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Novel dermatitis and relative viral nucleic acid tissue loads in a fin whale (*Balaenoptera physalus*) with systemic cetacean morbillivirus infection

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Abstract:	<p>Summary</p> <p>Cetacean morbilliviruses (CeMV) are significant causes of mortality affecting many cetacean species in epizootics and smaller outbreaks. Despite skin lesions being prominent in seals and terrestrial animals, including humans, affected by other morbilliviruses, they have not been reported in CeMV-infected cetaceans. Here we report CeMV-associated skin lesions in a fin whale (<i>Balaenoptera physalus</i>) with sub-acute, systemic CeMV infection that live-stranded in Scotland, UK. Grossly, the skin was sloughing in large sheets, presumed due to autolysis but histological examination showed syncytia formation below the dermo-epidermal junction that were strongly positive for morbillivirus antigen by immunohistochemistry, as were syncytia in other organs. By PCR, the relative load of CeMV-specific RNA was largest in the liver and urinary bladder, even in formalin-fixed, paraffin-wax embedded samples. Levels were low in skin and only detectable in frozen samples. Genetic comparison of the CeMV showed close alignment with isolates from fin whales in the North Atlantic Ocean and the Mediterranean Sea but distinct from the porpoise CeMV clade. These findings show skin samples can be used to diagnose CeMV infection in cetaceans highlighting the potential of ante-mortem sampling to monitor disease in current populations and assessment of changes in host and pathogen genetics.</p>

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1 **Novel dermatitis and relative viral nucleic acid tissue loads in a fin whale**
2 **(*Balaenoptera physalus*) with systemic cetacean morbillivirus infection**

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17 Key words: cetacean morbillivirus, dermatitis, fin whale, viral load.

18

19 **Summary**

20 Cetacean morbilliviruses (CeMV) are significant causes of mortality affecting many cetacean
21 species in epizootics and smaller outbreaks. Despite skin lesions being prominent in seals and
22 terrestrial animals, including humans, affected by other morbilliviruses, they have not been
23 reported in CeMV-infected cetaceans. Here we report CeMV-associated skin lesions in a fin
24 whale (*Balaenoptera physalus*) with sub-acute, systemic CeMV infection that live-stranded
25 in Scotland, UK. Grossly, the skin was sloughing in large sheets, presumed due to autolysis
26 but histological examination showed syncytia formation below the dermo-epidermal junction
27 that were strongly positive for morbillivirus antigen by immunohistochemistry, as were
28 syncytia in other organs. By PCR, the relative load of CeMV-specific RNA was largest in the
29 liver and urinary bladder, even in formalin-fixed, paraffin-wax embedded samples. Levels
30 were low in skin and only detectable in frozen samples. Genetic comparison of the CeMV
31 showed close alignment with isolates from fin whales in the North Atlantic Ocean and the
32 Mediterranean Sea but distinct from the porpoise CeMV clade. These findings show skin
33 samples can be used to diagnose CeMV infection in cetaceans highlighting the potential of
34 ante-mortem sampling to monitor disease in current populations and assessment of changes in
35 host and pathogen genetics.

36

37 **Introduction**

38 Cetacean morbillivirus (CeMV) is a distinct species made up of three well characterised
39 strains named after the species in which they were initially found (porpoise morbillivirus,
40 dolphin morbillivirus [DMV] and pilot whale morbillivirus) plus one recovered later from a
41 Longman's beaked whale (*Indopacetus pacificus*), collectively called CeMV-1. Two more
42 strains, recovered more recently from a Guiana dolphin (*Sotalia guianensis*) and two Indo
43 Pacific bottlenose dolphins (*Tursiops aduncus*), are collectively called CeMV-2 (Van

