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A COMPARISON OF FOUR SEROLOGIC ASSAYS IN SCREENING FOR *BRUCELLA* EXPOSURE IN HAWAIIAN MONK SEALS

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ABSTRACT: A survey for *Brucella* spp. antibodies was undertaken on 164 serum samples from 144 Hawaiian monk seals (*Monachus schauinslandi*) from the northwestern Hawaiian Islands collected between 1995 and 2002. The buffered antigen plate agglutination test (BPAT), the indirect enzyme immunoassay (I-ELISA), the competitive enzyme immunoassay (C-ELISA), and the fluorescence polarization assay (FPA) were compared with regard to their ability in detecting antibodies to *Brucella* spp. in the serum samples. Overall, antibodies were detected in 28 (17.1%) animals, using the BPAT test, 25 (15.2%) by the C-ELISA, and 19 (11.6%) in the I-ELISA and the FPA test, using thresholds established for cattle. No evidence of gross pathology consistent with clinical brucellosis was noted in any of the seropositive animals tested. Although further work would be necessary to validate these tests for use with monk seals it appears that both the C-ELISA and the FPA tests would be appropriate as diagnostic screening tests for detection of antibodies to *Brucella* spp. in this species.

Key words: *Brucella* spp., buffered antigen plate agglutination test, comparative serologic diagnostic tests, competitive ELISA test, fluorescent polarization test, Hawaiian monk seal, *Monachus schauinslandi.*

INTRODUCTION

Hawaiian monk seals (HMS; *Monachus schauinslandi*) presently number around 1,400 animals and are the most endangered species of marine mammal that lives entirely within the United States (Carretta et al., 2001). There are six main reproductive populations of HMS in the northwestern Hawaiian Islands, which include French Frigate Shoals (23°45′N, 166°10′W), Laysan Island (25°42′N, 171°44′W), Lisianski Island (26°02′N, 174°00′W), Pearl and Hermes Reef (27°55′N, 175°45′W), Midway Atoll (28°15′N, 177°35′W), and Kure Atoll (28°25′N, 178°10′W). A decline in the population since the late 1950s led to their being listed as an endangered species under the U.S. Endangered Species Act in 1976. Since 1985, the average rate of decline has been approximately 3% per year although counts have remained stable since the early 1990s. Further declines due to high juvenile mortality and an inverted age structure are predicted in the largest colony on the French Frigate Shoals. The annual number of births has varied over the last few decades and is expected to decline in the future due to poor recruitment expected at French Frigate Shoals (Carretta et al., 2002). Approximately 90% of HMSs remain near their natal birth site throughout their life, but the remaining 10% frequent the other islands (Carretta et al., 2002).

Known causes of mortality in HMSs include emaciation of juveniles, shark predation, male aggression, collisions with boats, and entanglement in marine debris such as fishing gear (Kenyon, 1981; Alcorn and Kam, 1986; Henderson, 1990; Nitta and Henderson, 1993; Hiruki et al., 1993). There is also evidence that human disturbance of pregnant and nursing females can cause them to abandon preferred pupping areas, resulting in decreased pup survival (Kenyon, 1981). Biotoxins such as ciguatoxin have been found in prey species throughout HMS habitat (National Marine Fisheries Service, unpubl. data) and were

implicated in a die-off of HMSs at Laysan Island in 1978 (Gilmartin, 1987).

The role of infectious diseases in the population decline of HMSs is not well understood, but is an active area of investigation. Serologic evidence of exposure to potential pathogens (*Leptospira* and *Chlamydophilus*) as well as isolation of a number of species of *Salmonella* and endoparasites has been reported in HMSs (Aguirre, 2000). Emerging diseases are now recognized by most wildlife biologists as a substantial threat to the conservation of global diversity (Daszak et al., 2000). Recent examples of infectious diseases causing catastrophic declines in populations of wildlife include the loss of amphibian species worldwide (Daszak et al., 1999) and the more subtle role that infectious diseases might be having on the decline of sea otters (*Enhydra lutra*) in California (Estes et al., 2003). In cases where an animal population is threatened or endangered the risk of extinction due to infectious diseases becomes even greater. Notable examples include an epidemic of canine distemper virus (CDV) in Serengeti lions (*Panthera leo*; Roelke-Parker et al., 1996) and the near extinction of black-footed ferrets (*Mustela nigripes*) by CDV (Thorne and Williams, 1988).

