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High proportion of mosquito vectors in Zika forest, Uganda, feeding on humans has implications for the spread of new arbovirus pathogens

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There is a steady increase in the contact between humans and wildlife, brought about by encroachment, destruction of natural forests, climatic and environmental changes. Mosquitoes get exposed to hosts and pathogens; creating possibilities for new disease patterns. Therefore, the identification of blood-meal sources is important to determine the linkages between hosts and vectors. Engorged mosquitoes were collected in Zika forest (Uganda) for a period of 12 months using carbon dioxide (CO_2)-baited light traps. Total genomic DNA was extracted from the abdominal contents of the mosquitoes and the diagnostic regions of the mitochondrial genes, *cytochrome oxidase subunit 1* (COI) and *cytochrome b* (cytb) sequenced. The sequences were subsequently blasted in the GenBank. Sequence analyses revealed that feeds were derived from mammalian and avian hosts. Blood-meal sources from *Aedes africanus* (Theobold) examined were entirely human. There were significant differences between host species from which the mosquito species fed (Krusakal Wallis test, χ^2 = 19.118, df = 5, p = 0.018). Several mosquitoes were considered as potential bridge vectors for a number of arboviruses and other diseases have been collected from the forest. Taking of mammalian origin blood-meals, including humans, may facilitate exposure to new pathogens and disease patterns.

Key words: Blood-meal, bridge vectors, cytochrome oxidase sub unit I, cytochrome b, Entebbe.

INTRODUCTION

Female mosquitoes take blood-meals from various vertebrates, in preparation for oviposition (Lefèvre et al., 2009). Consequently, humans are at risk of infections with pathogens circulating within the blood system of other

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animals especially mammals (Taylor et al., 2001; Lambin et al., 2010). Mosquitoes are bridge vectors of emerging and re-emerging parasitic (Apperson et al., 2002; Kent and Norris, 2005; Diallo et al., 2012) and arboviral pathogens causing infections including West Nile virus (Apperson et al., 2004; Turell et al., 2005; Hamer et al., 2008) and Chikungunya (Diallo et al., 1999; 2012) responsible for millions of infections and deaths of humans and animals globally. Knowledge of host choice and preferences is critically required for effective vector-based disease control. Host choice depends on a number of factors including; host availability, abundance, flight behaviour, feeding periodicity and region which vary according to innate, seasonal, and environmental conditions (Hamer et al., 2008; Molaei et al., 2008). The basis for blood-meal host's choice, a consequence of a mosquito feeding and pathogen transmission in the process, currently remains unclear. Despite their relevance to public and veterinary health, knowledge of mosquito feeding patterns in Uganda is still poor.

In Uganda, earlier mosquito studies from forests including Zika focused on vertical feeding patterns of especially the genera *Aedes*, *Coquillettidia* and *Mansonia*. These studies reported many anopheline and culicine species preferring avian, amphibian, reptilian, or mammalian blood-meals (Williams, 1964; Haddow and Ssenkubuge, 1965; Mukwaya, 1972). Blood-meal sources could be directly estimated by analyzing the abdomen contents of field-derived females. All estimations during earlier studies were drawn using the precipitin test, visual observations and attraction to animal bait traps (Gilles and Wilkes, 1974; Savage et al., 2008). The precipitin test has a disadvantage of forming a cloudy antibody - antigen complex at the confluence of sera and antisera and only a small number of hosts can be identified due to limitations of commercially available reagents. Thus, serological tests would be limited in identifying hosts to species level. Visual observations and animal attractions may not be true to source.

