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A Naturally Occurring Outbreak of Tuberculosis in a Group of Imported Cynomolgus Monkeys (*Macaca fascicularis*)

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This case report describes the diagnosis of tuberculosis (caused primarily by *Mycobacterium bovis*) in a group of newly imported Chinese origin cynomolgus monkeys. We also describe the use of sedation to enhance the accuracy of evaluation of the intrapalpebral tuberculin skin test using the mammalian old tuberculin reagent and report the first known diagnosis of *Mycobacterium paraffinicum* in a nonhuman primate. By 48 h after injection during the second tuberculin skin test, 6 of the 80 macaques had developed eyelid reactions ranging from mild (grade 1) to severe (grade 4). Given the range and severity of reactions, we suspected an outbreak of tuberculosis in the group. Because of the nature of the reactions, we sedated the animals at the 72-h evaluation to more closely observe and then palpate the injected eyelid. Evaluation of unsedated animals revealed 22 with a reaction to mammalian old tuberculin. We confirmed these 22 cases and identified an additional 11 animals with reactions when the monkeys were sedated. Mycobacterial culture of tissue from 6 macaques with reactions confirmed *M. bovis* in 3 animals. In addition, 1 of these 3 animals was culture-positive for both *M. bovis* and *M. paraffinicum*, and another was culture-positive for *M. avium* complex only. The addition of sedation to facilitate visual inspection and then palpation of the injected eyelid of these macaques increased the accuracy of evaluation and understanding of the number and severity of reactions to tuberculin skin testing.

Abbreviations: TST, tuberculin skin test; MTC, *Mycobacterium tuberculosis* complex; NVSL, National Veterinary Services Laboratories.

Tuberculosis is a significant bacterial disease in laboratory animal medicine. The zoonotic potential of tuberculosis and its effect on laboratory animals remain causes for concern.[6,7,14,15] The tuberculin skin test (TST) was established as a diagnostic screening tool for tuberculosis in humans and animals and continues as the first-line diagnostic test for animals. Previous reports have concluded that the sensitivity of the TST ranges from 80% to 100%.[7,16] In addition, in vitro cell-mediated and humoral-based diagnostic tests are currently available for use in humans (for example, QuantiFERON, Cellestis, Valencia, CA) and animals (for example, Primagam, Prionics USA, La Vista, NE). The application and diagnostic value of the animal-specific tests have been reported previously.[1,7,9]

The case reported here describes the detection of tuberculosis in a recently imported group of 80 Chinese origin cynomolgus monkeys during import (primary) quarantine. Here we describe the detection of tuberculosis in a group of newly imported Old World (Chinese) monkeys by using the intrapalpebral TST.[14] We also review and discuss the use of sedation to evaluate reactions to TST, and document the first diagnosis of *M. paraffinicum* in a cynomolgus monkey.

Case Report

A group of 80 (male, 40; female, 40: age, 2 to 4 y) cynomolgus monkeys (*Macaca fascicularis*) of Chinese origin were imported into the United States and held in primary quarantine at our institution (Buckshire, Perkasie, PA), which is licensed by the United States Department of Agriculture and registered as an import quarantine facility with the Centers for Disease Control and Prevention. Animals were held and cared for according to US Department of Agriculture regulations and recommendations from the Centers for Disease Control and Prevention. The macaques were fed a commercial diet (Teklad Global Primate Diet 2050, Harlan Laboratories, Indianapolis, IN) and were provided with well water. Animals were maintained on an approximately 10:14-h light:dark cycle. Room temperature was maintained between 17.8 and 28.9 °C (64 and 84 °F).

Important history on the group prior to shipment includes the administration of measles vaccine on 15 July 2008 and again on 27 August 2008. In addition the animals received several treatments with an agricultural synthetic pyrethroid (Sumicidin, Sumitomo Chemical, Tokyo, Japan) for ectoparasites and albendazole for endoparasites. All diagnostic test results from original colony health reports were negative, including tuberculosis testing.

