

Noninvasive Test for Tuberculosis Detection among Primates

**Tiffany M. Wolf, Lawrence Mugisha,
Fernanda Miyagaki Shoyama,
Melanie J. O'Malley, JoAnne L. Flynn,
Benon Asimwe, Dominic A. Travis,
Randall S. Singer, Srinand Sreevatsan**

Traditional testing methods have limited epidemiologic studies of tuberculosis among free-living primates. PCR amplification of insertion element IS6110 of *Mycobacterium tuberculosis* from fecal samples was evaluated as a noninvasive screening test for tuberculosis in primates. Active tuberculosis was detected among inoculated macaques and naturally exposed chimpanzees, demonstrating the utility of this test.

The susceptibility to tuberculosis (TB) of nonhuman primates in captivity is established (1,2), although the extent of the disease among free-living primates remains unclear. Much of our understanding of primate TB is based on documentation of *Mycobacterium tuberculosis* transmission in captive primates (1,2), but TB caused by *M. bovis* spillover dominates among populations of free-living monkeys (3,4). Research demonstrates increases in *M. tuberculosis* complex (MTC) infections among free-ranging macaques in areas of frequent human contact and high human TB prevalence (5). The first evidence of TB in a free-living ape was reported in 2009 in West Africa; the infectious agent was identified upon routine necropsy of a chimpanzee as a novel MTC strain closely related to lineage 6 (i.e., *M. africanum* West-Africa type-2) (6).

Epidemiologic studies of TB among free-living primates have been limited by existing diagnostic technologies. Diagnosis of disease in primates traditionally relies upon procedures that identify tissue lesions or demonstrate host immune responses or upon culture of the organism (1), methods that are generally not feasible for free-living species because of the need for handling and anesthesia. To overcome this challenge, we evaluated a novel approach using molecular detection of MTC-specific DNA in noninvasively collected fecal samples. This approach has shown excellent sensitivity among humans with active pulmonary TB (7,8).

Author affiliations: Minnesota Zoological Gardens, Apple Valley, Minnesota USA (T.M. Wolf); University of Minnesota, St. Paul, Minnesota, USA (T.M. Wolf, L. Mugisha, F.M. Shoyama, D.A. Travis, R.S. Singer, S. Sreevatsan); Conservation & Ecosystem Health Alliance, Kampala, Uganda (L. Mugisha); Makerere University, Kampala (L. Mugisha, B. Asimwe); University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA (M.J. O'Malley, J.L. Flynn)

DOI: <http://dx.doi.org/10.3201/eid2103.140052>

Our objective was to evaluate the performance of PCR amplification of IS 6110 of *M. tuberculosis* in fecal samples (fecal IS6110 PCR) for noninvasive TB detection in inoculated and naturally exposed primates.

The Study

Fecal IS6110 PCR was first evaluated by using samples from primates with known TB infection status. Fecal samples were collected from 41 adult (>4 years) cynomolgus macaques (*Macaca fascicularis*) included in experimental *M. tuberculosis* infection studies and 13 uninfected rhesus macaques (*M. mulatta*) included in diabetes studies. All experiments and protocols were approved by institutional animal care and use committees at the University of Pittsburgh School of Medicine or University of Minnesota.

For concurrent studies, 36 cynomolgus macaques were inoculated with a low or mid dose (≈ 25 or 50–100 colony-forming units, respectively) *M. tuberculosis* Erdman strain by bronchoscopic instillation, as described (9); 5 animals were uninfected controls. Samples from 10 macaques that had active disease, 23 animals characterized as latently infected, and 3 infected animals classified as subclinically diseased or “percolators” (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/3/14-0052-Techapp1.pdf>) (9). Fecal samples were collected from all macaques on a single day, coinciding with varying durations of infection, ranging from 63 to 286 days (Table 1). The online Technical Appendix includes details on disease development and infection status classification.

