolve the trypanozoon species using ITS1 primers, which give 480-bp PCR product sizes (Njiru *et al.*, 2005), two samples positive for *Trypanozoon*, one from camel and the other from hippoboscid, were amplified using ILO 7957F and ILO 8091R primers (Table 1) targeting RoTat 1.2 VSG gene described by Urakawa *et al.* (2001).

To identify the *Anaplasma* species associated with the HRM peaks observed, amplicons of two samples positive for *Anaplasma* spp., one from camels and one from camel ked, were selected for sequencing using AnaplasmaJVF and *Anaplasma*JVR targeting 300-bp of *Anaplasma* 16S rRNA genes. These primers could not resolve *Anaplasma* to species level. To resolve the *Anaplasma* to species level, a longer 1000-bp fragment of *Anaplasmataceae* 16S rRNA gene was further amplified by conventional PCR using published primers EHR16SD and 1492R (Parola *et al.*, 2000; Reysenbach *et al.*, 1992, Table 1), and sequenced. The PCR amplifications were performed in a ProFlex PCR system (Applied Biosystems by life technologies) with the following cycling conditions: 95°C for 15 min; two cycles of 95°C for 20 sec, 58°C for 40 sec, and 72°C for 90 sec; three cycles of 95°C for 20 sec, 57°C for 30 sec, 35 cycles of 95°C for 20 sec, 56°C for 40 sec and 72°C for 90 sec, and a final extension at 72°C for 10 min (Bastos *et al.*, 2015).

Sequences obtained in the study were deposited in GenBank database with the following accession numbers: short 16S

rRNA of *Anaplasma* spp. in camel (MN306317) and camel ked (MN306316); full length 16S rRNA of "*Candidatus* Anaplasma camelii" (MN306315), *T. vivax* ITS1 in camel (MK880188), and camel ked (MK880189); RotTat 1.2 VSG gene of *T. evansi* in camel (MK867833) and camel ked (MK867832).

Data analysis

Data on sampled camels and hippoboscids were entered into Microsoft Excel spreadsheet, version 12.3.1. Georeferenced data of the sampling sites and administrative boundaries data from Kenya Open Data in shapefile data format, were loaded into the ArcMap component of ArcGIS 10.6 software. The component was then used to design and generate the map layout of the sampling sites.

Using the MAFFT plugin in Geneious Prime 2019.1.1 software version (created by Biomatters) (Kearse *et al*., 2012), all study nucleotide sequences were edited and aligned with related sequences identified by querying in the GenBank nr database using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST/).

Results

Detections of pathogens

Out of 249 camel samples screened, 102 (40.96%) tested positive for trypanosomes by ITS1 PCR (Table 2). All trypanosome positive samples were infected with *T. vivax* showing an expected band of 250 bp and confirmed by amplicon sequencing. Mixed infections with *T. vivax* (250-bp band) and *T. evansi* (480-bp band) were detected in three camels (1.2%).

Out of 117 *H. camelina* samples, 53 (45.30%) were infected with trypanosomes, all of which had *T. vivax*. Three flies (2.56%) had mixed infections with *T. evansi* and *T. vivax*. Additionally, one fly had double infection of *T. vivax* and *Trypanosoma* sp. amplicon of about 400 bp. The 400 bp *Trypanosoma* sp. was sequenced using the ITS1 marker and shared 98.14% identity with *Trypanosoma melophagium* (GenBank accession HQ664851) that was sequenced from *Melophagus ovinus*, sheep ked, in Croatia. Figure 3 shows pairwise alignment of *T. melophagium* sequence from this study and that from GenBank.

In total 98% of all monoconical trap catches were *Stomoxys calcitrans*, with the remaining 2% consisting of Tabanidae and *H. camelina* (Table 3). None of the traps caught tsetse (0%) in all sampling locations.

"*Candidatus* Anaplasma camelii" was detected in 68.67% (n = 171/249) of dromedary camels and 16.24% (n = 19/117) of *H. camelina* (Figure 4). Though the 300-bp *Anaplasma* 16S rRNA sequences could not resolve the *Anaplasma* spp. to species level, analysis of the 1000-bp 16S rRNA nucleotide sequence showed 100% identity with "*Candidatus* Anaplasma camelii" sequenced from camels in Saudi Arabia (GenBank accession numbers KF843824-KF843825) and Iran (GenBank accession KX765882) (Figure 5). Piroplasms (*Theileria* spp. and *Babesia* spp.), *C. burnetii*, *Ehrlichia* spp., *Brucella* spp., and camel pox were not detected either in camels or keds collected from them.

Discussion

We report the occurrence of similar blood-borne pathogens in dromedary camels and in *H. camelina* flies collected from the same herds. The high infection rates of pathogens in camels (*T. vivax* = 41%, *T. evansi* = 1.2%, and *Anaplasma* spp. = 68.67%) and flies (*T. vivax* = 45.3%, *T. evansi* = 2.56%, and *Anaplasma* spp. = 16.24%) suggest the potential of these camel biting flies in disease transmission as well in diagnosis of haemopathogens found in camels. Thus, our findings show *T. vivax* as the most predominant species causing trypanosomiasis in camels sampled in September 2017 from Koya and its surroundings. Similarly, we recorded high fly infection rates of 45.3% caused by the same parasite, *T. vivax*. These high *T. vivax* infection rates could be attributed to mechanical transmission by several biting flies such as *Tabanus* spp. and *Stomoxys* species (Baldacchino *et al*., 2013) that were collected in this study using monoconical traps (Table 3). We hypothesize that camels are initially infected with trypanosomes when nomadic pastoralists occasionally move their livestock into distant neighbouring tsetse-infested regions in search of pasture and water, and thereafter maintenance of pathogen transmission among camels continues throughout the year via mechanical transmission in the process of bloodmeal acquisition by biting flies such as camel hippoboscids.

Tsetse flies were not caught despite repeated attempts to trap them in major sampling sites using monoconical traps with cow urine and acetone (Table 3). This ASAL region in Marsabit south is generally arid, hot, and dry (low humidity) with poor vegetation cover that presumably renders it uninhabitable for tsetse flies. However, by using robust landscape and climatic data modeling, Marsabit has generally been predicted as a region with potential risk of tsetse infestation (Moore & Messina, 2010).

Table 2. Summary of selected pathogens detected in camels and *Hippobosca camelina*.

Pathogen	Prevalence in camels (*n* = 249)	Prevalence in *H. camelina* (*n* = 117)
Trypanosoma vivax	102 (41%)	53 (45.3%)
Trypanosoma evansi	3 (1.2%)	3 (2.56%)
Trypanosoma melophagium	0 (0%)	1 (0.85%)
"*Candidatus* Anaplasma camelii"	171 (68.67%)	19 (16.24%)

Figure 3. Pairwise alignment of ITS1 sequences of _T. melophagium_. ITS1 sequence of _T. melophagium_ in study sample H63 was aligned with highly identical sequence (HQ664851) from GenBank. At position 32, there is a nucleotide change from C in the sequence from GenBank to Y in the sequence from this study.

