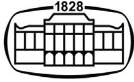

AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

69 (2021) 2, 204–210

DOI: 10.1556/004.2021.00028

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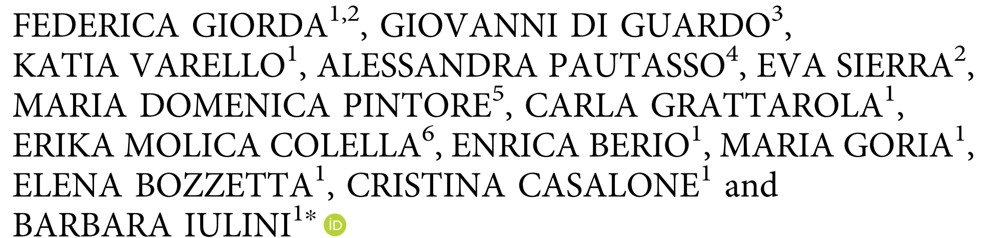
SHORT COMMUNICATION

# Retrospective immunohistochemical investigation on dolphin morbillivirus infection by comparing the performance of heterologous monoclonal and polyclonal antibodies – Short communication



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Received: 1 November 2020 • Accepted: 27 May 2021
Published online: 23 July 2021

## ABSTRACT

Dolphin morbillivirus (DMV) is a pathogen of great concern in free-ranging cetaceans. Confirmation and staging of morbillivirus infections rely on histology and immunohistochemistry (IHC), following molecular detection. As at the present time no specific antibodies (Abs) against DMV are available, two heterologous Abs have been used worldwide for the examinations of morbillivirus infections of cetaceans. One is a monoclonal Ab (MoAb) prepared against the N protein of canine distemper virus (CDV), whereas the other is a polyclonal Ab raised in rabbits against rinderpest virus (RPV). Both Abs are known to show cross-reactivity with DMV. In this study we compared the labelling quality and the neuroanatomical distribution of staining with these two Abs by means of IHC analysis. To this end, serial sections of the target organs from ten free-ranging stranded cetaceans, previously diagnosed as being infected with DMV by PCR and/or serology, were subjected to IHC. The brain, lungs and lymph nodes of one animal were found to be positive with both Abs. From two other animals, the brain and the spleen, respectively, tested positive only with the polyclonal Ab. In the positive brain tissues, multifocal immunostaining was observed, with similar staining location and extent, with the two antibodies tested. Our results suggest that the polyclonal anti-RPV Ab might have a stronger binding activity to DMV than the anti-CDV MoAb. Nevertheless, the elaboration and use of specific anti-DMV Abs might be essential to guarantee conclusive results in diagnostic and pathogenetic investigations.

## KEYWORDS

cetaceans, dolphin morbillivirus, immunohistochemistry, monoclonal antibody, polyclonal antibody

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Dolphin morbillivirus (DMV), officially classified into the virus species *Cetacean morbillivirus* within the genus *Morbillivirus*, causes infection and disease in different cetacean animals. The pathogen has been found to be responsible for several epizootic and enzootic outbreaks worldwide. Over the last 30 years, the virus has caused two epizootics and three unusual mortality events in the Mediterranean basin, the most recent along the Italian coastline in 2016 (Pautasso et al., 2019).

The gold standard for the diagnosis remains virus isolation, which can be challenging when dealing with stranded animals. Indeed, very few reports on successful virus isolation are available (Peletto et al., 2018). Reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing the PCR product is a specific and sensitive diagnostic method; however, histology and immunohistochemistry (IHC) should be performed to confirm morbillivirus infection (MI) and to obtain information on the stage of the disease. Negative antigen immunolabelling is commonly obtained in subacute-to-chronic or subclinical MI (Van Bressem et al., 2014).

Since no homologous antibody (Ab) against DMV is available, two Abs prepared against other members of the genus *Morbillivirus* have been used worldwide in the IHC diagnosis. One of them is a monoclonal antibody (MoAb) against the N protein of canine distemper virus (CDV). The other is a polyclonal Ab raised in rabbits against rinderpest virus (RPV) (Van Bressem et al., 2014). In the present study, we performed IHC analysis of cetacean tissue samples from animals that had been found DMV positive by other techniques, such as PCR and/or serology. We compared the performance of the two Abs for positivity detection, labelling quality, and the neuroanatomical distribution of the staining.

