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Fluorescent microbead-based immunoassay for anti-*Erysipelothrix rhusiopathiae* antibody detection in cetaceans

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ABSTRACT: A fluorescent microbead-based immunoassay (FMIA) for detection of anti-*Erysipelothrix rhusiopathiae* antibodies in pigs was adapted for use in cetaceans. The FMIA was validated and adjusted using serum samples from 10 vaccinated captive bottlenose dolphins *Tursiops truncatus* collected between 1 and 13 mo after immunization. The technique was then used to analyze specimens from 15 free-ranging cetaceans stranded alive on the Valencian Mediterranean coast between 2006 and 2014: 11 striped dolphins *Stenella coeruleoalba*, 3 Risso's dolphins *Grampus griseus* and 1 bottlenose dolphin *Tursiops truncatus*. One of these wild animals was confirmed to have died from *E. rhusiopathiae* septicemia, but no anti-*E. rhusiopathiae* antibodies were detected in its serum, pericardial fluid or milk samples. Another free-ranging individual, which lacked any signs or lesions that might be indicative of *E. rhusiopathiae* infection, showed high fluorescence intensity similar to that measured in captive dolphins at 6–13 mo after vaccination. These results suggest that this animal underwent an *E. rhusiopathiae* infection several months before stranding. The findings in the present study suggest that FMIA can be useful for detecting anti-*E. rhusiopathiae* antibodies in cetaceans, and its application to free-ranging animals is particularly interesting because of the great value of these specimens. Furthermore, the FMIA can be multiplexed to allow the determination of up to 100 analytes per sample in a single well, thereby reducing the cost, time and sample volume needed.

KEY WORDS: *Erysipelothrix rhusiopathiae* · Erysipelas · Antibodies · Fluorescent microbead-based immunoassay · Cetaceans

INTRODUCTION

Erysipelothrix rhusiopathiae is a Gram-positive, non-acid-fast, rod-shaped bacterium that is a facultative anaerobe and does not form spores (Brooke & Riley 1999, Wang et al. 2010). *E. rhusiopathiae* is ubiquitous and may persist in the environment, including marine systems (Wang et al. 2010), for long periods in the presence of organic matter (Opriessnig & Wood 2012). It has been isolated from the cutaneous slime of both fresh- and saltwater fish, insects, mollusks and crustaceans (Seibold & Neal 1956, Lauckner 1985, Kinsel et al. 1997, Brooke & Riley 1999, Wang et al. 2010, Opriessnig et al. 2013).

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E. rhusiopathiae is the causative agent of erysipelas, a disease that affects several species of mammals and birds, but mainly swine, turkeys (Kinsel et al. 1997, Dunn et al. 2001, Wang et al. 2010) and humans, in which *E. rhusiopathiae* infection is known as erysipeloid (Wang et al. 2010). In cetaceans, only the dermatologic and acute septicemic forms have been reported (Seibold & Neal 1956, Chastel et al. 1975, Thurman et al. 1983, Buck & Spotte 1986, Kinsel et al. 1997, Melero et al. 2011). Thrombosis of peripheral arteries and local tissue infarction cause pathognomonic rhomboid-shaped skin lesions (Sweeney & Ridgway 1975).

The main source of infection in cetaceans is ingestion of contaminated fish (Geraci et al. 1966, Lauckner 1985, Suer & Vedros 1988, Higgins 2000), but other sources can include humans with erysipeloid, contaminated food preparation sites, opportunistic colonization of wounds, and flying insects that serve as vectors of the pathogens between contaminated areas (Wood & Shuman 1981). Therefore, most cases of erysipelas in cetaceans have been reported to occur in captive animals (Seibold & Neal 1956, Geraci et al. 1966, Medway & Schryver 1973, Thurman et al. 1983, Buck & Spotte 1986, Bossart & Eimstad 1988, Kinsel et al. 1997, Dunn et al. 2001), with infections rarely reported in free-ranging cetaceans (Chastel et al. 1975, Melero et al. 2011).