Table 3. Biting flies were trapped using monoconical traps with cow urine and acetone as attractants. Daily trap collections were pooled, fly species sorted, counted, and preserved in absolute ethanol for transportation to the Nairobi-based laboratories at icipe. Majority of the fly collections comprised of _Stomoxys calcitrans_. Tsetse flies (genus _Glossina_) were absent in all traps.

Sampling site	Day	*Daily fly captures	Sex (M = male; F = female)
Kula pesa 01° 35’ 44.9” N, 037° 48’ 35.8” E	Day 1	12	12 F – _Stomoxys calcitrans_
	Day 2	8	5 F – _S. calcitrans_; 1 M & 2 F – _Hippobosca camelina_
	Day 3	4	3 F – _S. calcitrans_; 1 F – _H. camelina_
	Day 4	12	9 F & 1 M - _S. calcitrans_; 1 F – _Tabanus_ spp.; 1 M – _H. camelina_
	Day 5	5	4 F – _S. calcitrans_; 1 F – _H. camelina_
	Day 6	11	1 M & 9 F – _S. calcitrans_; 1 M – _H. camelina_
	Day 7	8	6 M & 2 F – _S. calcitrans_
Soweto 01° 35’ 43.1” N, 037° 48’ 35.7” E	Day 1	2	2 F – _S. calcitrans_
	Day 2	9	2 M & 7 F – _S. calcitrans_
	Day 3	7	1 M & 6 F – _S. calcitrans_
	Day 4	7	2 M & 4 F – _S. calcitrans_; 1 F – _H. camelina_
	Day 5	2	2 F – _S. calcitrans_
	Day 6	9	9 F – _S. calcitrans_
	Day 7	0	Biting flies count = 0 (Only house flies were trapped)
Naigero 01° 35’ 49.7” N, 037° 49’ 58.1” E	Day 1	33	8 M & 25 F – _S. calcitrans_
	Day 2	14	2 M & 12 F – _S. calcitrans_

*Daily fly captures: represent pools of three trap catches per site per day.
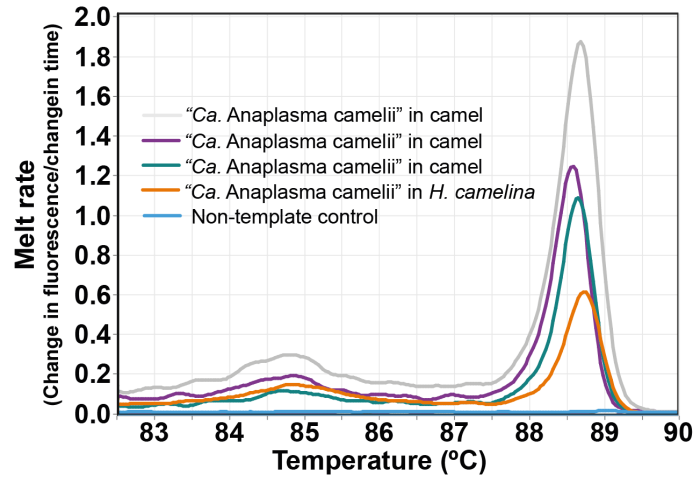
Figure 4. Melt curves of amplification of 16S rRNA of "*Candidatus* Anaplasma camelii" in camels and camel keds.
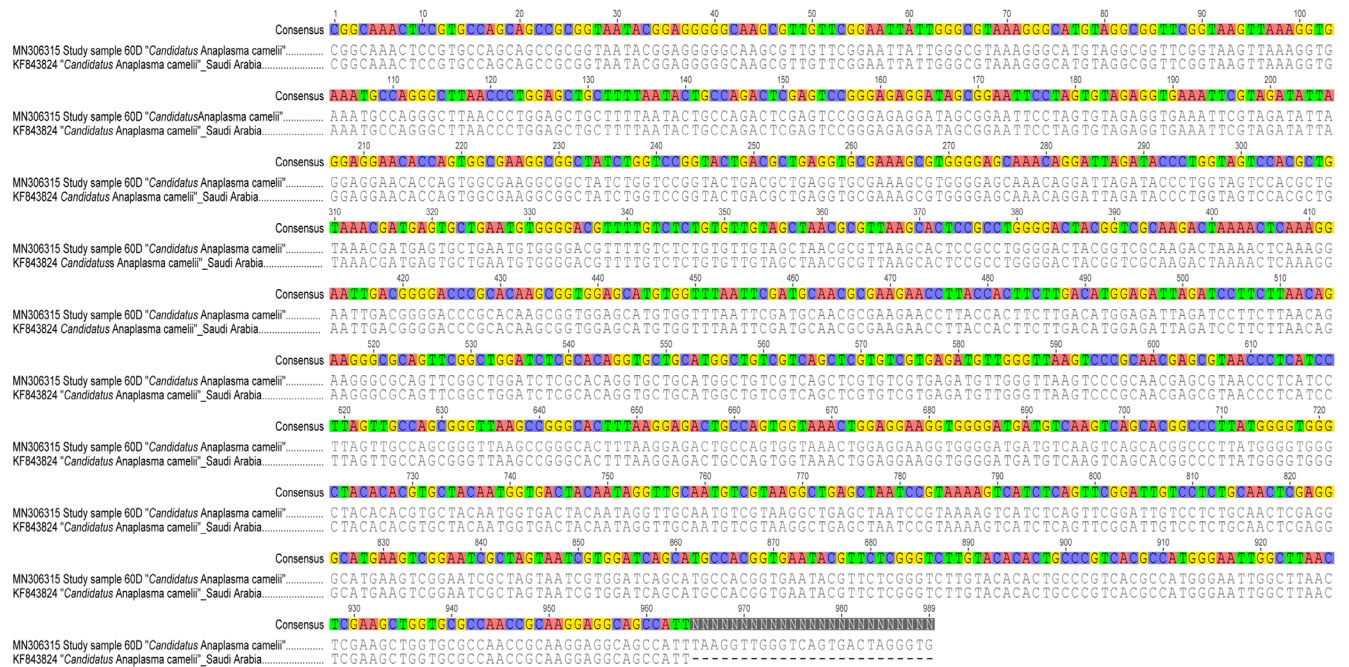

Figure 5. Pairwise alignment of 16S rRNA sequences of "*Candidatus* Anaplasma Camelii" amplified from camel blood sample 60D with GenBank-retrieved nucleotide sequence (KF843824) showed 100% identity. N is ambiguous nucleotide.

