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Incidence of *Brucella* species in marine mammals of the German North Sea

E. Prenger-Berninghoff[1,*], U. Siebert[2], M. Stede[3], A. König[1,4], R. Weiß[1], G. Baljer[1]

[1]**Institut für Hygiene und Infektionskrankheiten der Tiere der Justus-Liebig-Universität Gießen, Frankfurter Strasse 85–89, 35392 Giessen, Germany**

[2]**Forschungs- und Technologiezentrum Westküste der Christian-Albrecht-Universität Kiel, Hafentörn 1, 25761 Büsum, Germany**

[3]**Staatliches Veterinäruntersuchungsamt für Fische und Fischwaren Cuxhaven, Lentzkai 1, 27472 Cuxhaven, Germany**

[4]***Present address:*** **Landesuntersuchungsamt Rheinland-Pfalz, Blücherstr. 34, 56073 Koblenz, Germany**

ABSTRACT: In this study, organ samples from 426 common seals *Phoca vitulina*, 298 harbour porpoises *Phocoena phocoena*, 34 grey seals *Halichoerus grypus* and 10 other marine mammals were assessed for the presence of *Brucella* species. Forty-seven common seals, 2 harbour porpoises and 1 grey seal were found to be positive for these bacteria. A total of 91 *Brucella* strains were successfully isolated, due to the fact that *Brucella* spp. were found in more than one organ sample in 15 animals. The primary organ in which the bacteria were present was the lung. In addition, 2 strains were isolated from lungworms (*Parafilaroides* spp.). Forty-nine of the isolated strains were selected for further analysis using conventional phenotyping methods. Molecular characterisation was carried out by analysing the *IS711* and *omp2* loci. With respect to the distribution of the *IS711* loci in the genome, the 49 field isolates differed strongly from the terrestrial *Brucella* species and marginally from the marine *Brucella* reference strain NCTC12890. Based on the results of the PCR restriction fragment length polymorphism (PCR-RFLP) investigation of the *omp2* locus, the majority of the *Brucella* field isolates were classified as *B. pinnipediae*, recently proposed *B. pinnipedialis*, possessing 1 *omp2a* gene and 1 *omp2b* gene. Two field isolates revealed the presence of 2 *omp2a* genes, as has been described for *Brucella ovis*. To our knowledge, these results confirm for the first time the presence of *Brucella* species in the marine mammal population of the German North Sea. These findings highlight the need for additional research on the relevance of these *Brucella* species for marine hosts and their environment.

KEY WORDS: *Brucella* species · Marine mammals · Phenotypic and molecular investigation · German North Sea

INTRODUCTION

Since 1994, *Brucella* species have been isolated from various marine mammal species inhabiting several geographic regions (Ewalt et al. 1994, Ross et al. 1994, 1996, Foster et al. 1996, 2002, Garner et al. 1997, Clavareau et al. 1998, Forbes et al. 2000). The marine *Brucella* isolates can be distinguished from the terrestrial *Brucella* species using conventional phenotypic investigation analyses of, for example, CO_2 requirement, urease activity, H_2S production, agglutination with monospecific antisera, growth in the presence of different dyes and oxidative metabolism pattern (Alton et al. 1988, Foster et al. 1996, Garner et al. 1997, Jahans et al. 1997, Clavareau et al. 1998, Forbes et al. 2000, Jacques et al. 2007).

Studies which employed fingerprinting of the genus-specific mobile genetic element *IS711* observed a difference in the number and distribution of *IS711* copies between the genome of the marine *Brucella* isolates and that of the terrestrial *Brucella* species. The marine mammal isolates contained more *IS711* copies and possessed at least one specific *IS711* copy present in all

*Email: ellen.prenger-berninghoff@vetmed.uni-giessen.de

marine *Brucella* isolates (Clavareau et al. 1998, Bricker et al. 2000, Cloeckaert et al. 2000).