Until the mid-1990s brucellosis was thought to be a disease of terrestrial animals only. Six nomen species of *Brucellae* are presently recognized and are distributed worldwide (Moreno et al., 2002). Since 1994, reports have described isolation and characterization of *Brucella* isolates from a number of marine mammal species from North America and Europe (Ewalt et al., 1994; Foster et al., 1996; Forbes et al., 2000). Evidence of *Brucella* antibodies has been reported in marine mammals from North America, the north Atlantic, and Antarctica, indicating that *Brucella* infections affect a large number of cetacean and pinniped species and are distributed worldwide (Tryland et al., 1999; Retamal et al., 2000; Nielsen et al., 2001). Some cetacean stranding events are associated with meningoencephalitis caused by *Brucella* infections, and accounts of reproductive failure in dolphins and baleen whales due to *Brucella* infections have been reported (Miller et al., 1999; González et al., 2002; Ohishi et al., 2003). Brucellosis is a zoonotic disease and there are two reports of the disease in humans that were acquired from marine mammals (Brew et al., 1999; Sohn et al., 2003).

There are three reasons why it is important to quickly and accurately screen HMS sera for *Brucella* antibodies. First, considerable rescue and rehabilitation work is done in Hawaii with this and other marine mammal species, and the risks of human exposure to animals with active brucellosis could be significant. Secondly, a rapid field test for identifying HMSs with brucellosis could also aid in diagnosis and subsequent treatment of sick and live-stranded animals. Thirdly, serologic identification of *Brucella* infections might help investigators determine cause of death of animals under examination. Results from such investigations could be useful in understanding the role that brucellosis might be contributing to HMS population decline. The present study was undertaken to compare four serologic tests for determining *Brucella* exposure in HMSs.

MATERIAL AND METHODS

Blood samples from 144 HMSs were collected as part of a health assessment study that was ongoing in the test area since 1998. Some seals were sampled on more than one occasion and nine captive animals held at the Kewalo Research Facility, Honolulu, Hawaii were opportunistically sampled between 1995–99. Wild seals were captured while hauled out on the beach. Mature seals were captured with a hoop net, immature seals (juveniles) were captured with a stretcher, and weaned pups were captured by hand. Diazepam (Steris Laboratories Inc., Phoenix, Arizona, USA) was given intravenously and following induction, blood was collected from the bilaterally divided extradural veins by inserting an 18 gauge, 3.5-in spinal needle between the dorsal spinous processes of the third, fourth, or fifth lumbar vertebrae (Geraci and Lounsbury, 1993). Blood tubes

were kept in the shade for 60 min to allow for normal coagulation processes to occur then transferred to a cooler with ice. Serum was separated by centrifugation at 1,000 × G for 10 min, 1.0 ml aliquots of serum were transferred to cryogenic vials and placed in liquid nitrogen in the field, and then transferred to a −86 C freezer. Serum samples (n=164) for this study were shipped on dry ice to the Animal Diseases Research Institute, Ottawa, Ontario, Canada for analysis.

The buffered antigen plate agglutination test (BPAT) was performed as described in the Office International Des Epizooties Manual of Standards for Diagnostic Tests and Vaccines (1996).