The study was performed in 2016 (the serum against RPV is no longer available due to the recent OIE procedures for RPV eradication) on 122 formalin-fixed target tissue samples (brain, lung, spleen, lymph nodes) from seven striped dolphins (*Stenella coeruleoalba*), two bottlenose dolphins (*Tursiops truncatus*), and a pilot whale (*Globicephala melas*). The samples were selected on the basis of the presence of suggestive microscopic lesions and/or molecular (Verna et al., 2017) and/or serological (Di Guardo et al., 2010) evidence of MI. In addition, a panel of 10 tissues sampled from two striped dolphins (ID 11 and 12), all found negative with PCR or serology and without histological lesions suggestive of MI, served as negative control (Table 1). All the animals included in this study were free-ranging cetaceans stranded along the Italian coast of the Pelagos Sanctuary (between 43°47′4″N, 7°31′49″E and 43°45′24″N, 10°16′25″E) in the period 2008–2015 and submitted to complete postmortem examination at the C.Re.Di.Ma. (Italian National Reference Centre for Diagnostic Activities in Stranded Marine Mammals) following standard procedures (Geraci and Lounsbury, 2005). The results obtained on a few specimens included in this study have also been published previously (Giorda et al., 2017).

In detail, lung, spleen, lymph nodes (prescapular, tracheobronchial, and mesenteric, according to tissue availability) and nine anatomic areas from the brain (basal nuclei, thalamus, mesencephalon, pons, obex, frontal, parietal, occipital and cerebellar cortex) were sampled and examined. Serial formalin-fixed, paraffin-embedded sections were processed by IHC alternately with an anti-CDV-NP MoAb (provided by VMRD, Pullman, WA, USA) (Stanton et al., 2004) and an anti-RPV polyclonal Ab (provided by the Pirbright Institute, Pirbright, UK) (Yang et al., 2006). Two biotinylated antibodies (Vectastain ABC method, provided by of Vector Laboratories, Burlingame, CA, USA) were used as secondary antibodies, namely a horse anti-mouse IgG for the MoAb and a goat anti-rabbit IgG for the polyclonal Ab. Negative internal controls were employed in which the primary antibody was replaced with a non-immune homologous serum.

Two pathologists independently reviewed all the slides and identified each tissue as negative or positive by the MoAb and/or polyclonal Ab staining. Tissue samples that tested positive by only one of the two Abs underwent additional evaluation by a board-certified pathologist. The results reported here are thus to be intended as an agreement reached by two or three pathologists. Moreover, the type(s) of cells that showed positive staining in each organ was/were recorded. Kappa statistic was calculated using the Epitools epidemiological calculator to test agreement between the two IHC protocols (Sergeant, 2018): if at least one organ was positive, the animal was classified as positive for DMV by IHC with the MoAb and/or the polyclonal Ab.

Only three out of the ten cetaceans showed positive IHC staining. These included ID1 (striped dolphin), ID 6 (bottlenose dolphin), and ID 8 (pilot whale). In detail, the brain, the lung and the tracheobronchial lymph node from ID1 tested positive with both Abs, whereas the brain of ID6 and the spleen of ID8 tested positive with the anti-RPV polyclonal Ab. Among the tissues testing positive with both Abs, labelling of morbillivirus antigen was demonstrated in the same types of cells, including astrocytes, neurons (soma and processes), meningeal and endothelial cells and mononuclear cells in the perivascular cuffs and the meninges in the brain; type I and II pneumocytes and multinucleated giant cells/syncytia (MGCS) in the lung; follicular lymphocytes and MGCS in the lymph nodes, and macrophages in the spleen. The brain of ID6 showed focal immunoreactivity in the inflamed meninges of the parietal cortex, with limited staining of mononuclear and meningeal cells (Fig. 1a), while scattered labelling of macrophages was evident in the spleen of ID8 (Fig. 1b). The lung of ID3 was not evaluable due to postmortem autolysis.

There was a similar pattern of immunoreactivity for both Abs characterised by diffuse or fine granular dark cytoplasmic and nuclear immunostaining. Generally, the MoAb showed a stronger staining intensity, free of non-specific signal. Differently, the polyclonal Ab showed strong, diffuse background labelling. In addition, the bradyzoites, belonging to multiple *Toxoplasma gondii* cysts, stained positive with this Ab in ID1 that presented a co-infection with this protozoan (data not shown). Such non-specific labelling was not observed in any section of the internal controls.