Currently, little is known about the prevalence and transmission of vector-borne diseases of livestock in northern Kenya, mostly because these animals belong to the marginalized poor nomadic pastoralists whose economic welfare is neglected. Thus, information about *T. vivax* in Kenyan camels is scarce. However, *T. vivax* has been reported in camels in Sudan, Ethiopia, and Nigeria (Fikru *et al.*, 2015; Mbaya *et al.*, 2006; Mossaad *et al.*, 2017). The pathogenicity of *T. vivax* infection in camels is not well understood, but it is known to be pathogenic to cattle, sheep, equines, and goats (Galiza *et al.*, 2011). Our finding of a high trypanosome infection rate (40.96%) in camels, consisting predominantly of *T. vivax*, suggests that infected camels could act as parasite reservoirs for other susceptible and often co-herded livestock species in the region.

Trypanosoma evansi infection rates of 1.2% in camels reported in this study is much lower than earlier reports of up to 46% infections in other regions of Kenya (Ngaira *et al.*, 2004; Njiru *et al.*, 2004). These regional variations in prevalence of *T. evansi* infection could result from seasonal disease outbreaks, variations

in the micro-climatic conditions, disease stability in endemic zones, and the presence of competent insect-vectors, among other factors, including differences in the study designs and time lapse. Our study design has key limitations in comparative studies because sampling was not done during wet season, thus the relationship between seasonality versus prevalence of haemoparasites and vector density could not be established. *Trypanosoma evansi* is considered the most important protozoan pathogen of dromedary camels in Kenya (Njiru *et al*., 2004) and has been reported to infect horses and donkeys in other parts of the world (Desquesnes *et al*., 2013). Additionally, cases of atypical *T. evansi* infections have been reported in humans (reviewed in Truc *et al*., 2013) and was specifically attributed to frameshift mutations of apolipoprotein L-I in one of the patients (Vanhollebeke *et al*., 2006), thus resulting into lack of immunity against African animal trypanosomes.

"*Candidatus* Anaplasma camelii" infection, which we report here for the first time in Kenyan camels, was the most prevalent (68.67%) amongst all detected pathogens in this study. This emergent *Anaplasma* pathogen was recently detected in 35.85% of Moroccan dromedary camels (Ait Lbacha *et al*., 2017). *Anaplasma* sp. isolated from Kenyan camels was 100% identical to the GenBank-retrieved "*Candidatus* Anaplasma Camelii" 16S rRNA nucleotide sequences from Saudi Arabia and Iran dromedary camels, hence suggesting a common origin of this pathogen (Figure 5). High prevalence of camel anaplasmosis could be attributed to ticks (the definitive vector of *Anaplasma*) that were present on 100% of camels, and in addition, biting flies such as *Stomoxys calcitrans* promote mechanical transmission (Scoles *et al*., 2005). Although previous studies could not prove the ability of cattle keds (*Hippobosca rufipes*) to transmit *Anaplasma marginale* (Potgieter *et al*., 1981), it is possible that in the process of bloodmeal acquisition, camel keds, *Hippobosca camelina*, could mechanically transmit anaplasmosis via contaminated mouthparts (unpublished study; authors from the present study). The clinical role of "*Candidatus* Anaplasma camelii" in camels is uncertain, but oedema has been observed in infected camels (Ait Lbacha *et al*., 2017).

The high prevalence of "*Candidatus* Anaplasma camelii" in healthy dromedary camels indicates the possible role of camels as reservoir hosts for maintaining its circulation. Further research is needed to determine the zoonotic potential of this tick-borne pathogen. This is important because cases of human infection with *Anaplasma platys* and *Ehrlichia canis,* that are closely related to the emergent "*Candidatus* Anaplasma camelii" pathogen, have been reported (Arraga-Alvarado *et al*., 2014; Doudier *et al*., 2010). This possibly zoonotic pathogen of camels (Lbacha *et al*., 2017) should stimulate the need for increased surveillance by veterinary and public health partners to mitigate spread of infection to humans and other animals.

We detected *T. vivax*, *T. evansi*, *T. melophagium*, and *Anaplasma* species in *Hippobosca camelina*. Detection of identical haemopathogens in *H. camelina* flies as well as in camels from which they were collected suggests that this fly could play role in the transmission of infectious agents amongst its bloodmeal hosts. The ability of *H. camelina* as efficient flier facilitates fast movements on the host or between camel hosts, hence increasing its chances of acquiring infected bloodmeal that could transmitted to the next host following interrupted feeding. Various hippoboscid species have been implicated in transmission of pathogens (Rahola *et al*., 2011). For instance, *Hippobosca longipennis* is thought to transmit the larva of filarial nematode *Acanthocheilonema dracunculoides* to hyenas and domestic dogs (Nelson, 1963; Rani *et al*., 2011). Louse flies, *Melophagus ovinus,* play a role in the transmission of *Bartonella* spp. among ruminants (Halos *et al*., 2004). Another louse fly known as *Icosta americana* is suspected to transmit West Nile virus in North America (Farajollahi *et al*., 2005). Further studies are needed to determine the vectorial competence of *H. camelina* in the transmission of pathogens.

Potential role of *H. camelina* in xenodiagnosis

Our findings consistently show that the blood-borne pathogens detected in camels are also present in *H. camelina* collected from them (i.e. sampled camels). It is likely that when keds bite camel hosts to acquire bloodmeals, they also take up haemopathogens if the camel is infected.

H. camelina acquires bloodmeals from camels for nutrition and reproduction. Adult stage of keds are obligate blood-feeding ectoparasites of camels that hardly leave their host, unless disturbed and even then, they quickly find the next host. Keds have claspers for firm attachment to the skin hairs of the host during feeding or resting. These flies that prefer to always remain on the vertebrate host, preferentially attach to specific body parts, commonly on the underbelly (Figure 2) of the camel, near or on the udder, or the perineal region where they are not easily disturbed during bloodmeal acquisition (Higgins, 1985). These features of camel keds make them good candidates for xenosurveillance and they can be collected easily for molecular screening to detect pathogens acquired from naturally infected camels in the process of feeding. Screening of camel keds for indirect detection of pathogens present in camels, from which they were collected, will save on time and cost. Collection of keds off camels was much easier and required relatively less time than blood sampling. We employed six field assistants to restrain each camel for blood collection, veterinary personnel who collected blood samples, and additional three assistants to carry cool boxes and consumables, ensure accurate labeling of samples and storage, and recording of baseline data. On the other hand, only about four field assistants were needed to collect keds from camel herds, resulting in >50% reduction in labour costs and the required human resource. Fly collection also took shorter time as it was not necessary to restrain camels. Importantly, this xenosurveillance detection provides a less invasive approach than the currently available painful blood collection procedures that pose huge risk to the handlers as camels could occasionally cause severe and even fatal injuries through bites (Abu-Zidan *et al*., 2012) or by kicking with their legs. In a similar indirect pathogen detection approach, previous reports showed the utility of mosquitoes in xenosurveillance of human pathogens (Grubaugh *et al*., 2015).