A further distinction of the marine *Brucella* species is possible when analysing the genus-specific *omp2*-locus. This locus consists of the 2 closely related genes *omp2a* and *omp2b*. Both share an approximated 85% DNA homology; they are separated by 900 bp and oriented in opposite directions (Ficht et al. 1989). Current data suggest the gene arrangement is conserved in all *Brucella* species. Therefore, distinction between the *Brucella* species and some of their biovars can be performed by PCR-restiction fragment length polymorphism (PCR-RFLP) analysis (Cloeckaert et al. 1995). In a further study, Cloeckaert et al. (2001) obtained data which led to a subdivision of the marine *Brucella* isolates into 2 groups. The strains isolated from seals and 1 otter possessed 1 *omp2a* and 1 *omp2b* gene, representing the formerly proposed species *B. pinnipediae*. In contrast, *Brucella* isolates from dolphins and porpoises possessed no *omp2a* but 2 *omp2b* genes, thus identifying these isolates as *B. cetaceae* (Cloeckaert et al. 2001). *Brucella* isolates from Pacific common minke whales *Balaenoptera acutorostrata* revealed *omp2a* and *omp2b* genes with motifs very similar to Atlantic marine strains as well as to terrestrial strains thus showing them to be chimeric between marine and terrestrial strains (Ohishi et al. 2005).

Oxidative metabolic profiles as well as variable number of tandem repeats (VNTR) typing and multilocus sequence analysis (MLSA) of marine *Brucella* isolates revealed the existence of 3 different groups, one comprising mainly pinniped isolates and the other 2 consisting of either porpoise or dolphin isolates (Groussaud et al. 2007, Jacques et al. 2007). The recently proposed classification of *Brucella* isolated from marine mammals is based on phenotypic and molecular methods and comprises *B. pinnipedialis* which prefer seal hosts, and *B. ceti* which prefer cetacean hosts (Foster et al. 2007). The pathogenic potential of these 2 *Brucella* species for marine mammals remains unknown. Reproductive disorders such as placentitis or abortion in connection with the isolation of *Brucella* species were only present in a few cases (Ewalt et al. 1994, Miller et al. 1999). The transmission to terrestrial animals has been shown to be possible in experiments performed with sheep and cattle (Rhyan et al. 2001, Foster et al. 2002). Furthermore, human infections with marine *Brucella* isolates have also been reported. A research laboratory worker was proved to be infected with a cetacean *Brucella* isolate (Brew et al. 1999), and *B. pinnipediae* was isolated from 2 Peruvian patients with central nervous system disease (Sohn et al. 2003). Characterisation of a *Brucella* strain isolated from a patient with spinal osteomyelitis in New Zealand revealed a close relationship with *Brucella* sp. originating from a United States bottlenose dolphin *Tursiops truncatus* and common seals *Phoca vitulina* (McDonald et al. 2006).

The aim of the present study was to determine whether *Brucella* species are present in marine mammals inhabiting the German part of the North Sea and to classify the obtained isolates via pheno- and genotyping.

MATERIALS AND METHODS

Sampling. The health status of marine mammals from the German North Sea was determined in the course of a national health monitoring program. The examined marine mammals were either stranded, accidentally caught in fishing nets or inhabitants of one of the 2 German seal rehabilitation centres. Tissue samples from 426 common seals *Phoca vitulina*, 298 harbour porpoises *Phocoena phocoena*, 34 grey seals *Halichoerus grypus*, 3 hooded seals *Cystophora cristata*, 3 common dolphins *Delphinus delphis*, 1 white beaked dolphin *Lagenorhynchus albirostris*, 1 ringed seal *Phoca hispida*, 1 pilot whale *Globicephala melas* and 1 minke whale *Balaenoptera acutorostrata* were evaluated for the presence of *Brucella* species. All animals originated from the German North Sea, except 1 common seal which was found in the port of Hamburg. Each individual underwent a full necropsy including further investigations (histopathological examinations, parasitological screening and morbillivirus immunohistochemistry) as described by Siebert et al. (2001). Organ samples and lungworms, if available, were stored at –20°C until further processing.

Bacterial culturing and phenotype analysis. Prior to preparing bacterial cultures, the thawed samples were flame-decontaminated and incised with sterile scissors. The freshly incised area was placed on Brain Heart Infusion agar (Oxoid) supplemented with 5 g yeast (Oxoid), 50 ml sheep blood and aqua destillata up to 1000 ml and *Brucella* agar (Merck) supplemented with 2 ampoules of *Brucella* selective supplement (SR0083, Oxoid), 1.4 ml crystal violet 0.1% (Merck) and aqua destillata up to 1000 ml. Lungworms were incised with a sterile scalpel and the freshly incised area was placed on the media described above. The cultures were incubated for at least 7 d at 37°C in an atmosphere of 10% CO_2.

