



Clinicoimmunopathologic findings in Atlantic bottlenose dolphins *Tursiops truncatus* with positive *Chlamydiaceae* antibody titers

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ABSTRACT: Sera from free-ranging Atlantic bottlenose dolphins *Tursiops truncatus* inhabiting the Indian River Lagoon (IRL), Florida, and coastal waters of Charleston (CHS), South Carolina, USA, were tested for antibodies to *Chlamydiaceae* as part of a multidisciplinary study of individual and population health. A suite of clinicoimmunopathologic variables was evaluated in *Chlamydiaceae*-seropositive dolphins (n = 43) and seronegative healthy dolphins (n = 83). Fibrinogen, lactate dehydrogenase, amylase, and absolute numbers of neutrophils, lymphocytes, and basophils were significantly higher, and serum bicarbonate, total alpha globulin, and alpha-2 globulin were significantly lower in dolphins with positive *Chlamydiaceae* titers compared with seronegative healthy dolphins. Several differences in markers of innate and adaptive immunity were also found. Concanavalin A-induced T lymphocyte proliferation, lipopolysaccharide-induced B lymphocyte proliferation, and granulocytic phagocytosis were significantly lower, and absolute numbers of mature CD 21 B lymphocytes, natural killer cell activity and lysozyme concentration were significantly higher in dolphins with positive *Chlamydiaceae* antibody titers compared to seronegative healthy dolphins. Additionally, dolphins with positive *Chlamydiaceae* antibody titers had significant increases in ELISA antibody titers to *Erysipelothrix rhusiopathiae*. These data suggest that *Chlamydiaceae* infection may produce subclinical clinicoimmunopathologic perturbations that impact health. Any potential subclinical health impacts are important for the IRL and CHS dolphin populations, as past studies have indicated that both dolphin populations are affected by other complex infectious and neoplastic diseases, often associated with immunologic perturbations and anthropogenic contaminants.

KEY WORDS: Bottlenose dolphin · *Chlamydiaceae* antibody · Seroepidemiology · Clinical pathology · Immunology

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INTRODUCTION

Chlamydiaceae are a family of obligate, Gram-negative bacteria that may cause ocular, pulmonary, genital, articular, and/or intestinal disease in humans and a wide range of domestic and wildlife animal species (Sprague et al. 2009). *Chlamydiaceae* infections are complex and may be subclinical, acute, or chronic (with or without intermittent bacterial shedding) (Longbottom & Coulter 2003). The various disease stages induced by infection with *Chlamydiaceae* are mediated by the immune response which in some instances may be ineffective in resolution of infection and actually contribute to disease progression (Mygind et al. 1998). Ultimately, the control of chlamydial infection is an immunologic challenge to the host given the agent's unique extracellular infectious and intracellular vegetative phases of development (Rodolakis & Yousef Mohamad 2010).

The Family *Chlamydiaceae* contains 2 genera, *Chlamydia* and *Chlamydophila*, with 9 species: 3 in the genus *Chlamydia* (*C.*), *C. trachomatis*, *C. muridarum*, and *C. suis*; 2 in the genus *Chlamydophila* (hereafter *Cp.*), *Cp. pneumoniae* and *Cp. pecorum*; and 4 species that corresponded to the previous *C. psittaci* (the avian pathogen *Cp. psittaci*, the ruminant pathogen *Cp. abortus*, the guinea pig pathogen *Cp. caviae*, and the feline pathogen *Cp. felis*) (Rodolakis & Yousef Mohamad 2010). Two species are of particular interest, namely *Cp. psittaci* and *Cp. abortus*, as they are zoonotic and may produce life-threatening pneumonia in humans (Longbottom & Coulter 2003). Additionally, *Cp. pneumoniae*, which is frequently isolated from humans, has been found in a range of species, including the horse, the koala *Phascolarctos cinereus* and in various amphibians and reptiles (Bodetti et al. 2002).

Clinical disease due to *Chlamydiaceae* has not been reported in marine mammals. However, antibodies to *Chlamydophila psittaci* were described in Steller sea lions *Eumetopias jubatus* in the northern Pacific with a seroprevalence of >60% in adults, suggesting that *Cp. psittaci*, or a closely related agent, was endemic in that population (Burek et al. 2005). Antibody to *Cp. abortus* was also detected in a seroepidemiologic study of Hawaiian monk seals *Monachus schauinslandi*, with a higher seroprevalence among adult seals (Aguirre et al. 2007). We recently reported the first evidence of exposure to *Cp. psittaci*, or a closely related *Chlamydiaceae*, in a cetacean species as part of a multidisciplinary and multi-institutional free-ranging Atlantic bottlenose dolphin *Tursiops truncatus* health assessment pro-

gram along the southeastern Atlantic coast of the USA (Schaefer et al. 2009). In this study, the seroprevalence of *Cp. psittaci* was 85.8% in bottlenose dolphins from the Indian River Lagoon, Florida (IRL), and 83.5% in the estuarine waters near Charleston (CHS), South Carolina. We postulated that the high seroprevalence of *Cp. psittaci* antibody in dolphins from these locations might be explained by extensive shedding of the agent among local bird populations, since *Cp. psittaci* has been identified in gulls, terns, waterfowl, and other shore birds and may be non-pathogenic in wild avian species (Hubálek 2004).

