

WORKSHOP REPORT

Bottlenecks in diagnostics of Mediterranean fish diseases

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Abstract

Three independent Horizon 2020 projects are prioritising a common task of solving diagnostic issues using different approaches and strategies. PerformFISH focuses its research objectives on diagnostic methods, MedAID has prepared a diagnostic manual for the most devastating fish diseases of the Mediterranean and ParaFishControl has prepared a diagnostic manual on parasitic diseases of European aquaculture species. Currently, the projects are investing large collaborative efforts to reinforce their tasks and to deliver mutual and cutting-edge material on the subject. Nevertheless, further efforts for the harmonisation, standardisation and validation of diagnostic methods for infectious diseases in Mediterranean aquaculture are needed, involving as many relevant stakeholders and experts as possible. The main goal of the workshop (WS) "Bottlenecks in diagnostics of Mediterranean fish diseases", organised during the 19th International EAFP conference on Diseases of Fish and Shellfish, was to present results already obtained from the projects and discuss the bottlenecks in diagnostic procedures of target groups of pathogens. The WS provided an ideal environment and opportunity to exchange and discuss experiences and data with international experts in the diagnostic field attending the conference. Interactive discussions on diagnostics for a specific group of pathogens have resulted in highlighting new initiatives needed in the management of diseases in Mediterranean aquaculture. Furthermore, the WS emphasised a need for the establishment of this strong interactive network which will collaborate to receive, collect, compile and analyse all information gathered from different parts of the region.

Introduction

Recent reports from different European events discussing Mediterranean marine aquaculture, such as the European Aquaculture Society (EAS) meeting in 2014 and EAFP meeting in 2015, highlighted the importance and industry need for healthier fish throughout the whole production cycle. Stakeholders are engaging tremendous efforts in striving to minimise disease impacts; therefore, disease control needs to be an overall priority for the development and improvement of the Mediterranean aquaculture sector (EATiP, 2014; Vendramin et al., 2016). A key element of the health management strategy is the availability of fast, reliable, efficient and validated diagnostic techniques capable to detect the presence of pathogens and diagnose diseases in fish stocks (Zrnčić et al., 2019).

Two currently active European Horizon 2020 projects, MedAID (Mediterranean Aquaculture Integrated Development) and PerformFISH (Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain), have similar goals to increase the overall competitiveness and sustainability of the Mediterranean marine fish-farming sector throughout the whole value chain, focusing especially on fish health. In addition, a third H2020 project, ParaFishControl (Advanced tools and research strategies for parasite control in European farmed fish), now in its finalising phase, aims to improve understanding of fish-parasite interactions and subsequently develop innovative solutions and tools for the prevention, control and mitigation of farmed fish parasitic diseases.

Interestingly, all three projects working independently from each other have prioritised a similar task of solving diagnostic issues using different approaches and strategies. PerformFISH is focusing its research on diagnostic methods and MedAID and ParaFishControl have prepared diagnostic manuals for the most devastating fish diseases of the Mediterranean. Currently, the projects are investing large collaborative efforts to reinforce their tasks and to deliver mutual and cutting-edge material on the subject. Despite this cooperation, further efforts for the validation of diagnostic methods of infectious diseases in Mediterranean aquaculture are needed, involving as many relevant stakeholders and experts as possible. In addition, harmonisation and standardisation of recommended procedures is necessary to enable the generation of meaningful, homogenous and reliable data from the field that is comparable among each other.

Considering future work that should still be done, the workshop “Bottlenecks in diagnostics of Mediterranean fish diseases” was arranged during the 19th International EAFP conference with collaboration amongst the three projects. The main goal of the workshop was to present results already obtained from the projects and discuss the bottlenecks in diagnostic procedures of target groups of pathogens. The workshop provided an ideal environment and opportunity to exchange and discuss experiences and data with international experts in the diagnostic field attending the conference. Such an interactive discussion on diagnostics for a specific group of pathogens will result in streamlining the future work in the ongoing projects and also point out new initiatives needed in the management of diseases in Mediterranean aquaculture.

