AAVLD

Journal of Veterinary Diagnostic Investigation
1–5
© 2017 The Author(s)
Reprints and permissions:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1040638717733024
jvdi.sagepub.com

Application of real-time quantitative PCR assays for detecting marine *Brucella* spp. in fish

Stephanie A. Norman,[1] Martha A. Delaney, Katherine H. Haman, Austen C. Thomas, Jacques Godfroid, Anett K. Larsen, Ingebjørg H. Nymo, Suelee Robbe-Austerman, Christine Quance, Jack C. Rhyan, Dyanna M. Lambourn, Steven J. Jeffries, Peter Rabinowitz

Abstract. *Brucella ceti* and *Brucella pinnipedialis* have been documented as occurring in marine mammals, and *B. ceti* has been identified in 3 naturally acquired human cases. Seroconversion and infection patterns in Pacific Northwest harbor seals (*Phoca vitulina richardii*) and North Atlantic hooded seals (*Cystophora cristata*) indicate post-weaning exposure through prey consumption or lungworm infection, suggesting fish and possibly invertebrates play an epizootiologic role in marine *Brucella* transmission and possible foodborne risk to humans. We determined if real-time quantitative PCR (qPCR) assays can detect marine *Brucella* DNA in fish DNA. Insertion sequence (IS)*711* gene and sequence type (ST)27 primer–probe sets were used to detect *Brucella* associated with marine mammals and human zoonotic infections, respectively. First, DNA extracts from paired-species fish (containing 2 species) samples were tested and determined to be *Brucella* DNA negative using both IS*711* and ST27 primer–probe sets. A representative paired-species fish DNA sample was spiked with decreasing concentrations of *B. pinnipedialis* DNA to verify *Brucella* detection by the IS*711* primer–probe within fish DNA. A standard curve, developed using isolated DNA from *B. pinnipedialis*, determined the limit of detection. Finally, the IS*711* primer–probe was used to test Atlantic cod (*Gadus morhua*) DNA extracts experimentally infected with the *B. pinnipedialis* hooded seal strain. In culture-positive cod tissue, the IS*711* limit of detection was ~1 genome copy of *Brucella*. Agreement between culture and PCR results for the 9 positive and 9 negative cod tissues was 100%. Although a larger sample set is required for validation, our study shows that qPCR can detect marine *Brucella* in fish.

Key words: *Brucella* spp.; cetacean; fishes; IS*711*; marine; pinniped; real-time PCR; ST*27*.

Brucella ceti and *Brucella pinnipedialis* are documented as occurring in marine mammals,[18] and in natural human infections.[14,23] However, their transmission and prevalence are poorly understood. In bottlenose dolphins (*Tursiops truncatus*), *Brucella* infection is similar to that in livestock, with possible horizontal dissemination between individuals through sexual contact or contact with aborted birth tissues or fetuses, or vertically from mother to fetus.[16] In earless seals, neither gross pathology nor abortion has been reported, whereas in eared seals, *B. pinnipedialis* has been detected in 6 northern fur seal (*Callorhinus ursinus*) placentas.[6] Infection with *B. pinnipedialis* has furthermore been documented in California sea lion (*Zalophus californianus*) placentas and fetuses (Sidor I, et al. Transplacental infection with *Brucella* in California sea lion (*Zalophus californianus*). Brucellosis Intern Res Conf; Egham, Surrey, England; 2008). In pinnipeds, the transmission route may include close contact between members of the same species at haul-outs[13] or ingestion of fish and/or their parasites.[13] Marine mammal parasites, such as lungworms (*Parafilaroides* spp., *Otostrongylus circumlitus*, and *Pseudalius inflexus*), can serve as vectors of marine brucellae.[5,10] Lungworms are shed in the feces of an

Marine-Med, Bothell, WA (Norman); Department of Comparative Medicine, University of Washington, Seattle, WA (Delaney); Washington Department of Fish and Wildlife, Olympia, WA (Haman); Smith-Root Inc., Vancouver, WA (Thomas); Arctic Infection Biology, Department of Arctic and Marine Biology, UiT–The Arctic University of Norway, Tromsø, Norway (Godfroid, Larsen, Nymo); U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Diagnostic Bacteriology Laboratory, National Veterinary Services Laboratories, Ames, IA (Robbe-Austerman, Quance); U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Fort Collins, CO (Rhyan); Washington Department of Fish and Wildlife, Marine Mammal Investigations, Lakewood, WA (Lambourn, Jeffries); and Center for One Health Research, Department of Environmental and Occupational Health Sciences, University of Washington School of Public Health, Seattle, WA (Rabinowitz).

