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Source: Journal of Wildlife Diseases, 44(2) : 237-246

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-44.2.237

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Journal of Wildlife Diseases, 44(2), 2008, pp. 237–246
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ISOLATION AND CHARACTERIZATION OF *BRUCELLA* FROM THE LUNGWORMS OF A HARBOR PORPOISE (*PHOCOENA PHOCOENA*)

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ABSTRACT: Adult female nematodes identified as *Pseudalius inflexus* were collected from the lungs of a juvenile male harbor porpoise (*Phocoena phocoena*) found dead on a beach in Cornwall, UK. Classic and molecular typing methods, immunologic and electron microscopy immunolabeling techniques, provided evidence of *Brucella* sp. infection within the uterine tissue of nematodes of this marine mammal. This finding presents further evidence to suggest parasites should be considered as a potential means of transfer of bacterial infection in marine mammals and highlights the zoonotic implications for humans exposed to marine mammals through occupation or leisure.
Key words: *Brucella*, lungworms, *Phocoena phocoena*, transmission electron microscopy.

INTRODUCTION

Brucella isolated from the organs of four common seals (*Phoca vitulina*), two harbor porpoises (*Phocoena phocoena*), and one common dolphin (*Delphis delphis*) originating from the Scottish coasts was first reported by Ross et al. (1994). Since then there have been numerous reports of culture and serologic evidence occurring in a range of species inhabiting many of the world's oceans. *Brucella* has been reported to cause abortion in bottlenose dolphins (*Tursiops truncatus*) in the USA (Ewalt et al., 1994; Miller et al., 1999). Studies using molecular techniques have shown isolates differ from the six recognized species originating from terrestrial mammals (Cloeckaert et al., 2001). Phenotypically, these strains can be differentiated with respect to their requirement for increased carbon dioxide (CO_2) for primary growth; generally isolates originating from pinnipeds require increased CO_2 whereas isolates from cetaceans do not. *Brucella* strains isolated from marine mammals have been known to infect humans in both occupational and natural settings. In 1995, a laboratory worker contracted brucellosis from a marine mammal strain, proving it can be pathogenic to humans (Brew et al., 1999). More recently, the first reported case of community-acquired human infections with marine mammal–associated *Brucella* species describes the identification of strains in two patients with neurobrucellosis and intracerebral granulomas (Sohn et al., 2003). Subsequently, a further report describes the isolation and the characterization of a *Brucella* strain from a New Zealand patient with osteomyelitis that appears closely related to marine mammal strains previously identified (McDonald et al., 2006). These cases highlight the zoonotic implications of infection with nonterrestrial strains in the absence of direct association with marine mammals.

Previously immunohistochemical techniques have provided evidence of *Brucella* organisms in the uterus and intestinal lumen of *Parafilaroides* lungworms in a Pacific harbor seal (*P. vitulina richardsi*; Garner et al., 1997). Subsequent light and electron microscopic studies with immunogold labeling demonstrated the intrauterine *Brucella* in adult *Parafilaroides* lungworms to be located largely in the membranes separating and surrounding the developing larvae. Heavy growth of *Brucella* was also isolated from the host's lung (Rhyan, 2000).

Previously, we reported the isolation of

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Brucella species from the lungworm species (*Pseudalius inflexus*) of a harbor porpoise (22/M39/01/04; Perrett et al., 2004). The life cycle of *P. inflexus*, as true of many species of nematodes of marine mammals, is unknown. The purpose of this was study was to complete a full characterization of *Brucella* isolates originating from this harbor porpoise to determine whether they were typical of those originating from this cetacean species (Dawson et al., submitted for publication). Furthermore, visual evidence of the bacteria and their localization within the lungworms using investigations by electron microscopy may offer an insight as to whether this *Pseudalius* nematode should be considered a possible route of transmission of the marine strain of *Brucella* commonly found in harbor porpoises.