Over the years, a new method of identifying blood-meals using polymerase chain reaction (PCR) was developed. During this process sized DNA fragments have been used to identify blood-meals (Kent and Norris, 2005), establish host choice (Garcia-Rejon et al., 2010; Lee et al., 2002) and feeding patterns of mosquitoes (Apperson et al., 2004). PCR has an advantage over older serological methods by being relatively simple to perform and more sensitive. With many DNA-size fragments, animal hosts of mosquitoes can be reliably identified to species level and if a complete match to the unknown DNA sequence is not present in the databases, the sequence data may be subjected to phylogenetic analyses. Once a given blood-meal is identified, the PCR product can be used to generate a new reference sample. Simple (Molaei et al., 2008) multiplex (Kent and Norris, 2005) and heteroduplex PCR assays (Lee et al., 2002) have been used with cytb determining hosts that play a significant part in vector borne diseases to species level through blood-meal analysis. On the other hand, (Alcaide et al., 2009) and (Roiz et al., 2012) have used the HOTSHOT protocol with *cytochrome oxidase subunit I* (COI) to identify blood-meal hosts. Along with these identifications, a number of arboviruses including Chikungunya (Weinbren et al., 1958), Zika (Weinbren and Williams, 1958; Haddow et al., 1964; Henderson et al., 1968), Rift Valley Fever (Woodall, 1964), O'nyong-Nyong (Rwaguma et al., 1997; Lanciotti et al., 1998; Kiwanuka et al., 1999), Sindbis (Smithburn et al., 1946), Bunyamwera (Smithburn et al., 1946), Ntaya (Smithburn and Haddow, 1951), Semliki Forest (Finter, 1964), West Nile and Usutu (Smithburn et al., 1940; Williams et al., 1964), Witwatersrand and Germiston (Monath et al., 1972), Uganda S virus (Dick and Haddow, 1952) and yellow fever (Kirya et al., 1977) were isolated from mosquitoes.

However, mosquito blood-meal studies from forests in Uganda including Zika, were discontinued after the 1970's due to instabilities in the country. From the 1980's, very little was documented about mosquito feeding. Presently, anthropogenic activity has greatly modified the environment around the forest. New homes and/or crop fields and plantations are adjacent to the forest and many are still coming up with increasing urbanization. Changes around the forest might have great implications on the blood-meal host sources, and the emergence or re-emergence of pathogens and infections. It is therefore necessary to closely examine the interaction between animals and man. The aim of this study was to examine mosquito blood meal sources from Zika forest and provide information on the potential interactions that could lead to transmission of zoonoses.

MATERIALS AND METHODS

Study area

Zika is a small isolated tropical forest found at 32° 30' E and 0° 7' N and approximately 11 km (6.2 mi) from Entebbe. It is located at Kisubi on Entebbe/Kampala road, Wakiso district, Uganda. The forest covers approximately 25 hectares (61.8 acres) and forms part of a narrow sinuous strip skirting the extensive grass and papyrus swamps of Waiya Bay, a sheltered inlet of Lake Victoria near Entebbe. Using GIS, Arcview and ArcInfo software, base maps of the collection points were produced to give a pictorial representation of the study area (Figure 1). Sixteen representative sites were selected along the vertical and horizontal gradients. Six sites were located along the tower platforms, seven along the horizontal gradient within the forest and three along the forest edge (Figure 1). The sites were selected based on distance from the open swamp and tower.

Mosquito sampling

Sampling was conducted weekly for 12 months along a vertical (steel tower) and horizontal (wet, raised and marginal forest, outliers, and grassland) gradient. Adult mosquitoes were collected using carbon dioxide (CO_2)-baited CDC light trap collections