Workshop organisation

The workshop was organised by members of the three projects and was an open event for all conference delegates. A total of 75 fish health experts originating from aquaculture farming companies, private veterinary practitioners, academia, diagnostic laboratories, National Reference Laboratories (NRLs) as well as early career scientists from the Mediterranean and non-Mediterranean countries such as Canada, Denmark, Ireland, Korea, Norway, Poland, The Netherlands, UK, Ukraine, and USA, participated in the event. The workshop was organised into four sessions:

1. Introduction and state of art

Diagnostic needs with respect to viral, bacterial and parasitic diseases in the Mediterranean basin, summarised from the work carried out in the three projects, were presented by Padrós. He highlighted how the three projects converged in a mutual endeavour to define the diagnostic needs in Mediterranean marine aquaculture. The goal of this was to understand the status of the diseases and to meet the industry needs, but also to assess the obstacles of different approaches applied in different countries/regions of the Mediterranean basin. The strategy to develop accurate and reliable diagnostics was based on components at three different levels:

a) Fieldwork is the first level where it is essential to evaluate the problem by implementing a holistic approach. By doing this, the choice of diagnostic procedures should be based on in-depth disease understanding and known epidemiological management. Sampling is a key element of accurate diagnostics that raises different questions such as where to

sample, which are appropriate hosts species or tissues to be sampled, appropriate sample size, sample packaging and packing, and transportation to the diagnostic laboratory.

b) Laboratory work depends on the technical capacity and the expertise of the staff. The level of pathogen detection is based on diagnostic tools employed and the criteria for a specific test that should be repeatable, reproducible, sensitive and specific, with high recovery and detection efficiency, as well as being fast and within an acceptable price range. Laboratory results should be robust, validated and reliable, and in respect to customers, accurate, confidential and at a moderate price.

c) Prescribing the solution for disease management should be based on accurate laboratory results and proper interpretation. An important remark is that data generated from industry related to fish diseases may require confidentiality, therefore legal aspects should be taken into consideration when disseminating such laboratory results. Currently all three projects have accomplished specific tasks and foreseen activities concerning the identification of different approaches to be considered at the fieldwork, laboratory work and solution prescription levels; the improvement of protocols and methodologies; and the new approaches to be developed in the future.

For the second part of the WS, updates on the diagnostic developments achieved in the three projects was presented, enabling the discussions by multistakeholders that followed.

2. Presentation of project goals and results

a) Presentation of the "Diagnostic manual for the main pathogens in the European sea bass and gilthead sea bream aquaculture" prepared by the MedAID consortium was given by Zrnčić.

The manual is conceived as a handbook for use by both field and laboratory specialists. It includes instructions for sampling, sample packaging/packing and delivery, receipt of samples, and it describes the requirements for virological and bacteriological laboratories, detailed protocols for diagnostics of the most important and emerging viral and bacterial pathogens, procedures in the case of mortalities caused by unknown pathogens, laboratory sensitivity and specificity testing, as well as guidelines for reporting the laboratory results. The manual will be updated continuously with new techniques and most likely with new pathogens.

b) The Diagnostic manual on parasitic diseases of European aquaculture species was presented by Palenzuela. This manual is conceived as a repository of recommended protocols for the diagnosis of the main parasites of concern in European aquaculture, and it is the direct result of one of the deliverables of the ParaFishControl project. In addition to the parasites and new methods developed or validated in the project, an effort has been put towards the coverage of additional parasites of interest, building a more comprehensive source of information on the matter. An agreement for the publication of this manual as an Open Access book is being arranged.

c) Mutual results of MedAID and PerformFISH on the evaluation of "Diagnostic capacities in Mediterranean basin" were presented by Fio-

ravanti. Special focus was placed on the results of an online questionnaire survey carried out to:

Set up a database of fish health experts working in diagnostics of marine fish diseases in Mediterranean aquaculture, to facilitate communication and collaboration activity; and

Define diagnostic capacities and methodologies applied for the most relevant pathogens, to give the basis for optimisation of diagnostic techniques.

In total 55 laboratories/fish health experts filled in the questionnaire answering five groups of questions: 1) general information on the laboratory; 2) methods applied to diagnose parasitic diseases; 3) methods applied to diagnose bacterial diseases; 4) methods applied to diagnose viral diseases; 5) other methods applied to diagnose all groups of aforementioned pathogens, such as histology, immunohistochemistry or other immunological methods, *in situ* hybridisation and NGS. Respondents originated from private and public bodies of mainly European and non-European Mediterranean countries. Interestingly, the lower rate of diagnostic capacities in some non-European Mediterranean countries contrasts with their high production volumes, potentially representing a significant regional risk that consequently points towards the necessity to address health management in the Mediterranean region in a more holistic, cooperative and harmonised way.