Determination of antibodies against *E. rhusiopathiae* in captive cetaceans allows for the assessment of vaccine efficiency (Lacave et al. 2001, Sitt et al. 2010, Bernal-Guadarrama et al. 2014). Its determination in unvaccinated captive and in free-ranging cetaceans may also allow detection of previous exposure and may assist in diagnosis. Main surface protective antigen (Spa) proteins have been described as suitable antigens for antibody detection against *E. rhusiopathiae* in pigs (Imada et al. 2003, To & Nagai 2007, Giménez-Lirola et al. 2012). Here, we adapted a fluorescent microbead-based immunoassay (FMIA) based on a Spa protein-derived recombinant polypeptide that was specifically designed for detection of anti-*E. rhusiopathiae* antibodies in cetaceans. The FMIA was validated using samples from vaccinated captive bottlenose dolphins. It was then used to detect anti-*E. rhusiopathiae* antibodies in samples from free-ranging cetaceans stranded alive in the Mediterranean Sea, including the first case of erysipelas reported in a free-ranging bottlenose dolphin (Melero et al. 2011).

The main advantage of the FMIA is the capacity to develop multiplex diagnostic assays (Elshal & McCoy 2006) for simultaneous determination of up to 100 analytes in the same sample, thereby reducing the cost, time and sample volume needed. FMIA can be more sensitive than traditional immunoassays (van Gageldonk et al. 2008, Giménez-Lirola et al. 2012, Sánchez-Matamoros et al. 2015). Although FMIA, based on Luminex's xMAP technology, has been used successfully in veterinary disease research (Clavijo et al. 2006, Anderson et al. 2011, Wagner et al. 2011, Giménez-Lirola et al. 2012, Christopher-Hennings et al. 2013, Sánchez-Matamoros et al. 2015), the present study is, to the best of our knowledge, the first application of FMIA to cetaceans.

MATERIALS AND METHODS

A recombinant polypeptide of 415 amino acids derived from the *E. rhusiopathiae spaA* gene (GenBank accession number AB019124.1) was selected and produced as an antigen for anti-*E. rhusiopathiae* antibody detection in cetaceans. This protein was previously described as a suitable antigen for anti-*E. rhusiopathiae* antibody detection in pigs (Imada et al. 2003, Ingebritson et al. 2010, Giménez-Lirola et al. 2012). The *spaA* gene fragment was commercially produced (GenScript), and the recombinant Spa protein-derived polypeptide was expressed in *Escherichia coli* by cloning into a pETDuet-1 expression vector (Novagen®). The resulting fusion protein was purified from a clarified extract of *E. coli* Rosetta BL21 (DE3) using a nickel-nitrilotriacetic acid chelate affinity chromatography kit (PrepEase® His-tagged protein purification kit, USB Corporation) according to the manufacturer's instructions. FMIA was performed as previously described in methods used for antibody detection in pigs (Giménez-Lirola et al. 2012). All incubations were carried out at room temperature (20–25°C) in the dark and under constant vortexing at 500 rpm (Fisher Scientific). During the coupling reaction, antigen was coupled to carboxylated fluorescent microbeads (25 µg antigen per 5 million beads; bead region 64) using a 2-step carbodiimide reaction as previously described for pigs (Staros et al. 1986).

For the FMIA, serum samples were diluted 1:25 in assay buffer (0.1 M phosphate-buffered saline [PBS], 10% goat serum [Gibco®, Life Technologies], 0.05% Tween-20, pH 7.2) and mixed with 50 µl of the suspension of antigen-coupled beads into each well of a 96-well flat bottom microtiter plate (Bio-Plex Pro™, Bio-Rad Laboratories). Plates were incubated for 60 min and washed 3 times with 0.1 M PBS containing 0.05% Tween-20 (PBST). Subsequently, 50 µl of biotinylated Protein A from *Staphylococcus aureus*

(Sigma-Aldrich) diluted 1:100 in assay buffer was added to each well, and the plates were incubated for another 30 min. After 3 washes with PBST, 50 µl of streptavidin-R-phycoerythrin (Sigma-Aldrich), diluted to 2.5 µg ml^{-1} in assay buffer, was added to each well and the plates were incubated for 30 min. After an additional wash step, the beads were resuspended in 100 µl of assay buffer and the reporter fluorescence of the beads was determined using a dual-laser Bio-Plex® 200 instrument (Bio-Rad) and analyzed using Bio-Plex Manager 6.0 software (Bio-Rad).

