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First isolation of *Brucella pinnipedialis* and detection of *Brucella* antibodies from bearded seals (*Erignathus barbatus*)

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ABSTRACT: *Brucella* species infecting marine mammals was first reported in 1994 and in the years since has been documented in various species of pinnipeds and cetaceans. While these reports have included species that inhabit Arctic waters, the few available studies on bearded seals (*Erignathus barbatus*) have failed to detect evidence of *Brucella* infection to date. We report the first isolation of *Brucella pinnipedialis* from a bearded seal. The isolate was recovered from the mesenteric lymph node of a bearded seal that stranded in Scotland and typed as ST24, a sequence type associated typically with pinnipeds. Furthermore, serological studies of free-ranging bearded seals in their native waters detected antibodies to *Brucella* in seals from Chukchi Sea (1990-2011; 19 %) and Svalbard (1995-2007; 8 %), whereas no antibodies were detected in bearded seals from the Bering Sea, Bering Strait or from captive bearded seals.

KEY WORDS: Antibodies · bearded seal · *Brucella pinnipedialis* · isolation · MLST

INTRODUCTION

The isolation of *Brucella* from marine mammals was first reported in 1994 from four free-ranging harbour seals (*Phoca vitulina*), two harbour porpoises (*Phocoena phocoena*) and a common dolphin (*Delphinus delphis*), all inhabiting Scottish coastal waters (Ross et al., 1994) and from an aborted foetus born to a captive bottlenose dolphin (*Tursiops truncatus*) in the USA (Ewalt et al., 1994). Since these initial reports, *Brucella* infection has become recognised in cetaceans and pinnipeds inhabiting many of the world’s oceans (Foster et al., 2002; Nymo et al., 2011) and two species, *Brucella ceti* and *Brucella pinnipedialis*, have been described for isolates with cetaceans and seals as preferred hosts, respectively (Foster et al., 2007).

With respect to Scottish coastal waters, *B. pinnipedialis* has been recovered from the other resident species, grey seals (*Halichoerus grypus*) as well as from hooded seals (*Cystophora cristata*), which are occasional visitors to the region (Foster et al., 1996; 2002). The isolation of *B. pinnipedialis* has also been reported from hooded seals in their native Arctic waters and from harbour and grey seals elsewhere in Europe (Nymo et al., 2011). Further afield, *B. pinnipedialis* has been cultured from other pinniped species including Pacific harbour seal (*Phoca vitulina richardsi*) (Garner et al., 1997), ringed seal (*Pusa hispida*), harp seal (*Pagophilus groenlandica*) (Forbes et al., 2000) and California sea lion (*Zalophus californianus*) (Goldstein et al., 2009). Serological studies provide further presumptive evidence that *Brucella* infections are widespread amongst other pinniped species, including some resident in the Southern Hemisphere (Nymo et al., 2011). Taken together, culture and serological evidence (Foster et al., 2002; Nymo et al., 2011), indicate that *Brucella* is endemic in many of the mammals that inhabit the world's open oceans and seas. Seropositive animals, however, can be due to immunological cross-reactions to an organism from a different genus; thus the isolation of *Brucella* by cultural methods, remains the gold standard of definitive proof of infection in different hosts and discrete populations of marine mammals.

There have been few reports on studies of *Brucella* infection in bearded seals (*Erignathus barbatus*) to date, but where performed, no evidence of exposure was found (Calle et al., 2008; Tryland et al., 1999). Bearded seals are members of the Phocidae family and represent the only species within the genus *Erignathus*. They have a patchy circumpolar distribution throughout the Arctic and subArctic between 45 and 85° N. Two sub-species are recognised, *Erignathus barbatus barbatus*, which ranges from the central Canadian Arctic eastwards to the central Eurasian Arctic and *Erignathus barbatus nauticus*, which ranges from the central Canadian Arctic westwards to the Laptev Sea, Russia. The availability of ice to breed, moult and rest on, in shallow water areas, is thought to be an important factor governing the distribution of this benthic-feeding seal (Kovacs, 2016). In a review of their extralimital records, bearded seals have been reported from the Netherlands, France and Spain in the Eastern Atlantic and the island of Rügen in the Baltic Sea (van Bree, 2000). Sightings in the UK are rare, with most modern reports occurring around the Scottish coast, including the Shetland and Orkney Islands and single sightings from the Isle of Mull, Aberdeenshire and Fife (JNCC/Defra, 2013).

