Viral Encephalo-Retinopathy Inter-laboratory Proficiency Test (VER-IPT): results from two years' experience

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Abstract

Viral Encephalo-Retinopathy (VER), also known as Viral Nervous Necrosis (VNN) is an important disease of marine finfish that severely impacts on the profitability of Mediterranean aquaculture. The first attempt to organise an interlaboratory proficiency test against VER (VER-IPT) was carried out in 2016, followed by a second and a third edition in 2018 and 2020. These last two editions of the VER-IPT required the participants to undergo investigation for the presence of VER in 10 unknown samples. Four different viral genotypes were included and viral strain identification was also requested, even if not mandatory. A large number of laboratories from all over the world took part in the exercise. Fifteen out of 29 laboratories participating in the 2nd VER-IPT and 17 out of 26 taking part in the 3rd VER-IPT obtained the maximum score. The overall observed percentage of agreement was equal to 85.5% and 92.7%, in the 2nd and 3rd VER-IPT respectively, indicating an overall improvement of the diagnostic laboratories capacities over time. On the other hand, complete and correct viral species identification was performed only by a small number of participants, despite the importance of this information for field operators.

Introduction

In 2016 the 1st Viral Encepalo-Rethinopathy Interlaboratory Proficiency Test (VER-IPT) was organised with the aim of assessing the capability of the laboratories working with this pathogen to detect a single *Betanodavirus* genotype (namely the red-spotted grouper nervous necrosis virus -RGNNV) by real time RT-PCR (rRT-PCR). In addition, an inventory of the diagnostic laboratories interested in taking part in such initiatives was made. The results of this first exercise were published in the Bulletin of the EAFP (Toffan et al., 2017a).

In light of the successful outcome of the first VER-IPT (26 participants from 15 different countries took part in the initiative) second and third editions were organised in 2018 and 2020, respectively.

In contrast to the first VER-IPT, in the 2nd and the 3rd VER-IPT editions all the laboratories performing molecular detection of VER (adopting both rRT-PCR, and conventional RT-PCR) could take part in the exercise. In addition, the participants were asked to perform characterisation of the different betanodavirus genotypes

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included in the panel, by either sequencing or using other identification methods.

Betanodavirus, the causative agent of VER, is a small non enveloped single strain +RNA virus. Its genome is extremely small and composed by only two separate genetic segments called RNA1 and RNA2, which encode for 4 different proteins: the RNA-dependent RNA polymerase (RdRp), the capsid protein (CP) and two small non-structural proteins called B1 and B2 (Bandin and Souto, 2020). Betanodaviruses can be classified into four different species, also called genotypes, based on the phylogenetic analysis of the RNA1 and RNA2 segments: the striped jack nervous necrosis virus (SJNNV), the tiger puffer nervous necrosis virus (TPNNV), the barfin flounder nervous necrosis virus (BFNNV) and the red-spotted grouper nervous necrosis virus (RGNNV). Additionally in the Mediterranean basin, the presence of reassortant viruses called RGNNV/SJNNV and SJNNV/RGNNV has been extensively documented (Bitchava et al., 2019; Olveira et al., 2009; Panzarin et al., 2012; Toffolo et al., 2007). One of these reassortants, the RGNNV/SJNNV, is currently considered an emerging pathogen for the gilthead sea bream (Toffan et al., 2017b; Volpe et al., 2020). Since the diversity among Betanodavirus strains is ascribable to diverse pathogenicity, host and temperature tropism, it is of utmost importance for the diagnostic laboratory working with fish diseases to be able to detect and correctly identify all the viral species. The inclusion of the viral species characterisation as part of the proficiency test was specifically inteded as an attempt to stimulate laboratories to include the identification of Betanodavirus genotype in their diagnostic workflow.

In the present paper, the results obtained from the 2nd and 3rd VER-IPT are summarised and discussed.