[1]Corresponding author: Stephanie A. Norman, Marine-Med: Marine Research, Epidemiology, and Veterinary Medicine, 24225 15th Place SE, Bothell, WA 98021. stephanie@marine-med.com

infected marine mammal host into the water, then eaten by coprophagic fish; the worms can then migrate from the host gastrointestinal tract to their lungs.[13]

Reports of *Brucella* spp. in fish are scarce.[7] Nile catfish (syn. North African catfish, *Clarias gariepinus*) experimentally infected with *B. melitensis* maintained positive titers up to 5 wk post-inoculation.[21] Nile catfish naturally infected with *Brucella melitensis* harbored *Brucella* spp. that could possibly be transmitted to other animals and humans.[8] Recently, Atlantic cod (*Gadus morhua*) experimentally infected with the *B. pinnipedialis* hooded seal strain sustained the infection for at least 28 d, suggesting fish as potential bacterial reservoirs for other *Brucella* spp.[17]

Marine brucellae were identified in 3 naturally acquired cases of human brucellosis in which the individuals either consumed or handled raw seafood products.[14,23] Two of these naturally acquired human cases, which were reported in Peru, contained *Brucella* strains that could not be speciated. These patients regularly consumed unpasteurized cheese products and reported no contact with marine mammals; however, both individuals did consume raw shellfish. The third human case, from New Zealand, had no direct exposure to marine mammals, but regularly engaged in fishing activities, and handled and consumed raw fish.[14] The isolates from all of the human cases were subsequently determined to be of marine origin and to be sequence type (ST) 27.[2,23,26] In addition, isolates recovered from 2 aborted bottlenose dolphin fetuses,[4,9] and a California sea lion (Sidor I, et al. Transplacental infection with *Brucella*) were also ST27. Given that fish (and possibly invertebrates) may transmit marine *Brucella* spp. to marine mammals and humans through ingestion of raw seafood, the objective of our study was to evaluate the use of real-time quantitative PCR (qPCR) technology to detect marine *Brucella* spp. in DNA from fish tissues. Specifically, our aims were to demonstrate that marine *Brucella* DNA is detectable in fish DNA; perform a serial dilution to determine the lowest detectable level of *Brucella* spp. in fish DNA; and determine the limit of detection (LOD) of marine *Brucella* spp. in fish DNA spiked with *B. pinnipedialis* DNA.

The strains *B. ceti* B92-1350 (isolated from an aborted bottlenose dolphin, *Tursiops truncatus*, on the U.S. west coast, also referred to as strain F5/99)[9] and *B. pinnipedialis* B04-0281 (isolated from a harbor seal from Camano Island, WA, field ID WDFW0104-03)[15] were both obtained from the National Veterinary Services Laboratories (Ames, IA) and served as positive controls. All primers and probes used in our study were as previously described,[27] and were supplied by Integrated Technologies (Coralville, IA). One primer–probe set targeted the IS*711* gene,[11] and the other targeted a ST27-specific IS*711* chromosomal locus (BCETI_7000072).[2]

Fish DNA were obtained as convenience samples from a previous harbor seal diet analysis study.[24] These samples included species known to be preferred harbor seal prey and also consumed by humans. The following samples were collected by trawl bycatch or purchased directly from fishermen in British Columbia, Canada: chum salmon (*Oncorhynchus keta*), coho salmon (*O. kisutch*), Pacific herring (*Clupea pallasii*), sardine (*Sardinops sagax caerulea*), Pacific hake (*Merluccius productus*), walleye pollock (*Gadus chalcogrammus*), and rock sole (*Lepidopsetta bilineata*). In this prior study,[24] paired-species (i.e., containing 2 species) extractions were created. Briefly, representative whole carcasses of each species were chopped, individually ground, and then homogenized in a food processor. Next, 4 g of paired-species tissue mixture (50/50 by biomass) was created by combining Atlantic chub mackerel (*Scomber colias*) homogenate with that of a second species in a 20-mL vial. Chub mackerel served as control material in the source study.[24] Nine paired-species samples (including 2 replicate salmon extracts) were used in the present study: chum salmon (n = 2); coho salmon (n = 2); Pacific herring (n = 1); sardine (n = 1); Pacific hake (n = 1); walleye pollock (n = 1); or rock sole (n = 1). The approximate DNA sequence proportions for each species paired with the mackerel were: chum salmon (50%); coho salmon (60%); Pacific herring (42%); sardine (54%); Pacific hake (40%); walleye pollock (20%); and rock sole (22%).