44 Bressems *et al.*, 2014). It is an unsegmented, linear, negative-sense, single-stranded, RNA
45 virus belonging to the genus *Morbillivirus*, family *Paramyxoviridae*, order Mononegavirales
46 and has been responsible for epizootics in odontocetes and mysticetes (Van Bressems *et al.*,
47 2014) including fin whales (*Balaenoptera physalus*) (Mazzariol *et al.*, 2016).
48
49 Beffagna *et al.* (2017) suggested that the lack of typical morbillivirus pathology on post-
50 mortem examination in a subset of stranded, CeMV-1 infected, striped (*Stenella*
51 *coeruleoalba*) and bottlenose (*Tursiops truncatus*) dolphins (Casalone *et al.*, 2014) may be
52 due to genomic variations in the virus compared to the CeMV-1 originally detected in
53 previous epizootics in the Mediterranean Sea, which affected mainly striped dolphins and
54 long-finned pilot whales (*Globicephala melas*). They also suggested the genomic variations
55 might have facilitated fin whale infections in the Mediterranean Sea resulting in several cases
56 where CeMV-1 specific RNA was found in the brain, lung and spleen of a stranded neonate
57 and the liver, spleen, lung, lymph nodes and skeletal muscle of older animals (Mazzariol *et*
58 *al.*, 2016). Previous reports of CeMV infection in fin whales have described lesions including
59 non-suppurative encephalitis, ‘bronchiolo-interstitial’ pneumonia, hepatitis, mild catarrhal
60 enteritis and lymphoid depletion of the spleen and pulmonary and prescapular lymph nodes
61 (Di Guardo *et al.*, 2013; Mazzariol *et al.*, 2016). Definitive CeMV-related skin lesions have
62 not been reported in cetaceans nor the relative amounts of CeMV specific RNA present in
63 different organs during systemic infection.

64

65 **Material and Methods**

66 An adult, male fin whale (M37/13) live-stranded in the Cree estuary, Dumfries and Galloway,
67 Scotland, UK (54°52'29.3"N and 4°22'38.8"W) and died within 12 hours of being found. A
68 standardised necropsy was performed 36 hrs after death (Kuiken and Hartman, 1991).

69 Samples for bacteriology (brain, cerebrospinal fluid (CSF), heart, lung, liver, spleen, kidney,
70 intestine, pulmonary-associated and mesenteric lymph node) were cultured (as detailed in
71 Davison *et al.*, 2015).

72 Tissue samples, including (part brain stem and cerebellum [sampled through the foramen
73 magnum], trachea, lung, myocardium and attached cyst, heart valve, liver, stomach [cardiac
74 region], spleen, three lymph nodes [two pulmonary-associated, one mesenteric], kidney,
75 urinary bladder, testis and skin) were fixed in 10% neutral-buffered formalin and processed
76 routinely through graded alcohols prior to embedding in paraffin-wax. Sections (4 µm) were
77 stained with haematoxylin and eosin (HE) and subjected to immunohistochemistry (IHC) for
78 morbillivirus antigen as described previously (Wessels *et al.*, co-submission). Sections were
79 graded for the extent of IHC labelling (0 = absent, 1 = perceptible, 2 = small, 3 = medium, 4
80 = large and 5 = very large).

81

82 Brain and skin samples were frozen (-80°C) and RNA was extracted from these and all
83 formalin-fixed, paraffin wax-embedded (FFPE) tissue samples using the RNeasy kit (Qiagen,
84 Manchester, UK) or RecoverAll™ Total Nucleic Acid Isolation Kit (ThermoFisher,
85 Loughborough, UK), respectively, according to manufacturers' protocols. For CeMV-1 PCR,
86 primers and probes were employed in a one-step RT-qPCR reaction targeting the
87 hypervariable C-terminal domain of the nucleocapsid (N) gene of DMV (Grant *et al.*, 2009).

88 The reaction was performed in a final volume of 25 µl containing 12.5 µl of reaction mix
89 with ROX (Invitrogen Superscript III Platinum One-Step Quantitative RT-PCR System), 200
90 nM of each primer, 240 nM probe, 0.5 µl of SuperScript™ III RT/Platinum™ Taq Mix
91 (Thermofisher) and 2 µl of total nucleic acid. Cycling conditions were: 50°C for 15 minutes
92 followed by 95°C for 2 minutes then 45 cycles each consisting of 95°C for 15s, and 60°C for
93 1 minute (ABI Prism 7500, Applied Biosciences, ThermoFisher). The fluorescence threshold

94 was set manually above the background level. Two technical replicates were analysed for
95 each sample. Positive target controls (DMV positive liver sample, sequenced) and no
96 template controls were included in all plates. A second RT-qPCR detecting a mammalian
97 housekeeping gene (β -actin) (as detailed in Thonour *et al.*, 2012, with 500 nM primers and
98 200 nM probe) was performed using the above conditions to confirm validity and suitability
99 of extracted nucleic acids. Positive results, expressed as CT (cycle threshold) values
100 indicating the number of cycles required for the fluorescent signal to cross the threshold (i.e.
101 exceed background level), are inversely proportional to the amount of target nucleic acid
102 present.

