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Detection and characterization of zoonotic pathogens of free-ranging non-human primates from Zambia

Jesca Nakayima[1,2], Kyoko Hayashida[1], Ryo Nakao[3], Akihiro Ishii[4], Hirohito Ogawa[4], Ichiro Nakamura[1], Ladslav Moonga[5], Bernard M Hang'ombe[5], Aaron S Mweene[6], Yuka Thomas[4], Yasuko Orba[7], Hirofumi Sawa[7*] and Chihiro Sugimoto[1*]

Abstract

Background: Wildlife may harbor infectious pathogens that are of zoonotic concern acting as a reservoir of diseases transmissible to humans and domestic animals. This is due to human-wildlife conflicts that have become more frequent and severe over recent decades, competition for the available natural habitats and resources leading to increased human encroachment on previously wild and uninhabited areas.

Methods: A total of 88 spleen DNA samples from baboons and vervet monkeys from Zambia were tested for zoonotic pathogens using genus or species-specific PCR. The amplified products were then subjected to sequencing analysis.

Results: We detected three different pathogenic agents, including *Anaplasma phagocytophilum* in 12 samples (13.6%), *Rickettsia* spp. in 35 samples (39.8%) and *Babesia* spp. in 2 samples (2.3%).

Conclusion: The continuously increasing contacts between humans and primate populations raise concerns about transmission of pathogens between these groups. Therefore, increased medical and public awareness and public health surveillance support will be required to detect and control infections caused by these agents at the interface between humans and wildlife.

Keywords: Non-human primates, Reservoir, Pathogens, Zoonosis, Zambia

Background

Wildlife poses a threat as a potential source of emerging infectious diseases (EIDs) to biodiversity conservation as well as human health. Three quarters of zoonotic EIDs are caused by pathogens in wildlife and the incidence of such diseases is increasing significantly in humans [1,2]. Human activities have contributed to a closer contact between humans and wildlife due to a complex relationship between social and environmental factors causing a major threat both to human health and biodiversity conservation mainly through disease transmission between the two groups [3-5].

The Order Primates has traditionally been divided into two main groupings: prosimians and anthropoids (simians). Non-human primates (NHPs) are a diverse group of animals. Generally, Old World monkeys (Catirrhini) and apes (Hominoidea) are those found in Africa, the Indian subcontinent and in East Asia. New World or neotropical NHPs (Platirrhini) are found in South and Central America. In Zambia, baboons and vervet monkeys are the major non-human primates not only in wildlife management regions, but even out of the management areas. Human-monkey conflicts in the form of crop damage, grabbing of personal effects and direct injury are reported [6].

Several hundred infectious diseases are classified as zoonotic diseases as they are caused by bacteria, viruses, fungi, prions or parasites that can be transmitted from animals to humans and vice versa [7]. Transmission can be direct or indirect, via another organism, either a vector or an intermediate host. Invertebrates spread pathogens

* Correspondence: h-sawa@czc.hokudai.ac.jp; sugimoto@czc.hokudai.ac.jp
[7]Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan
[1]Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan
Full list of author information is available at the end of the article

by two main mechanisms, either through their bite, or their feces, thus, transmission occurring mechanically or biologically. Tick-borne microbial pathogens, which cause human and zoonotic diseases such as Lyme disease, anaplasmosis, ehrlichiosis, babesiosis, Q ("query") fever, tick-borne encephalitis, Crimean–Congo hemorrhagic fever, Rocky Mountain spotted fever, Colorado tick fever, tick typhus and tularemia, have enormous negative impacts on human health and economic development worldwide. Other zoonotic disease vectors include tsetse flies (*Glossina* spp.) transmitting trypanosomiasis, sand flies (*Phlebotomus* spp.) transmitting leishmaniasis and mosquitoes (*Culicidae* spp.) transmitting malaria.

The hotspots of zoonotic disease transmission include livestock markets, urban and peri-urban wildlife and farming on fragments and edges of wildlife conservation areas and buffer zones. We hypothesized that a possible interaction between human and simian pathogens coming from a zoonotic cycle cannot be disregarded because simians that live in the areas of the disease endemic foci of Africa could play a role as reservoir for urban cycle disease transmission.

Therefore, we undertook a study of the sylvatic cycle zoonotic pathogens that can threaten humans in Zambia. The pathogens tested here included: *Anaplasma* spp., *Trypanosoma* spp., *Rickettsia* spp., *Coxiella burnetii*, *Leishmania* spp., *Babesia* spp., *Plasmodium* spp., *Ehrlichia* spp. and *Borrelia* spp. in African NHPs.