Unanswered questions regarding the pathogenesis of *Chlamydiaceae* infection in dolphins remain, including the clinicoimmunologic effects of subclinical infection and the potential secondary impacts these changes may have on dolphin and ecosystem health. Additionally, nothing is known about protective immunity against chlamydial infection in dolphins. Answering these questions is especially important for the IRL and CHS dolphin populations, as past studies indicate that the prevalence of disease is high among dolphins at both sites (Reif et al. 2008). The purpose of this report was to evaluate the clinicoimmunopathologic findings in IRL and CHS bottlenose dolphins with positive *Chlamydiaceae* antibody titers.

MATERIALS AND METHODS

Free-ranging dolphins

The Atlantic bottlenose dolphin *Tursiops truncatus* Health and Environmental Risk Assessment (HERA) project was initiated as a multidisciplinary, integrated, collaborative effort in 2003 to assess individual and population health in 2 southeast coastal regions of the USA: CHS and the IRL (Bossart et al. 2006). In part, the goals of the project are to develop standardized tools for health and risk assessment and to explore associations between health status and environmental stressors. As an apex predator, bottlenose dolphins serve as a sentinel species for monitoring the health of the environment and may provide valuable information for evaluating the relationship between exposure to biological and chemical agents and the adverse health effects for these populations (Bossart 2011).

The IRL is a shallow-water ecosystem that comprises 40% of Florida's central east coast. The lagoon is an aggregate of 3 estuarine water bodies, the Indian and Banana Rivers and the Mosquito Lagoon,

and extends 250 km from Ponce De Leon Inlet in the north to Jupiter Inlet in the south. The CHS site is an estuarine environment in the central region of South Carolina's coastal zone that includes the Charleston Harbor, as well as portions of the Ashley River, Cooper River, Wando River, and Stono River estuary. Photo-identification survey data indicate that dolphins at both sites display long-term residency patterns and site fidelity (Speakman et al. 2006, Mazzoil et al. 2008).

Dolphins were captured in the IRL during June each year from 2003 to 2010 and in the waters near CHS during August each year from 2003 to 2005. Sampling was conducted in specific areas within each site. Standard operating protocols and techniques used for capture, sample collection, and release of dolphins are described in detail elsewhere (Fair et al. 2006a). Health status was determined by a panel of marine mammal veterinarians, as previously described, and classified as clinically healthy, possibly diseased, or definitely diseased (Reif et al. 2008). Health status was based on physical and ultrasound examinations, hematology, serum chemistry, gastric, blowhole and fecal cytology, urinalysis, and microbiologic evaluation of blowhole and rectal contents. Age was estimated by counting post-natal dentine layers in an extracted tooth (Hohn et al. 1989). All methods used in HERA for capture and sample collection were approved under National Marine Fisheries Service Scientific Research Permit Nos. 998-1678 and 14352-02 issued to G. Bossart and Florida Atlantic University IACUC Protocol Number A10-13.

Blood collection

Blood samples were drawn from the periarterial venous rete in the flukes (Bossart et al. 2001), generally within the first 15 min of capture, with a 19-gauge, 1.9 cm butterfly catheter (Becton Dickinson). Serum was collected in 10 ml separator vacutainer tubes (Becton Dickinson), placed in a cooler for 20 to 40 min, and centrifuged for 15 min at 1200 rpm (350 ×g). Samples for hematology and immunology were collected in a vacutainer tube with ethylene diamine tetraacetic acid (EDTA) or sodium heparin as an anticoagulant, respectively (Becton Dickinson). Samples for hematology, serum chemistry, and serum protein electrophoresis analyses were stored in an insulated cooler and shipped overnight to the Cornell University Veterinary Diagnostic Laboratory in Ithaca, New York, USA. Samples for immune assays (heparinized whole blood or serum as re-

quired) were stored in an insulated cooler and shipped overnight to Mystic Aquarium, a division of Sea Research Foundation, Mystic, Connecticut, USA, and to the NOAA Ocean Service, Charleston, South Carolina, USA, for analysis.

Hematology, serum chemistry, and serum protein electrophoresis

For the complete blood count, relative leukocyte proportions were performed by microscopic examination of modified Wright-stained blood smears (Bayer Healthcare). A microhematocrit tube was centrifuged for 5 min at 11 700 rpm (28 977 ×g), and the manual hematocrit was interpreted by visual inspection against a standard calibration. Automated hemoglobin, red blood cell count, mean corpuscular platelet volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), white blood cell count (WBC), and total platelets were determined by an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics). The concentrations of serum chemistry analytes were determined with an automated analyzer (Hitachi 917, Roche) and included analyses for sodium, potassium, chloride, bicarbonate, anion gap, blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus, magnesium, glucose, total direct and indirect bilirubin, cholesterol, triglycerides, and iron. Enzyme activity was determined for alanine aminotransferase (ALT), alkaline phosphatase, aspartate amino transferase (AST), creatine phosphokinase (CPK), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), amylase, and lipase. Fibrinogen concentration was determined by the method of Schalm using heat precipitation. Serum protein electrophoresis was done on an automated analyzer (Rapid Electrophoresis, Helena Laboratories). Hemolyzed or lipemic samples were not included.