MATERIALS AND METHODS

Gross pathology

On 7 January 2004, a juvenile male harbor porpoise (22/M39/01/04) was found dead at Top Tiebe beach, Marazion Cornwall, UK. It was taken to the Veterinary Laboratories Agency (VLA) in Truro, UK on 8 January 2004 and a necropsy was done the same day. The animal, considered to be in good nutritional state, was 132 cm long and weighed 41 kg. There was one linear depression on the side of the tail fluke. There was scavenger damage over some of the carcass, which was most marked on the right side, particularly around the eye (which was absent) and thorax. A hole was present into the thorax at the level of the heart and the pericardium was punctured. No abnormalities were seen in the body orifices, blubber, or musculoskeletal system, and no ectoparasites were seen. Aside from the punctured pericardium, no other abnormalities were seen in the cardiovascular system. A large amount of froth was present in the trachea and the bronchi. Numerous long slender nematodes identified as *P. inflexus* (Gibson et al., 1998) were present in the lower airways. Both lungs were hyperemic. Three whole herring (*Cuplea harengus*) were present in the cardiac section of the stomach with tails extending into the esophagus. No abnormalities were seen in the alimentary, urogenital, lymphatic, or endocrine systems. The gross postmortem examination findings were consistent with death due to entanglement in fishing gear. The animal had recently fed and was in good body condition. The lungs were congested and the froth present in the airway suggested death caused by asphyxiation. The linear depression on the tail fluke was suspicious of monofilament net damage. Tissue samples of lung, liver, kidney, spleen, testes, chest fluid, and lungworms were submitted to the VLA laboratory at Weybridge, UK for analysis.

Serology

The Rose Bengal test (RBT) was used to test for agglutination with the use of equal volumes of antigen to serum (Nielsen et al., 2004). A competitive and two indirect ELISAs (cELISA and iELISA) were used to detect the presence of *Brucella* antibodies in the marine mammal serum. The cELISA described by MacMillan et al. (1990) uses a lipopolysaccharide (LPS) *Brucella melitensis* 16M antigen and a monoclonal antibody BM40 (Greiser-Wilke et al., 1985) labeled with horseradish peroxidase (Nakane et al., 1974). Of the two indirect ELISAs, *B. melitensis* 16M antigen was used for the first (as described for the cELISA), and the other a *Brucella abortus* LPS antigen was used. The iELISAs require antiglobulin conjugate with specificity for the immunoglobulin (Ig) isotypes of the species under test; however, protein A has been shown by Sikkema (1989) and Eliasson et al. (1989) to bind to the IgG of a range of marine mammals. Positive/negative thresholds for these assays were set with some uncertainty but are based on those used for testing a wide range of terrestrial mammals for brucellosis worldwide. Currently, thresholds are set at $\geq$10% of the optical density (OD) of the positive control for both iELISAs, and $\leq$60% of the OD of the conjugate control for the cELISA are considered positive.

Isolation

Tissue samples were prepared by a method similar to one described by Foster et al. (2002). Lungworms were washed in sterile distilled water before maceration and culture with Farrell's media (Farrell, 1994). Sample inocula were added to Brodie and Sinton broth (Brodie and Sinton, 1975) in order to boost low *Brucella* numbers in competition with bacterial contaminants, nutrient broths were also inoculated. Media plates and broths were incubated at 37 C in the presence of increased (10%) CO_2 for intitial isolation and subcultured weekly to Farrell's and/or serum dextrose agar (SDA) media with the use of a 10-µl loop, for up to a maximum 4 wk.

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Phenotypic characterization

Phenotypic characterization of the isolates originating from the lungworm and kidney was carried out with the use of classic biotyping methods including serotyping, phage typing, dye sensitivity, CO_2 requirement, and H_2S production (Alton et al., 1988).

