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Short Communication

Noninvasive Tuberculosis Screening in Free-Living Primate Populations in Gombe National Park, Tanzania

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Abstract: Recent advances in noninvasive detection methods for mycobacterial infection in primates create new opportunities for exploring the epidemiology of tuberculosis in free-living species. Chimpanzees (*Pan troglodytes schweinfurthii*) and baboons (*Papio anubis*) in Gombe National Park, Tanzania, were screened for infection with pathogens of the *Mycobacterium tuberculosis* Complex using Fecal IS*6110* PCR; none was positive. This study demonstrates the feasibility of large-scale mycobacterial screening in wild primates.

Keywords: *Mycobacterium tuberculosis* Complex, Chimpanzees, Baboons, fecal IS*6110* PCR

Background

Anthropogenic impacts such as deforestation, agriculture, ecotourism, and research have led to new contact patterns among humans, domesticated animals, and human-habituated primates (i.e., primates conditioned to human observers) resulting in the emergence of infectious diseases, including those of pathogenic mycobacteria. There is evidence of *Mycobacterium bovis* morbidity among wild monkey populations, and recent research demonstrates *Mycobacterium tuberculosis* Complex (MTC) infections among free-ranging macaques in areas of frequent human contact and high human tuberculosis prevalence (Keet et al. 2000; Sapolsky and Else 1987; Wilbur et al. 2012). Further, a novel pathogenic MTC strain has been diagnosed in a free-living chimpanzee in western Africa (Coscolla et al. 2013). This finding introduces the possibility of a chimpanzee-associated tuberculosis strain, circulating at low, undetected levels within free-living populations (Coscolla et al. 2013). The disease caused by and risks of MTC infection among primates are thoroughly reviewed in Wolf et al. (2014).

Unfortunately, the true extent of tuberculosis (TB) and its impacts on free-living primates are unknown due to challenges in effectively screening for infection. Traditionally, TB diagnosis in primates required multiple modalities, such as demonstration of tissue lesions, host immune responses, or culture of the organism (Lerche et al. 2008; Lin et al. 2008). Such approaches are generally infeasible in free-ranging species given logistical and ethical challenges

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of handling and anesthesia. Detection has been limited to post-mortem diagnosis, which is complicated by timely carcass retrieval. Consequently, a mycobacterial pathogen of low prevalence could go undetected. The noninvasive monitoring of systemic diseases, especially those caused by respiratory pathogens, is established for free-living great apes (Gillespie et al. 2008; Köndgen et al. 2010; Köndgen et al. 2008; Leendertz et al. 2006), which is critical to understanding the etiologic agents of disease outbreaks in the wild. Advances in molecular technology enable the detection of TB infection among free-ranging species (Wilbur et al. 2012), although these technologies have yet to be combined with noninvasive sampling. Our team validated a noninvasive approach for MTC DNA detection in feces of primates (Wolf et al. 2015) and applied it to screen free-living, human-habituated populations of chimpanzees (*Pan troglodytes schweinfurthii*) and baboons (*Papio anubis*) in Gombe National Park, Tanzania. This population of chimpanzees was considered important for MTC screening due to recognition of a wasting syndrome (not unlike primate TB infection) and infection with pathogenic simian immunodeficiency virus (Keele et al. 2006; Rudicell et al. 2010; Williams et al. 2008).

The Study

Gombe National Park is a small park, with land mass 35 km^2, on the eastern edge of Lake Tanganyika in Tanzania, bordered by villages to the north and south. The park is home to seven species of nonhuman primates (Wallis and Lee 1999) and is the site of the longest continuous research of free-living chimpanzees in the world. Approximately 100 chimpanzees comprise three communities, two of which are habituated to human observers: Kasekela, which is centrally located and visited by researchers and tourists, and Mitumba, which ranges in the northern part of the park and visited only by researchers (Table 1). Researchers follow chimpanzees daily, from nest to nest, as part of ongoing behavioral studies. There are also six habituated olive baboon troops centrally located within the park, totaling 189 individuals (Table 2). Habituated baboons are followed over several hours, as part of ongoing behavioral studies.

Noninvasive fecal sampling of individually identified chimpanzees and baboons was conducted from January to June 2014. Sampling goals were two fecal samples per individual of habituated chimpanzee communities (n = 162 samples from 81 individuals), and a single fecal sample from a representative number of individuals from six habituated baboon troops (n = 70). Upon defecation, a sample ca. 2 g was collected in a 15-ml tube containing 4.5 ml RNA. Later (Qiagen, Inc). Samples were stored frozen at −20°C for up to 9 months until testing.

Testing was conducted in a dedicated Mycobacterium research lab at the University of Minnesota's College of Veterinary Medicine. DNA was extracted using the QIAMP Fast DNA Stool Mini Kit (Qiagen, Inc) with minor modifications to manufacturer's instructions to optimize mycobacterial cell wall lysis, as previously described (Wolf et al. 2015). A portion of the IS*6110* insertion sequence was amplified with realtime PCR (rtPCR) (Wolf et al. 2015). Samples were tested in duplicate; every reaction included positive and negative controls. Positive or suspect samples were reamplified and assessed by Tm analysis with positive controls for confirmation.

