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Serological Evidence of Exposure to Selected Viral, Bacterial, and Protozoal Pathogens in Free-Ranging Atlantic Bottlenose Dolphins (*Tursiops truncatus*) from the Indian River Lagoon, Florida, and Charleston, South Carolina

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Abstract

Sera from free-ranging Atlantic bottlenose dolphins (*Tursiops truncatus*) in the Indian River Lagoon, Florida (IRL) (n = 122), and the estuarine waters near Charleston, South Carolina (CHS) (n = 82) were collected from 2003 to 2007 and analyzed for antibodies to several bacterial and viral pathogens. Serological evidence of exposure to *Chlamydophila psittaci*; Eastern, Western, and Venezuelan equine encephalitis viruses; and West Nile virus represents the first reports of these pathogens in cetacean populations. Antibodies to Eastern and Venezualan encephalitis viruses and to West Nile virus were detected only in IRL dolphins. Positive titers to *Toxoplasma gondii* and *Brucella abortus* (rivanol and card tests) were identified in dolphins from both locations. The prevalence of antibodies to *Brucella* spp. on the card test was significantly higher in bottlenose dolphins sampled in the IRL compared to the CHS location. This study establishes baseline seroprevalence for several zoonotic pathogens in these two populations.

Key Words: *Tursiops truncatus*, *Chlamydophila psittaci*, West Nile virus, equine encephalitis, *Toxoplasma gondii*, *Brucella* spp., viral pathogens, bacterial pathogens, zoonoses

Introduction

The prevalence of antibodies to several recognized or potential marine mammal pathogens, including the arthropod-borne encephalitis viruses and *Chlamydophila psittaci*, has not been widely reported in marine mammals. Antibody surveys for *Brucella* spp. and *Toxoplasma gondii* have been reported in free-ranging cetaceans, but their role in disease causation is not fully established. The collection of baseline seroprevalence data for these agents in bottlenose dolphins (*Tursiops truncatus*) would be useful in expanding understanding of their epidemiology and potential for zoonotic transmission. Atlantic bottlenose dolphins serve as a sentinel species for ecosystem health and are important barometers for assessing the burden of emerging pathogens in the environment (Bossart, 2006). Seroepidemiology is an essential component for studying the extent and distribution of exposure to pathogens in free-ranging bottlenose dolphin populations. Therefore, a seroepidemiologic investigation was undertaken to establish baseline seroprevalence of antibodies to *Brucella* spp.; Eastern, Western, and Venezuelan equine encephalitis virus; West Nile virus; *C. psittaci*; and *T. gondii* in dolphins in the Indian River Lagoon, Florida, and the estuarine waters around Charleston, South Carolina.

Materials and Methods

Dolphin HERA Project

Sera were collected from wild bottlenose dolphins inhabiting the Indian River Lagoon, Florida (IRL), during June 2003 to 2007 and the estuarine waters of Charleston, South Carolina (CHS), during August 2003 to 2005 as a part of the Dolphin Health and Risk Assessment (HERA) Project. The HERA Project is a collaborative effort between the Harbor Branch Oceanographic Institute and the National Ocean Service's Center for Coastal Environmental Health and Biomolecular Research designed to assess the health status of these two free-ranging bottlenose dolphin populations (Fair et al., 2006). A comprehensive clinical assessment was conducted, and individual dolphins

were categorized by health status. A panel of five marine mammal veterinarians classified dolphins as clinically normal, possibly diseased, or definitely diseased on the basis of results of physical and ultrasonographic examinations, hematologic and serum biochemical analyses, and cytologic and microbiologic evaluations of gastric contents and swab specimens (Reif et al., 2008).

Serological Tests

Blood samples were drawn from the periarterial venous rete in the flukes within the first 10 min of capture with a 19-gauge, 1.9-cm, butterfly catheter (Becton Dickinson, Franklin Lakes, NJ, USA). Serum was collected in 10-ml separator vacutainer tubes (Benton Dickinson), placed in a cooler for 20 to 40 min, and centrifuged for 15 min at 1,200 rpm (Goldstein et al., 2006). Serum was separated, packed in dry ice, and sent to multiple laboratories for analyses (Table 1). Virus neutralization tests (VNT) were conducted to detect antibodies to Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), and Venezualan equine encephalitis (VEE) viruses. A titer of > 1:10 was considered positive for each virus based on previously established laboratory cutoffs. The rivanol (> 1:25 positive) and card tests were used to test for antibody to *B. abortus*. *C. psittaci* antibody was detected using an indirect fluorescent antibody (IFA) test, with titers of > 1:5 defined as a positive result. The indirect hemagglutination test (IHAT) (> 1:64 positive) was conducted to detect antibodies to *T. gondii* in dolphin sera.