Median fluorescence intensity (MFI) was estimated from at least 50 beads; samples were evaluated in triplicate and the result was taken as the average of the 3 measurements. To assay levels of non-specific binding, we included on each plate 3 'background wells' consisting of coupled microspheres incubated with serum diluent in the absence of sample. The MFI response was corrected by subtracting the background well signal (the mean of the 3 wells) from the signal obtained for each sample.

The Spa-based FMIA was validated for cetaceans with serum samples from 10 captive bottlenose dolphins *Tursiops truncatus* vaccinated with *Erysipelothrix*-based inactivated vaccines, originally designed for use in pigs. Animals were housed at Oceanografic (Valencia, Spain) where a 12-mo booster schedule is followed. Serum samples were collected at intervals of 1, 2, 6, 12, and 13 mo after annual revaccination (or after the primo-vaccination, in the case of 2 individuals). All dolphins were vaccinated with ER BAC PLUS (Pfizer), with the exception of 1 individual that was vaccinated with ERYVAC (Intervet UK). For negative controls, serum samples from 20 unvaccinated captive bottlenose dolphins were also analyzed. Additionally, serial decimal dilutions of positive samples were tested.

After validation with samples of vaccinated animals, the FMIA was used to analyze serum samples from 15 free-ranging cetaceans which live-stranded (and later died) on the Mediterranean coast of Valencia Community (Spain). These specimens came from 11 striped dolphins *Stenella coeruleoalba*, 3 Risso's dolphins *Grampus griseus*, and the first case of erysipelas reported in a free-ranging bottlenose dolphin *T. truncatus*, stranded in the Mediterranean Sea (Melero et al. 2011).

Necropsy, histopathology, virology and bacteriology analysis were performed on all 15 free-ranging cetaceans. However, descriptive case evaluation included only the bottlenose dolphin and one Risso's dolphin that tested positive for anti-*E. rhusiopathiae* antibodies. Necropsies were performed following the standard protocols of the European Cetacean Society (Kuiken & García-Hartmann 1991). Three sets of samples were prepared: one set was preserved in transport medium for bacteriological assays, a second set was stored at −20°C for virological assays, and the third was preserved in formalin for histopathology examination.

For bacteriological analysis, swabs were collected from the mouth, blowhole, liver, spleen, lung, kidney and several sections of intestine from 4 striped dolphins and 2 Risso's dolphins; swabs were also collected from the cardiac valves of one Risso's dolphin. Each swab was preserved in 1 cryotube with FBP medium (see Gorman & Adley 2004) containing 0.5% active charcoal (Sigma-Aldrich), and the tubes were frozen at −20°C until analysis. Samples processed for aerobic culture were inoculated onto Columbia agar with 5% sheep blood (CBA; Oxoid) and MacConkey agar plates (Oxoid), and then incubated overnight at 35°C. For anaerobic culture, swabs were inoculated onto CBA and tryptose sulphite cycloserine agar plates (Oxoid) and incubated overnight at 35°C in anaerobic jars using the AnaeroGen system (Oxoid). Intestinal swabs were further analyzed using protocols based on ISO 10272-1:2006 (ISO 2006a,b) to detect *Campylobacter* and protocols based on ISO 6579:2002/Amd 1:2007 (ISO 2007) to detect *Salmonella*. Finally, swabs collected from liver, spleen, lung, and kidney were inoculated into 5 ml of brain heart infusion (BHI) broth medium (Difco Laboratories) containing 0.1% Tween 80, 5% horse serum, 50 µg ml^{-1} gentamicin, 0.1% sodium azide, and 0.001% crystal violet. After incubation at 37°C for 24 h, 1 loopful of broth culture was streaked onto BHI agar containing 0.1% Tween 80, 50 µg ml^{-1} gentamicin, and 0.1% sodium azide. The agar plates were incubated at 37°C for 48 h in an atmosphere of 5–10% CO_2 and then examined for the presence of typical *Erysipelothrix* colonies (Hassanein et al. 2001). All isolates considered significant after culture were further characterized using standard diagnostic techniques, including the API 20E, API 20NE, and API 20STREP systems (BioMérieux) and PCR-based analysis of 16S rRNA gene sequences when necessary. Bacteriology analysis of specimens from the bottlenose dolphin was previously described in Melero et al. (2011).