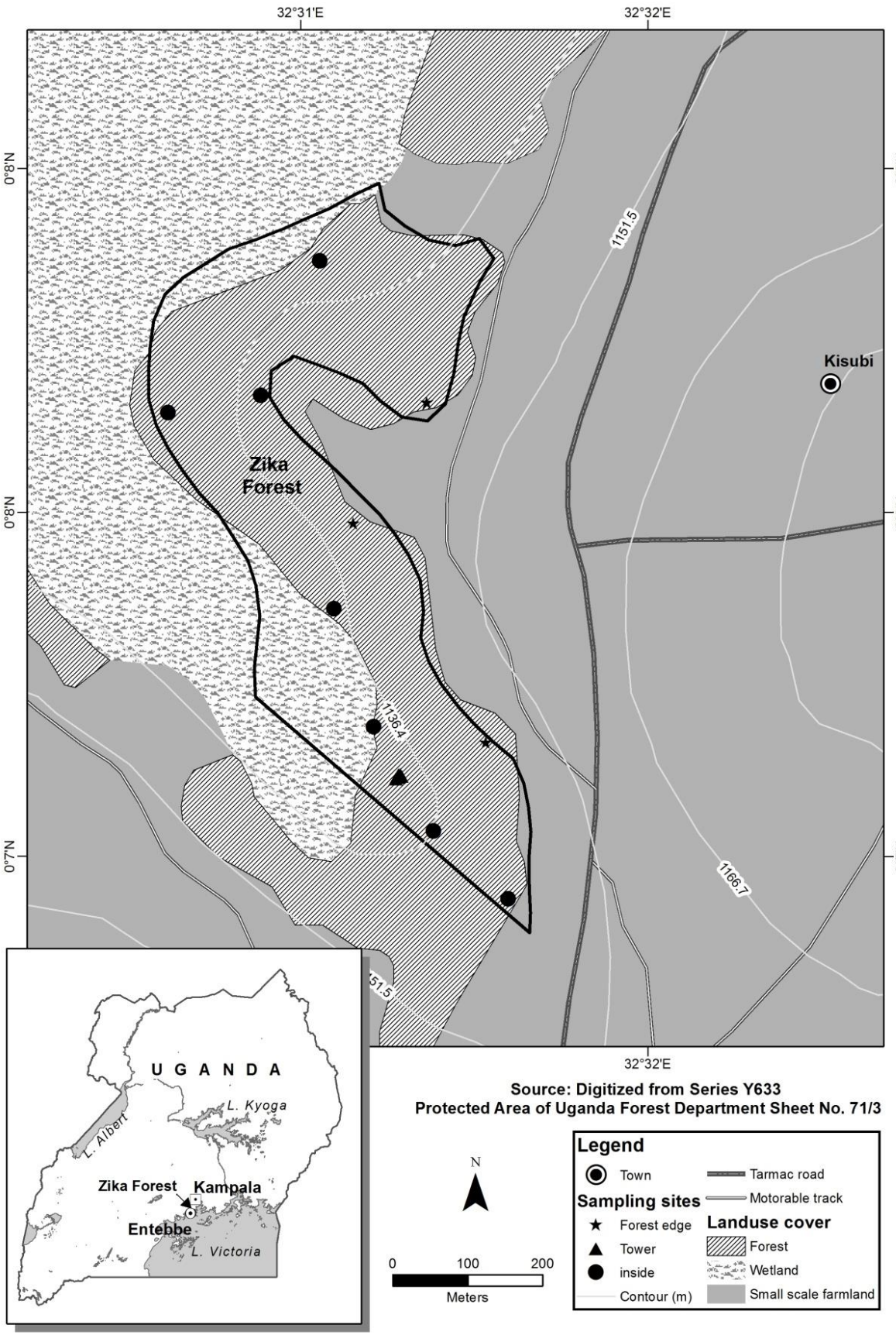

Figure 1. Sampling sites in Zika forest.

(Service, 1993). The light/CO_2 trap collections were done from the steel tower platforms (vertical) at 20 ft (6 m) intervals from 20 ft upwards to 120 ft and along the horizontal transect at 10 m intervals. Light/CO_2 trap were set weekly over 12 h period (6.00 pm to 6.00 am) just before sunset and collected early in the morning. After collection mosquitoes were quickly counted, put in vials and placed on dry ice and transported to the laboratory for identification. Mosquito species identifications were done with available keys (Edwards, 1941; de Meillon, 1947; Gillett, 1972; Gillies and Coetzee, 1987; Jupp, 1996). We used the nomenclature of (Knight and Stone, 1977) supplemented with notes and updates from the Walter Reed Biosystematics Unit website (http://www.wrbu.org/docs/mq ClassificationTraditional201307.pdf). After mosquito sorting, we selected engorged mosquitoes in the laboratory at the Uganda Virus Research Institute (UVRI). Mosquitoes were inspected for a visible blood-meal and positive samples stored at -80°C in individual cryo-vials with silica gel. Voucher specimens for each species are currently stored at the Uganda Virus Research Institute, Entebbe, Uganda.