The phenotype analysis (of carbon dioxide requirement, H_2S and urease production, dye sensitivity and agglutination with the 3 *Brucella* monospecific antisera A, M and R; Federal Institute for Risk Assessment) was performed as described by Alton et al. (1988). Dye sensitivity was tested using basic fuchsin, thionin and safranin supplemented in a concentration of 10, 20 and 40 µg per ml of tryptose soy agar (Merck), respectively.

Molecular analysis. DNA was isolated using the DNEasy Tissue Kit (Qiagen) and precipitated with ethanol according to the manufacturer's instructions. Fingerprinting of the *IS711* locus was carried out with minor variations as described previously (Southern 1975, Chomczynski 1992, Bricker & Halling 1994, Clavareau et al. 1998, Bricker et al. 2000). Briefly, 1 µg of genomic DNA was enzymatically digested overnight at 37°C with 40 U *Eco*RI. The resulting fragments were separated by electrophoresis in a 0.8% agarose gel. After exposure to ultraviolet light (254–312 nm; 60 mJ cm^{-2}), washing twice in 0.25 N HCl and denaturing (1.5 M NaCl, 0.5 M NaOH), the DNA was then transferred to a nylon membrane (Roche). Following neutralisation (1.5 M NaCl, 0.5 M Tris pH 8, 1 mM EDTA pH 8) the blot was dried and baked at 120°C for 30 min. Prehybridisation was performed for 1 h in aqueous hybridisation buffer (50% formamide, 5× saline sodium citrate (SSC), 2% blocking reagent, 0.1% N-lauroylsarkosin, 0.02% sodium dodecyl sulphate, SDS). The digoxigenin (DIG)-labelled *IS711* probe was prepared by PCR amplification of the *IS711* loci of the marine *Brucella* reference strain NCTC12890 according to the manufacturer's instructions (DIG DNA-Labelling Kit, Boehringer). The labelled probe was denatured (10 min boiling) and then added to the hybridisation buffer. Hybridisation was performed for 18 h at 42°C. The membrane was then washed, blocked, incubated with anti-DIG alkaline phosphatase-conjugated Fab-antibody and washed according to the manufacturer's instructions for the DIG Luminescent Detection Kit (Boehringer). Luminescence was recorded on Cronex medical radiographic films 4 (Siemens) for 1 to 2 h.

The *omp2* PCR was performed with primer pairs 2aA, 2aB (*omp2a*) and 2bA, 2bB (*omp2b*) as well as the amplification protocol described by Cloeckaert et al. (1995) (for details see Table 1). Amplification reactions were performed in 50 µl volumes containing 20× *Tfl* reaction buffer (Biozym), 25 mM $MgCl_2$, 16 mM deoxyribonucleotide triphosphate (dNTP), 20 µM primer, 1 U $µl^{-1}$ *Tfl*-DNA-polymerase (Biozym), 100 ng genomic DNA and aqua destillata.

The restriction digestion was carried out according to Cloeckaert et al. (1995). The restriction enzymes *Alu*I, *Cla*I, *Hae*III, *Hinf*I, *Kpn*I and *Pvu*II were purchased from Amersham; *Ban*I, *Nco*I and *Sty*I from New England Biolabs; *Bgl*II, *Eco*RI and *Pst*I from MBI Fermentas; and *Taq*I from Sigma. Buffers and incubation temperatures were used as recommended by the manufacturers. Agarose gel electrophoresis was performed as described previously (Cloeckaert et al. 1995).

The amplicons were sequenced based on the dideoxy-chain-termination method (Sanger et al. 1977). In addition to the primers applied above (2aA, 2aB, 2bA and 2bB), 2 further primers were established (2b470 and 2b609; Table 1) to verify sequence readout. The primers 2b470 and 2b609 were established with OLIGO® primer analysis software 4.0. Sequences were aligned using LASERGENE® Seqman II software. The statistical evaluation of the constructed phylogenetic tree was performed by bootstrap analysis (Felsenstein 1985) using the program 'Seqboot' (PHYLIP-package) with 100 replicates. All primers used in the present study were purchased from Roth.

