Inter-Laboratory Ring Trial to Evaluate Reverse Transcription Polymerase Chain Reaction Methods Used for *Dolphin Morbillivirus* Detection in Italy

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**Abstract**

*Dolphin Morbillivirus* (DMV) is one of the most frequently detected pathogens in stranded cetacean specimens worldwide as well as in Italy. Due to the persistence of DMV in the Mediterranean Sea and to the lack of information about the efficiency of the available diagnostic techniques, the Italian National Reference Centre for diagnostic activities on dead stranded marine mam-
Dolphin Morbillivirus (DMV), a single negative stranded RNA virus within the genus Morbillivirus, subfamily Paramyxovirinae, family Paramyxoviridae [1], is included in the cluster of Cetacean Morbillivirus (CeMV) [2]. DMV infection, in a similar manner to many other Morbillivirus genus members, affects mainly the upper respiratory tract as well as the central nervous system and the immune system of marine mammals [3] [4], having been associated with high mortality rates and stranding of cetaceans in different regions of the world [5]. In the Mediterranean Sea, two well documented DMV outbreaks occurred between 1990 and 1992 [6] as well as between 2006 and 2008 [7]. Moreover, DMV was deemed as the most likely cause of three cetacean unusual mortality events (UME s) oc-
Viral isolation and subsequent PCR identification are usually considered the “gold standard” for the definitive diagnosis of morbilliviral infections [4]. Nevertheless, this is often a challenging issue given the poor preservation of virus-targeted tissues when dealing with stranded cetacean carcasses; therefore, direct detection in tissues by means of reverse transcription-PCR (RT-PCR) followed by sequencing represents the first rapid, sensitive and specific tool for DMV detection. In the last few years, many conventional and Real-time RT-PCR methods have been developed to detect the presence of DMV worldwide [11][12][13].

In Italy, the National Reference Centre for diagnostic activities on dead stranded marine mammals (C.Re.Di.Ma) coordinates post mortem investigations on stranded cetaceans along the entire Italian peninsula, promoting the application of standardized guidelines regarding sampling and diagnostic techniques and the sharing of diagnostic results. In regard to direct DMV detection in tissues, C.Re.Di.Ma developed an RT-PCR restriction fragment length polymorphism (RFLP) technique, based upon the use of an RT-PCR with degenerate primers targeting a 287 bp fragment of the nucleoprotein (N) gene, followed by MseI RFLP analysis [14]. This work is aimed at assessing the performances of different diagnostic methods routinely applied in Italy for DMV infection’s diagnosis. In this respect, identical panels of ad hoc samples were analyzed by the whole Italian dead stranded marine mammals’ diagnostic network. More in detail, the accuracy (Se = sensitivity and Sp = specificity) and precision (reproducibility) of the method described by Verna et al. 2017 [14] were verified and, at the same time, they were also compared with the accuracy of other 7 biomolecular techniques routinely applied for DMV detection in Italy [11][12][15]-[20].

2. Material and Methods

Twelve Public Diagnostic Laboratories belonging to the Italian diagnostic network on stranded cetaceans, listed in Table 1, took part in the ring trial. They constantly remained anonymous, thereby respecting privacy requirements and were named as L1 to L12. All the participating Laboratories were asked to apply the method proposed by C.Re.Di.Ma [14] on the first panel of samples. That method [14] was developed to detect a 287 bp sequence of a highly conserved region of Canine Distemper Virus (CDV) nucleoprotein (NP). Considering the relevant polymorphism of the target region, primers developed by Frisk et al. 1999 [16] were redesigned through an in silico analysis. C.Re.Di.Ma, as the ring trial organizer, and in order to ensure the most consistent homogeneity of results, provided all of the participating Laboratories with detailed instructions and reagents to carry out the protocol described by Verna et al. More in detail, for total RNA extraction the Laboratories used the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions and reverse transcription...
Table 1. Laboratories involved in the ring trial listed in alphabetical order.

