New Approaches to Tuberculosis Surveillance in Nonhuman Primates

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Abstract

Despite significant progress in reducing the incidence of tuberculosis in nonhuman primates (NHPs) maintained in captivity, outbreaks continue to occur in established colonies, with potential serious consequences in human exposures, animal losses, disruption of research, and costs related to disease control efforts. The intradermal tuberculin skin test (TST) using mammalian old tuberculin (MOT) has been the mainstay of NHP tuberculosis surveillance and antemortem diagnosis for more than 60 years. But limitations of the TST, particularly its inability to reliably identify animals with latent TB infections, make it unsuitable for use as a single, standalone test for TB surveillance in nonhuman primates in the 21st century. Advances in technology and the availability of *Mycobacterium* spp. genomic sequence data have facilitated the development and evaluation of new immune-based screening assays as possible adjuncts and alternatives to the TST, including in vitro whole blood assays that measure the release of interferon gamma in response to stimulation with tuberculin or specific mycobacterial antigens, and assays that detect antibodies to highly immunogenic secreted proteins unique to *M. tuberculosis*, *M. bovis*, and other species belonging to the *M. tuberculosis* complex. It is becoming apparent that no single screening test will meet all the requirements for surveillance and diagnosis of tuberculosis in nonhuman primates. Instead, the use of several tests in combination can increase the overall sensitivity and specificity of screening and surveillance programs and likely represents the future of TB testing in nonhuman primates. In this article we describe the characteristics of these newer screening tests and discuss their potential contributions to NHP tuberculosis surveillance programs.

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Introduction

Despite significant reductions in the incidence of tuberculosis (TB[1]) among captive nonhuman primates (NHPs[1]) since the 1970s, due in large part to adherence to guidelines established by the Centers for Disease Control and Prevention (CDC 1991) and the widespread implementation of surveillance programs in virtually all primate facilities in the United States (Butler et al. 1995; Kaufmann 1971; Roberts and Andrews 2008), tuberculosis remains a serious threat to the health of nonhuman primates and their human caretakers. Outbreaks of tuberculosis continue to occur in established colonies of nonhuman primates (Table 1) and can have severe economic consequences due to the loss of animals, disruption or confounding of ongoing research protocols, and costs associated with disease control efforts.

Early detection is critical to minimizing the adverse effects of a tuberculosis outbreak, but in nonhuman primates it poses significant challenges, among them the necessity of detecting both active and latent infections. Infected animals with active TB may show no overt signs of disease for weeks or months (Gibson 1998), during which time they can transmit infection to other colony animals. Animals with latent TB are not infectious and may appear healthy for years, but eventual reactivation of latent TB can result in secondary transmission and outbreaks of disease in established colonies. Reactivation of latent infections that were not detected using traditional screening methods during primary quarantine is emerging as an important factor in the epidemiology of TB in nonhuman primates.

Tuberculosis in NHPs is caused primarily by infection with *Mycobacterium tuberculosis* or, less commonly, *M. bovis*. Virtually all species of nonhuman primates are susceptible to tuberculosis, although there is a general hierarchy of susceptibility: Old World monkeys are considered more susceptible than apes, which appear to be more sus-

Table 1 Recent outbreaks of tuberculosis in nonhuman primates

Year	Species	# Exposed	# Culled	# Confirmed	Location
2001	Baboon	30	30	7	Quarantine
2002[a]	Rhesus	22	11	8	Postquarantine[b]
	Cynomolgus	58	58	19	
2003	Rhesus	53	53	10	Postquarantine[b]
2004	Rhesus	27	1	1	Postquarantine[b]
2004	Rhesus	45	23	10	Postquarantine[b]
2004	Rhesus	52	5	5	Quarantine

[a]Data from Garcia MA, Bouley DM, Larson MJ, Lifland B, Moorehead R, Simkins MD, Borie DC, Tolwani R, Otto G. 2004. Outbreak of *Mycobacterium bovis* in a conditioned colony of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques. Comp Med 54:578-584.
[b]No external human or animal source of TB infection identified.

ceptible than New World monkeys (Montali et al. 2001). In an experimental challenge model, infection of three species of Old World monkeys revealed that African green monkeys (*Chlorocebus aethiops*) are exquisitely sensitive to infection, showing uniformly rapidly progressive disease. Rhesus macaques *(Macaca mulatta)* showed a more variable clinical course, while cynomolgus macaques *(M. fascicularis)* experienced a more chronic course of infection (Motzel et al. 2003).