RESULTS

Specimens from 426 common seals, 298 harbour porpoises, 34 grey seals and 10 other marine mammals were examined to detect *Brucella* species. Each of the 91 *Brucella* isolates was obtained from an organ sample taken from common seals (n = 47), grey seal (n = 1) and harbour porpoises (n = 2). Most of these animals were suffering from verminous bronchopneumonia. From the common seals, 39 *Brucella* isolates were obtained from lung tissue or lung lymph nodes, 19 isolates from the digestive tract, 11 strains from the spleen, 7 strains from the kidneys and 7 strains from the liver. One isolate was found in tongue and skin samples. Two isolates were obtained from lungworms

Table 1. Primer sequences used for PCR and sequencing (targeting the *omp2a* and *omp2b* genes)

Primer	Sequence (5′–3′)
2aA	GGCTATTCAAAATTCTGGCG
2aB	ATCGATTCTCACGCTTTCGT
2bA	CCTTCAGCCAAATCAGAATG
2bB	GGTCAGCATAAAAAGCAAGC
2b470	ATTCGGATTCGTCGATAC
2b609	CGCTCTCGAACAGGGTGG

Table 2. *Brucella* spp. infecting *Phoca vitulina*. Number of strains isolated and analysed from common seals, and the locus of isolation

Locus of isolation	No. isolated strains	No. analysed isolates
Lung	32	22
Lung lymph nodes	7	3
Digestive tract	10	4
Digestive tract lymph nodes	9	3
Spleen	11	5
Kidney	7	3
Liver	7	3
Tongue and skin	2	2
Lungworm	2	2
Total	87	47

of *Brucella*-positive seals (Table 2). A single isolate from a grey seal was found in lung tissue and 3 isolates from harbour porpoises were obtained from lung, kidney and mesenteric lymph nodes. All isolates became apparent on BHI- and *Brucella*-agar after 3 to 5 d of incubation at 37°C in 10% CO_2-enriched atmosphere, the latter condition being essential for bacterial growth.

Forty-nine isolates from 2 lungworms and different organs of 28 common seals (Table 2), 1 grey seal and 1 harbour porpoise were selected as representative strains for further biotyping and molecular analysis. All tested isolates produced urease and grew in the presence of the dyes fuchsin, thionin and safranin (except strain no. 8: sensitive to 40 µg/ml thionin). None of the isolates showed H_2S production. All tested isolates agglutinated in the presence of monospecific antiserum A, whereas no agglutination was observed in the presence of monospecific antiserum M or R.

The number and distribution of *IS711* copies in marine *Brucella* genomes determined by Southern blot of *Eco*RI-digested DNA probes with DIG-labelled *IS711* are shown in Fig. 1. All investigated marine *Brucella* field isolates (n = 49) contained at least 23 *IS711* copies in their genome and showed identical hybridisation patterns. Furthermore, the marine-specific *IS711* copy on the 1.7 kb *Eco*RI fragment was observed in the genome of the 49 field isolates. In contrast to the marine reference strain NCTC12890, all tested isolates lacked an *IS711* fragment in the range of the 3 kb *Eco*RI fragment.

Previous studies observed different restriction amplification patterns for *Brucella* isolates of the *omp2* locus determined by PCR-RFLP and used letters to classify these patterns (Cloeckaert et al. 1995, 2001). Following this classification, all tested marine field isolates displayed the restriction pattern I for their *omp2a* gene. For the *omp2b* gene, 43 isolates showed restriction pattern L (Fig. 2a), 1 grey seal isolate (no. 9) pattern O and 1 common seal isolate (no. 44) pattern M. The common seal isolate no. 22 could not be classified because of an *Eco*RI restriction pattern different from those described by Cloeckaert et al. (2001). Two common seal field isolates (nos. 8 and 10) represented an '*omp2b*' restriction pattern (quotation marks are used here to distinguish this apparent *omp2a* gene positioned at the locus of the *omp2b* gene

