Case Report

Brucella ceti from two striped dolphins stranded on the Apulia coastline, Italy

Giuliano Garofolo,[1] Katiuscia Zilli,[1] Pasquale Troiano,[2] Antonio Petrella,[2] Francesca Marotta,[1] Gabriella Di Serafino,[1] Massimo Ancora[1] and Elisabetta Di Giannatale[1]

Correspondence
Giuliano Garofolo
g.garofolo@izs.it

[1]Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise 'G. Caporale', National and OIE Reference Laboratory for Brucellosis, Via Campo Boario 64100, Teramo, Italy

[2]Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Diagnostic Unit, Via Manfredonia 20, 71100, Foggia, Italy

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Since 1994, when *Brucella ceti* was first isolated from an aborted dolphin fetus, several cases have been reported worldwide. The first case of *B. ceti* in the Mediterranean (and in Italy), however, was recorded only in 2012, off the coast of Tuscany. Extensive studies, using serological and microbiological methods, have documented this bacterium in dolphins and demonstrated its zoonotic potential. We describe the typing of two *B. ceti* strains isolated from striped dolphins (*Stenella coeruleoalba)* stranded on the southern Apulia coastline. *B. ceti* isolates were conventionally typed, and then genotyped by both the multilocus sequence typing (MLST) and the multilocus variable number of tandem repeats typing (MLVA) methodologies to infer phylogeny and potential epidemiological links between the two cases. The two isolates were identified through MLST analysis as belonging to the common sequence type 26 (ST26), while MLVA analysis, having established that the two isolates have identical profiles, assigned them to a novel genotype within cluster A – a unique representative of a new Mediterranean subcluster. The results thus revealed a link between the two cases studied, demonstrating the usefulness of MLST and MLVA for the epidemiological investigation of brucellae among marine mammals.

Introduction

Information on stranded marine mammals has been collected systematically in Italy since 1986, when a nationwide network was established to address the problem of stranded animals, identify the species involved and discover the causes of death. The network's online database is freely accessible at http://mammiferimarini.unipv.it/. To date, 4396 records have been entered, with no cases of brucellosis reported before 2012. Close contact with stranded cetaceans exposes humans to potential health hazards, including the risk of transmission of pathogenic agents such as brucellae (McDonald *et al.*, 2006).

Brucella spp. is a genus of bacteria that infects many terrestrial and aquatic vertebrates (Pappas, 2010). The two *Brucella* species isolated from marine mammals are *B. ceti* from cetaceans and *B. pinnipedialis* from pinnipeds. Cross-species infections have also been reported. The true occurrence of brucellosis among marine mammals remains unknown, however, as the target animals, having extensive home ranges and distributions, are not easy to monitor and are thus not included in brucellosis national control programmes (Guzmán-Verri *et al.*, 2012).

The first documented case of *B. ceti* in Italy occurred in February of 2012, and involved a striped dolphin found along the coast of Tuscany on the Tyrrhenian Sea (Alba *et al.*, 2013). We report on two brucellae isolated from stranded striped dolphins along the Apulia coastline in March and November of 2012, respectively. These two cases thus occurred during the same general period as the first, but in a different geographical area.

We used two genomic techniques: multilocus sequence typing (MLST), to identify the pathogens' species; and variable number of tandem repeats analysis (MLVA), to infer the phylogeny and evolution of the *B. ceti* isolates, and to investigate possible epidemiological links between the cases. This methodology is proposed as a systematic approach for the identification and characterization of brucellae from marine mammals.

Abbreviations: MLST, multilocus sequence typing; MLVA, multilocus variable number of tandem repeats analysis; RFLP, restriction fragment length polymorphism; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the MLST gene sequences obtained are KF314739–KF314756.

Case report

In March 2012, a young male striped dolphin (*Stenella coeruleoalba*), 192 cm long and weighing 79.20 kg, was

found dead at Gallipoli Lido Pizzo on the Ionian coast (southern Mediterranean) (Fig. 1). Post-mortem examination revealed moderate nutritional status with several subcutaneous and abdominal parasitic cysts (*Phyllobotrium* sp. and *Monorygma* sp., respectively; data not shown). The salient gross pathological findings were intense meningeal hyperaemia, cerebral oedema, lung oedema and petechial haemorrhages in the spleen. Eight months later, in November 2012, an adult female striped dolphin (*S. coeruleoalba*), about 180 cm long and weighing 60 kg, was recovered dead on the shores of Porto Cesareo Bacino Grande on the Ionian coast (Fig. 1). The post-mortem examination was limited, due to the state of the carcass. A poor nutritional status was observed, and no relevant gross pathological lesions. In both cases, initial diagnostic screening yielded negative results for *Toxoplasma* and *Morbillivirus* (data not shown). A subsequent bacteriological examination allowed us to isolate *Brucella* from the brain and spleen of the first animal (strain i.d. 10759) and from the brain of the second (strain i.d. 28753). A single colony from each animal was cultured on serum glucose agar and tested for Gram stain reaction as well as for catalase, oxidase and urease activities. Strains were identified through phage typing, CO_2 requirements, H_2S production, slide agglutination tests with specific *Brucella* antisera A, M and R, and dye sensitivity tests performed in duplicate according to international recommendations [World Organisation for Animal Health (OIE), 2012], and compared with four reference strains (Table 1).

