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BRUCELLA PINNIPEDIALIS IN GREY SEALS (*HALICHOERUS GRYPUS*) AND HARBOR SEALS (*PHOCA VITULINA*) IN THE NETHERLANDS

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ABSTRACT: Brucellosis is a zoonotic disease with terrestrial or marine wildlife animals as potential reservoirs for the disease in livestock and human populations. The primary aim of this study was to assess the presence of *Brucella pinnipedialis* in marine mammals living along the Dutch coast and to observe a possible correlation between the presence of *B. pinnipedialis* and accompanying pathology found in infected animals. The overall prevalence of *Brucella* spp. antibodies in sera from healthy wild grey seals (*Halichoerus grypus*; *n*=11) and harbor seals (*Phoca vitulina*; *n*=40), collected between 2007 and 2013 ranged from 25% to 43%. Additionally, tissue samples of harbor seals collected along the Dutch shores between 2009 and 2012, were tested for the presence of *Brucella* spp. In total, 77% (30/39) seals were found to be positive for *Brucella* by IS*711* real-time PCR in one or more tissue samples, including pulmonary nematodes. Viable *Brucella* was cultured from 40% (12/30) real-time PCR-positive seals, and was isolated from liver, lung, pulmonary lymph node, pulmonary nematode, or spleen, but not from any PCR-negative seals. Tissue samples from lung and pulmonary lymph nodes were the main source of viable *Brucella* bacteria. All isolates were typed as *B. pinnipedialis* by multiple-locus variable number of tandem repeats analysis-16 clustering and matrix-assisted laser desorption ionization-time of flight mass spectrometry, and of sequence type ST25 by multilocus sequence typing analysis. No correlation was observed between *Brucella* infection and pathology. This report displays the isolation and identification of *B. pinnipedialis* in marine mammals in the Dutch part of the Atlantic Ocean.

Key words: *Brucella pinnipedialis*, *Halichoerus grypus*, MALDI-TOF MS, marine mammals, MLST, MLVA-16, *Phoca vitulina*, the Netherlands.

INTRODUCTION

In the last two decades, the presence of *Brucella* spp. has been reported in marine mammals in various geographic waters (Foster et al. 2002; Maquart et al. 2009). Depending on differences in genotype and phenotype, especially host preference, two separate groups within the marine *Brucella* spp. were defined: strains from cetaceans were named *Brucella ceti* (Cloeckaert et al. 2001), whereas isolates from pinnipeds were designated as *Brucella pinnipedialis* (Foster et al. 2007).

The classification of *Brucella* species correlates roughly with the taxonomic divisions of their preferred hosts (Guzman-Verri et al. 2012; Olsen and Palmer 2014). The currently accepted taxonomic *Brucella* grouping shows not only a differentiation into species but also a subgrouping of strains into biovars (Moreno et al. 2002). Depending on the genotyping technique used, several subgroups within the marine *Brucella* strains were identified, although not outlined as being biovars (Cloeckaert et al. 2001; Maquart et al. 2009). Based on the identification of single nucleotide

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polymorphisms by the 9-scheme multilocus sequence typing (MLST) analysis, pinniped isolates clustered into two distinct sequence types, ST24 and ST25 (Groussaud et al. 2007). A different approach is the branching of these strains by using the first panel of the multiple-locus variable number of tandem repeat analysis (MLVA)-16 clustering, finding a C1 group to be in correspondence with the ST24 type, and a C2 and C3 cluster to align with the sequence type ST25 (Maquart et al. 2009).

In humans, brucellosis is a major (reemerging) contagious zoonotic disease, mainly caused by *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, and occasionally by *Brucella canis* and *Brucella ceti* strains (Nymo et al. 2011; Moreno 2014; Olsen and Palmer 2014). Transmission of *Brucella* occurs through inhalation or ingestion of infected material into the respiratory or gastrointestinal tract (Moreno 2014). Mucous membranes and lesions of the skin are also known to be routes of entry for *Brucella* infection (Pappas 2010). A small number of human brucellosis cases have been linked to *Brucella* spp. associated with marine mammals (Brew et al. 1999; Sohn et al. 2003; McDonald et al. 2006). The exact nature of transmission between mammals and the zoonotic potential of *Brucella* isolates originated from cetaceans and pinnipeds needs further investigation.