103

104 Total RNA extracted from frozen samples of brain and skin was subjected to RT-PCR
105 amplification of the CeMV-Hemagglutinin binding protein gene, using DMV-F10 and DMV-
106 R10 primers as described by Mazzariol *et al.* (2016). A 718 bp PCR fragment was purified
107 followed by bidirectional Sanger sequencing using the DVM-10 primers. A full length
108 consensus sequence was generated from independent PCR fragments amplified from RNA
109 isolated from brain and skin tissues using SeqMan Pro 15 software within the DNASTAR
110 Lasergene package. Sequence identity was confirmed and similarity to other CeMV-H gene
111 sequences was determined by a BLAST search of the NCBI nucleotide database.

112

113 A maximum likelihood phylogenetic tree predicting the relationship between closely related
114 CeMV-H gene sequences was constructed in IQ-TREE (Trifinopoulos *et al.*, 2016) using a
115 multiple alignment generated using CLUSTAL Omega (Sievers *et al.*, 2011). The Rinderpest
116 morbillivirus H gene (Accession MN632637) was included in the alignment to provide a root
117 for the tree. The model selection tool (Kalyaanamoorthy *et al.*, 2017) within IQ-TREE was
118 used to select the optimum substitution models, prior to phylogenetic tree estimation. The

119 optimum substitution model selected for the CeMV sequences was the Kimura 3 parameter
120 model (K3P, Kimura, 1980). Tree topology was tested with 10,000 bootstrap replicates using
121 the ultrafast bootstrap method of Minh *et al.* (2013). The tree was visualised and prepared for
122 publication using Dendroscope 3 (Huson and Scornavacca, 2012).

123

124 **Results**

125 The animal measured 1,755 cm from tip of upper jaw to fluke notch; the girth measurement
126 immediately rostral to the dorsal fin was 490 cm. The mean of three standard blubber
127 thickness measurements (immediately rostral to the dorsal fin at the dorsal, lateral and ventral
128 aspects) was 57mm. This and reduced mass of the longissimus dorsi muscles indicated poor
129 nutritional condition. Mild excoriations of the fins and fluke (presumed due to live-stranding
130 process), skin sloughing in large sheets (initially presumed autolysis) and opacity of the lens
131 in the left eye were seen but no indication of entanglement. Fluid was present in the nasal
132 cavity and the trachea (presumed seawater) with large amounts of stable foam present in the
133 bronchi. The lungs were congested equally and fluid oozed from the cut surface of the
134 parenchyma. The pulmonary-associated lymph nodes were deep red with dark red-black
135 centres on sectioning. A single, smooth parasitic cyst was present in the myocardium of the
136 left ventricle. The fundic stomach contained several full thickness ulcers (10-155 mm
137 diameter) and the entire gastro-intestinal tract was empty. Liver and kidneys were yellow and
138 the urinary bladder contained a small amount of red-tinged translucent fluid. Bacteriology
139 revealed only post mortem invaders.

140

141 Histological examination showed the tracheal, bronchial and bronchiolar sub-mucosa
142 contained large numbers of syncytia, lymphocytes and macrophages (Figure 1), and a similar
143 infiltration was present in the sub-serosa. Large amounts of bile were present in the hepatic

144 bile ducts. Large numbers of lymphocytes, macrophages and syncytia infiltrated the hepatic
145 parenchyma, sub-mucosa of the stomach, renal cortico-medullary region and formed a band
146 within the urinary bladder detrusor muscle. The spleen was devoid of peri-arteriolar
147 lymphoid sheaths, the red pulp severely depleted of erythrocytes and an inflammatory cell
148 infiltrate similar to that in the other tissues was present primarily in the sub-capsular and
149 trabecular regions. The paracortices of all lymph nodes were mild-moderately depleted of
150 lymphocytes and contained, variably, small to large numbers of syncytia. The dermis of the
151 skin contained a medium to large number of lymphocytes, macrophages and syncytia (Figure
152 2). A small number of randomly scattered mononuclear glial foci within the caudal medulla
153 oblongata.

154

155 Immunohistochemistry for morbillivirus antigen showed intense cytoplasmic labelling in all
156 tissues except the heart, which was devoid of labelling. Principally, syncytia were labelled
157 (Figures 1 and 3, Table 1), except in the medulla oblongata where neurones and their
158 processes were positive but the cerebellum was devoid of labelling. All negative control
159 preparations were devoid of labelling.

