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Source: Journal of Wildlife Diseases, 31(3) : 410-415

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-31.3.410

Journal of Wildlife Diseases, 31(3), 1995, pp. 410–415

Postmortem Diagnosis of Morbillivirus Infection in Bottlenose Dolphins (*Tursiops truncatus*) in the Atlantic and Gulf of Mexico Epizootics by Polymerase Chain Reaction-Based Assay

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ABSTRACT: Lung tissue from 39 bottlenose dolphins (*Tursiops truncatus*) found dead off the U.S. Atlantic and Gulf of Mexico coasts from 1987 to 1994 was examined for the presence of morbillivirus using a reverse transcriptase polymerase chain reaction (RT-PCR) technique. Of the Atlantic cases examined, six of six were positive using this assay; 18 of 25 Gulf of Mexico cases with amplifiable RNA also were found to be positive, and eight additional specimens had no amplifiable RNA. The RT-PCR allowed the diagnosis of morbillivirus infection to be made from either sections of paraffin-embedded formalin-fixed material or from unfixed tissue. Confirmation of diagnosis was made by subsequent hybridization of the amplified products with a dolphin morbillivirus specific probe using the Southern blot technique. Application of this method to autolyzed post-mortem tissues allows diagnoses of morbillivirus infection to be made in specimens which cannot be evaluated by histologic and immunocytochemical techniques.

Key words: Atlantic bottlenose dolphin, *Tursiops truncatus*, morbillivirus, Atlantic Ocean, Gulf of Mexico, polymerase chain reaction, diagnostic techniques.

Within the last 6 yr, morbillivirus infections have been implicated in epizootics among several marine mammal populations in Europe, Asia, and North America, including populations of Lake Baikal seals (*Phoca sibirica*) (Grachev et al., 1989), European harbor seals (*P. vitulina*) (Osterhaus et al., 1988), and Mediterranean striped dolphins (*Stenella coeruleoalba*) (Domingo et al., 1990). Morbillivirus infection recently has been documented in the Atlantic bottlenose dolphin (*Tursiops truncatus*) epizootic of 1987 and 1988 (Lipscomb et al., 1994a), and in a single bottlenose dolphin found dead off the Florida coast of the Gulf of Mexico (Lipscomb et al., 1994b).

Diagnosis of morbillivirus infection in pinniped and cetacean species has been made by histopathologic and immunocytochemical methods (Lipscomb et al., 1994a), antigen capture enzyme linked immunoabsorbent assay (ELISA) (Van Bressem et al., 1991), and polymerase chain reaction (PCR) of viral isolates from infected Vero cells (Barrett et al., 1993). The PCR technique allowed subsequent sequencing of the amplified region to be performed, enabling construction of probable phylogenetic trees with these recently isolated members of the morbillivirus family. Using primers derived from conserved portions of the morbillivirus phosphoprotein (P) gene (Cattaneo et al., 1989), Barrett et al. (1993) derived species-specific sequence data for the dolphin morbillivirus (DMV) isolated from the Mediterranean striped dolphin, *S. coeruleoalba*.

Diagnosis of morbillivirus infection in postmortem specimens can be hampered by postmortem autolysis of the tissue. Detection of morbillivirus antigen using immunocytochemistry on formalin-fixed paraffin-embedded tissue was reported in 42 (53%) of 79 cases from the 1987 to 1988 U.S. Atlantic coast dolphin epizootic (Lipscomb et al., 1994a). Forty (95%) of these 42 cases had histologic evidence of morbilliviral infection including syncytia and inclusion bodies. However, one third of the

specimens with histologic features of viral infection were negative with the immunocytochemical technique. As an aid to diagnosis, a PCR-based assay was developed.