Although *B. ceti* is related to clinical manifestations in reproductive organs, cardiovascular and respiratory systems, bones, joints, and skin, and causes chronic diseases in cetaceans, no apparent pathology in a wide range of pinnipeds was ever associated with the isolation of *B. pinnipedialis* (Nymo et al. 2011; Guzman-Verri et al. 2012). *Brucella pinnipedialis* bacteria were isolated from a range of different seal species: grey seal (*Halichoerus grypus*), harbor seal (*Phoca vitulina*), harp seal (*Pagophilus groenlandicus*), hooded seal (*Cystophora cristata*), Pacific harbor seal (*Phoca vitulina richardii*), and ringed seal (*Pusa hispida*), as well as from California sea lions (*Zalophus californianus*). In addition, there have been several reports on the presence of *B. pinnipedialis* in various organs of seals such as digestive tract, kidney, liver, lung, pulmonary lymph node, placenta, and spleen (Nymo et al. 2011). *Brucella pinnipedialis* was also found in nematodes located in the lungs of pinnipeds (Garner et al. 1997; Maratea et al. 2003).

Although pathology appeared to be absent, serologic responses against *Brucella*, most likely *B. pinnipedialis*, were observed in seals. This seroprevalence of *Brucella* antibodies in pinnipeds is highly related to geography and seal species (Lambourn et al. 2013; Nymo et al. 2013). In seals located in the western part of the Atlantic Ocean, prevalence can be as high as 35%, whereas seals in other parts of the world, including the eastern side of the Atlantic Ocean, were as low as 5% (Nielsen et al. 2001; Lambourn et al. 2013). Around Antarctica, seroprevalences from 4.7% to 65.6% were found, depending on the serologic test used and the seal species studied (Tryland et al. 2012; Jensen et al. 2013).

Little is known about the presence of *Brucella* species in marine mammals living in the Dutch North Sea. Recently, *B. ceti* was detected in harbor porpoises (*Phocoena phocoena*) stranded on the Dutch coast (Maio et al. 2014). In addition, in the bordering German North Sea, grey seals and harbor seals tested positive for *Brucella*-specific DNA in one or more organs (Prenger-Berninghoff et al. 2008). The aim of the present study was to assess the presence of *B. pinnipedialis* in marine mammals living along the Dutch coast and to examine a possible correlation between *B. pinnipedialis* and accompanying pathology in infected animals.

MATERIALS AND METHODS

Animals and tissue samples

Harbor seals that stranded along the Dutch shore died either naturally or were euthanized due to poor prognosis at the seal rehabilitation center Ecomare (De Koog, the Netherlands). Carcasses were necropsied either fresh and cooled (n=6), or first stored at −20 C and defrosted (n=33) at the Department of Pathobiology, Faculty of Veterinary Medicine at Utrecht University. Briefly, necropsy included macroscopic evaluation of the animal, followed by tissue sampling based on the Decomposition Condition

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Code (DCC). For histopathologic evaluation, tissues fixed with 10% neutral buffered formalin (VWR International, Radnor, Pennsylvania, USA) were embedded in paraffin (VWR International), cut in 3 μm sections, and stained with H&E (VWR International). Macroscopic and histopathologic changes were grouped and scored according to Siebert et al. (2007). Frozen samples of 132 tissues: kidney (n=7), liver (n=28), lung (n=26), pulmonary lymph node (n=15), reproductive tract (n=23), spleen (n=28), and lung nematodes (*Otostrongylus circumlitus*; n=5; Supplementary Material Table S1) were submitted to the Dutch *Brucella* reference laboratory at the Wageningen Bioveterinary Research of Wageningen University and Research (WBVR) for detection of *Brucella* spp.

Additionally, sera from healthy wild grey seals (n=11) and harbor seals (n=40) were collected between 2007 and 2013 by the Wageningen Marine Research of Wageningen University and Research from two geographic locations (Texel and Dollard Bay) in the northern part of the Netherlands (Wadden Sea). Sera were tested for the presence of *Brucella* antibodies by enzyme-linked immunosorbent assay (ELISA) and two dissimilar agglutination assays at the WBVR.