Material and Methods

VER-IPT panels preparation

In both rounds the sample panel was composed of 10 ampoules. The viral strains used were propagated on the E-11 cell line (Iwamoto et al., 2000) either at 20°C or 25°C (depending on the viral species). Cell culture supernatants were harvested at completion of the cytopathic effect (CPE) and finally clarified by centrifugation (2700*g* for 10 min). Viruses were titrated in E-11 cells before being heat inactivated. The pre–inactivation titre was calculated according to the Reed and Muench formula (Reed and Muench, 1938). Where necessary, viral productions characterised by a high pre-inactivation titer were diluted with sterile MEM (Merck, former Sigma-Aldrich) to reach the desired titer. Inactivation was performed at 70° for 1 h. The complete inactivation of the virus was confirmed by three blind passages of 10 days each in SSN-1 cells (Frerichs et al., 1996) performed according to the standard procedure (OIE, 2019). Negative ampoules contained different kinds of reagents namely: sterile MEM, sterile MEM conditioned with 10% yeast extract (CONDA-Pronadisa) or negative serum collected from healthy rainbow trout (*Oncorhynchus mykiss*) reared in the IZSVe experimental facilities. All the positive samples, as well as the negative ones, were mixed with equal volumes of 20% w/v lactalbumin hydrolysate solution (Merck, former Sigma-Aldrich) and lyophilised in 2 mL glass ampoules. A requirement was to reconstitute the ampoules with 0.5 mL sterile saline solution or sterile cell culture medium before use, in order to extract the RNA and analyze it according to the standard methods in place in the users' laboratory.

Table 1. Contents of the vials included in the 2nd VER–IPT panel.

Vial n°	Contents (genotype)	Viral titre ($TCID_{50}$/mL)	Reference
1	Sterile MEM	-	-
2	389/I96 (SJ/RG)	$10^{6.30}$	Panzarin et al., 2012
3	283.2009 (RGNNV)	$10^{3.60}$	Panzarin et al., 2012
4	Sterile MEM	-	-
5	Rainbow trout negative serum	-	-
6	283.2009 (RGNNV)	$10^{3.60}$	Panzarin et al., 2012
7	484.2.2009 (SJNNV)	$10^{6.55}$	Panzarin et al., 2012
8	367.2.2005 (RG/SJ)	$10^{4.55}$	Panzarin et al., 2012
9	Sterile MEM	-	-
10	389/I96 (SJ/RG)	$10^{6.30}$	Panzarin et al., 2012

Table 2. Contents of the vials included in the 3rd VER-IPT panel.

Vial n°	Contents (genotype)	Viral titre ($TCID_{50}$/mL)	Reference
1	283.2009 (RGNNV)	$10^{7.30}$	Panzarin et al., 2012
2	367.2.2005 (RG/SJ)	$10^{7.55}$	Panzarin et al., 2012
3	484.2.2009 (SJNNV)	$10^{9.55}$	Panzarin et al., 2012
4	389/I96 (SJ/RG)	$10^{8.30}$	Panzarin et al., 2012
5	Sterile MEM+ 10% yeast extract	-	-
6	367.2.2005 (RG/SJ)	$10^{4.55}$	Panzarin et al., 2012
7	Sterile MEM	-	-
8	Sterile MEM	-	-
9	Sterile MEM+ 10% yeast extract	-	-
10	Negative rainbow trout serum	-	-

Details about the contents, the viral titer before inactivation and the references of viral strains are presented in Table 1 for the VER-IPT 2018 and in Table 2 for the VER-IPT 2020.

Prior to distribution, homogeneity and stability of the ampules were tested. Homogeneity was checked by testing 3 ampoules per batch of sample. Stability was tested at different time points (at least 7 times) before and after the completion of the exercise. Positive vial contents were checked by different assays: rRT-PCR targeting RNA1 (Baud et al., 2015); rRT-PCR targeting RNA2 (Panzarin et al., 2010), conventional end point RT-PCR and sequencing for the confirmation of viral genotype/species (Bovo et al., 2011; Toffolo et al., 2007). To further ascertain the stability of the ampoule contents after thermal stress, one additional panel was tested after a 2-week storage at 25°C for the VER-IPT 2018 and after 10 days at 37°C for the VER-IPT 2020. Analyses of viral titre always provided satisfactory results, and the PT was finally delivered.

Application, shipment, results collection and interpretation

Applications were sent via e-mail and participants were informed about the shipping date one month before the dispatch of the samples. In order to guarantee the anonymity of the participants, a new alphanumerical code was assigned to each laboratory every time. All the parcels were delivered by courier and, whenever possible, participants were provided with the AWB number for their own tracking online. The panel was shipped at room temperature. The deadline for the submission of results was set 3 months after the delivery of the parcel. Participants were asked to complete a spreadsheet with the obtained results and any other relevant information about the diagnostic approaches applied. Correct results (Positive/Negative for the presence of Betanodavirus) scored 1, while wrong, missing or doubtful results were scored 0. Viral species identification was not subject to evaluation. For the evaluation of agreement, the Cohen's K coefficient was estimated (Landis and Koch, 1977).