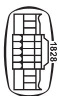


*Table 1.* Data of the stranded cetaceans involved in this study, along with the results of the histopathological, molecular, serological and immunohistochemical (IHC) analyses. IHC was performed by using an anti-canine distemper virus (CDV) monoclonal antibody (MoAb) or an anti-rinderpest virus (RPV) polyclonal antibody on selected tissue samples as shown

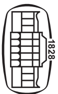
| ID animal – YS | Species | Age | NuS | DC | Tissues | Microscopic lesions suggestive of MI | RT-PCR | Anti-CDV Abs in serum | IHC results | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | anti-CDV MoAb | anti-RPV polyclonal Ab |
| 1–2008 | Striped dolphin | Adult | Poor | 2 | Brain | NS meningoencephalitis | + | - | + | + |
| | | | | | Lung | Bronchointerstitial pneumonia | + | | + | + |
| | | | | | Spleen | Lymphoid depletion | n. p. | | – | – |
| | | | | | Tracheobronchial LN | Lymphoid depletion | n. p | | + | + |
| 2–2011 | Striped dolphin | Adult | Poor | 4 | Brain | NS meningoencephalitis | – | 1:16 | – | – |
| | | | | | Lung | – | – | | NVA | NVA |
| | | | | | Spleen | Germinal centre hyperplasia | – | | – | – |
| | | | | | Prescapular LN | Lymphoid depletion | n. p. | | – | – |
| 3–2012 | Striped dolphin | Adult | Good | 4 | Brain | NS meningoencephalitis | + | – | – | – |
| | | | | | Lung | – | – | | – | – |
| | | | | | Spleen | – | + | | – | – |
| 4–2012 | Striped dolphin | Adult | Good | 2 | Brain | NS meningoencephalitis | – | 1:8 | – | – |
| | | | | | Lung | – | – | | – | – |
| | | | | | Spleen | – | n. p. | | – | – |
| | | | | | Prescapular LN | – | – | | – | – |
| | | | | | Tracheobronchial LN | – | – | | – | – |
| 5–2012 | Striped dolphin | Adult | Good | 3 | Brain | NS meningoencephalitis | – | 1:32 | – | – |
| | | | | | Lung | Bronchointerstitial pneumonia | – | | – | – |
| | | | | | Spleen | – | – | | – | – |
| | | | | | Prescapular LN | – | – | | – | – |
| | | | | | Tracheobronchial LN | – | – | | – | – |
| 6–2012 | Bottlenose dolphin | Juvenile – subadult | Good | 3 | Brain | NS meningoencephalitis | – | 1:32 | – | + |
| | | | | | Spleen | – | – | | – | – |
| | | | | | Tracheobronchial LN | – | – | | – | – |
| 7–2013 | Bottlenose dolphin | Juvenile – subadul | Good | 3 | Brain | – | – | 1:8 | – | – |
| | | | | | Lung | – | – | | – | – |
| | | | | | Spleen | Germinal centre hyperplasia | – | | – | – |
| | | | | | Tracheobronchial LN | Germinal centre hyperplasia | – | | – | – |
| | | | | | Mesenteric LN | Germinal centre hyperplasia | – | | – | – |

*(continued)*

**Table 1.** Continued

| ID animal – YS | Species | Age | NuS | DC | Tissues | Microscopic lesions suggestive of MI | RT-PCR | Anti-CDV Abs in serum | IHC results | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | anti-CDV MoAb | anti-RPV polyclonal Ab |
| 8–2013 | Pilot whale | Juvenile – subadult | Good | 3 | Brain | Mild and focal NS encephalitis | - | 1:8 | - | - |
| | | | | | Lung | – | - | | - | - |
| | | | | | Spleen | Germinal centre hyperplasia and hyalinosis | n. p. | | – | + |
| | | | | | Prescapular LN | Hyperplastic lymphadenitis | n. p. | | – | – |
| | | | | | Tracheobronchial LN | - | n. p. | | – | – |
| | | | | | Mesenteric LN | - | n. p. | | – | – |
| 9–2015 | Striped dolphin | Adult | Good | 2 | Brain | NS encephalitis | + | 1:32 | – | – |
| | | | | | Lung | Bronchointerstitial pneumonia | – | | – | – |
| | | | | | Spleen | Lymphoid depletion | – | | – | – |
| | | | | | Prescapular LN | – | + | | – | – |
| 10 –2015 | Striped dolphin | Adult | Poor | 3 | Brain | NS meningoencephalitis | – | – | – | – |
| | | | | | Lung | Interstitial pneumonia | + | | – | – |
| | | | | | Spleen | Lymphoid depletion | – | | – | – |
| 11–2008 NC | Striped dolphin | Adult | Poor | 3 | Brain | – | – | n. p. | – | – |
| | | | | | Lung | NVA | – | | – | – |
| | | | | | Spleen | – | – | | – | – |
| | | | | | Prescapular LN | – | – | | – | – |
| | | | | | Tracheobronchial LN | – | – | | – | – |
| 12–2008 NC | Striped dolphin | Juvenile – subadult | Good | 2 | Brain | – | – | n. p. | – | – |
| | | | | | Lung | – | – | | – | – |
| | | | | | Spleen | – | – | | – | – |
| | | | | | Prescapular LN | – | – | | – | – |
| | | | | | Tracheobronchial LN | – | – | | – | – |