Fecal IS6110 PCR was also evaluated in primates under conditions of natural exposure and infection. Fecal samples were collected from 36 juvenile and adult (7–27 y, mean 15 y) chimpanzees (*Pan troglodytes*) managed in 2 sanctuaries and 1 zoo in East Africa. Housing and management are described in the online Technical Appendix. All animals were considered to be clinically healthy during sampling. Fecal PCR results of sanctuary chimpanzees were compared with their most recent tuberculin skin test (TST) responses (10). TSTs were performed opportunistically on 27 chimpanzees during routine exams on the same day as fecal collection. For the remaining 9 animals, TST results were available from 9 months before fecal collection for 3 chimpanzees and from 2 years before for 6 chimpanzees. In addition to TST, results from the PrimaTB STAT-PAK (ChemBio Diagnostic Systems, Inc., Medford, New York, USA), a field-based serologic assay, were also available for 6 animals.

We extracted DNA from fecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Inc., Valencia,

Table 1. Fecal IS6110 PCR results for detection of tuberculosis among cynomolgus and rhesus macaques, by infection status, inoculation dose, and time to sampling

| Species and infection status | Inoculation dose | No. animals | Time postinoculation for sampling, mo | No. PCR positive | |
|------------------------------|------------------|-------------|---------------------------------------|------------------|---|
| Cynomolgus | | | | | |
| Active | Mid | 4 | 2 | 2 | |
| | Mid | 1 | 5 | 1 | |
| | Mid | 1 | 6 | 0 | |
| | Low | 2 | 7 | 1 | |
| | Low | 1 | 8 | 1 | |
| | Low | 1 | 9 | 0 | |
| | Latent | Mid | 4 | 2 | 0 |
| | | Mid | 1 | 5 | 0 |
| | | Mid | 4 | 6 | 0 |
| | | Low | 3 | 7 | 0 |
| | | Low | 4 | 8 | 0 |
| | | Low | 4 | 9 | 1 |
| | | Low | 3 | 10 | 1 |
| | Subclinical | Low | 2 | 7 | 1 |
| | | Low | 1 | 8 | 0 |
| Uninfected | N/A | 5 | NA | 0 | |
| Rhesus | | | | | |
| Uninfected | N/A | 13 | NA | 0 | |

CA, USA). Feces-free negative controls were included in all extraction procedures. Conventional and real-time PCR were used to amplify a portion of the IS6110 insertion sequence. Primers, master mixes, and thermocycling conditions are included in Table 2. For conventional PCR, amplicons of target size were confirmed as IS6110 by Sanger sequencing (University of Minnesota Genomics Center, St. Paul, Minnesota, USA). Nuclease-free water (QIAGEN) negative controls were included in all amplification reactions. The online Technical Appendix contains additional methodological details.

Conclusions

Fecal IS6110 PCR was effective in identifying 5 of 10 inoculated macaques with active disease and 8 of 36 total infected macaques. No uninoculated macaques were positive by results of IS6110 PCR. Conventional PCR identified 3 actively infected macaques and real-time PCR identified 2 additional active infections. Two latently infected macaques and 1 with subclinical infection were also positive by using IS6110 PCR. Overall sensitivity for this testing method was 22% (95% Wilson CI 12%–38%) and specificity

was 100% (95% Wilson CI 82%–100%). Sensitivity of detection of active infections was estimated at 50% (95% Wilson CI 24%–76%). The latter sensitivity estimate is equivalent to that of gastric aspirate of children with radiographic evidence of pulmonary TB (11).

The observed sensitivity of fecal IS6110 PCR is limited by several factors. Unlike immunologic tests, the success of this approach relies on bacterial shedding in sputum, subsequent swallowing, and excretion in feces; hence, active infection. Thus, most latent infections may go undetected, as observed in this study. Aside from outbreaks, identifying large numbers of actively infected primates for test validation is challenging. We sampled animals in experimental infection studies, but even so, active infections were few. Also, low numbers of organisms are likely shed intermittently in feces; thus, serial testing of multiple fecal samples may improve diagnostic sensitivity. PCR may also be paired with mycobacterial culture of feces for further molecular characterization of infection (8). Overall, this study demonstrates that fecal detection of mycobacterial DNA is best suited for identifying actively infected primates, which are crucial in TB transmission.

