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Identification of Three *Brucella ceti* Genotypes in Bottlenose Dolphins (*Tursiops truncatus*) Using a Multiplex SYBR Green Real-Time PCR

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Abstract

Three sequence types (ST) of *Brucella ceti* (ST23, ST26, and ST27) in marine mammals have been identified using multilocus sequence analysis and multilocus variable number tandem repeat analysis. This study reports a multiplex SYBR green real-time PCR assay and melting curve analysis for rapid identification of these *B. ceti* strains and for application to test clinical samples from 272 bottlenose dolphins (*Tursiops truncatus*) stranded in the coastal region of northern Florida, South Carolina, and Virginia in the United States. The multiplex real-time PCR assay detected all *B. ceti* ST23, ST26, and ST27 strains and field isolates, and none of the other *Brucella* spp. and non-*Brucella* pathogens tested. The limit of detection was 15 genome copies from *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) per PCR volume. *Brucella* DNA fragments specific for ST26 and ST27 were found in 15% (41/272) and 7% (20/272) of dolphin samples, respectively. No specific fragment of *Brucella* DNA for ST23 was detected in these samples. The presence of the gene fragments specific for ST26 and ST27 in positive samples observed with multiplex real-time PCR was further confirmed by conventional PCR, consisting of a set of six specific PCRs, targeting IS*711*-specific chromosomal locations for *Brucella* in marine mammals. To our knowledge, this study is the first report on identification of *B. ceti* genotypes ST26 and ST27 in dolphins using a multiplex real-time PCR assay. The results in this study indicate that the assay may be used as a fast and reliable alternative approach for identification of *B. ceti* in samples from dolphins.

Key Words: real-time PCR, multiplex, *Brucella* spp., bottlenose dolphins, *Tursiops truncatus*

Introduction

Since the isolation of *Brucella* from bottlenose dolphins (*Tursiops truncatus*) for the first time in 1994 (Ewalt et al., 1994), *Brucella* strains have been identified and isolated from a variety of free-ranging marine mammals from most parts of the world (Nymo et al., 2011). Two species, *Brucella ceti* and *Brucella pinnipedialis*, are the proposed taxon names for the *Brucella* isolates from cetaceans and seals, respectively (Foster et al., 2007). Gross pathology of *Brucella* infection in marine mammals is found mostly in cetaceans and has been observed in the central nervous system, joints, reproductive organs, lung, cardiovascular system, liver, spleen, lymph nodes, skin, and bones (Nymo et al., 2011; Guzman-Verri et al., 2012).

Several subgroups of marine mammal *Brucella* spp. have been identified by multilocus sequence analysis (MLSA) and multilocus variable number tandem repeat analysis (MLVA) methods (Groussaud et al., 2007; Whatmore et al., 2007; Maquart et al., 2009). There are five documented sequence types (ST) within *Brucella* spp. in marine mammals: ST24 and ST25, which are predominantly associated with the *B. pinnipedialis* sp.; and ST23, ST26, and ST27, which are most common within *B. ceti* sp. (Whatmore et al., 2007). The mobile genetic element IS*711* gene is specific for the *Brucella* genus (Ocampo-Sosa & Garcia-Lobo, 2008). The sequence types can be further identified based on IS*711*-specific chromosomal locations using conventional PCR, consisting of a set of six specific PCRs, targeting specific chromosomal

locations of IS*711* for *Brucella* in marine mammals as described previously (Zygmunt et al., 2010; Cloeckaert et al., 2011). Fragment I was found in all *Brucella* strains in marine mammals studied. Fragment II was detected to be specific to *B. pinnipedialis* but was not observed in hooded seal (*Cystophora cristata*) isolates. Fragments III, IV, and V were detected to be specific to *B. ceti*, with III and IV for ST23 strains and V for ST26 strains. A ST27 specific chromosomal locus of IS*711* (BCETI_7000072) has been previously discovered in an extensive panel of marine and terrestrial *Brucella* spp. (Cloeckaert et al., 2011). Some findings suggest that genotype ST27 has zoonotic potential for infection in humans (Brew et al., 1999; Sohn et al., 2003; McDonald et al., 2006; Whatmore et al., 2008). The strain isolated from a 1999 laboratory-acquired marine mammal *Brucella* infection in a human was identified to be ST23 (Whatmore et al., 2008). However, marine-associated brucellosis in humans has not been documented in the U.S.