Results

Stability and homogeneity tests always provided satisfactory results. Notably, once lyophilised the vials contents were stable even after thermal stress, with minor and not significant losses of threshold values (Ct) (data not shown).

The participation to VER-IPT was considerable, and included countries from all over the world. A map summarising all the countries which took part in both VER-IPT is reported in Figure 1.

Results of the 2nd VER-IPT

Twenty-nine laboratories out of 32 applicants (29/32), which received the panel, submitted

Figure 1. Distribution of the participants to the VER-IPT in 2018 and 2020.

results within the deadline, while 3 were not able to complete the exercise in time. Twenty (20) laboratories were from EU countries while 9 were from non-EU countries. Both public/research and private laboratories were represented. Nineteen (19) of the laboratories taking part in the initiative belonged to countries surrounding the Mediterranean basin. Both public/research and private laboratories were equally represented.

Fifteen out of the 29 (15/29) participating laboratories obtained the maximum score. The remaining 14 participants produced a percentage of correct answers ranging from 90% to 40%. In detail, 3 laboratories scored 90% of correct results, 5 laboratories scored 80%, 2 scored 70% and finally 1 and 3 laboratories scored only 50% and 40% correct results, respectively.

The mean and median percentage of correct answers were 85.5% and 100%, respectively.

The overall observed percentage agreement was equal to 85.5%, while the overall calculated Cohen's Kappa (K) was 0.5387 ($p<0.00001$) meaning a "moderate agreement" according to the Landis and Koch scale (Landis and Koch, 1977). Detailed results are reported in Table 3 and Figure 2.

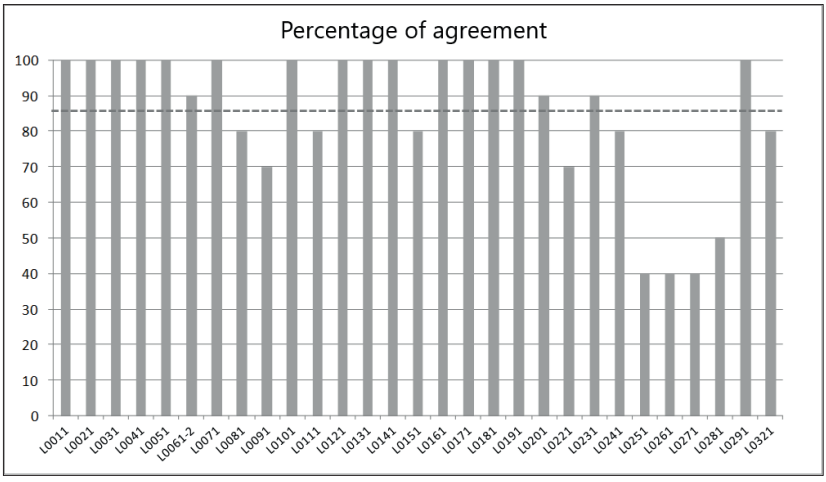

Figure 2. Observed percentage of agreement per laboratory participating in the 2nd VER-IPT. The mean no. of correct results (85.5%) is represented by a dashed line. Median and Mode = 100% are not plotted in the graph.

With reference to the ampoules contents, sample no. 4 (negative) was correctly identified by all the laboratories, while sample no. 2 (SJNNV/RGNNV) appeared to be the specimen with the highest error rate (only 65.5% of correct results).

Samples no. 2 and 10, as well as samples no. 3 and 10, which were replicates of the same SJNNV/RGNNV and RGNNV viruses, obtained different averages of correct results, meaning that for some laboratories results were not reproducible.

Results of the 3rd VER-IPT

Twenty-six laboratories out of 27 applicants (26/27), which received the panel, submitted results within the deadline and only 1 was not able to complete the exercise in time.

Nineteen (19) laboratories were from EU countries, while the remaining seven (7) were from extra-EU countries. Fifteen (15) of the participants belonged to countries surrounding the Mediterranean basin. Both public/research and private laboratories were equally represented.