Immune assays

The methods used for immune assays and the sources of chemicals, reagents, and antibodies were described previously (Reif et al. 2009, Bossart et al. 2011). For assessment of natural killer (NK)-cell activity, dolphin major histocompatibility complex (MHC) Class II molecules, immunophenotyping, and lymphocyte proliferation, peripheral blood leukocytes (PBLs) were isolated by a slow-spin technique within 36 h of blood collection, as previously

described (Keller et al. 2006) with minor modifications, counted and assessed for viability, and subsequently diluted as described for each endpoint.

Immunophenotyping

Lymphocyte subsets were labeled and analyzed according to methods described previously (Romano et al. 2004, Bossart et al. 2011). Briefly, 1×10^6 cells ml^{-1} were labeled with 50 μl of monoclonal supernatant for 30 min at 4°C , followed by fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-mouse F(ab)'2 immunoglobulin G (IgG) for 30 min at 4°C in the dark. Cells were resuspended in 500 μl of 1% paraformaldehyde for analysis by flow cytometry and analyzed on an LSR flow cytometer (BD Biosciences). Tenthousand lymphocyte-gated events were analyzed by histogram statistics.

Phagocytosis

The percent phagocytosis for phagocytic cell types (i.e. granulocytes and monocytes) was determined using a technique previously described (Keogh et al. 2011). Briefly, cells were incubated with 10 μl of 2.1×10^9 units ml^{-1} of heat-killed *Staphylococcus aureus* previously labeled with 100 $\mu\text{g ml}^{-1}$ of propidium iodide added to each tube for a 25:1 bacteria:cell ratio and incubated in a shaking 37°C water bath (100 rpm) for 0 and 75 min. The experiment was stopped by adding 10 μl of 1 mM N-ethylmaleimide (NEM) to each tube, and red blood cells were lysed by the addition of 1 ml of lysis buffer (0.01 M Tris/0.001 M EDTA/0.17 M NH_4Cl , pH 7.4). Cells were resuspended in 250 μl of 1% cold paraformaldehyde, pH 7.4, and analyzed on an LSR flow cytometer (BD Biosciences). A total of 100 000 gated cells (granulocytes and monocytes) were analyzed by histogram statistics.

Lysozyme activity

Lysozyme activity, another measure of innate immunity, was assessed using slight modifications of a standard turbidity assay (Keller et al. 2006). A solution of *Micrococcus lysodeikticus* was prepared fresh daily by dissolving 50 mg of the lyophilized cells in 100 ml of 0.1 M phosphate buffer (pH 5.9). Hen egg lysozyme (HEL; Sigma) was serially diluted in phosphate buffer to produce a standard curve of 40, 20,

10, 5, 2.5, 1.25, 0.6, 0.3, and 0 $\mu\text{g ml}^{-1}$. Aliquots of each concentration (25 $\mu\text{l well}^{-1}$) were added to a 96-well plate in triplicate along with 25 μl of test plasma in quadruplicate; 175 $\mu\text{l well}^{-1}$ of *M. lysodeikticus* (50 mg of cells in 100 ml of 0.1 M phosphate buffer [pH 5.9]) was added to 3 sample wells and to each of the standard wells, while the fourth well served as a blank containing only phosphate buffer. Plates were assessed for absorbance at 450 nm with a spectrophotometer (SpectraCount, Packard) immediately (T0) and again after 5 min (T5). Absorbance unit values were converted to HEL concentration ($\mu\text{g ml}^{-1}$) via linear regression of the standard curve.

Mitogen-induced lymphocyte proliferation (LP)

The LP response was measured using methods optimized previously (Peden-Adams & Romano 2005). Briefly, isolated viable PBLs (1×10^5 cells well^{-1}) were incubated in 96-well plates with 2.5 $\mu\text{g ml}^{-1}$ concanavalin A (Con A; Type IV-S; a T-cell mitogen), 120 $\mu\text{g ml}^{-1}$ lipopolysaccharide (LPS; *Escherichia coli* 055:B5; a B-cell mitogen), or supplemented RPMI-1640 (unstimulated wells) in triplicate. Plates were incubated for 96 h at 37°C and 5% CO_2 followed by addition of 0.5 μCi of tritiated thymidine well^{-1} . Then, 16 h later, cells were harvested and analyzed using a Packard Top Count™-NXT scintillation counter (Packard).

NK cell activity

NK cell activity was assessed via an *in vitro* cytotoxicity assay using ^{51}Cr -labeled Yac-1 cells, as described previously with slight modifications (Peden-Adams et al. 2007). PBLs (1×10^7 nucleated cells ml^{-1}) and ^{51}Cr -labeled Yac-1 cells were prepared, in triplicate, in ratios of 100:1, 50:1, and 25:1. After 6 h of incubation at 37°C and 5% CO_2 , the plates were centrifuged ($377 \times g$; 5 min), and 25 μl of supernatant was then transferred to a 96-well plate containing solid scintillant (LumaPlate™). Plates were air dried overnight, and then counted for 5 min, after a 10 min dark delay, on the Packard Top Count™-NXT (Packard).