Morbillivirus can have immunosuppressive effects (Domingo et al. 1992, Schulman et al. 1997, Heaney et al. 2002). In order to examine whether infection with this virus could affect anti-*E. rhusiopathiae* antibody production, total RNA was extracted from skin, brain, lung, kidney, tonsil, prescapular and pulmonar lymph node tissues of all 15 free-ranging cetaceans

using the NucleoSpin RNA II Kit (Macherey-Nagel). Cetacean morbillivirus (CeMV) diagnosis was performed using a real time RT-PCR assay as previously described (Rubio-Guerri et al. 2013).

During necropsy, tissues of all major organs and lesions were collected and stored in a fixative solution of 10% neutral buffered formalin for histological analysis. Fixed tissue samples were trimmed, processed by routine methods, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin for examination by light microscopy.

RESULTS AND DISCUSSION

The presence of anti-*Erysipelothrix rhusiopathiae* antibodies in serum samples from all vaccinated dolphins was confirmed by Spa-based FMIA (Table 1). MFI values varied between individuals and decreased in most animals (except for Dolphins 5, 8, and 10) over time after vaccination (Table 1). No differences in MFI were related to the time of sample collection (after primo-vaccination or annual booster) (Table 1). Dolphin 2 displayed high MFI values only at 1 and 2 mo after vaccination, and Dolphin 1 presented the highest MFI values of all evaluated animals (except for Dolphin 6 at 12 mo after vaccination). However, a larger number of primo-vaccinated animals should be evaluated to establish the relationship between MFI value and the number of previous immunizations.

MFI values in negative controls were between −97.25 and 117.75 (mean = −54.89). Serial decimal dilutions showed that most positive samples could be diluted 100 times before becoming negative, while serum samples with MFI values near 1000 could not be diluted (Fig. 1).

Among free-ranging cetaceans, only 1 individual Risso's dolphin *Grampus griseus* (i.e. 6.7% of dolphins sampled) showed positive levels of anti-*E. rhusiopathiae* antibodies by Spa-based FMIA (Table 2).

The bottlenose dolphin *Tursiops truncatus* that died from an *E. rhusiopathiae* septicemia (Melero et al. 2011) tested negative for anti-*E. rhusiopathiae* antibodies in its serum, pericardial fluid, and milk specimens. Rhomboid-shaped skin lesions, which are a pathognomonic sign of erysipelas in many species (Wang et al. 2010), were observed in this animal (Fig. 2). In addition, *E. rhusiopathiae* was successfully cultured and isolated from skin and brain samples. Blood analysis revealed leukocytosis (48 600 leukocytes μl^{-1}) characterized by a neutrophilia (48 200 neutrophils μl^{-1}), based on reported normal ranges in healthy animals of 5600–12 400 leukocytes μl^{-1} and 2540–6140 neutrophils μl^{-1} (Bossart et al. 2001). The test for CeMV was negative. Histopathological analysis revealed erysipelas-like lesions consisting of multisystemic intravascular bacteria and mild suppurative inflammatory reaction in affected tissues. Based on external examination, bacteriology and histopathology findings, a final diagnosis of acute *E. rhusiopathiae* septicemia was made (Melero et al. 2011). *E. rhusiopathiae* infection can progress rapidly in immunocompromised individuals

Table 1. Median fluorescence intensity (MFI) of serum from 10 vaccinated bottlenose dolphins sampled at different times after annual revaccination. Dolphin 1 was vaccinated with ERYVAC (Intervet), and Dolphins 2–10 with ER BAC PLUS (Pfizer). For Dolphins 1 and 2, time after vaccination was counted after receiving the booster dose at 21 d. MFI values in **bold** are lower than the MFI value obtained from the free-ranging Risso's dolphin with a positive MFI value (shown in Table 2)