The indirect enzyme immunoassay test (I-ELISA) was done as described by Nielsen et al. (1994) with the following modification. The I-ELISA uses smooth lipopolysaccharide (s-LPS) from *B. abortus* strain 1119.3 as the antigen, adsorbed onto polystyrene microplates (NUNC 2-69620 from Gibco-BRL, Burlington, Ontario, Canada), followed stepwise by the application of the diluted serum samples (1:50), then universal protein A and G binding reagent (Pierce, Rockford, Illinois, USA) conjugated with horseradish peroxidase and substrate/chromogen. Divalent cation chelating agents (EDTA/EGTA) were added to the serum diluent to reduce nonspecific protein interactions (Nielsen et al., 1994) and the microplates were washed between each step with 0.01 M, pH 7.2 phosphate buffered saline containing 0.15 M NaCl and 0.05% Tween-20 (PBS/T). Optical density readings at 414 nm were obtained after 10 min and a positive result relative to a strongly positive bovine serum was based on the cut-offs obtained in previous experiments with cattle (inhibition ≥46% was considered positive).

The competitive enzyme immunoassay (C-ELISA) was performed as described by Nielsen et al. (1996a). Again, sLPS antigen was adsorbed onto the polystyrene microplates. After incubation and washing of the adsorbed microplates, serum samples diluted 1:10, were added immediately followed by the addition of a murine monoclonal antibody specific for a common epitope of *Brucella* O-polysaccharide (M-84). Serum samples and M84 were diluted in PBS/T containing EDTA/EGTA (Nielsen et al., 1994). The serum samples and the monoclonal antibody were mixed for 3 min in the microplate and were incubated for 30 min. After incubation and washing, commercially available goat anti-mouse IgG horseradish peroxidase-conjugated antibody (heavy and light chain specific; Jackson ImmunoResearch Labs Inc., West Grove, Pennsylvania, USA) was added followed by the addition of substrate chromogen after incubation and washing. Optical density readings at 414 nm were obtained after 10 min and a positive result was based on the cut-off obtained in previous experiments with cattle (positivity ≥30%).

The fluorescence polarization assay (FPA) was performed as described by Nielsen et al. (1996b). The assay used *B. abortus* O-polysaccharide conjugated with fluorescein isothiocyanate. The assay tested serum at 1:100 in 1.0 ml of 0.01M Tris, pH 7.0 containing 0.15M NaCl, 10 mM EDTA, and 0.05% Igepal CA 630 (Sigma–Aldrich Canada Ltd., Mississauga, Ontario, Canada). The sample was measured in a fluorescence polarization analyser, (SENTRY from Diachemix Corporation, White Fish Bay, Wisconsin, USA) to obtain a baseline fluorescence measurement. A predetermined amount of conjugated antigen in 0.01 M sodium phosphate, pH 7.0 containing 0.15 M NaCl and 0.1% sodium azide was added to each sample, mixed and incubated for approximately 2 min to allow for the interaction of antigen and any antibody present. After incubation, the sample was again measured in a fluorescence polarization analyser. In the presence of significant antibody, a high millipolarization (≥90 mP) result was obtained; in the absence of significant antibody a low value was obtained.

RESULTS

When all tests are considered together, the prevalence of brucellosis in HMSs in all the serum samples was 28/164 or 17.1%. Positive animals were detected in all six locations with the highest prevalence recorded at Midway Atoll (9/30 or 30%) and the lowest prevalence at Lisianski Island (1/17 or 6%; Table 1). Among the animals tested from Midway Atoll, a juvenile female was negative on all four tests on two separate occasions (1999 and 2000) whereas one adult female tested in 1998, 1999, and 2001 was positive all three times in all four tests. One weaned male from the French Frigate Shoals was positive on all tests except the BPAT, but was negative on all four tests 6 mo later. This latter result was possibly due to the presence of maternal antibody. Positive animals were identified among both sexes and in all age classes. All nine captive animals were seronegative in the four tests.

In comparing the tests, the BPAT had the most positive reactions (28/164 or

TABLE 1. Prevalences of serum antibodies to *Brucella* spp. in Hawaiian monk seals from the northwestern Hawaiian Islands.