Blood meal identification assay

The abdomens of engorged mosquitoes were carefully detached from the thorax using sterile fine forceps and scalpel blades. Each abdomen was macerated in 200 µl 0.01 M Phosphate Buffer Saline (PBS), pH 7.4 in 1.5 ml Eppendorf tube using a sterile micro-pestle for 20 min.

DNA extraction

DNA was isolated from the abdominal contents using the QIAGEN DNeasy blood and tissue kit (Qiagen Inc., Maryland, CA, USA), following manufacturer's instructions. DNA extracts from the mosquito blood-meals served as the DNA template in a standard polymerase chain reaction (PCR) assay (Kumar et al., 2007; Cywinska et al., 2006). The protocol run was revised by eluting in 32 µl AE buffer. The mixture was incubated for 1 min at room temperature followed by centrifuging at 8000 rpm and the process repeated thrice for maximum yield. Success of the DNA extracts was tested by electropheresing 5 µl of total genomic DNA on 1% NuSeive agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light. Extracted DNA was stored at -20°C until needed.

DNA Amplification

Blood meal sources were assessed using standard PCR on a 9800 Fast thermal cycler (Applied Biosystems). Amplification of the fragment of a vertebrate COI mitochondrial gene using previously described primers (VFd1_t1 and VRd1_t1, Ivanova et al., 2007) (Barcode of Life Database (BOLD) and cytb gene (L14181 and H15149) was done. This assay was a modification of previously published protocols (Kocher et al., 1989; Ngo and Kramer, 2003). Universal mammalian-specific primers that amplified the 648-bp region of the COI gene VF1d_t1 5'-TAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG-3' and VR1d_t1 5'-AGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA-3' (Kocher et al., 1989); and the 358-bp region from the cytb gene L14181 5'- CCATCCAACATCTCAGCATGATGAAA-3' and H15149 5'- GCCCCTCAGAATGATATTTGTCCTCA-3' (Ngo and Kramer, 2003) were used. A 30 µl reaction volume was prepared with 1 µl template DNA, 1 µl each primer (0.1 to 0.5 pmol/L), 6 µl 5X HF buffer (comprising of 1.5 mM $MgCl_2$), 0.6 µl dNTP mix (10 mM/L each), 0.25 µl Phusion High Fidelity DNA polymerase (Thermal Scientific, Finland) and 19.15 µl double distilled water. The thermo-cycling conditions consisted of initial denaturation at 98°C for 30 s, 39 cycles at 98°C for 10 s, annealing at 57°C for 30 s, primer extension of 72°C for 1 min and a final extension of 72°C for 7 min for COI. Conditions for cytb were similar, apart from annealing temperature at 61°C for 20 s and the cycles repeated 35 times.

Amplicon purification and sequencing

To detect the presence of amplified DNA fragments, eight micro litres of PCR products were electrophoresed on separate 1% agarose gels (NuSieve, FMC) in 40 mM Tris acetate (pH 8.0) and stained with 0.5 µg/ml EtBr. Products were run at 120 V for 30 to 60 min and viewed under Ultra Violet light with a 100-bp Gene Ruler (Fermentas Life Science, Hanover, MD, U.S.A.). Expected amplicon products from the reactions were purified using the Gen Script kit (GenScript USA Inc) following manufacturer's instructions. Purified products were commercially sequenced for both forward and reverse strands at the World Meridian Venture Centre Macrogen, Seoul, Korea. The same PCR amplification primer pairs were re-synthesized for sequencing.