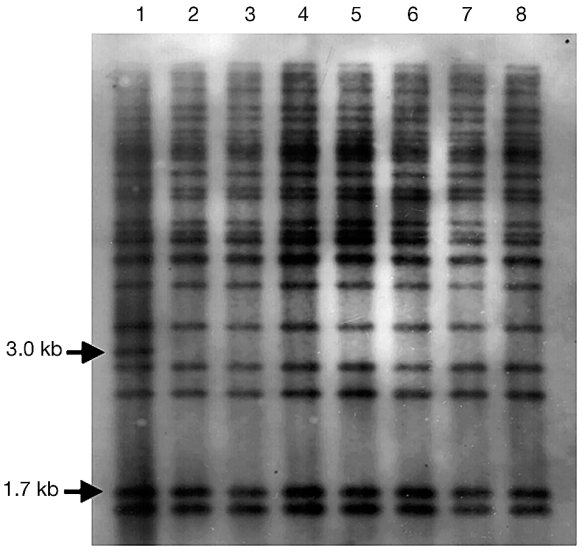

Fig. 1. Southern blot of *Eco*RI-digested DNA from *Brucella* reference strain NCTC12890 (Lane 1) and 7 *Brucella* field isolates (Lanes 2–8) probed with *IS711*-DNA including isolates no. 8 (Lane 3) and 17 (Lane 4). All field isolates demonstrated the same hybridisation pattern including the marine *Brucella* isolate-specific 1.7 kb band. The reference strain displayed 1 additional band in the range of 3 kb

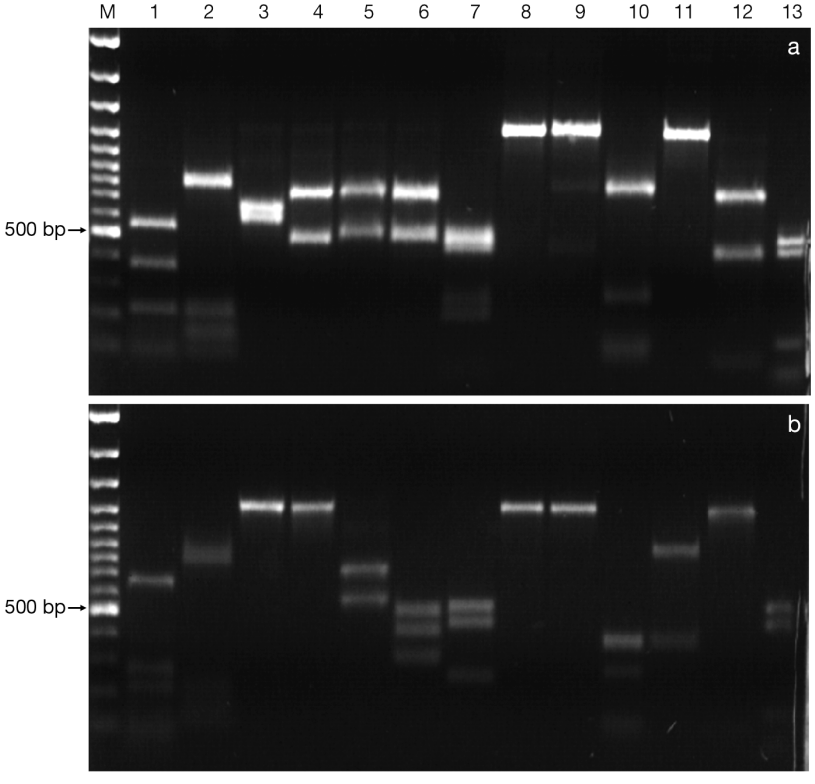

Fig. 2. Restriction patterns of PCR-amplified *omp2b* gene of the marine *Brucella* isolates no. 17 (a) and 8 (b) cut by *Alu*I (Lane 1), *Ban*I (Lane 2), *Bgl*II (Lane 3), *Cla*I (Lane 4), *Eco*RI (Lane 5), *Hae*III (Lane 6), *Hinf*III (Lane 7), *Kpn*I (Lane 8), *Nco*I (Lane 9), *Pst*I (Lane 10), *Pvu*I (Lane 11), *Sty*I (Lane 12) and *Taq*I (Lane 13). Note the differences between the restriction patterns of the *omp2b* gene from isolate no. 17 and '*omp2b*' from isolate no. 8. Lane M: marker (100 bp ladder plus)

from the usual *omp2b* gene) which was nearly identical with their *omp2a* restriction pattern (Fig. 2b).