Morbilliviruses are negative stranded ribonucleic acid (RNA) viruses which lack a deoxyribonucleic acid (DNA) intermediate (Fields and Knips, 1991). Thus, morbillivirus PCR first requires RNA reverse transcription with subsequent amplification of the first strand complementary DNA (cDNA) produced. The reverse transcriptase-PCR (RT-PCR) assay developed utilized morbillivirus consensus primers derived from highly conserved sequences of the morbillivirus phosphoprotein (P) gene (Barrett et al., 1993). The primer used for reverse transcription (primer 1) is the same as the primer used by Barrett et al. (1993). The second PCR primer was designed based on the published phosphoprotein gene sequence (Barrett et al., 1993). This primer set allowed RT-PCR amplification of dolphin morbillivirus and human measles virus, yielding a 78 base pair product. An internal oligonucleotide probe also was designed and used for subsequent confirmation of DMV-specific amplification by Southern blotting. Barrett's amplification strategy was designed to amplify a 429 nucleotide template from high quality RNA isolated from DMV-infected Vero cells (Barrett et al., 1993). Our protocol was designed to be used as a diagnostic test on samples containing degraded RNA from either formalin-fixed paraffin-embedded tissue or from unfixed autolyzed tissue samples.

We devised a general method for enrichment of RNA from formalin-fixed paraffin-embedded tissue for RT-PCR. This technique also has been applied to unfixed tissue. The RNA available for RT-PCR in such samples is degraded and primers must be designed to amplify templates no greater than approximately 150 nucleotides. This procedure is similar to a published procedure (Fincke et al., 1993), but the current protocol has been optimized for use in our laboratory. We have compared this procedure to guanidinium-based extractions (Sambrook et al., 1989, pp. 7.18–7.25), and find it superior (J. K. Taubenberger, unpubl.). In our method, RNA-containing extracts from formalin-fixed paraffin-embedded tissue were prepared by first deparaffinizing four to eight 6 μm sections in 800 μl Hemo-De (Fisher Scientific, Pittsburgh, Pennsylvania, USA), a deparaffinization agent. After mixing on a vortexer at full speed for 5 sec, 400 μl of 100% ethanol was added, and the preparation was mixed on a vortexer for an additional 5 sec. The samples then were centrifuged at 10,000 $\times$ *G* in a microcentrifuge for 5 min. The liquid was carefully decanted, the pellet washed with 800 μl 100% ethanol, and centrifuged for 5 min. The pellet was dried for 5 min at 80 C. Care was taken not to overly dry the pellet. The extraction buffer was composed of 20 mM Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl), pH 7.6, 20 mM ethylenediaminetetraacetic acid, disodium salt (EDTA), 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, Missouri, USA). The addition of RNase inhibitors at this stage other than SDS and proteinase K was not necessary. The extraction buffer was added, 600 μl/pellet, and the sample was incubated in a 55 C water bath for 4 hr. After incubation, two phenol/chloroform extractions were performed (Sambrook et al., 1989, pp. E.3–E.4). The aqueous phase from the final extraction was placed in a fresh tube, and nucleic acids were precipitated with 60 μl 3 M sodium acetate, pH 5.5, 20 μg glycogen, and 0.6 volumes isopropanol. The solutions were mixed well, and placed on ice for a minimum of 5 min. The precipitate was collected by centrifugation for 5 min, and the pellet washed twice with 80% ethanol. The pellets were rehydrated with 10 to 20 μl of diethylpyrocarbonate (DEPC)-treated water (Sambrook et al., 1989, pp. 7.3–7.4). Total nucleic acid yield was calculated by absorbance at 260 nm with a GeneQuant

spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, England). For each RT-PCR assay, 0.5 μg nucleic acid was used. Extracts were stored at −70 C.

Reverse transcription was performed with a specific primer (primer 1) from a conserved region of the morbillivirus phosphoprotein (P) gene (Barrett et al., 1993). Reaction conditions were as follows: each 20 μl reaction contained 0.5 μg nucleic acid, 50 U M-MLV reverse transcriptase (Life Technologies, Gaithersburg, Maryland, USA), 14 pmol primer 1 (5′-ATTGGGTTGCACCACTTGTC-3′), 500 μM deoxynucleotide triphosphates (dNTP), 6.5 mM $MgCl_2$, 2 μl 10-fold concentrated PCR buffer (Perkin Elmer, Norwalk, Connecticut, USA). Tubes were incubated at 37 C for 1 hr, and were then transferred to a thermal cycler (Gene Amp 9600, Perkin Elmer). Polymerase chain reaction on the first strand cDNA was performed in a 50 μl total reaction volume. A PCR reaction mixture, containing 14 pmol primer 2 (5′-ATTAAAAAGGG(G/C)ACAGGAGAGAGATCAGCC-3′), 1.5 U Taq polymerase (Perkin Elmer), and 3 μl 10-fold concentrated PCR buffer was added to each tube. Amplification was carried out in the thermal cycler using the following conditions: 94 C for 5 min; 40 cycles of 94 C/1 min, 55 C/1 min, 72 C/1 min; followed by a final 7 min 72 C extension. The products were analyzed by gel electrophoresis with 2.5% agarose, and ethidium bromide staining (Sambrook et al., 1989). Southern blot tests were performed (Sambrook et al., 1989), using a DMV-specific oligonucleotide probe (5′-CGGAGACCGAGTCTTCATT-3′) which hybridized to a sequence contained within the amplified 78 base pair product.

