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NOTE

Detection of *Brucella* spp. in bottlenose dolphins *Tursiops truncatus* by a real-time PCR using blowhole swabs

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ABSTRACT: Blowhole swabs are a simple and non-invasive method for collecting samples from cetaceans and can be used for screening large numbers of animals in the field. This study reports a real-time PCR assay for the detection of *Brucella* spp. using blowhole swab samples from bottlenose dolphins *Tursiops truncatus* stranded in the coastal region of Virginia, South Carolina and northern Florida, USA, between 2013 and 2015. We used real-time PCR results on lung samples from the same dolphins in order to estimate the relative sensitivity and specificity of real-time PCR of blowhole swabs. *Brucella* DNA was detected in lung tissue of 22% (18/81) and in blowhole swabs of 21% (17/81) of the sampled dolphins. The relative sensitivity and specificity of real-time PCR on blowhole swabs as compared to the real-time PCR on lung samples was 94% (17/18) and 100% (63/63), respectively. These results indicate that real-time PCR on blowhole swabs may be used as a non-invasive test for rapid detection of *Brucella* spp. in the respiratory tract of dolphins. To our knowledge, this is the first report on the use of blowhole swabs for detection of bacterial pathogens by real-time PCR in bottlenose dolphins.

KEY WORDS: Bottlenose dolphins · *Brucella* spp. · Real-time PCR · Blowhole swab · Lung

INTRODUCTION

Since the first isolation of *Brucella* species from marine mammals in 1994 (Ewalt et al. 1994), *Brucella* strains have been isolated from and detected in a variety of marine mammals from most parts of the world (Nymo et al. 2011). Two species, *B. ceti* and *B. pinnipedialis*, are the proposed taxon names for the cetacean and pinniped *Brucella* species, respectively (Foster et al. 2007). Gross pathology in association with *Brucella* infection in marine mammals is seen mostly in cetaceans and has been found in reproductive organs, central nervous system, lung, cardiovascular system, liver, spleen, lymph nodes, skin, bones and joints (Nymo et al. 2011, Guzmán-Verri et al. 2012). Some reports indicate that certain types of marine mammal *Brucella* spp. have zoonotic potential (Brew et al. 1999, Sohn et al. 2003, McDonald et

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al. 2006). The transmission routes of *Brucella* infection in cetaceans are not fully understood, but aerosols generated in microbiology laboratory exposures have caused a few cases of *Brucella* infections in humans (Staszkiewicz et al. 1991). The infectious dose of *B. melitensis* is 100 organisms by aerosol route in laboratory animals (Mense et al. 2004).

Microbiological culture is considered a gold standard to confirm the presence of *Brucella* in order to make a definitive diagnosis of brucellosis. Recently, real-time PCR assays have been used for screening for *Brucella* spp. in cetaceans because of their high sensitivity and specificity (Sidor et al. 2013, Maio et al. 2014, Wu et al. 2014). Different tissue and body fluid samples from bottlenose dolphins stranded in the coastal region of South Carolina, USA, between 2010 and 2013 were tested with the IS*711* real-time PCR assay for the detection of *Brucella* spp. (Wu et al. 2014). *Brucella* DNA was detected in 31% (55/178) of the clinical samples including lung, brain, brain stem, spleen, right ventricle, mesenteric and pulmonary lymph nodes, urine and amniotic fluid. Additionally, the detection of *Brucella* spp. was compared between real-time PCR assay and the culture method, with *Brucella* DNA found in 73% (27/37) of the tissue samples tested by real-time PCR, versus 24% (9/37) tested by culture, and the diagnostic sensitivity of the real-time PCR for tissue samples was 100% (Wu et al. 2014). However, similar to microbiological culture, PCR assays have generally been applied mostly to dead animals because they require sampling of potentially infected internal organs (e.g. lung). The best tissues and lesions for detecting *Brucella* are often inaccessible from live animals (Meegan et al. 2012).

Blowhole swabs are a simple and non-invasive method for collecting samples from cetaceans and can be used for screening large numbers of animals in the field. Bacterial and fungal pathogens have been isolated previously from swabs of blowhole, gastric fluid and feces from free-ranging Atlantic bottlenose dolphins *Tursiops truncatus* (Morris et al. 2011), but the significance of the detected pathogens was unclear. While these results indicate that blowhole swabs may be used as a sampling method for detection of bacterial pathogens, an understanding of how the detection in blowhole swabs correlates with presence of the pathogen in lung tissues is needed. In order to investigate the correlation between blowhole swab and lung samples, we analyzed both blowhole swab and lung samples using a real-time PCR assay for the detection of *Brucella* spp. Real-time PCR results on lung samples from the same dolphins were used as an estimation of the relative sensitivity and specificity of real-time PCR on blowhole swabs.

MATERIALS AND METHODS

Sample collection

Blowhole samples with sterile cotton or synthetic swabs and lung samples were collected at necropsy from bottlenose dolphins that stranded dead or stranded live and then died in the coastal region of Virginia, South Carolina and northern Florida, USA, between 2013 and 2015. Carcasses were necropsied according to standard procedures described previously (McFee & Lipscomb 2009). The swab (Puritan sterile, 15 cm Guilford, ME) was aseptically opened from the tip end to expose the plastic shaft of the applicator. The tip was inserted into the blowhole, and the walls of the blowhole were swiped down to about 1–3 inches (~2.5–7.5 cm). The applicator was withdrawn; the tip was then placed in a sterile cryovial and stored at −80°C until shipment on dry ice to the lab. Fifty to 400 g of lung samples were taken from dolphins, and samples were collected from the left or right lung including the pleural surface and parenchyma. The samples were stored at −20°C or −80°C for 2 wk to 18 mo until shipment on dry ice to the lab. These dolphins stranded prior to and during a dolphin morbillivirus outbreak along the Atlantic coast, and several of them tested positive for dolphin morbillivirus via PCR (Van Bressem et al. 2014). Dolphins were separated into age categories based upon standard length (Read et al. 1993).