Age Determination

Age was determined following dental extraction of the left 15th mandibular tooth under local anesthesia (3% mepevicaine). Post-natal dentine layers were counted to determine age (Hohn et al., 1989). All dolphins 6 y and older were defined as adults.

Statistical Analysis

Prevalence of antibody to each organism was stratified by age class, sex, and year of collection. Seroprevalence was compared between the IRL and CHS populations using Fisher's exact test or chi-square (χ^2) for each pathogen from samples taken between 2003 and 2005. A Fisher's exact test was used when over 20% of the cells had an expected count of less than five or any cells had an expected count of less than one. The effect of year of capture on antibody prevalence was also examined using χ^2. Multivariate analysis, using unconditional logistic regression, was conducted to estimate odds ratios (OR) with their 95% confidence intervals (CI) and to examine the effect of age class, sex, and health status on antibody prevalence to each pathogen. Statistical analyses were conducted using *SPSS 16 for Windows* (SPSS Inc, Chicago, IL, USA) and *Epi Info* (CDC). Results were considered statistically significant at $p < 0.05$.

Results

Serologic data from captures of 122 individual dolphins (76 males and 46 females) from the IRL and 82 individual dolphins (48 males, 34 females) from CHS were evaluated (some dolphins were recaptured in subsequent years). The mean age (+ SD) was 11.6 + 5.3 y and 13.9 + 8.0 y in IRL and CHS, respectively (Table 2). Health status did not differ significantly between study sites. The prevalence of antibody to all antigens tested was not significantly different across categories of health status (data not shown).

The prevalence of antibodies to *B. abortus* at the two study sites was significantly different (rivanol: $p < 0.006$, card: $p < 0.001$). IRL dolphins had a higher prevalence of antibody to *Brucella* spp., 34.0% and 66.7% compared to CHS, 9.0% and 7.3% for the rivanol and card tests, respectively (Table 3). The results of testing in 2006 and 2007 are included in Table 3, but statistical analyses comparing prevalence across capture sites was

Table 1. Summary of serological tests and laboratories

Laboratory	Pathogen	Test	Positive titer
National Veterinary Services Laboratories (USDA)	EEE[1]	VNT[5]	≥ 1:10
	WEE[2]	VNT	≥ 1:10
	VEE[3]	VNT	≥ 1:10
	WNV[4]	VNT	
	Brucella spp.	Rivanol, card	≥ 1:25
University of Miami Clinical Laboratory	*Chlamydophila psittaci*	IFA[6]	≥ 1:50
	Toxoplasma gondii	IHAT[7]	≥ 1:64

[1]Eastern equine encephalitis, [2]Western equine encephalitis, [3]Venezuelan equine encephalitis, [4]West Nile virus, [5]virus neutralization test, [6]indirect fluorescent antibody test, [7]indirect hemagglutination test

Table 2. Characteristics of Atlantic bottlenose dolphins in the IRL and CHS

	IRL (n = 122)	CHS (n = 82)
Gender		
Male	76 (62.3)	48 (58.5)
Female	46 (37.7)	34 (41.5)
Mean age (y)	11.36 + 5.39	13.80 + 8.05
Age class		
Adult	79 (84.0)	55 (78.6)
Juvenile	15 (16.0)	15 (21.4)
Clinical assessment		
Normal	48 (39.3)	41 (50.0)
Concerned	33 (27.0)	25 (30.5)
Diseased	39 (28.4)	16 (19.5)
Not assigned	2 (1.6)	0

* 94 IRL and 70 CHS dolphins with known ages

restricted to the years when results were available for both sites. Antibodies to EEE, VEE, and WNV were detected in the IRL but not in dolphins from CHS. Low levels of seropositivity were found to WEE at both sites. The prevalence of antibodies to *C. psittaci*, and *T. gondii* was similar at both sites.