Additionally, a novel *Trypanosoma* sp. closely related to *Trypanosoma melophagium* was detected in one camel ked,

H. camelina (1/117) , but not in camels. This host-specific parasite of sheep, called *T. melophagium*, has never been reported to cause camel infections. Interestingly, *T. melophagium* is known to be solely transmitted by wingless sheep ked called *Melophagus ovinus* (Gibson *et al.*, 2010). We conducted a survey of sheep keds among small ruminants in our study area in northern Kenya and found that they are absent in the region (unpublished study; authors from the present study). Thus, molecular detection of *T. melophagium* in a single camel-specific ked that was collected from camel raises an interesting question about the origin of this parasite. *H. camelina* acquired contamination possibly from *T. melophagium*-infected vertebrate host through bloodmeal. Further studies are needed to determine the vectorial competence of *H. camelina* in transmission of *T. melophagium*.

Conclusions

Our findings suggest the potential role of *H. camelina* in xenodiagnosis for detection of haemopathogens in camels, thus bypassing the need to obtain blood samples via jugular venipuncture for pathogen detection. Further studies to profile additional blood-borne pathogens including viral diseases occurring both in camels and *H. camelina* that fed on them, will be crucial for supporting usage of hippoboscids in xenomonitoring of camel diseases.

Data availability

Underlying data

16S r RNA of *Anaplasma* sp. in camel, Accession number MN306316: https://www.ncbi.nlm.nih.gov/nuccore/MN306316

16S r RNA of *Anaplasma* sp. in camel ked, Accession number MN306317: https://www.ncbi.nlm.nih.gov/nuccore/MN306317

16S rRNA of *Candidatus* Anaplasma camelii, Accession number MN306315: https://www.ncbi.nlm.nih.gov/nuccore/MN306315

T. vivax ITS1 in camel, Accession number MK880188: https://www.ncbi.nlm.nih.gov/nuccore/MK880188

T. vivax ITS1 in camel ked, Accession number MK880189: https://www.ncbi.nlm.nih.gov/nuccore/MK880189

RotTat 1.2 VSG gene of *T. evansi* in camel, Accession number MK867833: https://www.ncbi.nlm.nih.gov/nuccore/MK867833

RotTat 1.2 VSG gene of *T. evansi* camel ked, Accession number MK867832: https://www.ncbi.nlm.nih.gov/nuccore/MK867832

Figshare: Detection of Anaplasma and Trypanosomes in camels and camel keds, https://doi.org/10.6084/m9.figshare.10050587 (Kidambasi *et al.*, 2019).

This project contains the following underlying data:
- Raw HRM Rotor-Gene Q data files of *Anaplasma* spp. amplification in camels and camel keds. HRM data files can be accessed using Rotor-gene Q software.

- Gel visualization images of resolved PCR amplicons for detection of African trypanosomes in camels and camel keds.

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 21 May 2020

https://doi.org/10.21956/aasopenres.14165.r27470

✔ **Dennis Muhanguzi** (iD)

School of Biosecurity, Biotechnical and Laboratory Science, College of Veterinary Medicine Animal Resources and Biosecurity, Makerere University, Kampala, Uganda

The authors have implemented all suggestions I made to Version 1 to my satisfaction. Where they were not able to implement the suggested changes, they provided satisfactory explanations or included these challenges as limitations to the study.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular epidemiology, Spatial epidemiology, Veterinary Sciences

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 21 May 2020

https://doi.org/10.21956/aasopenres.14165.r27469

✔ **Elizabeth A. Opiyo** (iD)

Department of Biology, Faculty of Science, Gulu University, Gulu, Uganda

No further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector biology and parasitology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

- -

Version 1

Elizabeth A. Opiyo (iD)
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Hippobosca camelina, also known as Keds sampled from camels and blood samples from the camels from which the Keds were samples, were examined for the presence of pathogens with a view of using Keds for xenodiagnosis of camel haemopathogens. The study took place in Laisamis, northern Kenya. The samples were screened using PCR and PCR-HRM and revealed the presence of epizootic pathogens in camels from Kenya and suggests that Keds have potential use in diagnosis of heamopathogens circulating in camels.

In the study design and sampling, no particular sample size or criteria appear to have been planned in advance. If this was opportunistic sampling then this should be stated and justified.

Regarding molecular diagnosis of pathogens, conventional PCR was used for detection of *C. burnetii*, *Anaplasma* spp, *Ehrlichia* spp, *Brucella* spp and for piroplasma belonging to *Theileria* and *Babesia* PCR-HRM and for trypanosomes conventional PCR was followed by visualization and later gel electrophoresis and sequencing. The lack of uniformity in the analysis of the pathogens requires some explanation/justification for the readers that may be interested in doing the same analysis for their studies.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector biology and parasitology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 07 May 2020

Joel L. Bargul, International Centre of Insect Physiology and Ecology, Kenya

Reviewer: In the study design and sampling, no particular sample size or criteria appear to have been planned in advance. If this was opportunistic sampling then this should be stated and justified.

Response: The type of sampling is now specified as opportunistic sampling and was adopted for convenience by sampling camels from diverse geographical locations as they converge at specific water drinking points. Otherwise it will be challenging to conduct daily sampling of camels considering that camel owners are nomadic pastoralists with busy lifestyles characterized by long distance movements together with their animals and other belongings.

Reviewer: Regarding molecular diagnosis of pathogens, conventional PCR was used for detection of *C. burnetii*, *Anaplasma* spp, *Ehrlichia* spp, *Brucella* spp and for piroplasma belonging to *Theileria* and *Babesia* PCR-HRM and for trypanosomes conventional PCR was followed by visualization and later gel electrophoresis and sequencing. The lack of uniformity in the analysis of the pathogens requires some explanation/justification for the readers that may be interested in doing the same analysis for their studies.

Response: PCR-HRM assays (i.e. combination of both conventional PCR and HRM) were used in detection of all listed pathogens above, except trypanosomes whose detection protocols by HRM are not established at present hence sufficiently screened using conventional ITS-1 PCR by Njiru et al (2005) followed by gene sequencing. The advantage of HRM is that helps in selection of representative samples producing unique HRM melting curves for gene sequencing thus saving on cost.

Competing Interests: No competing interests were disclosed.

Reviewer Report 18 November 2019

? **Dennis Muhanguzi** iD

School of Biosecurity, Biotechnical and Laboratory Science, College of Veterinary Medicine Animal Resources and Biosecurity, Makerere University, Kampala, Uganda

Reviewer's Summary

Kidambasi K.O *et al.* set out to identify the most important Arthropod-Vectored *C. dromedarius* pathogens and those in *Hippobosca camelina* [Camel Keds] as well as to asses the potential use of camel keds in xenodiagnosis of *C. dromedarius haemo*pathogens in Marsabit county, Northern Kenya. They used a battery of molecular techniques to identify the most important parasites circulating in camels [n=249] and Camel Keds [n=117] as *T. vivax and T. evansi.* As well, *Candidatus A. camelli* was detected in camels and not Camel keds while *T. melophagium* was detected in Camel keds and not Camels. Entomological techniques were used to identify *Stomoxys calcitrans* as the major biting fly in this region. Given a close match of pathogens detected in camels and camel keds that were sampled from them, the authors discuss herein the potential use of camel keds in xenodiagnosis of camel haemopathogens and the animal and public health roles of the identified hemopathogens.