Molecular detection and characterization

PCR amplification of an IS*711* element downstream of the base-pair (bp) 26 gene was carried out as described by Cloeckaert et al. (2000) to assess whether the isolates possessed the specific marker of *Brucella* spp. unique to isolations from marine mammals. PCR amplification of the outer membrane proteins (*omp*) 2 locus and subsequent restriction digestion with the use of a selection of enzymes as described by Cloeckaert et al. (2001) was carried out on the lungworm and kidney isolates in order to characterize the strain. The IS*711* fingerprint analysis was generated by the digestion of genomic DNA with Eco R1, the products separated on a 0.8% agarose gel and southern blotted with the use of a vacuum method. The membrane-bound DNA was probed with the use of a digoxigenin (DIG) –labeled IS*711* probe generated with the use of primers and sequences derived from methods previously described by Halling et al. (1993) and Ouahrani et al. (1993). The hybridized probe was detected with the use of anti-DIG monoclonal antibody conjugated to alkaline phosphatase. The membrane was immersed in CSPD chemiluminescence substrate to allow visualization of the probe hybridization by exposure to X-ray film. The images were analyzed with the use of Bionumerics software (Applied Maths, Belgium) and a dendrogram analysis of the profiles was produced with the use of the coefficient of Jaccard to calculate similarities between the fingerprint patterns.

Light microscopic examination

Interpretation of lungworm anatomy under light microscopy (LM) was assisted by published data (Arnold et al., 1975). Several examples of the lungworm *P. inflexus* of the *Metastrongyloidia* family were selected and mature females examined by transmission electron microscopy (TEM) for *Brucella* spp.

Lungworm transmission electron microscopy

For TEM, lungworms were fixed in 3% glutaraldehyde prepared in a 0.1 M phosphate buffer. Selected lungworms were then washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated through a gradual series of alcohol concentrations up to 100% alcohol and placed in propylene oxide prior to embedding in araldite resin. The resin was polymerized at 60 C for 48 hr. One-micron sections, stained with toluidine blue were prepared for LM examination. Areas showing bacteria in close association with the uterus of *P. inflexus* were selected for ultrastructure examination. Ultrathin sections at 70–90-nm thickness were then prepared onto copper grids with the use of a diamond knife, and contrasted with uranyl acetate and lead citrate prior to examination under a Phillips CM10 TEM.

Immuno-electron microscopy

In order to confirm the observed bacteria were *Brucella* spp., further ultrathin sections were collected onto nickel grids and immunogold labeled. Briefly, etching of the sections was achieved with the use of saturated filtered sodium periodate for 1 hr, and in order to block endogenous peroxidase and to deosmicate, the grids were subjected to filtered 6% hydrogen peroxide for 10 min. Nonspecific labeling was blocked with the use of blocking buffer (PBS containing 2% normal goat serum, 0.1% Tween 20, 1% bovine serum albumen, and 0.1% sodium azide, pH 8.2) for 1 hr. Grids were then transferred to the primary antibody, a polyclonal anti-*Brucella* antibody (monospecific anti-A serum). The serum was raised in rabbits that had been inoculated with *B. abortus* strain 544 and absorbed with a concentrated *B. melitensis* (16M) suspension. The optimum dilution had been previously chosen when tested against all *Brucella* spp. reference strains held at the VLA. For this assay the serum was diluted 1:100 in blocking buffer and the grids incubated overnight. After six 10-min washes in blocking buffer, the sections were then incubated for 2 hr in the secondary antibody, a 10-nm gold conjugated goat anti-rabbit IgG, diluted 1:50 in blocking buffer (Auroprobe One). After a final rinse with filtered blocking buffer, the grids were postfixed in 2.5% glutaraldehyde for 10 min. The grids were then counterstained with uranyl acetate and lead citrate and examined under a Phillips CM10 TEM.

RESULTS

Antibody detection

Analysis of the chest body fluid by the RBT test gave a positive reaction indicat-

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ing the sample contained anti-*Brucella* antibodies (Nielsen et al., 2004). Serologic analysis by cELISA and the two iELISAs confirmed the sample was strongly positive by all three assays.

Isolation

Bacterial growth identified as *Brucella* spp. was visible 5 days after incubation on Farrell's media. Fourteen, 34, eight, four, two, and one colony counts originated from lung, liver, kidney, spleen, testes, and chest fluid, respectively. The most prolific growth, however, originated from the lungworms with colony counts exceeding 200. *Brucella* spp. were reisolated at 17 days' postincubation from the Brodie and Sintons and nutrient broths of all tissue samples, including lungworms.

