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Identification and typing of *Brucella* spp. in stranded harbour porpoises (*Phocoena phocoena*) on the Dutch coast

Elisa Maio [a], Lineke Begeman [b], Yvette Bisselink [a], Peter van Tulden [a], Lidewij Wiersma [b], Sjoukje Hiemstra [b], Robin Ruuls [a], Andrea Gröne [b], Hendrik-Ido-Jan Roest [a], Peter Willemsen [a], Joke van der Giessen [a,c,*]

[a] Central Veterinary Institute, Wageningen University (CVI), Edelhertweg 15, PO Box 65, 8200 AB Lelystad, The Netherlands
[b] Dept. of Pathology, Faculty of Veterinary Medicine, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[c] National Institute of Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3520 BA Bilthoven, The Netherlands

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ABSTRACT

The presence of *Brucella* (*B.*) spp. in harbour porpoises stranded between 2008 and 2011 along the Dutch coast was studied. A selection of 265 tissue samples from 112 animals was analysed using conventional and molecular methods. In total, 4.5% (5/112) of the animals corresponding with 2.3% (6/265) *Brucella* positive tissue samples were *Brucella* positive by culture and these were all confirmed by real-time polymerase chain reaction (real-time PCR) based on the insertion element 711 (IS*711*). In addition, two more *Brucella*-positive tissue samples from two animals collected in 2011 were identified using real-time PCR resulting in an overall *Brucella* prevalence of 6.3% (7/112 animals). *Brucella* spp. were obtained from lungs ($n = 3$), pulmonary lymph node ($n = 3$) and lungworms ($n = 2$). Multi Locus Variable Number of Tandem Repeats (VNTR) Analysis (MLVA) typing based on the MLVA-16 showed that the *Brucella* isolates were *B. ceti.* Additional in silico Multi Locus Sequence typing (MLST) after whole genome sequencing of the 6 *Brucella* isolates confirmed *B. ceti* ST 23. According to the *Brucella* 2010 MLVA database, the isolated *Brucella* strains encountered were of five genotypes, in two distinct subclusters divided in two different time periods of harbour porpoises collection. This study is the first population based analyses for *Brucella* spp. infections in cetaceans stranded along the Dutch coast.

1. Introduction

In 1994, *Brucella* spp. were isolated from marine mammals for the first time coincidently in two different locations. These strains originated from common seals (*Phoca vitulina*), harbour porpoises (*Phocoena phocoena*), a common dolphin (*Delphinus delphis*) in Scotland (Ross et al., 1994), and a captive bottlenose dolphin (*Tursiops truncatus*) in the USA (Ewalt et al., 1994). Since these first reports, marine mammal *Brucella* strains have been isolated from a wide range of marine mammal species originating from different geographic regions (Foster et al., 2002, 2007; Dawson et al., 2008; Maquart et al., 2009).

Detection and identification of *Brucella* spp. was historically based on culture, phenotypic and biochemical analyses (biovar typing) (Godfroid et al., 2011). PCR methods based on the detection of specific sequences of *Brucella* spp., including the IS*711* Insertion Sequence (also

* Corresponding author at: Central Veterinary Institute (CVI) Edelhertweg 15, PO Box 65, 8200 AB Lelystad, The Netherlands.
Tel.: +31 302743926.
E-mail address: joke.van.der.giessen@rivm.nl (J. van der Giessen).

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called IS*6501*), have been established (Ocampo-Sosa and García-Lobo, 2008) but limitations of these techniques, for example failure to discriminate among biovars within a species, stimulated the development of additional molecular typing techniques such as the Multiple Locus Variable Number of Tandem Repeats (VNTR) Analysis (MLVA). MLVA measures the number of tandem repeats at a given locus and can differentiate between isolates within a given *Brucella* biovar (Le Flèche et al., 2006; Maquart et al., 2009; Whatmore, 2009). The MLVA assay has been shown to be rapid, highly relevant and efficient for typing and clustering *Brucella* strains (Le Flèche et al., 2006; Al Dahouk et al., 2007; Maquart et al., 2009). Additionally, Multilocus Sequence typing (MLST), sequencing of multiple genetic loci in bacteria, has rapidly gained acceptance as a tool for characterization of microbial populations. The unambiguous and defined nature of sequence typing is ideal to address the overall genetic structure of the *Brucella* populations (Whatmore et al., 2007).