M. tuberculosis infection of nonhuman primates occurs by inhalation of infectious aerosols expelled from the respiratory tract of infected animals or humans with active disease, although ingestion is a rare alternative route (Sapolsky and Else 1987). After primary infection, a spectrum of clinical outcomes is possible, including no overt disease, rapidly progressive disease, or, more commonly, a chronic debilitating disease course. As in humans, not all primary infections in nonhuman primates result in active TB, and the development of latent infections without overt disease is well documented (Capuano et al. 2003; Gormus et al. 2004). Although animals with latent TB are not infectious, there is a significant risk of reactivation and the development of active tuberculosis (Capuano et al. 2003; Flynn and Chan 2001). Detection of latent TB infections is therefore a high priority in the control and prevention of the disease in nonhuman primates.

Standard Surveillance Practices

The intradermal tuberculin skin test (TST[1]), which detects delayed-type hypersensitivity (DTH) to tuberculin antigens, has been the mainstay of tuberculosis screening and antemortem diagnosis in nonhuman primates since the 1940s, and is currently the only ILAR/CDC-approved method for tuberculosis testing of animals in primary import quarantine (Kennard et al. 1939; NRC 1980; Roberts and Andrews 2008).

The TST entails injection of 0.1 ml of mammalian old tuberculin (MOT[1]) at the eyelid or a demarcated site on the abdomen according to established protocols, with reactions measured 24, 48, and 72 hours after injection (Richter et al. 1984). A standardized scoring system for intrapalpebral reactions, developed jointly by the California and Oregon National Primate Research Centers, uses a 0 to 5 grading system (Richter et al. 1984); abdominal skin reactions are scored on a similar 0 to 5 scale, as described by Staley and colleagues (1995). Reactions graded 0, 1, or 2 are considered negative, a grade 3 reaction is considered suspect, and grades 4 and 5 are considered positive. Although once deemed an appropriate follow-up test to a reactive palpebral TST, there is accumulating evidence that the abdominal TST in nonhuman primates may be significantly less sensitive than the palpebral test and may therefore be of limited utility in TB surveillance programs (Capuano et al. 2003; Motzel et al. 2003).

The TST is administered at minimum intervals of 2 weeks in animals either in quarantine or in outbreak situations, and at longer intervals (e.g., quarterly or annually) for routine surveillance of established colonies (NRC 1980). Efforts to confirm a positive TST result often involve additional, more definitive diagnostic methods such as thoracic radiographs, and/or more invasive methods such as bronchoalveolar lavage and gastric aspiration to obtain samples for acid-fast staining, mycobacterial isolation in culture, or amplification of TB-specific nucleic acids by polymerase chain reaction (PCR) (Rock et al. 1995). Although these confirmatory tests are important and valuable diagnostic tools, they are not suited for large-scale screening in routine surveillance. Frequently, however, animals with strong positive TST reactions or repeat positive reactions are euthanized and necropsied so that scientists can obtain a histologic diagnosis of tuberculosis by examination of postmortem tissue samples. Isolation in culture and definitive identification of *M. tuberculosis* or *M. bovis* is still considered the diagnostic "gold standard."

Limitations of the TST

Although the TST has been a valuable tool in efforts to control tuberculosis in nonhuman primates, it has a number of limitations with regard to sensitivity and specificity that reduce its utility as a standalone test. Chief among these limitations are the well-documented tendencies for the TST to give only intermittently positive results in serial testing of infected animals (Garcia et al. 2004b; Walsh et al. 1996) and for TST-positive infected animals (even those with radiographic evidence of lung disease) to eventually give negative TST results on serial testing due to the development of latent infections, anergy associated with progressive disease, or other mechanisms that are poorly understood (Heywood et al. 1970; Mayall et al. 1981; Motzel et al. 2003). Additional limitations include the lack of specificity of the mycobacterial antigens that constitute MOT, the required tuberculin preparation for use in nonhuman primates, resulting in false positive TST reactions in animals sensitized to other, nontuberculous environmental mycobacteria (Brammer et al. 1995; Goodwin et al. 1988; Soave et al. 1981).

The concentration of antigens required to elicit a positive TST reaction is higher in nonhuman primates than in humans. Although MOT meets the requirements for this higher concentration, it is a rather crude culture filtrate preparation that contains antigens common to many mycobacterial species, including those not associated with TB. Because of this antigenic cross reactivity, the TST suffers from low specificity, and false positive reactions are not uncommon. In addition, commercial production of MOT is a rather cumbersome process, and the resulting product, although critical for TB surveillance of primates, is not particularly profitable; as a result, there is only a single manufacturer of MOT in the United States (Synbiotics, Inc.). In the early 1990s periodic shortages of MOT led to a suspension of routine TB surveillance in many primate facilities and longer stays for animals temporarily unable to clear primary import quarantine. The periodic unavailability of MOT has been a prime motivating factor in recent efforts to develop and validate additional methods for TB surveillance in nonhuman primates.