Phenotypic characterization

The isolate originating from the lungworm was confirmed initially as *Brucella* spp. by classic biotyping methods (Alton et al., 1988). The characteristics were identical to those of nine additional harbor porpoises and two Atlantic white-sided dolphins (*Lagenorhynchus acutus*) previously analyzed at this laboratory. Characteristics of the lungworm isolate (22/M39/01/04) (VLA04/6) and an example of another typical *Brucella* isolate originating from the lungs and lungworms of a harbor porpoise (22/M21/4/04) are shown in Table 1. Isolates are shown in comparison to reference strains of *B. melitensis* biovar 1, *B. abortus* biovar 1, and *Brucella suis* biovar 1. None of the isolates were found to require additional CO_2 for growth when classically biotyped.

Molecular detection and characterization

PCR amplification of an IS*711* element downstream of the bp 26 gene (Cloeckaert et al., 2000) confirmed isolates originating from the lungworms and kidney of the harbor porpoise possess the specific marker of *Brucella* spp. unique to isolations from marine mammals. Both isolates

TABLE 1. Characteristics of *Brucella* species by classical biotyping.

	Hydrolyzation of urea	H_2S	CO_2		Th[b]	Monospecific sera		Lysis by phage at RTD[c]				
		Produced	Required	BF[a]	A	M	Wb	Tb	BK_2	Fi	R/C
Harbor porpoise from lungworms of 22/M39/01/04	+	–	–	+	+	–	PL	NL	PL	NL	NL
Harbor porpoise from lung and lungworms of 22/M21/04/04	+	–	+	+	+	–	PL	NL	PL	NL	NL
Brucella melitensis (Biovar 1)	+	–	+	+	–	+	NL	NL	CL	NL	NL
Brucella abortus (Biovar 1)	+	+	+	–	+	–	CL	CL	CL	CL	NL
Brucella suis (Biovar 1)	++	+	–	+	+	–	CL	NL	CL	PL	NL

[a] BF = basic fuchsin at 20 µl/ml (1/50,000 w/v).

[b] Th = thionin at 20 µl/ml (1/50,000 w/v).

[c] CL = confluent lysis, PL = partial lysis, NL = no lysis, Wb = Webridge, Tb= Tibilisi, BK_2 = Berkeley, Fi = Firenze, R/C = phage for identifying rough strains of *Brucella*.

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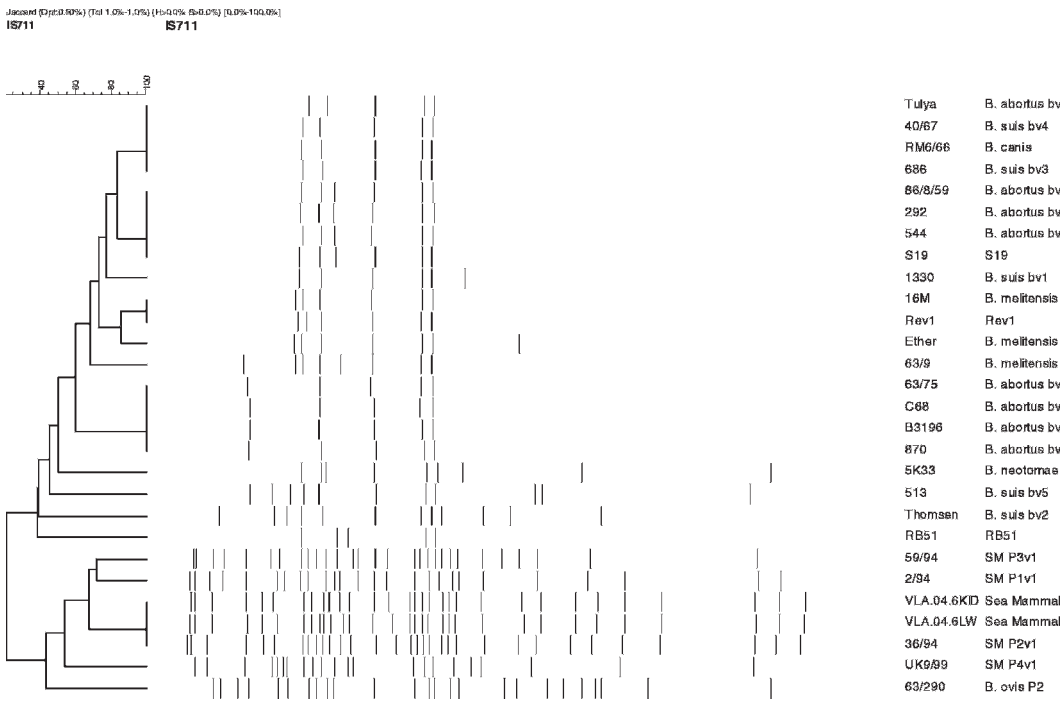