The frequency of finding antibody-positive dolphins for each pathogen was compared between capture years (Table 4). Annual seropositivity to all pathogens tested did not differ between capture years in the IRL or CHS populations individually. Pooled analysis for IRL and CHS data showed that the prevalence of antibody to *Brucella* using the card test was significantly different across years (p = 0.03) with a higher seroprevalence in 2004. The only evidence of antibody to EEE was obtained in the IRL in 2004, and antibodies to WNV were found only in 2003 in the IRL. In humans, *T. gondii* is widely distributed and poses a public health risk due to complications from infection during pregnancy (Elsheikha, 2008).

The relationship between antibody prevalence and gender and health status was assessed for each pathogen and was not found to be statistically significant. However, age class was a significant factor on the risk of seropositivity to *C. psittaci* with juveniles 3.6 × more likely (95% CI 1.35, 9.43) to test positive compared to adults from both study sites (data not shown).

Sera from recaptured dolphins were screened for seroconversion; only two animals (one from IRL and one from the CHS) converted from negative to positive titers against *C. psittaci*. No seroconversions to any other pathogens were detected among the 12 dolphins from which multiple sera were available.

Table 3. Statistical comparison of seroprevalence by capture site from 2003 to 2005

Pathogen	IRL*	CHS	p value**
Brucella abortus			
Rivanol			
Total tested	46	78	
Positive	17 (37.0)	7 (9.0)	0.01**
Card			
Total tested	82		120
Positive	80 (66.7)	6 (7.3)	< 0.01**
Eastern equine encephalitis			
Total tested	116	75	***
Positive	2 (1.7)	0	
Western equine encephalitis			
Total tested	116	75	0.67
Positive	3 (3.34)	3 (4)	
Venezuelan equine encephalitis			
Total tested	115	75	***
Positive	10 (8.7)	0	
West Nile virus			
Total tested	118	82	***
Positive	5 (4.24)	0	
C. psittaci			
Total tested	113	79	0.95
Positive	97 (85.84)	66 (83.54)	
T. gondii			
Total tested	86	79	0.51
Positive	8 (9.3)	12 (15.2)	

* Frequency reported from 2003 to 2007

**p values from chi-square tests for seroprevalence from 2003 to 2005 at both sites

*** Not calculated

Discussion

Serological evidence of exposure to all pathogens examined was found in one or both study populations of bottlenose dolphins. Of particular interest was the finding of antibodies to *Brucella* spp. in 37% of IRL dolphins. The marine strain of *Brucella* spp. was first isolated from common seals (*Phoca vitulina*), harbor porpoises (*Phocoena phocoena*), and a common dolphin (*Delphinus delphis*) off the coast of Scotland (Ross et al., 1994). The first isolate from a bottlenose dolphin was obtained from an aborted fetus in California (Ewalt et al., 1994). These isolates were genetically distinct from those previously described in terrestrial species. Characterization of *Brucella* from marine mammals has shown that separate species infect cetaceans and pinnipeds (Jahans et al., 1997; Kennedy, 1998; Cloeckaert et al., 2001; Bourg et al., 2007). Recent molecular typing suggests that at least three groups of marine *Brucella* exist and that a separate strain

Table 4. Pooled seroprevalence for IRL and CHS by pathogen for each year of collection*

	2003	2004	2005	*p* value**
Brucella abortus				
Rivanol	9 (13.6)	3 (11.5)	8 (33.3)	0.62
Card	27 (31.0)	29 (59.2)	9 (30.0)	0.03
Eastern equine encephalitis	0	1 (2.3)	0	***
Western equine encephalitis	2 (2.3)	3 (6.8)	0	0.21
Venezuelan equine encephalitis	1 (1.1)	4 (9.1)	0	0.02
West Nile virus	5 (5.7)	0	0	0.09
C. psittaci	72 (84.7)	40 (81.6)	27 (84.4)	0.89
T. gondii	8 (9.4)	9 (18.7)	3 (9.4)	0.31

* Number of positives and percent positive in parentheses
** *p* values from chi-square and Fisher's exact tests
*** Not calculated

infects dolphins and porpoises (Groussard et al., 2007).