The group of macaques arrived at our facility on 16 September 2008 and was split into 4 groups and housed in isolation in 4 separate rooms. The overall clinical health of the animals was good, with no obvious signs of disease in the group, except as noted in the current report. According to our facility standard operating procedure and Centers for Disease Control recommendations, TST was initiated 7 d after arrival and repeated at a minimal interval of 14 d in alternating upper eyelids. Animals were sedated with ketamine (10 mg/kg IM), 0.1 mL mammalian old tuberculin (Synbiotics) injected intradermally into the middle of the upper eyelid using a 27-gauge 1/2-in. needle attached to a 1-mL syringe, and reactions were evaluated at 24, 48, and 72 h after injection by the same technician.[3] Animals were weighed

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at every TST; with few exceptions, body weights were either stable or increasing. No animal was noted to have a cough or significant diarrhea. The results of the first TST were negative at all 3 time points in all 80 macaques. During evaluation of the second TST (which occurred 14 d after the first) at the 24-h time point, 4 animals developed reactions ranging from grade 1 to 3. By 48 h, the reaction in 1 of these 4 macaques progressed from grade 3 to 4 and from grade 2 to 3 in another.

All animals were sedated at the 72-h evaluation, which was performed by the attending veterinarian. The eyelid was first observed before palpating it to prevent causing mild erythema. At the 72-h evaluation of the second TST, visual observation identified that 6 of the 80 animals with a reaction ranging from grade 1 to 4. During palpation of sedated animals, the reactions in these 6 macaques were confirmed, and 2 additional animals were noted to have grade 3 reaction (generalized lid thickening with minimal erythema) that were not noted by visual observation only. Therefore, 8 of the 80 macaques had a reaction to the second TST.

In light of the severity of reactions, the decision was made to euthanize all animals with a reaction of any grade (8 macaques) and 5 cagemates. The 13 animals were necropsied. Two of these each had a single small nodule (diameter, 2 and 3 mm) in a lung lobe. Fresh and formalin-fixed sections of lung, liver, spleen, and hilar–gastrointestinal lymph nodes from 5 macaques were submitted for further testing (National Veterinary Services Laboratories, Ames, IA). Histology identified reactions consistent with mycobacteriosis in 3 of the 5 macaques. PCR analysis identified *Mycobacterium tuberculosis* complex in one of the animals identified with histologic evidence of mycobacteriosis. Culture results confirmed *M. bovis* in 3 of the 5 animals, excluding the PCR-positive macaque. The remaining animal was coinfected with *M. bovis* and *M. paraffinicum* (Table 1).

A commercial ELISA test liscenced for macaques (PrimaTB Stat-Pak Assay, Chembio Diagnostic Systems, Medford, NY) was performed on K_3·EDTA-anticoagulated whole blood from 10 animals at the second TST; these 10 animals included 5 macaques that provided the samples submitted to NVSL. At the time of the ELISA, the identity of the infectious organism was unknown. Overall the test correctly identified 2 of the 3 macaques that were culture-positive for *M. bovis*; the test did not identify the animal coinfected with *M. bovis* and *M. paraffinicum*. In addition, one macaque that was culture- negative and PCR-positive for *M. bovis* and had histologic evidence of tuberculosis was ELISA-negative. The remaining 5 animals were ELISA-negative, even though 3 of them had grade 1 or 3 reactions by TST; we did not submit samples for culture from these 5 macaques. In light of these results, we were not confident that further ELISA testing would enhance the diagnosis of tuberculosis over that afforded by TST, and we therefore ceased ELISA testing.

The third TST (which occurred 14 d after the second and involved 67 macaques) revealed similar results as the second. Two macaques had grade 1 reactions at 24 and 48 h but no reaction at 72 h and therefore were not euthanized. By visual inspection at the 72-h time point, 6 of the remaining 65 macaques had reactions ranging from grade 1 to 5. The addition of sedation for palpation of the test site confirmed the 6 previously recognized positive reactions and identified an additional 4 animals with grade 3 reactions characterized by minimal erythema and a uniformly thickened eyelid. These 10 macaques and 1 cagemate were euthanized and necropsied. Necropsy revealed a grossly visible (diameter, 4 mm) lesion in a lung lobe of an animal with a grade 5 reaction and a 2-mm lesion in a lung lobe of 1 of the 4 animals that were newly identified (grade 3 reaction) after sedation and palpation. Significant histologic results from the latter macaque (animal 5102) included granulomatous pneumonia with foreign material, negative acid-fast staining, and positive culture for *M. avium* complex (Table 1).

