A simultaneous diagnosis and genotyping method for global surveillance of cetacean morbillivirus

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Cetacean morbillivirus (CeMV) is considered one of the most important viral pathogens in cetaceans. CeMV outbreaks of lethal disease have repeatedly been observed in Europe, the Americas, and Australia, while large herds of gregarious species were found to be the likely reservoirs and sources of CeMV infection to susceptible species in the Atlantic and Pacific Oceans. Furthermore, three new strains were detected recently in Hawaii, Brazil and Australia. To clarify the real global distribution of CeMV and possible carriers, we showed a novel technique successfully diagnosing and distinguishing different virus strains (DMV, PWMV and novel CeMVs) using FFPE samples from 1996 to 2011. This efficient method that combines qRT-PCR and high resolution melting (HRM) could be applied to the future retrospective global studies for better understanding of different prevalence and outbreak conditions among ocean basins and the mechanism of variable host response to pathogens.

Cetacean morbillivirus (CeMV) is considered one of the most important viral pathogens in cetaceans. It includes three well-characterized strains (porpoise morbillivirus, PMV; dolphin morbillivirus, DMV; pilot whale morbillivirus, PWMV) and three new strains from Hawaii, Brazil, and Australia detected by RT-PCR. In infected cetaceans, the virus causes serious respiratory, central nervous system disease and immunosuppression that lead to serious secondary bacterial and fungal infections. Outbreaks of lethal CeMV infection have recurrently been noticed in Europe, the Americas, and Australia since the late 1980s. It is intriguing that some cetaceans are positive for CeMV by RT-PCR without typical pathological findings resulting from CeMV infection, indicating possible subclinical CeMV infection in certain species and areas. For greater understanding of the role of potential carriers and would clarify the global distribution of this important pathogen, a reliable method for conducting retrospective survey of CeMV is essential. As compared to ultracold frozen tissues, formalin-fixed paraffin-embedded (FFPE) samples that represent recent and historical stranding events are more widely available for retrospective survey. However, the regular RT-PCR method for CeMV detection is not suitable for FFPE samples because formaldehyde creates cross-links between nucleic acids and proteins resulting in short amplicons (<200 bp). Krafft et al. designed a protocol using RT-PCR with Southern blot that allows CeMV RNA amplification in FFPE samples. However, this method is laborious and time-consuming, particularly when a large number of samples need to be tested. We describe a novel scheme that combines real-time RT-PCR (qRT-PCR) and high resolution melting assay (HRM) for rapid genotyping using FFPE samples stemming from 1996 to 2011 that efficiently detects and differentiates the global spectrum of CeMV strains.

Results and Discussion

Two primer sets (P1 and P2) were designed for use with the qRT-PCR to amplify phosphoprotein (P) gene of CeMV. The lower limit of detection of P1 and P2 was both 10^1 copies/reaction (P1: R^2 = 0.997, efficiency = 103.37%; P2: R^2 = 0.988, efficiency = 107.24%). Both primer sets successfully detected different CeMV strains in the 7 cases previously confirmed as CeMV infections while the other 20 samples of other stranded cetaceans in western Pacific collected from 2005 to 2013 were negative. We are confident that high-quality RNA was extracted from all of the FFPE samples since all 27 samples were positive (Cq = 19–29) with the ß-actin

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primer set. In the difference graph of HRM analysis, the melting profiles of each sample were compared to the pilot whale morbillivirus (PWMV) that was converted to a horizontal line as reference. The HRM profiles with P1 primer set showed five distinct groups: (I) Hawaii 2010 CeMV (II) Spain 2011 DMV (III) Spain 2005, 2007 and 2008 DMV (IV) Brazil 2010 CeMV (V) PWMV (Fig. 1A). The HRM profiles with P2 primer set showed four distinct groups: (I) Hawaii 2010 CeMV (II) Spain 2005, 2007 and 2011 DMV (III) Brazil 2010 CeMV (IV) PWMV (Fig. 1B).

Single-nucleotide differences (Table 1) resulting in slight shifts in the melting domain were clearly detected. The melt profiles within one CeMV strain were consistent in all samples and replicates. Table 2 shows the Cq and melt peak temperature (Tm) of the positive samples.

It was proposed that there are two CeMV lineages: CeMV-1 includes DMV, PMV, PWMV, and Hawaii strain; CeMV-2 includes strains from Brazil and Australia. The representative CeMV strains from nearly all regions worldwide were included in this study, and the results provide evidence that the HRM-based method presents a valid and efficient approach to the detection and subtyping of the CeMV P gene of these two lineages. This method does not need specific probes in addition to the specific primers for each morbillivirus strain. A cheap

![Derivative high-resolution melting curves comparing individual cetacean morbillivirus strains.](image)

Pilot whale morbillivirus was converted to a horizontal line as reference. (A) P1 segment, (B) P2 segment.

