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An Evaluation of the Oral Microbiome and Potential Zoonoses of the Southern Thick-Tailed or Greater Galago (*Otolemur crassicaudatus*)

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Abstract: As the southern thick-tailed or greater galago (*Otolemur crassicaudatus*) is ubiquitous throughout parts of southern Africa they are thought to be of little conservation risk. As such, this species is seldom studied and relatively little is known of their behavior and biology. This research partially addresses this lack of knowledge by investigating the oral microbiome for potential zoonoses. Real-time PCR was used for the detection of *Mycobacterium tuberculosis* and *M. leprae* whereas next generation MiSeq Illumina sequencing was conducted on the 16S ribosomal RNA gene (16S rRNA) with a universal primer set which amplified the variable V3 and V4 regions to quantify the amount of different bacterial taxa. Major bacterial taxa of each tested oral microbiome were isolated, and those with potential for causing disease in humans and domestic animals were identified. The results illustrated a potential core microbiome for all *O. crassicaudatus* consisting of three separate bacterial taxa: *Mannheimia caviae*, *Porphyromonas catoniae* and *Gemella cunicula* that were present in all samples. The qPCR analysis did not detect the presence of mycobacteria. Several potentially pathogenic bacterial strains were observed that are known to result in disease in human and domestic animals. These findings thus form an important basis for several future avenues of potential research to assess any real zoonotic risks associated with *O. crassicaudatus* either to or from human and/or domestic animal populations of southern Africa.

Key words: *Otolemur crassicaudatus*, microbiome, 16s, zoonoses, next-generation sequencing

INTRODUCTION

The greater galago (*Otolemur crassicaudatus*) has a large distribution over most of southern Africa. A total of three subspecies of *O. crassicaudatus* has been described: *O. c. crassicaudatus*, which is reported only in the KwaZulu-Natal region; *O. c. kirkii* Gray, 1863, which ranges from Massangena, Mozambique, in the south to southern Malawi in the north; and *O. c. monteiri*, which is found in the *Brachystegia miombo* woodland zone from Angola in the west, to Zambia, Malawi and northern Mozambique in the east and north to Rwanda. However, Grubb *et al.* (2003) reported that *O. c. monteiri* can further be divided into two groups: the *monteiri* group, which is found from Angola through

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the southern Democratic Republic of the Congo, Zambia, Zimbabwe, northern Mozambique, Malawi and southern Tanzania (Tabora), and the *argentatus* group, which has an unknown distribution, although reported in Rwanda, Kenya and Tanzania (Bearder 2008). *O. crassicaudatus* is found in coastal forest and riparian bushland in the southern parts of its range and in bushland and open woodland in the northern parts of its range (Skinner & Chimimba 2005).

The greater, or thick-tailed, galago (*O. crassicaudatus*), which averages a weight of approximately 1,384 g (Nekaris & Bearder 2011), feeds primarily on gums and fruits and supplements its diet with insects (Nekaris & Bearder 2011). This species is found primarily in rural areas where they have been found in small family groups (Clark 1985), often in larger vegetation such as tall trees routinely surrounded by lower scrub foliage and acacia trees (Nekaris & Bearder 2011).

Zoonotic transmission represents a major source of novel and emerging pathogenic infections worldwide (Wolfe *et al.* 1998). It has thus become increasingly important to investigate potential reservoirs of zoonotic disease for novel pathogenic involvement in addition to those that are already known to harm humans and livestock. Nonhuman primates are important reservoirs of zoonoses. They are taxonomically and genetically close to humans and as such their parasites and pathogens are more likely to cross the species boundary than are those from other taxonomic groups (Guerrera *et al.* 2003). Rising human populations and anthropogenic pressures are resulting in humans and nonhuman primates being more frequently in close proximity creating increasing opportunities for pathogenic transfer (Chapman *et al.* 2005; Engel & Jones-Engel 2012). In addition, nonhuman primates are frequently relocated from wild populations (De Thoisy *et al.* 2001) and are kept in zoos, homes, and research centers that increase the amount of contact with humans (Guerrera *et al.* 2003). This is often done as a result of habitat loss, which is also one of the major causes of zoonotic transfer and emergence of novel pathogens (De Thoisy *et al.* 2001).