Serologic examination

Serum samples of seals were tested for *Brucella* spp. antibodies by a Rose Bengal agglutination test (IDEXX, Westbrook, Maine, USA) and a serum agglutination test (WBVR, Lelystad, the Netherlands). Both tests were performed as described in the World Organisation for Animal Health (OIE) manual (2016). Sera were also screened using the Svanovir *Brucella*-Ab competitive ELISA (C-ELISA; Boehringer Ingelheim Svanova, Uppsala, Sweden) according to the manufacturer's instructions (Nielsen et al. 1995). Serum samples exhibiting any degree of clumping of colored antigen in the Rose Bengal agglutination test, or a 25% agglutination reaction at a concentration of 1:15 or above in the serum agglutination test were considered positive. All three serologic tests used a *B. abortus* antigen.

Analysis of serologic data by the binormal mixture model

A cut-off value of 30% is recommended by the manufacturer to interpret the Svanovir *Brucella*-Ab C-ELISA test results. Because this value is optimized for other host species, the optical density (450 nm) values were analyzed in a binormal mixture model (Opsteegh et al. 2010) to determine the optimal cut-off value for pinnipeds. The sampled Dutch seal population was implied to consist of seronegative and seropositive animals (Nymo et al. 2011, 2013), and the optical density values of both groups were assumed to be normally distributed. Using maximum likelihood, a binormal mixture was fitted to the observed frequency distribution of the 51 ELISA values. The mixing parameter provided the prevalence estimate, and the optimal cut-off value was determined by maximizing the sum of the sensitivity and the specificity.

Brucella reference strains

The following reference strains from the National Collection of Type Cultures (NCTC) were used during culture, real-time PCR, and genotyping experiments: *B. melitensis* biovar 1 (NCTC10094; 16M), *B. pinnipedialis* (NCTC12890), and *B. ceti* (NCTC12891). A collection of 18 *Brucella* strains were added to those three to generate a matrix-assisted, laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) library: *B. neotomae* (NCTC10084), *B. abortus* biovar 1 (NCTC10093), *B. suis* biovar 1 (NCTC10316), *B. abortus* biovar 2 (NCTC10501), *B. abortus* biovar 3 (NCTC10502), *B. abortus* biovar 4 (NCTC10503), *B. abortus* biovar 5 (NCTC10504), *B. abortus* biovar 6 (NCTC 10505), *B. abortus* biovar 9 (NCTC10507), *B. melitensis* biovar 2 (NCTC10508), *B. melitensis* biovar 3 (NCTC10509), *B. suis* biovar 2 (NCTC10510), *B. suis* biovar 3 (NCTC10511), *B. ovis* (NCTC10512), *B. canis* (NCTC10854), *B. melitensis* biovar 1 (NCTC11362), *B. suis* biovar 4 (NCTC11364), and *B. suis* biovar 5 (NCTC11996). All strains were purchased from the Culture Collections of the Public Health England (Porton Down, Salisbury, UK), and handled according to the enclosed general terms of usage.

Detection of *Brucella* spp. in tissue and culture by real-time PCR

Tissue samples from seals were subjected to a *Brucella* real-time PCR (Maio et al. 2014) and to culturing as described in the OIE manual (2016). Briefly, samples of 2×2×2 cm were cut into small pieces and macerated with 20 mL of Castañeda's medium (WBVR) using a peddle blender. Solid and liquid Castañeda's selective media with antibiotics (2.5 IU/mL polymyxin B: Fagron B.V., Rotterdam, the Netherlands; 12.5 IU/mL bacitracin: Thermo Fischer, Waltham, Massachusetts, USA; 50 μg/mL cycloheximide: Applichem GmbH, Darmstadt, Germany; 2.5 μg/mL nalidixic acid: Sigma-Aldrich, St. Louis, Missouri, USA; 50 IU/mL nystatin: Sigma-Aldrich; 10 μg/mL vancomycin, Certa S.A., Braine l'Alleud, Belgium) were inoculated with the blended tissues (1 drop and 1

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mL, respectively), and incubated at 37 C in a 10% CO_2 incubator. Every seventh day, for 3 successive wk, liquid media were subcultured on solid Castañeda's selective media with antibiotics and incubated for 7 d under the same conditions. *Brucella* suspected colonies were confirmed by real-time PCR targeting the IS*711* sequences of *Brucella* spp. (Ocampo-Sosa and Garcia-Lobo 2008) as described by Maio et al. (2014). Samples were considered positive if the presented sigmoid curves showed threshold cycle (Ct) values $\leq$36, doubtful if 36$<$ Ct values $\leq$40, or negative with Ct values $>$40 or no Ct at all.