Marine mammal *Brucella* spp. are distinct from classically recognized *Brucella* spp. based on biochemical and genetic characteristics (Foster et al., 2007; Groussaud et al., 2007) and were identified as *B. ceti* in cetaceans and *B. pinnipedialis* in seals in over thirty isolates collected in several European countries (Foster et al., 2007). Within the cetacean group Maquart et al. (2009) identified two distinct genetic clusters. One cluster A divided into two subclusters, A1 and A2, from genotype 1 to 18, corresponding to MLST sequence type (ST) 26 (Whatmore et al., 2007) and composing various dolphin and one minke whale isolates; the second cluster B from genotype 19 to 74, corresponding to MLST ST23 (Whatmore et al., 2007) of which most were composed of porpoises.

The harbour porpoise has a northern hemisphere circumpolar distribution (Gaskin, 1984). It is the smallest and also the most common cetacean in the North Sea and adjacent waters (Hammond et al., 2002; Thomsen et al., 2006) and the only resident cetacean in Dutch coastal waters (Camphuysen and Peet, 2006). It has a wide continuous but uneven distribution throughout European waters, which is presumably related to the distribution of prey (Koopman, 1998; Santos and Pierce, 2003; Sveegaard, 2011). Movements of harbour porpoises are complex and although limited seasonal movements have been found, no organised seasonal migration pattern have been found and consequently most animals utilise the same area year round (Sveegaard, 2011). From the end of the nineties, numbers of harbour porpoises observed in Dutch waters increased rapidly associated with a concurrent increase in the numbers of strandings (Osinga et al., 2008).

B. ceti was detected in North Sea harbour porpoises in Scotland (Dagleish et al., 2008; Maquart et al., 2009) and Belgium (Jauniaux et al., 2010). Thus far, one *B. ceti* strain from a single Dutch harbour porpoise has been described (Maquart et al., 2009). Therefore, not much is known about *Brucella* spp., in particular *B. ceti*, in stranded harbour porpoises in the Netherlands.

The aim of this study was to evaluate the presence of *Brucella* spp. in harbour porpoises stranded on the Dutch coast between 2008 and 2011, to identify the *Brucella* species by conventional and molecular methods, and classify the obtained isolates through molecular typing methods.

2. Materials and methods

2.1. Animals

Harbour porpoises (374) that stranded at different locations along the Dutch coast between 2008 and 2011 were collected and submitted for necropsy at the Department of Pathobiology of the Faculty of Veterinary Medicine at Utrecht University. At necropsy, carcasses were sexed, weighed, measured, photographed, evaluated for macroscopic lesions and age was estimated. Samples for further testing for *Brucella* spp. were selected based on freshness of the carcass and from organs previously shown to harbour *Brucella*. Lung (88), lungworms (52), pulmonary lymph node (LN, 52), liver (18), reproductive tract (28), genital LN (1), abscesses (3) and skin lesions (23) were collected, transferred to clean bags and frozen at −20 °C. Samples were sent to the *Brucella* reference laboratory at the Central Veterinary Institute (CVI), part of Wageningen University, in Lelystad.