This is a good manuscript in its area but needs major changes to further improve its quality and scientific merit.

Minor Changes [discretionary]

Please consider implementing the following minor changes

1. Throughout this manuscript, change the phrase…."disease pathogens" to 'pathogens' because all pathogens cause disease

2. Please consider removing absolute fractions in the results section of the abstract given that you made mention of the number of samples analysed in the methods section of this abstract. If you decide to maintain them, please write them as; Trypanosoma vivax; 41 % [102/249], Trypanosoma evansi; 1.2 % [3/249]. You have indicated that only 200 camel blood samples were analysed to arrive at the prevalence of *Candidatus A. camelli*. I was unable to find a reason for this in the methods and materials section. Please cross check that this was not quoted in error. In case you did analyze 200 instead of 249 camel blood samples please explain this choice in your materials and methods section.

3. Please consider changing ---unpredictable rainfalls to … unpredictable rainfall

4. Rephrase the first sentence of the third paragraph of this section. You can as well break this sentence into two sentences i.e. Hippoboscids (keds) are obligate hematophagous ectoparasites of mammals and birds. They belong to the family Hippoboscidae within the superfamily Hippoboscoidae (Petersen et al., 2007[1]; Rahola et al., 2011[2]).

Methods and materials

1. **Weather conditions**: ' ….short and long rains to'……short and long wet seasons

2. **Study design and sample collection**: '……samples were collected after receiving informed verbal consent from camel keepers. All camel keepers were neither able to read nor write, thus verbal rather than the written consent was adopted as the pragmatic approach…' This sentence should be moved to Ethical approval on page 4 of 11.

3. **Ethical approval [page 4 of 11]**: please delete the sentence that begins with JB…the principal investigator…

4. **DNA extraction [Page 4 of 11]**: unless it is the journal requirement, 2.0-mm or 1.5-mL; change this to 2.0 mm or 1.5 mL

5. **Detection of pathogen DNA [page 5 of 11]:** Anaplasma and Ehrlichia were detected by PCR amplification using genus-specific primers…. should be changed to …. Anaplasma and Ehrlichia species were detected by PCR amplification using genus-specific primers. Please rewrite the sentence……….. Brucella arbutus forward primer, B. melitensis forward primer, and Brucella spp. universal reverse primer targeting the IS711 gene (Probert et al., 2004), 5.5 µL PCR water and 1.0 µL of template DNA. …..Camel pox…. in the last paragraph of this section as well as table 1 should be changed to Camel pox Virus [CMLV]. As you will remember, you did not detect the disease but CMLV genetic material.

6. **DNA purification and sequencing [page 5 of 11].** Second sentence of this section …..The PCR amplicons….is incomplete. Please complete this sentence.

7. **Data analysis**: Please delete the sentence that begins with….. Ground truthing….Ground truthing applies more to remote sensing and machine learning. You just need to explain how the map in figure 1 was drawn in the sentence that follows. Here you will need to mention the ArcGIS v. 10.6 extension that you used to complete this map.

8. **Results:** Please transfer contents of paragraph 4 that start with ….sequences obtained in the study… to an appropriate section under methods and materials. As well, explanatory text of Table 3 on page 8 of 11 sounds like methods and materials information. Please keep that in methods and materials and provide a stand-alone legend for this table if required.

9. **Major comments: Methods and Materials**: There is need to include sections on Sample size determination and sampling strategy as well as to improve the current sub-sections under this section. In your introduction section, you indicated that about 3 million camels are kept in northern Kenya. Under methods and materials, there is no explanation of how many of these 3 million camels are kept in Laisamis or even Marsabit County. Reading this manuscript the following questions arise. Are 249 camels sampled over 5 days period representative of **n** camels in the study country or Laisamis zone? Are the Laisamis camels representative of all the 3 million camels in Nothern Kenya? Were all the camels presented in the 5 sampling days sampled so long as their owners consented to the study?, If not, how were the 249 camels sampled from **n₂** camels presented during the 5 sampling days? Why was sampling only done in September [short wet season]? How were the 21 sampled herds [at 4 sites] arrived at? How many herds were there in the county and how were the 21 herds selected from all the county camel herds? What is the definition of a herd given that animals that are owned in a communal pastoral husbandry obtaining mix-up? What was the sampling unit? How were the sites for biting fly trapping selected and what was the inter trap distance? etc…\

10. Data analysis needs to be revisited. The fly apparent density in table 3 can be well presented spatially. To be able to discuss possibility of mechanical transmission of different hemopathogens by different biting flies e.g. Stomoxys the association between fly apparent density and hemopathogen prevalence needs to be adjusted for potential spatial dependence. This you can do using generalized least squares model with a Gaussian spatial correlation structure to quantify the

effect or other appropriate models.

11. Please include a pairwise alignment of *Candidatus A. camelli* from this study and those from the GenBank as was done for *T. melophagium*

12. **Discussion:** I would like to draw the authors' attention to some of the following discussion sections which I strongly believe they need to revisit.

13. First paragraph [Page 7 of 11]: Associating the high prevalence of hemopathogens in camels to the potential of camel keds in hemopathogen transmission is not supported by the results of this study. Note that keds were only less than 2% of all biting flies trapped. This can only be attributed to mechanical transmission of these hemopathogens by *Stomoxys Calicitrans* and Tabanids which were >98% of all biting flies trapped. You can be authoritative about these associations if you improve data analysis as recommended in comment II above

14. Pursuant to comment 13 above, you need to rewrite the hypothesis you make at the end of paragraph 1 of discussion section on page 7 of 11. If Mechanical transmission of camel hemopathogens were important in this region, it would rather be by Stomoxys and other Tabanids and not camel keds [camel keds were <2% of all biting flies trapped]; moreover you did not rule out or in potential spatial dependence between hemoparasite prevalence and fly apparent density.

15. Paragraph 2 of discussion [page 7 of 11]. There is mention of reports that support absence of tsetse flies in the study area and yet no references of such reports are included. When I checked this fact myself, I found that this study area has recently been cited as an area with high risk of tsetse infestation using robust landscape and climatic data modeling[3].

16. Last paragraph on page 7 of 11; The variations in the micro-climatic conditions, differences in the study designs and time lapse are the most likely explanations for the differences in *T. evansi* prevalence in camels previously reported in other parts of Kenya and in this study.

17. There is need to include a discussion of the limitations of this study [see major comment I & VII above. Think of snap short sampling during short wet season? seasonality vs hemoparasite and vector density etc

18. There is need to nuance the recommendation about heightening public and veterinary surveillance of *T. evansi* as a zoonotic hemoparasite [first paragraph, page 9 of 11] because there are no reported major outbreaks of this atypical human African trypanosomiasis either in Kenya or elsewhere ever reported. The only cases of atypical *T. evansi* human infections have been reported in either immunocompromised or accidental infections[4] that do not warrant setting up veterinary and public health surveillance programs.