**Figure 1.** Derivative high-resolution melting curves comparing individual cetacean morbillivirus strains. Pilot whale morbillivirus was converted to a horizontal line as reference. (A) P1 segment, (B) P2 segment.

|          | Hawaii 2010 | Spain 2011 | Spain 2005 | Spain 2007 | Spain 2008 | PWMV   | Brazil 2010 |
|----------|-------------|------------|------------|------------|------------|--------|-------------|
| Hawaii 2010 | —           | 1          | 1          | 1          | 1          | 5      | 4           |
| Spain 2011 | 2           | —          | 0          | 0          | 0          | 6      | 5           |
| Spain 2005 | 1           | 1          | —          | 0          | 0          | 6      | 5           |
| Spain 2007 | 1           | 1          | 0          | —          | 0          | 6      | 5           |
| Spain 2008 | 1           | 1          | 0          | 0          | —          | 6      | 5           |
| PWMV      | 2           | 2          | 3          | 3          | 3          | —      | 5           |
| Brazil 2010| 6           | 6          | 7          | 7          | 7          | 6      | —           |

**Table 1.** Pairwise comparison of nucleotide substitutions for the cetacean morbillivirus strains included in this study, based on P1 (below diagonal) and P2 (above diagonal) fragment of the phosphoprotein gene.
fluorescent molecule (Eva-Green®), which is readily available to diagnostic laboratories, was used. By using two primer sets targeting two different sites for increasing the detection ability, and HRM that is able to differentiate sequences differing by one single nucleotide, it is theoretically possible to detect small variations in the P gene among virus strains. Because the decomposition status and formalin fixation time of these samples are varied, β-actin was used as a control for the loading and integrity of the RNA. The organs tested in this study except lymph nodes and spleen from the Longman’s beaked whale (Table 2) present typical morbillivirus lesions, including non-purulent encephalitis, interstitial bronchopneumonia, syncytia, eosinophilic inclusion bodies, and lymphoid depletion with germinal center necrosis. It is imperative that the morbilliviral RNA was detected in every FFPE sample with adequate Cq value, representing the high sensitivity of this method. Furthermore, the PWMV sample collected in 1996 can be used for diagnosis, indicating that it is feasible to conduct retrospective study of CeMV infection with this method. Therefore, it can represent a valuable screening method for the initial detection of CeMV strains worldwide, especially for the ocean basins that no mass mortality has been reported and outbreak conditions between Atlantic and Pacific oceans, but also the mechanisms of variable host response strains from nearly all regions worldwide. High sensitivity enables reliable detection of this virus in FFPE samples and in low viral load samples. Through investigation of the relationship between virus variation and responsive-...
to pathogens. Future studies based on this method could elucidate the role of virus strains in disease susceptibility, resistance, and progression.

**Methods**

Primers were selected on the basis of alignments of P gene sequences of CeMV strains from GenBank. The amplification length of two primer sets (P1 and P2) was 85 bp and 72 bp, respectively (Fig. 2) (5′-3′: P1F-TTGAAGGAGTCAAGGATGCTG; P1R-GAGCTCTCATCTCCGTCCTGT; P2F-AGGGCCACAGGAGAGATCA; P2R-ATTGGGTGCACACCTTGTC). To assess the sensitivity of the assay, we constructed a plasmid that contains the P gene of CeMV (Fig. 2). The fragment of the P gene inserted into the plasmid was synthesized according to a dolphin morbillivirus (DMV) strain (GenBank accession number: HQ829973). The plasmid was serially diluted 10-fold in sterile water and used to test the assay. The linear range of sensitivity was $10^1$–$10^7$ copies. Seven FFPE samples diagnosed with CeMV infection from cetaceans stranded in Spain, Hawaii and Brazil from 1996 to 20116,11–15 and 20 FFPE samples from lung or lymphoid organs were used as controls.

Seven FFPE samples diagnosed with CeMV infection from cetaceans stranded in Spain, Hawaii and Brazil from 1996 to 20116,11–15 and 20 FFPE samples from lung or lymphoid organs were used (Table 2). The fixative of these samples was 10% neutral buffered formalin. These samples were processed according to routine histopathological methods and stained with haematoxylin and eosin for microscopic examination. Total RNA of the FFPE samples were extracted using RNeasy FFPE kit (Qiagen). RNA integrity was monitored routinely using denaturing gel electrophoresis. Total RNA concentration ranged from 5.4 to 8.9 ng/μl in the extracted samples, as measured using fluorescence-based quantitation method (Quibit fluorometer with a Quan-tiT RNA Assay Kit, Invitrogen) suggested in Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline46 for RNA quantity and purity determination. β-actin was used as an internal RNA quality control (β-actin-F-AGGACCTCTATGCCAACACG; β-actin-R-CCTTCTGCATCCTGTCAGC).

RNA was reverse transcribed in a final volume of 20 μl including 5 μl of QuantiTect reverse-transcription master mix (Qiagen), 0.7 μM of gene-specific primers and 14 μl of RNA. The reaction was performed at 42 °C for 15 min followed by inactivation of the reverse transcriptase by heating at 95 °C for 3 min. The qPCR assay was performed in a 10 μl volume containing 5 μl of IQ2 Fast qPCR System master mix (Biogenesis), 0.4 μM of each primer and 2 μl of diluted cDNA. The qPCR cycling was performed using the Eco Real-Time PCR System (Illumina) as follows: 95 °C for 2 min followed by 45 cycles, each consisting of 95 °C for 5 s, 60 °C for 30 s. After completion of the qPCR step, the amplified target was gradually denatured by increasing the temperature from 55 °C to 95 °C at 0.1 °C/s to produce a characteristic melting profile. Significant deviations from the horizontal line in the difference graph were indicative of sequence changes within the amplicon of the target gene analyzed. To assess reproducibility, every sample was analyzed in triplicate and on three different plates. Tm and the standard deviation (SD) were determined for replicate analyses of each specimen using tools provided in the software. The amplified products were cloned into TA plasmid vector. The nucleotide sequences were determined using Sanger sequencing, and were compared to the P gene sequences of CeMV from GenBank.

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K.-W.C. and B.-J.W. performed all the experimental procedure, data analysis, and manuscript writing. E.S., A.F., K.R.G., J.L.C.-D. and K.W. participated in the experimental process, providing samples, and commenting on the study design and data analysis. W.-C.Y. supervised the study design, experimental procedure and data analysis, and was the primary author of the manuscript. All authors reviewed the manuscript.

Additional Information
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