Conventional PCR is laborious and susceptible to PCR product carryover contamination (Barletta et al., 2013). A multiplex real-time PCR is time-saving and cost-effective and has a low risk of cross contamination (Lefterova et al., 2013). Some PCR and real-time PCR assays have been described for identification of *Brucella* spp. (Lopez-Goni et al., 2008; Mayer-Scholl et al., 2010; Winchell et al., 2010; Kang et al., 2011; Schmoock et al., 2011; Wattiau et al., 2011), but most of them cannot differentiate subgroups of *B. ceti* and none of them have been validated in clinical samples. The ligase chain reaction (LCR) (Wattiau et al., 2011) can identify all *Brucella* spp., including *B. ceti* genotypes ST23, ST26, and ST27, but the assay was conducted in three steps and requires analysis of LCR products using capillary electrophoresis.

We report the development of a multiplex SYBR green real-time PCR assay in a single tube for identification of *B. ceti* (ST23, ST26, and ST27) by targeting specific chromosomal IS*711* locations, which were used for identification of *B. ceti* ST23, ST26, and ST27 with a set of conventional PCRs described previously (Zygmunt et al., 2010; Cloeckaert et al., 2011). Clinical samples collected from bottlenose dolphins stranded in the coastal region of northern Florida, South Carolina, and Virginia in the U.S. were analyzed using our assay.

Methods

Reference Strains

The field isolates and reference strains of *Brucella* spp. were used in this study (Table 1). Non-*Brucella* pathogens (*Escherichia coli, Enterococcus faecalis*, *Leptospira interrogans* serovar Bratislava,

Table 1. Real-time PCR results in *Brucella* strains and field isolates

Strain	Host	Origin	MLSA	Real-time PCR		
				ST23	ST26	ST27
B. abortus 544	Bovine	Type strain	1	--	--	--
B. pinnipedialis 17A1	Hooded seal	Norway	NT	--	--	--
B. pinnipedialis B2/94	Common seal	Scotland	25	--	--	--
B. ceti B1/94	Harbor porpoise	Scotland	23	+	--	--
B. ceti B202R	Minke whale	Norway	23	+	--	--
B. ceti B14-94	Common dolphin	Scotland	26	--	+	--
B. ceti 03-0312	Dolphin vertebrae	USA	27	--	--	+
B. ceti M04-0174	Dolphin placenta	USA	27	--	--	+
B. ceti LA001	Bottlenose dolphin	USA	27	--	--	+
B. ceti IFAW12-087Dd	Common short beard dolphin	USA	27	--	--	+
B. ceti LA002	Bottlenose dolphin	USA	28	--	--	--
B. ceti 021MMS	Bottlenose dolphin	USA	28	--	--	--
B. ceti SC1135	Bottlenose dolphin	USA	27	--	--	+
B. ceti SC1304	Bottlenose dolphin	USA	26	--	+	--

Note: MLSA = Multilocus sequence analysis, according to Wu et al. (2014); and NT = Not tested.

L. interrogans serovar Canicola, *L. interrogans* serovar Copenhageni, *L. interrogans* serovar Grippotyphosa, *L. interrogans* serovar Hardjo, *L. interrogans* serovar Pomona, *Pseudomonas aeruginosa, Staphylococcus aureus, Cryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii*) were included in this study to test the specificity of the multiplex real-time PCR assay for *Brucella* spp. because these pathogens have been found previously in marine mammals (Higgins, 2000; Fayer et al., 2004). We developed and tested the multiplex real-time PCR assay in dolphins only and, thus, terrestrial *Brucella* spp. and other *Brucella*-closely related microorganisms were not included in the study.

Sample Collection

The carcasses of bottlenose dolphins stranded in the coastal region of northern Florida, South Carolina, and Virginia between 2011 and 2015 were necropsied by members of the U.S. Marine Mammal Stranding Network according to standard procedures described previously (McFee & Lipscomb, 2009; Wu et al., 2014). Tissue samples were aseptically collected and placed in a cryovial tube and stored at -80° C until use. Tissue samples included brain, lung, amniotic fluid, blood, joint fluid, liver, lymph node, spinal cord, spleen, thymus, blowhole swab, bronchus swab, and tracheal swab. Carcasses were categorized into age classes based on carcass length: perinate, < 115 cm; calve, < 210 cm; subadult, 210 to 240 cm; and adult, > 240 cm.