Emerging Risks

A scenario that is now occurring with some frequency involves outbreaks of tuberculosis in cohorts of imported nonhuman primates that have completed the CDC-mandated 31-day primary import quarantine and the required three consecutive negative TSTs at 2-week intervals (CDC 1993; Garcia et al. 2004a; Lerche, unpublished data). Recognition of active tuberculosis in the postquarantine period occurs even after additional negative TSTs and in virtually every instance no human or animal source of TB exposure has been identified at the new facility. There is accumulating evidence that these cases likely represent reactivation of latent infections that were undetected during primary quarantine due to false negative TST reactions. The apparent inability of the TST to reliably detect latent infections is thus emerging as an important issue in screening for and diagnosing TB in nonhuman primates, and has become another major motivation, along with past MOT availability issues, for the development of new, more sensitive screening and diagnostic methods.

Adjuncts and Alternatives to the TST

Since the 1970s a number of immune-based assays have been proposed for use in TB surveillance of nonhuman primates, including in vitro lymphoproliferation assays (Chaparas et al. 1970; Muscoplat et al. 1975) and tests for detection of mycobacterial antibodies (Affronti et al. 1973; Corcoran and Thoen 1991). Although several of these approaches showed promise, for a variety of reasons—including the requirements of a laboratory-based test methodology and lack of sufficient specificity—these tests were not widely adopted (Garnier et al. 2003).

Recent technological advances have enabled the development of new rapid in vitro techniques for TB surveillance and diagnosis, including cell-mediated immunity-, antibody-, and nucleic acid–based methods. The complete sequencing of the *M. tuberculosis* (Cole et al. 1998) and *M. bovis* genomes (Garnier et al. 2003) has enabled the identification of proteins unique to the *M. tuberculosis* complex (Harboe et al. 1996). Immunodominant antigens have been identified and evaluated for use in in vitro TB diagnostics, both as stimulatory antigens in CMI-based assays (Wang et al. 2007) and as antigenic targets for specific antibody detection (Brusasca et al. 2003; Kanaujia et al. 2003, 2004; Lyashchenko et al. 2000, 2006).

In Vitro Assays of Cellular Immune Responses to TB Antigens

Interferon-γ Assays

In vitro assays for interferon gamma (IFN-γ[1]) response to tuberculin antigens represent a refinement of the lymphocyte stimulation assay. Because *M. tuberculosis* and *M. bovis* are intracellular pathogens, the most significant response in immunocompetent hosts is cell mediated (Andersen et al. 2000), while in immunocompromised hosts an antibody response may be dominant (Meya and McAdam 2007). In experimental primate infection models, cell-mediated immune responses are detectable in vitro 2 to 4 weeks after infection (Capuano et al. 2003; Lin et al. 2006). IFN-γ is a critical cytokine in the cell-mediated immune response to tuberculin antigens, including the DTH response measured by the TST, and in the host immune response to infection with tuberculous mycobacteria (Collins and Kaufmann 2001; Fletcher 2007; Flynn et al. 1993; Lin et al. 2006).

Assessing the IFN-γ response to TB antigens in vitro provides an alternative method for screening and diagnosis.

Investigators have evaluated the utility of whole blood IFN-γ assays (WB-IFNγ[1]) for TB surveillance and diagnostics in a variety of species, including humans (Detjen et al. 2007), cattle (Wood and Jones 2001), and nonhuman primates (Garcia et al. 2004b; Vervenne et al. 2004). A commercial version of this assay, PRIMAGAM® (Prionics USA Inc., La Vista, NE), received provisional USDA licensure for use in nonhuman primates in 2002. WB-IFNγ assays are based on the response of memory T cells to stimulation with either tuberculin or TB-specific antigens, resulting in a release of IFN-γ. Briefly, within 24 hours of its collection, 0.5 to 1.0 ml aliquots of whole blood in 24-well culture plates are stimulated with nil antigen control, purified protein derivative (PPD) of *Mycobacterium bovis* (bPPD[1]), or *M. avium* PPD (aPPD[1]). Although not included in the commercial version, some users of the assay add a mitogen-stimulated well as a positive control. After 24 hours of incubation at 37°C and 5% CO_2 in a humidified atmosphere, the concentration of IFN-γ in the supernatant plasma of each aliquot is determined by enzyme immunoassay.