Table 2. Fecal IS6110 conventional and real-time PCR master mixes and reaction conditions for investigation of noninvasive tuberculosis detection in primates

| PCR type | Primers, 5' → 3' | Master mix | Reaction conditions |
|--------------|--|--|---|
| Conventional | Forward: TTCAGGTCGAGTACGCCTTC | 12.5 µL HotStarTaq Master Mix;* 8 µL RNase-free water,* 0.4 µM of each primer, 1.25 µL DMSO, 0.25 µL 1% BSA, 1 µL DNA template. Total volume 25 µL | 95°C for 15 min/DNA polymerase activation; 40 cycles: 94°C for 30 s/denaturation; 56°C for 30 s/annealing, 72°C for 1 min/extension. Termination at 72°C for 10 min |
| | Reverse: CGAACTCAAGGAGCACATCA | | |
| Real-time | Forward: AGAAGGCGTACTCGACCTGA Reverse: CCGGATCGATGTGTACTGAG | LightCycler 480 Probes Master:† 0.2 mM of each primer, 0.2 mM of the FAM-labeled IS6110 probe,† 5 µL DNA template. Total volume 25 µL | 95°C for 5 min; 45 cycles: 95°C for 10 s/denaturation; 56°C for 30 s/annealing; 72°C for 1 s/extension. Termination at 65°C–95°C at 2.2°C/s/melting curve analysis |

*QIAGEN, Inc., Valencia, CA, USA.
†Roche, Indianapolis, IN, USA.

TST conversion was not observed in any chimpanzees; however, IS6110 DNA was detected in 3 chimpanzee fecal samples. TST was conducted the same day as fecal sampling for 1 of these animals, 9 months before for 1 animal, and 2 years before for 1 animal. TST is a common TB screening method used in primate sanctuaries but it is limited by sensitivity and specificity (1). Although this limitation can be overcome with Bayesian methods to estimate sensitivity and specificity for test validation purposes, the challenge remains in effectively identifying populations of captive primates with TB. Unfortunately, confirmation of infection status by additional diagnostic testing modalities of the 3 fecal PCR-positive chimpanzees has been limited.

Test results for 1 fecal PCR-positive chimpanzee demonstrated an immunological response to *M. tuberculosis* antigen by using the PrimaTB STAT-PAK, but culture of a bronchoalveolar lavage (BAL) sample was unsuccessful. Another chimpanzee, positive by fecal PCR, retested positive the next year by fecal IS6110 PCR. The body size of this 14-year-old male that was historically TST negative was stunted (e.g., reduced growth) compared with other male chimpanzees of similar age.

These circumstances demonstrate the complexity of TB diagnosis and the challenges surrounding successful validation of TB tests in the natural setting. To reach a more complete understanding of diagnostic performance of fecal IS6110 PCR in a natural setting where disease prevalence is low, large-scale and long-term testing across many captive primate populations is still needed.

Fecal IS6110 PCR is a novel approach to the noninvasive detection of TB infection in primates, offering a new opportunity to screen for TB in free-living primates. IS6110 detection is advantageous for its MTC specificity, which is optimal given the known susceptibility of primates to *M. bovis*, *M. tuberculosis*, and the recently discovered strain known as chimpanzee bacillus. This approach offers new direction for the epidemiologic investigation of tuberculosis in free-living primate populations.

Acknowledgments

We thank the staff of each of the laboratories where this work was carried out for their assistance and contribution of expertise: the Tuberculosis lab of the Medical Microbiology Department at Mulago Medical School in Kampala, Uganda, the Schultz Diabetes Center, Wallin Medical Biosciences BSL-3 lab and Sreevatsan lab at the University of Minnesota, and the Flynn lab at the University of Pittsburgh School of Medicine. We also thank the participating sanctuaries for their support during sample collection and Uganda Wildlife Authority and Uganda National Council of Science and Technology for permit approval to undertake the research.