A key way to understand what pathogens a given species harbors is through investigation of their microbiomes. Studies have been conducted on both *Homo* and *Pan* salivary and oral microbiomes in order to understand bacterial diversity and individual health (Li *et al.* 2013). The oral cavity is usually selected both for ease of sampling - as it is minimally invasive - as well as for the propensity of different bacteria to colonize the oral cavity due to the presence of both hard tissue surfaces and mucosal surfaces (Zaura *et al.* 2009). However, use of the oral microbiome as a predictor of zoonoses is a new and potentially important research approach, and has been applied to few nonhuman primate taxa (Venezia *et al.* 2012, Mugisha *et al.* 2014). Information on the oral microbiome of *O. crassicaudatus* has not been noted previously. This species has not been reported to be linked with pathogen transmission and was selected as it is very distantly related to humans and can live in close proximity with humans. Thus, this study provides baseline knowledge in order to understand the type and diversity of bacterial groups found within an isolated population of wild *O. crassicaudatus* as well as to identify any known pathogens.

MATERIALS AND METHODS

Sample collection

Wild *O. crassicaudatus* individuals were sampled at the Lajuma Research Centre, which forms part of the Luvhondo Nature Reserve within the UNESCO Vhembe Biosphere Reserve in Limpopo Province, South Africa, during the months of June and July, 2013. This site consists of a mosaic of habitats that includes marshland, thicket and riverine forest (Willems 2007). The areas adjacent to the reserve, however, have been altered by humans, with a ranch along one border and a tourist lodge along another. Additionally, one area of the reserve was formerly used as a fruit plantation. Havahart™ live traps and custom-made Chardonneret traps were zip-tied into trees and were baited with a mixture of bananas and honey. Once secured in a trap, individuals were anesthetised by a certified wildlife veterinarian as prescribed in Larsen *et al.* (2011). The oral microbiome was sampled in two ways: with a Thermo Scientific AssayAssure™ kit and using a cheek swab (Epicenter) that was then placed in 700 ul of buffer containing 50 mM Tris (pH 8.0, 50 mM EDTA, 50 mM sucrose, 100 mM NaCl and 1% SDS (for collection of salivary mycobacterial samples, see Abusleme *et al.* 2014). Both sides of the cheek were swabbed as well as under the pseudo-tongue, which is where the majority of saliva pooled (Figure 1).

16 S DNA extraction and detection

DNA extraction was conducted using the ZR Genomic DNA™ Tissue MiniPrep kit from Zymo Research following the protocol as outlined by the manufacturer. Each sample was screened for the

Figure 1. Oral swab of wild *Otolemur crassicaudatus* at Lajuma Research Centre. Photograph by M. Sauther.

presence of bacteria by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA) with a universal primer set which amplified the variable V3 and V4 regions of the 16S rRNA gene in single amplicon of 460 bp (Frank *et al.* 2008). Samples (n=8) were submitted to the Agricultural Research Center (ARC, South Africa) for analysis using an Illumina MiSeq high-throughput sequencing platform. Analysis was conducted on BaseSpace, a MiSeq Reporter Metagenomics workflow which performs a taxonomic classification using an Illumina-curated version of the Greengenes 16S rRNA gene database providing information on genus or species level classification. In addition, samples were analysed in Genomics Workbench where primer sequences were removed and reads were grouped into operational taxonomic units (OTUs). In order to compare OTU prevalence and to identify the top eight contributions, the percentage abundance was determined (# of reads for strain/total # of reads), in all of the *O. crassicaudatus* samples.