Seventeen out of the twenty-six (17/26) participating laboratories obtained the maximum score. The remaining nine (9) participants produced an observed percentage of agreement ranging from 90% to 50%. In detail, 5 laboratories scored 90% of correct results, 1 laboratory scored 80%, 1 scored 70%, 1 scored 60% and 1 scored 50%.

The mean of correct results was 92.7%, while median and mode were equal to 100%. Detailed results are reported in Table 4 and Figure 3.

The overall percentage of agreement was equal to 92.7%, while the calculated Cohen's Kappa (K) was 0.7298 ($p<0.00001$) which means that a "substantial agreement" was achieved according to the Land and Koch scale (Landis and Koch, 1977).

With reference to the ampoules contents, sample no. 1 and 2 (containing RGNNV and RGNNV/SJNNV, respectively) were correctly identified

Table 3. Second VER-IPT results and scores obtained by participants. P= positive; N= negative. Incorrect results are in **underlined bold**.

Laboratory code number	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	Vial 7	Vial 8	Vial 9	Vial 10	n° correct answers per lab	% correct results per lab	Cohen's Kappa (K) value	Relative p-value per laboratory
L0011	N	P	P	N	N	P	P	P	N	P	10	100	1.000	0.0008
L0021	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0031	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0041	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0051	N	P	P	N	N	P	P	P	N	P	10	100	1.000	0.0008
L0061-2	N	P	P	N	N	P	P	P	**P**	P	9	90	0.7826	0.0056
L0071	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0081	N	**N**	P	N	N	P	P	P	N	**N**	8	80	0.6154	0.0175
L0091	N	**N**	P	N	N	P	**N**	P	N	**N**	7	70	0.4444	0.0455
L0101	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0111	N	P	**N**	N	**P**	P	P	P	N	P	8	80	0.5833	0.0325
L0121	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0131	N	P	P	N	N	P	P	P	N	P	10	100	10.000	0.0008
L0141	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0151	**P**	**N**	P	N	N	P	P	P	N	P	8	80	0.5833	0.0325
L0161	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0171	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0181	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0191	N	P	P	N	N	P	P	P	N	P	10	100	1.0000	0.0008
L0201	N	P	P	N	N	P	P	P	N	**N**	9	90	0.8000	0.0049
L0221	N	**N**	P	N	N	P	**N**	P	N	**N**	7	70	0.4444	0.0455
L0231	N	P	P	N	N	P	P	P	N	**N**	9	90	0.8000	0.0049
L0241	N	**N**	P	N	N	P	P	P	N	**N**	8	80	0.6154	0.0175
L0251	N	**N**	**N**	N	N	**N**	**N**	**N**	N	**N**	4	40	0.0000	n.c.
L0261	**P**	**N**	**N**	N	N	**N**	**N**	P	N	**N**	4	40	-0.0714	0.6266
L0271	N	**N**	**N**	N	N	**N**	**N**	**N**	N	**N**	4	40	0.0000	n.c.
L0281	N	**N**	**N**	N	N	**N**	**N**	P	**P**	P	5	50	0.0741	0.3891
L0291	N	P	P	N	N	P	P	P	N	P	10	100	10.000	0.0008
L0321	N	**N**	**N**	N	N	P	P	P	N	P	8	80	0.6154	0.0175
GOLD	**N**	**P**	**P**	**N**	**N**	**P**	**P**	**P**	**N**	**P**	-	-	**Overall Agreement**	**Overall p-value**
n° correct answers per vial	27	19	23	29	28	25	23	27	27	20	-	-	0.5387	0.0000
% correct results per vial	93.1	65.5	79.3	100	96.6	86.2	79.3	93.1	93.1	69.0	-	-	-	-

Table 4. Third VER-IPT results and scores obtained by participants. P= positive; N= negative. Incorrect results are in **underlined bold**.