Location	Year	I-ELISA[a]	BPAT[a]	C-ELISA[a]	FPA[a]	Number of samples	Positive[b]
French Frigate Shoals	1998–2002	4	4	4	4	27	5 (19)
Laysan Island	2000–01	5	10	9	5	52	11 (21)
Lisianski Island	2000–01	0	1	1	1	17	1 (6)
Pearl and Hermes Reef	2001	1	1	1	0	5	1 (20)
Midway Atoll	1998–2001	9	9	9	8	30	9 (30)
Kure Atoll	2001	0	3	1	1	20	3 (15)
Captive animals	1995–99	0	0	0	0	13	0
Total		19 (12)	28 (17)	25 (15)	19 (12)	164	28 (17.1)

[a] I-ELISA=indirect enzyme immunoassay, BPAT=buffered antigen plate agglutination test, C-ELISA=competitive enzyme immunoassay, FPA=fluorescence polarization assay.
[b] Number positive in BPAT, C-ELISA, I-ELISA, and FPA (percent positive).

17.1%) whereas the FPA and the I-ELISA were positive in 19/164 or 11.6% animals. The C-ELISA was positive in 25/164 or 15.2% of the sera (Table 1). The highest numbers of positive animals were detected by the BPAT test, although some of the animals that were negative by the BPAT test were positive by the other three tests and visa versa.

DISCUSSION

We found evidence of exposure to *Brucella* spp. is relatively high in the HMSs throughout their range in the northwestern Hawaiian Islands. Overall prevalence was 17.1% and this result is in the same range as has been reported in other seal species from North America, Europe, and Antarctica (Jepson et al., 1997; Retamal et al., 2000; Nielsen et al., 2001). Finding serologic evidence of *Brucella* exposure in HMSs in the northwestern Hawaii Islands was not unexpected and it is likely most marine mammal species worldwide are enzootically infected (Van Bressem et al., 2001). It is unknown at this time whether brucellosis in HMSs or other endangered species of seals constitutes a significant risk to long-term survival. No evidence of lesions consistent with clinical brucellosis was found in the animals sampled in this survey. However, because only live animals were sampled, overt signs of brucellosis could easily have been missed. Isolation of *Brucella* spp. has not been associated with clinical signs of disease in any seal species from which it has been isolated (Foster et al., 1996; Forbes et al., 2000).

The decline in HMSs since the 1980s and the low recruitment of animals, especially in the French Frigate Shoals (Carretta et al., 2001) might be interpreted as evidence that brucellosis is playing a role in the decline of these stocks (Aguirre, 2000). The first step in determining the role that brucellosis might be having in the decline of the HMS population is use of a reliable serologic test, in conjunction with bacterial isolation attempts from tissues from dead stranded seals (Forbes et al., 2000). Unfortunately, most serologic tests used for wild species have been directly transposed from use in domestic livestock species without proper validation. Therefore, in deciding which test should be adopted for routine screening for brucellosis in HMSs, some consideration of the underlying chemistry and history of each test should be taken into account.

The BPAT test was developed for detection of antibody to *Brucella* spp. in bovine serum (Angus and Barton, 1984). In this test an acidified antigen preparation is used and therefore reduces the final antigen/serum mixture to approximately pH 3.65. At this pH fibrinogen can be converted to an insoluble fibrin that could be interpreted by the investigator as aggluti-

nation, thereby giving rise to a false positive result. The BPAT test is also unable to distinguish antibody from cross-reacting organisms such as *Yersina enterocolitica* 0:9 which might be present in test sera and this would also lead to false positive results (Samartino et al., 1999).

