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SERUM ANTIGEN 85 LEVELS IN ADJUNCT TESTING FOR ACTIVE MYCOBACTERIAL INFECTIONS IN ORANGUTANS

A. M. Kilbourn,[1] H. P. Godfrey,[2] R. A. Cook,[1] P. P. Calle,[1] E. J. Bosi,[3] S. I. Bentley-Hibbert,[2] K. Huygen,[4] M. Andau,[3] M. Ziccardi,[5] and W. B. Karesh[1,6]

[1] Wildlife Health Sciences, Wildlife Conservation Society, 185th St. and Southern Blvd., Bronx, New York 10460, USA
[2] Department of Pathology, New York Medical College, Valhalla, New York 10595, USA
[3] Sabah Wildlife Department, W.D.T 200, Sandakan, Sabah 90009, Malaysia
[4] Pasteur Institute of Brussels, 1180 Brussels, Belgium
[5] Lincoln Park Zoo, 2300 North Cannon Drive, Chicago, Illinois 60614, USA
[6] Corresponding author (e-mail: wkaresh@wcs.org)

ABSTRACT: Diagnosis of active mycobacterial disease in orangutans (*Pongo pygmaeus*) has been impeded by high levels of non-specific intradermal skin test reactivity to mycobacterial antigens. This may be due in part to cross reactivity between antigens, tuberculin concentrations used or other species-specific factors. Antigen 85 (Ag85) complex proteins are major secretory products of actively growing mycobacteria, and measurement of serum Ag85 could provide a method for determining active mycobacterial infections that was not dependent on host immunity. Serum Ag85 was measured by dot-immunobinding assay using monoclonal anti-Ag85, purified Ag85 standard and enhanced chemiluminescence technology in coded serum samples from 14 captive orangutans from a zoo in Colorado, 15 semi-captive orangutans in Malaysia, and 19 free-ranging wild orangutans in Malaysia. Orangutans from Colorado (USA) were culture negative for *Mycobacterium tuberculosis* and *M. avium*, although all had laboratory suspicion or evidence of mycobacterial infection; median serum Ag85 was 10 μU/ml (range, <0.25–630 μU/ml). Of the semi-captive orangutans, six were skin test reactive and two were culture positive for *M. avium* on necropsy. Median serum Ag85 for this group was 1,880 μU/ml (0.75–7,000 μU/ml), significantly higher than that of Colorado zoo or free-ranging Malaysian orangutans. Median serum Ag85 in the latter group was 125 μU/ml (range, 0.75–2,500 μU/ml). These data suggest that suggest that additional studies using more specific reagents and more samples from animals of known status are appropriate.

Key words: Antigen 85, diagnosis orangutan, *Pongo pygmaeus, Mycobacterium tuberculosis.*

INTRODUCTION

Orangutans (*Pongo pygmaeus*) are threatened throughout their range by the clearing of land for agriculture and by the pet trade (Andau et al., 1994). Those confiscated from private homes or plantations where they have been kept illegally and with a history of human contact, malnutrition, and stress are at risk from potential exposure to zoonotic diseases, including mycobacterial infections. Southeast Asia, where the remaining free-ranging populations of orangutans are found, reports nearly 3 million tuberculosis cases per year in the human population (Wolfe et al., 1998).

Mycobacterium tuberculosis and other pathogenic mycobacteria can easily be transmitted to captive or semi-captive orangutans via aerosolization or contact and the potential for these animals to transmit these diseases to native wild populations after their release is thus of great concern (Karesh, 1995). Mycobacterial infections of orangutans have resulted in both morbidity and mortality, demonstrating this species' susceptibility to infection and disseminated disease (Calle et al., 1989; Calle, 1999). It has therefore been recommended that all confiscated or newly acquired semi-captive orangutans be skin tested for tuberculosis and quarantined prior to their release. However, healthy orangutans (of both subspecies as well as hybrids) with no known exposure to pathogenic mycobacteria will frequently respond positively to tuberculin skin testing and show lower specificity to routine primate tuberculosis testing protocols than gorillas (*Gorilla gorilla*) or chimpanzees (*Pon* spp.) (Calle et al., 1989).