Antibody titers against marine bacteria

As an indication of a general humoral response to common marine bacteria, specific antibody titers

against select common marine bacteria were determined in an ELISA system as described previously (Bossart et al. 2008). Cultures of the following bacteria were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia, USA) and cultured in their recommended broths: *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *Mycobacterium marinum*, *Vibrio cholerae*, *V. carchariae*, *V. vulnificus*, and *V. parahemolyticus*. Calculations of serum antibody titers from individual dolphins following ELISAs were then expressed in terms of antibody titers at a 1:200 serum dilution (Karsten & Rice 2004).

***Chlamydiaceae* serology**

Traditional indirect fluorescent antibody methods for *Chlamydiaceae* serology were utilized at the Avian and Wildlife Laboratory at the University of Miami's Miller School of Medicine as previously described (Cray & Bonda 2005). Briefly, *Chlamydia trachomatis*-infected McCoy cells (ATCC) were grown on multimicrowell indirect fluorescent antibody (IFA) slides under 5% CO₂ and 37°C in supplemented Eagles media. After reaching the inclusion stage of infection, slides were fixed in acetone and stored at -70°C until use. To perform the test, slides were warmed to room temperature and phosphate-buffered saline (PBS)-diluted samples were overlaid. After incubation at 37°C and a PBS wash, a FITC-conjugated polyclonal antiserum was incubated for an additional period. Slides were washed and counterstained with Evan's blue (Sigma). The antiserum was purchased commercially as FITC-conjugated rabbit anti-dolphin IgG—heavy and light chain (Bethyl Laboratories). Specific reactivity was evaluated by fluorescence microscopy versus positive and negative controls.

Chlamydia trachomatis was selected for its ease of growth and lipopolysaccharide and outer membrane protein 2 (Omp2) which are shared with other species of *Chlamydia* and *Chlamydophila* (Brade et al. 1987, Vanrompay et al. 1995, Mygind et al. 1998). These genus-specific antigens are known to be the primary targets of cell-mediated immune responses and resultant neutralizing antibodies. This method has been used in a reference laboratory with samples from confirmed cases of *C. psittaci* and also reported to be correlative with the *Chlamydophila* elementary body agglutination serology assay and other alternate research serological methods (Salinas et al. 1993, Cray & Bonda 2005). In that setting, titers of >1:5 were considered to be positive. In this study,

titers of ≥1:50 were evaluated, since these higher titers were considered to be more indicative of recent infection, re-infection, or chronic persistent infection (C. Cray pers. obs.). In an avian study using the indirect immunofluorescent method, higher titers were associated with the isolation of the organism by cell or egg culture (Salinas et al. 1993). Additionally, higher titers were associated with active infection in birds using the complement-fixation method (Grimes 1984).

Study design and statistical analyses

For purposes of this study, 126 animals were selected from dolphins sampled during the HERA study between 2003 and 2010 using a case control approach. Affected dolphins (n = 43) were defined as those with a positive antibody titer of ≥1:50 to *Chlamydiaceae*. A healthy control group (n = 83) was selected from seronegative animals after eliminating those with definite or probable evidence of disease based on clinicopathologic parameters (Bossart et al. 2008, Reif et al. 2008). All analyses were done using SPSS Version 20 (IBM). Mean ages of the groups were compared with a *t*-test, and the distributions of gender and study site were compared by chi-squared analysis to check for potentially confounding results. Descriptive statistics including means and standard deviations of clinical and immunologic parameters were determined for animals with antibodies to *Chlamydiaceae* and healthy controls. Differences between groups for clinical and immunologic parameters were compared initially using univariate analysis of variance after determining whether the data were normally distributed by the Shapiro-Wilk test. The Mann-Whitney *U*-test was used to compare the distributions of variables that were not normally distributed. Because the mean age of seropositive cases and healthy controls was significantly different (p = 0.04), a multivariable analysis of variance (MANOVA) was then conducted including age as a covariate. A p-value of ≥0.05 was considered statistically significant.

RESULTS

Forty-three dolphins *Tursiops truncatus* (31 IRL/12 CHS) had a positive antibody titer of ≥1:50 to *Chlamydiaceae*, representing 26 males and 17 females with a similar site prevalence. Eighty-three dolphins (50 IRL/33 CHS) represented the healthy *Chlamydi-*

aceae seronegative control group. The mean ages (\pm SD) of seropositive dolphins and healthy seronegative dolphins were 14.40 ± 7.72 and 11.20 ± 6.37 yr, respectively. Dolphins with a positive antibody titer of $\geq 1:50$ to *Chlamydiaceae* had multiple clinicoimmunologic abnormalities when compared to healthy dolphins.