Laboratory code number	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	Vial 7	Vial 8	Vial 9	Vial 10	n° correct answers per lab	% correct results per lab	Cohen's Kappa (K) value	Relative p-value per laboratory
20LAB01	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB02	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB04	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB05	P	P	P	P	**P**	P	N	N	N	N	9	90	0.8000	0.0049
20LAB06	P	P	P	P	N	P	**P**	**P**	**P**	**P**	6	60	0.2000	0.1459
20LAB07	P	P	P	P	N	P	N	**P**	N	N	9	90	0.8000	0.0049
20LAB08	P	P	P	P	**P**	P	**P**	**P**	**P**	**P**	5	50	0.0000	n.c.
20LAB10	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB11	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB12	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB13	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB14	P	P	P	P	N	P	N	N	N	P	9	90	0.8000	0.0049
20LAB15	P	P	P	P	N	P	N	**P**	N	N	9	90	0.8000	0.0049
20LAB16	P	P	P	**N**	N	P	N	N	N	N	9	90	0.8000	0.0049
20LAB17	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB18	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB19	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB20	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB21	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB22	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB23	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB24	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB25	P	P	**N**	**N**	N	P	N	**P**	N	N	7	70	0.4000	0.0984
20LAB26	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB27	P	P	P	P	N	P	N	N	N	N	10	100	1.0000	0.0008
20LAB28	P	P	P	**N**	N	**N**	N	N	N	N	8	80	0.6000	0.0192
GOLD	**P**	**P**	**P**	**P**	**N**	**P**	**N**	**N**	**N**	**N**	**-**	**-**	**Overall Agreement**	**Overall p-value**
n° correct answers per vial	26	26	25	23	24	25	24	21	24	23	-	-	0.7298	0.0000
% correct results per vial	100	100	96.2	88.5	92.3	96.2	92.3	80.8	92.3	88.5	-	-	-	-

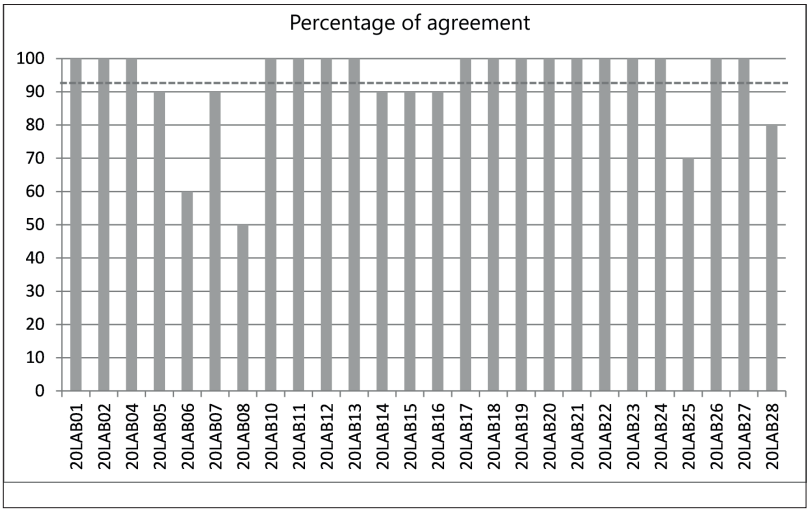

Figure 3. Observed percentage of agreement per laboratory participating to the 3rd VER-IPT. The mean no. of correct results (92.7%) is represented as a dashed line. Median and Mode = 100% are not plotted in the graph.

by all the laboratories, while the remaining samples were wrongly identified by at least 1 participant.

Surprisingly vial no. 8, a negative sample composed of only sterile MEM, was the most frequently incorrectly assigned, scoring only 80% of the correct answer. Vials no. 2 and 6 contained the same virus, namely the reassortant RGNNV/SJNNV strain, and in fact they were both correctly identified by the all the participants, barring one single exception.

Viral species identification

In both editions of VER-IPT, participants were asked to identify the genotype of positive samples according to the procedures in place in their laboratory, although the task was not mandatory. In the 2nd VER-IPT, thirteen out of twenty-nine participants (13/29) corresponding to 44.8% of the total reported a partial (only 1 genetic segment) or a complete (both RNA1 and RNA2) characterisation of the detected viruses. Ten (10) provided viral identification according to both RNA1 and RNA2 genes, while 3 laboratories used the RNA2 gene. Only 2 laboratories provided the correct and complete viral genotype of all the viruses included in the panel.

In the 3rd VER-IPT, eleven laboratories out of twenty-six (11/26) participants, corresponding to 42.3% of the total, identified viral genotype. Out of these, 11 participant provided the RNA2 genes identity, while 9 laboratories provided only the RNA1 gene. Eight (8) laboratories generated complete and correct characterisation of all the viruses included in the panel.