Interpretation of the WB-IFNγ assay is based on the difference in IFN-γ response to bPPD and aPPD; the suggested criterion for a positive test is OD bPPD–OD aPPD ≧ 0.05 OD units. A positive reaction to bPPD is interpreted as an indication of sensitization to antigens of either *M. tuberculosis* or *M. bovis*. However, apparent species-specific differences in IFN-γ response, specifically a significantly muted response observed in cynomolgus macaques *(Macaca fascicularis)*, may necessitate some fine-tuning of the interpretive criteria (Garcia et al. 2004b).

Incorporation of the aPPD antigens addresses the specificity issue of cross reactivity among mycobacterial PPD antigens. A stronger response to aPPD than to bPPD is often interpreted as sensitization to *M. avium* or other nontuberculous environmental mycobacterial species. This interpretation, however, should be based on test results from more than a single time point, as a small subset of nonhuman primates in the early stages of *M. tuberculosis* or *M. bovis* infection may show a stronger IFN-γ response to aPPD than to bPPD. Subsequent testing of these animals shows conversion to a dominant IFN-γ response to bPPD (Lerche, unpublished data; Vervenne et al. 2004). To avoid misdiagnosis, animals showing high avian PPD responses should be retested more than 2 weeks after the original test (Vervenne et al. 2004). Patterns of response in WB-IFNγ assay and their interpretation are summarized in Figure 1.

The ELISPOT Assay

A variation on the theme of in vitro IFN-γ response to mycobacterial antigens is the enzyme-linked immunosorbent spot (ELISPOT[1]) assay. The ELISPOT uses a known concentration of Ficoll-separated peripheral blood mononuclear cells rather than whole blood for incubation with stimulating antigens. Rather than measuring IFN concentration in plasma supernatants, the ELISPOT assay enables enumeration of the cells releasing IFN-γ by counting the "spots" of labeled IFN-γ through a stereomicroscope or automated ELISPOT plate reader (Lin et al. 2006; Wang et al. 2007). The ELISPOT IFN-γ assay has been used primarily for research purposes in experimental models, but a commercially available version (T SPOT-TB, Oxford Immunotec Ltd., Oxford, UK) has been licensed for use in human testing (Wang et al. 2007); to date, there is no commercial ELISPOT-based assay for use in nonhuman primates.

Advantages and Weaknesses of IFN-γ Assays

The in vitro IFN-γ assays have a number of advantages over the TST, including a quantitative result, thus avoiding the subjectivity involved in scoring the TST. Additional advantages include the need to access the animal only once for blood collection, the availability of results in less than 36 hours, the ability to immediately retest if necessary (as animals are not inoculated with antigens), and built-in comparative testing with nontuberculous mycobacterial antigens.

But the in vitro IFN-γ assays (as well as more standard lymphocyte proliferation assays) also share some of the weaknesses of the TST, particularly the tendency to give only intermittent or transient positive results over the course of infection (Capuano et al. 2003; Lerche, unpublished data; Lin et al. 2006; Motzel et al. 2003; Walsh et al. 1996). Accumulating evidence suggests that the ability of these cellular response assays to detect latent TB infections is unreliable. Preliminary data from a cynomolgus macaque model of tuberculosis developed by Flynn and colleagues at the University of Pittsburgh (Capuano et al. 2003) demonstrate an inability of the WB-IFNγ assay to detect latent TB in this species (Figure 2).