Sequence analysis

Peak identification and fragment sizing of the chromatograms for the blood meal sequences was done using the programme Peak Scanner^{TM} v1.0 (Applied Biosystems). The resulting sequences were edited and stored in Bioedit software (Hall, 1999). Sequences were then aligned using the programme Codon code aligner. Within the same programme, sequences were used to query the Genbank database using the BLAST algorithm (Altschul et al., 1990) and the barcode of life database (BOLD) (Ratnasingham and Herbert, 2007); BOLD-ID platform (http://www.barcodinglife.org/views/idrequest-php) for specific identification of hosts. All the host species identified from mosquitoes were the most similar species at more than 99% sequence identity. Sequences which did not meet the level were left out of the analysis. Collection sites were categorized according to 3 groups; within forest, forest edge and tower and host species according to 4 groups (Human, Bovid, Suid, and Aves). Differences between the mosquito blood meal host species were examined using the Mann-Whitney U test at $p < 0.05$.

RESULTS

Species composition and relative abundance of mosquitoes collected from this study are shown by Kaddumukasa et al. (2014). Sequencing of COI and cytb from 56 mosquito blood meals yielded a number of hosts. From the 56 wild-caught mosquitoes, 47 amplified with cytb and 9 from COI. Sequences obtained from the mosquito blood meal sources from Zika forest showed two main groups; human sources and the other group containing animal sequences (Figure 2). The sequences show that blood was obtained by mosquitoes from all sites in the forest and mosquitoes were able to access the different sites for their blood meal sources. After identification, the obtained mosquito blood meal sequences showed a distribution of blood meal hosts from all points of collection within the forest. Table 1 shows the vertebrate blood meal sources from engorged mosquito species detected by PCR. Blood meal sources were most identified from sites within the forest,

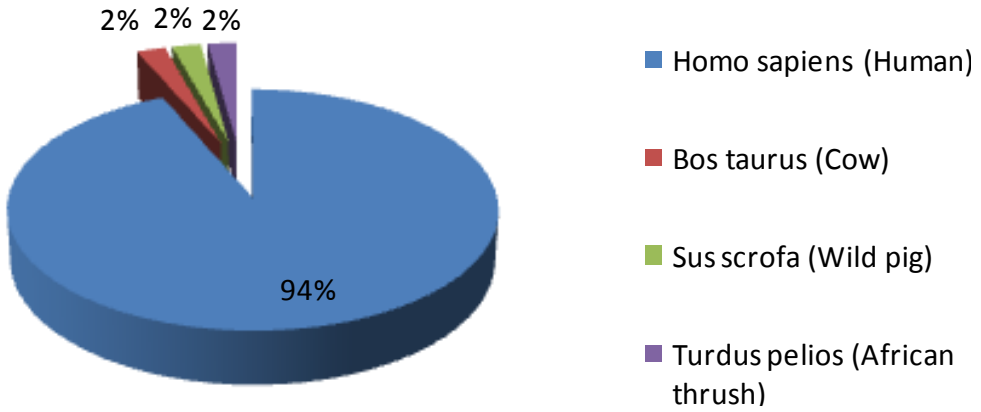

Figure 2. Blood meal sources identified with cytochrome b from Zika forest mosquitoes.

Table 1. Mosquito species and their blood meal sources identified by PCR and sequencing.

Primer	Habitat	Species	Human	Pig	Cow	Bird	No.
Cytochrome b	Forest edge	*A. coustani*	1				1
		A. implexus	2				2
		C. fuscopennata	15				15
		M. africana nigerrima	1				1
	Within Forest	*C. fraseri*	1				1
		C. fuscopennata	10		1		11
		C. pseudoconopas	1				1
		C.annulioris	1	1			2
		C. antennatus	2				2
		C. insignis	5				5
		C. univittatus	1				1
		M. africana africana	1				1
	Tower	*A. africanus*	2				2
		C. aurites				1	1
Total			43	1	1	1	46
Cytochrome oxidase I	Forest edge	*A. implexus*			3		3
		C. fuscopennata			1		1
		C. annulioris			1		1
	Within Forest	*C. pseudoconopas*			1		1
		C. annulioris			1		1
	Tower	*C. fuscopennata*			2		2
Total					9		9