As a control for amplifiable RNA, RT-PCR for beta-actin was performed in each case. The control primers and internal oligonucleotide probe were designed from the nucleotide sequences of the human cytoplasmic beta-actin gene, spanning intron C, and were derived from regions of the gene which are highly conserved in human, rat, and chicken (Nakajima-Iijima et al., 1985). The 5′-primer spans nucleotides 1,485–1,504 (in exon 3), the internal probe spans nucleotides 1,561–1,583 (in exon 3), and the 3′-primer spans nucleotides 2,062–2,081 (in exon 4) (Nakajima-Iijima et al., 1985). Thus, the spliced RNA product can be distinguished from a contaminating DNA template. The RT-PCR control product was 156 base pairs (within the amplifiable range from degraded RNA samples), whereas the DNA template would yield a 597-base-pair product. This gene was used since no genomic sequence data have been published for any dolphin species, only intronless mitochondrial cytochrome genes (Irwin et al., 1991). However, the highly conserved nature of the beta-actin gene in mammals and chickens permitted a control RT-PCR assay to be developed which worked with the dolphin specimens.

For the beta-actin control amplification, reverse transcription and PCR reactions were performed as described above with the following modifications: 15 pmol 3′ primer (5′-ACAGCCTGGATAGCAACGTA-3′) and 7 mM $MgCl_2$ were added to the reverse transcriptase reaction. The PCR reaction contained 15 pmol of the 5′ primer (5′-CCACACCTTCTACAATGAGC-3′). The thermal cycle profile was: 94 C for 5 min; 40 cycles of 94 C/1 min, 50 C/2 min, 72 C/1 min; followed by a final 7 min 72 C extension. The products were analyzed by gel electrophoresis with 2.5% agarose, and ethidium bromide staining. Southern blot tests were performed using a beta-actin specific oligonucleotide probe (5′-AAGGCCAACCGCGAGAAGATGAC-3′) which hybridized to a sequence contained within the amplified 156-base-pair product.

Formalin-fixed paraffin-embedded sections of lung from six dolphins found dead during the Atlantic coast epizootic were analyzed by histologic and immunocytochemical methods for evidence of morbillivirus infection as reported by Lipscomb et al. (1994a). These cases repre-

sented a fraction of the cases recovered from August of 1987 through April of 1988 along the Atlantic coast of the United States between 39°50′N, 74°20′W and 28°24′N, 80°37′W. Of these, all were positive by histologic criteria and four of six were positive using the immunocytochemical method. In addition to these six cases, six negative control specimens were analyzed in a blind fashion with the RT-PCR technique. The negative cases, representing specimens unrelated to the Atlantic epizootic, included sections of lung from five bottlenose dolphins and one beluga whale (*Delphinapterus leucas*). All of these cases were analyzed histologically and three of the cases were analyzed for immunocytochemical evidence of morbillivirus infection. None were positive. After the RT-PCR assay was performed on this group of cases, the code was broken and the results compared with the case histories. Reverse transcriptase-polymerase chain reaction amplification of beta-actin, the control gene, was positive in all cases. The morbillivirus assay was positive for all dolphin specimens from the Atlantic epizootic, and negative in all negative control specimens.