Methods

Sample collection and DNA extraction

Spleen samples were obtained from 48 yellow baboons (*Papio cynocephalus*) and 40 vervet monkeys (*Chlorocebus pygerythrus*) in 2008. The sampling was conducted at Mfuwe in South Luangwa National park, Zambia (13°14′ 42.00′′ S, 31°38′54.07′′ E) (Figure 1). Eighty eight spleen DNA samples were analyzed in the current study for *Anaplasma* spp., *T. brucei rhodesiense* and *T. brucei gambiense*, *Rickettsia* spp., *Coxiella burnetii*, *Leishmania* spp., *Plasmodium* spp., *Babesia* spp., and *Borrelia* spp. DNA was extracted from these organs by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Ethical clearance

The culling was conducted under the permission from the Zambia Wildlife Authority (ZAWA) and the Institutional

Figure 1 Map of Zambia showing the sampling site. NLNP: North Luangwa National Park, SLNP: South Luangwa National Park of the Luangwa valley ecosystem a Human African Trypanosomiasis (HAT) focus.

Ethical and Animal Care guidelines were adhered to during the culling and sampling exercise.

Molecular identification of pathogens

PCR amplifications

PCR reactions were conducted using Amplitaq Gold® 360 reagent (Applied Biosystems, Foster City, CA) in a 20 μl reaction volume. All the primer sets employed in this study and PCR conditions can be found in Table 1 [8-15]. The PCR products were electrophoresed in a 1.5% agarose gel stained with Gel-Red™ (Biotium, Hayward, CA) and were visualized under UV light.

Sequencing

The amplified PCR products for *Babesia* spp. *Rickettsia* spp. and *Anaplasma* spp. were subjected to direct sequencing and phylogenetic analysis. The amplicons were treated with ExoSAP-IT (USB Corporation, Cleveland, OH). The sequencing reaction was carried out with the BigDye terminator kit version 3.1 and resolved with a 3130 ABI (Applied Biosystems) capillary sequencer. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos. AB844434 to AB844437. Phylogenetic analysis of the pathogens (*R. africae*:A, 426 bp, 16S rRNA; *A. phagocytophilum*:B, 345 bp 16S rRNA and *B. microti*:C, 238 bp, 18S rRNA) detected in primates from Zambia was based on 16S rRNA or 18S rRNA sequences respectively. The tree was constructed using the neighbor-joining method and ClustalW alignment.

Statistical analysis

Statistical analysis of prevalence data was done using Chi-square statistical test. The chi-square test was meant to test the null hypothesis, which states that there is no significant difference between the expected and observed result.

Results

PCR assays using genus- or species-specific primers for the selected zoonotic pathogens detected *Rickettsia* spp. in 35 samples (39.8%), *Anaplasma* spp. in 12 samples (13.6%) and *Babesia* spp. in 2 samples (2.3%). However, *Borrelia* spp., *Trypanosoma* spp., *Plasmodium* spp., *Leishmania* spp., and *Coxiella burnetii* were not detected. Important to note, *Babesia* spp. was only detected in baboons (Table 2). There was no significant difference between the infection prevalence, primate species and sex

Table 1 Primers and conditions for PCR detection of pathogen DNA

Organism	Target gene	Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
Rickettsia spp	*gltA*	RpCS.780p	GACCATGAGCAGAATGCTTCT	600	48	[8]
		RpCS.877p	GGGGACCTGCTCACGGCGG	480	54	
		RpCS.1273r	CATAACCAGTGTAAAGCTG			
Anaplasma sp..	16S rDNA	EHR16SD	GGTACCYACAGAAGAAGTCC	345	53	[9]
		EHR16SR	TAGCACTCATCGTTTACAGC			
Coxiella burnetii	IS1111	Trans 1	TATGTATCCACCGTAGCCAGTC	687	60	[10]
		Trans 2	CCCAACAACACCTCCTTATTC			
Borrelia spp.	fla gene	BflaPAD	GATCA(G/A)GC(T/A)CAA(C/T)ATAACCA(A/T)ATGCA		55	[11]
		BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC			
		BflaPBU,nest	GCTGAAGAGCTTGGAATGCAACC	340	55	
		BflaPCR,nest	TGATCAGTTATCATTCTAATAGCA			
B. microti	18S rDNA	BabI	CTTAGTATAAGCTTTTATACAGC	238	55	[12]
		Bab4	ATAGGTCAGAAACTTGAATGATACA			
Trypanosoma spp.	ITS1 rDNA	ITS1 CF	CCGGAAGTTCACCGATATTG	Variable	58	[13]
		ITS1 BR	TTGCTGCGTTCTTCAACGAA			
Leishmania spp.	kDNA minicircle	L.MC-1S	CTRGGGGTTGGTGTAAAATAG-	700	55	[14]
		L.MC-1R	TWTGAACGGGRTTTCTG			
Plasmodium spp.	Cytb	DW2 &DW4	DW2; TAATGCCTAGACGTATTCCTGATTATCCAG	1253	60	[15]
			DW4; TGTTTGCTTGGGAGCTGTAATCATAATGTG			
		Cytb1 & Cytb2	CYTb1; CTCTATTAATTTAGTTAAAGCACA	939	50	
			Tb2; ACAGAATAATCTCTAGCACC			