3. Working groups discussion

Participants were divided into four working groups according to their expertise or interest in specific diseases: viral nervous necrosis (VNN); *Tenacibaculum* spp. & *Vibrio* spp.; parasitic dis-

eases; and diseases of unknown origin. Working groups discussions were moderated by experts for each particular disease group. Moderators agreed on the discussion scheme as follows: Methods currently used (clinical examination, sampling, necropsy, screening and confirmatory methods);

- Methods sensitivity under different circumstances and fish age, with listing of weaknesses of the methods applied.
- Critical issues that should be improved.
- General conclusions and remarks.

3.1. Viral Nervous Necrosis (VNN) discussion

Group discussing VNN was moderated by Toffan, Panzarin and Ciulli and the group elaborated the following remarks.

In general, laboratories involved in the detection of VNN are mostly using methods appropriate for the detection of pathogen virus (Toffan, 2018). Different PCR protocols are in use, such as reverse transcription-PCR (RT-PCR), nested RT-PCR, real-time RT-PCR and LAMP (Loop-mediated isothermal amplification) RT-PCR. Real-time RT-PCR assays are the most largely applied protocols due to their sensitivity and fast execution, and some are specific for a single genotype (Grove et al., 2006; Hick and Whittington, 2010), while others can detect all known viral genotypes (Dalla Valle et al. 2005; Panzarin et al., 2010; Hodneland et al., 2011; Baud et al., 2015). Since there is room for improvement in the harmonisation of methods, this has already been initiated under the MedAID project's interlaboratory proficiency testing for detection of nervous necrosis

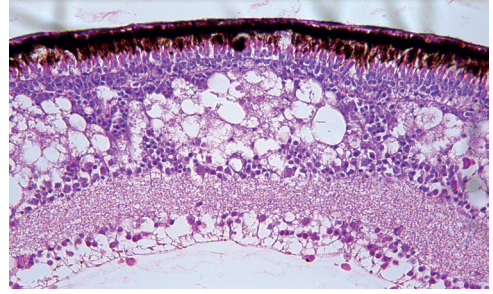


Figure 1. Vacuolisation of Gilthead sea bream (*Sparus aurata*) retina infected with nervous necrosis virus (NNV).

virus (NNV)(Figure 1) and differentiation of its genotypes (Toffan et al., 2018). Indeed, a rapid and reliable genotyping method is advisable to identify the viral genotype, which can help to know strain features (e.g. host range, optimal temperature) and to arrange the best control strategy. Another important future need is the development of a rapid test for the field diagnosis. Until now few are available, and all of them show low sensitivity. Surveillance is carried out on both larvae and broodstock, but time and frequency of sampling are variable in different countries (e.g. in Greece NNV is suspected only when the water temperature is $>26^{\circ}\text{C}$), which may create differences in the sensitivity of the surveillance programs. Non-lethal sampling methods for screening broodstock are lacking.

Enzyme Linked Immunosorbent Assay (ELISA) tests aiming to detect specific antibodies could be useful in broodstock screening, but it has the limitation of needing to be repeated more than once to give reliable outcomes. Vaccines are broadly used in Greece (80% of the farms are vaccinated), only sporadically in Italy, while they are not used at all in Croatia.



Figure 2. Lesion on the skin of European sea bass (*Dicentrarchus labrax*) infected with *Tenacibaculum maritimum*. Courtesy of Dražen Oraić, Croatian Veterinary Institute, Zagreb.

Moreover, at present, there is no method to differentiate antibodies of infected from vaccinated fish and the development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy could improve significantly the usefulness of the vaccination approach.

3.2. Discussion on the bacterial diseases

Tenacibaculum spp. and *Vibrio* spp. infections were moderated by LeBreton and Fouz.

3.2.1. Diagnostics of *Vibrio* spp.

Generally, laboratory capacities for diagnostics of *Vibrio* spp. infections (vibriosis) are overall satisfactory, mainly those caused by *V. anguillarum* and *V. vulnificus* (Haenen et al., 2014). For *Vibrio* spp. infections clinical signs are usually pathognomonic with haemorrhages on the skin, fin basis, internal organs such as liver, intestine etc. However, in case of *V. harveyi*, clinical signs are obvious above 20°C and specific ones such as neurological signs and excess mucus in the intestine have been reported (Lee et al., 2002). This species is relevant because it causes emerging vibriosis diseases in European sea bass *Dicentrarchus labrax* (Fouz et al., 2017). Isolation of the bacteria from kidneys on marine agar (MA) and Tryptic

soy agar (TSA) supplemented with 1 % NaCl is appropriate for isolation, the same for all vibrios. For *V. harveyi* additional samples should be taken from the brain (Pretto, 2018).