Mycobacterium tuberculosis and *M. leprae* detection

Prior to the extraction of DNA, each sample (n=24) was incubated at 80°C for a period of one hour to kill any remaining harmful viable bacteria. After incubation, 25 µL of Proteinase K (10 mg.mL) was added in order to lyse cells. Samples were then placed into a rotisserie incubator at 55 - 65°C for a period of one hour to catalyze the lysis process. Extraction was performed using the Phenol-Chloroform method. The TaqMan qPCR tests were conducted at Arizona State University using protocols described in Harkins *et al.* (2015). Four separate qPCR tests were run, two for *M. tuberculosis* DNA and two for *M. leprae* DNA. Of the two *M. tuberculosis* qPCRs, one targeted the single-copy rpoB gene (the rpoB2 qPCR) and one targeted the *M. tuberculosis* complex-specific multi-copy IS6110 insertion element (Klaus *et al.* 2010; Harkins *et al.* 2015). To detect the presence of *M. leprae* DNA, qPCRs targeting the 85B single-copy gene and the rlep multi-copy gene were used (Martinez *et al.* 2006; Truman *et al.* 2008). The four primer sets used are listed in Appendix 1. Each qPCR reagent set consisted of varying concentrations of primer, probe, Rat Serum Albumin and Taq Master Mix. Standards were included in duplicate with concentrations varying from 5e-1 – 5e-5 ng/µL. Two non-template controls were also included to test for contamination and samples were run in triplicate. The qPCR thermocycling conditions for the ABI7900 consisted of one cycle of two min at 50°C, one cycle of 10 min at 95°C followed by 50 cycles at 95°C for 15 sec and 60°C for one min.

RESULTS

In this study, the overall number of bacterial taxa, both indefinable and non-identifiable, in randomly selected oral microbiomes was parsed from the samples. Both the MiSeq Reporter results as well as analysis on Genomics Workbench used to group OTUs with at least 97% identity provided similar results. In addition, the top eight bacterial taxa were identified (Table 1) and abundance of each bacterial taxon per sample was determined (Table 2). The highest bacterial taxon in each sample was unknown and could not be identified to genus or species level (Table 1.). A total of four bacterial strains that were identified have been reported to have pathogenic implication in humans and/or livestock (Table 3). Amplification of *M. leprae* and *M. tuberculosis* DNA via qPCR was not detected in any of the tested samples. However, qPCR was considered successful as amplification occurred for the standards and was absent in the non-template control.

DISCUSSION

The identifiable oral microbiome of wild *O. crassicaudatus* was found to consist of three bacterial species: *Mannheimia caviae, Gemella cunicula,* and *Porphyromonas catoniae*. This finding is similar to previous studies of human populations where they found a core microbiome in unrelated healthy humans. A study on the oral microbiome of humans conducted by Zaura *et al.* (2009) in three healthy individuals found that the individuals shared 1660 of 6315 unique sequences of which the core microbiome was observed in 66% of the reads. A core microbiome in five healthy individuals has also been reported by Lazarevic *et al.* (2010). These three bacterial taxa observed in our study can thus potentially be considered the "universal core", meaning all *O. crassicaudatus* will exhibit these bacteria in addition to their individually diverse loads. However, microbiome studies are in their infancy and larger scale projects would be required in order to both identify core and individual microbiomes. Thus, the results reported here may only pertain to animals sampled from the Lajuma study site and further larger scale analysis could elucidate whether this proves to be true in other regions as well.

No mycobacterium loads were detected. While this means that this particular population of sampled individuals is not harboring these mycobacteria, it does not preclude the possibility that *O. crassicaudatus* may be able to do so elsewhere, i.e., in closer proximity to human populations or where human populations utilizing bushmeat. Thus, we cannot discount the possibility of *O. crassicaudatus* acting as a reservoir for these two pathogens elsewhere (Deredec & Courchamp 2003).