This work was supported by the Zoetis/Morris Animal Foundation Veterinary Research Fellowship (D10ZO-902) (to T.M.W.);

the Consortium on Law and Values in Health, Environment & the Life Sciences of the University of Minnesota (T.M.W.); the Ulysses S. Seal Conservation Fund of the Minnesota Zoo (T.M.W.); the USDA-NIFA Specials grant on bovine tuberculosis (61-4056A) (to S.S.); and the Veterinary Population Medicine Department of the University of Minnesota's College of Veterinary Medicine.

Dr. Wolf is a practicing zoo and wildlife veterinarian for the Minnesota Zoo in Apple Valley, Minnesota. She is also a PhD candidate in the Comparative and Molecular Biosciences program of the College of Veterinary Medicine at the University of Minnesota. The study described in this article is a component of her doctoral research into the epidemiology of respiratory diseases among free-living great apes.

References

1. Lerche NW, Yee JL, Capuano SV, Flynn JL. New approaches to tuberculosis surveillance in nonhuman primates. *ILAR J*. 2008;49:170–8. <http://dx.doi.org/10.1093/ilar.49.2.170>
2. Loomis M. Great apes. In: Fowler M, Miller R, editors. *Zoo and wildlife medicine, current therapy*. 5th ed. St. Louis (MO): Saunders; 2003. p. 381–96.
3. Keet DF, Kriek NP, Bengis RG, Grobler DG, Michel A. The rise and fall of tuberculosis in a free-ranging chacma baboon troop in the Kruger National Park. *Onderstepoort J Vet Res*. 2000;67:115–22.
4. Sapolsky RM, Else J. Bovine tuberculosis in a wild baboon population: epidemiological aspects. *J Med Primatol*. 1987;16:229–35.
5. Wilbur AK, Engel GA, Rompis A, A Putra I, Lee BP-H, Aggimarangsee N, et al. From the mouths of monkeys: detection of *Mycobacterium tuberculosis* complex DNA from buccal swabs of synanthropic macaques. *Am J Primatol*. 2012 Jul;74(7):676–86. <http://dx.doi.org/10.1002/ajp.22022>
6. Coscolla M, Lewin A, Metzger S, Maetz-Renning K, Calvignac-Spencer S, Nitsche A, et al. Novel *Mycobacterium tuberculosis* complex isolate from a wild chimpanzee. *Emerg Infect Dis*. 2013;19:969–76. <http://dx.doi.org/10.3201/eid1906.121012>
7. Cordova J, Shiloh R, Gilman RH, Sheen P, Martin L, Arenas F, et al. Evaluation of molecular tools for detection and drug susceptibility testing of *Mycobacterium tuberculosis* in stool specimens from patients with pulmonary tuberculosis. *J Clin Microbiol*. 2010;48:1820–6. <http://dx.doi.org/10.1128/JCM.01161-09>
8. El Khéchine A, Henry M, Raoult D, Drancourt M. Detection of *Mycobacterium tuberculosis* complex organisms in the stools of patients with pulmonary tuberculosis. *Microbiology*. 2009;155:2384–9. <http://dx.doi.org/10.1099/mic.0.026484-0>
9. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun*. 2009;77:4631–42. <http://dx.doi.org/10.1128/IAI.00592-09>
10. Bernacki B, Gibson S, Keeling M, Abee C. Nonhuman Primates. In: Fox J, Anderson L, Loew F, Quimby F, editors. *Laboratory animal medicine*. 2nd ed. New York: Academic Press; 2002. p. 676–791.
11. Abadco DL, Steiner P. Gastric lavage is better than bronchoalveolar lavage for isolation of *Mycobacterium tuberculosis* in childhood pulmonary tuberculosis. *Pediatr Infect Dis J*. 1992;11:735–8.