ID	Months after vaccination				
	1	2	6	12	13
1	9671.21	8373.75	8321.75	4356.50	
2	4968.67	2398.75	**113.75**	**36.00**	**−64.00**
3		6819.67	3078.00	**313.50**	
4	4710.17	**974.67**	**788.25**	**399.25**	
5	2471.50		3079.17	2454.75	
6	6827.17		5389.00	4746.25	
7		5277.50	1830.00	**780.00**	**387.75**
8		3982.67	1934.00	2867.00	
9	2979.50	3001.00	2339.50	**1457.33**	
10		**1273.50**	**1251.25**	**1508.00**	

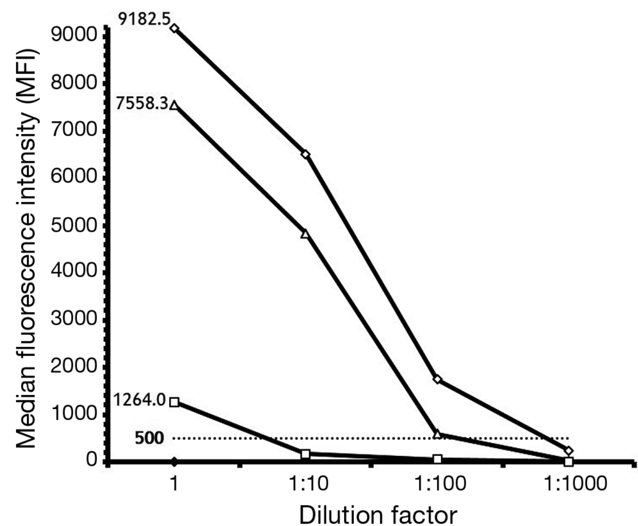

Fig. 1. Median fluorescence intensity (MFI) values of serial decimal dilutions from 3 serum samples taken from vaccinated bottlenose dolphins

Table 2. Median fluorescence intensity (MFI) of samples from 15 free-ranging cetaceans stranded alive in the Mediterranean Sea between 2006 and 2014. Free-ranging Risso's dolphin *Grampus griseus* with a positive MFI value shown in **bold** (see 'Results and Discussion' section). The *Tursiops truncatus* specimen died from acute *Erysipelothrix rhusiopathiae* septicemia

Individual ID	Tissue	MFI
Tursiops truncatus		
Tt14.07.2010	Serum	−110.75
	Pericardial fluid	−113.75
	Milk	−107.50
Grampus griseus		
Gg25.04.2006	Serum	−58.50
Gg11.12.2011	**Serum**	**1649.75**
Gg29.03.2013	Serum	−57.50
Stenella coeruleoalba		
Sc09.07.2007 neonate	Serum	−21.25
Sc09.07.2007 adult	Serum	−11.75
Sc15.07.2007	Serum	−13.25
Sc12.07.2007	Serum	−12.25
Sc18.07.2007	Serum	−14.25
Sc25.08.2007	Serum	−19.75
Sc25.03.2011	Serum	−118.50
Sc04.12.2012	Serum	−133.25
Sc13.02.2013	Serum	180.75
Sc12.05.2013	Serum	−49.00
Sc09.02.2014	Serum	−107.00

Fig. 2. Rhomboid-shaped skin lesions observed during an external examination of a bottlenose dolphin *Tursiops truncatus* stranded alive in the Mediterranean Sea

from other species (Sawada et al. 1987, Foster et al. 2012, Sinclair et al. 2013). In the present adult female dolphin, however, immunosuppression seems unlikely based on the marked neutrophilia and negative CeMV diagnosis. Therefore, we hypothesize that one explanation for the absence of anti-*E. rhusiopathiae* antibodies could be the possibility that the septicemia progressed too quickly to allow the animal to mount a specific antibody response.