Hematology and serum chemistry results for dolphins with positive *Chlamydiaceae* antibody titers were generally similar to those of healthy dolphins. However, after adjusting for age, statistically significant changes were found for 7 variables (Table 1). Fibrinogen, LDH, amylase, and absolute numbers of neutrophils, lymphocytes, and basophils were significantly higher, and serum bicarbonate was significantly lower in dolphins with positive *Chlamydiaceae* titers compared with healthy dolphins. No significant differences were found in the remaining hematological and serum chemistry results.

Several differences in markers of innate and adaptive immunologic were found (Table 2). Con A-induced T lymphocyte proliferation, LPS-induced B lymphocyte proliferation, and granulocytic phagocytosis were significantly lower in dolphins with positive *Chlamydiaceae* titers compared to healthy dolphins. Notably, LPS stimulation of B cells was reduced to approximately 23% of the value for healthy dolphins ($p < 0.01$), while Con A stimulation of T cells was reduced to approximately 65% of the healthy value ($p = 0.02$). Absolute numbers of mature CD 21 B lymphocytes, NK activity, and lysozyme concentration were significantly higher in dolphins with positive *Chlamydiaceae* antibody titers compared to healthy dolphins.

Dolphins with positive *Chlamydiaceae* antibody titers had significant increases in ELISA antibody titers to *Erysipelothrix rhusiopathiae* compared to seronegative healthy dolphins (Table 3). No significant differences in antibody titers to *Escherichia coli*, *Mycobacterium marinum*, *Vibrio cholerae*, and *V. para-*

hemolyticus were found. Antibodies to *V. carchariae* and *V. vulnificus* were not found in affected dolphins.

Serum protein electrophoresis data are shown in Table 4. Total alpha globulin and alpha-2 globulin were significantly lower dolphins with positive *Chlamydiaceae* titers compared with healthy dolphins. No significant differences were found in the remaining serum protein electrophoresis data.

Table 1. *Tursiops truncatus*. Mean (\pm SD) values for hematology and serum chemistry analytes in bottlenose dolphins seronegative and seropositive ($\geq 1:50$) for antibodies to *Chlamydiaceae*. **Bold values:** statistically significant at $p \leq 0.05$

Variable	Seronegative (n = 83)	Seropositive (n = 43)	Age-adjusted p-value
Glucose (mg dl ⁻¹)	95.44 (15.42)	96.39 (17.05)	0.38
Sodium (mEq l ⁻¹)	155.10 (0.35)	155.31 (0.53)	0.68
Potassium (mEq l ⁻¹)	3.84 (0.04)	3.93 (0.06)	0.29
Chloride (mEq l ⁻¹)	114.12 (0.40)	113.87 (0.57)	0.77
Bicarbonate (mEq l ⁻¹)	22.16 (0.50)	20.80 (0.74)	0.02
Anion gap	22.69 (0.67)	24.47 (0.96)	0.13
Blood urea nitrogen (mg dl ⁻¹)	60.34 (1.05)	63.31 (1.49)	0.14
Creatinine (mg dl ⁻¹)	1.13 (0.03)	1.03 (0.04)	0.15
Fibrinogen (mg dl ⁻¹)	96.12 (11.33)	218.80 (14.47)	<0.01
Total bilirubin (mg dl ⁻¹)	0.10 (0.01)	0.09 (0.01)	0.36
Direct bilirubin (mg dl ⁻¹)	0.02 (0.04)	0.01 (0.01)	0.66
Indirect bilirubin (mg dl ⁻¹)	0.08 (0.01)	0.08 (0.01)	0.64
Calcium (mg dl ⁻¹)	9.18 (0.06)	9.18 (0.08)	0.97
Phosphorus (mg dl ⁻¹)	4.91 (0.09)	4.98 (0.13)	0.69
Magnesium (mg dl ⁻¹)	1.42 (0.02)	1.46 (0.02)	0.20
Uric acid (mg dl ⁻¹)	0.73 (0.08)	0.05 (0.54)	0.20
Alkaline phosphatase (U l ⁻¹)	259.31 (14.64)	283.31 (21.28)	0.48
Alanine aminotransferase (U l ⁻¹)	41.57 (2.17)	40.93 (8.70)	0.94
Aspartate aminotransferase (U l ⁻¹)	234.37 (9.41)	237.14 (10.36)	0.78
Lactate dehydrogenase (U l ⁻¹)	492.04 (20.31)	715.18 (139.49)	<0.01
Creatine phosphokinase (U l ⁻¹)	161.80 (5.22)	157.12 (7.76)	0.52
Amylase (U l ⁻¹)	1.85 (0.34)	14.80 (2.15)	<0.01
Lipase (U l ⁻¹)	12.32 (3.04)	19.04 (15.57)	0.17
Gamma glutamyltransferase (U l ⁻¹)	26.00 (0.58)	27.97 (0.87)	0.07
Cholesterol (mg dl ⁻¹)	156.34 (4.96)	178.78 (31.37)	0.52
Triglyceride (mg dl ⁻¹)	82.36 (3.58)	85.69 (4.94)	0.63
Iron (μ g dl ⁻¹)	94.26 (4.19)	90.33 (6.17)	0.58
White blood cells (10 ³ cells μ l ⁻¹)	10.55 (0.33)	10.90 (0.44)	0.48
Neutrophils (10 ³ cells μ l ⁻¹)	4.29 (0.86)	8.36 (1.45)	0.03
Band neutrophils (10 ³ cells μ l ⁻¹)	0.08 (0.05)	0.02 (0.09)	0.37
Lymphocytes (10 ³ cells μ l ⁻¹)	2.24 (0.25)	3.27 (0.41)	0.05
Monocytes (10 ³ cells μ l ⁻¹)	0.30 (0.03)	0.33 (0.04)	0.57
Eosinophils (10 ³ cells μ l ⁻¹)	3.81 (0.60)	6.06 (1.10)	0.07
Basophils (10 ³ cells μ l ⁻¹)	0.07 (0.01)	0.03 (0.01)	0.01
Erythrocytes (10 ⁶ cells μ l ⁻¹)	3.60 (0.03)	3.49 (0.04)	0.07
Hemoglobin (g dl ⁻¹)	14.10 (0.13)	13.86 (0.18)	0.30
Manual hematocrit (%)	40.49 (0.38)	39.46 (0.54)	0.14
Mean corpuscular volume (fl)	112.63 (0.91)	113.40 (1.32)	0.70
Mean corpuscular hemoglobin (pg)	39.23 (0.29)	39.67 (0.41)	0.45
Mean corpuscular hemoglobin concentration (g dl ⁻¹)	34.81 (0.18)	35.11 (0.27)	0.40
Platelets (10 ³ μ l ⁻¹)	187.90 (5.79)	187.93 (8.32)	0.98