*Legend:* YS: year of stranding; NuS: nutritional status; DC: decomposition code (2: fresh; 3: moderate autolysis; 4: decomposed); Abs: antibodies; MI: morbillivirus infection; −: Negative sample; +: Positive sample; n. p.: not performed; LN: lymph node; NS: non-suppurative; NVA: not evaluable due to autolysis; NC: negative control



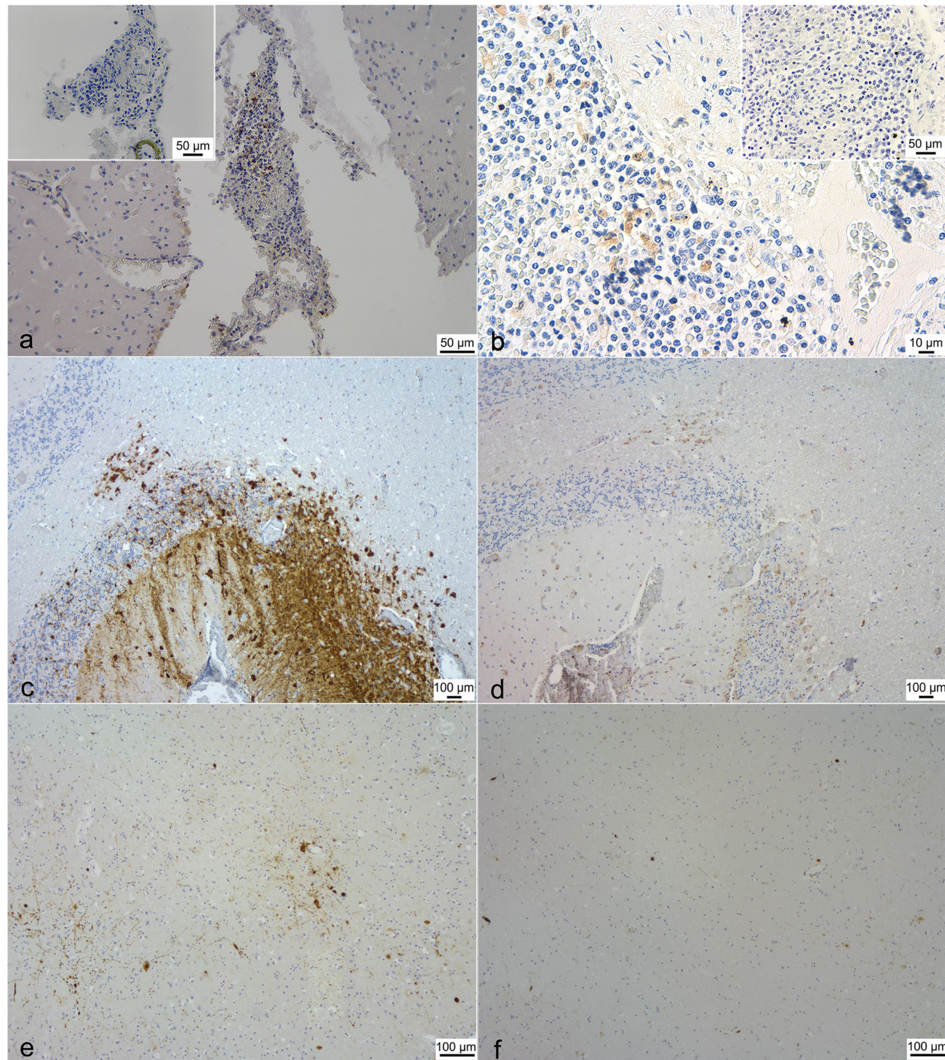



*Fig. 1. a)* Meninges of the parietal cortex (ID6). Positive labelling in mononuclear and meningeal cells. Anti-RPV polyclonal Ab. Upper left inset: meninges of the parietal cortex (ID6). Absence of staining (minimal unspecific signal in a blood vessel). Anti- CDV MoAb. *b)* Spleen (ID8). Positive labelling in macrophages. Anti-RPV polyclonal Ab. Upper right inset: Spleen (ID8). Absence of staining. Anti-CDV MoAb. *c)* Cerebellar cortex (ID1). Intense and extensive positive labelling of Purkinje cells, molecular and granular layers, and endothelial cells. Anti-CDV MoAb. *d)* Cerebellar cortex (ID1). Positive staining of molecular and granular layers and scattered endothelial and Purkinje cells. Anti-RPV polyclonal Ab. *e)* Occipital cortex (ID1). Intense immunoreaction of neurons (soma and processes) and glia cells. Anti-CDV MoAb. *f)* Positive labelling of scattered neurons (soma and processes), glia and endothelial cells. Anti-RPV polyclonal Ab

The neuroanatomical localisation of the staining by the two Abs could be compared only in ID1, since the brain of this animal was the only tissue from the nervous system that tested positive with both Abs. Histologically, there was a mild, multifocal non-suppurative sub-acute meningoencephalitis with minimal demyelination, without MGCS or inclusion bodies. Multifocal immunostaining was present with both Abs in all the neuroanatomical areas examined, including both the grey and the white matter, and generally involving scattered cells. The areas most affected were the mesencephalon and the cerebral and cerebellar cortex. The extent and location of the regions of immunostaining were similar, except for the cerebellum and the occipital cortex, in which positivity with the MoAb was more intense and widespread compared to the polyclonal Ab (Figs. 1c, 1d, 1e and 1f).

The agreement between the two IHC techniques was moderate (Cohen's kappa = 0.4118, SE for non-zero kappa = 0.2557). Of particular interest is that fewer tissues tested positive with the MoAb compared to the polyclonal Ab (4 vs. 6). This difference may have stemmed from the relatively small amount of antigen detected within the sections that stained positive only with the polyclonal Ab (brain of ID6 and spleen of ID8), as reported in a similar study of West Nile infection (Smedley et al., 2007). Furthermore, since the MoAb is directed against a single epitope of the N protein, its binding with the antigen may be influenced by the formalin fixation, the heat retrieval and the autolysis.