followed by the edge of the forest and least at the tower platforms (Table 1). All species of *Aedes, Anopheles, Coquillettidia,* and *Mansonia* were found to feed on mammalian hosts. *Coquillettidia fuscopennata* mosquitoes showed the highest composition of blood meals from within the forest and at the forest edge. The number of blood meal hosts was higher in the forest by this species than at the edge of the forest. In addition, *C. fuscopennata*'s blood source was identified from *Bos taurus*, *Cq. aurites* from *Turdus pelios* and *Culex annulioris* from *Sus scrofa.* For both COI and Cytb, majority of hosts identified were mammals and found to represent the most common host class (Table 1). Cytochrome b sequences represented 47 samples which were identified as 44 human, one (cow, pig and bird) respectively. All the nine COI sequences were identified as cow blood sources from four mosquito species. From means of hosts identified man, bovids and birds were major hosts. However, hosts from which

mosquitoes derived their blood-meals differed significantly (χ^2 = 19.118, df = 5, p = 0.0018). Sites inside the forest had the greatest variety of hosts followed by sites along the forest edge and least was tower sites. Non-parametric Kruskal Wallis test was used to examine variation of hosts because data did not conform to a normal distribution. There were no significant differences amongst sites within, at the forest edge and along the tower, in the number of mosquito blood-meal hosts identified (χ^2 = 0.301, df = 2, p = 0.861). To explore the differences between host species, Mann-Whitney U test was used and significant differences were observed between man and other hosts ($p < 0.05$).

DISCUSSION

This study has shown the first molecular analysis of mosquito blood-meal sources from Zika Forest with two important findings. First, from the host variety revealed, the majority of the blood meals were from human sources. The big number of positive blood-meals from humans than other hosts is of particular interest as humans may pick infections from animals. The close association of humans with forest habitats may easily promote emergence of new pathogens. Intra-population variation within the human sequences was revealed, showing the diversity of people interacting with the forest. The cytb assay detected more sources from the sequences from each blood meal taxon, representing different individuals; human, cow, pig and bird, which were different from each other. In contrast, for COI all the cow sequences were identical. Our study was able to identify a larger number of blood meals with cytb than COI probably because smaller products are detectable over a longer time of degradation than longer ones (Fornadel et al., 2008). Negative results may have included blood-meals that were too small or probably completely too digested for identification. Blood meals have been noted to degrade quickly (Fornadel et al., 2008). A small delay of hours may be the difference between an identifiable blood meal and one that will not be identified.

A variety of host feeding preferences namely human and a number of animals even for those species previously assumed to feed exclusively on birds or mammals was presented in this study (Table 1). For example, *C. fuscopennata* was reported to mainly have preference for man (Mukwaya, 1972). However, this species presented more than one host in this study. *C. fuscopennata* had more than one host choice from its amplifications (Table 1). This may open the possibility for transferring pathogens across distantly related vertebrate hosts, including humans. For the *Anopheles* spp., in particular, *An. implexus* bovine–derived blood sources were revealed from this study. Other studies showed *Anopheles* species host sources were mainly human sources showing their anthropophilic nature (Haddow and Ssenkubuge, 1973; Fornadel et al., 2010). Other authors however, reported *Anopheles* host sources as zoophilic, taking blood from cows and a few other wild mammalian sources (Muriu et al., 2008). This may suggest that the blood-seeking activities of this species from Zika extend beyond the forest's confines. In other studies, increased interaction between man, domestic animals and the forest was reported by Hamer et al. (2008) while examining *Culex pipiens* (Linnaeus). Humans served as a bigger blood source and more readily available target than the normal wild animal sources. No non-human primates were found as sources of blood for the collections examined. Other members of genus *Coquillettidia* (*Cq. aurites and Cq. pseudoconopas)* fed almost exclusively on birds. Cows were an additional host for *Cq. pseudoconopas* from this study. The results of this study agree with a previous study that reported this species to be primarily ornithophilic (Mukwaya, 1972). Other mosquito species in the genus *Coquillettidia* have been reported to take blood from avian and primate hosts (Mukwaya, 1972). Blood-meals from birds may be taken at any available opportunity. In other parts of the world, *Coquillettidia* species have been reported to feed predominately upon mammalian hosts (Molaei et al., 2008).