Few studies have provided direct comparisons of the performance of the TST and WB-IFNγ assay in any of the three situations where these screening tests are employed: routine screening of established colonies, screening of animals in quarantine, and identification of infected animals in naturally occurring outbreaks. In one direct comparison of serial tests during an outbreak of *M. bovis* infection in macaques, agreement between results of the two tests was 75%; the WB-IFNγ assay was less sensitive (68% vs. 84%) but more specific (97% vs. 87%) than the TST (Garcia et al. 2004b). It is worth noting that, in this outbreak, use of both the TST and WB-IFNγ assay in parallel identified all infected animals, increasing the overall sensitivity of screening to 100% (Garcia et al. 2004b). Vervenne and colleagues (2004) reported that all five confirmed TB cases diagnosed among rhesus macaques in a quarantine facility were detected using the PRIMAGAM® WB-IFNγ assay, whereas only four were detected by the TST (Vervenne et al. 2004). Thus available data indicate that the use of the WB-IFNγ assay in conjunction with the TST can increase the overall
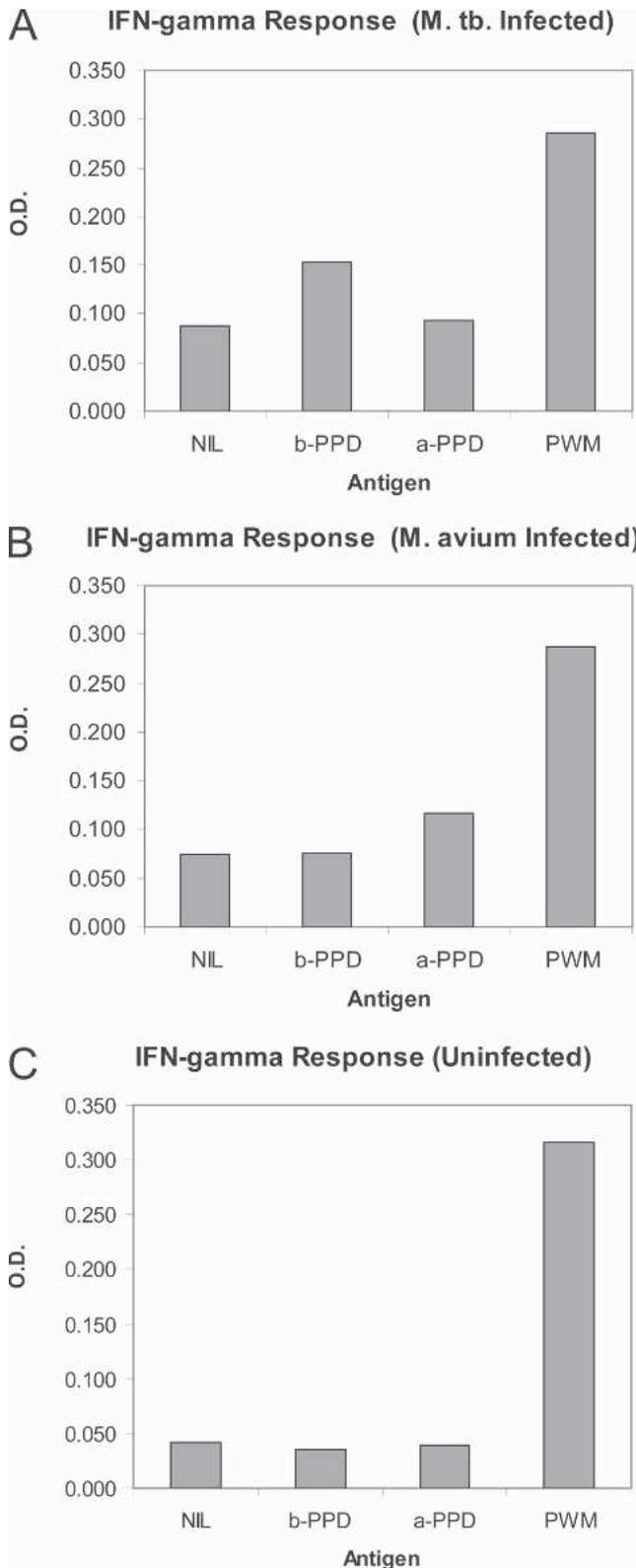
Figure 1 Idealized patterns of reactivity in the WB-IFNγ assay in relation to infection status. (A) Expected reactivity pattern of an animal infected with *Mycobacterium tuberculosis* or *M. bovis*. (B) Expected reactivity pattern of an animal infected with or sensitized to *M. avium* or other nontuberculous environmental mycobacteria. (C) Expected reactivity pattern of an uninfected animal.
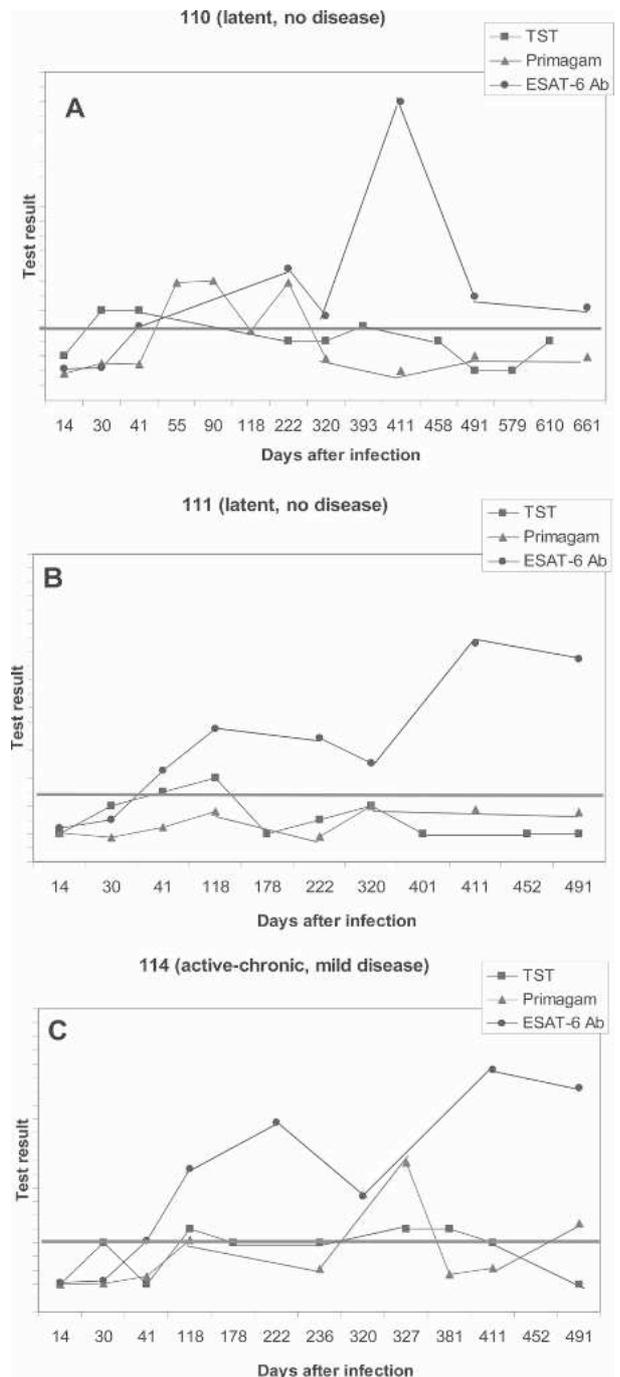
Figure 2 Sequential ESAT-6 antibody, TST, and WB-IFNγ assay results in cynomolgus macaques *(Macaca fascicularis)* developing latent (A, B) and chronic active (C) tuberculosis after experimental infection with *Mycobacterium tuberculosis*. The dark horizontal line represents an arbitrary threshold for a positive result for each of the three tests, corresponding to a TST score of 3 or greater; a delta value for OD bPPD – OD aPPD ≥ 0.05 units in the WB-IFNγ assay; and a delta value for MFI ESAT-6 – MFI BSA ≥ 500 MFI units in the ESAT-6 antibody assay. aPPD, *M. avium* purified protein derivative; bPPD, *M. bovis* purified protein derivative; BSA, bovine serum albumin; ESAT-6, early secreted antigenic target 6; MFI, mean fluorescence index; OD, optical density; TST, tuberculin skin test; WB-IFNγ, whole blood interferon-γ.