19. Second last sentence; paragraph 2, page 9 of 11….it is conceivable …. . This needs to be reinterpreted. Finding *Candidatus A. cameli* in keds and camels certainly means that keds are feeding predominantly on camels positive for *Candidatus A. cameli. This study design was* not to prove mechanical transmission of *Candidatus A. cameli* by Keds; given that previous mechanical transmission studies were not able to prove that, it is not conceivable in a study of such a design to make this assertion.

20. Second sentence of paragraph 4 page 9 of 11…….detection of similar haemopathogens in these camel flies…… This needs to be reinterpreted. Finding a similar repertoire of hemoparasites in keds and in camels only indicates that keds were feeding on hemoparasite positive camels. Only 2 % of the caught biting flies were keds and you can't emphasize ked mechanical transmission than that of Tabanids and Stomoxys which were > 98% of all biting flies trapped; with known mechanical transmission potential. The only application you can make out of this result is about xenodiagnosis and not mechanical transmission of hemoparasites by keds unless this is proven in study with suitable study design or you can refer to literature!

21. Page 10 of 11, first sentence: The point you make about xenodiagnosis saving time and money needs to be substantiated. It takes as much time to collect keds from camels as it takes to take blood samples from camels. If similar diagnostic methods are used to detect pathogens in keds and camels, I would not anticipate pathogen detection in keds to be any cheaper than pathogen detection in camels? can you please discuss how xenodiagnosis would be cheaper and shorter than detection of pathogens directly from camel blood?

22. Last paragraph of discussion section, page 10 of 11. ; …..Molecular detection of *T. melophagium* ….

Note that detection of this parasite in a ked does not mean that such a ked was infected with *T. melophagium*. Unless proven, it would mean that it had consumed a blood meal from a host [might not be camel at all since no camel was found positive for genetic material of this parasite] that had been positive for *T. melophagium* genetic material. This has nothing to do with *H. camelina* being able to transmit [biologically or mechanically] *T. melophagium.*

Conclusion
This needs to be refined after refining the discussion. Blood samples need not to be taken from the Jugular. You can these days take blood samples [125 ul] from ear veins and have them preserved on FTA cards. Unless substantiated as in comment XII, this conclusion has to be rewritten so that it is supported by the findings of this study.

References
As indicated in my comments above, the attention of the authors is drawn to some of the key literature they were not able to refer to in their discussion section e.g Truc *et al*.[4], Moore *et al*.[3].

References
1. Petersen I, Johnson AM, Islam A, Duckworth G, et al.: Protective effect of antibiotics against serious complications of common respiratory tract infections: retrospective cohort study with the UK General Practice Research Database.*BMJ*. 2007; **335** (7627): 982 PubMed Abstract | Publisher Full Text
2. Rahola N, Goodman SM, Robert V: The Hippoboscidae (Insecta: Diptera) from Madagascar, with new records from the "Parc National de Midongy Befotaka".*Parasite*. 2011; **18** (2): 127-40 PubMed Abstract | Publisher Full Text
3. Moore N, Messina J: A landscape and climate data logistic model of tsetse distribution in Kenya.*PLoS One*. 2010; **5** (7): e11809 PubMed Abstract | Publisher Full Text
4. Truc P, Büscher P, Cuny G, Gonzatti MI, et al.: Atypical human infections by animal trypanosomes. *PLoS Negl Trop Dis*. 2013; **7** (9): e2256 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular epidemiology, Spatial epidemiology, Veterinary Sciences

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 May 2020
Joel L. Bargul, International Centre of Insect Physiology and Ecology, Kenya

Minor Changes [discretionary]
Please consider implementing the following minor changes
1.   Throughout this manuscript, change the phrase…."disease pathogens" to 'pathogens' because all pathogens cause disease

Response: This has been corrected throughout the manuscript.

2.   Please consider removing absolute fractions in the results section of the abstract given that you made mention of the number of samples analysed in the methods section of this abstract. If you decide to maintain them, please write them as; *Trypanosoma vivax*; 41 % [102/249], *Trypanosoma evansi*; 1.2 % [3/249]. You have indicated that only 200 camel blood samples were analysed to arrive at the prevalence of "*Candidatus* A. camelii". I was unable to find a reason for this in the methods and materials section. Please cross check that this was not quoted in error. In case you did analyze 200 instead of 249 camel blood samples please explain this choice in your materials and methods section.

Response 1: absolute fractions have been removed from the results section of the Abstract.

Response 2: we analyzed a total of 249 samples as specified under 'Materials and Methods' section, and not 200 as quoted in the Abstract. We apologize for this error that is now corrected.

3.   Please consider changing ---unpredictable rainfalls to … unpredictable rainfall

Response: done.

4.   Rephrase the first sentence of the third paragraph of this section. You can as well break this sentence into two sentences i.e. Hippoboscids (keds) are obligate hematophagous ectoparasites of mammals and birds. They belong to the family Hippoboscidae within the superfamily Hippoboscoidae (Petersen et al., 20071; Rahola et al., 20112).

Response: done.

Methods and materials
5.   Weather conditions: ' ….short and long rains to'……short and long wet seasons

Response: Done

6.   Study design and sample collection: '……samples were collected after receiving informed verbal consent from camel keepers. All camel keepers were neither able to read nor write, thus verbal rather than the written consent was adopted as the pragmatic approach…' This sentence should be moved to Ethical approval on page 4 of 11.

Response: Above sentences have now been moved to "Ethical approval" section.

7.   Ethical approval [page 4 of 11]: please delete the sentence that begins with JB…the principal investigator…

Response: Deleted

8.   DNA extraction [Page 4 of 11]: unless it is the journal requirement, 2.0-mm or 1.5-mL; change this to 2.0 mm or 1.5 mL

Response: Done

9.   Detection of pathogen DNA [page 5 of 11]: *Anaplasma* and *Ehrlichia* were detected by PCR amplification using genus-specific primers…. should be changed to …. *Anaplasma* and *Ehrlichia* species were detected by PCR amplification using genus-specific primers.

Response: Done

Please rewrite the sentence……….. *Brucella arbutus* forward primer, *B. melitensis* forward primer, and *Brucella* spp. universal reverse primer targeting the IS711 gene (Probert et al., 2004), 5.5 µL PCR water and 1.0 µL of template DNA. …..Camel pox…. in the last paragraph of this section as well as table 1 should be changed to Camel pox Virus [CMLV]. As you will remember, you did not

detect the disease but CMLV genetic material.

Response: Rephrased

10. DNA purification and sequencing [page 5 of 11]. Second sentence of this section …..The PCR amplicons….is incomplete. Please complete this sentence.

Response: we apologize for this mistake and thank you for being very keen; the word 'were' is now inserted to complete the sentence.