2.2. Detection and identification of Brucella spp. from tissue by culture, agglutination test and phage typing

All selected samples were cultured using methods described for isolation of *Brucella* spp. by OIE (2012). Briefly, tissue samples of 2 cm × 2 cm were cut into small pieces and macerated with 20 ml of beef broth using a 'Stomacher' machine. The mixture was inoculated on to solid (1 drop) and liquid (1 ml) Castañeda's selective medium (Castañeda's medium with appropriate antibiotics added). Both solid and liquid media were incubated at 37 °C in 10% CO_2. Two times with 1 week intervals, liquid media were re-cultured on solid Castañeda's selective media and incubated under the same conditions. All plates were inspected weekly for 3 weeks and this procedure was repeated for real-time PCR positive samples after 6 weeks. Suspicious colonies were screened for *Brucella* spp. by slide agglutination tests using *Brucella* Agglutinating Sera (*B. abortus* and *B. melitensis*) (Remel Europe Ltd., UK), and its susceptibility to lysis by *Brucella* specific Weybridge (Wb) and Tbilisi (Tb) bacteriophages.

2.3. Brucella spp. reference strains

B. ceti type strain NCTC 12891 (harbour porpoise), *B. ceti* M83/07/1 MLVA 16 clusterA1, *B. ceti* M260/03/1 MLVA-16 cluster B (both field isolates received from Sac Vet. Services originating from Atlantic white-sided Dolphins (*Lagenorhynchus acutus*) Maquart et al., 2009), *B. pinnipedialis* NCTC 12890 (common seal) and *B. melitensis* 16 M were used as reference strains.

2.4. Detection and identification of Brucella spp. by real-time PCR, MLVA typing and MLST analysis

2.4.1. DNA isolation from tissue and colonies

DNA from lung, lungworms, pulmonary LN and skin lesions collected in 2010 and 2011 was extracted using a

DNA tissue kit (DNeasy Blood and Tissue Kit; QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA from a single colony was extracted by suspending the colony in 200 μL nuclease free water (Sigma–Aldrich, MO, USA) and boiling at 100 °C for 8 min, followed by centrifuging for 2 min at 20,000 × *g*. The clarified supernatant was used for real-time PCR and MLVA typing.

2.4.2. Real-time PCR

DNA isolated from all tissue samples and all colonies were tested by a real-time PCR targeting the IS*711* sequences of *Brucella* spp. (Ocampo-Sosa and García-Lobo, 2008) and a synthetic internal positive control (IPC) was added to detect possible inhibition of the reaction. The 20 μL PCR mixture contained 1 μL of the DNA extract, 1× TaqMan Universal MasterMix (Applied Biosystems, CA, USA), 1 mM (each) of IS*711* primers, 0.2 mM (each) of IPC probes, 1 U of uracil DNA glycosylase (UDG), 1 μL of IPCtemplate DNA and nuclease free water (Sigma–Aldrich, MO, USA). The performance of the PCR was monitored using a blank (nuclease free water; Sigma–Aldrich, MO, USA) and a positive control (*B. abortus* ref. 544, biotype 1) at different concentrations. Amplification was carried out using a 7500 Fast real-time PCR System (Applied Biosystems, CA, USA) under the following standard conditions: an initial UDG incubation step at 37 °C for 5 min, denaturation step at 95 °C for 20 s and 50 cycles with two steps of 95 °C for 3 s and 60 °C for 30 s. Results were analysed with 7500 System SDS Software version 1.4 (Applied Biosystems, CA, USA). Tissue samples and colonies were considered positive after real-time PCR if the results presented a $C_{T\ \ value} \leq 38$ (with sigmoid curve), doubtful if $38 < C_{Tvalue} < 40$ (with doubtful sigmoid curve) and negative if $C_{Tvalue} \geq 40$.

