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Short communication

Evidence of *Brucella* strain ST27 in bottlenose dolphin (*Tursiops truncatus*) in Europe

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ABSTRACT

Marine mammal brucellosis has been known for more than 20 years, but recent work suggests it is more widespread than originally thought. *Brucella (B.) pinnipedialis* has been isolated from pinnipeds, while *B. ceti* strains have been associated with cetaceans. Here we report a *Brucella* strain isolated from multiple lymph nodes of one bottlenose dolphin (*Tursiops truncatus*) during routine examination of dolphin carcasses found in the Croatian part of the northern Adriatic Sea during the summer of 2015. Classical bacteriological biotyping, PCR-based techniques (single, multiplex, PCR-RFLP) and 16S rRNA DNA sequencing were used to identify *Brucella* spp. Multiple-locus variable number tandem repeat analysis of 16 loci and multilocus sequence typing of 9 loci were used for genotyping and species determination. The combination of bacteriological, molecular and genotyping techniques identified our strain as ST27, previously identified as a human pathogen. This report provides, to our knowledge, the first evidence of ST27 in the Adriatic Sea in particular and in European waters in general. The zoonotic nature of the strain and its presence in the Adriatic, which is inhabited by bottlenose dolphins, suggest that the strain may pose a significant threat to human health.

1. Introduction

Brucellosis is disease known to occur not only in terrestrial animals and humans but also in whales, dolphins, seals, sea lions, walruses and otters. It is widespread among marine mammals, which may or may not show clinical manifestations (Guzmán-Verri et al., 2012; Hernández-Mora et al., 2013). Isolates from cetaceans are classified as *Brucella (B.) ceti*, while isolates from pinnipeds are classified as *B. pinnipedialis* (Foster et al., 2007). *B.ceti* strains have been isolated from striped dolphins (*Stenella coeruleoalba*) in the Tyrrhenian Sea (Alba et al., 2013) and Ionian Sea (Garofolo et al., 2014). In the Spanish Mediterranean Sea, *B. ceti* has been isolated from two striped dolphins as well as one bottlenose dolphin (*Tursiops truncatus*) (Isidoro-Ayza et al., 2014). All Mediterranean *B. ceti* strains isolated to date belong to sequence type (ST) 26, based on multilocus sequence typing (MLST) (Garofolo et al., 2014). ST27 was first isolated from an aborted bottlenose dolphin foetus at an aquarium in San Diego, California, USA (strain F5/99) (Whatmore, 2009), and more recent work has identified it in the foetuses and neonates of bottlenose dolphins in South Carolina, USA (Wu et al., 2014). ST27 appears to be the only ST with the proven ability to naturally infect humans (Cloeckaert et al., 2011; Whatmore, 2009). So far, all ST27 strains have been found only in Pacific Ocean species (Guzmán-Verri et al., 2012; Whatmore, 2009). This raises the question of whether ST27 strains are present in other waters.

In this paper we describe a novel *Brucella* sp. strain isolated from a bottlenose dolphin from the northern Adriatic Sea in Croatia. Phenotypic and molecular methods were used to identify the strain as ST27 and classify it relative to known *B. ceti* and *B. pinnipedialis* strains.

2. Materials and methods

Four bottlenose dolphins found dead between June and August 2015 on the eastern coast of the Adriatic Sea were examined post mortem and sampled as part of a long-term project to investigate

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marine mammal strandings along the Croatian coast of the Adriatic Sea. Species, sex, body mass and external measurements were recorded and standard post mortem procedures were performed (Kuiken and García Hartmann, 1991). Animal **348** was an adult male of unknown weight. A stomach full of prey and post mortem removal of tail fluke indicated bycatch. Animal **350** was a juvenile male weighing 108 kg. Foam in the lungs and post mortem removal of tail fluke indicated bycatch. Animal **355** was a female calf weighing 51 kg, which died of unknown causes. Animal **358** was a female calf weighing 33.5 kg, which died of pulmonary parasitism and parasitic pneumonia (Supplementary Fig. 1).