FIGURE 1. IS*711* fingerprinting.

produced a PCR product sized at 1,900 bp; isolates originating from terrestrial species produce a product sized at 1,029 bp.

PCR amplification of the outer membrane proteins (*omp*) 2 locus of *Brucella* and subsequent restriction digestion with the use of a selection of enzymes (Cloeckaert et al., 2001) enabled analysis of DNA polymorphisms. The overall pattern profile demonstrated the isolate to be unique from the profiles produced from isolates originating from terrestrial mammals. Characterization of the lungworms and kidney isolates of 22/M39/01/04 (VLA04/6) revealed the type to be J and M, which refer to the profiles of omp2a and omp2b genes, respectively. This overall profile is associated with minke whale (*Balaenoptera acutorostrata*), common dolphin (*D. delphis*), white-sided dolphin, white-beaked dolphin (*Lagenorhynchus albirostris*), common seal, harbor porpoise, and bottlenose dolphin (*T. truncatus*). Isolates with this pattern profile have been shown to possess two omp2b gene copies instead of one omp2a and one omp2b gene copy as observed in isolates originating from pinniped species and those of terrestrial mammals. (Cloeckaert et al., 2001).

Molecular characterization of the isolates by IS*711* fingerprinting based on the number and distribution of IS*711* copies within the bacterial genome produced a pattern profile similar to those described for *Brucella* isolated from marine mammals (Bricker et al., 2000). Each genome contained 25 copies of the IS*711* element sized between 20,000 and 1,520 bp. The higher number of IS*711* copies and pattern profile labeled pattern 2 variant 1 (P2V1) demonstrated the isolate was unique from strains originating from terrestrial mammals. The isolate was, however, identical to further marine mammal *Brucella* strains originating from harbor porpoises also analyzed in as yet unpublished VLA data (Fig. 1).

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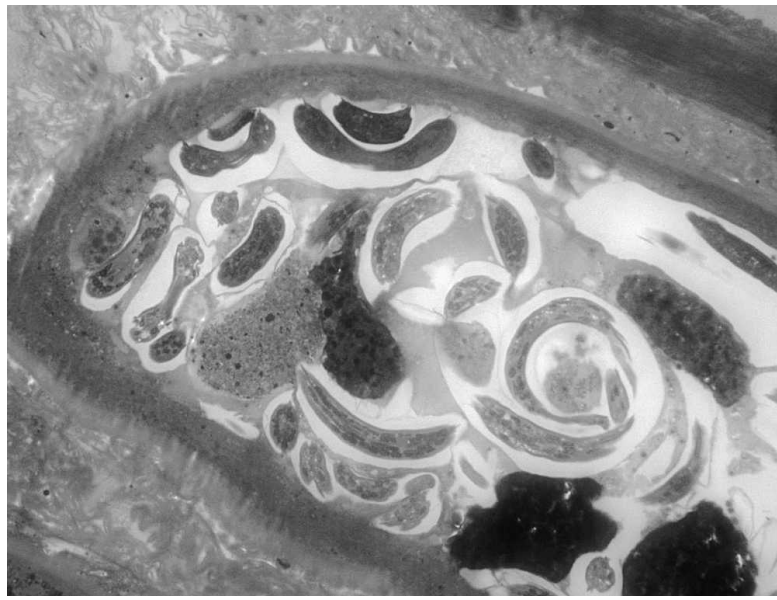

FIGURE 2. Mature female *Pseudalius inflexus* nematodes containing larvae.