Culture of Brucella *spp. from Tissue Samples*—Tissue samples from dolphins were submitted to the National Veterinary Services Laboratories (Ames, IA, USA) for identification of *Brucella* spp. in these samples using the culture method.

DNA Extraction—The QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) was used for genomic DNA extraction from *Brucella* spp., non-*Brucella* spp., and tissue samples as described previously (Wu et al., 2014).

Design of PCR Primers

Specific IS*711* chromosomal locus has been previously reported for identification and classification of *Brucella* strains in marine mammals (Zygmunt et al., 2010; Cloeckaert et al., 2011). Fragment III (GenBank Accession Number HM352554) and V (HM352556) were specific to *B. ceti* ST23 and ST26, respectively (Zygmunt et al., 2010). A chromosomal locus (JN383971) was found to be specific for the *B. ceti* ST27 (Cloeckaert et al., 2011). The primers targeting these fragments were designed using PrimerQuest (www.idtdna.com/Primerquest/Home/Index) so that amplicons would be produced having melting temperatures (Tm) ranging from 79° to 85° C, with > 1° C between peaks. The theoretical Tm of the amplicon sequences was estimated using the MBCF Oligo Calculator (http://mbcf.dfci.harvard.edu/docs/oligocalc.html). One ST23 specific forward primer (5'-GCCTGGACCAAGATCGTCAC-3'), one ST26 specific forward primer (5'-CCTGA GGGTGCTGTTCAT-3'), one ST27 specific forward primer (5'-GGCGGGCGGAATACAATTA-3'), and one generic reverse primer (5'-AAGG GAATCCTTGAATGCAGA-3') were used in the reaction. The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer specificities in *Brucella* spp. were confirmed by *BLASTN* analysis against all GenBank entries and assessed using DNA extracted from the *Brucella* strains and field isolates listed in Table 1.

Multiplex Real-Time PCR Analyses

Multiplex real-time PCR was run in a volume of 15 µl with primer concentration of 200 nM, 7.5 µl of Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA), and 1.5 µl DNA template in an Eco™ PCR system (Illumina, San Diego, CA, USA). Multiplex real-time PCR was performed in a single tube with three specific forward primers and one generic reserve primer. The multiplex real-time PCR was conducted under conditions: one cycle of 10 min at 95° C followed by 40 cycles of 10 s at 95° C and 60 s at 60° C and a melting curve analysis (from 60° C; gradually increasing 0.3° C/s to 95° C). DNA extracted from *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) were used as the positive template controls. Nuclease-free water (Qiagen) instead of DNA was used as negative control. All clinical samples were analyzed in triplicate with both positive and negative controls. Amplification efficiency (E) was estimated according to the formula $E = 10^{-1/slope} - 1$ (Bustin et al., 2009).

Analytical Sensitivity

Genomic DNA extracted from *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) were used to determine the analytic sensitivity of the assay. *Brucella* genomic DNA was quantified by measuring absorbance of DNA using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Assuming a genome size of 3.28 Mbp (Ancora et al., 2014), 3.6 fg DNA was considered to be one genome equivalent. Standard curves were generated using dilution series of DNA from *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27), ranging from 1.5 to 1.5×10^6 copies per PCR volume.

Sequencing Amplicons from Multiplex Real-Time PCR-Positive Tissue Samples

The amplicons from *B. ceti* B1/94, two ST26, and two ST27 multiplex real-time PCR-positive tissue samples were purified using the QIAquick PCR purification kit and then analyzed by Agilent Bioanalyzer 2100 (Santa Clara, CA, USA) to verify the presence of a single product prior to sequencing by Eurofins Genomics (Huntsville, AL, USA) with the same forward and reverse primers as those used in multiplex real-time PCR. The sequences of the amplicons were analyzed by running *BLASTN* to evaluate homologies with sequences in the GenBank database.