This paper documents the first recovery and characterisation of *B. pinnipedialis* from a bearded seal. The results of a serological study of free-living bearded seals in Arctic waters and captive members of the species kept at the aquarium 'Polaria' in Tromsø, Norway are also presented.

MATERIALS AND METHODS

Bearded seal necropsy

In early February, 2012, a stranded bearded seal (M61/12) was reported to the Scottish Marine Animal Strandings Scheme (SMASS). The juvenile male animal had stranded dead at Annachie Lagoon, St Fergus on the Aberdeenshire coast of the north-eastern Scottish mainland (57°34′10.74″N 001°49′22.02″W) and represented the first report of a stranded bearded seal in Scotland since records began in 1992. The carcase was transported to SAC Consulting Veterinary Services, Inverness for a post mortem examination performed according to a standard protocol (Dierauf, 1994). Samples of brain, lung, liver, spleen, kidney, mesenteric lymph node, urinary bladder and small intestine were cultured on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK) and Farrell's medium (FM) (Farrell, 1974), incubated at 37 °C in air with 5 % added CO_2 as described previously (Foster et al., 2002). Plates were examined for growth, daily, for 4 days and at frequent intervals thereafter up to 14 days. Isolates with colonial appearance typical of *Brucella* were tested initially for Gram reaction, cellular morphology, acid-fastness with the modified Ziehl-Neelsen stain, agglutination with *Brucella abortus* antiserum (Remel, Basingstoke, UK) and ability to grow in air without added CO_2. Further testing included urea hydrolysis, H_2S production, inhibition by basic fuchsin at 1/50,000 and 1/100,000, agglutination with monospecific antisera A and M and lysis by phages TB, Wb, BK2, Fi, Iz and R/C all at Routine Testing Dose. Multilocus sequence typing (MLST) using a 9 locus scheme was performed as described previously (Whatmore et al., 2007). Samples for histological examination (whole brain, trigeminal ganglion, skin, thyroid gland, adrenal gland, urinary bladder, spleen, lung, kidney, heart, and pancreas) were collected, trimmed and processed routinely through graded alcohols and embedded in paraffin wax prior to sectioning (5µm), mounting on glass microscope slides and staining with haematoxylin and eosin. Blood was collected for serology and urine analysis was performed with the Combur 9 Test (Roche, Burgess Hill, UK).

Serology

The Alaska Department of Fish and Game Ice Seal program collected heart blood samples from bearded seals during the hunt by Alaska Native subsistence hunters. Seals were shot on sea ice in the Chukchi and Bering Strait off the north and northwest coasts of Alaska. In addition, 17 seals were sampled immediately post mortem during 1978-1979 scientific collections conducted by the Outer Continental Shelf Environmental Assessment Program during United States National Oceanographic and Atmospheric Administration cruises in the Bering Sea (Figure 1). The Svalbard bearded seals were sampled as described previously (Tryland et al., 1999). Sex and age category (pup < 1 year, juvenile < 3 years, adult > 3 years) were known for some or all of the seals at each location (Table 1). Furthermore, blood samples were obtained from 5 bearded seals kept in captivity at the aquarium "Polaria" in Tromsø. These animals, initially captured in the wild in Svalbard, had been kept in captivity since they were approximately 5 weeks of age; the seals interact extensively with humans through training and feeding (Stokke, 2010). They were 9-10 years of age at the time of sampling and had been trained to tolerate handling and blood sampling (Table 1).