First, the paired-species fish DNA extracts were determined to be negative for brucellae by using the IS*711* primer–probe sets. All reactions were performed following PCR conditions described previously.[27] Total PCR reaction volumes were 15 μL with 4.5 μL of DNA template, 100 μM primers and probe, and a commercial master mix (TaqMan Universal Master Mix II, Life Technologies, Carlsbad, CA). DNA quantities ranged from 3 to 140 ng. Real-time qPCR was completed using the ABI PRISM 7900HT Fast real-time PCR system (Thermo Fisher Scientific, Waltham, MA). Amplification began with an activation step at 95°C for 10 min, then 40 cycles of 10 s at 95°C for denaturation, and annealing/extension for 30 s at 60°C.[27] Positive controls included *B. pinnipedialis* B04-0281 DNA and *B. ceti* B92-1350 DNA as template. RNase-free water served as a negative control. All samples and controls were run in triplicate.

From these, selected DNA extracts were inoculated with decreasing amounts of control *B. pinnipedialis* B04-0281 DNA. Specifically, *B. pinnipedialis* DNA dilutions were created using the fish DNA (mackerel-to-chum salmon) mixtures as: 9 μL of *Brucella* DNA in 1 μL of fish DNA (equivalent to 2441.7 ng of bacterial DNA); 8 μL of *Brucella* DNA in 2 μL of fish DNA; and so on until the final (lowest) concentration of 1 μL of *Brucella* DNA in 9 μL of fish DNA (equivalent to 271.3 ng of bacterial DNA). These dilutions were used as template DNA for triplicate reactions with the IS*711* and ST27 primer–probe sets. Positive and negative controls were the same as described above, and all samples and controls were run in triplicate.

In addition, a standard curve was developed in order to determine bacterial DNA quantities within fish DNA. Briefly, the DNA concentration was determined (NanoDrop 2000, Thermo Fisher Scientific) and, with a genome size of

~3.41 Mb,[1] the genome copy number per mL DNA was calculated: number of copies = (amount × [6.022 x 10^{23}])/(length × [1 × 10^9] × 650; https://goo.gl/sNfbUb). Based on the known hooded seal *Brucella* genome size of ~3.41 Mb, the number of genome copies was estimated to be 7.38 × 10^7 copies/µL DNA. Using this number, an initial DNA dilution was made containing 1 × 10^6 genome copies/µL *Brucella* DNA, after which serial 1:10 dilutions were made to a final concentration of 1 genome copy/µL. RNase-free water was used as the negative control. These serial dilutions and water (negative control) were used as template for PCR reactions as described above, and threshold cycle (Ct) values were obtained. In a base-10 semi-logarithmic graph, Ct values were plotted versus the dilution factor, then to a straight line using linear regression. The LOD was calculated by interpolating the linear regression of percent amplification versus the log transformed concentration at the 3 lowest concentrations.

To demonstrate that the IS711 primer–probe set detects *Brucella* DNA following systemic infection in fish, we utilized DNA extracts incidental to a previous experimental study, in which Atlantic cod were infected with a *B. pinnipedialis* hooded seal field isolate (17a-1).[17] The experiment was conducted in Norway in accordance with the Norwegian Animal Welfare Act (approved by the Norwegian Animal Research Authority, permit 7265). Tissue samples, previously cultured to determine colony-forming units (CFUs), were stored at −20°C. *Brucella*-positive samples (0.05–0.5 g of tissue diluted in 0.5–1.0 mL of sterile phosphate-buffered saline) were heat-treated in 1.5-mL microcentrifuge tubes at 80°C for 15 min using a heat block to inactivate bacteria.