2.1. Bacteriological investigation

The following organs from all four dolphins were analysed: cervical superficial lymph nodes (LN), tracheobronchial LN, marginal pulmonary LN, caudal mediastinal LN, cranial mesenteric LN, caudal mesenteric LN, renal LN, and hypogastric LN, liver, spleen, brain, testes and prostate. Ovaries and uterus were sampled only in dolphins 355 and 358. Samples were tested for *Brucellae* using classical microbiology as described (Alton et al., 1988). Material was cultured on Farrell selective medium and Brucella agar (Brucella medium base, Oxoid Ltd, UK) supplemented with 25 mL of horse serum, 12,500 IU of bacitracin and 3000 IU of polymyxin B sulphate per 0.5 L of agar. Inoculated agarswere incubated at 37 °C in both ambient air and 10% CO_2. Five strains were isolated only from dolphin **350** and were labelled 350/1, 350/2, 350/3, 350/4, and 350/5. These strains were tested for their ability to cause agglutination of anti-A, -M and -R monospecific sera (Veterinary Laboratories Agency, UK). Isolated strains were also biotyped based on metabolic activity usingthe BBL CRYSTAL™ System(Enteric/Nonfermenter ID System, Becton Dickinson, USA) and VITEK® 2 System with VITEK® 2 g-Negative identification card (GN; bioMerieux, France).

2.2. Bacterial strains and DNA isolation

The five *Brucella* isolates were analysed together with 11 known *Brucella* strains: *B. abortus* S99, *B. melitensis* 16M, *B. suis* reference strains bv.1 to bv. 5, *B. canis* RM 6/66, *B. ovis* REO 198, and one strain each from dolphin (*B. ceti*) and seal (*B. pinnipedialis*) obtained in 2002 from the Unidad de Sanidad Animal (CITA), Zaragoza Spain. DNA was isolated for classical PCR reactions by adding a loopful of culture to 100 μL of distilled water (AccuGENE Molecular Biology Water, Lonza, Verviers, Belgium), incubating at 95 °C for 20 min with shaking at 400 rpm (Thermomixer Comfort, Eppendorf), and then centrifuging at 14,000*g* for 1 min (SL8, Thermo Scientific, Germany). Supernatant was used as DNA template in PCR reactions. DNA was isolated for genotyping purposes using the QIAcube DNA Mini Kit and QIAcube system (QIAGEN, Hilden, Germany).

2.3. Molecular identification

Genus of the isolates was identified based on the presence of the *BCSP-31*gene (Serpe et al., 1999) and on 16S rRNA gene sequencing (Bricker et al., 2000). These results were analysed usingBioNumerics Software (version 7.5, Applied Maths, Gent, Belgium) against megablast results in the GenBank (National Center for Biotechnology Information), EMBL, RefSeq Nucleotide, DDBJ, and PDB databases. *Brucella* isolates were identified to species level using the Bruce-ladder (López-Goñi et al., 2008). *B. ceti* and *B. pinnipedialis* were also distinguished using multiplex PCR (Isidoro-Ayza et al., 2014). Possible additional differences between our isolates and reference strains were explored using multiplex-suis (Suis-ladder) (López-Goñi et al., 2011) (Table 1). All PCR reactions except for Bruce-ladder involved the same PCR reaction mixture (20 μL): 10 μL of HotStarTaq Master Mix (Qiagen, Hilden, Germany), 6 μL of RNase-free water (Qiagen), 0.5 μM of each primer pair specific for the target locus (Invitrogen, Paisley, UK; or Macrogen, Amsterdam, Netherlands), and 2 μL of template DNA. Cycling regimes varied according to the target and were performed as described in previous studies. The PCR reaction mix for Bruce-ladder (20 μL) consisted of 10 μL of Multiplex PCR Master Mix (Qiagen), 2.5 μL of RNase-free water (Qiagen), 0.4 μM BMEI0998f and BMEI0997r primers (Invitrogen or Macrogen), 0.1 μM of each remaining primer (López-Goñi et al., 2008), and 2 μL of DNA. Amplification products were analysed using capillary electrophoresis on the QIAxcel system (Qiagen) against size markers ranging from 100 to 2500 bp.