Microscopic observations

Examination of sections by LM offered an insight into the anatomy of the lungworm. A longitudinal section (LS) through female nematode tissue revealed vacuoles containing multiple structures identified as larvae, as previously observed in *Parafilaroides* nematodes (Garner et al., 1997) and in nematodes of harbor porpoise (Jauniaux et al., 2002) (Fig. 2). Examination (LM) of 1-μm sections identified large numbers of bacteria in an area outside the gut in transverse section. This area had previously been identified as the uterus and associated connective tissue by Garner et al. (1997). Examination of this area by TEM confirmed the presence of bacteria and their association with the uterus, although at this stage it was not possible to determine the species as *Brucella* (Fig. 3). Further TEM examination of a lungworm uterine section revealed the presence of bacteria uniformly appose to the epithelial cell lining.

Immunogold labeling of the lungworm uterine and associated connective tissue sections containing bacteria with the use of the polyclonal *Brucella* antibody revealed small amounts of gold labeling, with typically only one–two gold particles per bacteria, although with very little nonspecific background gold. Gold labeling was not observed in sections processed minus the polyclonal *Brucella* antibody. In order to confirm the specificity of the *Brucella* antibody, *Brucella* cells isolated from a common dolphin originating from Spain (VLA Ref F5/06), were harvested after 5 days' growth, heat inactivated, and processed in a similar way to the lungworms. These cells were then immunolabeled in an identical way and large amounts of specific gold labeling was observed on these *Brucella* cells. Again, gold labeling was not observed in cells labeled without the polyclonal *Brucella* antibody that served as a negative control.

DISCUSSION

In this study, the phenotypic, serologic, molecular, and microscopic detection together with molecular characterization provides reasonable indication that the bacteria present within the uterus of *P. inflexus* originating from the lungs of this harbor porpoise are most likely *Brucella*

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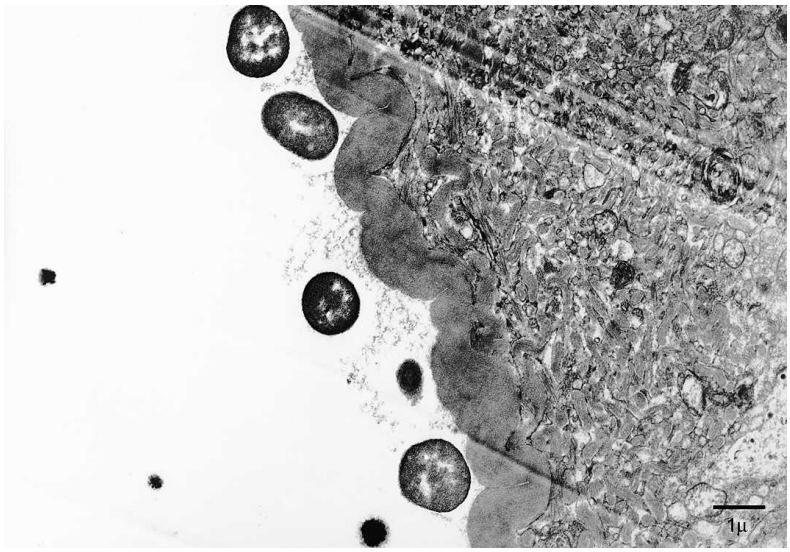

FIGURE 3. Transmission electron micrograph showing bacteria associated with the uterus of *Pseudalius inflexus*.

species. The results of the TEM immunolabeling method described proved inconclusive in this study, with only very small numbers of gold particles observed on the bacteria associated with the uterus. As a full EM examination was not originally envisaged for the lungworms used in this study, it is suggested the immunogold labeling was less successful because of the nonideal collection and fixation of the worms and the subsequent processing of tissue areas into araldite resin. All of which may have had an adverse effect on the level of labeling by immuno-electron microscopy. However, the *Brucella* cells originating from the common dolphin, used as controls for the method, were processed successfully, and therefore, the TEM immunolabeling method described was determined a suitable technique for the detection and confirmation of *Brucella* spp. in tissues. Little is known about the route of transmission of *Brucella* spp. infection of marine mammals. Opportunities to be considered are during social activity, breeding, maternal transfer, physical trauma, ingestion and through feeding and by the carriage of parasites. The previous reports of *Brucella* infection in *Parafilaroides* lungworms in a Pacific harbor seal (Garner et al., 1997; Rhyan, 2000) have provided corroborative evidence to suggest this nematode species should be considered as a possible vector of brucellosis between harbor seals. *Parafilaroides decorum* is the most common lungworm species found in young Californian sea lions (*Zalophus californianus*), the intermediate host of which is the opal-eyed feces-eating fish (*Girella nigricans*). The heavy growth of a *Brucella* spp. upon bacteriologic culture of the feces of a Pacific harbor seal suggests another opportunity for transmission of infection originating from this marine mammal species exists, via feces-feeding fish (Rhyan, 2000).