sensitivity of TB surveillance programs, and that the WB-IFNγ may be a suitable alternative to the TST for detection of active TB in nonhuman primates if MOT is unavailable.

Newer versions of WB-IFNγ assays for use in humans (Quantiferon Gold, Celestis, Australia) and cattle (Bovigam, CSL Ltd., Australia) have substituted TB-specific antigens such as early secreted antigen target 6 (ESAT-6[1]) and culture filtrate protein 10 (CFP-10[1]) for PPD, with a resulting increase in specificity. In the bovine assay, however, substitution of specific proteins for PPD resulted in an apparent slight decrease in sensitivity (Pollock et al. 2000). Although research evaluating the use of TB-specific proteins in WB-IFNγ assays for nonhuman primates is ongoing, to date there is no commercial assay utilizing these antigens.

In Vitro Assays of Humoral Immune Responses to TB Antigens

Earlier efforts to include mycobacteria-specific antibody testing with enzyme immunoassays (ELISA) in TB surveillance and diagnosis were limited mainly by insufficient specificity to distinguish reactivity to tuberculous from nontuberculous mycobacteria (Corcoran and Thoen 1991; Rock et al. 1995). Recent advances in the sequencing of the *M. tuberculosis* and *M. bovis* genomes (Cole et al. 1998; Garnier et al. 2003) have facilitated the identification of a number of secreted proteins that are unique to mycobacterial species of the *M. tuberculosis* complex (Amor et al. 2005; Harboe et al. 1996). Two of these proteins, ESAT-6 and CFP-10, are highly immunogenic and have been evaluated as antigen targets for detection of TB-specific antibodies in nonhuman primates. Gennaro and colleagues (Brusasca et al. 2003) reported that sera from 100% of 17 nonhuman primates from three species (cynomolgus, rhesus, and African green monkeys) experimentally infected with *M. tuberculosis* had detectable ESAT-6 antibodies, and 90% were reactive against two additional proteins, CFP-10 and α-crystallin. Although animals tended to be positive by palpebral TST earlier than in the antibody test, the levels of specific antibody remained elevated over the course of infection, whereas TST reactivity was intermittent or waned (Brusasca et al. 2003).