160

161 Most FFPE tissues were CeMV RNA positive but relative levels varied with the largest
162 amounts in the urinary bladder (CT 22.0) and liver (CT 22.4) but none in the heart, skin or
163 cerebellum (Table 1). Frozen medulla oblongata contained relatively large amounts of
164 CeMV-specific RNA (CT 25.2) whereas frozen skin contained only small amounts (CT 40.2,
165 Table 1). A definitive diagnosis of severe, sub-acute, systemic CeMV-1 infection was made.

166

167 CeMV-1 was confirmed by sequence analysis of the morbillivirus H-gene fragment amplified
168 independently from brain and skin tissue. After removal of the primer sequences, the

169 remaining 678 bp sequence was submitted to the European nucleotide archive (Accn.
170 LR877357). Pairwise comparison with a CeMV-1 sequence derived from a fin whale from
171 Denmark (Accn. MH430939) identified two synonymous substitutions while comparison
172 with a Mediterranean fin whale sequence (Accn. MH430938) also identified two
173 substitutions, one was identical to the Danish sequence while the second was unique and non-
174 synonymous. Phylogenetic analysis of all CeMV-H gene sequences in the NCBI database
175 indicated a high degree of conservation between viruses isolated from different cetacean
176 species. However, two distinct virus clades were identified, one included North Atlantic and
177 Mediterranean whales and dolphins while the second included North Atlantic porpoises
178 (Figure 4).

179

180 **Discussion**

181 Although not the first case of CeMV in a fin whale, this is the first report of skin lesions
182 definitively co-localised with CeMV-antigen in cetaceans. Gross skin lesions suspicious of
183 CeMV were suspected in Guiana dolphins (*Sotalia guianensis*) (Flach *et al.*, 2019) but never
184 proven, and an extensive review of cetacean skin lesions failed to suggest any link with
185 CeMV (van Bresseem *et al.*, 2015). Conversely, seals with phocine distemper virus (PDV),
186 which is very closely related to canine distemper virus (CDV) and occasionally presents with
187 skin lesions, showed epidermal and follicular hyperplasia, hyperkeratosis, necrosis and
188 morbillivirus-antigen IHC-positive syncytia (Lipscombe *et al.*, 2001). Measles virus, the
189 morbillivirus type species, causes skin lesions that coincide with the formation of large
190 numbers of syncytia (Griffin, 2007). As syncytia are present in cetaceans with CeMV it is
191 surprising skin lesions have not been reported previously. These differences may be due to
192 known genetic variations in cetacean signalling leukocyte adhesion molecules (SLAM/F1) to
193 which CeMV binds (Shimizu *et al.*, 2013) or, possibly, the presence or absence of poliovirus

194 receptor-like 4 (PVRL4/nectin-4) that is expressed in epithelial cells to which morbilliviruses
195 bind (Noyce *et al.*, 2011).

196

197 The relative amounts of CeMV-1 RNA in different tissues during systemic infection have not
198 been reported previously. Typically, in the per-acute stage morbilliviruses infect and replicate
199 in the respiratory mucosa and local immune cells prior to spreading to the immune organs
200 and other viscera in the acute phase. The skin is usually last and this coincides with rash
201 formation in the subacute stage, with the brain variably affected in the subacute-chronic
202 stages (Griffin, 2007). This case highlights the urinary bladder and liver as ideal samples for
203 PCR CeMV diagnosis plus a range of other tissues that even after FFPE, which compromises
204 RNA integrity (Relova *et al.*, 2018), remain suitable and when combined with the distribution
205 of CeMV-associated lesions aids accurate disease staging.

206

207 Comparative analysis of the CeMV-H-gene identified a high degree of conservation between
208 viruses isolated from cetacean species distributed across the North Atlantic and the
209 Mediterranean. Interestingly, CeMV-H-gene sequence similarity was highest between whales
210 and dolphins compared to CeMV isolated from porpoises, suggesting a more recent or more
211 efficient exchange of CeMV between whales and dolphins compared to porpoises. This may
212 reflect differences in the efficiency of binding of CeMV H protein to its cell surface receptor,
213 a key early event in virus infection (Shimizu *et al.*, 2013).