Amplification products of *omp2a* and *omp2b* genes were analysed for restriction fragment length polymorphism (RFLP) using 6 restriction endonucleases (Cloeckaert et al., 1995): *Hae*III, *Sty*I, *Nco*I, *Alu*I, *Eco*RI, *Kpn*I. The RFLP reaction mixture contained

Table 1
Summary of molecular identification and genotyping results.

Reference	Method(s)	Results shown	Typing result
Serpe et al. (1999)	BCSP-31		*Brucella* sp.
Bricker et al. (2000)	16SrRNA	GenBank KU324483	*Brucella* sp.
Cloeckaert et al. (1995)	PCR-RFLP *omp2a*	data not shown	*B. pinnipedialis* (*Hae*III, *Sty*I, *Nco*I, *Alu*I, *Eco*RI, *Kpn*I)
	Sequencing *omp2a*		Terrestrial *Brucella* species[a] (*Alu*I, *Eco*RI,)
			B. ceti
	PCR-RFLP *omp2b*	data not shown	*B. ceti* (*Hae*III); *B. pinnipedialis* (*Sty*I, *Kpn*I, *Nco*I); unique pattern (*Eco*RI); terrestrial Brucella species (*Hae*III/*B.m*[a]/*B.s*[a], *Alu*I)
	Sequencing *omp2b*		*B. pinnipedialis*
López-Goñi et al. (2008)	Bruce ladder		*B. ceti*/*B. pinnipedialis*
Isidoro-Ayza et al. (2014)	Multiplex PCR		Unique strain
López-Goñi et al. (2011)	Suis-ladder		Unique strain
Le Flèche et al. (2006), Al Dahouk et al. (2007)	MLVA 16		Unique genotype, closer to *B. pinnipedialis*
Whatmore et al. (2007)	MLST 9		ST27

[a] Terrestrial *Brucella* species: *Brucella melitensis* 16M (*B.m.*), *Brucella abortus* S99, and *Brucella suis* bv.1. (*B.s.*).

5 μL of DNA amplified as described above, 5 U of restriction enzyme (Fermentas, Burlington, Canada; or Takara Bio, Otsu, Japan), 2 μL of buffer (Fermentas or Takara Bio) and 12.5 μL of AccuGENE Molecular Biology Water (Lonza). DNA was digested at 37 °C for 3 h. Restriction products were analysed using capillary electrophoresis as described above. Undigested *omp2a* and *omp2b* sequences were submitted for sequencing, and the results were compared using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

In order to identify our isolates to species level and compare them with other *B. ceti* and *B. pinnipedialis* strains worldwide, isolates were genotyped using multiple-locus variable number tandem repeat analysis (MLVA) and MLST. MLVA genotyping was performed on 16 loci (Al Dahouk et al., 2007; Le Flèche et al., 2006) in the following order: panel 1, *Bruce06-08 -11 -12-42-43-45-55*; panel 2a, *Bruce18-19-21*; and panel 2b, *Bruce04-07-09-16-30*. *B. melitensis* 16M served as the reference strain. This approach was applied to our isolates from dolphin (350/1-5) and to reference strains from dolphin (*B. ceti*) and seal (*B. pinnipedialis*). Analogously, MLST was performed on 9 loci (Whatmore et al., 2007) in the order *gap-aro*A-*glk-dna*K-*gyr*B-*trp*E-*cob*Q-*omp*25- *int-hyp (orf1)*.