DNA extraction from the cod tissues was performed (Maxwell 16, Promega, Madison, WI) according to the manufacturer's instructions. The DNA quality and quantity were assessed by measuring absorbance at 230, 260, and 280 nm (NanoDrop 2000, Thermo Fisher Scientific). DNA extracts from head kidney (n = 3), liver (n = 3), and spleen (n = 3) of *B. pinnipedialis*–positive Atlantic cod (collectively from a total of 7 fish) were analyzed using only the primer–probe set targeting the *Brucella* IS*711* gene. Additionally, an equivalent number of DNA extracts from the same tissue types from non-infected, culture-negative cods from the same experiment were obtained and analyzed. We estimated the agreement between the cod qPCR and culture results using the Cohen kappa statistic (κ),[3] The diagnostic sensitivity and specificity of the PCR assay was calculated and compared to culture results.

The IS*711* primer–probe successfully detected control *Brucella* DNA (*B. ceti* B92-1350 and various concentrations of *B. pinnipedialis* B04-0821), determined as genome equivalents, spiked into the paired-species fish DNA samples, demonstrating a lack of inhibition of bacterial DNA amplification among fish DNA. Dilutions up to 1:10^5 were considered positive. The LOD of marine *Brucella* in fish DNA spiked with *B. pinnipedialis* DNA was 1.5 genome copies

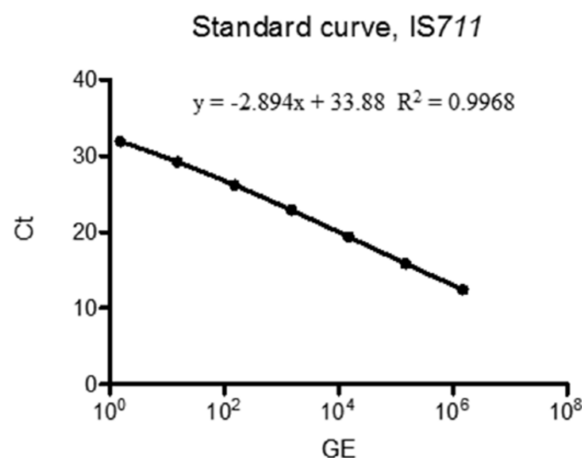

Figure 1. Standard curve demonstrating the range of threshold cycle (Ct) values plotted versus genome equivalents (GE) of *Brucella pinnipedialis* strain B04-0821 per PCR reaction volume (1.5 µL of DNA). The regression line represents data that were in the linear range. Each point represents the mean value for triplicate runs at each dilution.

per reaction, as the lowest concentration (highest dilution) contained 4.5 µL of bacterial DNA within the original mixture, from which triplicate aliquots were taken and placed in individual wells (reactions), thus each reaction contained 1.5 µL of bacterial DNA (concentration = 1 genome copy/µL). The ST27 primer–probe set only detected *B. ceti* (dolphin isolate, marine mammal-specific zoonotic type), and not *B. pinnipedialis* DNA, as expected. Similar Ct values were achieved with the lower dilutions in fish DNA, as were found with the *B. pinnipedialis* and *B. ceti* controls (Ct = 5 and 6, respectively). Although the Ct values did increase as the amount of *Brucella* DNA decreased, these values are still considered positive, given that negative controls (RNase-free water) only yielded negative (i.e., not determined) or greater than the cutoff for negative Ct values (e.g., >35). Only the negative control in the original run yielded a high Ct value in the negative range (Ct = 36), whereas the negative control in subsequent runs yielded "not determined," and thus were considered negative.

Assays performed on extracts from infected, *B. pinnipedialis* culture-positive cod samples revealed that all 9 culture-positive tissues were positive by PCR and all 9 culture-negative samples were negative. The range of Ct values for the infected, culture-positive cod samples was 24–33. Based on the standard curve, the amplification efficiency ($10^{(-1/\text{slope})} - 1$) of the assay was 1.12 with a model fit of 0.997 (Fig. 1). Quantitative PCR testing and culture agreed in 100% of the cases (both tests were either positive or negative; κ = 1.00, $p < 0.001$). Diagnostic sensitivity and specificity were 100%.