Bruce-ladder PCR and restriction fragment length polymorphism analysis (RFLP) of the *omp2a* and *omp2b* genes using, respectively, *Pst*I and *Hin*fII restriction endonucleases (Promega) were carried out to define *Brucella* species (López-Goñi *et al.*, 2008; Cloeckaert *et al.*, 1995). Lastly, isolates were genotyped using two methods: (1) the nine-loci MLST scheme, comprising seven housekeeping genes (*gap*, *aroA*, *glk*, *gyrB*, *trpE*, *cobQ*, *dnaK*), the *omp*25 gene and the hypothetical int-*hyp* protein gene (Whatmore *et al.*, 2007); and (2) the MLVA-16 panel (Le Flèche *et al.*, 2006) with the modifications proposed by Al Dahouk *et al.* (2007), performed by capillary electrophoresis, as previously described (Garofolo *et al.*, 2013).

MLST sequences were queried against the NCBI nucleotide sequence database through the BLAST program; sequence types (STs) were compared with the data available from Whatmore *et al.* (2007), and assigned a genotype accordingly.

The MLVA profiles of the two isolates were compared with genotypes available in the online Brucella MLVA database (http://mlva.u-psud.fr/) following a hierarchical approach dividing the loci into different panels with increasing levels of discrimination: MLVA-11 (microsatellites), and MLVA-16

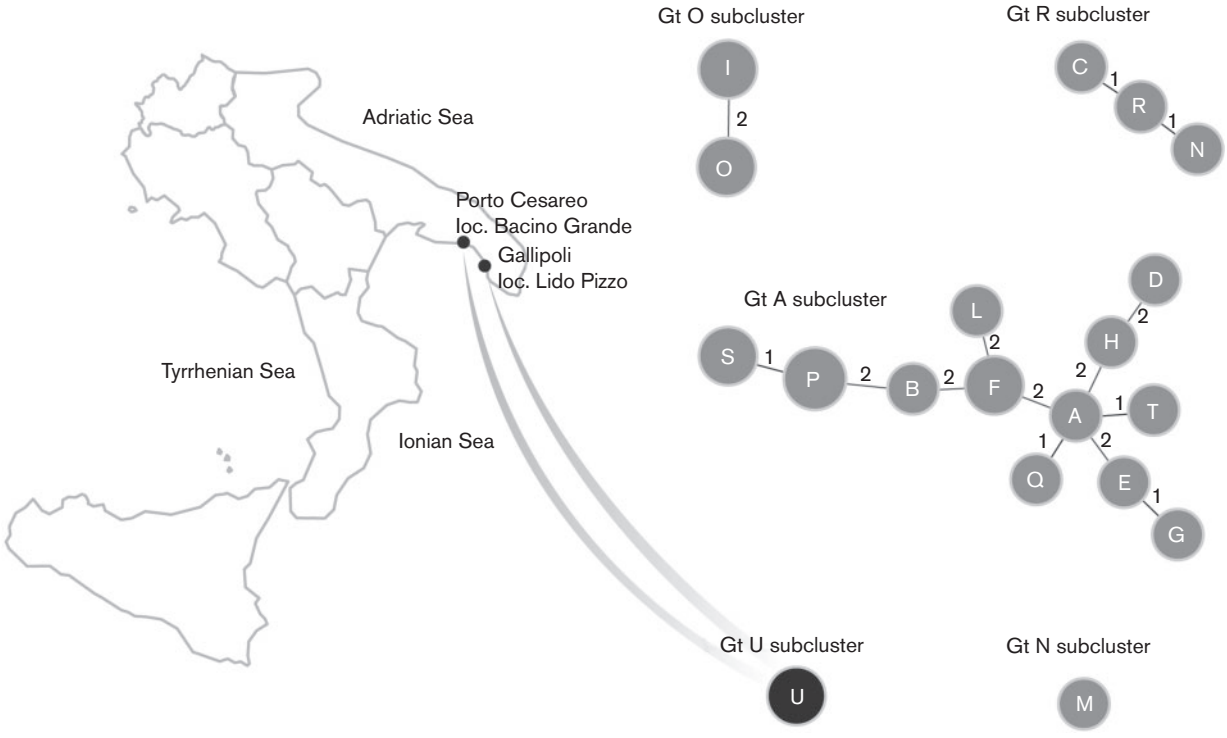

Fig. 1. Minimum spanning tree for *B. ceti* using MLVA-16 data from the international database, aggregating all available cluster A isolates (right). Isolates from the Atlantic Ocean are marked in grey, while the Mediterranean isolates analysed in the present study are marked in black. Gt, Genotype. The geographical locations of the strandings on the Apulia coastline are shown on the left.