Fragment-Specific PCR

A conventional PCR assay targeting IS*711*-specific chromosomal locations for identification and classification of *Brucellae* in marine mammals was performed as described previously (Zygmunt et al., 2010; Cloeckaert et al., 2011) to confirm the results of our multiplex real-time PCR in samples from dolphins, including ST26 positive, ST27 positive, and negative samples observed with multiplex real-time PCR assay. The amplicon from positive samples in the conventional PCR was purified using the QIAquick PCR purification kit and analyzed by Agilent Bioanalyzer 2100 to confirm the presence of a single amplicon.

Results

Sensitivity and Specificity of the Multiplex Real-Time PCR Assay

We identified the primer concentrations that optimize amplification of target gene fragments. The amplification efficiency of multiplex SYBR green real-time PCR assay for *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) was 0.948, 0.947, and 0.910 with all correlation coefficients beyond 0.99, respectively. The limit of detection of the multiplex PCR assay was 15 genomic DNA copies of *B. ceti* B1/94, *B. ceti* B14-94, and *B. ceti* SC1135 per PCR volume. Observed (estimated) Tm values of *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) were 84.6° C (85° C), 79.5° C (79° C), and 83.1° C (83° C), respectively. All strains and field isolates of *B. ceti* ST23, ST26, and ST27 were positive using our multiplex real-time PCR assay (Table 1). No amplification of non-*Brucella* pathogens (listed in the "Methods" section) and other *Brucella* spp. (Table 1) was observed. No nonspecific products were found in the negative control using the multiplex real-time PCR assay.

Detection and Identification of Brucella *in Clinical Samples*

The samples from 272 dolphins were tested by the multiplex SYBR green real-time PCR assay for the detection of marine mammal *Brucella* spp. and the identification of *B. ceti* genotypes ST23, ST26, and ST27. Specific fragments of *Brucella* DNA for ST26 and ST27 were detected in 15% (41/272) and 7% (20/272) of the individual bottlenose dolphins tested by multiplex real-time PCR (Table 2), respectively. *Brucella* DNA was detected in samples of brain, lung, spleen, spinal cord, lymph node, joint fluid, and blowhole swab. All positive detections of multiplex real-time PCR for ST27 were in samples from perinates (Table 2). No amplification product of the fragment specific for *B. ceti* genotype ST23 was detected in these dolphin samples. In comparison to the analysis of 53 tissue samples for the detection of *Brucella* spp. by both multiplex real-time PCR assay and the culture method, we detected *B. ceti* in 66% (35/53) of the tissue samples by multiplex real-time PCR vs 40% (21/53) by culture (Table 3). Our multiplex real-time PCR assay detected *Brucella* spp. in all of the samples for which *Brucella* spp. was detected by culture method.

Conventional PCR assays described previously (Zygmunt et al., 2010; Cloeckaert et al., 2011) were used to confirm the results of multiplex real-time PCR observed in 45 dolphin samples, including 15 ST26 positive, 15 ST27 positive, and 15 negative samples, which had been analyzed using multiplex real-time PCR. Fragment I (common for marine mammal *Brucella*) was detected in all 30 positive samples. Fragment V, specific for *B. ceti* ST26, was detected in 15 ST26

Table 2. Multiplex real-time PCR results (% positive) in clinical samples from bottlenose dolphins (*Tursiops truncatus*)[a]

Age class	ST26	ST27
Perinate	4% (2/52)	38% (20/52)
Calve	18% (16/91)	0 (0/91)
Subadult	17% (9/53)	0 (0/53)
Adult	18% (14/76)	0 (0/76)
Total	15% (41/272)	7% (20/272)

[a]No amplification product of the fragment specific for *B. ceti* genotype ST23 was detected in these dolphin samples.

Table 3. Comparison of culture and multiplex real-time PCR results in 53 tissue samples from bottlenose dolphins tested

	Real-time PCR			
Culture	ST26 positive	ST27 positive	Negative	Total
Positive	17	4	0	21
Negative	7	7	18	32
Total	24	11	18	53

positive dolphins, and a fragment specific for *B. ceti* ST27 (BCETI_7000072) was observed in 15 ST27 positive samples. Fragment II, specific for *B. pinnipedialis*, and fragments III and IV, specific for *B. ceti* ST23, were not found in these ST26 or ST27 positive dolphin samples. No amplification of fragments I through V and fragments specific for ST27 was detected in 15 negative dolphin samples. Figure 1 showed the gel-like image of combined products of the conventional PCR amplified from *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), *B. ceti* SC1135 (ST27), one ST26 positive, and one ST27 positive dolphin samples in multiplex real-time PCR, respectively.