2.4. Analysis of MLVA-16 and MLST-9 data

Band sizes from MLVA-16 results were translated into numbers of individual repeats (Le Flèche et al., 2006), and results were presented in the form of 16-digit numerical codes based on the *Brucella* allele assignment table, version 3.6 (http://mlva.u-psud.fr) (Grissa et al., 2008). Codes were analysed using the categorical coefficient and unweighted pair group method with arithmetic mean (UPGMA) in BioNumerics software (version 7.5; Applied Maths). Allelic profiles of our isolates were compared to those deposited in the Brucella2012 database (Grissa et al., 2008). MLST sequences were assembled, processed and compared using BioNumerics software. Alleles and STs were identified by comparison against published profiles (Whatmore et al., 2007) in BioNumerics software and the web-based *BrucellaBase* platform (http://59.99.226.203/brucellabase/mlst.html) (Sankarasubramanian et al., 2016). Alleles were assigned 9-digit numerical codes, which were compared using the categorical coefficient and UPGMA. The STs of our isolates were compared to those deposited in the Brucella2012 database. The 16S rRNA gene sequences in this study have been deposited in GenBank under accession number KU324483. Sequences at MLST loci were deposited under the following accession numbers (Table 1): *gap*, KU500420; *aroA*, KU500421; *glk*, KU500422; *dnaK*, KU500423; *gyrB*, KU500424; *trpE*, KU500425; *cobQ*, KU500426; *omp25*, KU500427; and *int-hyp*, KU500428.

3. Results

3.1. Bacteriological identification

Suspected *Brucella* sp. strains were isolated from the following organs of dolphin **350**: hypogastric LN (350/1), caudal mediastinal LN (350/2), tracheobronchial LN (350/3), marginal pulmonary LN (350/4) and cervical superficial LN (350/5). Strains were isolated after 5–7 days of incubation both in the presence and absence of CO_2. Colonies were small, convex, translucent, and shiny on both Farrell selective medium and Brucella agar supplemented with 5% dextrose and 5% horse serum. Agglutination was observed exclusively with anti-M monospecific serum. In the BBL CRISTAL E/NF System, activity was observed with p-nitrophenyl α-ß glucoside (BGL) and proline (PRO) in panel 2, and with urea (URE), glycine (GLY) and arginine (ARG) in panel 1. The strain was defined as *Flavimonas oryzihabitans* with 66% probability. The VITEK®2 System with VITEK® 2 GN identification card identified the isolated strains as *B. melitensis* with 99% probability. Strains were also determined to be oxidase-positive, catalase-positive, and H_2S-negative.

3.2. Molecular identification

Conventional PCR to detect the *Brucella*-specific *BCSP-31* gene was positive for all isolates, and the 16SrRNA gene sequences (GenBank KU324483) were identical to published sequences from terrestrial *Brucella* species as well as marine mammal *Brucellae*, including *B. abortus* strain M5, *B. ceti* strain TE10759-12,and *B. pinnipedialis* strain 6/566. Using the Bruce-ladder, we identified the five strains as marine *Brucella* spp., but we were unable to differentiate between *B. ceti* and *B. pinnipedialis*. Therefore we performed a multiplex PCR previously shown to differentiate *B. ceti* and *B. pinnipedialis* (Isidoro-Ayza et al., 2014). Amplification product was obtained for only one region of ~660 bp using theforward primer CGT CAA CTC GCT GGC CAA GAG and the reverse primer GCA GGA GAA CCG CAA CCT AA. We were unable to confirm the existence of the second region, amplified by the forward primer TCA ACT GCG TGA ACA ATG CT and reverse primer GCG GGC TCT ATC TCA AGG TC. As a result, we were unable to identify our isolates to single-species level using these primers. Similarly, multiplex-suis (Suis-ladder) testing of our strains did not give the same pattern as reference strains *B. ceti* and *B. pinnipedialis*. In fact, the pattern of our isolates did not correspond to those of any reference strains for other species or biovars that we examined. Amplification products were obtained for the regions BMEI 1426/1427 and BMEI 0205f/r, but not for regions 1080f/r or BMEI 1688/1687. RFLP analysis of *omp2a/b* sequences based on 6 restriction endonucleases was consistent with that of *B. ceti* or *B. pinnipedialis* reference strains, depending on the gene and enzyme used. BLAST analysis of *omp2a* sequences showed greater similarity with *B. ceti* B1/94 (97.89%), while analysis of *omp2b* sequences showed greater similarity with *B. pinnipedialis* strain 37a-1 (97.43%). MLVA-16 typing allowed the assignment of a unique 16-digit code to all five strains: 2-5-5-8-3-2-5-3-5-10-9-5-4-6-4-4. Comparison of our results with a public database (Grissa et al., 2008) indicated differences from *B. pinnipedialis* M292/94/1 in at least 6 loci and differences from *B. ceti* 02/611 in 7 loci (Maquart et al., 2009) (Supplementary Fig. 2). The code for the reference seal strain was 3-5-6-13-3-2-5-4-7-42-9-5-5-5-3-3, while that for the reference dolphin strain was 2-5-8-13-2-2-4-2-6-12-9-8-7-8-14-3. These codes were identical to those of the reference dolphin strain *B. ceti* M9/02/01 (Maquart et al., 2009) and seal strain *B. pinnipedialis* REF Seal (Le Flèche et al., 2006). MLST-9 was performed on three strains (350/1, 350/4, 350/5). Strains were identified as ST27 based on the 9 loci (code: 1-2-4-3-1-1-5-2-1) (Table 1).