Genotyping of *Brucella* spp. by MLVA-16 clustering and MLST analysis

Multiple-locus variable number of tandem repeat analysis-16 clustering was performed using a selection of 16 different repeat loci markers to differentiate isolates into *Brucella* species and biovars (Le Fleche et al. 2006; Al Dahouk et al. 2007a; Maquart et al. 2009). Briefly, PCR amplification was performed using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, California, USA) in a total volume of 25 µL containing 1× reaction buffer (Thermo Fisher), 0.1 U/µL TrueStart Taq DNA polymerase (Thermo Fisher), 2 mM $MgCl_2$ (Thermo Fisher), 0.4 mM of each nucleotide (dATP, dCTP, dGTP, dUTP; Thermo Fisher), 1 µM of each primer (Eurogentec S.A., Liège, Belgium), 0.1 U/µL UDG (New England Biolabs, Ipswich, Massachusetts, USA), 1 µL template, and nuclease-free water (Sigma-Aldrich). An initial UDG incubation for 5 min at 37 C and denaturation/activation for 2 min at 96 C was followed by 40 cycles of denaturation for 30 s at 96 C, annealing for 30 s at 60 C, elongation for 30 s at 72 C, and finalized by an extension step of 5 min at 72 C. The PCR products with different fluorescent dyes were diluted depending on the PCR efficiency, and pooled. From these pooled PCR products, 2 µL was mixed with 15 µL of Hi-Di formamide (Applied Biosystems) and 0.5 µL of GeneScan 600 LIZ Size Standard (Applied Biosystems). Samples were denaturated for 5 min at 98 C and separated on a 3130 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined using Peak Scanner version 1.0 software (Applied Biosystems). The number of repeats for each locus was determined on the basis of published data (Al Dahouk et al. 2007b). Further MLVA-16 clustering was carried out as described previously (Al Dahouk et al. 2007b) using Bionumerics version 6.3 (bioMérieux, Marcy l'Etoile, France). For MLST analysis, fragmented libraries were constructed using Nextera DNA sample preparation kit (Illumina, San Diego, California, USA). Next generation whole genome sequencing was performed by paired-end sequencing using the Illumina technology on the MiSeq instrument (Illumina). De novo assembly of the quality filtered reads was performed using ABySS-pe version 1.3.3. (Simpson et al. 2009). Bowtie2 version 0.2 (Johns Hopkins University, Baltimore, Maryland, USA) aligning was used for curation of the contigs quality by Tablet version 14.04.10 (Milne et al. 2013). Additionally, MLST typing was performed in silico with a set of MLST specific primers (Whatmore et al. 2007) and the assembled contigs as input.

Identification and typing of *Brucella* spp. by MALDI-TOF MS

A MALDI-TOF MS procedure using the microflex LT instrument (Bruker, Billerica, Massachusetts, USA) was performed according to the instructions of the manufacturer. From frozen stocks, bacteria were cultured on Castañeda's selective plates with antibiotics for at least 4 d at 37 C in the presence of 10% CO_2. Briefly, one colony was suspended in 300 µL of water, 900 µL of absolute ethanol (Merck, Kenilworth, New Jersey, USA) was added, and the suspension was mixed thoroughly. After centrifugation for 3 min at 10,000 × G, supernatants were discarded. To remove residual ethanol, a short spinning step was introduced, and the remaining supernatant was removed carefully. Subsequently, 50 µL of 70% formic acid (Sigma-Aldrich) was added to the pellet, and mixed by vortexing. Next, 50 µL of pure acetonitrile (Sigma-Aldrich) was added, and the suspension was mixed carefully. The particulate matter that could not be dissolved was spun down by centrifugation for 2 min at 10,000 × G. Finally, 1 µL of supernatant was used to fix a spot onto a MALDI-TOF MS target polished steel plate (Bruker) and air-dried at room temperature. Each spot was overlaid with 1 µL of a saturated solution of alpha-cyano-4-hydroxy cinnamic acid (Bruker) in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid; both Sigma-Aldrich), and air-dried at room temperature. Mass spectra acquisition was performed in linear mode using the following parameters under the control of flexControl version 3.4 (Bruker): 20–36% laser intensity, positive polarity, 120 ns PIE delay, 20 kV source voltage 1, 18 kV source voltage 2, 6 kV lens voltage, 2.6 kV linear detector voltage, and 2,000–20,000 Da detector gating. The instrument was calibrated externally with a bacterial test standard (Bruker). Each spot was measured using the standard flexControl method (MBT_FC.par; linear and positive mode) and the auto executes method (MBT_autox.axe).