Address for correspondence: Tiffany M. Wolf, 205 Veterinary Sciences Building, University of Minnesota, 1971 Commonwealth Ave, St. Paul, MN 55108, USA; email: Wolfx305@umn.edu

Noninvasive Test for Tuberculosis Detection in Primates

Technical Appendix

Additional Methods

Fecal samples for IS6110 PCR were collected from 41 adult (>4 years) cynomolgus macaques (*Macaca fascicularis*) included in experimental *M. tuberculosis* infection studies and 13 uninfected rhesus macaques (*Macaca mulatta*) included in diabetes studies. Uninfected animals were housed under BSL-2 conditions; infected animals were housed in BSL-3 level facilities.

Thirty-six cynomolgus macaques were infected with a low or mid dose (≈ 25 or 50–100 colony-forming units, respectively) *M. tuberculosis* Erdman strain by bronchoscopic instillation, as described (1). Infection was confirmed by tuberculin skin test (TST) conversion, lymphocyte proliferation assay, and gamma interferon enzyme-linked immunosorbent spot of peripheral blood mononuclear cells (PBMC) (1). In the cynomolgus macaque low-dose model, half of the animals develop active tuberculosis, typically 4–12 months post-infection, with substantial variability in timing and presentation of clinical signs. (1) The other half develop clinically latent infection and have no signs of disease. Included in this study were ten macaques that had developed active disease, as determined by clinical signs and disease on thoracic radiography in association with *M. tuberculosis* growth from gastric aspirate (GA) or bronchoalveolar lavage (BAL). *M. tuberculosis* culture methods have been described previously. (1) Briefly, GA sample culture was performed by the University of Pittsburgh Medical College clinical microbiology laboratory. The samples were subcultured onto LJ slants for *M. tuberculosis*. BAL samples were cultured on 7H11 plates. Twenty-three animals were characterized as latently infected based on absence of both clinical signs and *M. tuberculosis* growth with a positive TST reaction and peripheral responses to *M. tuberculosis* antigen (1). Three infected animals were classified as subclinically diseased based on the absence of clinical disease but intermittent *M. tuberculosis* growth on GA or BAL (1).

Fecal samples were collected from 36 juvenile and adult (7–27yrs, mean=15yrs) chimpanzees (*Pan troglodytes*) managed in two chimpanzee sanctuaries and one zoo in East Africa. Many chimpanzees were raised from infant stage by the sanctuaries as a result of confiscation. All are managed in social groups in indoor/outdoor housing. Human contact includes direct, intimate contact up until age 5yrs, followed by protected, close contact at older ages with regular visitation by tourists and researchers. Contact with free-ranging primate species is variable. TST is a routine tuberculosis screening test employed by the chimpanzee sanctuaries during routine examinations, offering a platform for comparison of the fecal IS6110 PCR. In general, any TST responders are followed up with additional tuberculosis diagnostic testing (e.g. culture of BAL or GA samples).

Fecal samples were stored frozen at -80°C for 1 week to 30 months until processing. DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Inc.) according to manufacturer's instructions, with a few exceptions. First, feces (180-220mg) was added to a 2.0ml microcentrifuge tube containing 0.1mm Zirconia/Silica beads (BioSpec Products, Inc.) in addition to 1.4ml ASL Buffer, which was vortexed for 3 minutes. Additionally, water bath temperatures were increased from 70°C to 95°C. These alterations were to optimize mycobacterial cell wall lysis. Following completion of the extraction protocol, samples were stored frozen at -20°C until PCR testing.

A portion of the IS6110 insertion sequence was amplified by conventional and real-time PCR. Primers, master mixes, and thermocycling conditions are included in Table 2. For conventional PCR, amplicons were visualized under UV illumination after electrophoresing through a 1% agarose gel containing ethidium bromide; products of target size were confirmed as IS6110 by Sanger sequencing (University of Minnesota Genomics Center, St. Paul, Minnesota, USA).

Reference

1. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. [Internet]. Infect Immun. 2009;77:4631–42 [PubMed http://dx.doi.org/10.1128/IAI.00592-09](http://dx.doi.org/10.1128/IAI.00592-09)