Relocation of orangutans to new habitat raises concerns regarding the simultaneous

translocation of disease (Woodford and Rossiter, 1993). Mycobacterial disease can pose a serious problem in orangutan management because of its chronicity and the challenge in diagnosis until late in the disease process or at post-mortem examination. For this reason, determining the antemortem presence of active mycobacterial infections and pinpointing which mycobacterial species is present is critical to eliminating the release of potentially infectious animals into free-ranging populations. A rapid diagnostic assay to evaluate active infection with pathogenic mycobacteria which could be run under limited laboratory conditions and which could be correlated with other diagnostic procedures would have great practical value for the management of high risk populations of orangutans.

Antigen 85 (Ag85) complex proteins are major secretory products of actively growing mycobacteria (Wiker and Harboe, 1992). Measurement of serum Ag85 by monoclonal antibody immunoassay could therefore provide a method for determining active mycobacterial infections that was not dependent on host immunity (Godfrey et al., 1992). This is the case in human culture-positive tuberculosis. Elevations of serum Ag85 in these patients are independent of skin-test status (Bentley-Hibbert et al., 1999). Similar results have been obtained in a mixed collection of captive animals [sable antelope (*Hippotragus niger*), eland (*Taurotragus oryx*), greater kudu (*T. strepsiceros*), sitatunga (*T. spekii*), cape buffalo (*Syncerus caffer*)] with a documented history of an *M. bovis* outbreak (Mangold et al., 1999). To determine whether this diagnostic immunoassay might be useful as an adjunctive tool for determining active mycobacterial disease in orangutans, serum Ag85 levels from captive orangutans in the USA with known medical histories were compared to those in semi-captive and free-ranging orangutans in Sabah (Malaysia).

MATERIALS AND METHODS

Sera from three discrete populations of orangutans were evaluated in this project. It was obtained from blood collected by venipuncture from the saphenous, femoral, or cephalic veins in anesthetized animals and frozen in aliquots in liquid nitrogen and stored at −80 C until tested. The first group contained 14 captive orangutans from Cheyenne Mountain Zoo (Colorado Springs, Colorado), a group of animals with minimal risk of exposure to *M. tuberculosis*, known medical histories and under close observation for an extended period of time. The second group contained 15 semi-captive animals at Sepilok Orangutan Rehabilitation Center (SOURC), Malaysia. Semi-captive orangutans were orphaned or injured individuals who, admitted as infants or juveniles, had undergone or were undergoing rehabilitation back into the wild at SOURC. These animals were sampled upon entering or exiting quarantine, during health evaluation or during any procedure requiring chemical immobilization. They had histories that included high human contact and potential exposure to pathogenic mycobacteria. Several had a history of respiratory disease and positive mycobacterial cultures on post-mortem examination. The third group consisted of 19 free-ranging orangutans whose contact with humans occurred only at the time of translocation procedures in Sabah, when sample collection was conducted. Most of the wild free-ranging orangutans were lean and all appeared healthy on physical exam.

Captive and semi-captive orangutans were given one or more routine intradermal tuberculin tests. Intradermal palpebral tuberculin testing was the most common test. Other test sites included the arm, chest or abdomen (Calle et al., 1989). Mammalian old tuberculin (MOT, Tuberculin Mammalian Human Isolates, Coopers Animal Health, Kansas City, Kansas, USA), 0.1 ml (13,500 Tuberculin Units), was injected intradermally and the site examined at 24, 48, and 72 hr. Positive responses were characterized by erythema, swelling, induration and ulceration, and serum exudation (Calle, 1999). Tests were classified as negative, suspicious or positive based on the extent of swelling around the injection site. A "comparative skin test" was also performed on some animals by intradermal injection of 0.1 ml of normal saline (control) and three different purified protein derivative tuberculin (PPD) products (PPD-Human, diluted, Parke-Davis, Ann Arbor, Michigan, USA; balanced PPD of *M. avium*, PPD-Avian, and balanced PPD of *M. bovis*, PPD-Bovine, National Veterinary Service, Ames, Iowa, USA) on the arms (Calle et al., 1989). Each site was evaluated at 24, 48, and 72 hr. A positive response was defined as swelling and hyperemia at an injection site.