Serum samples (n = 205) were analyzed for anti-*Brucella* antibodies with a Protein A/G indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Nymo et al., 2013a).

RESULTS

Bearded seal necropsy

The carcase of M61/12 was fresh and had been chilled, but not frozen, prior to necropsy, two days after notification. The animal was 149 cm in total length, 79 cm girth behind the front flippers and in moderate to poor body condition with a mid-sternal blubber thickness of 16 mm. A bilateral symmetrical alopecia was noted over the pelage and the skin was markedly thickened on the ventral surface. The oesophagus and stomach contained a notable amount of sand, 1 cm diameter stones and small fragments of marine debris. There was no evidence of recent successful feeding. The lungs and cerebral vessels were markedly congested, the bladder mucosa was grossly reddened and the urine was turbid and dark red in colour and a high level of haemoglobin was detected with the Combur 9 Test. The brain showed diffuse dilation of cerebral vessels but the cerebrospinal fluid was unremarkable.

Bacteriology

Small numbers of colonies typical of *Brucella* were recovered from the mesenteric lymph node on CSBA and FM after four days. In addition, *Vibrio alginolyticus* was recovered from multiple tissues. Cells of suspect *Brucella* colonies were tiny Gram negative cocco-bacilli, which were acid-fast when tested in the modified Ziehl-Neelsen stain. Agglutination was obtained in slide tests with *B. abortus* antiserum. The strain required CO_2 for growth, was urease positive, H_2S negative and A dominant. Growth was inhibited by basic fuchsin at 1/50,000 and 1/100,000 and cultures were completely lysed by Tb phage, partially lysed by Wb, BK2 and Iz, with no lytic effect with Fi and R/C. The strain was identified by MLST as *Brucella pinnipedialis* sequence type (ST) 24.

Histopathology

The most significant histological change in M61/12 consisted of moderate, multifocal granulomatous and eosinophilic meningo-encephalitis within the brain, often centred on degenerate or intact nematode parasite larvae, with perivascular cuffing and multifocal haemorrhages. Mild, multifocal histiocytic and eosinophilic pneumonia (likely parasitic) was also noted along with moderate splenic histiocytosis with mild lymphodepletion. The skin lesions consisted of mild epidermal hyperplasia with follicular atrophy. Moderate to marked thyroid follicular hyperplasia and moderate to marked bilateral adrenocortical hyperplasia were suspected to be associated with the skin lesions. The most significant lesions and likely cause of death, were multiple granulomatous foci in many regions of the brain consistent with migrating nematode larvae. Overall, the seal appeared to have indications of chronic morbidity and malnutrition/pica which, given the extralimital nature of this case, could be due to pathogen exposure and/or inadequate feeding capacity.

Serology

Antibodies to *Brucella* were detected in 22 of 200 (11 %) serum samples collected from wild bearded seals in Alaska and Svalbard (Table 1). Sixteen of the seropositive seals came from 86 (19 %) animals that were hunted in the Chukchi Sea between 1990 and 2011; one juvenile female, two juvenile males, four adult females, one adult male, five females with unknown age class, one male of unknown age and two animals of unknown sex and age (Table 1). The other seropositive bearded seals, 6 of 76 (8 %), were all captured in the Svalbard archipelago during the period 1995 to 2007. The positive animals were three female and two male pups and the mother of one of the female seropositive pups. It is not known whether the mothers of the other seropositive pups were amongst the animals sampled.

Antibodies to *Brucella* were not detected from any of the 38 bearded seals hunted in the Bering Strait or collected in the Bering Sea or from the five animals kept in captivity at “Polaria” (Table 1). *Brucella* antibodies were detected in the blood collected from the necropsied animal (M61/12).