Table 2. *Tursiops truncatus*. Mean (\pm SD) values for immunologic parameters in bottlenose dolphins seronegative and seropositive ($\geq 1:50$) for antibodies to *Chlamydiaceae*. **Bold values:** statistically significant at $p \leq 0.05$

Immune parameter	Seronegative (n = 83)	Seropositive (n = 43)	Age-adjusted p-value
CD2 T cells (absolute nos.)	867.09 (77.77)	856.90 (11.24)	0.96
CD4 helper T cells (absolute nos.)	387.79 (34.79)	436.40 (46.32)	0.38
CD 19 B cells — immature (absolute nos.)	519.71 (54.01)	427.91 (83.89)	0.35
CD 21 B cells — mature (absolute nos.)	708.98 (82.62)	1129.81 (162.07)	0.03
CD2/CD4 ratio	2.50 (1.17)	2.40 (1.27)	0.30
CD2/CD21 ratio	1.48 (0.20)	1.85 (1.09)	0.39
MHCII+ (absolute nos.)	1565.91 (126.71)	1909.70 (183.35)	0.13
T cell proliferation (concanavalin A, 2.5 $\mu\text{g ml}^{-1}$)	551.64 (47.32)	356.82 (67.22)	0.02
B cell proliferation (lipopolysaccharide, 120 $\mu\text{g ml}^{-1}$)	111.97 (17.31)	25.83 (23.66)	<0.01
Immunglobulin G1 (mg ml^{-1})	11.73 (0.62)	10.98 (0.41)	0.18
Granulocytic phagocytosis (%)	23.74 (1.83)	17.91 (2.21)	0.05
Monocytic phagocytosis (%)	18.65 (1.71)	21.67 (2.08)	0.28
Natural killer cell activity (100:1)	6.80 (1.26)	11.53 (1.56)	0.02
Lysozyme concentration ($\mu\text{g } \mu\text{l}^{-1}$)	6.22 (0.41)	8.84 (0.54)	<0.01

Table 3. *Tursiops truncatus*. Mean (\pm SD) antibody titers ($\text{U } \mu\text{l}^{-1}$) against common marine bacteria in bottlenose dolphins seronegative and seropositive ($\geq 1:50$) for antibodies to *Chlamydiaceae*. **Bold values:** statistically significant at $p \leq 0.05$

Antigen	Seronegative (n = 83)	Seropositive (n = 43)	Age-adjusted p-value
<i>Escherichia coli</i>	151.28 (21.28)	182.52 (84.29)	0.77
<i>Erysipelothrix rhusiopathiae</i>	143.80 (20.20)	309.24 (76.36)	0.05
<i>Mycobacterium marinum</i>	142.04 (19.83)	234.54 (77.29)	0.28
<i>Vibrio carchariae</i>	230.02 (2.78)	—	—
<i>Vibrio cholerae</i>	155.50 (122.04)	223.60 (84.88)	0.44
<i>Vibrio parahaemolyticus</i>	166.67 (23.99)	204.96 (92.61)	0.75
<i>Vibrio vulnificus</i>	231.71 (5.15)	—	—

Table 4. *Tursiops truncatus*. Mean (\pm SD) values for serum protein electrophoresis analytes in bottlenose dolphins seronegative and seropositive ($\geq 1:50$) for *Chlamydiaceae*. **Bold values:** statistically significant at $p \leq 0.05$