IZS Abruzzo and Molise—Department of Teramo
IZS Lazio and Toscana—Department of Roma
IZS Mezzogiorno—Department of Portici
IZS Piemonte, Liguria and Valle d’Aosta—Department of La Spezia
IZS Piemonte, Liguria and Valle d’Aosta—Department of Torino
IZS Puglia and Basilicata—Department of Foggia
IZS Sardegna—Department of Sassari
IZS Sicilia—Department of Palermo
IZS Umbria and Marche—Department of Ancona
IZS Venezia—Department of Legnaro
University of Padova—Dpt of Comparative Biomedicine and Nutrition—Pathological Anatomy Laboratory
University of Teramo—Virology and cell cultures laboratory

with simultaneous cDNA amplification was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen). Eight Laboratories that routinely utilized a different method also examined a second panel of samples using their own protocols [11] [12] [15]-[20] whose essential details are reported in Table 2.

2.1. Samples Preparation

Each laboratory was provided with a panel composed of 40 samples to be analyzed with the method proposed by C.Re.Di.Ma (Panel 1: Samples from 1 to 40). Each panel consisted of 27 positive samples and 13 negative ones. C.Re.Di.Ma expected that its own method was able to reach values of Se ≥ 80% and Sp ≥ 90%, therefore the number of positive and negative samples included in the study had to be sufficient to ensure that performance, with a limit lower than the 95% confidence interval for both indices was set at over 60%. Laboratories that routinely employed other detection methods were also provided with a second panel containing the same number of samples, with distinguishable header and numbering (Panel 2: Samples from 51 to 90). The positive samples consisted in viral suspensions at different concentrations obtained from a cell culture infected with a given DMV isolate [21], recognized as the “gold standard”. (GenBank Acc. No. MF589987). The virus was propagated on Vero/dogSLAM cell line [21] and after 6 days, when the cytopathic effect (CPE) affected 80% - 90% of the cells, supernatants were collected and clarified by centrifugation at 3500 rpm. The initial concentration of the DMV isolate was determined on Vero/dogSLAM cell line with preformed monolayer and the final count of 103.5 TCID50/25 μL was calculated with the Reed-Muench method. Positive samples were set up at different viral concentrations (level 1 = 1:20 of the original supernatant, level 2 = 1:100 and level 3 = 1:500). As negative control, a non-inoculated Vero/dogSLAM cell line supernatant was used under the same conditions. Samples were distributed
Table 2. Method used by laboratories participating in the DMV ring trial.

| Methods                                      | Target amplicon | primers                                                                 | Laboratory |
|----------------------------------------------|-----------------|------------------------------------------------------------------------|------------|
| RT-PCR (Verna et al. 2017) [14]              | Gene N—287 bp   | MvF 5'—ACAGGATTGCKGAGGACCTAT-3' MvR 5'—VARGATAACCATGTACAGGTC-3'          | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 |
| Semi Nested RT-PCR [19]                      | Gene P—300 bp   | MVP2202 5'—KKCTCRTGTGWCWRCAGAGG-3' MVP2480R 5'—TCTCTYCTGCGCCCTTTTATGAG-3' | 1          |
| Real-time RT-PCR TaqMan [17] [18]            | Gene F—192 bp   | DMV Fu-F 5'—GGCACCATAATTAGCCAGAGG-3' DMV Fu-R 5'—GCCCGAGTTTGTGCTCAT-3'  | 2          |
|                                             |                 | probe unpublished                                                      |            |
| RT-PCR [12]                                  | Gene N—173 bp   | DMV-N-FP 5'—TGCCAGTACTCCCGAGGAAATCCCTT-3' DMV-N-RP 5'—TTGGGTCTCGCTACGTGCTTATC-3' | 3, 8       |
| Real-time RT-PCR [20]                        | Gene F—45 bp    | DMV-F3 5'—TCGCCGCAAGACAAATG-3' DMV-R1 5'—TCCCTGGGACACTTGGG-3'           | 4          |
|                                             |                 | probe                                                                  |            |
| RT-PCR [15]                                  | Gene P—429 bp   | DMV-C 5'—ATGTTTTATGATCCACAGGGT-3' DMV-P2 5'—ATGGGTTGCACTTGG-3'           | 5          |
| Real time RT-PCR sybrgreen [16]              | Gene N—287 bp   | P-1 (CDV) 5'—ACAGGATTGCTGAGGACCTAT-3' P-2 (CDV) 5'—CAAGATAACCATGTACAGGTC-3' | 9          |
| Nested PCR [11]                              | Gene H—612 bp   | DMV-11F 5'—CCGAACCTGGATCCATTT-3' DMV-11R 5'—CGTAAATGTCATGCTTTGG-3'      | 11         |
|                                             | nested 200 bp   | DMV-13F 5'—CATCATAGGGGGTTGTGTATGAC-3' DMV-13R 5’—GGGTTGGTCATCTCTTTGCAC-3' |            |