2.4.3. MLVA typing

MLVA for both typing and species identification was performed based on methods reported previously (Le Flèche et al., 2006; Al Dahouk et al., 2007). A selection of 16 repeat loci markers was used in the *Brucella* MLVA (MLVA-16) assay consisting of two panels of 8 markers each, according to the MLVA databases for genotyping. The markers from panel 1, 8 minisatellite loci (bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45 and bruce55), have good species identification capability and the panel 2 markers have discriminatory features. MLVA panel 2 (8 microsatellite loci) was split into 2 groups, panel 2A (bruce18, bruce19, bruce21) and panel 2B (bruce04, bruce07, bruce09, bruce16 and bruce30) (Le Flèche et al., 2006; Al Dahouk et al., 2007; Maquart et al., 2009). PCR amplification was performed using an Applied Biosystems 9700 thermocycler in a total volume of 25 μL containing 2.5 μL 10× reaction buffer, 0.5 U True Start Taq DNA polymerase (Fermentas, MD, USA), 2 mM $MgCl_2$, 0.4 mM of each nucleotide (dATP, dGTP, dCTP, dUTP), 1 μM of each primer, 0.5 U UDG (New England Biolabs, MA, USA), 1 μL template and nuclease free water (Sigma–Aldrich, MO, USA). 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, followed by a final extension step for 5 min at 72 °C. Up to 4 different 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, CA, USA) and 0.5 μL of GeneScan 600 LIZ Size Standard (Applied Biosystems, CA, USA). Samples were denaturated for 5 min at 98 °C and separated on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA). The fragment size was determined using Peak Scanner version 1.0 software (Applied Biosystems, CA, USA). The number of repeats for each locus was determined on the basis of the published data (Al Dahouk et al., 2007). Estimated PCR fragment sizes were converted to a number of units within the dataset used by the software Peak scanner version 1.0. Reproducibility was checked with reference strains. Further MLVA-16 clustering analysis was carried out as previously described (Al Dahouk et al., 2007) by the use of Bionumerics (version 6.3) (Applied Math, Belgium).

2.4.4. MLST analysis

Fragmented libraries were constructed using Nextera DNA sample preparation kit (Illumina). Next generation whole genome sequencing was performed by paired-end sequencing using the Illumina technology on the MiSeq. 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), paired end mapping was used for curation of the contigs quality by Tablet (version 14.04.10, Milne et al., 2013). MLST typing was performed *in silico* using the isPCR tool (BLAT version 33build5, Kent, 2002) with a set of MLST specific primers (Whatmore et al., 2007) and the assembled and curated contigs as input. The results are processed by custom scripts and MLST types were assigned according to the combination of single nucleotide polymorphisms (SNPs) as described by Whatmore et al. (2007).

3. Results

3.1. Animals

From 2008 until 2011, a total of 112 out of 374 stranded and necropsied harbour porpoises were tested for *Brucella* (Table 1). In 7 animals (6.3%, 7/112) at least one of the used methods detected *Brucella* (Table 2). Harbour porpoises #1 and #2, two juvenile males stranded in 2009, were in very good nutritive condition. Animal #1 exhibited moderate nematode infestation of the lungs. Animal #2 showed mild nematode infestation of lungs and ears. Harbour porpoises # 3, 4, 5, 6 and 7 stranded in 2011. Animal #3, a juvenile male, was found in good nutritive condition and no parasites were detected. Animal #4, a juvenile female, in poor condition, exhibited moderate ear nematode infestations and moderate to severe cestode infestations of the intestines. Animal #5, an adult female, was found in poor nutritive condition with moderate nematode infestations of lungs, mild infestations of the ears, and evidences of recent calving and lactating. Animal #6, a juvenile female, was found to be in very poor nutritive condition with mild

Table 1
Description of the stranded harbour porpoises (*Phocoena phocoena*) and their analysed samples.

Stranded year	No. animals	Sex		Age			No. samples	Tissue samples
		Male	Female	Neonate	Juvenile	Adult		
2008–2009	46	37	9	0	38	8	103	Lung, lungworms, liver, reproductive tract, genital LN and abscesses (lung and laryngeal area)
2010–2011	66[a]	33	31	9	38	18	162	Lung, lungworms, pulmonary LN and skin lesions
Total	112						265	

[a] 2 animals, unknown sex; 1 animal, undetermined age.

to severe nematode infestations in the heart, lungs and the ears respectively. Animal #7, a juvenile male with a normal nutritive condition, had mild nematode infestations in the lungs and ears.