Analyte	Seronegative (n = 83)	Seropositive (n = 43)	Age-adjusted p-value
Total protein (g dl^{-1})	7.25 (0.07)	7.20 (0.09)	0.73
Albumin (A) (g dl^{-1})	3.64 (0.04)	3.70 (0.05)	0.38
Total globulin (G) (g dl^{-1})	2.78 (0.07)	2.90 (0.09)	0.31
A:G ratio	1.73 (0.59)	1.51 (0.35)	0.21
Total alpha globulin (g dl^{-1})	1.31 (0.03)	1.13 (0.03)	<0.01
Alpha-1 globulin (g dl^{-1})	0.39 (0.02)	0.39 (0.03)	0.94
Alpha-2 globulin (g dl^{-1})	0.92 (0.03)	0.74 (0.04)	<0.01
Total beta globulin (g dl^{-1})	0.46 (0.01)	0.44 (0.01)	0.38
Gamma globulin (g dl^{-1})	1.78 (0.06)	1.95 (0.09)	0.12

DISCUSSION

Multiple statistically significant differences between seronegative and seropositive dolphins were found in hematological, serum chemistry, and serum protein electrophoresis analytes. This constitutes the first evidence that infection of wild bottlenose dolphins *Tursiops truncatus* with *Chlamydiaceae* induces a variety of clinicopathologic and immunologic disturbances in this cetacean species. Fibrinogen, LDH, amylase, and absolute numbers of neutrophils and lymphocytes were significantly higher, and bicarbonate, total alpha globulin, alpha-2 globulin, and basophils were significantly lower in dolphins with positive *Chlamydiaceae* antibody titers compared to healthy dolphins. However, with the exception of amylase, the mean values for dolphins with positive *Chlamydiaceae* antibody titers and healthy dolphins were within the range for healthy dolphins from the same populations determined in previous studies (Fair et al. 2006b, Goldstein et al. 2006). Thus, the findings for these markers are probably not clinically significant. The cause of elevated serum amylase in dolphins with positive *Chlamydiaceae* antibody titers is unknown, as this analyte is not

well-studied in dolphins. The amylase elevation could reflect low-grade pancreatic inflammation based on limited past studies (Bossart et al. 2001).

In contrast, several important immunologic differences between dolphins with positive *Chlamydiaceae* antibody titers and healthy seronegative dolphins were found, suggesting that infected dolphins demonstrate immunologic perturbations that may impact health. With respect to innate immunity, plasma lysozyme and NK activity were significantly increased, and granulocytic phagocytosis was significantly decreased in dolphins with positive *Chlamydiaceae* antibody titers. Plasma lysozyme concentration is a marker for pro-inflammatory responses with antibacterial functions, but it has not been extensively studied in dolphins (Bossart et al. 2011). We recently reported higher lysozyme concentrations in HERA dolphins with lobomycosis (lacaziosis) and subclinical cetacean morbillivirus infection and postulated an up-regulation of innate immunity associated with these infections (Reif et al. 2009, Bossart et al. 2011). In *Chlamydia trachomatis* infection in rabbits (experimental infection) and cattle (natural infection), plasma lysozyme concentrations were reported to be unchanged and elevated, respectively (Pawlikowska & Deptuła 2007). The significance of elevated lysozyme concentrations in dolphins with positive *Chlamydiaceae* antibody titers is unknown but suggests up-regulation of innate immunity and a pro-inflammatory response to *Chlamydiaceae* infection.

An up-regulation of innate immunity due to *Chlamydiaceae* infection was supported by increased NK activity. NK cells are an important component of the immediate immune response to infections, including infection by intracellular bacteria like chlamydiae (Vanrompay et al. 1995). Altered NK activity was not reported in HERA dolphins with orogenital papillomas, lobomycosis, or subclinical cetacean morbillivirus infection (Bossart et al. 2005, 2011, Reif et al. 2009). However, in mice infected with *Chlamydia muridarum*, NK activity was increased (Williams et al. 1987). Similarly, in mice with *C. trachomatis* infection, NK cells were activated to produce interferon (IFN)- γ when peripheral blood mononuclear cells were stimulated with *C. trachomatis* organisms (Tseng & Rank 1998). Tseng & Rank (1998) suggested that early NK production of IFN- γ down-regulated the Th2 response, thereby allowing expression of a strong Th1 response which has been shown to be essential for resolution of the infection. A similar cytokine-mediated mechanism may occur in dolphins.

Alternatively, decreased granulocytic phagocytosis in dolphins with positive *Chlamydiaceae* antibody titers suggested impairment of this component of innate immunity (Keogh et al. 2011). As with the other markers of innate immunity, granulocytic phagocytosis has not been extensively studied in dolphins. Impairment of granulocytic phagocytosis in HERA dolphins was not reported with lobomycosis or subclinical cetacean morbillivirus infection (Reif et al. 2009, Bossart et al. 2011). However, HERA dolphins with orogenital papillomas had significantly increased granulocytic phagocytosis suspected to be associated with viral-associated tumorigenesis (Bossart et al. 2005). Interestingly, decreased granulocytic phagocytosis was also reported in rabbits with experimental *Chlamydia trachomatis* infection (Pawlikowska & Deptuła 2007). Additionally, *Chlamydo-phila pneumoniae* infection in humans impacts granulocytes by extending the life span of neutrophils, making them suitable host cells for organism survival and multiplication within the first hours/days after infection (van Zandbergen et al. 2004). It is possible that chlamydial infection in dolphins also extends neutrophil life span which, in turn, compromises function, leading to the observed impairment of phagocytosis. Further studies are required to support this hypothesis.