Table 1. Phenotypic features and *omp* restriction pattern for the *Brucella* strains studied

Strain A	Lysis by phages				Oxidase	Catalase	Urease	H_2S production	Thionin	Basic fuchsin	Agglutination with monospecific antisera			*omp2b* restriction pattern
	Tb	Wb	Iz	R/C							A	M	R	
B. ceti strain i.d. 10759	−	+	+	−	+	+	+	−	+	+	+	−	−	N (K)
B. ceti strain i.d. 28753	−	+	+	−	+	+	+	−	+	+	+	−	−	N (K)
B. ceti NCTC 12891	+	+	+	−	+	+	+	−	+	+	+	−	−	N (K)
B. pinnipedialis NCTC 12890	+	+	+	−	+	+	+	−	+	+	+	−	−	NP*
B. melitensis biovar 3 NCTC 8334	−	−	+	−	+	+	+	−	+	+	+	+	−	B (B)
Brucella abortus biovar 1 NCTC 624	+	+	+	−	+	+	+	+	−	+	+	−	−	A (A)

*NP, Not performed.

(minisatellites). A minimum spanning tree was generated aggregating the MLVA data obtained from the new Mediterranean strains with all other MLVA profiles belonging to cluster A from the international database, using the goeBURST algorithm with PHYLOViZ 1.0 (Francisco *et al.*, 2012). A subcluster was defined as either an independent isolate, or a group of isolates with MLVA profiles sharing identical alleles at 13 or more loci. Each subcluster was named after the genotype identified as the putative founder of the group by goeBURST, followed by the word 'subcluster'. MLVA-11 genotypes refer to the international database, while MLVA-16 genotypes were arbitrarily assigned the letters from A to U.

Primary cultures of the suspected isolates revealed smooth, convex, circular colonies, 0.5–10 mm in diameter, of Gram-negative, urease- and oxidase-positive coccobacilli with phenotypic tests as shown in Table 1.

Through Bruce-ladder PCR, the isolates were identified as belonging to the *Brucella* genus, and to the species that infect marine mammals. *Pst*I and *Hin*fII restriction patterns were in line with those reported by Cloeckaert *et al.* (2001) for marine brucellae, showing the N (K) pattern for the *omp*2 gene (Table 1).

MLST assigned the strains to the *B. ceti* species, ST26 (Table 2). MLVA revealed an identical profile in both cases. Querying against the international database under the MLVA-11 microsatellite panel identified the isolates as belonging to genotype 8, while a query under the MLVA-16 panel identified them as belonging to a novel genotype (Table 2). Clustering the MLVA-16 profile with the 24 cluster A (Maquart *et al.*, 2009) strains currently available in the database revealed five subclusters, including the new genotype as the single representative of the novel Ionian genotype U subcluster (Fig. 1).

Discussion and Conclusions

Two cases of brucellosis in dolphins from the northern Ionian Sea have been reported. Classical biotyping was performed and compared with the results obtained by new molecular typing tools. Biotyping methods are time-consuming and require skilled technicians as well as reagents that are not always readily available. Moreover, in the case of marine mammals, classical typing methods may give inconclusive or atypical results (Dawson *et al.*, 2008), especially if, as often happens, the carcass is in an advanced state of decomposition. Molecular typing methods represent a solution to this problem.

In this study, *omp*-based PCR RFLP was able to identify the *omp* N (K) profile, typically associated with *Brucella* from *Delphinidae*. This method, however, can only broadly assign strains to one of two groups having either dolphins or porpoises as their preferred host. Moreover, should new RFLP profiles occur, this may result in ambiguous diagnoses.