From the 15 free ranging cetaceans evaluated for the presence of anti-*E. rhusiopathiae* antibodies, only 1 of the 3 Risso's dolphins was identified as seropositive according to Spa-based FMIA (MFI = 1649.75) (Table 2). This MFI value was markedly higher than the MFI values obtained for the other 2 free ranging Risso's dolphins evaluated in this study (Table 2). Although this animal belongs to a different species than the vaccinated cetaceans, the MFI obtained was similar to the values obtained for the vaccinated animals evaluated at 6 mo after immunization (when 30% of vaccinated animals showed lower MFI value than the Risso's dolphin) and at 12 mo after immunization (when 60% of vaccinated animals showed lower MFI value) (Table 1). Detailed comparison of the MFI value from the free-ranging Risso's dolphin with the mean MFI values from vaccinated dolphins at each time point after immunization shows that the value for the free-ranging animal fell between those for the vaccinated dolphins at 12 mo (mean MFI = 1891.86) and at 13 mo (mean MFI = 161.88) (Fig. 3).

The seropositive free-ranging Risso's dolphin that was stranded alive in December 2011 was an adult male. External examination of the stranded dolphin revealed poor body condition, a moderate amount of external parasites, and the presence of secretion and parasites in the pterygoid sinuses and the tympanic area, which may have made echolocation difficult. Thus, the ability of the animal to feed effectively may have been compromised, potentially leading to it becoming weak. Nevertheless, the final cause of stranding remains unclear. This animal tested negative for CeMV, and histopathological analysis revealed no microscopic lesions compatible with *E. rhusiopathiae* infection in any tissues examined. Bacteriology analysis revealed negative results for *E. rhusiopathiae*, *Salmonella* spp. and *Campylobacter* spp. in this animal, as well as in the other free-ranging cetaceans evaluated.

Stenotrophomonas maltophilia was isolated from the cardiac valves of the seropositive Risso's dolphin. *Streptococcus* sp. and *Staphylococcus* sp. were isolated from mouth swabs of many of the free-ranging cetaceans, and bacterial species *Clostridium perfringens*, *E. coli*, and *Enterobacter aerogenes* were frequently isolated from intestinal samples.

The MFI value obtained from the wild Risso's dolphin (1649.75), when compared to the values obtained during several months post vaccination in 10 vaccinated bottlenose dolphins, may suggest that the Risso's dolphin was previously in contact with *E. rhusiopathiae*, though, based on the bacteriology and histopathology results, it seemed unlikely that the animal was undergoing the disease at the time of death.

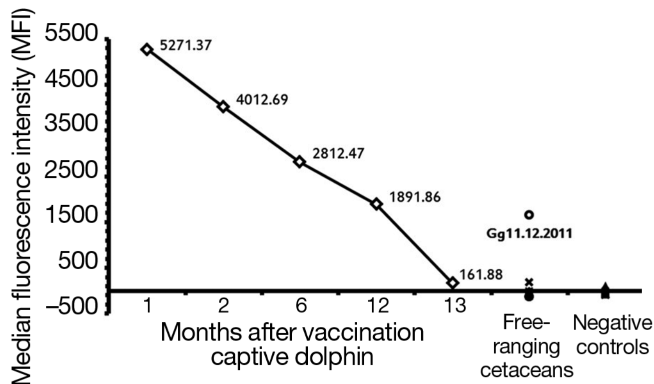

Fig. 3. Average of median fluorescence intensity (MFI) values of serum samples taken from vaccinated bottlenose dolphins at different times after annual revaccination (n = 10), free-ranging cetaceans stranded alive in the Mediterranean Sea between 2006 and 2014 (n = 15), and unvaccinated bottlenose dolphins (negative controls) (n = 20). MFI value of 1649.75 from 1 free-ranging Risso's dolphin is labeled with its individual identifier (Gg11.12.2011)

CONCLUSIONS

The present study validates a fluorescent microbead-based immunoassay, based on *E. rhusiopathiae* Spa protein, to detect antibodies against the pathogen in cetaceans. The assay was validated in serum samples from vaccinated bottlenose dolphins, and then used successfully to assay samples from 3 species of free-ranging cetaceans: striped dolphin, Risso's dolphin and bottlenose dolphin.

FMIA can be a useful tool for vaccine efficiency assessment. This technique may be particularly valuable for studying diseases in cetaceans not only because it can be applied to free-ranging animals, but also because it allows for multiplexing. Up to 100 analytes in the same sample can be simultaneously determined, reducing the time, costs and sample volume required. This makes FMIA well suited to studies of wild dolphins, where sample collection is difficult, specimens have a great value, and where rapid, reliable diagnosis is essential.

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