DISCUSSION

This study documents for the first time the recovery of *Brucella* from a bearded seal, as well as the first serological evidence of *Brucella* exposure in this host. Antibodies were detected in sera from two of the four groups of free-ranging bearded seals sampled; the Chukchi Sea (19 %) and the Svalbard archipelago (8 %), however, they were not detected from 38 bearded seals from the Bering Strait region or the Bering Sea (Table 1). A previous small-scale study also failed to detect *Brucella* antibodies from six bearded seals taken during a subsistence hunt at St Lawrence Island in the Bering Sea (Calle et al., 2008), so evidence of exposure to *Brucella* in this region remains lacking (Figure 1). The Pacific bearded seals are not distinct populations, they move from the Bering Sea however and move through the Bering Strait with the advancing and retreating ice edges. The detection of seropositive bearded seals from the Chukchi Sea therefore may be significant for *Erignathus barbatus nauticus* across their entire area. Another serological study for *Brucella* in bearded seals did not detect antibodies from two locations in the North Atlantic, while antibodies were detected in the other three sympatric species sampled; hooded, harp and ringed seals (Tryland et al., 1999).

Typing of the *Brucella* isolate by MLST demonstrated that it belonged to the ST24 lineage of *B. pinnipedialis*. Sequence type 24 is the less common of two STs isolated predominantly from pinnipeds (Groussaud et al., 2007) and has previously been found associated with harbour seals, grey seals and a minke whale (*Balaenoptera acutorostrata*) which stranded in Scotland and from harbour seals and a beluga whale (*Delphinapterus leucas*) from North America (Groussaud et al., 2007; Whatmore, submitted for publication).

Brucella-associated pathology was not found either grossly or histologically, although histology was not performed on the lymph node and an association of *B. pinnipedialis* with the death of this animal was not established. This is in line with previous findings, which have revealed a paucity of pathologies following *Brucella* isolation from pinnipeds, including several apparently healthy harbour seals which had been shot by fishermen (Foster et al., 2002). In contrast, a broad range of pathologies have been reported for *Brucella* infection of various cetacean species which include lymphocytic meningoencephalitis, sub-cutaneous lesions, blubber abscessation, liver abscess, hepatic and splenic necrosis, macrophage infiltration in liver and spleen, lymph node inflammation, pneumonia, peritonitis, mastitis, osteomyelitis, spinal discospondylitis, diseased atlanto-occipital joint, endocarditis, epididymitis and abortion (Foster et al., 2002; Nymo et al., 2011).

In vitro work has revealed differences between the classical terrestrial *Brucella* strains and *B. pinnipedialis*. The *B. pinnipedialis* reference strain NCTC 12890 and *B. pinnipedialis* hooded seal strains were eliminated from murine and human macrophage cell lines, and a human epithelial cell line within 72-96 h (Larsen et al., 2013b). Even more rapid elimination patterns were observed in hooded seal primary alveolar macrophages (Larsen et al., 2013a) and epithelial cells (Larsen et al., 2016). *Brucella pinnipedialis* NCTC 12890 was also found to be attenuated in the BALB/c *Brucella* mouse model (Nymo et al., 2016). The reduced virulence in these models, when compared to the terrestrial virulent strain *Brucella suis* 1330 (Larsen et al., 2013b; Nymo et al., 2016), is in line with the limited virulence of the *B. pinnipedialis* strains in their natural hosts (Foster et al., 2002).

Brucella infection was suggested as a possible cause of abortion in the otariid species, California sea lion (Goldstein et al., 2009), following recovery of *Brucella* from the placenta and stomach contents of an aborted foetus, raising concerns that *Brucella* may be capable of causing reproductive problems in populations of free-ranging pinnipeds, including bearded seals. Seropositive pups were detected in the present study, however, seals have an endotheliochorial placenta (Stewart and Stewart, 2009), where 5-10 % of the maternal antibodies are transferred to the fetus *in utero* while the rest are transferred through the colostrum. The immunity transmitted by the colostrum is determined by the level of systemic immunity in the mother (Tizard, 2000). At least one of the seropositive pups in the present study was the pup of a seropositive mother suggesting a transfer of maternal antibodies.