214

215 In conclusion, this case shows cetaceans develop CeMV-associated skin lesions and even
216 somewhat autolytic skin samples are useful for definitive diagnosis. This will be especially
217 useful in large wildlife species as skin is relatively more resistant to autolysis/putrefaction
218 than visceral organs and easier/safer to sample. Furthermore, skin biopsies taken from live

219 free-ranging animals may be useful for determining morbillivirus status as well as genetic
220 studies of host and pathogen.

221

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228 Institute and the Royal Zoological Society of Scotland (RZSS).

229

230 **Table 1:** Relative amounts of CeMV-1 specific RNA (CT-value) detected by real-time RT-
231 PCR (the lower the number the greater the amount of RNA) in the various formalin-fixed,
232 paraffin-wax embedded (FFPE) and frozen tissue samples (where stated) and the
233 corresponding results for immunohistochemistry (FFPE only) along with the principal cell
234 type labelled and amount of labelling (0 – 5). UD = undetermined. NA = not applicable.

235 **Figure legends**

236 **Figure 1:** Histological preparation of tracheal mucosa; note autolytic loss of epithelium (*)
237 and the very large numbers of large syncytia (black arrows) and lymphocytes (yellow arrows)
238 greatly expanding the sub-mucosa. Stain: H&E. Bar = 100µm. Insert. Semi serial section of
239 tracheal mucosa; note extensive cytoplasmic labelling of syncytia for morbillivirus antigen
240 (brown pigment). Immunohistochemistry for morbillivirus antigen counterstained with
241 haematoxylin. Bar = 100µm.

242 **Figure 2:** Skin; note syncytia in dermis (yellow arrows) just below the dermo-epidermal
243 junction (black arrows). Stain H&E. Bar = 200µm.

244 **Figure 3:** Skin; note syncytia (black arrows) in H&E stained insert (bar = 100µm) and
245 intense labelling of morbillivirus antigen (brown pigment) in the cytoplasm of the syncytia in
246 the main plate (semi-serial section). Immunohistochemistry for morbillivirus antigen
247 counterstained with haematoxylin. Bar = 100µm.

248 **Figure 4:** Maximum likelihood phylogenetic tree estimating the relationship between 20
249 CeMV-H gene sequences. The H gene sequence derived from fin whale M37/13 is marked
250 with an asterisk *. For each sequence, the species of origin, geographic origin and GenBank
251 accession numbers are shown. The Rinderpest morbillivirus H gene (Accession MN632637)
252 roots the tree.