As humans could also be at risk from galago-borne diseases, especially in the context of keeping galagos as pets, consuming them as bushmeat, or other documented contact (Linden 2015, Tshikudo 2015), the investigation of the oral microbiome revealed many bacterial strains (Table 3) that are opportunistically pathogenic to humans, primarily those which cause endocarditis, a potentially fatal inflammation of cardiac tissues due to injury or severe illness (Mayo Clinic 2014). Another key result is the suspected interplay in bacterial ecology between *O. crassicaudatus*, domesticated ungulates, and humans. One potential bacterial microbiome species, discovered in 40% of tested samples, was *Streptococcus bovis*, a bacterium that is commonly found in the alimentary tract of ruminants and causes neonatal septicemia and meningitis in humans (Russell & Hino 1985). The source of this bacterium is unknown, but there were several free ranging horses at the research centre during the research period and there are also farms with cattle around the surrounding area. There is thus a potential of pathogen transmission to humans and domestic animals, but this would require further research.

The large number of previously unidentified bacterial strains is also an important finding. It is because of this gap in our knowledge that we are unable to say if those strains are potentially hazardous or benign in this population. This study thus offers important baseline data to provide a greater understanding of how susceptible nonhuman primates are to human pathogens and vice versa, something of great concern to conservationists, wildlife biologists, and zoo health officials (De Thoisy *et al.* 2001; Engel & Jones-Engel 2012).

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Table 1. Top eight contributors of distinct bacterial taxa for each oral microbiome sample.

Sample	# of Contributors	1st Highest	2nd Highest	3rd Highest	4th Highest	5th Highest	6th Highest	7th Highest	8th Highest
C35F	703	Unspecified at Species Level	*Mannheimia caviae*	*Gemella cunicula*	*Moraxella caviae*	*Porphyromonas catonaie*	*Streptococcus bovis*	*Butyrivibrio proteoclasticus*	*Rothia aeria*
3C15	790	Unspecified at Species Level	*Mannheimia caviae*	*Porphyromonas catoniae*	*Gemella cunicula*	*Streptococcus bovis*	*Butyrivibrio proteoclasticus*	*Sneathia sanguinegens*	
5F87	682	Unspecified at Species Level	*Mannheimia caviae*	*Gemella cunicula*	*Porphyromonas catoniae*	*Streptococcus bovis*	*Lautropia mirabilis*	*Moraxella caviae*	*Planococcus maritimus*
58A3	652	Unspecified at Species Level	*Mannheimia caviae*	*Gemella cunicula*	*Porphyromonas catoniae*	*Fusobacterium navidorme*	*Sneathia sanguinegens*	*Ureaplasma gallorale*	*Streptococcus peroris*
98C7	554	Unspecified at Species Level	*Mannheimia caviae*	*Gemella cunicula*	*Porphyromonas catoniae*	*Ureaplasma gallorale*	*Butyrivibrio proteoclasticus*	*Streptococcus peroris*	*Fusobacterium naviforme*
2979	684	Unspecified at Species Level	*Mannheimia caviae*	*Butyrivibrio proteoclasticus*	*Porphyromonas catoniae*	*Gemella cunicula*	*Fusobacterium naviforme*	*Aggregatbacter aphrophilus*	*Leptotrichia goodfellowi*
5010	792	Unspecified at Species Level	*Mannheimia caviae*	*Porphyromonas catoniae*	*Butyrivibrio proteoclasticus*	*Gemella cunicula*	*Fusobacterium naviforme*	*Sneathia sanguinegens*	*Aggregatibacter aphrophilus*
8123	901	Unspecified at Species Level	*Mannheimia caviae*	*Porphyromonas catoniae*	*Gemella cunicula*	*Butyrivibrio proteoclasticus*	*Streptococcus bovis*	*Moraxella caviae*	*Lautropia mirabilis*
Cap1	887	Unspecified at Species Level	*Mannheimia caviae*	*Neisseria lactamica*	*Aggregatbacter aphrophilus*	*Streptococcus sanguinis*	*Rothia aeria*	*Streptococcus peroris*	*Gemella cunicula*
Cap3	883	Unspecified at Species Level	*Mannheimia caviae*	*Gemella cunicula*	*Streptocaucus peroris*	*Corynebacterium durum*	*Aggregatibacter aphrophilus*	*Veillonella dispar*	*Avibacterium avium*

Table 2. Abundance (number of reads identified/total number of reads) of bacterial species.