DNA extraction and real-time PCR analyses

Genomic DNA was extracted from blowhole swab and lung samples using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Twenty to 25 mg of lung samples were cut using carbon-steel scalpel blades for DNA extraction. Yield and purity of DNA extracted from blowhole swab and lung samples were analyzed by Spectrophotometer ND-1000 (Thermo Fisher Scientific). Each sample was analyzed for detection of *Brucella* DNA using a real-time PCR assay targeting the IS*711* gene as described previously (Wu et al. 2014). Both blowhole swab and lung samples were extracted from the same dolphins and assays were run at the same time. An internal amplification control was included in the

assay as described previously (Wu et al. 2014) to assess false-negative real-time PCR results due to inhibition. DNA extracted from *B. ceti* reference strain (NCTC 12891T, BCCN 94-74T) was used as the positive template control. Nuclease-free water (Qiagen) was used as the no template control or negative control. DNA from a *Brucella*-negative dolphin, which was confirmed by both real-time PCR and the culture method, was often used as a biological negative control in the study. All clinical samples and both negative and positive controls were run in triplicate. A 2-sample *t*-test was used to analyze differences in the threshold cycle (Ct) between samples analyzed and the limit of detection (LOD) of the real-time PCR assay. Statistical significance was considered for p-values <0.05.

RESULTS AND DISCUSSION

Eighty-one pairs of blowhole swab and lung samples from stranded bottlenose dolphins were tested for the detection of *Brucella* spp. using the real-time PCR assay. *Brucella* DNA was detected in lung tissue of 22% (18/81) and in blowhole swabs of 21% (17/81) of the sampled dolphins. The relative sensitivity and specificity of real-time PCR on blowhole swabs as compared to the real-time PCR on lung tissue was 94% (17/18) and 100% (63/63), respectively. The means for Ct values of positive samples from blowhole swabs and lung were 32.6 (17.7–37.3) and 32.7 (18.8–37.4), respectively. Standard deviation of triplicate run from all positive samples was less than 0.3. These positive samples of both blowhole swab and lung in real-time PCR were collected from 13 stranded dead dolphins and 4 stranded live dolphins which then died. The single discordant result was from 1 stranded dead dolphin that was negative on the blowhole swab and positive on the lung sample. For this dolphin, the Ct value (37.37 ± 0.28, mean ± SD) for the lung sample was close to the Ct value (37.43 ± 0.26, $p = 0.7991$) for the assay LOD, indicating a low concentration of the target gene. There were 7 perinates (<115 cm), 39 calves (<210 cm), 10 subadults (210–240 cm) and 25 adults (>240 cm) tested. The highest percentage of positive blowhole swabs was found in subadults (40%, 4/10), followed by perinates (29%, 2/7), calves (18%, 7/39) and adults (16%, 4/25).

We report detection of *Brucella* spp. in bottlenose dolphins by a real-time PCR assay using blowhole swabs that is highly concordant with real-time PCR results from lung samples. Blowhole swabs are relatively easy to collect from stranded (live or dead) dolphins in the field and can also be collected using the same or similar methods from live dolphins that are temporarily captured for health assessment studies (Stewart et al. 2014). While the microbial flora in live versus dead dolphins may differ, our result indicates that detection of *Brucella* from a blowhole swab correlates well with detection in internal lung tissue, suggesting that real-time PCR with blowhole swab may be used as a non-invasive substitute for rapid detection of *Brucella* spp. in the respiratory tract of dolphins when lung tissue cannot be obtained. To our knowledge, this is the first report on the use of blowhole swabs for rapid detection of bacterial pathogens by real-time PCR assay in bottlenose dolphins.

The cell numbers of *Brucella* in tissues are frequently very low (Al Dahouk et al. 2003). The real-time PCR assay used in the study was highly sensitive (Wu et al. 2014); however, 1 dolphin exhibited a negative blowhole swab sample but a positive lung sample. The concentration of *Brucella* DNA detected in the lung sample was low, close to the limit of detection for the assay.

The cetacean respiratory tract may be a primary source of *Brucella* infection (Guzmán-Verri et al. 2012), so we used blowhole swabs and lung samples as a comparison in our study to evaluate the use of blowhole swab for detection of *Brucella* spp. in dolphins. *Brucella* organisms have been isolated from a variety of organs including lung in apparently healthy marine mammals (Nymo et al. 2011, Guzmán-Verri et al. 2012). Therefore, it should be noted that a positive real-time PCR result only indicates the presence of the *Brucella* DNA; it does not necessarily indicate an active infection. Regardless of the source of the tissue in which *Brucella* is detected, additional evidence based on clinical symptoms in live animals or characteristic pathologic lesions from histological evaluation of internal tissues in dead animals, is needed for definitive diagnosis of active infection. Further investigation with a large sample size from dolphins may be needed to better understand correlation between the result of real-time PCR using blowhole swabs and brucellosis in lung and other organs of dolphins.

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