Nucleotid sequencing of the *omp2a* and '*omp2b*' genes of the *Brucella* field isolate no. 8 (GenBank accession numbers DQ059380 and DQ0593801) demonstrated a homology of 95.2%. Construction of the phylogenetic tree demonstrated the highest degree of relationship of the isolate no. 8 '*omp2b*' gene to the marine *Brucella omp2a* gene of a harbour seal (Fig. 3). Comparison of the *omp2a* gene of isolate no. 8 with the *omp2a* sequence AF300819 from the common seal strain NCTC12890 resulted in 100% similarity, whereas comparison of the '*omp2b*' gene with the *omp2b* sequence AF300818 showed a lower similarity of 91.3%.

DISCUSSION

There have to date been a number of reports of *Brucella* infections in marine mammals living in different marine ecosystems of the southern and northern hemisphere (reviewed in Foster et al. 2002). The aim of the present study was to identify and characterise *Brucella* strains isolated from marine mammals of the German North Sea and clarify how they correspond to marine *Brucella* isolates from other geographical regions. *Brucella* species were detected in 11% of the investigated common seals, 0.7% of the harbour porpoises and 2.9% of the grey seals investigated. The greater number of *Brucella* isolates recovered from common seals may be related to their social behaviour. They gather in large numbers on sandbanks to rest and give birth to their offspring; this crowding might enhance the risk of an infection with *Brucella* species.

We obtained a significant number of strains from lung tissue which corresponded to data from previous studies (Garner et al. 1997, Foster et al. 2002). In most cases, pathological examinations diagnosed a suppurative, necrotising or granulomatous bronchopneumonia in association with lungworm infection and secondary bacterial infections by β-haemolytic streptococci (Swenshon et al. 1998, Siebert et al. 2001). Because of the occurrence of other bronchopneumonia-causing agents the significance of *Brucella* isolates remains unclear. The isolation of marine *Brucella* sp. from lungworms (*Parafilaroides* spp.) as well as from marine mammals with verminous bronchopneumonia suggests that transmission of marine *Brucella* strains by lungworms is possible. Garner et al. (1997) detected marine *Brucella* strains in uterine and gut lumen of lungworms. However, the possibility of *Brucella* infection prior to the entry of lungworms, which then ingest the bacteria, should be kept in mind.