3.2. Culture, agglutination test and phage typing

Brucella spp. colonies were cultured from 5 (4.5%) of the 112 harbour porpoises tested for *Brucella*. Of the 265 tissue samples analysed, 6 samples (2.3%) were culture positive corresponding to the five animals as described above (Table 2). *Brucella* spp. isolates were obtained from samples S034, S035, S058, S136 and S137 after 3 weeks of broth incubation. Based on the results of the real-time PCR one more culture positive isolate (S133) was obtained after 7 weeks of culture. Tissue samples S136 and S137 belonged to the same animal (#6). *Brucella* spp. isolates were obtained from lungs (S034, S035 and S136), pulmonary LN (S058) and lungworms (S133 and S137) (Table 2). All the *Brucella* spp. isolates showed typical characteristics for the genus *Brucella*; translucent colonies with a pale honey colour when viewed in the daylight and convex and pearly white when viewed from above (OIE, 2012).

All *Brucella* spp. isolated cultures were agglutinated by both *B. abortus* and *B. melitensis* agglutinating sera after 1 min, showing the presence of A and M antigens, respectively. Only *Brucella* strain S133 showed doubtful results for *B. melitensis* agglutinating sera. Regarding to the phage typing, both *Brucella* spp. isolates S058 and S136 were lysed by Wb phage and had doubtful results for Tb phage. No lysis occurred on *Brucella* spp. isolates S34, S35, S133 and S137 for Wb and Tb phages (Table 2). These results show that phage typing did not give consistent results, in contrast to the agglutination test results.

3.3. Real-time PCR

Brucella spp. was confirmed by positive amplification curves on DNA of tissue samples and colonies of 7 (6.3%) of the 112 harbour porpoises (# 1, 2, 3, 4, 5, 6 and 7). Two additional animals (#4 and 7) were *Brucella* spp. positive by real-time PCR but not by culture (Table 2). In total, DNA of 6 (3.7%) of the 162 tissue samples analysed were real-time PCR positive (S058, S073, S133, S136, S137 and S158). DNA was extracted from lung (S136), pulmonary LN (S058, S073 and S158) and lungworms (S133 and S137). All these samples presented a $C_{T\ value}$ lower than 38 (Table 2). All 6 *Brucella* spp. isolates obtained by culture from samples S034, S035, S058, S133, S136 and S137 tested positive using real-time PCR.

Table 2
Culture, agglutination test (Agg.), phage typing, real-time PCR, MLVA typing and MLST analysis *Brucella* spp. positive results of 112 harbour porpoises (*Phocoena phocoena*) stranded on the Dutch coast.

Animal ref. (stranded year)	Sample ref.	Sampled tissue	Culture	Agg. test[a]	Phage typing[b]	Real-time PCR ($C_{T\ value}$ of the tissue samples)	MLVA typing	MLST analysis
#1 (2009)	S034	Lung	Positive	A: + M: +	Wb: NL Tb: NL	n.a.	*B. ceti*	ST23
2 (2009)	S035	Lung	Positive	A: + M: +	Wb: NL Tb: NL	n.a.	*B. ceti*	ST23
#3 (2011)	S058	Pulmonary LN	Positive	A: + M: +	Wb: L Tb: D	Positive (C_T = 37.33)	*B. ceti*	ST23
#4 (2011)	S073	Pulmonary LN	Negative	n.a.	n.a.	Positive (C_T = 36.54)	n.a.	n.a.
#5 (2011)	S133	Lungworms	Positive[c]	A: + M: D	Wb: NL Tb: NL	Positive (C_T = 23.43)	*B. ceti*	ST23
#6 (2011)	S136	Lung	Positive	A: + M: +	Wb: L Tb: D	Positive (C_T = 34.31)	*B. ceti*	ST23
#6 (2011)	S137	Lungworms	Positive	A: + M: +	Wb: NL Tb: NL	Positive (C_T = 25.60)	*B. ceti*	ST23
#7 (2011)	S158	Pulmonary LN	Negative	n.a.	n.a.	Positive (C_T = 33.84)	n.a.	n.a.

n.a., not applicable.
[a] A, A antigen (*B. abortus* Agglutinating sera); M, M antigen (*B. melitensis* Agglutinating sera); D, doubtful.
[b] Wb, Weybridge; Tb, Tbilisi; NL, no lysis; L, lysis; D, doubtful.
[c] Growth after 7 weeks.

