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Evidence of Exposure to *Brucella* sp. in Harbor Porpoises (*Phocoena phocoena*) from the Bay of Fundy, Canada

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ABSTRACT: Novel strains of *Brucella* recently have been discovered in marine mammals. To investigate *Brucella* exposure and infection in a general population of cetaceans, blood and tissue samples were collected and analyzed from wild harbor porpoises (*Phocoena phocoena*) incidentally caught in fishing gear in the Bay of Fundy, Canada. Two of 170 (1.2%) animals had detectable antibodies against *Brucella,* but no organisms were isolated from genital swabs or tissues from 22 and 8 porpoises, respectively. Genetic analysis of inflamed testes from 20 animals yielded no amplification of *Brucella* DNA. This is the first evidence of exposure to *Brucella* in porpoises from the western North Atlantic, and the prevalence is much lower than documented for conspecifics from the eastern North Atlantic.

Key words: Atlantic, *Brucella*, brucellosis, Canada, harbor porpoise, marine, *Phocoena phocoena*, serology.

Brucella species long have been recognized as significant pathogens in terrestrial mammals, and they most commonly cause reproductive disease (Carter et al., 1995). Over the past decade *Brucella* species that are genetically and biologically distinct from terrestrial pathogens have been isolated from numerous marine mammals (Foster et al., 2002). If serologic evidence also is considered, *Brucella* exposure occurs around the world in a variety of marine mammal species (Nielsen et al., 2001; Van Bressem et al., 2001; Foster et al., 2002). To date, the effect of *Brucella* infection on marine mammal populations is unknown.

It has been suggested that, in many marine mammal species, *Brucella* infection may be enzootic and host-adapted (Foster et al., 2002). However, *Brucella* does have the potential to cause disease in some individuals. For example, *Brucella* was isolated from two aborted bottlenose dolphin fetuses (*Tursiops truncates;* Miller et al., 1999), and it was demonstrated in lesions of meningoencephalitis in three stranded striped dolphins (*Stenella coeruleoalba*) (Gonzalez et al., 2002).

Serological evidence of exposure to *Brucella* and isolation of *Brucella* have been documented in harbor porpoises (*Phocoena phocoena*) from the eastern North Atlantic (Jepson et al., 1997; Foster et al., 2002; Perrett et al., 2004), but little is known about this agent in other harbor porpoise populations. The goal of this study was to examine *Brucella* exposure and infection in a general population of wild harbor porpoises from the western North Atlantic.

From 1985 to 2004, blood, tissues, or both were collected from 196 harbor porpoises from waters around northern Grand Manan Island in the Bay of Fundy, NB (44°48′N, 66°41′W), and tissue samples were collected from two carcasses from the Gulf of St. Lawrence. One was found dead on the shores of Bouctouche, New Brunswick (46°29′N, 64°43′W), and the other was killed in a gill net in Chaleur Bay, New Brunswick (48°00′N, 65°45′W). The majority of porpoises from the Bay of Fundy were caught in local fishing weirs (n=176; 149 were alive and 27 were

dead), and handled through the Harbor Porpoise Release Program (HPRP) (Neimanis et al., 2004). Another 19 were killed incidentally in gill nets, and the remaining animal was found dead on shore in the Bay of Fundy. Sex, length, girth, and weight were collected routinely for live animals, and necropsies were performed on all dead animals. Not all samples could be collected from all animals due to varying field conditions.

All blood samples ($n=170$) were collected from 1993 to 2004 through the HPRP. These consisted of serum from 114 live animals and 25 dead animals, and plasma from 31 live animals. Blood was collected from live animals from a fluke blood vessel (Koopman et al., 1999) and was sampled directly from the heart in dead animals. All samples were centrifuged to obtain serum or plasma, which then was stored at −20 C until analysis.

Serologic testing was performed at Ottawa Laboratory (Fallowfield; OLF), Canadian Food Inspection Agency, Ottawa, Ontario. Competitive enzyme-linked immunosorbent assay (cELISA) was used to detect anti-*Brucella* antibodies in all samples ($n=170$) following methods outlined in O. Nielsen et al. (1996). Test results of ≥30% were considered positive. For samples with detectable antibodies, results were confirmed using a fluorescence polarization assay (FPA; positive samples had high millipolarization of ≥90 mP) and an indirect ELISA (iELISA; positive samples had an inhibition of ≥46%) according to methods described in K. Nielsen et al. (1996) and Nielsen et al. (2005), respectively. Porpoises were considered to have been exposed to *Brucella* sp. only if the serum or plasma samples had detectable antibodies in all three tests. Tests were carried out with and without bovine serum antibodies to *B. abortus* as positive and negative controls.