11. Data analysis: Please delete the sentence that begins with….. Ground truthing….Ground truthing applies more to remote sensing and machine learning. You just need to explain how the map in figure 1 was drawn in the sentence that follows. Here you will need to mention the ArcGIS v. 10.6 extension that you used to complete this map.

Response: We thank the Reviewer for this suggestion that we have effected to improve the manuscript.

12. Results: Please transfer contents of paragraph 4 that start with ….sequences obtained in the study… to an appropriate section under methods and materials.

Response: this paragraph is now appropriately placed under 'methods and materials' section.

As well, explanatory text of Table 3 on page 8 of 11 sounds like methods and materials information. Please keep that in methods and materials and provide a stand-alone legend for this table if required.

Response: Many thanks for this comment, we have re-written heading of Table 3.

13. Major comments: Methods and Materials: There is need to include sections on Sample size determination and sampling strategy as well as to improve the current sub-sections under this section. In your introduction section, you indicated that about 3 million camels are kept in northern Kenya. Under methods and materials, there is no explanation of how many of these 3 million camels are kept in Laisamis or even Marsabit County.
Reading this manuscript the following questions arise. Are 249 camels sampled over 5 days period representative of n camels in the study country or Laisamis zone? Are the Laisamis camels representative of all the 3 million camels in Nothern Kenya? Were all the camels presented in the 5 sampling days sampled so long as their owners consented to the study?, If not, how were the 249 camels sampled from n2 camels presented during the 5 sampling days? Why was sampling only done in September [short wet season]? How were the 21 sampled herds [at 4 sites] arrived at? How many herds were there in the county and how were the 21 herds selected from all the county camel herds? What is the definition of a herd given that animals that are owned in a communal pastoral husbandry obtaining mix-up? What was the sampling unit? How were the sites for biting fly trapping selected and what was the inter trap distance? etc…

Response: more information is now provided, for instance, a recent study reported a total population of 203,320 camels in Marsabit County, where our present study was conducted (SSFR, 2017).

This field study was cross-sectional in design and involved opportunistic sampling, whereby we sampled camels converging at the water drinking points. This type of sampling was most convenient due to the nomadic pastoralist lifestyle involving frequent long distance movements. We sampled all camels in the herds found at water drinking points for 5 consecutive days in the dry season (September 2017). Due lack of data, we could not calculate the sample sizes, thus we collected as many samples as possible during the sampling duration.

Sampling was preferred in dry season of September because then the camel ked densities are high, unlike during wet season.

Flies were randomly collected from 21 camel herds (that was possible in 5 days) and we targeted to collect as many keds infesting camels as possible. The herds were from four sites. We do not have data on the number of herds in this community whose main occupation of the Household Heads is livestock herding at 87%, followed by Casual Labor (SSFR, 2017). We defined a camel herd as one under care of a specific farmer and it comprises of camels that graze and stay together most of the time. Much as we tried to avoid sampling of camel herds that mostly co-graze, this did not affect our objective of studying pathogens in camels and keds kept under natural setting. All camels, in each herd that ranged from 8 – 90 camels, were sampled and we aimed to collect all camel keds from the sampled camel herds.

The sites for biting fly trapping were selected near livestock pens and next to watering points along Laisamis and Koya Rivers. The inter trap distance was at least 100 meters.

14. Data analysis needs to be revisited. The fly apparent density in Table 3 can be well presented spatially. To be able to discuss possibility of mechanical transmission of different haemopathogens by different biting flies e.g. *Stomoxys* the association between fly apparent density and hemopathogen prevalence needs to be adjusted for potential spatial dependence. This you can do using generalized least squares model with a Gaussian spatial correlation structure to quantify the effect or other appropriate models.

Response: It is not possible to do these analyses with our limited data. Our key focus was on camel keds infesting camels. Since our preliminary data showed high prevalence of camel trypanosomiasis in the study region, we therefore wanted to check whether tsetse flies are present to cause disease transmissions. Three traps were deployed per site on daily basis. Daily trap collections were pooled, fly species sorted, counted, and preserved in absolute ethanol ready for transportation to the Nairobi-based laboratories at icipe (Table 3).

15. Please include a pairwise alignment of "*Candidatus* A. camelii" from this study and those from the GenBank as was done for *T. melophagium*

Response: we thank the reviewer for this helpful addition. This alignment is now provided as Figure 5.

16. Discussion: I would like to draw the authors' attention to some of the following discussion sections, which I strongly believe they need to revisit.

17. First paragraph [Page 7 of 11]: Associating the high prevalence of hemopathogens in camels to the potential of camel keds in hemopathogen transmission is not supported by the results of this study. Note that keds were only less than 2% of all biting flies trapped. This can only be attributed to mechanical transmission of these hemopathogens by *Stomoxys Calcitrans* and Tabanids which were >98% of all biting flies trapped. You can be authoritative about these associations if you improve data analysis as recommended in comment II above

Response: As described under 'materials and methods' and 'discussion' sections, camel keds do not normally leave their host (unless when disturbed) as they firmly attach to the hairs on the camel's skin by their tarsal claws during feeding or resting. During our study, keds were common on the camels which was not the case for other biting flies. The keds can hop from one camel to another when disturbed and if they are contaminated they could transmit the pathogens to the next host as shown by preliminary findings of our ongoing studies (Bargul et al., unpublished). Thus, our intention to deploy fly traps was mainly to catch tsetse flies and other biting fly species but not keds as we understand at present that efficient traps for keds are not available and the monoconical traps we deployed are efficient at trapping tsetse flies, as well as biting flies such as *Stomoxys* and Tabanids, with house flies often being non-targets. Our ongoing studies aim at designing ked-specific traps. It is very likely that the few trapped keds comprising of 0 - 2% of total biting fly catches were off targets.

18. Pursuant to comment 13 above, you need to rewrite the hypothesis you make at the end of paragraph 1 of discussion section on page 7 of 11. If Mechanical transmission of camel haemopathogens were important in this region, it would rather be by *Stomoxys* and other Tabanids and not camel keds [camel keds were <2% of all biting flies trapped]; moreover you did not rule out or in potential spatial dependence between haemoparasite prevalence and fly apparent density.

Response: please refer to #13 above that partially addresses this question.
Although *Stomoxys* and Tabanids are potential mechanical vectors of pathogens as previously reported, we do not have data to affirm their vectorial competence in disease transmission among camels in northern Kenya. The major focus of our study was on the ectoparasitic camel keds, but not on the other biting flies that were often absent on camels, unlike keds. In fact, our motivation to deploy traps was to determine occurrence of tsetse flies (definitive biological vectors of African trypanosomes) as camel trypanosomiasis was detected in almost half of the sampled camels. Our preliminary findings from ongoing studies show evidence of *Anaplasma* transmission by camel keds from naturally infected dromedary camels to laboratory-reared mice and rabbits (Bargul et al., unpublished). We are also testing trypanosome transmission capacity of camel keds as keds are the closest tsetse relatives both belonging to same superfamily.