Our study demonstrated that marine *Brucella* DNA is detectable in fish DNA using PCR primers and a hydrolysis probe previously developed to detect the IS*711* gene, specific for marine *Brucella* spp. The qPCR assay performed

well analytically in fish tissues, detecting very low concentrations (~1 genome copy) of *Brucella* genomic DNA. The LOD of the *B. pinnipedialis* DNA was comparable to that reported for other brucellae assays.[22] Although developed to detect marine *Brucella* spp. in marine mammal tissues, the results demonstrate the utility of this primer–probe combination in fish.

Limitations to our study include small sample size and the possibility of PCR inhibition. Given that the sample size was small and fixed, the power to detect a positive specimen would have been small (<5%). However, even if *Brucella* spp. are present in fish populations at a low prevalence (i.e., 1%), the probability of the bacteria being transmitted from an infected prey fish to a marine mammal could be high given that large quantities of fish are consumed by marine mammals.[19] For instance, the estimated per capita fish consumption rate of harbor seals in the Salish Sea (Washington State) is 2.1 kg/day/seal.[12]

The presence of impurities left after DNA extraction can lead to PCR inhibition problems.[20] We evaluated this issue by spiking the paired-species samples with positive *B. ceti* and *B. pinnipedialis* controls—with no evidence of PCR inhibition detected. The method of DNA extraction may influence PCR inhibition. However, multiple extraction methods were not tested in our study given practical limitations and the use of convenience samples from other studies.[17,24]

Evaluating the ability of marine *Brucella* spp. to survive and be detected in fish tissues will greatly improve the understanding of *Brucella* transmission in the marine environment. The present findings suggest that qPCR assays might be appropriate initial screening tests for detecting marine *Brucella* spp. in fish. These results would also afford animal and human health professionals the opportunity to rapidly screen for the bacteria to detect potential sources of infection, and mitigate and reduce their transmission within ex-situ marine mammal settings, and during consumption of fish products by humans. Although only selected marine mammal isolates of *Brucella* are known to infect humans, the zoonotic potential of *Brucella* spp. must be considered when handling or consuming marine mammals.[14,23] ST27, implicated in the zoonotic cases, may be more pathogenic to humans, or may be linked with natural or intermediate hosts that promote a greater likelihood of contact with humans.[25]

Future work should include evaluating the assays with other extraction methods to more fully determine PCR reaction efficiency and LODs. The *Brucella*-specific qPCR assay used in our work would appear to be useful in detecting low numbers of organisms within fish tissues, and in conjunction with culture surveys, can confirm the presence of marine *Brucella* in fish. Efficient detection of *Brucella* infection by PCR will enhance detection in a timely manner and improve our epidemiologic knowledge of these organisms within the marine environment.

Acknowledgments

We thank Dr. Qingzhong Wu of Hollings Marine Laboratory, National Centers for Coastal Ocean Science (NOAA Fisheries) for helpful suggestions regarding *Brucella* spp. rtPCR assays, and Dr. Joe Gaydos of the Karen C. Drayer Wildlife Health Center–Orcas Island Office, UC Davis School of Veterinary Medicine, for valuable insights on *Brucella* in marine mammals.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The University of Washington Center for One Health Research provided funding for this project. Support for DML and SJJ was through a Prescott Marine Mammal Stranding grant to Washington Department of Fish and Wildlife. The Norwegian experimental infection was financed by the German Federal Institute for Risk Assessment, Berlin, Germany (grant 1329-520 and 1322-578).