Some captive and semi-captive animals had

additional tests including mycobacterial culture and smears of tracheal washes, gastric lavages, and post-mortem examination. Mycobacterial cultures on semi-captive, Malaysian orangutans were performed using standard clinical laboratory techniques at the Tuberculosis Laboratory (Duchess of Kent Hospital, Sandakan, Malaysia). Acid-fast staining, mycobacterial cultures and determination of serum anti-*M. bovis* and anti-*M. avium* antibody levels by enzyme-linked immunosorbent assays (ELISA) on captive orangutans in Colorado were performed as previously described (Calle et al., 1989). Antibody results are reported as − (negative), ± (suspicious), or + (positive).

Serum Ag85 was measured by dot immunobinding on 5-fold serial dilutions of 100 μl aliquots of coded samples (diluted in phosphate buffered saline, pH 7.2, lowest dilution tested, 1:25) using IgG_1 monoclonal anti-*M. bovis* BCG Ag85, clone 17/4 (Drowart et al., 1992), purified BCG Ag85 complex standard containing 1 mU immunoreactive Ag85 complex/mg protein, peroxidase-conjugated, affinity purified goat anti mouse IgG (H+L) with minimal reactivity to human, bovine, horse, rabbit, swine serum proteins (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), and enhanced chemiluminescence technology (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and standard X-ray film (Fisher Scientific, Springfield, New Jersey) (Godfrey et al., 1992). Clone 17/4 displays broad cross-reactivity with Ag85 complex proteins from a variety of pathogenic and non-pathogenic mycobacteria (*M. tuberculosis, M. avium, M. kansasii, M. xenopi, M. gordonae, M. fortuitum, M. phlei, M. smegmatis*) (Drowart et al., 1992). Positive and negative controls were included on each blot. The assay was quantitated by visual comparison with dilutions of Ag85 standard included on each blot; this method has been shown to give comparable results to those obtained using densitometric methods (Van Vooren et al., 1988). No immunoreactivity was detected in dot blots of orangutan sera when mouse monoclonal IgG_1 control was substituted for specific IgG_1 monoclonal anti-Ag85 complex antibody. Ag85 can be detected by this technique in sera from captive animals with culture-positive tuberculosis even after prolonged storage at −80C (B. J. Mangold, R. A. Cook and H. P. Godfrey, unpubl. obser.). Results are reported as geometric means of one to three determinations in duplicate in μU/ml.

Differences in median serum Ag85 among groups were analyzed by the Kruskal-Wallis nonparametric ANOVA test and Dunn's multiple comparison post-test. For purposes of statistical analysis, samples not reactive at 0.25 μU/ml were assumed to react at 0.025 μU/ml Ag85. The probability that the data were drawn from a population fitting a normal (Gaussian) distribution was estimated by the method of Dallal and Wilkinson (1986).

RESULTS

All captive orangutans in Colorado were healthy with no clinical evidence of disease, but all had laboratory evidence or suspicion of mycobacterial infection (Table 1). *Mycobacterium tuberculosis* or *M. avium* were not cultured from any animal. They have remained in good health, and none have come to necropsy. Median serum Ag85 in this group was 10 μU/ml with a range of <0.25 to 630 μU/ml. Eight of these 14 animals had serum Ag85 <30 μU/ml. An animal from which *M. nonchromogenicum* was cultured had a serum Ag85 of 630 μU/ml, but two others from which *M. terrae* and *M. fortuitum* were cultured had serum Ag85 of <3 μU/ml.

Six of the semi-captive orangutans from SOURC were positive on one or more tuberculin skin tests, and two were culture positive for *M. avium* at necropsy (Table 2). One of these culture-positive animals had a serum Ag85 of 2,800 μU/ml, the other, 310 μU/ml. Median serum Ag85 for the semi-captive orangutans was 1,900 μU/ml (0.75–7,000 μU/ml). Only one animal had serum Ag85 <30 μU/ml. Serum Ag85 in Malaysian semi-captive orangutans was significantly higher than levels found in Colorado zoo orangutans ($P < 0.001$) or Malaysian free-ranging orangutans ($P < 0.05$). In free-ranging orangutans, median serum Ag85 was 130 μU/ml (range 0.75-2,400 μU/ml) (Table 3), not significantly different from the values found in captive animals in Colorado.