In 2002–2004, 16, five, and one genital swabs were collected for *Brucella* culture from live animals from weirs, dead animals from weirs, and an animal killed in a gill net, respectively. A suite of internal tissues was collected from eight additional carcasses. Five carcasses were retrieved from weirs, one was caught in a gill net, and two were found dead on shore. Tissues collected included vagina, cervix, uterus, ovary, mammary, testis, epididymus, penis, liver, spleen, kidney, bladder, heart, lung, brain, lymph nodes (prescapular, mediastinal, mesenteric, colonic, inguinal, sublumbar, lung-associated), thymus, muscle, blubber, thyroid gland, adrenal gland, pancreas, and a blubber lesion. Swabs and tissues were frozen at −20 C until cultured for *Brucella* at OLF, using standard protocols outlined in Alton et al. (1975).

Testes from 20 dead porpoises collected from waters around Grand Manan Island from 1985 to 1995 had microscopic evidence of mild to moderate chronic orchitis ($n=19$) or mild suppurative orchitis ($n=1$). Nineteen and one inflamed samples were from animals killed incidentally in gill nets and weirs, respectively. Ten to 12 sections 10 μm thick were collected from each of 20 paraffin blocks containing samples of inflamed testes and sent to OLF for amplification of *Brucella* DNA using polymerase chain reaction (PCR). DNA extraction followed the nonheating DNA extraction protocol outlined in Shi et al. (2002). Following resuspension in lysis buffer, samples were incubated at 60 C for 60 min before extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and 0.1% 8 hydroxyquinoline. Nucleic acids were precipitated with two volumes of ice-cold 95% ethanol; the pellet was washed with 1 ml 70% ethanol and then resuspended in 100 μl of water.

PCR was carried out using primers and protocols for both terrestrial and marine *Brucella.* All PCRs included positive and negative controls. A terrestrial *Brucella* strain was used as a positive control for all reactions because a marine isolate was unavailable. The enhanced AMOS PCR assay containing eight primers (Bricker

TABLE 1. Harbor porpoises (*Phocoena phocoena*) from the Bay of Fundy, Canada with positive serologic test results for anti-*Brucella* antibodies.

Identification number	Date sampled	Sex	Length (cm)	Maturity status[a]	cELISA result	FPA[b] result	iELISA result
GM 01 70	4 August 2001	F	133.0	Immature	Positive	Positive	Positive
GM 01 110	11 August 2001	F	150.0	Mature	Positive	Positive	Positive

[a] Based on Read and Gaskin (1990).

[b] FPA = fluorescence polarization assay.

and Halling, 1995) was used to detect terrestrial *Brucella* with the following modifications. Five microliters of extracted DNA were used as the template in the PCR containing 250 nmol of AMOS-A, AMOS-S, and AMOS-RB51/2308 primers, 500 nmol of AMOS-eri1 and AMOS-eri2 primers, 750 nmol of AMOS-M and AMOS-O primers, 1 250 nmol of AMOS-IS711 primer, 2.5 U of Taq DNA polymerase, 1.5 mmol of $MgCl_2$, 60 nmol of Tris-Cl (pH 9.0), 15 nmol of ammonium sulfate, and 250 µl each of the dNTPs. Samples were held at 95 C for 5 min, then subjected to 40 cycles of (60 sec at 94 C, 90 sec at 60 C, 90 sec at 72 C), with a final extension of 10 min at 72 C. PCR products were visualized on a 2.5% agarose gel stained with ethidium bromide.

The PCR to amplify marine *Brucella* was carried out using two different methods. The first method targeted the bp26 gene using primers and the thermocycling protocol described in Cloeckaert et al. (2000). A High Fidelity PCR Expand Plus (Roche Applied Science, Indianapolis, Indiana, USA) kit was used with 1 µl of 10mM dNTP, 1 µl of 25 µM of each primer 26A and 26B, 10 µl of 10X buffer, and 0.5 µl of DNA mix, in a total volume of 50 µl. The second method targeted omp2a and omp2b genes using primers and protocols as outlined in Cloeckaert et al. (2003).