in aliquots of 250 μl, identified individually with numerical code and stored at −80°C.

2.2. Homogeneity and Stability Tests on Ring Trial Samples

Homogeneity in sample preparation was evaluated by analyzing with the method Verna et al. 2017 [14] five replicates for each level of positivity under the same conditions planned for the execution of ring trial analyses (Figure 1). Extractions were carried out using the QIAamp viral RNA mini kit QIAGEN and retrotranscription and amplification kit OneStep RT-PCR Qiagen. Evaluation of homogeneity was performed at the end of the preparation of all samples and prior to their shipment to all laboratories involved in the ring trial. Five aliquots for each virus concentration were randomly selected from every group of samples ready for distribution. In order to eliminate possible confounding factors, tests were carried out under strict standard conditions; therefore they were performed by the same operator and in the same analytical session. Acceptability criteria for homogeneity tests were set up at reproduction, on each selected aliquot, of the same amplicon yield, corresponding to their respective reference samples for each viral dilution, including negative ones. With reference to stability tests, aliquots for each level of virus concentration were used to perform two different trials. In order to simulate the effect of potential temperature failure
occurrences during shipment on virus samples, a stability test was designed to evaluate the resistance of samples to stress temperature for each level of positivity, including the negative ones and it was carried out before the shipment. Analyses were performed on three replicates for each virus concentration maintained at room temperature at: $t_0 =$ corresponding to homogeneity test day; $t_1 =$ day1; $t_2 =$ day2; $t_3 =$ day3 (Figure 2). A second stability test was carried out to verify the sample stability at the storage conditions (-80°C), during the entire period of execution of the ring trial. In this case, three replicates for every single virus concentration (including negative controls) were analyzed, every 15 days, from the day of shipment until the defined closing time for results submission. Acceptability criteria for stability tests were also set up at reproduction, during all ring-testing period under all storage and stress conditions, on each selected aliquot, of the same amplicon yield, corresponding to their respective reference samples for each viral dilution, including negative ones.

2.3. Statistical Analysis

Performances of each laboratory in the application of the method described by Verna et al. were evaluated in terms of both accuracy and precision (reproducibility). Conversely, for each of the other 7 methods routinely applied throughout the Country, accuracy was evaluated. In order to assess the accuracy, Sensitivity (Se) and Specificity (Sp), positive (PPV) and negative (NPV) predictive values were estimated, as well as their 95% confidence intervals (95% CI). To assess precision Cohen’s kappa was pairwise estimated for each participant towards everyone, as well as for each participant with respect to the majority judgment (MJ, i.e. the outcome given by most of participants for each sample examined).
Moreover, the k-combined was calculated [22] to evaluate the agreement between all the participants in the trial. Statistical analysis was carried out using the software STATA 15 software (StataCorp, College Station, Texas, USA).