MALDI-TOF MS data analysis

For the construction of the custom *Brucella* reference library of 21 reference strains, at least

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20 independent measurements for each reference strain were created according to company guidelines (Bruker) using default settings. The initial data analysis and selection of raw spectra was performed with the flexAnalysis version 3.4 (Bruker). Main spectra were assembled using a fully automated process in MALDI Biotyper version 3.1. from a selection of 20 raw data spectra that were automatically preprocessed in a five-step approach: 1) mass adjustment, 2) smoothing, 3) baseline subtraction, 4) normalization, and 5) peak detection. Next, from all pinniped field isolates, spectra were generated and matched with the main spectra in the reference database of the manufacturer (Bruker) and the newly generated custom *Brucella* reference library using default settings.

Correlation of histopathologic lesions with *Brucella* detection

Animals and organs were grouped according to the abovementioned *Brucella* detection: PCR-positive, culture-positive (double positive) or PCR-negative, culture-negative (double negative). From the necropsy reports, pathologic lesions of double negative organs of double negative animals were compared to double positive organs from double positive animals.

RESULTS

Serologic analysis of grey seals and harbor seals for *Brucella* spp.

The presence of antibodies against *Brucella* in wild healthy seals was studied by analyzing a collection of 51 sera from two different seal species (grey seals, n=11; harbor seals, n=40) by two different agglutination assays and one ELISA (Table S2). The Rose Bengal agglutination test showed positivity in 53% (21/40) sera from harbor seals, whereas 40% (16/40) sera were *Brucella*-positive in the serum agglutination test. Only 9% (1/11) sera from grey seals showed a positive agglutination, and was positive in both tests. In the competitive ELISA testing, 36% (4/11) sera from grey seals showed inhibition percentages higher than 30% (the cut-off value according to the manufacturer), whereas 60% (24/40) sera from harbor seals yielded a positive serologic response. When combined, the seroprevalence of *Brucella* antibodies in these seals based on these ELISA results was 55% (28/51). In all three assays, a *B. abortus* antigen was used to capture pinniped antibodies (Meegan et al. 2010). Because the ELISA cut-off values for pinniped sera are unknown, the seroprevalence, and hence the optimal cut-off value, was determined in a binormal mixture model (Fig. 1). The population mixture that best described the observations had an estimated seroprevalence of 25% (95% confidence interval: 12–40%). The optimal cut-off that maximizes the test characteristics is 50%, with a corresponding sensitivity of 88% and specificity of 93%. Overall, the seroprevalence in 51 seal sera was 43, 33, and 25% in the Rose Bengal agglutination test, the serum agglutination test, or in the ELISA, respectively.

Presence of *Brucella* in tissues of harbor seals

Postmortem examinations were performed on 39 harbor seals, comprising 25 females and 14 males, consisting of 31 juveniles, seven adults, and one neonate. The animals were stranded along the Dutch shore between September 2009 and December 2012 (Table 1).

Of the necropsied harbor seals, 77% (30/39) tested positive for *Brucella* by PCR in one or more tissues or lung nematodes. For nine animals, all tissues were observed negative in the real-time PCR. *Brucella* bacteria were cultured from 40% (12/30) of the PCR-positive seals and 14% (19/132) examined seal tissue samples, including lung nematodes. Positive samples included lung (31%, 8/26 organs tested), pulmonary lymph node (27%, 4/15), lung nematodes (60%, 3/5), spleen (11%, 3/28), and liver (4%, 1/28). Tissues from reproductive tract (n=23) and kidney (n=7) tested negative for *Brucella* (Table S1).

Detection and identification of *Brucella* genotypes

All *Brucella*-isolates were subjected to MLVA-16 clustering and were closely related to *B. pinnipedialis* strains in the publicly available MLVA database for *Brucella* (Grissa et al. 2008; Table S1), despite the fact that different MLVA profiles were found among