Use of species independent competitive immunoassays such as C-ELISA and I-ELISA would eliminate some false positives, and would therefore be more specific because production of fibrinogen is not an issue. The reactivity of the protein A/G HRPO conjugate with seals has not been reported and the lower number of reactors identified by the I-ELISA might be due to the conjugate not reacting with some isotypes of seal antibody. Another advantage of the C-ELISA is that it is possible in most cases to distinguish between antibodies to *Brucella* spp. and antibodies from other cross-reacting Gram negative bacteria (Nielsen, 1990). Both enzyme immunoassays have been used successfully to identify *Brucella* serologic reactors among a variety of marine mammal species (Tryland et al., 1999; Nielsen et al., 2001). A further drawback of the BPAT test is that it requires the use of good quality serum, whereas whole blood and hemolysed serum do not interfere with the detection of serum antibodies in the FPA and enzyme immunoassays. Rarely are good quality serum samples available from wildlife for serologic screening and this is especially true when the animals are found dead.

It is unlikely that the specificity and sensitivity of the BPAT, the two immunoassays, and the FPA will ever be determined for detecting *Brucella* antibodies in HMS serum. This would involve the analysis of a statistically significant number of sera in comparison with another "gold standard" test such as isolation of the causative organism under controlled conditions. Sensitivity and specificity of each test could be determined by comparing the results from animals known to be positive and for those known to be negative by bacterial isolation. It would not be feasible to carry out this conclusive validation process for every species of marine mammal, and for routine screening, it is not necessary. No attempt was made to isolate *Brucella* from HMSs sampled in this study because bacterial isolation also has some limitations, especially when used in free-ranging animals. Recovered carcasses are usually in some state of decomposition and *Brucella* is notoriously fragile, making recovery difficult. Maratea et al. (2003) reported they were only able to recover isolates from two of five stranded harp seals (*Phoca groenlandica*) showing serologic evidence of *Brucella* exposure using the BAPA, rivanol, and card test, a sensitivity of only 40%.

The C-ELISA, I-ELISA, and FPA perform well with regards to sensitivity and specificity in sera from wild species whereas the BPAT performed relatively poorly (Gall et al., 2001). Therefore, it is assumed that the BPAT test overestimated the number of positive HMSs in this survey. The FPA and I-ELISA tests each detected the fewest number (19) of *Brucella* reactors whereas C-ELISA detected 25 (Table 1). Given the small number of animals tested, no one test can be judged superior to another and validation of each of these tests is impractical for the reasons stated above. In cases where an accurate estimation of *Brucella* antibodies is required, C-ELISA is qualitatively at least the most suitable choice. The I-ELISA is not as specific as C-ELISA and FPA because it cannot be used to discriminate between antibodies that cross-react with other Gram negative bacteria. For this reason, it too should be considered unsuitable for the identification of *Brucella* reactors in wildlife. A disadvantage of both C-ELISA and I-ELISA tests is the considerable expertise and equipment required to perform the test in order to obtain reliable results. This is not always possible in wildlife monitoring situations.

As with other wild species, the FPA test is the diagnostic test of choice for detection of exposure to *Brucella* in HMSs (Gall et al., 2001). It has the ability, in some cas-

es, to distinguish antibody from cross-reacting organisms (e.g., *Y. enterocolitica* 0:9) from antibody against *Brucella* spp. and is marginally better at it than the C-ELISA test; it is technically simple to do; it is adaptable to field use even with hemolysed sera, milk and whole blood; and is relatively inexpensive (Gall et al., 2001). The results we obtained were based on cutoff values established for cattle, but they are probably sufficient for preliminary screening for evidence of *Brucella* exposure in HMSs. These cutoff points might differ between cattle and HMSs as well as other marine mammal species.

Once reliable species-independent serologic testing is adopted for routine screening of HMSs, identification of presumptively *Brucella*-positive stranded or sick seals will assist in guiding treatment of affected animals and alert health care workers to take measures to prevent infection of themselves and uninfected animals with which affected seals might come in contact. At present the policy is to not return *Brucella* seropositive animals to the wild, but this decision was based on use of the BPAT that is unsuitable for determining serologic status. Adopting FPA and C-ELISA for a more accurate determination of *Brucella* status would make this policy less error prone and will result in reintroduction of healthy HMSs back into the wild.

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