Table 2 The prevalence of zoonotic pathogens in non-human primates in Zambia

	Baboon (n = 48)			Vervet monkey (n = 40)		
	Sex		Sub-total (%)	Sex		Sub-total (%)
	M (39)	F (9)		M (33)	F (7)	
Anaplasma spp.	3	2	5 (10.4%)	6	1	7 (17.5%)
Babesia spp.	1	1	2 (4.2%)	0	0	0
Borrelia spp.			0			0
Coxiella burnetii			0			0
Leishmania spp.			0			0
Plasmodium spp.			0			0
Rickettsia spp.	14	2	16 (33.3%)	15	4	19 (47.5%)
Trypanosoma spp.			0			0

of the primates as tested by Chi square test (data not shown). Information on age of the primates was not available.

Some of the positive samples with genus-specific primers were further subjected to direct sequencing and the BLAST sequence homology searches were performed. Two *Rickettsia* spp.-positive samples had 99% identity with *R. africae* from Nigerian ticks [16]. Two *Anaplasma* spp.-positive samples were sequenced and showed 100% similarity with *A. phagocytophilum* from various host species and geographical regions. Two *Babesia* spp.-positive samples from baboons showed the highest sequence similarity with *Babesia* spp. KMG-2009a from baboons with 100% identities, and also showed 98% similarity with *B. leo*-K8, the isolate from a domestic cat in South Africa, and 99% similarity with *Babesia* spp. from a laboratory raised baboon in USA [17].

Discussion

An investigation of pathogens in wild NHPs found in habitats close to human settlements is of importance in the control and eradication of probable human zoonotic pathogens.

We detected *Rickettsia* spp. in a total of 35 samples (39.8%). Further sequencing analysis revealed that some of the sequences were highly similar to that of *R. africae* (Figure 2). This is an agent of African tick bite fever, an acute and flu-like illness that is frequently accompanied by severe headache, inoculation eschars with regional lymphadenitis, vesicular cutaneous rash, and aphthous stomatitis [18,19]. The disease is transmitted in rural sub-Saharan Africa by ungulate ticks of the *Amblyomma* genus, mainly *Amblyomma hebraeum* in southern Africa and *Amblyomma variegatum* in west, central, and east Africa [20]. Phylogenetic comparisons between our obtained sequence and previous studies worldwide revealed a close relationship between Zambian and Nigerian *R. africae* isolates, suggesting general occurrence of rickettsioses in African continent.

We also obtained the sequences associated with *A. phagocytophilum* from both baboons and vervet monkeys (Figure 2). *A. phagocytophilum*, an obligate intracellular bacterium, is the agent of human granulocytic anaplasmosis, formerly known as human granulocytic ehrlichiosis [21]. This bacterium can infect humans and numerous animal species, including horses, cats, dogs, ruminants, and wildlife. In our analysis of *A. phagocytophilum* 16S rRNA gene, we found that the sequences of 16S rRNA were very conserved not only between African isolates but also between the other isolates of world-wide origin and this was in agreement with previous studies [22].

The rodent parasite *B. microti* and the bovine pathogen *Babesia divergens* appear to be responsible for virtually all of the known human zoonotic *Babesia* cases [23,24]. We detected a *B. microti*-like parasite from Zambian primates at a prevalence of 2.3% from baboons. Because *B. microti* shares a vertebrate host reservoir, the white-footed mouse (*Peromyscus leucopus*) and tick vector (*Ixodes dammini*) with *B. burgdorferi*, it might be expected that the caseload for human babesiosis will parallel the rise in the number of cases of Lyme disease in endemic areas [25-27].