Discussion

The implementation of the 2nd and 3rd VER-IPT was made possible thanks to the support provided by the European project MedAID (Mediterranean Aquaculture Integrated Development, Grant No. 7217315). Among its main objectives this project aims to increase the overall diagnostic capacity of laboratories working in

the Mediterranean region. One of the ways to achieve this goal is the organisation of international proficiency tests. The participation to IPTs is in fact one of the most effective ways to compare the abilities of the participants. On the other hand, IPTs are a test bench for measuring participants skills and provide reference materials to be used for validating or improving their analytical methods. Beside the VER-IPT organisation, under the framework of the MedAID project, a handbook collecting the most updated and reliable laboratory methods for the diagnosis of the main bacterial and viral diseases affecting European sea bass and gilthead sea bream was published (Zrncic, 2020). This manual was made available online free of charge just before the organisation of the 3rd VER-IPT, and it could have been used as reference for the selection of the methods of choice for completing the exercise.

Overall both editions of VER-IPT turned out to be a great success, with the participation of many laboratories coming from all over the world. Noteworthy, the participation to the 3rd VER-IPT was only minimally impacted by the COVID-19 pandemic that affected the world in 2020. Both IPTs were similar in the number and in the composition of the panel, meaning that their results were comparable.

With reference to the 2nd VER-IPT, we can summarise that overall the diagnostic capacity of the participants was good, but with a large gap between the participants scoring very well and those scoring poorly. Significant differences in the capacity of detecting betanodaviruses were also observed among laboratories located in different geographic regions (data not shown). By analysing the incorrect results, it seems that test inclusivity was the major problem of this edition. This may have been related to the inclusion of different genotypes in the panel. As a matter of fact, ampoules containing *Betanodavirus* SJNNV/RGNNV and SJNNV genotypes were the most commonly misdiagnosed. In fact, not all the laboratories chose to test the panel with molecular methods capable of recognising all betanodaviruses species. Our suggestion was then to select, as a first screening method, a molecular protocol capable of recognising all VER genotypes.

With reference to viral species identification, less than half of the participants completed this part of the exercise and only 2 laboratories correctly and completely identified all the viruses included in the panel test.

With reference to the 3rd VER-IPT, more than half of the participants (17/26) obtained the maximum score, while the remaining 9 participants produced a percentage of correct results ranging from 90% to 50%. Noteworthy, if we consider 90% of correct answer as an acceptable level of results, only 4 participants (15.3% of the total) were unable to reach this score. This is overall a very good result, especially if we consider that the majority of the participants had also taken part in the 2nd VER-IPT.

By analysing the incorrect results of this latter edition, it seems that specificity was the major issue. This was somehow expected as negative vials did not contain only sterile saline solution, but complex protein solutions (i.e. obtained by adding yeast extract to the cell culture medium) which could produce non-specific fluorescence signals. Furthermore in the 2020 edition, some participants not only detected fluoresce signals

in negative vials, but they were also able to provide an identification of the viral strain, and this could be attributed only to cross contamination between vials. *Betanodavirus* can reach really high viral loads in the brain of the infected fish (even higher than the viral load present in some of the IPT vials), therefore the prevention of cross-contamination must always be considered a priority when testing these kind of samples.

With reference to viral species identification and if compared to the 2nd VER-IPT, this time an increasing number of participants (n=11) completed this task, and 8 out of them correctly identified all the viruses included in the panel. This means that those who completed the viral identification scored quite well, although their number is still too low to be considered satisfactory.

In conclusion, results from these two editions of the VER-IPT indicated that laboratories working with fish disease overall have a good diagnostic capacity. However, laboratories should work towards improving their capacity to correctly identify the betanodavirus genotype, as viral identification is essential information for the management of the disease in the field.

Since there are several published molecular protocols for the detection of *Betanodavirus* in fish samples, it can be difficult for a laboratory to select the most suitable and best performing one. In this view, the protocols reported in the VER dedicated chapter of the "Diagnostic Manual for the main pathogens in European sea bass and Gilthead sea bream aquaculture" proved to be reliable and reproducible since the laboratories which reported the use of those methods were the best performing ones (personal observation-data not show). Finally, the regular participation of these laboratories to proficiency tests can be an invaluable tool for self-monitoring the quality of their analytical results.

Acknowledgements

The work has received funding from the European Union's Horizon 2020 research and innovation programme MedAID under grant agreement No 72731.

All laboratories participating in the VER-IPT are kindly acknowledged. It is not possible to list all of them, but without their collaboration and suggestions these results could not have been possible. A special thank you goes to Francesca Ellero for administrative and logistic support and to Paola Mozzi for the graphical work.

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