The results of the fourth TST (which occurred 23 d after the third and involved 56 macaques) were similar to those for the previous 2 tests. Visual evaluation of unsedated animals revealed 6 with grade 1 or 2 reactions. Evaluation after sedation confirmed the observations for 4 macaques, led to upgrading for 2 animals (1 from grade 1 to grade 2 and the other from grade 2 to grade 3 or 4), and identified 3 additional animals with grade 1 or 2 reactions. After the fourth TST, the owner of the animals, attending veterinarian, and facility owner discussed whether to continue TST. Because of the newly identified animal with a grade 3 to 4 reaction, mandatory tuberculosis testing would have to continue until there were 5 additional consecutive negative results.[3] Given the large number of animals with reactions by this time (22 of 80), the decision was made to euthanize and necropsy all except 9 of the remaining animals. Of the necropsied animals, one macaque had multiple nodules (diameter, 1 to 3 mm) throughout the liver and was TST-negative. Histology revealed granulomatous and eosinophilic hepatitis, negative acid-fast staining, and negative culture. An additional animal had a whole-body maculopapular rash and was TST-negative.

The 9 remaining macaques were followed for an additional 3 mo. These 9 animals had exhibited the most consistent reactions to TST (8 with grade 1 or 2 reactions; 1 with a grade 3 to 4 reaction). Serial TST continued at approximately 2-wk intervals, with blood sampling for in vitro diagnostics.[10] Testing ended after an additional 6 TST (tests 5 through 10), at the end of February 2009. Over the course of the 6 additional TST, 4 of the 9 macaques had intermittent reactions, 4 exhibited a grade 1 reaction, and 1 animal consistently had a reaction of grade 2 to 4. Reaction grade did not differ between visual inspection in unsedated macaques and with examination of sedated animals in this series of tests. At the final TST evaluation, the remaining animals were euthanized and necropsied. Significant necropsy findings were one animal (reaction grade 1) with a 2-mm surface nodule on the spleen.

Two of the 9 macaques died during the final set of 6 TST. Just prior to the eighth TST, one animal (reaction grade 2 to 4) was found dead in its cage, with no prior clinical signs noted. Necropsy revealed enlarged mediastinal–hilar lymph nodes. The second macaque did not recover from sedation during the eighth TST, although supportive care was provided. This animal demonstrated no reactions during TST evaluation. Necropsy revealed liquid green contents in the large intestine.

Management of the macaques did not change substantially throughout the course of the outbreak. At our facility, standard personal protective equipment for working with nonhuman primates includes a company-provided uniform, high-density polyethylene suit with hood, double-gloving, N95 respirator, safety glasses, face shield or powdered air-purifying respirator, and rubber boots. Rooms were attended to beginning with the cleanest room in the group and ending with the most contaminated based on disease status. Personnel attending these animals showered after exiting the last room in the group. Access was restricted to the rooms and involved the fewest personnel necessary to achieve appropriate care. All personnel exposed to the animals underwent repeat tuberculosis testing using purified protein derivative, with no human reactors detected. Animals were not moved until they were consolidated into a single room for the final 6 TST. Once tuberculosis was suspected, the Centers

Table 1. Test results from the 6 cynomolgus macaques from which samples were submitted to the National Veterinary Services Laboratories

	TST reaction (grade)								
	Visual observation only (in unsedated animal)			By visual observation and palpation at 72 h in sedated animal					
Animal	24 h	48 h	72 h		Gross necropsy	Commercial ELISA assay	PCR analysis	Acid-fast staining	Culture
2223	–	–	–	3[a]	No visible lesions	–	Not done	–	–
7863	3	4	4	4	No visible lesions	–	Positive for *M. tuberculosis* complex (*M. avium*-negative)	–	–
1159	2	3	3	3	Single nodule (diameter, 3 mm) in left middle lung lobe	+	Not done	+	*M. bovis*
4031	–	1	2	2	No visible lesions	+	Not done	+	*M. bovis*
9435	1	2	1	1	Single nodule (diameter, 2 mm) in right middle lung lobe	–	Not done	+	*M. bovis* and *M. paraffinicum*
5102	–	–	–	3[a]	Single nodule (diameter, 2 mm) in margin of left middle lung lobe	–	Not done	–	*M. avium* complex

–, negative; +, positive
[a]General eyelid induration with minimal erythema (no drooping)

for Disease Control and Prevention was informed, and reports regarding disease status were made regularly.