Table 2. Characteristics of the *B. ceti* strains included in the minimum spanning tree: MLVA and MLST genotypes, geographical location and host

Strain	Gt MLVA16	Gt MLVA11	Gt MLST9	Ocean/Sea	Country	Host
M18/96/1	A	8	NA	Atlantic	Scotland	AWSD
M2194/94/1	B	8	26	Atlantic	Scotland	Striped dolphin
M654/99/1	C	7	26	Atlantic	Scotland	Striped dolphin
M642/99/2	D	8	26	Atlantic	Scotland	Striped dolphin
M9/02/01	E	8	NA	Atlantic	Scotland	Striped dolphin
M656/99/2	F	8	26	Atlantic	Scotland	Striped dolphin
M656/99/1	F	8	26	Atlantic	Scotland	Striped dolphin
M13/05/1	G	8	26	Atlantic	Scotland	Striped dolphin
M42/07/1	H	8	NA	Atlantic	Scotland	AWSD
M83/07/1	I	7	NA	Atlantic	Scotland	AWSD
M83/07/3	I	7	NA	Atlantic	Scotland	AWSD
M158/06/1	L	8	NA	Atlantic	Scotland	AWSD
M222/07/1	M	8	NA	Atlantic	Scotland	AWSD
M231/07/3	N	7	NA	Atlantic	Scotland	Bottlenose dolphin
M267/05/4	O	7	NA	Atlantic	Scotland	AWSD
M267/05/1	O	7	NA	Atlantic	Scotland	AWSD
M52/07/9	P	8	NA	Atlantic	Scotland	AWSD
M52/07/1	P	8	NA	Atlantic	Scotland	AWSD
M52/07/7	P	8	NA	Atlantic	Scotland	AWSD
M57/07/1	Q	8	NA	Atlantic	Scotland	AWSD
M231/07/2	R	7	NA	Atlantic	Scotland	Bottlenose dolphin
M52/07/4	S	8	NA	Atlantic	Scotland	AWSD
M52/07/3	S	8	NA	Atlantic	Scotland	AWSD
M260/03/1	T	8	NA	Atlantic	Scotland	AWSD
10759	**U***	**8**	**26**	**Ionian**	**Italy**	**Striped dolphin**
28753	**U**	**8**	**26**	**Ionian**	**Italy**	**Striped dolphin**

The two Italian isolates are marked in bold. Abbreviations: AWSD, Atlantic white sided dolphin; Gt, genotype; NA, not available.
*MLVA genotype from this study.

Most of the molecular methods available for the identification of *Brucella* are either unable to detect marine strains, such as the classical AMOS PCR (Bricker & Halling, 1994), or relatively cumbersome, such as IS*711* typing (Zygmunt *et al.*, 2010).

We believe the solution to lie in a two-step procedure involving Bruce-ladder PCR coupled with multilocus typing. The Bruce-ladder multiplex PCR can be easily set up by any laboratory familiar with PCR methods, and its ability to reliably identify marine mammal brucellae has been demonstrated (López-Goñi *et al.*, 2008). Within this group, however, Bruce-ladder PCR cannot distinguish between the different species. MLST analysis may then be used to assign the strains to either the *B. ceti* or *B. pinnepedialis* species. Furthermore, a comparison with the 27 previously identified STs has the added advantage of being a valuable tool for the identification of unknown *Brucella* isolates. In our case, MLST analysis revealed that the Ionian isolates, like the Tyrrhenian isolate found earlier that year (Alba *et al.*, 2013), belong to ST26.

The subsequent MLVA analysis, yielding identical profiles for the two Ionian isolates, suggested that the two cases are closely related. It also confirmed – in line with Maquart *et al.* (2009) – that these ST26 isolates group with cluster A strains.

Brucellosis is a chronic disease that can be asymptomatic or cause only mild symptoms for long periods of time, but may also result in severe complications. Therefore, despite the 8 months between the two cases of stranding, it is plausible that the animals, found less than 50 km apart, were members of the same social unit.

Minimum spanning tree analysis identified the genotype U subcluster within cluster A as a unique representative of Mediterranean *Brucella* strains.

The fact that no cases of marine brucellosis have been reported in Italy in 26 years highlights the need for a systematic approach to the research of brucellae among marine mammals. In 2012, our local diagnostic unit detected *B. ceti* in two out of five cases of stranding, suggesting that this event may not be rare in the Mediterranean Sea. This is all the more important in light of the recent stranding emergency in Italy, with 114 cases reported during the first 3 months of 2013. Marine contamination with heavy pollutants such as dioxins and

dioxin-like compounds may endanger cetaceans, jeopardize their nutrition and compromise their immune systems. Increased susceptibility to specific and non-specific risks could result in a rise in outbreaks with more virulent patterns.

This is the first study to our knowledge to assess fine-scale genetic relationships between *B. ceti* isolates from the Mediterranean Sea. Interestingly, in almost 8 months no genetic variation was detected between the strains implicated in the two cases, confirming the power of MLVA-16 to assess epidemiological links between pathogens. Nevertheless, further studies on *Brucella* isolates from the Mediterranean are needed to elucidate the molecular evolution of brucellae in this marine basin.

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