253

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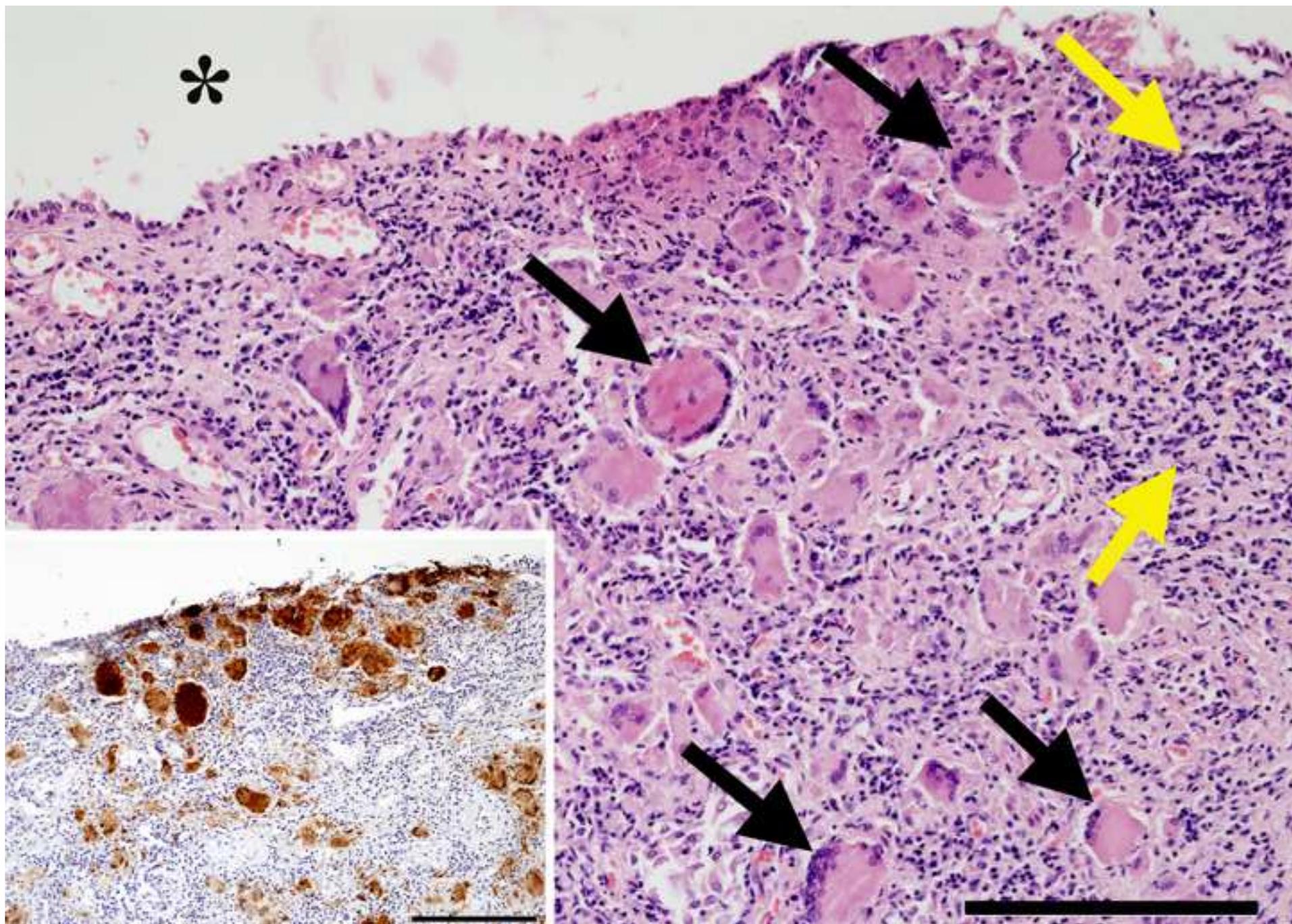
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