Serum ag85 in captive and semi-captive orangutans correlated poorly with culture-positive mycobacterial infection or other laboratory diagnostic tests but were normally distributed. The distribution of serum Ag85 in free-ranging orangutans was bi-modal. Statistical analysis confirmed that the serum Ag85 values in captive and

TABLE 1. Skin reactivity, anti-mycobacterial antibodies, mycobacterial culture and serum Ag85 in captive orangutans in Colorado.

Studbook number	Skin test reactivity[a]				Culture and smear of gastric lavage or tracheal wash[b]	ELISA reactivity[c]		Serum Ag85 (μU/ml)
	MOT	PPD-human	PPD-avium	PPD-bovis		Anti-*M. avium*	Anti-*M. bovis*	
352	+	−	+	+	O	−	±	5.6
815	−	ND	−	−	N	−	±	13
907	−	−	ND	ND	ND	−	±	190
1051	+	−	+	+	O, C, N	−	+	130
1277	+	ND	ND	ND	*M. terrae*	−	−	2.5
1344	+	ND	+	+	*M. fortuitum*	−	+	0.75
1367	ND	ND	−	−	C	−	−	240
1529	+	−	+	−	C, N	−	−	240
1605	ND	ND	+	+	O, N, N	−	+	<0.25
1702	ND	ND	ND	ND	N	−	±	<0.25
1744	+	−	+	−	O, N	−	−	5.9
1814	+	−	+	+	*M. nonchromogenicum*	−	−	630
1934	ND	ND	+	+	N	−	−	7.5
1977	ND	ND	−	−	C	−	−	63

[a] Skin reactivity to mammalian Old Tuberculin (MOT), or to Tuberculin, purified protein derivative, prepared from *M. tuberculosis* (PPD-human), *M. avium* (PPD-avium), *M. bovis* (PPD-bovis): +, positive; −, negative. ND, not done.
[b] Results of one or more repeated attempts. O, acid-fast bacteria observed in stained smear, no growth in culture. C, acid-fast bacteria cultured but not identified due to overgrowth of contaminants. N, no growth of acid-fast bacteria in culture.
[c] Antibodies as detected by ELISA to indicated antigen: − (negative), ± (suspicious reactivity), + (positive).

semi-captive orangutans were likely to have been drawn from populations with a normal distribution, while those in free-ranging orangutans were not ($P < 0.025$).

DISCUSSION

Mycobacteriosis is a significant health problem in many species, and especially in primates. Orangutans are susceptible to tuberculosis and a positive response to tuberculin testing cannot be ignored (Calle et al., 1989; Calle, 1999). Observations that positive skin tests and antibody titers may not be reliable diagnostic indicators for active disease in orangutans (Calle et al., 1989; Calle, 1999) have added complexity to wildlife management decisions regarding this endangered species.

Development of alternate diagnostic methods not dependent on host immune competency offers the promise of early, antemortem diagnosis of individuals actively infected with pathogenic mycobacteria so that they can be separated from those responding to non-pathogenic environmental mycobacteria. Measurement of serum Ag85 in culture- and/or histologically-positive human and animal hosts with active tuberculosis has suggested that elevated levels of this mycobacterial secretory protein are associated with active disease (Bentley-Hibbert et al., 1999; Mangold et al., 1999). In the present study, it was not possible to demonstrate any consistent correlation between serum Ag85 levels and active mycobacterial infection. This may be a consequence of the broad species specificity of the anti-Ag85 monoclonal antibody employed or of differences in mycobacterial number or tissue localization in orangutans as compared to other hosts. Another possible source of interference is the presence of natural, cross-reactive anti-mouse Ig antibodies in orangutan serum. The lack of immunoreactivity in blots of orangutan sera treated with control monoclonal mouse IgG_1 makes this alternative less likely.