3. Results

3.1. Samples Homogeneity and Stability

Outcomes satisfied the acceptability requirements, thereby confirming expectations, as summarized in Figure 1 and Figure 2.

All dilution samples gave back the expected results. Pictures show: in Figure 1(a) five replicates of negative samples (lanes 1 to 5); five replicates of DMV at 1:500 dilution (lanes 6 to 10); five replicates of DMV at 1:100 dilution (lanes 11 to 15); in Figure 1(b) five replicates of DMV at dilution 1:20 (lanes 16 to 20) (Amplicon size: 287 bp, Agarose gel: 2%, DNA ladder: AmpliSize Molecular Ruler 50 bp - 2000 bp BioRad, USA)

Replicates of each viral dilution, kept at room temperature, were examined in triplicates at the onset (t₀) and at 24 (t₁), 48 (t₂) and 72 (t₃) hours from the shipment. Presence of viral RNA was always confirmed in all replicates containing DMV with the appropriate yield at each time interval. Figure 2 only shows amplicons obtained from each DMV dilution, as well as negative replicates, extracted and amplified after storage at room temperature for 72 hours (t₃): Negative samples (lanes 1 to 3); DMV at 1:500 dilution (lanes 4 to 6); DMV at 1:100 dilution (lanes 7 to 9); DMV at dilution 1:20 (lanes 10 to 12). (Amplicon size: 287 bp, Agarose gel: 2%, DNA ladder: AmpliSize Molecular Ruler 50 bp - 2000 bp BioRad, USA)

For stability test at storage conditions, replicates of each viral dilution, stored at ~−80°C, were examined after 0, 15 and 30 days to cover all testing period until the deadline for results’ submission. Presence of viral RNA was confirmed in all replicates containing DMV at each time interval.

3.2. PCR Results

This study allowed us to verify the performances of most of molecular detection
methods applied within the Italian dead stranded marine mammals’ diagnostic network. In particular, the technique by Verna et al. 2017, proposed by C.Re.Di.Ma, showed high accuracy values; sensitivity, specificity and predictive values obtained by each participating Laboratory are shown in Figure 3 and Figure 4. The average accuracy values assessed on the Verna et al. method resulted as Se = 97.8% (95% CI 84.2% - 99.3%), Sp = 98.1% (95% CI 72.5% - 99.9%), PPV = 99% (95% CI 85.4% - 100%), NPV = 96.6% (71.4% - 99%).

With reference to the other methods, the Nested PCR method (Gene H-612 bp - 200 bp) showed difficulties both in identifying positive samples (Se = 66.7%; CI = 46% - 83.5%) and negative ones (Sp = 76.9%; CI = 46.2% - 95%), although there was not enough significative statistical evidence to infer that the performances were lower than the method by Verna et al. For the rRT-PCR SYBR green method only it was possible to state that the ability to correctly identify negative samples (Sp = 15.4%; CI = 1.9% - 45.4%) was significantly lower than the other methods. The estimates of Cohen’s kappa calculated either for each pair of participants or for each participant versus the majority judgment, using the Verna et al. technique, are shown in supplementary material (Table S1, Table S2). The precision of the results obtained by all the Laboratories involved in the herein reported ring trial using the method by Verna et al. 2017 (k combined) was equal to 0.91 (95% CI 0.87 - 0.95).