The use of screening serological assays developed for antibodies to *Brucella* in terrestrial animals is known to have limitations when applied to marine mammals and the marine strains of *Brucella*. Enzyme-linked immunosorbent assay (ELISA) tests based on antigens obtained from marine *Brucella* show higher seropositivity rates than found with the terrestrial tests with seroprevalence approximately twice as high (Dunn et al., 2001; Meegan et al., 2006). The seroprevalence rate reported here is similar to that reported for three bottlenose dolphin populations (including the IRL) using the rivanol and card assays but lower than those found by indirect or competitive ELISA assays (Meegan et al., 2006). Thus, seroprevalence reported here may represent an underestimate of the true extent of exposure in the population.

The routes of transmission for marine *Brucella* spp. may include ingestion of feces-feeding fish and infestation with parasitic lungworms (*Parafilaroides inflexus*) (Dawson et al., 2008). Human infection with marine strains of *Brucella* is uncommon but has been reported in several patients (Sohn et al., 2003; McDonald et al., 2006; Whatmore et al., 2008). Potential sources of exposure for humans include consuming raw fish or shellfish, handling infected bait, and swimming (Whatmore et al., 2008). In one case in which the marine strain was identified, the likely source of infection did not include direct marine mammal contact (McDonald et al., 2006). Serological evidence of *Brucella* infection in IRL dolphins may indicate the potential for zoonotic transmission to humans who use the same resources for recreational activities such as fishing, boating, and swimming.

To our knowledge, these results represent the first serological evidence of EEE, WEE, VEE, and WNV exposure in cetaceans. Previous descriptions of arbovirus exposure in marine mammals have been limited to pinnipeds. Equine encephalitis virus was previously transmitted to the elephant seal (*Mirounga leonia*) population of Macquarie Island, Australia, by lice, suggesting that other marine mammals may be at risk for infection with arboviruses (La Linn et al., 2001; Kuno & Chang, 2005). WNV infections have been reported in two pinnipeds: (1) a fatal infection in a captive 12-y-old harbor seal in a New Jersey aquarium and (2) a nonfatal infection in a captive seal at the Detroit Zoo (Del Piero et al., 2006). The public health risk posed by these arboviruses has been well-documented.

The arboviruses examined in this study are commonly found in Florida; however, WEE and VEE are not common in South Carolina (Day et al., 1996). Infection with the VEE II strain has been frequently described in the Florida Everglades in humans (Calisher, 1994). High antibody prevalence in the absence of clinical disease found in long-term human residents of south Florida suggests that this endemic strain of VEE virus has low virulence in humans (Calisher, 1994). A similar situation may occur in marine mammals in coastal areas of Florida. The finding of antibodies to WEE could represent a cross-reaction with Highlands J virus (HJV) in the VNT (Douglas Pederson, pers. comm., 2008). HJV, unlike WEE, has previously been identified in the southeastern United States (Day et al., 1996). Serological evaluation of small mammals from Indian River County, Florida, which borders the IRL, showed neutralizing antibody titers to SLE (46%), EEE (24%), HJV (3.2%), and Everglades virus, a member of the VEE complex (14%) (Day et al., 1996). Further investigations should include screening for HJV and WEE to distinguish between these two closely related alphaviral infections, searching for active infections by repeat sampling, histologic

examination of brain tissue, and virus isolation attempts from selected animals.

The only previously reported alpha-virus infection in a cetacean was a single case of St. Louis encephalitis virus (SLE) isolated from a captive killer whale (*Orcinus orca*) (Buck et al., 1993). The possibility exists that our reported titers to WNV could represent a cross-reaction on the VNT. The probability of a cross-reaction with another flavivirus is dependent on how active other viruses are in the IRL region (D. Pederson, pers. comm., 2008). The Centers for Disease Control and Prevention (CDC) (2008) did not report any human cases of SLE during the study period in counties adjacent to the IRL and CHS study sites, but human cases of WNV infection and nonhuman WNV activity (sentinel birds) occurred throughout Florida in 2001, 2002, and 2003. Therefore, it is unlikely that WNV titers represent cross-reactions with SLE or other arboviruses. SLE and WNV are zoonotic pathogens that cause the highest morbidity and mortality rates in children and older adults (CDC, 2006, 2008). Evidence of exposure to these pathogens in the dolphin population may indicate mosquito bite transmission to dolphins from infected bird reservoirs in the same geographical regions as human case activity.