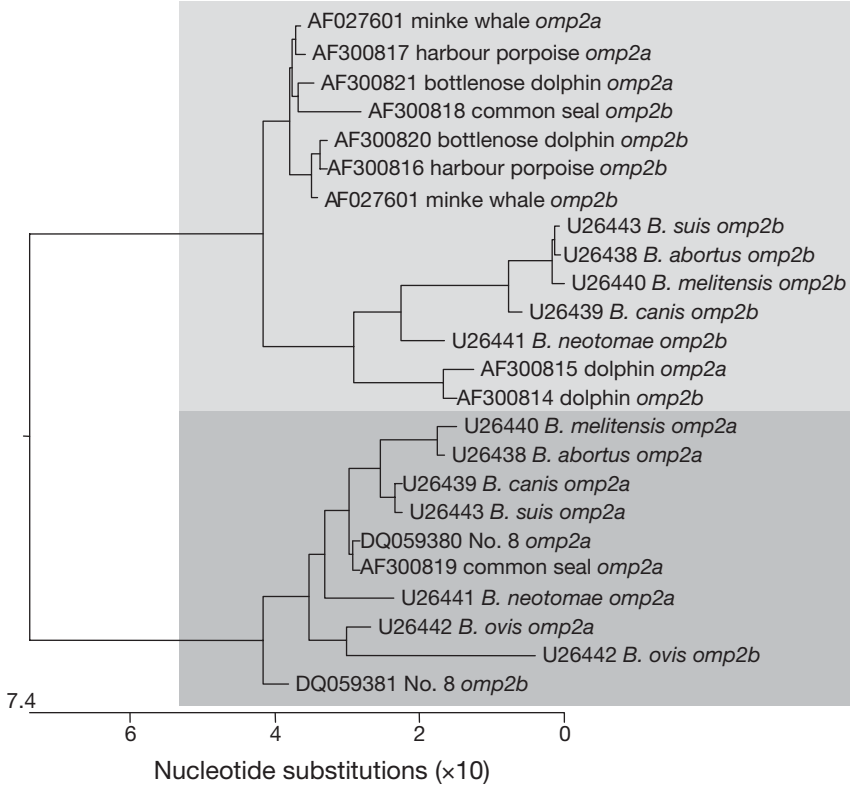

Fig. 3. Phylogenetic tree of different *Brucella* isolates after alignment of *omp2a* and *omp2b* gene sequences (sequence alignment with ClustalW [Slow/Accurate, International Union of Biochemistry standard genetic codes], construction of the phylogenetic tree with the neighbor-joining-method. Light grey shading comprises *omp2b* and dark grey shading *omp2a* gene sequences

For further characterisation, 49 of the isolated marine *Brucella* strains were investigated by conventional phenotypic methods. All strains demonstrated a CO_2-dependent growth, a phenomenon previously described in *Brucella* isolates from seals (Foster et al. 1996, 2002, Jahans et al. 1997, Payeur et al. 1998, Forbes et al. 2000). Cetacean strains are usually able to grow without CO_2 supplement; however, the only cetacean strain involved in the present study did not show this ability.

In terms of their positive urease activity, missing H_2S production, A-dominant LPS profile and low sensitivity to the dyes fuchsin, thionin and safranin (except strain no. 8), none of the tested isolates differed from earlier reports (Garner et al. 1997, Jahans et al. 1997, Clavareau et al. 1998, Payeur et al. 1998, Forbes et al. 2000). To further dif-

ferentiate the marine *Brucella* strains, *IS711* fingerprinting was carried out. Compared to classical *Brucella* strains (except *B. ovis*), all 49 marine isolates from the German North Sea possessed a higher number of *IS711* copies in their genome. These findings correspond to the results of Bricker et al. (2000), as does the detection of the marine specific *IS711* copy on the 1.7 kb *Eco*RI fragment. The hybridisation patterns indicate a close relationship between the investigated *Brucella* isolates and the reference strain NCTC12890 which originates from a common seal from the British coast.

To detect polymorphism between the German isolates, PCR-RFLP of the *omp2* locus was performed based on the data from previous studies (Cloeckaert et al. 1995, 2001). All tested isolates revealed *omp2a* restriction pattern I, which has also been detected in the majority of *omp2a* genes from other North Sea seal isolates, including the reference strain NCTC12890 (Cloeckaert et al. 2001). Furthermore, the *omp2a* sequence from this reference strain has recently been proven to be identical to that of North Pacific minke whales (Ohishi et al. 2004). With regard to the *omp2b* gene, 5 isolates differed from the most frequently detected pattern L, which has also been des-cribed for the seal reference strain (Cloeckaert et al. 2001). Interestingly, 2 isolates showed an '*omp2b*' gene pattern very similar to their *omp2a* pattern, suggesting the presence of 2 *omp2a* genes. The sequence alignment results support this hypothesis, as we were able to demonstrate a high similarity between both *omp2* genes. Furthermore, the close relationship of both genes was confirmed by the phylogenetic tree. To date, only the *omp2* locus of *Brucella ovis* has been described as consisting of 2 *omp2a* genes.

Phenotyping and genotyping methods revealed no differences between the German seal isolates and the cetacean strain investigated in this study. One potential reason might be the close vicinity of seals and cetaceans in the restricted area of the German North Sea. The results of this study might suggest that *Brucella* is endemic in this region. Infection of non-preferred host species has been described for *Brucella* in endemic regions (Verger et al. 1989).

In summary, we were able to classify 47 of the 49 marine *Brucella* isolates as members of the formerly proposed species *B. pinnipediae*. The rarity of detecting differences between the reference strain NCTC12890 and the German *Brucella* isolates might result in their classification as *B. pinnipedialis*. This study confirms for the first time the presence of *Brucella* species in the marine mammal population of the German North Sea. These findings highlight the urgent need for additional research on the transmission, zoonotic potential and pathogenic relevance of these *Brucella* species for marine hosts and their environment.

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Editorial responsibility: Michael Moore, Woods Hole, Massachusetts, USA

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