In summary we identified 33 cynomolgus macaques among a shipment of 80 from China that demonstrated a reaction during TST. Whereas 22 of these 33 reactions were recognized through visual observation of unsedated macaques, examination (visualization followed by palpation) of the injected eyelid in sedated macaques identified 11 additional animals with previously undocumented reactions. Overall, visual observation coupled with palpation in sedated macaques led to diagnosis of 33% more reactions as compared with results after evaluation of unsedated animals.

Discussion

This report describes the procedure performed to make a tentative diagnosis of tuberculosis in imported cynomolgus macaques by using mammalian old tuberculin in the intrapalpebral TST, the standard and time-honored test for tuberculosis surveillance in nonhuman primates in the United States.[3,11,13] We suspected an outbreak of tuberculosis after the 24-h evaluation of the second TST, for which 3 animals had suspect (grade 3) reactions and 1 animal had a positive (grade 4) reaction. Tuberculosis was diagnosed by culture after necropsy of the suspect animals from the second TST. In addition, to our knowledge, we have documented the first reported case of *M. paraffinicum* in a nonhuman primate.

The use of sedation to allow closer visualization and then the palpation of TST sites during this outbreak enhanced the overall identification of reactions: examination after sedation revealed 33% more macaques with TST reactions than those identified by visual assessment of unsedated animals. The use of sedation during evaluation of the TST in the eyelid or abdominal skin in nonhuman primates is not novel.[2,4] In these reports the use of restraint or sedation (or both) was done under experimental conditions. We did not perform comparative testing in the abdomen because intradermal abdominal testing may be less accurate in nonhuman primates than was once thought.[2] The authors of the cited study suggest that sedation may be useful for identifying reactions that cannot be observed in animals that are not sedated.

Several questions arose during the course of the outbreak. None of the 80 macaques reacted to the first TST, which was performed 27 d after the last measles vaccine, which perhaps led to false-negative results. One report[15] states that the first TST should be performed at least 4 wk after the last measles vaccine. We could have waited one more day before initiating TST. Additional causes of false-negative reactions include early-, middle-, or late-stage infection (anergy) and vaccination, specifically for measles.[1,7,12,15] False-positive reactions may occur after exposure to nontuberculous bacteria and vaccination with Freund complete adjuvant.[1,12,15]

We were unable to determine the percentages of false-negatives and -positives among the reactors in this group because we did not submit samples for culture from all reactors or all 80 macaques. Instead we submitted 6 sets of samples (from 6 reactors) for histology and culture to confirm tuberculosis in the group as a whole. Of the 5 macaques sampled after yielding reactions to the second TST, 3 were culture-positive for *M. bovis* (with 1 animal coinfected with *M. paraffinicum*). In addition, we submitted samples from one macaque with a grade 3 reaction to the third TST; this animal was culture-positive for *M. avium* complex. Due to the difficulty in growing *Mycobacterium*, an additional burden during an outbreak of tuberculosis is the lag time (which typically exceeds 1 mo) between submitting tissue to receiving culture results.[8] During this lag time, TST continued in the remaining animals, with additional animals developing reactions. In practice, the TST testing cycle continues as long as reactions cause the attending veterinarian to suspect that tuberculosis is still active in the group.

An additional concern arose when the TST results were paired with the culture results. During the course of this outbreak, 2 macaques were culture-positive for *M. bovis*, with 1 animal (which was coinfected with *M. paraffinicum*) having

only a grade 1 reaction to TST and the other having a grade 2 reaction. According to the current grading system,[13] reactions of grades 0 to 2 are considered negative, grade 3 reactions are rated suspect, and reactions of grades 4 or 5 are deemed positive for tuberculosis. As in the current report, nonhuman primates with reactions considered negative under the current grading system but with positive culture results in their group should heighten suspicion during subsequent evaluations. When TST reactions vary widely across a group of cynomolgus monkeys, the clinician may want to consider even animals with low-grade reactions to be suspect for tuberculosis, particularly when some animals in the group exhibit grade 4 or 5 reactions.