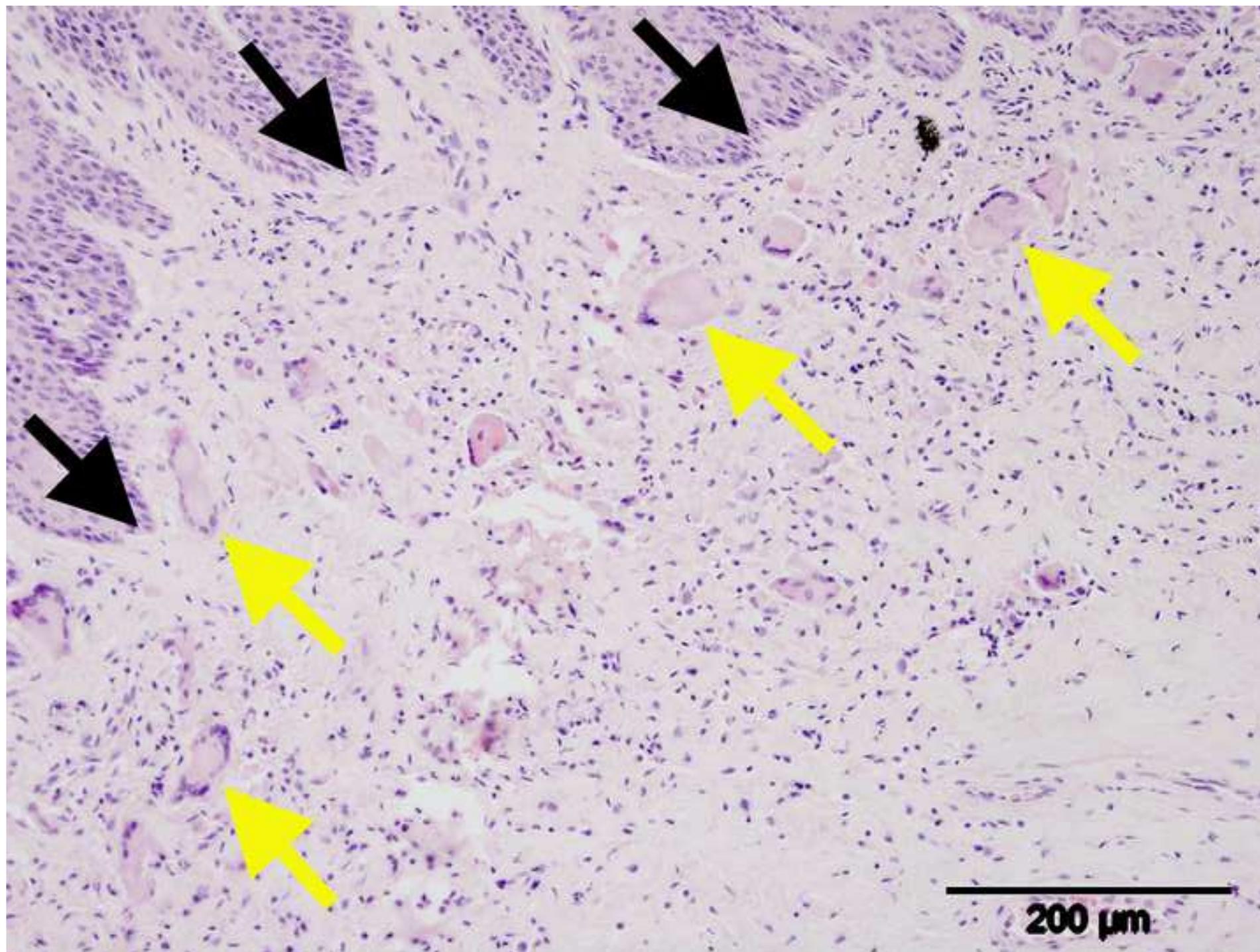
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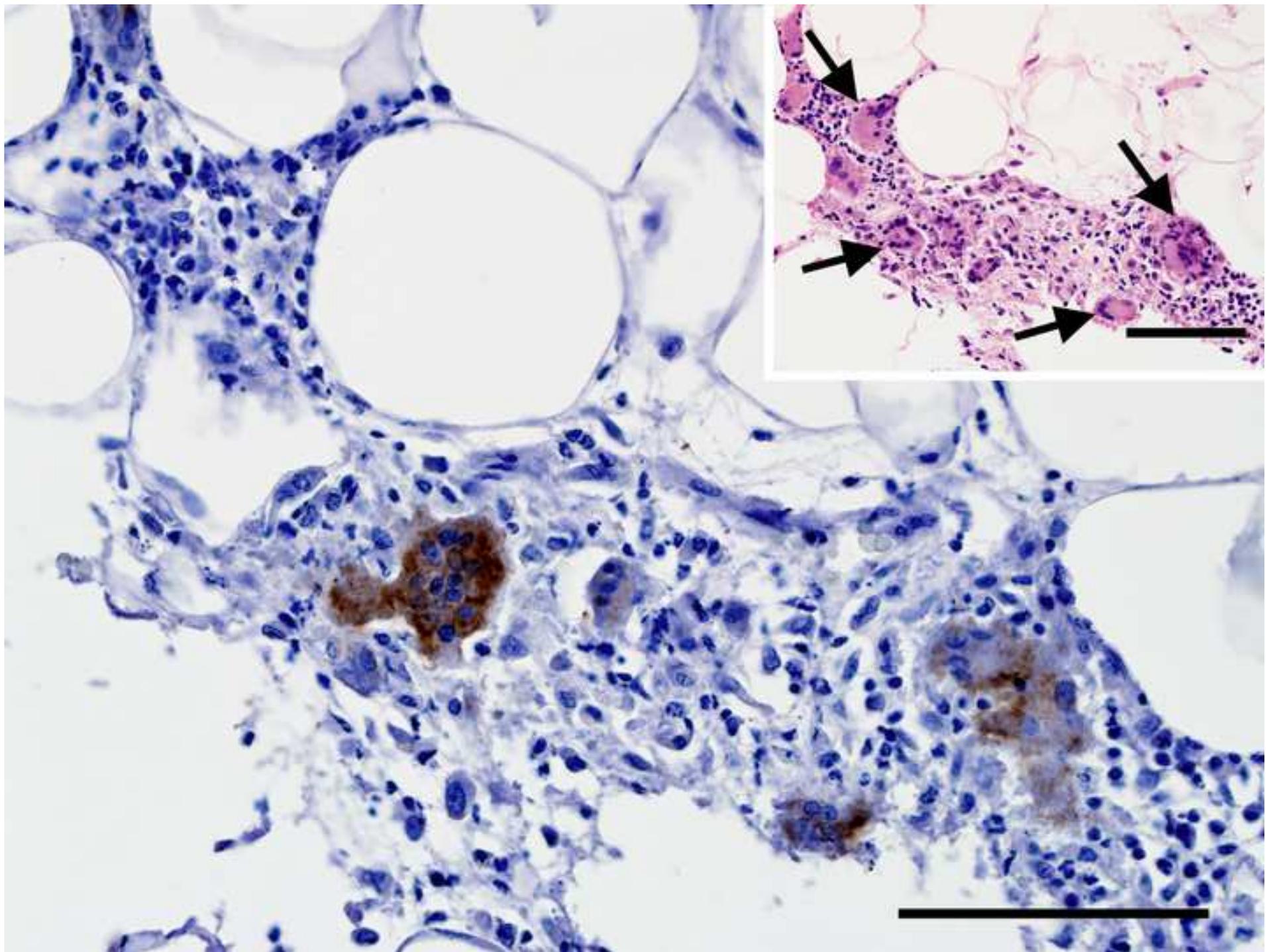