3.4. MLVA typing

MLVA typing results were obtained for the six strains isolated from the five culture positive harbour porpoises (Table 2). According to the Brucella2010 MLVA database (http://mlva.u-psud.fr/mlvav4/genotyping/index.php?&largeur=1229) all 6 *Brucella* positive isolates (S034/lung, S035/lung, S058/pulmonary LN, S133/lungworms, S136/lung and S137/lungworms) were most closely related to reference strain *B. ceti* type strain NCTC 12891 (*harbour porpoise*) and presented the same MLVA-16 panel 1 genotype 23. Based on all 16 loci, we distinguished 5 genotypes in the 6 isolates. Strains S136 and S137 (lung and lungworms, respectively) had the same MLVA profile and originated from the same harbour porpoise (#6). The genetic relationship between the genotypes of all strains is shown in Fig. 1a, including the genotype of the reference strains (*B. ceti* NCTC 12891 (harbor porpoises), *B. ceti* M260/03/1 MLVA-16 cluster A1, *B. ceti* M83/07/1 MLVA-16 cluster A2 (Atlantic white-side Dolphins), *B. pinnipedialis* NCTC 12890 (common seal) and *B. melitensis* 16 M. The 5 *B. ceti* genotypes were separated in 2 subclusters. The first subcluster contained 3 genotypes: strains S058, S133 and S136 and S137. The second subcluster was distinctly separated, representing 2 genotypes: strains S034 and S035. This was also confirmed by the parsimony tree as shown in Fig. 1b.

3.5. MLST analysis

MSLT analyses confirmed that the 5 *B. ceti* genotypes were sequence type 23 (ST23) (Table 2) corresponding to *B. ceti* cluster B according to Maquart et al. (2009).

4. Discussion

This study comprises the first population based analyses for *Brucella* spp. infections in harbour porpoises stranded along the Dutch coast. The presence of *Brucella* in harbour porpoise tissue samples after classical bacteriological biotyping techniques was subsequently confirmed by IS*711* real-time PCR and MLVA typing. Five of the 112 animals tested positive for *Brucella* spp. by culture and two additional animals were positive by real-time PCR only, resulting in an overall *Brucella* prevalence of 6.3%. MLVA typing was applied for the *Brucella* spp. isolates and it confirmed the presence of *B. ceti* in 5 (4.5%) of the 112 harbour porpoises. Prevalence reported from stranded animals may be an overestimation of the true prevalence in the population because health status may affect the incidence of stranding (Neimanis et al., 2008).

The MLVA-16 clustering analysis of the six isolates from harbour porpoise tissue samples distinguished 5 genotypes and their relative position to reference strains. These genotypes are in agreement with the currently recognized