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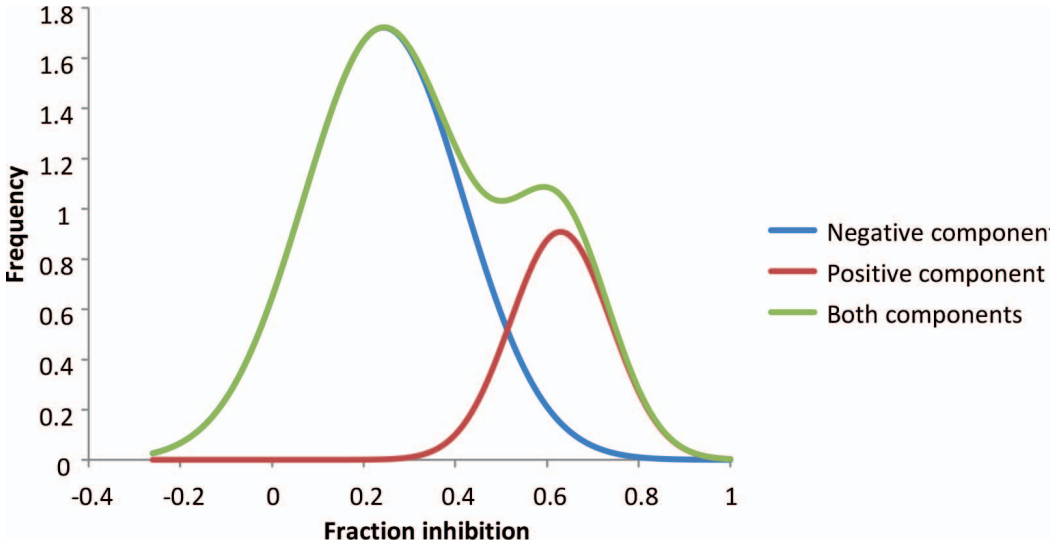


FIGURE 1. Analysis of Svanovir *Brucella*-Ab competitive enzyme-linked immunosorbent assay (C-ELISA) results testing the presence of antibodies against *Brucella* in wild healthy seals by a binormal mixture model. Frequency distribution of observed inhibition percentages (n=51) and fitted normal distributions (lines) are used to calculate the cut-off value as being 50%. Accordingly, ELISA results were reclassified showing animals as being positive or negative for *Brucella* antibodies.

the 19 isolates. When more than one sample from the same animal was culture positive, either from seal tissue or lungworm, the MLVA profiles proved to be identical (maximum of three loci with a different number of repeats), suggesting that one genotype was present within the animal. The genotypic relationship between the strains and the reference strains *B. melitensis* biovar 1, *B. ceti*, and *B. pinnipedialis* is shown in Figure 2. Using maximum parsimony analysis on these MLVA profiles as a taxonomic confirmation, all strains clustered around the reference strain *B. pinnipedialis* and were located neighboring subcluster C2 of the marine mammals group (Groussaud et al. 2007). Further confirmation of these genotypic relationships was done by MLST

TABLE 1. Stranding year, sex, age, PCR-, and culture-positivity for *Brucella* spp. of harbor seals (*Phoca vitulina*) stranded along the Dutch coast from 2009–12 and subjected to postmortem examination.

	No.					
		Sex		Age		
	Total	Male	Female	Neonate	Juvenile	Adult
Year stranded						
2009	6	3	3	0	5	1
2010	8	1	7	0	4	4
2011	13	6	7	1	11	1
2012	12	4	8	0	11	1
Total	39	14	25	1	31	7
Brucella status						
PCR-positive	30	12	18	0	26	4
Culture-positive	12	6	6	0	12	0