As MTC infection was expected to be rare, if present, advanced statistical methods for rare diseases were employed. Wilson score confidence intervals were chosen as an exact method for small n and rare disease (Brown et al. 2001; Newcombe 2012). Additionally, Agresti–Coull prevalence shrinkage estimators and respective confidence

Table 1. Demographics of the Habituated Chimpanzee Communities in Gombe National Park, Tanzania, During the Study Sampling Period, January–June 2014.

Age–sex classes	Kasekela	Mitumba	Total
Adult males	13	6	19
Adult females	26	9	35
Immature males	8	3	11
Immature females	8	8	16
Total	55	26	81

Immature animals include ages of 0–11 years and 11 months; adult animals aged 12 years and older.

Table 2. Demographics of the Habituated Baboon Troops in Gombe National Park, Tanzania, During the Study Sampling Period, March–April 2014

Age–sex classes	BB	DB	AC	DA	BA	DC	Total
Adult males	1	2	5	3	10	11	33
Adult females	7	8	13	7	18	19	72
Immature males	2	6	11	8	7	16	50
Immature females	5	0	3	6	2	18	34
Total	15	16	32	24	38	64	189

The table contains the number of baboons in each age–sex class in each group as documented on October 31, 2013. Males are classified as adults at the age in which they have emigrated and joined a new troop as a breeding male; females are classified as adults at the age in which they have their first vaginal swelling cycle (Collins, unpublished data).

intervals were compared with the maximum likelihood estimator (Newcombe 2012). The Agresti–Coull estimate is appropriate for situations where disease of low prevalence may be more easily missed in surveillance. Since the shrinkage estimator approaches 0.5 where n is small (Newcombe 2012), this comparison was made for population-level prevalence estimates (Newcombe 2012). Finally, true population prevalence was estimated in Epitools using sensitivity (Se) and specificity (Sp) estimates from our previous validation study (Sergeant 2015). "Appendix 1" contains a complete description of rare disease statistics and true prevalence calculation.

A total of 207 fecal samples were collected, including 144 chimpanzee (Kasekela: n = 105, Mitumba: n = 39) and 62 baboon samples. In Kasekela, 84% of the community was sampled at least once, 60% was sampled twice, and 31% was sampled more frequently. Thirty-three adults and 12 immatures were sampled, accounting for 85 and 75% of Kasekela adults and immatures, respectively. Twenty (95%) Kasekela males and 25 (74%) females were sampled. During the study, two adult males (ages 42, 14 years) were chronically ill with clinical signs of weight loss and wasting. Before study conclusion, one individual died and the other disappeared and presumed dead. One male was sampled once; the other was sampled three times. In Mitumba, 85% of the community was sampled at least once, 42% was sampled twice, and 23% was sampled more frequently. Fifteen adults and seven immatures were sampled, accounting for 94 and 70% of Mitumba adults and immatures, respectively. All Mitumba males and 12 (71%) females were sampled. Sixty-two baboon samples originated from five habituated troops, including 25 from troop DC, 17 from BA, 11 from AC, five from DA, and four from DB.

All chimpanzee and baboon samples tested negative by IS*6110* rtPCR (Table 3). Agresti–Coull shrinkage estimates for prevalence are compared to maximum likelihood estimates in Table 4. Adjusted prevalence estimates were 2.8 and 3% for the chimpanzee and baboon populations, respectively. Considering the estimator approaches 0.5 with smaller n (Newcombe 2012), it is likely that small population size is impacting the precision of this method. True prevalence was estimated as 0 (95% Wilson CI 0–0.11) and

Table 3. Results of Tuberculosis Testing of Chimpanzee Communities and Baboon Troops in Gombe National Park, TZ.

Species	Community or troop	Sample size (proportion of community)	Sample prevalence	Wilson score confidence interval
Chimpanzees	Kasekela	46 (0.84)	0	0–0.07
	Mitumba	22 (0.85)	0	0–0.15
Baboons	AC	11 (0.34)	0	0–0.26
	BA	17 (0.45)	0	0–0.18
	DA	5 (0.21)	0	0–0.43
	DB	4 (0.25)	0	0–0.49
	DC	25 (0.39)	0	0–0.13

Table 4. Comparison of Maximum Likelihood and Agresti–Coull Estimators of Prevalence and Respective Confidence Intervals.

Species	Sample size	Maximum likelihood estimate (P)	Agresti–Coull shrinkage estimate (P_{AC})	Wilson score confidence interval for P	Agresti–Coull confidence interval for P
Chimpanzees	68	0	0.028	0–0.05	−0.01–0.06
Baboons	62	0	0.030	0–0.06	−0.01–0.07

0 (95% Wilson CI 0–0.12) for the chimpanzee and baboon populations, respectively. True prevalence confidence intervals are wider than those of maximum likelihood estimators and considered more precise as the calculation method accounts for uncertainty associated with Se and Sp estimates (Rogan and Gladen 1978). While the adjusted prevalence estimates and confidence intervals suggest the possibility of a low prevalence of MTC infection, the degree to which chimpanzee health is monitored (Lonsdorf et al. 2006; Terio et al. 2011), coupled with the absence of positive fecal samples with high sampling coverage in this study, makes a stronger argument for zero prevalence.