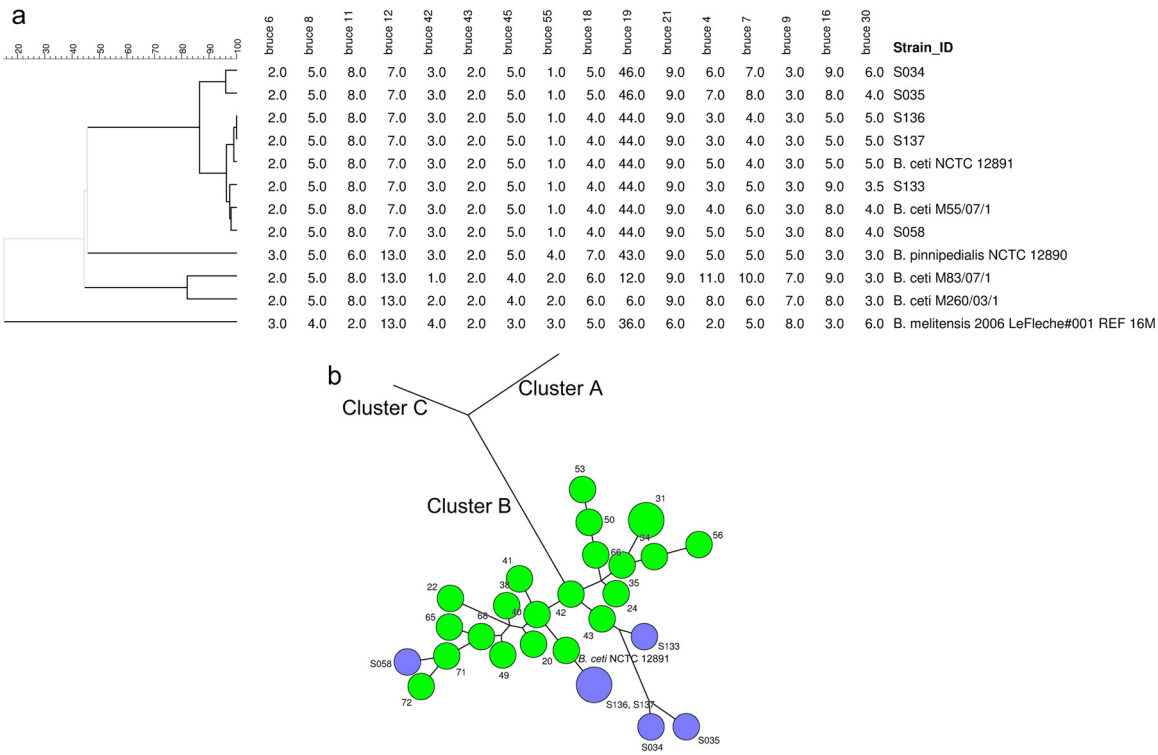

Fig. 1. (a) MLVA-16 clustering analysis of 6 *B. ceti* isolates from 265 analysed samples belonging to 112 harbour porpoises. Five genotypes were determined corresponding to samples S034, S035, S058, S133 and S136 and S137 (same genotype), and two subclusters were identified, one of which clustered most closely to *B. ceti* type NCTC 12891 (harbour porpoises) and strain M55/07/1 (Maquart et al., 2009). (b) Maximum parsimony analysis of 6 *B. ceti* isolates in conjunction with reference strains and previously described (Maquart et al., 2009) marine mammal genotypes (genotype numbers in black).

and published marine mammal *B. ceti* typing data. *Brucella* strains (S136 and S137) were isolated from a single animal (#6) and were shown to have the same MLVA profile. Identical genotypes were also shown previously in two strains analysed from one harbour porpoise in the Netherlands (strain M55/07/1) (Maquart et al., 2009). The 5 genotypes were separated into 2 subclusters. The first group (strains S058, S133, S136 and S137) clustered most closely to the *B. ceti* type strain NCTC 12891 (*harbour porpoises*) and strain M55/07/1. Strains S034 and S035 belonged to the second subcluster. Interestingly, subcluster analysis revealed association with the stranding year. Animals belonging to the second subcluster (#1 and 2) stranded in 2009. Harbour porpoises # 3, 5 and 6 (first subcluster) stranded in 2011. Additionally, strain M55/07/1 was isolated from one harbour porpoise stranded in 2007. This indicates that several strains are present in the Dutch harbour porpoises and it may also indicate that the porpoises stranded may come from different geographic regions as strains clustering together after MLVA typing frequently have a close or identical geographic origin (Le Flèche et al., 2006; Al Dahouk et al., 2007). Despite a decline in the mid-twentieth century, harbour porpoises are now at times quite abundant in the southern North Sea (Thomsen et al., 2006). Therefore, changes in occurrence of the species in parts of the North Sea might result from a recruitment of porpoises from other areas, which might in turn be caused by environmental factors such as the reduced availability of prey (Camphuysen, 2004). It is known that the harbour porpoise distribution is divided into several spatially separated populations. Three populations have been genetically recognized from the North Sea to the Baltic Sea, with putative borders in the Kattegat, and western Baltic Sea (Andersen et al., 2001; Teilmann et al., 2004; Wiemann et al., 2010). In addition, slight differences between isolates obviously of the same origin may be explained by microevolution from a step-wise mutation event of an individual locus (Al Dahouk et al., 2007). How these different genotypes of *B. ceti* can be connected to different groups of harbour porpoises should be subject to further research, preferably in collaboration with neighbouring countries.