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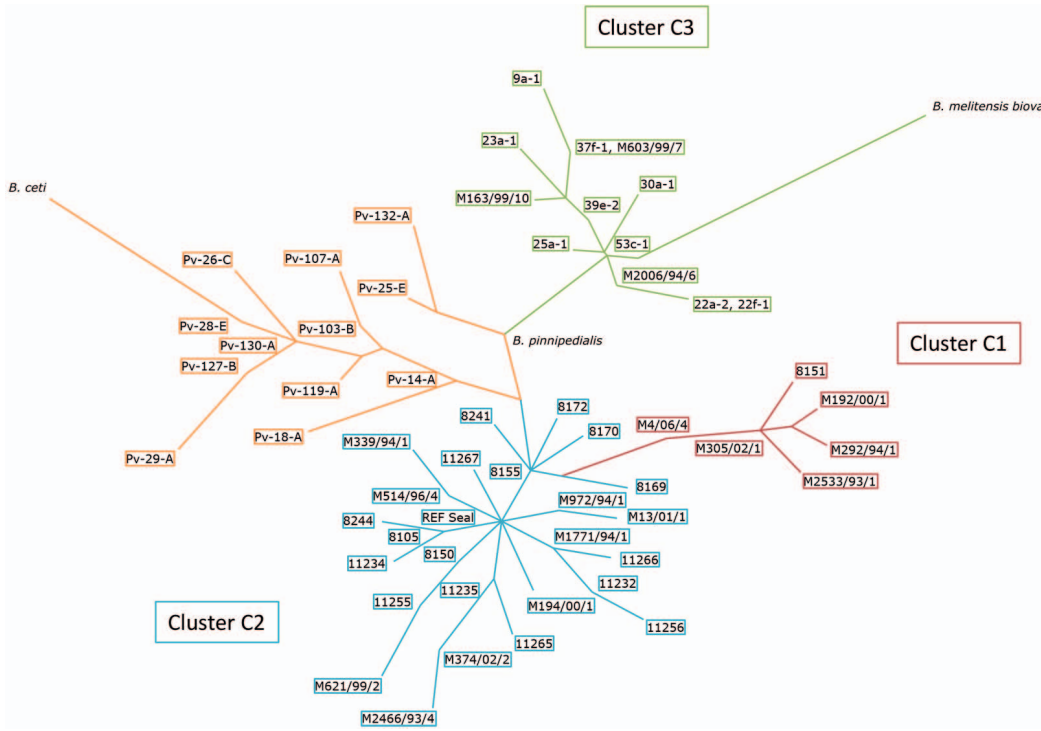


FIGURE 2. Genotypic clustering of *Brucella pinnipedialis* strains isolated from harbor seals (*Phoca vitulina*; Table S1) using maximum parsimony analysis of multiple-locus variable number of tandem repeat analysis-16 clustering determined genotypes. All isolates (orange) grouped within the *B. pinnipedialis* cluster identified by Groussaud et al. 2007 (C1 strains in blue, C2 strains in red, and C3 strains in green). Reference strains *Brucella melitensis* biovar 1 (NCTC10094), *Brucella ceti* (NCTC12891), and *B. pinnipedialis* (NCTC12890) were included. Genotypes numbers correspond to sample references in Table S1.

analysis on a selected number of isolates: Pv-14A (2009), Pv-18A (2010), Pv-107A (2011), Pv-119A (2012), together with the reference strains *B. melitensis* biovar 1, *B. ceti*, and *B. pinnipedialis*. All pinniped strains including the *B. pinnipedialis* reference strain were sequence type ST25 (Whatmore et al. 2007) corresponding to the aforementioned subcluster C2 (Groussaud et al. 2007). Reference strain *B. melitensis* biovar 1 and *B. ceti* were identified as sequence type ST7 and ST23, respectively. All *Brucella*-positive strains displayed highly similar proteomic spectra in the MALDI-TOF MS analysis and aligned best with the *B. pinnipedialis* reference strain (NCTC12890) present in the customized *Brucella* reference library. All scores of the MALDI-TOF MS analysis are summarized in Table S3.

Analysis of histopathologic lesions related to *Brucella* presence

The DCC of carcasses varied between DCC 1 (very fresh, not frozen; *n*=6), DCC 2 (fresh, frozen; *n*=29), and DCC 3 (putrefied, frozen; *n*=4). From 12 *Brucella* double positive (PCR- and culture-positive) seals and nine double negative (PCR- and culture-negative) seals, pathologic lesions were compared between double positive versus double negative organs (Table S4): lungs double positive (*n*=8)/double negative (n=6), pulmonary lymph nodes double positive (n=4)/double negative (n=4), spleens double positive (n=3)/double negative (n=2), and livers double positive (n=1)/double negative (n=6). *Brucella*-positive nematodes (*n*=3) were only detected in double positive seals. *Brucella*-negative organs from double negative animals also included kidney (*n*=1) and reproductive

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tract (*n*=6). In the *Brucella*-positive organs, histopathologic lesions were detected in the lung (*n*=8/8), pulmonary lymph node (*n*=3/4), and liver (*n*=1/1), but not in the spleen (*n*=0/3). In the *Brucella*-negative organs, histopathologic lesions were detected in lung (*n*=3/6), pulmonary lymph node (1/4), and liver (*n*=2/6), and again not in the spleen. In both groups, the majority of histologic lesions were matching symptoms of a lungworm infection: pneumonia, hyperplasia of bronchial lymph nodes, secondary hepatitis, and ischemic liver necrosis. Hence, it was not possible to test for a correlation because in the dataset there were hardly any observations without pathologic lesions.