Secondly, the *Aedes africanus* mosquitoes examined had human-derived blood-meals. Interestingly, *A. africanus* mosquitoes have been known to take primarily primate blood (Mukwaya, 1972). Opportunistic and easy human blood feeding may be provided by the available people living close to the edge of the forest than the primates. However, since only two mosquitoes were examined we may not draw conclusions. Blood-meal sources from other *A. africanus* have revealed host varieties including entirely bovine (Muriu et al., 2008; Linthicum et al., 1985), avian and mammalian sources. Blood-meal sources from *Mansonia* mosquitoes were mainly from humans. This may have serious implications because this is one of the main vectors of Rift Valley fever in Kenya (Lutomiah et al., 2011). Previously, *Mansonia* species records have reported reptilian blood-meal sources. *Culex* blood-meals were recorded from human and other animal sources (Table 1). *Culex annulioris* had human and pig sources from cytb and cow hosts from the COI amplifications but had not been reported in any previous study. *Culex* species from West Africa have been reported to feed on a variety of mammal and bird sources (Snow and Boreham, 1973). *Culex quinquefasciatus* was reported to take mainly animal sources (Lee et al., 2002), while *C. pipiens* fed exclusively on bird sources (Hamer et al., 2008). Along with identifications of blood meals, arboviruses were isolated from mosquitoes. From earlier studies, some of these arboviruses include; Chikungunya (Weinbren et al., 1958), Zika (Haddow et al., 1964), Rift Valley Fever (Woodall, 1964), O'nyong-Nyong (Rwaguma et al. 1997; Lanciotti et al., 1998), and yellow Fever viruses (Kirya et al., 1977).

The mosquitoes therefore pose a serious threat to communities living around the forest and need to be constantly monitored. Variations of the host species selected for feeding may be brought about by several factors namely; the availability of host-species at the time of feeding, innate behaviour of each species selecting for particular blood types and location of host. Species in one particular part of the world specialize in certain hosts while others feed on a variety of animals. Types of hosts may be chosen as a result of environmental conditions, such as environmental temperature which later determines disease outbreak (Molaei et al., 2008). The emergence of a disease in a particular region is associated with changes that influence people's livelihood strategies, their rapid conversion of natural habitats, and urbanization (Smith, 1975). Host specificity may be influenced by factors such as; host type present, mosquito behaviour and environment (Patz et al., 2008; Norris, 2004), namely visual, thermal, and olfactory stimuli (Costantini et al., 1996). These direct a mosquito to which species to feed on and in so doing pass on its pathogens.

Based on our results, more than half of the blood meals were derived from humans (Figure 2). In addition to a better understanding of mosquito blood feeding habits for ecological and disease transmission purposes, cytb and COI should be used to explore more interactions between man and the mosquito. Further investigations using a range of collection methods, targeting ecologically diverse sites should be done to determine mosquito contribution as reservoirs or amplifying hosts in the transmission of arboviruses. Special precaution should be taken when interpreting sequence data for mammals where a limited number of specimens were obtained. Some mammals had not been examined before and most bird species in Uganda are not recorded in the BOLD or GenBank database. There is a possibility that the blood-meals were derived from vertebrates for which *cytochrome b* and *cytochrome oxidase subunit I* sequences are not yet available because some failed to yield a host and were left out of the analysis.

Conflict of interests

The authors did not declare any conflict of interest.

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Abbreviations: UVRI, Uganda virus research institute; **PCR,** polymerase chain reaction; **COI,** cytochrome oxidase subunit 1; **cytb,** *cytochrome b*; **PBS,** phosphate buffer saline; **EtBr,** ethidium bromide; **BOLD,** barcode of life database.

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