Babesia microti, long considered on morphological grounds to be a single species found only in rodents, is now thought to consist of a complex of closely related subspecies, many of which are found in non-rodent hosts. Goethert and Telford III [28] identified 3 clades based on analysis of the 18S rRNA and beta-tubulin genes, with one (Clade 1) containing the majority of strains thought to be zoonotic. This clade includes the American zoonotic strains that have caused most babesiosis cases worldwide, but there are also separate zoonotic strains occurring in Japan ('Kobe' and 'Hobetsu') and Taiwan [29]. Strains of unknown zoonotic potential but closely related to the zoonotic American strains, according to 18S rRNA or beta-tubulin gene analysis, have been isolated in Germany (Hannover), central and eastern Russia (Mis, near Berezniki, Perm region and Vladivostok), Japan, South Korea and north-east China (Xinjiang) [28,30-32]. The zoonotic potential of Zambian *B. microti*-like parasite found calls for further investigation.

The genus *Borrelia* comprises of 37 known species of which 12 species are known to cause Lyme disease and are transmitted by ticks. *Borrelia burgdorferi* sensu *lato* complex, which is related to Lyme disease, is classified into four genospecies on the basis of genetic, phenotypic, and immunological properties [33]. The endemic tick-borne relapsing fever spirochetes are transmitted through the bites of soft ticks of the genus *Ornithodoros*; *O. sonrai* serves as