Figure 4

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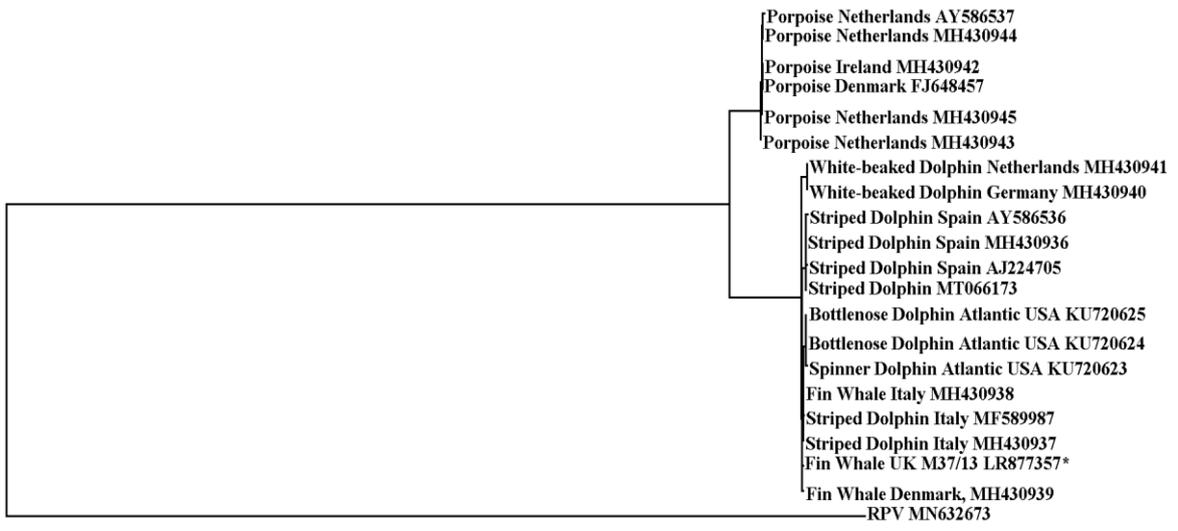


Table 1: Relative amounts of CeMV-1 specific RNA (CT-value) detected by real-time RT-PCR (the lower the number the greater the amount of RNA) in the various formalin-fixed, paraffin-wax embedded (FFPE) and frozen tissue samples (where stated) and the corresponding results for immunohistochemistry (FFPE only) along with the principal cell type labelled and amount of labelling (0 – 5). UD = undetermined. NA = not applicable.

Tissue	No. of samples	Real-Time rt-PCR mean CT value	Morbillivirus IHC (principal cell type positive, overall score)
Brain stem (frozen)	1	25.2	not applicable
Brain stem	3	32.1	positive (neurones, 3)
Cerebellum	4	Undetected	negative
Trachea	1	23.9	positive (syncytia, 5)
Lung	2	24.4	positive (syncytia, 3)
Heart	1	Undetected	negative
Heart valve	1	Undetected	negative
Heart cyst	1	Undetected	negative
Liver	1	22.4	positive (syncytia, 5)
Stomach	1	24.5	positive (syncytia, 3)
Spleen	1	23.6	positive (syncytia, 5)
Lymph node	3	25.0 (range 23-26)	positive (syncytia, 3)
Kidney	1	24.1	positive (syncytia, 4)
Urinary bladder	1	22.0	positive (syncytia, 4)
Testis	1	26.3	positive (UD, 1)
Skin (frozen)	1	40.2	NA
Skin	1	Undetected	positive (syncytia, 2)