Little is known of the life cycle of many lungworm species, including the species *P. inflexus*; therefore, it remains difficult to determine the originator of infection associated with the relationship between parasite and host. In experimental transmission of *Pharurus pallasii* (*Nematoda*; *mestastrongyloidea*; *Pseudaliidae*), a lungworm of the cranial sinuses of Beluga whales (*Delphinapterus leucas*), first-stage larvae were removed from the uterus of

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gravid female pseudaliid originating from dead Beluga whales. The larvae were exposed to marine organisms such as fish, crustaceans, and molluscs. Although the first-stage larvae failed to develop in the experimentally exposed invertebrates, the first-stage molt occurred in the intestinal wall of American plaice (*Hippoglossoides platessoides*) and Artic sculpins (*Myoxocephalus scorpoides*). Survival and development of larvae to the second stage in fish suggests they are a suitable intermediate host (Houde et al., 2003).

The diet of harbor porpoises consists of a variety of fish species, squid, octopus, and shellfish. Grey mullet (*Mugil* sp.), Atlantic mackerel (*Scomber scombrus*), herring and sand eels (*Ammodytidae* sp.) have been observed in the stomach contents of harbor porpoises assessed at postmortem at VLA Truro. Adult nematodes (*Halocercus lagenorhynchi*) collected from the lungs of four bottlenose dolphins aged between newborn and 3 wk of age from the Atlantic and Gulf coasts of Florida, USA, provides evidence of prenatal infection with the lungworms of this cetacean species (Dailey et al., 1991).

In an experimental study, the blood of predatory Nile catfish were found to have produced *Brucella* antibodies after 7 days postinoculation with *B. melitensis* biovar 3 and the antibody titer was maintained throughout the period of observation. The organism was recovered from visceral organs of infected fish whilst control fish showed no serologic or bacteriologic response. These results suggest that fish could be considered as a susceptible species to brucellosis and act as reservoirs of infection for other species and man (Salem and Mohsen, 1997).

Cases of community-acquired human infections with marine-mammal–associated *Brucella* spp. include a report of these strains in two Peruvian patients with neurobrucellosis with intracerebral granulomas. Both of these patients denied any direct contact with marine mammals though the diets of both included raw shellfish and one patient had frequently swum in the Pacific Ocean (Sohn et al., 2003). A further report describes the isolation and characterization of a strain of *Brucella* spp. from a New Zealand patient with spinal osteomyelitis. This isolate was identified as one closely related to a *Brucella* spp. originating from a bottlenose dolphin from the USA. The patient had not been exposed to marine mammals, but had been exposed to several species of uncooked fish bait and had consumed raw freshly caught snapper (McDonald et al., 2006).

Isolations of *Brucella* spp. from the lungworm species *P. inflexus* of a further two harbor porpoises and from two common dolphins have since been completed at VLA Weybridge. The tissues from these animals also originated from VLA Truro, Cornwall; further studies are in progress.

This case provides further evidence to suggest parasites should be considered as a potential means of transfer of bacterial infection in marine mammals and highlights the potential means of spread by scavenging animals and zoonotic implications for human health. Further studies should be completed to determine the life cycles of lung nematodes of marine mammals and establish the risks of consumption and handling of uncooked fish and shellfish products.

ACKNOWLEDGMENTS

We would like to thank C. Marins, CITA, Spain for supplying the common dolphin isolate F5/06 used in this study. We also thank Defra (Ministry of the UK parliament) for funding this work. This manuscript is under British Crown Copyright.

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Received for publication 28 March 2007.

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