Strandings investigations are opportune in nature and restricted largely to material that washes ashore in suitable condition for necropsy and further investigations, including bacteriological and histological studies. Detection of evidence of the impact of *B. pinnipedialis* on reproductive success, should it occur, is likely to rely on additional input from researchers from other fields, which study pinnipeds from sources other than strandings. It may be worth noting that for hooded seals, however, no relation was found between *Brucella* serostatus and ovulation rate or neonatal body condition (Nymo et al., 2013b).

Accurate population estimates for bearded seals are lacking due to their low-density occurrence, widespread distribution and a relative lack of research attention, but they are listed as a species of least concern on the IUCN Red List of Threatened Species (Kovacs, 2016). Their preferred habitat is drifting pack ice in areas over shallow coastal shelves and monitoring of their populations is recommended due to the ongoing impacts of climate change on sea-ice conditions.

Bearded seals are largely solitary animals (Kovacs, 2016). Hooded seals and ringed seals, from which *B. pinnipedialis* has been isolated and anti-*Brucella* antibodies detected (Forbes et al., 2000; Nymo et al., 2013b) are also generally described as being largely solitary (Kovacs, 2002; Miyazaki, 2002), though all three of these species do gather in areas where habitat is suitable for breeding, moulting and foraging. Contrary to what has been documented in cetaceans, no evidence of vertical transmission of *Brucella* in true seals has been reported. Furthermore, the solitary behaviour of bearded seals suggests that opportunities for *Brucella* transmission between conspecifics are restricted. Altogether, this re-enforces the possibility that *Brucella* infection may be acquired from the environment, possibly via diet, as suggested previously (Lambourn et al., 2013; Nymo et al., 2013b). In contrast, harp seals have also been shown to harbour infections with *B. pinnipedialis* (Forbes et al., 2000; Tryland et al., 1999) but this species demonstrates a much stronger tendency to congregate (Lavigne, 2002) and transmission between conspecifics cannot be excluded.

Brucellosis is a significant zoonotic infection, which causes a broad range of manifestations, especially associated with farmed animals and their products, infected with *Brucella melitensis, B. abortus* and *B. suis*, but also *Brucella canis* contracted from dogs. Whilst, there have been three reports of human infections with marine mammal *Brucella*, none have involved *B. pinnipedialis*. Human infection has been reported in a laboratory infection scenario with ST23, a clade predominantly associated with porpoises, while naturally occurring infections have been reported only with ST27 (Whatmore et al., 2008), only isolated thus far from bottlenose dolphins (*Tursiops truncatus*) and California sea lions in the USA (Whatmore et al., submitted for publication) and recently, from a single bottlenose dolphin in the Mediterranean (Cvetnik et al., 2016).

While the lack of human infections with *B. pinnipedialis* are in contrast to the findings with *B. ceti* and the classical *Brucella* spp. mentioned above, the zoonotic potential of *B. pinnipedialis* remains unknown at present. Notably, however, *Brucella* strains isolated from pinnipeds are able to enter and survive in human macrophage-like cell lines *in vitro*, highlighting their potential virulence for humans (Larsen et al., 2013b; Maquart et al., 2009). It is advisable, therefore, that those working with bearded seals and other pinniped species consider the infectious nature of the genus and follow appropriate safety procedures. Commercial sealing of bearded seals was undertaken in the past by Russia reaching 10,000 animals in the 1950s and 1960s in the Pacific Arctic but this harvest tailed off in the 1970s and 1980s and has since ceased. Bearded seals are, however, regarded as an important subsistence species for indigenous peoples, providing food and clothing, boat leather, strapping ropes etc. (Kovacs, 2016). Others at risk of infection include those working with captive bearded seals. The aquarium, “Polaria” (Tromsø, Norway), with more than 100 000 visitors annually, has been a pioneer when it comes to care and handling of bearded seals and still represents one of very few facilities that keep this species. Concerns associated with climate change and growing interest in the Arctic, also increase the interest for including bearded seals among captive Arctic seal species.

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