To our knowledge, this is the first reported evidence of exposure to *C. psittaci* in a cetacean species. Prevalence of antibody to *C. psittaci* was high, 85.8% and 83.5% for IRL and CHS dolphins, respectively. Antibodies to *C. psittaci* have been described in Steller sea lions (*Eumetopias jubatus*) in the northern Pacific with seroprevalence increasing to > 60% in adults (Burek et al., 2005). Antibody to *Chlamydophila* was also detected in a seroepidemiologic study of Hawaiian monk seals (*Monachus schauinslandi*) with higher seroprevalence among adult seals (Aguirre et al., 2007). *C. psittaci* causes a potentially fatal infection in humans and is transmitted by inhalation and contact with or ingestion of infected tissues (Eugster, 1980). Migratory birds could play a major role in the distribution of *C. psittaci* as chlamydiosis has been identified in gulls, terns, waterfowl, and other shore birds and may be nonpathogenic in wild avian species (Hubalek, 2004). The high seroprevalence of antibody to *C. psittaci* in both dolphin populations might be explained by extensive shedding of the agent among local bird populations. It is unclear as to why younger animals were at a greater risk of exposure. Future studies to establish prevalence of exposure in local bird populations could provide insight on the source of exposure for dolphins and the mechanisms of transmission between reservoirs and marine mammals.

Toxoplasma infection is widely distributed in marine mammals, including sea otters (*Enhydra lutris*), dolphins, seals, and whales (Dubey et al., 2003; Kreuder et al., 2003; Conrad et al., 2005; Thomas et al., 2007). Clinically apparent toxoplasmosis has been reported in bottlenose dolphins from the United States (Migaki et al., 1977; Inskeep et al., 1990; Dubey et al., 2003) and Italy (Di Guardo et al., 1995a, 1995b), and may be accompanied by immune suppression due to morbillivirus infection (Shulman et al., 1997). Recently, *T. gondii* was isolated from stranded dolphins along the South Carolina coast by bioassay in mice and cats, but test sensitivity for cetaceans was not described (Dubey et al., 2008). Serological evidence of infection (13.3% using IHAT) was previously described in dolphins sampled in the IRL and Charleston Harbor in 2004 (Dubey et al., 2005). In this more expanded sampling, antibodies were found in 15.2% of IRL dolphins and 9.3% of CHS dolphins.

High rates of seropositivity among dolphins in the IRL and CHS populations suggest that exposure could be the result of contamination of coastal estuaries with effluents containing oocysts. Similarly, *T. gondii* infection and mortality occurs frequently in southern sea otters (*E. l. nereis*) along the California coast in proximity to human population centers (Miller et al., 2004; Jessup et al., 2007). These findings suggest that environmental contamination with *T. gondii* oocysts may also pose a public health risk for humans in adjacent areas due to complications from infection during pregnancy (Elsheikha, 2008).

The HERA Project was designed as a cross-sectional study with corresponding limitations, including limited opportunity for resampling and longitudinal follow-up for individuals. Therefore, it is difficult to establish rates at which new infections occur and to evaluate rates over time. The ability to assess the clinical effects of pathogen exposure directly was not possible since antibodies may represent infections that occurred years ago and have resolved. The lack of differences in health status between seropositive and seronegative individuals at the group level suggests that none of the agents studied played an important role in population health. However, the possibility that exposure to these agents results in differential mortality and that affected animals are not sampled cannot be excluded. In addition, the serologic tools used in this study have not been adequately validated in marine mammals and, therefore, their sensitivity and specificity are unknown. It is conceivable that some of the positive tests were false positives due to crossreactions or other, nonspecific responses. It is also possible that some tests such as the rivanol and card tests used for *Brucella* serology were not adequately sensitive (Meegan et al., 2006). We were limited by lack

of availability of newer, validated screening tests specific to marine mammals for these assays and, therefore, serological data must be interpreted cautiously.

Despite these limitations, baseline seroprevalence data such as those reported here are useful in establishing prior exposures and reinforce the importance of sentinel species such as bottlenose dolphins for monitoring marine ecosystem health (Bossart, 2006). The proximity of these coastal dolphin populations to human habitation suggests that detection of antibodies to zoonotic pathogens may provide a warning system for human infection. Future collections of serological data for pathogen surveillance will aid in determining the potential for epizootics or more gradual changes in the prevalence of infection that have implications for the health of the surrounding ecosystem.

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