In a naturally occurring outbreak of *M. bovis* infection in rhesus and cynomolgus macaques, 22 of 25 animals with tuberculosis lesions identified at necropsy had detectable immunoglobulin G (IgG) ESAT-6-specific antibodies, and the sensitivity and specificity of the antibody ELISA (88% and 84%, respectively) were comparable to the sensitivity (84%) and specificity (84%) of the TST (Kanaujia et al. 2003). Three confirmed positive cases in this outbreak did not have detectable antibody in early serum samples, and 5 of 32 animals (16%) without lesions were antibody-test positive (Kanaujia et al. 2003). Although seroconversion to ESAT-6, the earliest recognized antigen, occurs 1 to 2 months after infection, the persistence of detectable antibody over the course of infection suggests that a significant improvement in TB surveillance programs may result from the addition of antibody testing for ESAT-6 and other specific antigens.

In addition to ELISA, several additional formats have been proposed for TB-specific antibody testing and may have applications for TB surveillance of nonhuman primates (Table 2). Two such approaches incorporate antigen arrays, allowing testing for antibodies against multiple antigens in a single assay. A third approach uses individual cassettes to test for serum antibodies to a "cocktail" of several TB-specific antigens.

Table 2 Tuberculosis-specific antibody assays for use in nonhuman primates

Assay	Format/platform	Result	Antigens	Information	Reference[b]
ELISA[a]	96-well plate	Optical density	Single	Positive/negative	Brusasca et al. 2003
MMIA	Microbead liquid suspension array; Luminex technology	Fluorescence intensity	Multiple	Pattern of Ag reactivity	Khan et al. in press
MAPIA	Solid phase multiple antigen array; nitrocellulose membrane	Visualized bands	Multiple	Pattern of Ag reactivity	Brusasca et al. 2003; Greenwald et al. 2007
Prima-TB STAT-PAK	Individual cassette; lateral flow technology	Visualized band	Multi-Ag "cocktail"	Positive/negative	Greenwald et al. 2007

[a]AG, antigen; ELISA, enzyme-linked immunosorbent assay; MAPIA, multiantigen press immunoassay; MMIA, multiplex microbead immunoassay.

[b]Brusasca PN, Peters RL, Motzel SL, Klein HJ, Gennaro ML. 2003. Antigen recognition by serum antibodies in non-human primates experimentally infected with *Mycobacterium tuberculosis*. Comp Med 53:165-172. Greenwald R, Lyashchenko K, Esfandiari J, Gibson S, Didier P, McCombs C, Stutzman L. 2007. Prima-TB STAT-PAK™ assay, a novel rapid test for tuberculosis in nonhuman primates [Abstract #182]. Am J Primatol 69(Suppl 1):120. Khan IH, Ravindran R, Yee JL, Ziman M, Lewinsohn D, Gennaro M, Flynn J, Goulding C, DeReimer K, Lerche NW, Luciw PA. Profiling antibodies to *Mycobacterium tuberculosis* by multiplex microbead suspension arrays for serodiagnosis of tuberculosis. Clin Vacc Immunol (in press).

Multiplex Microbead Immunoassay (MMIA)

Recent advances in the sequencing of the *M. tuberculosis* genome and the development of multiplex microbead assays have provided the opportunity to simultaneously determine antibody profiles against multiple *M. tuberculosis* antigens. Investigators have reported antibody responses to recombinant antigens unique to *M. tuberculosis* in infected macaques (Brusasca et al. 2003), as well as the value of antibody profiles in humans against multiple *M. tuberculosis* antigens in TB serodiagnosis and disease states (Davidow et al. 2005).

In our laboratory we have preliminary, proof-of-concept data that support the use of the multiplex microbead suspension array for defining antibody profiles in *M. tuberculosis* infection and disease (Khan et al., in press). In this assay, six specific purified recombinant antigens (ESAT-6, CFP-10, hspX, Ag85B, MPT53, and MPT63) and control antigens are coated onto sets of microbeads that each have a unique spectral signature determined by an internal red/infrared dye mixture. Microbeads are incubated with diluted serum samples, washed, and reacted with phycoerythrin-conjugated goat antihuman immunuglobulin. After additional washing, independent gated events for each bead are counted and analyzed in a Luminex cytometer. The median fluorescence intensity (MFI) of antigen-coated bead sets was compared to control coated beads in each serum. *M. tuberculosis* antibody profiles from five experimentally infected macaques 1 year after inoculation were as follows: five of five animals were ESAT-6 positive, four of five animals were hspX positive, and one of five animals was CFP-10 positive. Antibody was not detected in either pre-inoculation plasma samples from these five macaques or additional negative control plasma samples from ten specific pathogen-free (SPF) animals that had received multiple TSTs collected six times over 2 years (Khan et al., in press).