Adaptive immunity was also impacted in dolphins with positive *Chlamydiaceae* antibody titers. The increased absolute numbers of mature CD 21 B cells in dolphins with positive *Chlamydiaceae* antibody titers suggested a partial up-regulation of humoral immunity that may be a response to *Chlamydiaceae* infection. However, mitogen-induced B and T lymphocyte proliferation responses were severely reduced in dolphins with positive *Chlamydiaceae* antibody titers, suggesting functional impairment of both humoral and cell-mediated immunity. Similar B and T lymphocyte proliferation depression was reported in HERA dolphins with lobomycosis that also had other immunologic impairments (Reif et al. 2009). Also, mitogen-induced T lymphocyte proliferation responses were significantly reduced in HERA dolphins with positive cetacean morbillivirus titers; these were postulated to be a result of the severe generalized immunosuppression caused by morbillivirus infection (Bossart et al. 2011). In immunologically competent mice, chlamydiae can act as stimulators of splenic B lymphocytes, inducing potent proliferation and differentiation to plaque-forming cells that result in a broad polyclonal antibody response and, ultimately, in elimination of chlamydial organisms (Levitt et al. 1986). Stimulation can be

affected in mice that are LPS non-responders, suggesting something other than the genus-specific LPS antigenic determinant is responsible for stimulation of B lymphocytes. However, B-cell deficient mice with chlamydial genital infection cannot eliminate chlamydial organisms (Morrison & Caldwell 2002). The inability to eliminate chlamydial organisms could occur in infected dolphins, since the LPS response is decreased. Comparison of the mouse and dolphin adaptive immunologic responses to *Chlamydiaceae* infection requires further investigation.

Significant increases in *Erysipelothrix rhusiopathiae* titers were found in dolphins with positive *Chlamydiaceae* antibody titers. *E. rhusiopathiae* infection in cetaceans (erysipelas) occurs in a dermatologic form and in an acute, typically fatal, septicemic form and is thought to be acquired from food fish (Dunn et al. 2001). *E. rhusiopathiae* can also cause epornitics or sporadic individual cases in mainly water-birds, including migratory species such as mergansers, ducks, geese, storks, gulls, and cranes (Jensen & Cotter 1976, Wobeser 1997). Both *E. rhusiopathiae* and *Chlamydophila psittaci* infections have been identified in gulls, terns, waterfowl, and other shore birds (Hubálek 2004). Thus, the observation of high seroprevalence of antibody to both *Chlamydiaceae* and *E. rhusiopathiae* in HERA dolphins might be explained by extensive shedding of the agents among local bird reservoir populations which, in turn, may be a potential source of both chlamydial and *E. rhusiopathiae* infection in wild dolphins.

Clinical disease due to *Chlamydiaceae* infection has not been reported in HERA dolphins. However, the present report suggests that *Chlamydiaceae* infection impacts health subclinically. Potential subclinical health impacts are important for the IRL and CHS dolphin populations, as past studies indicate that both dolphin populations are impacted by other complex infectious and neoplastic diseases, often associated with immunologic perturbations and anthropogenic contaminants (Bossart et al. 2005, 2008, Reif et al. 2006, 2008, 2009, Fair et al. 2009, 2010, 2012, 2013, Schaefer et al. 2009, 2011, Bossart 2011, Goldstein et al. 2012). The intricate dynamic interactions between various infectious agents and environmental factors highlight the complexity of evaluating health in dolphins. Additionally, these interactions may further complicate our understanding of disease pathogenesis and the detection of the contributing factors in population morbidity and mortality events, since the effect of any single factor may be obscured or confounded by other contributors. The latter is especially important to understand with

Chlamydiaceae infections, since the organisms can exist in latent forms in other species. The latency state raises the question of whether the organisms can alter immune responses if released during other disease processes (Levitt et al. 1986). A potential impact on dolphin fertility also exists, since *Chlamydiaceae* infection in other species may produce reproductive disease and abortion (Rodolakis & Yousef Mohamad 2010).

To address these complex issues we plan several new lines of investigation in future studies. The methodology in the present report used genus-specific antigens shared with other species of *Chlamydia* and *Chlamydophila*. Thus, we will first attempt, by polymerase chain reaction, to determine the presence of the specific *Chlamydiaceae* agent(s) infecting HERA dolphins. Second, the temporal issues of dolphin *Chlamydiaceae* infection will be examined by evaluating trends in antibody titers in infected individuals over time. Both new lines of investigation may help define whether serologic evidence of infection represents a subclinical, acute, chronic (with or without with intermittent shedding), or a resolving condition. Finally, we will further characterize the immune function in infected dolphins by evaluation of a panel of cytokines. Such data may shed additional light on the immunopathogenesis of *Chlamydiaceae* infection in this species.

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