4. Discussion

As previously stated, DMV represents one of the most relevant threats to free-ranging cetaceans. Different RT-PCR assays have been used for DMV and,
more in general, for CeMV detection from stranded cetaceans worldwide. Within such context, the consistent advances in molecular biology have allowed a progressively faster, easier and more reliable development of biomolecular protocols for DMV infection’s laboratory diagnosis [4]. The main goal of the herein reported interlaboratory ring trial was to assess the diagnostic performances of molecular assays to detect DMV used by 12 different Public Laboratories belonging to the Italian diagnostic stranding network. To the best of our knowledge, this is the first study performed to evaluate the accuracy of different biomolecular methods for DMV detection worldwide. The aforementioned network has been recently involved in the management of 2 cetacean UMEs [9] [10] respectively caused by the Mediterranean and the North-Eastern Atlantic DMV strains. The method described by Verna et al. 2017, applied by C.Re.Di.Ma routinely as well as during both the aforementioned UMEs, turned out to be a reliable and relatively inexpensive diagnostic tool, easily applicable and suitable to characterize different morbilliviral genotypes, as amplicons are appropriate to be submitted to RFLP analysis by mean of MseI Enzyme. First of all, our study was aimed at evaluating the accuracy and reproducibility of such technique. The method was successfully applied by all the participating Laboratories and high accuracy values were obtained. Even though two Labs (L10-L11) faced sensitivity-related issues and three others (L4-L9-L10) specificity-related ones, there was no statistically significant evidence to infer that the performances among the participants were different. In fact, the concordance values obtained by the various participants were fully satisfactory, provided that the lower limit of the confidence interval was at levels considered by the literature from “almost perfect”
to “excellent” [22]. The accuracy values of the other 7 methods tested were satisfactory too. Most laboratories obtained high values. We additionally noted that the Laboratory that applied the Nested PCR method (Gene H-612 bp - 200 bp) showed difficulties both in identifying positive samples (Se = 66.7%; CI = 46% - 83.5%) and negative ones (Sp = 76.9%; CI = 46.2% - 95%), but, even in this case, there was not enough statistical evidence to infer that the concerned Laboratory had lower performances than with the method by Verna et al. 2017. Only for the Real-time RT-PCR (rtRT-PCR) SYBR green (Gene N-287 bp) method it was possible to verify that the ability to correctly identify the negative samples (Sp = 15.4%; CI = 1.9% - 45.4%) was lower than the other methods. This might have resulted from cross-reaction or cross contamination, which are potential hazards in routine laboratory diagnostics, as also reported in other ring tests. Comparison of the sensitivities of different methods showed that the probability that the positive result given by the rtRT-PCR SYBR green (Gene N-287 bp) method was correct was 71% (CI = 52% - 84%), whereas for the method by Verna et al. 2017 it was at least 95% (CI = 85% - 100%). With reference to the negative results that were generally obtained by means of the rtRT-PCR SYBR green method (Gene N-287 bp), they also had low reliability: the probability that a negative sample was correctly classified could vary from 10% to 100%. The experimental conditions under which every test was carried out showed good reliability of the diagnostic methods. Notwithstanding the above, the results of laboratory analyses under field conditions could be affected by other factors, such as the genetic variability within between the different morbillivirus genus members and CeMV/DMV strains, along with the poor preservation status of tissues frequently found on stranded animals. Within such context, the stability test allowed us to highlight the remarkable resistance of morbilliviral agents under both correct and stressed preservation conditions. In this respect, the good stability of viral genomes, observed under unsuitable storage conditions, leads us to suppose that the “morbillivirus-related samples” preservation degree may not be “dramatically” affected during transfer between different laboratories for confirmatory analyses and comparison of results.

5. Conclusion

Based upon the results of the herein described ring trial, we can conclude that most of the biomolecular techniques used in our Country for DMV infection’s diagnosis show a satisfactory reliability and reproducibility, while an adequate level of expertise appears to be also present in all of the participating Laboratories regarding the application of the method by Verna et al. 2017, which could be recommended as a reliable RT-PCR protocol to be used for the routine laboratory diagnosis of DMV infection in Italy.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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### Supplementary Materials