We have also used the MMIA in preliminary retrospective studies to assess the ESAT-6 antibody status of a small number of cynomolgus macaques that developed latent and active TB in an experimental *M. tuberculosis* infection model (Capuano et al. 2003). In six of seven animals examined so far, ESAT-6 antibody was detectable approximately 4 weeks after inoculation (p.i.) and persisted throughout the course of infection, up to 500 days p.i. One of three latently infected animals had no detectable antibody at any time point. In two animals with latent infection, detectable antibody persisted throughout the follow-up period, although the TST and WB-IFNγ assay became consistently negative at about 150 and 300 days p.i. respectively (Figure 2A,B). In four animals with active infection, antibody levels persisted although TST and WB-IFNγ assays were only intermittently positive (Figure 2C). Thus, because the antibody-based assay gives consistent positive results whereas the cellular immune response assays are only intermittently positive, it may be prudent to use both cellular and antibody-based assays to detect infected animals (active or latent).

These preliminary data support previous observations of transient or intermittently positive results with cell-mediated immune-based assays (Garcia et al. 2004b) and of the potential utility of antibody tests using ESAT-6 antigen for detection of both active and latent TB infections (Brusasca et al. 2003; Kanaujia et al. 2003).

Multiantigen Print Immunoassay (MAPIA)

Another assay format that allows for the simultaneous evaluation of the pattern of reactivity to multiple TB-specific antigens is the multiantigen print immunoassay (MAPIA) (Lyashchenko et al. 2000). In this assay multiple antigens are immobilized in solid phase on nitrocellulose membranes as narrow bands using a semiautomated airbrush printing device (Linomat IV, CAMAG Scientific, Wilmington, NC). The antigen-coated nitrocellulose membrane is cut into 4 mm-wide strips that are then used for antibody detection by Western blot–like chromogenic immunodevelopment. Presence of a visible band is interpreted as a positive result (Lyashchenko et al. 2000).

Use of multiple antigen arrays such as MMIA and MAPIA on sequential serum samples enables the assessment of changing patterns of immunoreactivity over the course of infection. This format can also serve as a confirmatory test for ELISA or rapid test reactive sera. In addition, multiple antigen arrays provide a powerful research tool for identifying novel immunodominant proteins, and may identify reactivity patterns that are predictive of disease progression or reactivation. MAPIA reactivity patterns have also been used to monitor response to therapy in animals undergoing treatment for tuberculosis (Lyashchenko et al. 2006).

Rapid Test Lateral Flow Immunoassay

The lateral flow assay utilizes a membrane impregnated with selected antigens combined with a sample pad, a conjugate pad, and a sink pad in individual plastic cassettes. Results can be obtained in 20 minutes and require a small volume (~30 µl) of serum, plasma, or whole blood. The rapid test can be performed "cage-side" and does not require laboratory equipment or specific technical training. One commercially available rapid test, Prima-TB STAT-PAK® (ChemBio Diagnostic Systems, Inc., Medford, NY) has recently been licensed by the USDA for use in nonhuman primates. This assay uses a "cocktail" of three immunodominant TB-specific antigens (ESAT-6, CFP-10, and MPB83), a combination that was sufficient to correctly identify 25 of 27 (93%) macaques (20 rhesus and 7 cynomolgus) experimentally infected with *M. tuberculosis* or *M. bovis*. Testing of 195 uninfected macaques produced 3 (1.5%) false positive results (Greenwald et al. 2007).

Multiple Assays in TB Surveillance— Where Do We Go from Here?

No single test currently available meets all the requirements for accurate and efficient TB screening in nonhuman pri-

mates. The earlier but transient cellular immune response and the later but persistent antibody response to infection with tuberculous mycobacteria in nonhuman primates clearly indicate the need to incorporate both cellular and humoral immune-based assays in testing algorithms to increase the sensitivity and specificity of TB surveillance. Testing for TB-specific antibodies using recombinant protein antigen targets appears particularly promising for identifying latent TB infections likely to be missed by TST or WB-IFNγ assays. The testing algorithms and their component tests will likely be different for various applications, such as routine testing of a closed colony with no history of TB, screening animals in primary import quarantine, or identifying infected animals in recognized outbreaks. Appropriate validation of these novel testing modalities will require access to samples from relatively large numbers of experimentally and naturally acquired cases of tuberculosis in nonhuman primates.

All screening and diagnostic tests have limitations, which should be considered when interpreting the results. In addition to test results, colony managers need to consider sound clinical evaluations and the history of the colonies or groups of animals being tested. The combinatorial use of both TB-specific antibody tests and in vitro WB-IFNγ assays as adjuncts to traditional TST can significantly improve the overall effectiveness of TB surveillance programs for nonhuman primates.

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