A previous study showed that *B. ceti* isolates, when genotyped by MLVA-16, break down in two clusters (Maquart et al., 2009). Cluster A mostly composed of dolphin isolates and cluster B which comprises isolates from porpoises as well from dolphins. To assign the newly isolated strains to one of the two clusters, an additional MLST typing was performed as both clusters are distinguishable by their unique MLST sequence type. All five *B. ceti* genotypes were typed as MLST sequence type 23 (ST23), which is included in cluster B of the cetacean group (Groussaud et al., 2007).

B. ceti has been isolated from different organs of harbour porpoises, such as lung, mesenteric lymph node, and in lungworms obtained from the respiratory tract (Dagleish et al., 2008; Jauniaux et al., 2010). In this study, *B. ceti* was isolated and identified in tissues of the respiratory system (lung and pulmonary lymph node) and lungworms. It is well known that respiratory exposure may represent a transmission route of classical *Brucella* spp. infection in livestock (Corbel, 2006). Nevertheless, the routes of *Brucella* spp. transmission between marine mammals are still unclear (Foster et al., 2002; Dawson et al., 2008). Macroscopically, five harbour porpoises exhibited lung nematode infestation. *B. ceti* was isolated from lungworms in two of these harbour porpoises. The isolation of marine *Brucella* spp. from lungworms indicates that transmission of marine *Brucella* strains by lungworms may be possible (Perrett et al., 2004; Dawson et al., 2008; Prenger-Berninghoff et al., 2008). Moreover, it has been suggested that lungworms carrying *Brucella* spp. are the means by which marine mammals become infected (Rhyan, 2000; Perrett et al., 2004). However, lungworms found in two other animals that had *B. ceti* in lung tissue, were negative to *Brucella* infection by culture. Therefore, other ways of transmission might be considered.

The recent isolation of marine mammal *Brucella* strains extends the ecologic range of the genus and, potentially, its scope as a zoonosis (Corbel, 1997). *Brucella* strains isolated from marine mammals have been known to infect humans associated with the consumption of raw fish or shellfish (Sohn et al., 2003; McDonald et al., 2006) and through laboratory work (Brew et al., 1999). These cases highlight the zoonotic implications of infection with agents originating from non-terrestrial species in the absence of direct association with marine mammals (Dawson et al., 2008). Although so far, only some marine mammal *Brucella* isolates appear to infect humans (Nymo et al., 2011) and to date no terrestrial animal nor human case nor infection from *B. ceti* from the Old World has been reported, *Brucella* spp. isolated from marine mammals have zoonotic potential and must be handled as such (Brew et al., 1999; Sohn et al., 2003; McDonald et al., 2006; Jauniaux et al., 2010). In addition, scavenging of infected harbour porpoises by terrestrial animals may allow spread of bacteria (Foster et al., 2002).

Marine mammals are considered sentinels of both the marine and coastal environments (Dagleish et al., 2008), therefore studies using microbiological methods (conventional and molecular) with associated and pathological analysis for *Brucella* spp. are useful to better understand the epidemiology of the infection in European populations of marine mammals and to improve knowledge of the impact of *B. ceti* on the health of marine and terrestrial animals and human beings.

Conflict of interest

All authors read and approved the final manuscript.

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