Serum Ag85 levels in healthy, tuberculin-positive human beings (Bentley-Hibbert et al. 1999) and in most captive animals involved in a *M. bovis* outbreak (Mangold et al., 1999) were <50 μU/ml. These were much lower than values seen

TABLE 2. Skin reactivity, mycobacterial culture, and serum Ag85 in semi-captive orangutans in Malaysia.

Animal number	Skin test reactivity[a]				Culture of gastric lavage, tracheal wash, or tissue from necropsy	Serum Ag85 (μU/ml)	Current status/cause of death
	MOT	PPD-human	PPD-avian	PPD-bovine			
s1	−	−	−	−	N[b]	0.75	Healthy/Treated for tuberculosis for over 1 yr
s2	−	−	−	−	ND	2,700	Healthy/Semi-captive
s3	−	−	−	−	N	79	Died/Melioidosis, liver disease
s4	−	ND	ND	ND	N	1,300	Died/Severe pneumonia
s5	−	ND	ND	ND	ND	2,000	High risk of acid-fast bacterial exposure
s6	[c]	[c]	[c]	[c]	N	2,200	Died/Severe chronic pneumonia, acute septic emboli
s7	−	−	−	−	ND	79	Healthy/Semi-captive
s8	−	ND	ND	ND	*M. avium*	2,800	Died/Pneumonia
s9	−	−	−	−	N	6,250	Died/Melioidosis, lung abscess
s10	ND	−	+	−	*M. avium*	310	Severe respiratory disease
s11	+	−	+	−	N	630	Chronic upper respiratory disease and cough
s12	+	−	+	−	ND	500	Healthy/Semi-captive
s13	+	−	+	−	N	1,900	Died/Severe enteritis
s14	+	−	+	−	ND	7,000	Healthy/Free ranging
s15	+	−	+	−	ND	3,800	Healthy/Free ranging

[a] Skin reactivity to indicated tuberculins: +, positive; −, negative. ND, not done. See footnote to Table 1 for details.
[b] Results of one or more repeated attempts. N, no growth of acid-fast bacteria in culture.
[c] Animal died within 48 hr of onset of respiratory distress and prior to reading of skin test results.

TABLE 3. Skin reactivity and bacterial culture of free-ranging orangutans in Malaysia.

Animal number	Skin test reactivity: MOT	Culture of tissue from necropsy	Serum Ag85 (μU/ml)	Status
p1	ND	ND	0.75	Transported and released
p2	ND	ND	130	Transported and released
p3	ND	ND	150	Transported and released
p4	ND	ND	0.75	Transported and released
p5	ND	ND	550	Transported and released
p6	ND	ND	6.3	Transported and released
p7	ND	ND	400	Transported and released
p8	ND	ND	180	Transported and released
p9	ND	ND	11	Transported and released
p10	ND	ND	250	Transported and released
p11	ND	ND	110	Transported and released
p12	ND	ND	2,400	Transported and released
p13	ND	ND	23	Transported and released
p14	ND	ND	2.2	Transported and released
p15	ND	ND	630	Transported and released
p16	ND	ND	120	Transported and released
p17	ND	ND	170	Transported and released
p18	−	ND	125	Moribund on arrival/ Released after recovery
p19	−	N[b]	7.8	Died/Renal failure, spinal injury

[a] Skin reactivity to mammalian Old Tuberculin (MOT): +, positive; −, negative. ND, not done.
[b] N, no growth of acid-fast bacteria in culture.

in about 20% of healthy tuberculin-positive captive orangutans and 30% of semi-captive orangutans. The reasons for very high serum Ag85 levels in some healthy semi-captive and many free-ranging Malaysian orangutans are unclear. They could indicate chronic-active mycobacterial infection acquired as a result of human exposure or transient infection with non-pathogenic environmental mycobacteria, but data to support this supposition are lacking.

The results of this research suggest that additional studies using more specific reagents and more samples from animals of known status are appropriate. Continued opportunistic collection and testing of serum from orangutans with confirmed positive *M. tuberculosis*, *M. bovis* or *M. avium* infections are needed to determine the sensitivity and specificity of this test and its possible role in diagnosis of these infections in the field.

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