DISCUSSION

The aim of the present study was to determine and identify the presence of *Brucella* spp. in the Dutch seal population and to observe a possible correlation between the presence of *B. pinnipedialis* and the accompanying pathology in infected seals. The analysis of the Svanovir *Brucella*-Ab competitive C-ELISA results with a binormal mixture model showed that the recommended cut-off value of 30% was not optimal for the serologic screening of *Brucella* in pinnipeds. This model allowed for the determination of a more appropriate cut-off value, but the positive predictive value of the test seems limited, reflected in less-than-perfect test characteristics (sensitivity of 88% and specificity of 93%). The seroprevalence using the binormal mixture model analysis was estimated to be 25%, with a substantial 95% confidence interval (12–40%) largely due to the limited sample size of 51 animals. This number was in agreement with seroprevalence numbers of marine *Brucella* spp. determined in other studies on pinnipeds roaming the western part of the Atlantic Ocean (Tryland et al. 1999, 2005; Nymo et al. 2011, 2013).

Brucella pinnipedialis was detected in tissue samples of stranded harbor seals by PCR (77%, 30/39) and culturing (31%, 12/39). Forty percent of the 30 real-time PCR-positive seals investigated also tested positive for *Brucella* spp. by bacterial culture. The lower recovery by culture might be due to the loss of viability of *Brucella* bacteria during storage of carcasses and tissue samples. Prenger-Berninghoff et al. (2008) showed a lower prevalence (11%) of *Brucella* spp. in harbor seals sampled in the neighboring German North Sea based on bacterial culture analysis. Given the continuous movement of seals between Dutch and German waters, the discrepancy in prevalence is unlikely due to geographic differences. More likely, this significant increase could represent a temporal change in the incidence of *Brucella* in the seal population of the international North Sea.

The age-dependent prevalence of *Brucella* was remarkable in both serology and in the investigation of tissues isolated from stranded animals. The PCR-positivity was 84% (26/31) in juveniles compared to 57% in adults (4/7; Table S1). More striking was the fact that *B. pinnipedialis* was cultured only from juveniles and not from adults. Out of the 33 adults 48% (16/33) were serologic negative in all three tests, whereas 67% (12/18) of the juveniles were seropositive in at least one serologic assay. These numbers substantiate data demonstrating that *Brucella* infection in pinnipeds occurs early in life and that loss of antibody levels is probably due to clearance of *Brucella* bacteria (Lambourn et al. 2013; Nymo et al. 2013; Hoover-Miller et al. 2017).

Genotyping by *Brucella*-specific PCR, MLVA clustering, MLST analysis, and proteomic typing by MALDI-TOF MS, revealed that all isolated strains from Dutch seals were *B. pinnipedialis*. All isolates grouped together and adjoined subcluster C2 with strains mainly from Germany and Scotland (Groussaud et al. 2007). Whether this observation is of any taxonomic importance within the genus *Brucella* or of any biological relevance within the seal population in the North Sea or the western part of the Atlantic Ocean could be a subject of future research, probably in combination with whole genome sequencing (Maquart et al. 2009). The analysis of MALDI-TOF MS spectra from pinniped field

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isolates were found to correlate well with MLVA-16 and MLST outcomes, suggesting that MALDI-TOF MS could be a useful tool for identifying and subtyping of *Brucella* species.

To assess whether potential pathologic lesions were associated with *Brucella* infection, histologic findings from *Brucella*-positive animals were compared to those from organs of negative animals. All observed pathologic lesions in the respiratory tissue samples could be explained by lungworm infections. No further indication of any *Brucella*-related pathology was observed in this study. However, it is remarkable that all *Brucella*-positive nematodes were observed consistently in *Brucella*-positive seals. Obviously, nematodes could be infected in the host, and consequently might play a role in the transmission of *Brucella* to other seals. It is hypothesized that infected nematode larvae leave the host, possibly via an intermediate host (fish), and are consumed by other seals (Garner et al. 1997; Dawson et al. 2008). Studies to investigate the transmission routes of marine *Brucella* strains should therefore consider fish and other natural hosts that could serve as possible reservoirs for *B. pinnipedialis* (Nymo et al. 2013).

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SUPPLEMENTARY MATERIAL

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