Confirmation of Amplicon Identity by DNA Sequence Analysis

Amplicons of multiplex real-time PCR from two ST26 and two ST27 positive dolphin samples were analyzed with the Agilent Bioanalyzer 2100 and showed a single band. Since no amplification product of the fragment specific for *B. ceti* genotype ST23 was detected in these dolphin samples tested, product amplified from *B. ceti* B1/94 in multiplex real-time PCR was also sequenced to identify the amplicon from the species. The purified products of multiplex real-time PCR were sequenced, and the outputs were compared with known sequence databases in the GenBank using *BLASTN*. Sequences of multiplex real-time PCR products from two ST26 positive dolphin samples aligned with 100% identity to the sequence of fragment V (HM352556), which is specific for *B. ceti* ST26. Sequences of PCR products from two ST27 positive dolphin samples matched 100% with the sequence of a fragment specific for *B. ceti* ST27 (JN383971). The sequence of PCR product from *B. ceti* B1/94 was the same as fragment III (HM352554), which is specific for *B. ceti* ST23. These results from DNA sequence analysis further indicate the assay specificity for identification of *B. ceti* ST23, ST26, and ST27.

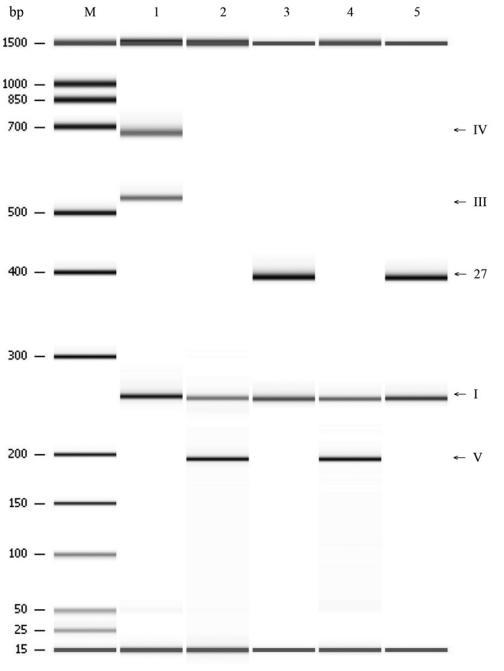

Figure 1. Gel-like image of products from a conventional PCR. Products of several PCRs amplified from same DNA samples using different primers were pooled together prior to the analysis. Lane M, 1,000 bp DNA ladder; lane 1, *B. ceti* B1/94 (ST23); lane 2, *B. ceti* B14-94 (ST26); lane 3, *B. ceti* SC1135 (ST27); lane 4, dolphin sample 1 (ST26 positive in multiplex real-time PCR); and lane 5, dolphin sample 2 (ST27 positive in multiplex real-time PCR). I, III, IV, and V represent fragments I, III, IV, and V, respectively. The number 27 represents a fragment specific to ST27.

Discussion

We report the development of a multiplex SYBR green real-time PCR assay in a single tube using primers for detecting the IS*711* chromosomal locations, which were specific for the *B. ceti* ST23, ST26, and ST27 (Zygmunt et al., 2010; Cloeckaert et al., 2011). Real-time PCR has been widely used for the detection and identification of pathogens in clinical samples. The multiplex real-time PCR assay developed in this study was shown to be highly specific for *B. ceti* ST23, ST26, and ST27 since the target gene sequence was detected in all strains and field isolates of *B. ceti* ST23, ST26, and ST27 tested but were not found in other *Brucella* spp. and non-*Brucella* pathogens included in the study. The amplicon sequences amplified from *B. ceti* B1/94 (ST23)

and two ST26 positive and two ST27 positive dolphin samples in the multiplex real-time PCR assay aligned with 100% identity to the sequences of gene fragments specific for *B. ceti* ST23, ST26, and ST27, respectively, further indicating the specificity of SYBR green real-time PCR assay for *B. ceti* subgroups.