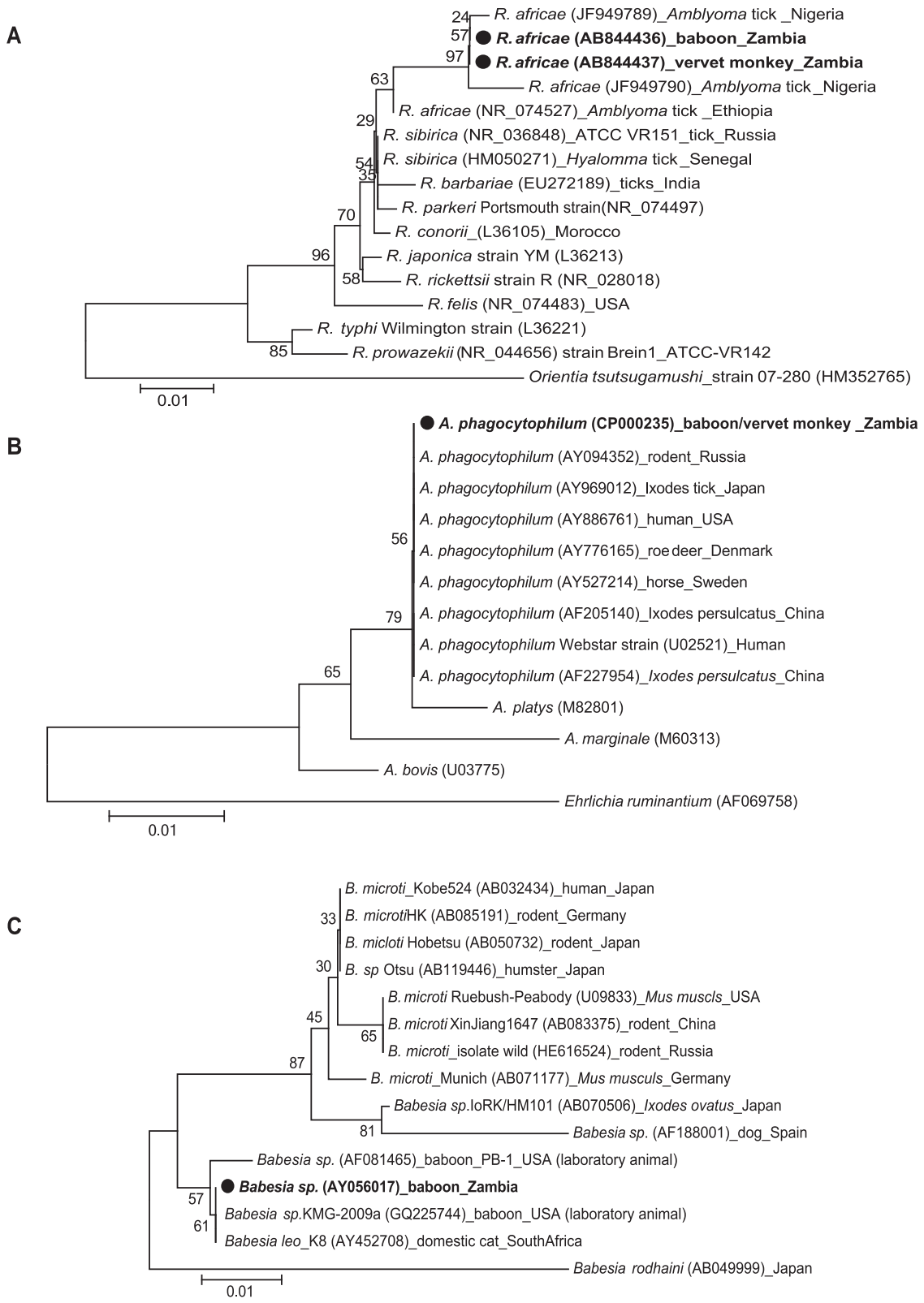

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Phylogenetic positions of the pathogens (*R. africae*: A, 426 bp, 16S rRNA; *A. phagocytophilum:* B, 345bp 16S rRNA and *B. microti* C, 238bp, 18S rRNA) detected in primates from Zambia based on 16S rRNA or 18S rRNA sequences respectively. The tree was constructed using the neighbor-joining method and ClustalW alignment, and numbers on the tree indicate 1000 bootstrap values for branch points. Accession numbers are indicated.

the principle vector for *Borrelia crocidurae* in West Africa, and *O. moubata* complex ticks effectively maintain these spirochetes in East Africa [34]. *Borrelia recurrentis* causes louse-borne relapsing fever and *B. duttonii* is the agent of East African tick-borne relapsing fever [35]. Some cases of Lyme disease have been reported in Kenya [36], but any *Borrelia* species were not detected in our study.

Although we have included several other pathogens, which have the potential for causing zoonoses, we could not detect those species in this study. Trypanosomes infect a wide range of wildlife species that constitute a reservoir of infection for both people and domestic animals. In Zambia, human African trypanosomiasis, caused by *T. brucei rhodesiense*, is endemic especially alongside the Luangwa Valley ecosystem [37]. However, active trypanosome infection was not demonstrated in sampled NHPs in our study, although the Luangwa Valley ecosystem is an active trypanosomiasis endemic focus with several human cases having been reported from the same area [38], and vervet monkeys are experimentally susceptible to African salivarian trypanosomes *T. b. rhodesiense* [39,40] and *T. b. gambiense* [41].

Plasmodium spp. was not detected in the current study. So far, the transmission of *P. knowlesi*, a malaria parasite of Southeast Asian macaques occurs from monkeys to humans in South-East Asia [42]. Coexistence of humans and monkeys in the same habitat has been driven in some cases by ecological conditions as observed in the transmission of *P. knowlesi* to the human population in Southeast Asia [43]. Recently, several additional *Plasmodium* species such as *P. cynomologi*, *P. inui*, *P. simium*, and *P. brasilianum* have been considered to be the zoonotic parasites from monkey to human, but none of them have been reported in Africa. Therefore, several authors hypothesized that monkeys may act as reservoirs for human malaria or vice versa [43]. In the wild, baboons harbour parasites closely related to *Plasmodium*, such as *Hepatocystis* spp., but they are not naturally susceptible to *Plasmodium* [44].

To the best of our knowledge, this is the first report of these potential zoonotic pathogens detected in non-human primates in Zambia. Therefore, zoonotic infections namely: human Babesiosis, Anaplasmosis and Rickettsiosis are suspected to be endemic in Zambia in humans and cases could be simply misdiagnosed especially as malaria due to the febrile nature of the illnesses.

Conclusion

Our study revealed that, potential zoonotic pathogens; *R. africae*, *A. phagocytophilum* and *B. microti*-like parasites exist in Zambian non-human primates. Zoonosis transmission involves interplay between humans, livestock and wildlife, making disease control complicated due to the lack of knowledge of the roles played by each. Better understanding of zoonotic pathogens harbored in non-human primates is necessary and must be adopted as a control measure in all regions inhabited by these animals or where they are in close proximity with human beings.

Abbreviations

EID: Emerging infectious diseases; NHP: Non-human primates; ZAWA: Zambia Wildlife Authority; TG: Typhus group; SFG: Spotted fever group.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HS and CS designed the study and performed data analysis. AI, HO, IN, LN, BMH, AM, YT and YO performed sample collection and processing. YO and AM identified research areas and contributed to obtaining the ethical clearance. JN, KH, RN and CS wrote the manuscript and all authors edited the manuscript. All authors read and approved the final manuscript.

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Author details

[1]Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan. [2]National Livestock Resources Research Institute (NaLIRRI), P. O. Box 96, Tororo, Uganda. [3]Unit of Risk Analysis and Management, Research Center for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan. [4]Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, University of Zambia, PO Box 32379 Lusaka, Zambia. [5]Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, PO Box 32379 Lusaka, Zambia. [6]Department of Disease Control, School of Veterinary Medicine, University of Zambia, PO Box 32379, Lusaka, Zambia. [7]Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan.

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