4. Discussion

Traditionally, *Brucella* strains associated with pinnipeds have been classified as *B. pinnipedialis* and strains associated with cetaceans, as *B. ceti* (Foster et al., 2007). More recent work on host preference, bacteriological properties and genetic traits suggests that this binary classification may be too simplistic (Guzmán-Verri et al., 2012). Guzmán-Verri et al. (2012) has proposed dividing *B. ceti* into three types: dolphin, porpoise, and human. *B. ceti* was recently found in striped and bottlenose dolphins in Italian and Spanish parts of the Mediterranean Sea (Alba et al., 2013; Garofolo et al., 2014; Isidoro-Ayza et al., 2014). In the present study, we describe the first case of marine *Brucella* spp. in bottlenose dolphins in the Adriatic Sea.

Our strains were isolated using selective media and did not require CO_2 for growth. This lack of CO_2 requirement is similar to dolphin strain F5/99 and contrasts with human strain 02/611 (McDonald et al., 2006). Most isolates derived from seals require additional CO_2 for growth, while nearly all isolates from cetaceans do not (Dawson et al., 2008). Our strains reacted only with anti-M monospecific sera, similar to the US bottlenose dolphin strain F5/99 and in contrast to the human *B. ceti* strain 02/611, which causes agglutination of both anti-A and -M sera (McDonald et al., 2006). Both *B. ceti* and *B. pinnipedialis* have been described as A antigen dominant (Foster et al., 2007), but Dawson et al. (2008) have described isolates containing both of these species that cause agglutination of both anti-A and -M sera. We were able to identify *B. melitensis* with 99% probability in our isolates using the commercial biotyping system VITEK®2 with the VITEK®2 GN identification card, which includes only terrestrial *Brucella* spp. Our results are consistent with the suggestion by Dawson et al. (2008) that phenotypic results on their own are insufficient for characterizing marine mammal strains. We relied on molecular methods to classify our isolates to genus and species level. Primers for identifying *Brucella* spp. detected the *BCSP-31* coding region (Serpe et al., 1999), and 16S rRNA gene sequencing (Bricker et al., 2000) suggested that our dolphin isolates belong to the same *Brucella* monospecies as the classical *Brucella* strains. The Bruce-ladder or multiplex PCR can usually differentiate *B. ceti* and *B. pinnipedialis* (Isidoro-Ayza et al., 2014; López-Goñi et al., 2008), but this was not the case in the present study. Our results with the Suis-ladder and multiplex PCR differed from those of other known *Brucella* spp. (Isidoro-Ayza et al., 2014; López-Goñi et al., 2011). Therefore we performed additional typing tests. PCR-RFLP analysis of *omp* genes, IS*711* fingerprinting, and Infrequent Restriction Site (IRS)-derivative PCR can differentiate marine mammal *Brucella* strains from terrestrial *Brucella* species (Dawson et al., 2008). Most restriction patterns that we examined were more similar to those of *B. pinnipedialis* than to those of *B.ceti*: this was the case for *omp2a* restriction using *Hae*III, *Sty*I, *Nco*I, *Alu*I, *Eco*RI, or *Kpn*I; and for *omp2b* restriction using *Sty*I, *Kpn*I, and *Nco*I. Cloeckaert et al. (2001) reported identical *Alu*I restriction patterns of *omp2b* for all marine mammal *Brucella* isolates, except one from hooded seal. The *Alu*I restriction patterns of *omp2b* and *omp2a* in our isolates differed from those of other marine mammal *Brucella* spp. and matched those of terrestrial mammal *Brucella* spp. The *Eco*RI restriction pattern of *omp2b* in our isolates differed from that of both *Brucella* groups. Only the *Hae*III restriction pattern of *omp2b* in our isolates was more similar to that of *B. ceti*. Taken together, our data suggest that our strains are more similar to *B. pinnipedialis* than to *B. ceti.