Sample	# of Contributors	1st Highest	%	2nd Highest	%	3rd Highest	%	4th Highest	%
C35F	703	Unspecified at Species Level	*66*	*Mannheimia caviae*	*19*	*Gemella cunicula*	*6*	Other	*9*
3C15	790	Unspecified at Species Level	*51*	*Mannheimia caviae*	*26*	*Porphyromonas catoniae*	*13*	*Other*	*10*
5F87	682	Unspecified at Species Level	*70*	*Mannheimia caviae*	*16*	*Gemella cunicula*	*7*	*Other*	*7*
58A3	652	Unspecified at Species Level	*76*	*Mannheimia caviae*	*14*			*Other*	*10*
98C7	554	Unspecified at Species Level	*45*	*Mannheimia caviae*	*32*	*Gemella cunicula*	*11*	*Other*	*12*
2979	684	Unspecified at Species Level	*65*	*Mannheimia caviae*	*20*	*Butyrivibrio proteoclasticus*	*5*	*Other*	*10*
5010	792	Unspecified at Species Level	*74*	*Mannheimia caviae*	*10*	*Porphyromonas catoniae*	*5*	*Other*	*9*
8123	901	Unspecified at Species Level	*52*	*Mannheimia caviae*	*16*	*Porphyromonas catoniae*	*13*	*Other*	*19*
Cap1	887	Unspecified at Species Level	*84*	*Mannheimia caviae*	*6*			*Other*	*10*
Cap3	883	Unspecified at Species Level	*62*	*Mannheimia caviae*	*21*	*Gemella cunicula*	*5*	*Other*	*12*

Table 3. Identified bacterial pathogens and their known effects.

Bacterial Strain	Human pathogenic involvement	Livestock pathogenic involvement
Gemella cunicula	Endocarditis, Meningitis, Arthritis and Pneumonia	No known pathogenic effects
Streptococcus bovis	Colorectal Cancer, Endocarditis, Meningitis and Neonatal Septicemia	Ruminal Acidosis and Feedlot Bloat
Fusobacterium naviforme	Lemierre Syndrome and Generalized Inflammation	No known pathogenic effects
Mannheimia caviae	No known pathogenic effects	Conjunctivitis

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Appendix 1: Primers and TaqMan probes used for mycobacterial qPCR.

Primer /probe name	Sequence
RpoB2_F	5'- CAA CGT CGA GGT GCT ATC G -3'
RpoB2_R	5'- CTC CAG GTC CTC GTC CTC A -3'
RpoB-Probe	5'- 6FAM-TCG CCG CAC CGT CAC T-MGBNFQ-3'
Rlep_F	5'-GCA GTA TCG TGT TAG TGA A-3'
Rlep_R	5'-CGC TAG AGG GTT GCC GTA TG-3'
Rlep_Probe	5'- 6FAM-TCG ATG ATC CGG CCG TCG GCG-MGBNFQ-3'
IS6110-F	5'-GGG TAG CAG ACC TCA CCT ATG TG-3'
IS6110-R	5'-CGG TGA CAA AGG CCA CGT A-3'
IS6110-Probe	5'- 6FAM-ACC TGG GCA GGG TT-MGBNFQ-3'
85B-F	5'-GTG GTC GGC CTC TCG AT-3'
85B-R	5'-CGA GCC AGC ATA GAT GAA CTG ATC-3'
85B-Probe	5'- 6FAM-CTC GGC CCT AAT ACT-MGBNFQ-3'