19. Paragraph 2 of discussion [page 7 of 11]. There is mention of reports that support absence of tsetse flies in the study area and yet no references of such reports are included. When I checked this fact myself, I found that this study area has recently been cited as an area with high risk of tsetse infestation using robust landscape and climatic data modeling3.

Response: Despite the high risk prediction for tsetse infestation in our study area (Moore and Messina, 2010), we did not collect any tsetse flies during the sampling period. Additionally, during our community and public engagement sessions, the camel farmers reported absence of these flies in Laisamis, but in the far regions such as Meru County, over 200 km away.

20. Last paragraph on page 7 of 11; The variations in the micro-climatic conditions, differences in the study designs and time lapse are the most likely explanations for the differences in *T. evansi* prevalence in camels previously reported in other parts of Kenya and in this study.

Response: these factors that could influence disease prevalence are now better reflected in the discussion, i.e.
"These regional variations in prevalence of *T. evansi* infection could result from seasonal disease outbreaks, variations in the micro-climatic conditions, disease stability in endemic zones, and the presence of competent insect-vectors, among other factors, including differences in the study designs and time lapse. Our study design has key limitations in comparative studies because sampling was not done during wet season, thus the relationship between seasonality versus prevalence of haemoparasites and vector density could not be established"

21. There is need to include a discussion of the limitations of this study [see major comment I & VII above. Think of snapshot sampling during short wet season? Seasonality vs haemoparasite and vector density, etc.

Response: limitations of our study design are now highlighted in the discussion. Please see #17 above.

22. There is need to nuance the recommendation about heightening public and veterinary surveillance of *T. evansi* as a zoonotic haemoparasite [first paragraph, page 9 of 11] because there are no reported major outbreaks of this atypical human African trypanosomiasis either in Kenya or elsewhere ever reported. The only cases of atypical *T. evansi* human infections have been reported in either immunocompromised or accidental infections that do not warrant setting up veterinary or public health surveillance programs.

Response: we fully agree that *T. evansi* human infections are very uncommon, thus we deleted our earlier suggestion proposing "increased surveillance by veterinary and public health partners to mitigate spread of *T. evansi* in humans"

23. Second last sentence; paragraph 2, page 9 of 11….it is conceivable …. . This needs to be reinterpreted. Finding "*Candidatus* A. camelii" in keds and camels certainly means that keds are feeding predominantly on camels positive for "*Candidatus* A. camelii". This study design was not to prove mechanical transmission of "*Candidatus* A. camelii" by Keds; given that previous mechanical transmission studies were not able to prove that, it is not conceivable in a study of such a design to make this assertion.

Response: yes, it is true that the focus of this study was not to prove mechanical transmission of pathogens. However, we show that identical *Anaplasma* species in camels and keds collected from them, suggesting that keds fed on *Anaplasma*-positive camels. Based on this finding, we hypothesize that in the process of blood-feeding, keds, just like *Stomoxys calcitrans*, could mechanically transmit anaplasmosis via contaminated mouthparts. We have preliminary data, from another ongoing study, to support "*Candidatus* A. camelii"-transmission by camel keds from naturally-infected camels to mice and rabbits (Bargul et al., unpublished).

24. Second sentence of paragraph 4 page 9 of 11…….detection of similar haemopathogens in these camel flies…… This needs to be reinterpreted. Finding a similar repertoire of haemoparasites in keds and in camels only indicates that keds were feeding on haemoparasite positive camels. Only 2% of the caught biting flies were keds and you can't emphasize ked mechanical transmission than that of Tabanids and *Stomoxys* which were > 98% of all biting flies trapped; with known mechanical transmission potential. The only application you can make out of this result is about xenodiagnosis and not mechanical transmission of haemoparasites by keds unless this is proven in study with suitable study design or you can refer to literature!

Response: please note that this is already addressed under response #13, #14, & #19 above.

25. Page 10 of 11, first sentence: The point you make about xenodiagnosis saving time and money needs to be substantiated. It takes as much time to collect keds from camels as it takes to take blood samples from camels. If similar diagnostic methods are used to detect pathogens in keds and camels, I would not anticipate pathogen detection in keds to be any cheaper than pathogen detection in camels? Can you please discuss how xenodiagnosis would be cheaper and shorter than detection of pathogens directly from camel blood?

Response: Collection of keds off camels was much easier and required relatively less time than blood sampling., We employed six field assistants to restrain each camel for blood collection, a veterinary personnel who collected blood samples, and additional three assistants to carry cool boxes and consumables, ensure accurate labeling of samples and storage, and recording of baseline data. On the other hand, only about four field assistants were needed to collect keds from camel herds, resulting in >50% reduction in labour costs and the required human resource. Fly collection also took shorter time as it was not necessary to restrain camels. Importantly, this xenosurveillance detection provides a less invasive approach than the currently available painful blood collection procedures that pose huge risk to the handlers as camels could occasionally cause severe and even fatal injuries through bites (Abu-Zidan *et al.,* 2012) or by kicking with their legs.

26. Last paragraph of discussion section, page 10 of 11. ; …..Molecular detection of *T. melophagium*….
Note that detection of this parasite in a ked does not mean that such a ked was infected with *T. melophagium*. Unless proven, it would mean that it had consumed a blood meal from a host [might not be camel at all since no camel was found positive for genetic material of this parasite] that had been positive for *T. melophagium* genetic material. This has nothing to do with *H. camelina* being able to transmit [biologically or mechanically] *T. melophagium*.

Response: we agree with the reviewer, and subsequently this sentence has been re-written to ensure accurate delivery of information.

Conclusion:
This needs to be refined after refining the discussion. Blood samples need not to be taken from the Jugular. You can these days take blood samples [125 ul] from ear veins and have them preserved on FTA cards. Unless substantiated as in comment XII, this conclusion has to be rewritten so that it is supported by the findings of this study.

Comment: with the above clarification on xenodiagnosis under #21, our conclusions are now well supported.

References

As indicated in my comments above, the attention of the authors is drawn to some of the key literature they were not able to refer to in their discussion section e.g Truc et al.4, Moore et al.3.

Comment: many thanks to the Reviewer for your helpful suggestions. We have now added the following references; Rahola et al., 2011, Nelson, 1963, Moore and Messina, 2010, Vanhollebeke et al., 2006, Truc et al., 2013

References

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2.   Rahola N, Goodman SM, Robert V: The Hippoboscidae (Insecta: Diptera) from Madagascar, with new records from the "Parc National de Midongy Befotaka".Parasite. 2011; 18 (2): 127-40 PubMed Abstract | Publisher Full Text (was already cited)
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4.   Truc P, Büscher P, Cuny G, Gonzatti MI, et al.: Atypical human infections by animal trypanosomes.PLoS Negl Trop Dis. 2013; 7 (9): e2256 PubMed Abstract | Publisher Full Text (was already cited)

Competing Interests: No competing interests were disclosed.