MTC-infected animals in advanced disease stages are expected to shed higher numbers of organisms, and thus detection is more likely to be successful in such animals (Lin et al. 2009). In this study, two chimpanzees with chronic, fatal diseases were screened for MTC infection by this method, both of which tested negative. Although necropsies could not be performed to positively identify underlying disease processes, one animal was screened three times by fecal PCR during clinical disease. Thus, TB was unlikely to be associated with death in at least one of these cases.

The success of screening fecal samples for pulmonary infection with *Mycobacteria* sp. relies on bacterial shedding in sputum with subsequent swallowing and excretion in feces; this only occurs during active infection (El Khéchine et al. 2009; Lin et al. 2008). Alternatively, fecal shedding may also occur with extra-pulmonary or systemic mycobacterial infection, depending on the location of infection in the body [e.g. TB in a wild chimpanzee included infection of the mesenteric lymph nodes (Coscolla et al. 2013)]. Thus, detection of mycobacterial DNA in feces is best suited for identifying primates with active pulmonary or systemic infection, which are most important in MTC transmission. This approach does not detect all stages of disease by measuring host immunologic responses (Lerche et al. 2008; Lin et al. 2008). Accordingly, animals with latent infection are expected to test negative by this method. In this study, we did not identify any individuals with active MTC infection. Combined with the pathology study conducted by Terio et al., which found no evidence of latent or active TB on post-mortem examination, our research reveals no evidence of MTC infection in the Gombe primate populations at this time (Terio et al. 2011).

Conclusions

In this study, we demonstrate the feasibility of large-scale, noninvasive screening of free-living primate populations for MTC infection using fecal IS*6110* PCR. We also demonstrate the diagnostic utility of this approach in disease investigation among individuals. This approach is invaluable to managers and researchers of primate populations where the epidemiology of MTC infection, because of spillover events or endemic infection, requires further investigation.

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Technical Appendix 1

Advanced Statistics for Prevalence Estimation of Rare Disease

MTC prevalence among chimpanzee and baboons populations was estimated from laboratory results. Wilson score confidence intervals were chosen as an exact method for small n and rare disease (Brown et al. 2001; Newcombe 2012). This method for estimating confidence intervals accounts for the limits of a proportion, being bound by 0 and 1, and also for interval asymmetry that occurs when estimating a low proportion, providing a more precise estimate for the upper limit. As MTC infection was expected to be rare, if present, in these primate populations, advanced statistical methods for rare diseases, where prevalence is expected to be close to 0, were also employed. Agresti–Coull prevalence shrinkage estimators and respective confidence intervals were compared with the maximum likelihood estimator. For comparison, the maximum likelihood prevalence estimator (P) is calculated as:

$$P = \frac{m}{n} \tag{1}$$

where m is the number positive and n is the number sampled. In contrast, the prevalence shrinkage estimator (P_Ψ) for rare disease is estimated as:

$$P_\psi = \frac{(m+\psi)}{(n+2\psi)} \tag{2}$$

where Ψ is a pseudo-frequency greater than 0, commonly set as $z^2/2$ (Newcombe 2012). When α is set to 0.05, $z = 1.96$ and $\Psi = 1.92$. Thus, by setting $\Psi = 2$, as done for the Agresti–Coull confidence interval (Newcombe 2012), the equation is reduced to:

$$P_{AC} = \frac{(m+2)}{(n+4)} \tag{3}$$

This method essentially adds two positive and two negative samples to the calculation of the prevalence estimate. The Agresti–Coull estimate is appropriate for situations where the true prevalence is expected to be close to 0, thus where disease may more easily be missed in surveillance; however, the shrinkage estimator approaches 0.5 where n is small (Newcombe 2012). Therefore, this comparison was made for the population-level prevalence estimates, as the Agresti–Coull prevalence estimator is expected to perform better for $n > 40$ (Newcombe 2012).

True Prevalence Estimation

True prevalence (TP) was calculated as:

$$\mathrm{TP} = \frac{\mathrm{AP} + \mathrm{Sp} - 1}{\mathrm{Se} + \mathrm{Sp} - 1} \tag{4}$$

where AP is the apparent or maximum likelihood prevalence, P (Dohoo et al. 2009; Rogan and Gladen 1978). Sensitivity (Se) and specificity (Sp) estimates from our previous validation study were used as inputs for the calculation (Wolf et al. 2015). Ninety-five percent Wilson score confidence intervals were calculated in Epitools and were based on variance estimates that incorporate additional uncertainty associated with the sample sizes used in Se and Sp estimation (Rogan and Gladen 1978; Sergeant 2015; Wolf et al. 2015).

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