Antibodies were detected in the sera of four porpoises by cELISA, but only two also had detectable antibodies by FPA and iELISA and were classified as positive (Table 1). These two positive samples were collected from live porpoises in 2001, and genital swabs had not been taken from them. Both were in good nutritional condition, and no abnormalities were noted when these animals were handled in the field.

None of the genital swabs or tissues cultured yielded *Brucella.* PCR analyses of inflamed testicular tissues did not amplify *Brucella.*

The two conclusively seropositive porpoises constitute the first evidence of *Brucella* sp. exposure in harbor porpoises from the western North Atlantic. Although serologic tests used in marine mammals have not been validated for these species, and the sensitivity and specificity of these tests in marine mammals are unknown, we are confident in the accuracy of our serologic results. Samples were considered positive only if they contained detectable antibodies in all three serologic tests. The cELISA and FPA tests used here have been shown to be very specific for *Brucella* in terrestrial species (Nielsen et al., 1992) and are the tests of choice for Hawaiian monk seals (Nielsen et al., 2005). Additionally, *Brucella* was cultured successfully from ringed seals and a harp seal that were positive for anti-*Brucella* antibodies using this same cELISA (Forbes et al., 2000).

In previously published reports, none of four harbor porpoises examined from the western North Atlantic had detectable anti-*Brucella* antibodies (Nielsen et al., 2001; Maratea et al., 2003). This is not surprising, given the low antibody preva-

lence found in this study (two of 170 or 1.2%). Antibody prevalence reported in other marine mammal species has ranged from 1.8% in harp seals (*Phoca groenlandica*) (n=453; Nielsen et al., 2001) to 78% in dusky dolphins (*Lagenorhynchus obscurus*) (n=27; Van Bressem et al., 2001). In populations with low *Brucella* seroprevalence, researchers have speculated that the organism may have been transmitted from another local species with enzootic infection (O. Nielsen et al., 1996). Further efforts to isolate the bacterium from Bay of Fundy porpoises therefore are needed for molecular characterization and comparison of this bacterium with other marine *Brucella* strains. Additionally, further genetic analyses of fresh tissues are warranted. Although we were unable to detect *Brucella* DNA in inflamed testes, the quality of these samples was compromised by formalin fixation (Greer et al., 1991).

The low number of seropositive animals from the Bay of Fundy is in sharp contrast to the antibody prevalences of 31% and 33% reported for harbor porpoises from the eastern North Atlantic (Jepson et al., 1997; Foster et al., 2002, respectively). Some of this variation may be explained by differences in serologic techniques used. However, sampling bias may also contribute to this difference in seroprevalence. The majority of porpoises sampled in the eastern North Atlantic had stranded on shore. Stranded animals often are ill or debilitated, and prevalence reported from stranded animals may overestimate the true prevalence in the general population. In contrast, porpoises caught incidentally in weirs and gill nets are considered healthy and are in significantly better nutritional condition than stranded and emaciated animals (Neimanis et al., 2004). However, weirs catch a disproportionate number of male yearlings (Neimanis et al., 2004), and results from weir-caught animals may underestimate the prevalence of *Brucella* exposure in this population if exposure or infection is more prevalent in other age or sex classes.

Other possible explanations for apparent differences in antibody prevalence between eastern and western North Atlantic porpoise populations include differences in exposure or susceptibility to *Brucella* infection. Perhaps *Brucella* is more prevalent or more easily transmitted in the marine ecosystem of the eastern versus western North Atlantic. The mode of transmission of *Brucella* in marine species is not known. Lung worms have been implicated as possible vectors, and lungworms infected with *Brucella* were found in a harbor porpoise from England (Perrett et al., 2004). Fish may also play a role in transmission, as *B. melitensis* was recovered from experimentally infected fish (Salem and Mohsen, 1997). Further research into *Brucella* transmission is needed to better understand the ecology of *Brucella* in different marine mammal populations.

Finally, *Brucella* strains identical to those isolated from marine mammals have infected humans (Brew et al., 1999; Sohn et al., 2003; McDonald et al., 2006). Our findings have implications for rehabilitators, researchers, and fishermen who handle live and dead porpoises from this population, and appropriate precautions should be taken to protect humans from potential exposure.

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