* McDonald et al. (2006) used different molecular tests to show that the *Brucella* isolates from human and US bottlenose dolphin are closely related to *B. pinnipedialis*. Sohn et al. (2003) concluded the same for *Brucella* isolates from two Peruvian patients; the isolates were found to be identical to a *Brucella* sp. from common seal. The existence of different subgroups of strains may reflect co-evolution of host and *B. ceti* in the host's geographic location (Ruiz-Villalobos et al., 2014). MLVA-16 typing of our strains shows the closest agreement with *B. pinnipedialis* strain M4/06/4 isolated from grey seal (*Halichoerus grypus*) in Scotland and ST27 strain 02/611 isolated from a New Zealand patient (Maquart et al., 2009). These results suggest that our isolates may cluster with the human New Zealand strain, which forms a distinct group from other *B. ceti* clusters based on MLVA-16. Maquart et al. (2009) used MLVA-16 typing to divide *B. ceti* strains into cluster A (with subclusters A1 and A2) and cluster B. They analysed 294 marine mammal *Brucella* strains collected in European waters (excluding the Mediterranean Sea) as well as a human isolate from New Zealand. The human isolate from New Zealand fell clearly outside clusters A and B, occupying an intermediate position within the marine mammal groups of isolates. Based on MLVA-16 data, our strains appear to be quite different from three *B. ceti* strains from the Ionian Sea and other *B. ceti* isolates from the Mediterranean Sea (Garofolo et al., 2014), as well as from *B. ceti* strains more recently isolated in Italy and Spain (G. Garofolo, personal communication). MLST-9 typing defined our strains as ST27 based on the criteria of Whatmore et al. (2007). This is in contrast to the classification of isolates from the Ionian Sea (Garofolo et al., 2014) and Tyrrhenian Sea (Alba et al., 2013), which belong to ST26. Our dolphin isolates correspond to the first ST27 strain described in Europe and the first ST27 found in bottlenose dolphins in the Adriatic Sea. So far, strain ST27 is the only marine *Brucella* strain known to have potential as a zoonotic pathogen capable of causing serious, naturally aquired infection in humans (Cloeckaert et al., 2011; McDonald et al., 2006; Sohn et al., 2003). The existence of a third marine mammal *Brucella* species or subspecies inside *B. ceti* has been proposed on the basis of different molecular analysis (Cloeckaert et al., 2011; Guzmán-Verri et al., 2012; Maquart et al., 2009). The extent of marine mammal brucellosis and its importance for human disease are only starting to become evident. Although human and animal brucellosis is rare in Croatia (Špičić et al., 2010), the expanding tourism industry and aquaculture production in the Adriatic Sea will bring people into closer contact with diverse marine organisms that may serve as sources of infection. Our findings also strengthen arguments in favour of dividing *B. ceti* species into additional subtypes and highlight the need for research into new ecological niches.

Authors' **contributions**

ZC and GT conceived the study. ZC, DS, DM, GT, RI, ZTM and SS designed the study. DM and GT organised collecting of material in the field and provided pathomorphological examination and tissue and organ determination for laboratory testing. ZC isolated strains and performed bacteriological investigation. DS, RI and ZTM performed bacteriological and molecular identification and genotyping. SS interpreted data and drafted the manuscript. ZC, DS, DM, GT, RI and ZTM revised the manuscript. All authors approved the final version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2016.10.013.

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