**Table S1.** Cohen's \( k \) estimated for each pair of participants using the method Verna et al.

| Lab A | Lab B | \( k \) Cohen | Lower Limit | Upper Limit |
|-------|-------|---------------|-------------|-------------|
| LO1   | LO2   | 1.00          | 0.69        | 1.00        |
| LO3   | LO4   | 1.00          | 0.69        | 1.00        |
| LO5   | LO6   | 0.94          | 0.63        | 1.00        |
| LO7   | LO8   | 0.94          | 0.69        | 1.00        |
| LO9   | LO10  | 0.64          | 0.34        | 0.94        |
| LO11  | LO12  | 0.94          | 0.63        | 1.00        |
| LO2   | LO3   | 1.00          | 0.69        | 1.00        |
| LO4   | LO5   | 0.94          | 0.63        | 1.00        |
| LO6   | LO7   | 1.00          | 0.69        | 1.00        |
| LO8   | LO9   | 0.94          | 0.69        | 1.00        |
| LO10  | LO11  | 0.64          | 0.34        | 0.94        |
| LO12  | LO1   | 1.00          | 0.69        | 1.00        |
| LO3   | LO4   | 0.94          | 0.63        | 1.00        |
| LO5   | LO6   | 1.00          | 0.69        | 1.00        |
| LO7   | LO8   | 1.00          | 0.69        | 1.00        |
| LO9   | LO10  | 0.94          | 0.63        | 1.00        |
| LO11  | LO12  | 0.94          | 0.63        | 1.00        |
| LO4   | LO5   | 0.94          | 0.63        | 1.00        |
| LO6   | LO7   | 0.94          | 0.63        | 1.00        |
| LO8   | LO9   | 0.64          | 0.34        | 0.94        |
| LO10  | LO11  | 0.94          | 0.63        | 1.00        |
| LO12  | LO1   | 1.00          | 0.69        | 1.00        |
| LO5   | LO6   | 1.00          | 0.69        | 1.00        |
| LO7   | LO8   | 1.00          | 0.69        | 1.00        |
| LO9   | LO10  | 0.94          | 0.69        | 1.00        |
| LO11  | LO12  | 1.00          | 0.69        | 1.00        |
| LO6   | LO7   | 1.00          | 0.69        | 1.00        |
| LO8   | LO9   | 0.94          | 0.63        | 1.00        |
| LO10  | LO11  | 0.94          | 0.63        | 1.00        |
| LO12  | LO1   | 1.00          | 0.69        | 1.00        |
| LO7   | LO8   | 0.94          | 0.63        | 1.00        |
| LO9   | LO10  | 0.64          | 0.34        | 0.94        |
| LO11  | LO12  | 0.94          | 0.63        | 1.00        |
| LO8   | LO9   | 0.94          | 0.63        | 1.00        |
| LO10  | LO11  | 0.64          | 0.34        | 0.94        |
| LO12  | LO1   | 1.00          | 0.69        | 1.00        |
| LO9   | LO10  | 0.94          | 0.63        | 1.00        |
| LO11  | LO12  | 0.94          | 0.63        | 1.00        |

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Table S2. Cohen’s k against the majority judgment (MJ) for each participant using the method Verna et al.

| Laboratory | Cohen’s kappa | confidence interval 95% |
|------------|---------------|-------------------------|
| L01        | 1.00          | 0.69 - 1.00             |
| L02        | 1.00          | 0.69 - 1.00             |
| L01        | 1.00          | 0.69 - 1.00             |
| L02        | 1.00          | 0.69 - 1.00             |
| L03        | 1.00          | 0.69 - 1.00             |
| L04        | 0.94          | 0.63 - 1.00             |
| L05        | 1.00          | 0.69 - 1.00             |
| L06        | 1.00          | 0.69 - 1.00             |
| L07        | 1.00          | 0.69 - 1.00             |
| L08        | 1.00          | 0.69 - 1.00             |
| L09        | 0.94          | 0.63 - 1.00             |
| L10        | 0.64          | 0.34 - 0.94             |
| L11        | 0.94          | 0.63 - 1.00             |
| L12        | 1.00          | 0.69 - 1.00             |