V. harveyi identification using only phenotypic methods (biochemical properties, API identification system or similar multi-test tools) may lead to misidentification with *V. alginolyticus* and other closely related vibrios from the *V. harveyi* clade. MALDI TOF could be a useful tool but more reference strains of *V. harveyi* and the *V. harveyi* clade need to be included into the database. Specific PCR methodology targeting the *toxR* gene is a highly specific method (Pang et al., 2006).

Vaccination trials for *V. harveyi* are in progress with serologically different isolates in several countries and it seems that intra/peritoneal application is required.

3.2.2. Diagnostics of *Tenacibaculum* spp.

The suspicion of tenacibaculosis is based on clinical signs and stained smears of the lesions in gills or skin. Diagnostics should be done by swabbing the lesions and seeding material inoculating onto FMM (*Flexibacter maritimum* medium) agar or

MA for bacterial cultivation (López et al., 2011). The identification of the genus is possible using API while species are identified by MALDI TOF (Fernandez-Alvarez et al., 2017) or 16S rRNA sequencing (Fernández-Alvarez & Santos, 2018).

Furthermore, some PCR protocols for *Tenacibaculum maritimum* (Figure 2) identification exists, but show different specificity (Fernandez-Alvarez and Santos 2018).

Antimicrobial sensitivity testing is carried out on FMM agar prepared with seawater and results can be obtained in two days.

Isolation of bacteria from the genus *Tenacibaculum* is not simple and seeding of the bacterial media should be optimised. Currently, the best results in the cultivation of this fastidious bacteria are obtained by taking swabs that should be sent into a transport medium (with gentamycin) for cultivation and identification in the laboratory (Le Breton, 2019).

3.3. Parasitic diseases discussion

Moderated by Palenzuela , Mladineo and Gustinelli.

Among the wide array of endo- and ectoparasites affecting Mediterranean aquaculture fish, the monogenean *Sparicotyle chrysophrii* seems to be the most detrimental. Although identification or diagnostics of *S. chrysophrii* in the field is straightforward by the naked eye or stereomicroscope (Figure 3), it is essential that the number of eggs, juveniles and adults is assessed in time to prevent outbreaks of clinical disease with the loss in fish weight and subsequent mortality. Even if industry already employs periodic enumeration of

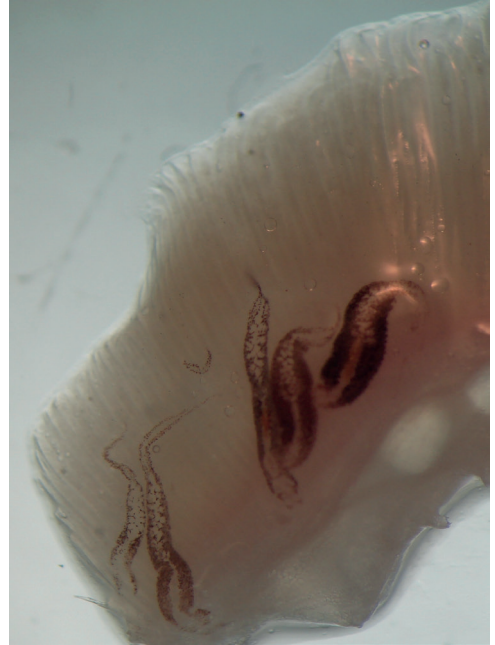


Figure 3. *Sparicotyle* spp. parasiting on the gills of Gilthead sea bream (*Sparus aurata*).

monogenean adults on certain gill arches in order to take appropriate measures to contain or treat the infection (Mladineo, 2018), the diagnostic approach should be harmonised across Mediterranean regions, setting up a shared standardised quantitative method aimed at scoring the infection level. Furthermore, indirect methods (e.g. blood parameters, gross signs, etc.) seems to be necessary to better assess the progress and impact of the disease.

In the case of intestinal parasites, in particular the microsporidian *Enterospora nucleophila*, (Palenzuela et al., 2014) and the coccidians, which are difficult to diagnose in routine analyses, more economic, fast and reliable methods should be developed and applied.

In conclusion, the prerequisites for successful diagnostics of all parasitic infections are:

Definition of tools for assessing the real-time and tangible impact of parasites;

Increased understanding and recognition of the importance of some parasites by industry and veterinary practitioners, which could also be facilitated through training activities;

Implementation of a targeted surveillance designed for specific areas that would ultimately encompass the whole heterogeneous Mediterranean area.