A highly sensitive method for detection of *Brucella* spp. in clinical samples is required because the cell numbers of *Brucella* in body fluids and tissues are usually very low (Bricker, 2002; Al Dahouk et al., 2003). The limit of detection of the multiplex real-time PCR assay was 15 genomic DNA copies of *B. ceti* B1/94 (ST23), *B. ceti* B14-94 (ST26), and *B. ceti* SC1135 (ST27) per PCR volume in this study. This level of sensitivity is comparable to the analytic limit of detection (at approximately seven bacterial genomic copies) reported from an assay for the detection of *Brucella* spp. at genus level from clinical samples in marine mammals using a multiplex real-time PCR with two internal controls (Sidor et al., 2013). All of the culture-positive samples were found to be positive by the multiplex real-time PCR assay, and we detected additional positive samples in which no isolation of *Brucella* spp. was obtained by culture. Multiplex real-time PCR positive but culture-negative samples may be due to either a higher sensitivity of the PCR assay, contamination, or the presence of nonviable *Brucella*, which cannot be cultured; results from other findings also indicate that real-time PCR assay has a higher detection sensitivity for *Brucella* than culture in marine mammals (Sidor et al., 2013; Wu et al., 2014).

According to MLSA, three *B. ceti* (ST23, ST26, and ST27) are associated with dolphins, although ST23 is predominantly associated with porpoises (75% of isolates) (Whatmore et al., 2007). Some *B. ceti* ST26 have been recently isolated from tissue samples of striped dolphins (*Stenella coeruleoalba*) (Alba et al., 2013; Garofolo et al., 2014), a long-finned pilot whale (*Globicephala melas*), and a Sowerby's beaked whale (*Mesoploden bidens*) (Foster et al., 2015). A *Brucella* isolate from a bottlenose dolphin in the U.S. and all three isolates from naturally acquired human infections with marine mammal *Brucella* spp. have been characterized as having the same genotype, ST27 (Whatmore et al., 2008). We detected *B ceti* ST26 and ST27 in 15 and 7% of the 272 dolphins sampled, respectively. However, no *B. ceti* ST23 was found in these samples. All positive samples for *B. ceti* ST27 were found in perinates, suggesting that perinatal dolphins may be more susceptible to genotype ST27 infections. A conventional PCR targeting the IS*711*-specific chromosomal locations further confirmed the presence of fragments specific for ST26 and ST27 in positive dolphin samples of multiplex real-time PCR. To our knowledge, this study is the first report on identification of *B. ceti* ST26 and ST27 in dolphins using multiplex real-time PCR.

Several molecular typing methods, including determination of IS*711* numbers and locations, MLSA, VNTR analysis (MLVA), and *omp2* locus gene analysis, have been reported for characterization of marine mammal *Brucellae* (Clavareau et al., 1998; Cloeckaert et al., 2001, 2003; Vizcaino et al., 2004; Bourg et al., 2007; Groussaud et al., 2007; Dawson et al., 2008; Maquart et al., 2009; Whatmore, 2009). Conventional PCRs targeting IS*711* specific chromosomal locations can distinguish three *B. ceti* subgroups: (1) the *B. ceti* porpoise type (ST23), (2) *B. ceti* dolphin type (ST26), and (3) *B. ceti* isolated from humans (ST27) (Groussaud et al., 2007; Whatmore et al., 2007; Zygmunt et al., 2010; Cloeckaert et al., 2011). However, these assays are time consuming, laborious, and may require analysis of products. None of these assays had been described in analysis for clinical samples. Real-time PCR is a useful tool in rapid detection and genotyping of microbial templates with reduced risk of cross contamination (Mackay, 2004). We selected SYBR green as a real-time PCR reporter molecule because it is cost-saving and a simple technical method in comparison to other real-time PCR formats such as beacons and TaqMan. The multiplex format of the assay in a single tube will reduce reagent cost and staff time required to perform testing for detection and identification of *Brucella* spp. The results in this study indicate that the assay may be used as a fast and reliable alternative approach for identification of *B. ceti* in samples from dolphins.

New *B. ceti* ST28 has been reported in bottlenose dolphins in the U.S. (Wu et al., 2014), but detailed information about the isolate is still not available. Thus, the assay in this study may need to be expanded in the future to include identification of *B. ceti* ST28 in order to identify all currently described *B. ceti* sp. in clinical samples from dolphins.

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