Specimens from the recent bottlenose dolphin epizootic of morbillivirus infection in the Gulf of Mexico also were analyzed using this technique. One dolphin was recovered near Panama City, Florida (30°7′N, 85°44′W) on 5 June 1993 (Lipscomb et al., 1994b). Two dolphins were recovered off the Gulf coast, one along Alabama (30°24′N, 88°15′W) on 7 November 1993 and another along Mississippi (30°14′N, 88°40′W) on 19 November 1993. Available tissue from these three specimens was fixed in formalin fixed and embedded in paraffin. Frozen unfixed tissue samples from 30 stranded dolphins were recovered from 27 March to 30 April 1994 along the Texas (USA) coast between 29°40′N, 94°03′W and 28°47′N, 95°36′W. We prepared RNA-containing extracts from approximately 1.5 mg of unfixed lung tissue homogenized in a sterile Dounce homogenizer (Fisher Scientific, Pittsburgh, Pennsylvania, USA) followed by the RNA extraction procedure used for formalin-fixed paraffin-embedded tissue sections. Histologic and immunocytochemical analyses as described by Lipscomb et al. (1994a) were performed on formalin-fixed and paraffin-embedded tissue from the first three dolphins. All three had evidence of morbilliviral infection using both criteria. They also were positive for morbillivirus using the RT-PCR technique. The specimens from the Texas coast were highly autolyzed, making histologic and immunocytochemical evaluation impossible. Of the 33 Gulf specimens analyzed for morbillivirus by RT-PCR, 25 contained amplifiable RNA and 18 (72%) of these were positive for morbillivirus. In eight cases, the RNA was badly degraded, precluding RNA amplification. Thus, 18 (55%) of 33 total cases were positive in this assay. Representative results of the assay are shown in Figure 1 with extracts from six of the 33 Gulf of Mexico cases amplified for beta-actin and morbillivirus. After gel electrophoresis and blotting, Southern hybridization with oligonucleotide probes internal to the primer sets in each case was performed. Cases 1 and 3 were negative for the control beta-actin amplification and could not be further evaluated for morbillivirus RNA (Fig. 1). Of the remaining ones, cases 5 and 6 were positive for morbillivirus.

Morbillivirus infections in cetacean and pinniped species have now been documented in North America, Asia, and Europe. We here report a readily performed diagnostic assay for morbillivirus infection suitable for use in postmortem diagnosis. Since tissue autolysis may limit histologic or immunocytochemical analysis of such specimens, this assay may provide a useful adjunct to other means of diagnosis. The primer set chosen could be used for diagnosis of other members of the morbillivirus family, and has been used to amplify human measles virus in addition to dolphin morbillivirus specimens (J. K.

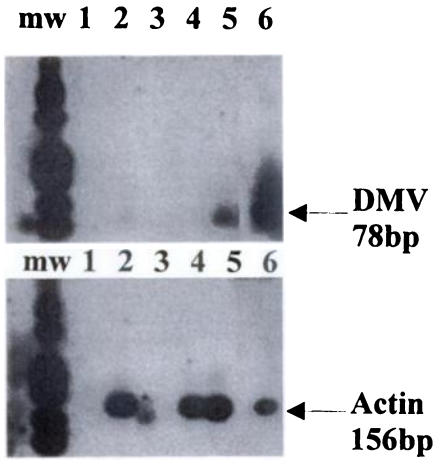

FIGURE 1. Representative cases amplified by reverse transcriptase polymerase chain reaction (RT-PCR) for the dolphin morbillivirus phosphoprotein gene and actin control gene are shown in relation to molecular weight (mw) markers. Lanes 1 to 6 represent six different dolphin specimens. The upper autoradiogram represents a Southern blot analysis of the samples amplified for the dolphin morbillivirus phosphoprotein gene and hybridized with a dolphin morbillivirus-specific probe (DMV). Specific amplification resulted in a 78 base pair (bp) product. Cases 5 and 6 were positive. The lower autoradiogram contains the same six cases as above but amplified and hybridized with the actin primers and probe. Specific amplification resulted in a 156 base pair product. Cases 2, 4, 5, and 6 were positive. Cases 5 and 6 were positive for morbillivirus, cases 2 and 4 were morbillivirus negative, and cases 1 and 3 could not be evaluated for morbillivirus.

Taubenberger, unpubl.). The amplified products can be sequenced, allowing identification of specific morbilliviruses, and allowing evaluation of strain variation or genetic drift between isolates to be performed.

The authors thank the National Marine Fisheries Service and the Marine Mammal Stranding Networks for provision of the specimens. This study was supported in part by the American Registry of Pathology, Washington, D.C., 20306-6000. T. P. Lipscomb is a lieutenant colonel in the U.S. Army. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official views of the Department of the Army or of the Department of Defense.

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Received for publication 28 July 1994.