The choice of the location for placement of TST was questioned during review of the outbreak. Facility standard operating procedures did not specify the exact location for the intradermal injection in the upper eyelid. The standard practice of our facility technicians was to make the injection in the middle of the eyelid. However, the 1980 Institute for Laboratory Animal Resources recommendations suggest placing the intradermal injection "into the edge of the upper eyelid."[11] The location of the injection into the middle of the eyelid may have contributed to inaccurate evaluation of reactions by visualization only (under-reporting of reactions). The cynomolgus upper eyelid appears to be recessed into the orbit, thus perhaps contributing to overlooked reactions. The facility standard operating procedure has now been altered to require placement of injections as close as possible to the edge of the eyelid.

An additional concern emerged regarding reactions noted after palpation of sedated macaques, especially the 6 animals with grade 3 reactions (generalized eyelid induration but minimal erythema) that were readily apparent only when animals were sedated and test sites palpated. One of these animals was culture-positive for *M. avium* complex. The 1980 Institute for Laboratory Animal Resources recommendations state "positive reactions may range from minimal reddening, usually with slight edema, through severe swelling with close of the eyelids and purulent discharge, to hemorrhage in the site of injection and necrosis in the most severe reactions."[11] This description of positive reactions can be interpreted to encompass the current grades 1 through 5. Therefore, unrealized reactions may occur and be unrecognized.

Most of the cases of tuberculosis described in this report were caused by *M. bovis*; one animal was coinfected with *M. bovis* and *M. paraffinicum*, and another animal was infected with *M. avium* complex. *M. bovis* is a component of the *Mycobacterium tuberculosis* complex, which also includes MTB, *M. africanum*, and other strains. *M. paraffinicum* is a nontuberculous *Mycobacterium* that is found in soil, was first identified in 1956, and utilizes paraffinic hydrocarbons except methane for growth.[5] A recent outbreak of *M. paraffinicum* was described in a human health care facility.[17] The organism was isolated from human patients and the water from an ice machine in the facility. *M. avium* complex includes both *M. avium* and *M. intracellulare*, tuberculous mycobacteria that infect many avian and mammalian species including humans.

The source of infection for these animals could not be determined specifically. The strain of *M. bovis* was genotyped and determined to be exogenous to Canada, the United States, and Mexico. Environmental swabs from the room housing the last 9 animals and that included the cages, watering devices, and room drains submitted for culture of *Mycobacterium* spp. were negative. Because the animals originated from China, we suspect that the *M. bovis*, *M. avium* complex, and *M. paraffinicum* are of Chinese origin. Subsequently, staff members who were exposed to this group of animals were retested by using purified protein derivative, with no reactors. At our institution, all staff members typically are screened for tuberculosis either by skin testing using purified protein derivative every 6 mo or by a yearly chest radiograph.

In conclusion, we suggest that during quarantine (primary or secondary) of cynomolgus monkeys, attention should be paid to previous recommendations[13] and the following considerations. First, the attending veterinarian should review the 1980 Institute for Laboratory Animal Resources guidelines pertaining to tuberculosis detection to be familiar with the broad range of descriptions regarding possible positive reactions. Second, standardize the location of the mammalian old tuberculin injection at (or as near as possible to) the edge of an upper eyelid. Third, maintain consistency among the personnel who perform TST and record reactions (the same person should make consecutive evaluations). Fourth, document reactions initially according to clinical signs, not grade. Thereafter, use the grading system as a means to categorize animals, but remain aware that animals exhibiting 'negative' reactions by grading schema actually may have tuberculosis. Finally, if positive TST reactions are noted, consider sedating the animals at 72 h after injection for evaluation by visualization and then palpation. Because palpation itself may cause erythema, remember to observe the eyelid for the presence of erythema before palpating for induration. When working with large groups or in 'all-in–all-out' situations, sedating all animals for the 72-h evaluation may not be advantageous. Although the actual number and extent of reactions may be greater than those apparent through visual examination only, the expense of and time for sedation has to be weighed against the possibility of salvaging individual animals or even the entire group.

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