3.4. Diseases of unknown origin

Moderated by Varvarigos and Zarza.

Due to the need for emergency activity, the discussion was mostly focused on 'petechial rash'. It was unanimously agreed that the following major points apply to marine fish petechial rash as well as rainbow trout red mark syndrome (RMS):

Petechial rash (commonly called red spot) (Figure 4) is important in several countries (mainly Greece, Italy and Spain) because it affects several species (e.g., gilthead sea bream *S. aurata*, European sea bass *D. labrax*, sharp snout bream *D. puntazzo*) with a negative impact on production (Schmidt et al., 2018);

The aetiology of the disease is still obscure; it is known that affected fish self-heal and that the symptoms may reappear in the same cage that had previously been affected.

Due to the lack of knowledge in respect to the causative agent, there is no diagnostic procedure for its confirmation;

Diagnosis is empirical, based on clinical appearance and epidemiology. There are no diagnostic tools to confirm the disease beyond doubt; the efforts focus on excluding other potential causes, such as handling trauma or pathogens associated with ulceration (e.g. *Aeromonas* spp., *Mycobacterium* spp.)

There is a need for systematic studies including histology and electron microscopy, in order to reveal and isolate the responsible pathogen. The association of a specific pathogen with the disease causing lesions observed in rainbow trout affected with RMS has been achieved, and the application of similar methodology that led to this understanding might provide useful data with marine fish;

As for RMS, which is an intracellular pathogen, oxytetracycline (OTC) proves helpful in most cases, although results following treatment are inconsistent across farms. Treatments are empirical, without the knowledge of OTC's function;

Funds are needed to focus research on Petechial rash as was done for RMS in trout. Research should be collaborative and scientists from different Mediterranean countries should coordinate work on diseased fish from different Mediterranean locations

4. Discussion and conclusions

The high number of attendees of the workshop illustrated the interest and relevance of the topics presented. The enthusiastic discussions in working groups further emphasised this interest. There is clearly a need to facilitate such a platform where diagnosticians and other experts can sit together and discuss diagnostics



Figure 4. Red rash syndrom in Gilthead sea bream (*Sparus aurata*).

at a practical level. MedAID has established an online forum to facilitate such a platform, an approach that needs to be further developed to become sustainable.

Throughout the Mediterranean, the aquaculture industry experiences many of the same diseases between different countries, although to a different degree. Being economically significant, aquaculture constantly transports live animals and equipment within and between EU- and non-EU Mediterranean boundaries, although the countries involved reported different capacities regarding fish health management and biosecurity. In-line with this, particular oceanographic, geographic, environmental and socioeconomic characteristics of the Mediterranean Sea and basin have significant implications in many epidemiological aspects of fish diseases in both wild and farmed species.

The issue of low diagnostic capacities in certain countries, which contrasts to their high production volumes, represents a significant regional risk that reinforces the necessity to

address health management in the Mediterranean region in a more holistic, cooperative and harmonised way. Furthermore, regional initiatives should strive to equalise the diagnostic capacities through capacity building in high producing countries.

The discussions on four topics revealed the need for updating on those specific diseases, as well as to harmonise terminology and applicable diagnostics. Establishing a network of experts in diagnostics and harmonised diagnostic methods, as well as respective protocols, are necessary steps towards a holistic health management approach. Moreover, this is also important in the context where these relevant pathogens are not listed/ notifiable at present. Understanding the diseases at sub regional, but also whole Mediterranean level, disease management and the performance of harmonised and trustworthy diagnostics is an essential platform to improve the health situation in farmed fish and the sustainable aquaculture industry. However, essential as it is, the work done through the current Horizon 2020 projects on diagnostic manuals and mapping of diag-

nostic capability is only a first step that needs to be followed up by supplementary support and continuous networking.

There are several key issues, which are beyond the capability of the activities of Horizon 2020 projects, that are markedly arising from the evaluation of the current situation in the Mediterranean basin. There are many different fish health experts working in their area of scientific or industrial interest, and developing the diagnostics required in either bacteriology, virology or parasitology. The outputs of the mentioned project will facilitate the improvement of diagnostic capacities in all fields, either by providing diagnostic protocols through diagnostic manuals, or through the educational workshops on different health issues. Still, there is a major need for the establishment of a strong interactive network which will receive, collect, compile and analyse all information from different parts of the region. In this manner the clear trends in disease impact, spreading, and diagnostic capacity will be visible and it would therefore be possible to pave the way to removing the gaps and obstacles currently facing successful regional health management.

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