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

Jesca Nakayima1,2, Kyoko Hayashida1, Ryo Nakao3, Akihiro Ishii4, Hirohito Ogawa4, Ichiro Nakamura1, Ladslav Moonga5, Bernard M Hang’ombe5, Aaron S Mweene6, Yuka Thomas4, Yasuko Orba7, Hirofumi Sawa7* and Chihiro Sugimoto1*

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 (Hominioidea) 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...
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...
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. africai*: 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             | GGGGACCTGTCACGGCCGG  | 480                | 54                          |                   |
|                  |             | RpCS.1273r            | CATAACCAGTGAAGAAGCTG |                    |                             |                   |
| *Anaplasma* spp. | 16S rDNA    | EHR16SD               | GGTCACCAGAAGAATGCTTC | 345                | 53                          | [9]               |
|                  |             | EHR16SR               | TAGCACCAGTTAGTACGC   |                    |                             |                   |
| *Coxiella burnetii* | IS1111     | Trans 1               | TATGTATCCACCTGACGTC  | 687                | 60                          | [10]              |
|                  |             | Trans 2               | CCAACAAACACCTTATATTC |                    |                             |                   |
| *Borrelia* spp. | *fla gene*  | BflaPAD               | GATCA(G/A)GC(T/A)CAA(C/T)ATACCA(A/T)ATGCA | 55 | Variable | [11] |
|                  |             | BflaPDU               | AGATTCAGTCTGGTTTGGAAAGC | 340 | 55 | |
|                  |             | BflaPBU,nest          | GCTGAAGAGCTTGAATGACCAACC | 350 | 55 | |
|                  |             | BflaPCR,nest          | TGATCGGTTATCTCTAATAGCA | 238 | 55 | |
| *B. microti*     | 18S rDNA    | Babl                  | CTTAGTATAAAGCTTTTATACGC | 238 | 55 | [12] |
|                  |             | Bab4                  | ATAGGTCAAGAACCTGATGATACCA | 238 | 55 | |
| *Trypanosoma* spp.| IT51 rDNA  | IT51 CF               | CCAGAAGTTCACCTGATATTG | Variable | 58 | |
|                  |             | IT51 BR               | TTGGCTGGTCTTCAACAGAA |                    |                             |                   |
| *Leishmania* spp.| kDNA minicircle | LMC-1S           | CTRGGGGTGGTGGTATAGTGTTACATCA | 700 | 55 | [14] |
|                  |             | LMC-1R                | TWTGACGGGGTTTCTGTG   |                    |                             |                   |
| *Plasmodium* spp.| Cytb        | DW2 & DW4             | DW2; TAATGCTTGACTATTTCCTGATTACCAG | 1253 | 60 | [15] |
|                  |             | Cytb1 & Cytb2         | Cytb1; CTCCTATGTTTGGTAAAGCACA | 939 | 50 | |
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].

**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 (%) |
| Sex              | M   | F   | Sub-total (%) | M   | F   | Sub-total (%) |
|                  | (39) | (9) | 5 (10.4%) | 6 | 1 | 7 (17.5%) |
| *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   | 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   |

**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 Vladvostok), 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.)
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 for 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. cynomolgi*, *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**

BD: 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**

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

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