References

1. Bohlin J, et al. Genomic comparisons of *Brucella* spp. and closely related bacteria using base compositional and proteome based methods. BMC Evol Biol 2010;10:249.
2. Cloeckaert A, et al. Novel IS*711* chromosomal location useful for identification of marine mammal *Brucella* genotype ST27, which is associated with zoonotic infection. J Clin Microbiol 2011;49:3954–3959.
3. Cohen JA. A coefficient of agreement for nominal scales. Educ Psychol Meas 1960;20:37–46.
4. Cvetnić Ž, et al. Evidence of *Brucella* strain ST27 in bottlenose dolphins (*Tursiops truncatus*) in Europe. Vet Microbiol 2016;196:93–97.
5. Dawson CE, et al. Isolation and characterization of *Brucella* from the lungworms of a harbor porpoise (*Phocoena phocoena*). J Wildl Dis 2008;44:237–246.
6. Duncan CG, et al. *Brucella* placentitis and seroprevalence in northern fur seals (*Callorhinus ursinus*) of the Pribilof Islands, Alaska. J Vet Diagn Invest 2014;26:507–512.
7. Eisenberg T, et al. Isolation of novel 'atypical' *Brucella* strain from a bluespotted ribbontail ray (*Taeniura lymma*). A Van Leeuw J Microb 2016;110:221–234.
8. El-Tras WF, et al. Brucella infection in fresh water fish: evidence for natural infection of Nile catfish, *Clarias gariepinus*, with *Brucella melitensis*. Vet Microbiol 2010;141: 321–325.
9. Ewalt DR, et al. Characteristics of a *Brucella* species from a bottlenose dolphin (*Tursiops truncatus*). J Vet Diagn Invest 1994;6:448–452.
10. Garner MM, et al. Evidence of *Brucella* infection in *Parafilaroides* lungworms in a Pacific harbor seal (*Phoca vitulina richardsi*). J Vet Diagn Invest 1997;9:298–303.
11. Hinić V, et al. IS*711*-based realtime PCR assay as a tool for detection of *Brucella* spp. in wild boars and comparison with bacterial isolation and serology. BMC Vet Res 2009;5:22.

12. Howard SMS, et al. Fish consumption by harbor seals (*Phoca vitulina*) in the San Juan Islands, Washington. Fish Bull 2013;111:27–41.
13. Lambourn DM, et al. *Brucella pinnipedialis* infections in Pacific harbor seals (*Phoca vitulina richardsi*) from Washington State, USA. J Wildl Dis 2013;49:802–815.
14. McDonald WL, et al. Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. J Clin Microbiol 2006;44:4363–4370.
15. Meegan J, et al. Development, validation, and utilization of a competitive enzyme-linked immunosorbent assay for the detection of antibodies against *Brucella* species in marine mammals. J Vet Diagn Invest 2010;22:856–862.
16. Miller WG, et al. *Brucella*-induced abortions and infection in bottlenose dolphins (*Tursiops truncatus*). J Zoo Wildl Med 1999;30:100–110.
17. Nymo IH, et al. Experimental challenge of Atlantic cod (*Gadus morhua*) with a *Brucella pinnipedialis* strain from hooded seal (*Cystophora cristata*). PLoS One 2016;11:e0159272.
18. Nymo IH, et al. A review of *Brucella* infection in marine mammals, with special emphasis on *Brucella pinnipedialis* in the hooded seal (*Cystophora cristata*). Vet Res 2011;42:93.
19. Olesiuk PF. Annual prey consumption by harbor seals (*Phoca vitulina*) in the Strait of Georgia, British Columbia. Fish Bull 1993;91:491–515.
20. Purcell MK, et al. Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting. J Aquat Anim Health 2011;23:148–161.
21. Salem SF, Mohsen A. Brucellosis in fish. Vet Med Czech 1997;42:5–7.
22. Sidor IF, et al. A multiplex real-time polymerase chain reaction assay with two internal controls for the detection of *Brucella* species in tissues, blood, and feces from marine mammals. J Vet Diagn Invest 2013;25:72–81.
23. Sohn AH, et al. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. Emerg Infect Dis 2003;9:485–488.
24. Thomas AC, et al. Quantitative DNA metabarcoding: improved estimates of species proportional biomass using correction factors derived from control material. Mol Ecol Resour 2015;16:714–726.
25. Whatmore AM, et al. Marine mammal *Brucella* genotype associated with zoonotic infection. Emerg Infect Dis 2008;14:517–518.
26. Whatmore AM, et al. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiol 2007;7:34.
27. Wu Q, et al. Real-time PCR assays for detection of *Brucella* spp. and the identification of genotype ST27 in bottlenose dolphins (*Tursiops truncatus*). J Microbiol Methods 2014;100: 99–104.