These conditions might exert less effect on the results obtained with the polyclonal Ab, which targets different epitopes of multiple structural proteins of the virus. Finally, Amude et al. (2010) has found that the chronicity of infections in CDV brain lesions may lead to antigen (N protein) clearance from the lesions and, as a consequence, to negative staining. Such aspects may explain the lower sensitivity of the MoAb compared to the polyclonal Ab in our study. We have no plausible explication for the more marked staining obtained with the MoAb in the cerebellum and the occipital cortex; additional positive tissues would be necessary to analyse this feature. Unlike Amude et al. (2010), we observed no non-specific intra-neuronal immunoreaction with the MoAb, but a minimal unspecific signal was present in the blood vessels (Fig. 1a, inset).

Interestingly, the MI of the two animals that tested positive by the polyclonal Ab only, was not confirmed by molecular methods previously. The RT-PCR test of the young bottlenose dolphin (ID6) was negative, whereas in the pilot whale (ID) the PCR was not performed. A similar situation has been reported before (Díaz-Delgado et al., 2017). In the animals examined by us, the diagnosis of MI was based on DMV-related microscopic lesions associated with the presence of specific serum antibodies (Di Guardo et al., 2010).

Our results suggest that a polyclonal Ab is more efficient than a MoAb in detecting DMV by IHC, though the staining quality is overall enhanced with the MoAb. Within this framework, future studies should be aimed at evaluating the cross-reactivity of the MoAb and polyclonal antibodies, raised against additional members of the genus *Morbillivirus*, with DMV-specific epitopes. Finally, because IHC should always be associated with RT-PCR and because this technique is essential for staging DMV infection (Van Bressem et al., 2014; Díaz-Delgado et al., 2019), considering that the use of both antibodies is not able to ensure conclusive results, the next step would be to produce a MoAb specific for DMV proteins, which would be fundamental for increasing the accuracy of diagnostic and pathogenetic investigations.

## ACKNOWLEDGEMENTS

This research was funded by the Italian Ministry of Health (Ricerca Corrente 2014 IZS PLV 08/14 RC). The authors are grateful to C. E. Di Francesco for her support in serological analysis, to R. Desiato and F. Ingravalle for their help in statistical analysis and to M. Giorda for his assistance with the microscopic images.

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