Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture

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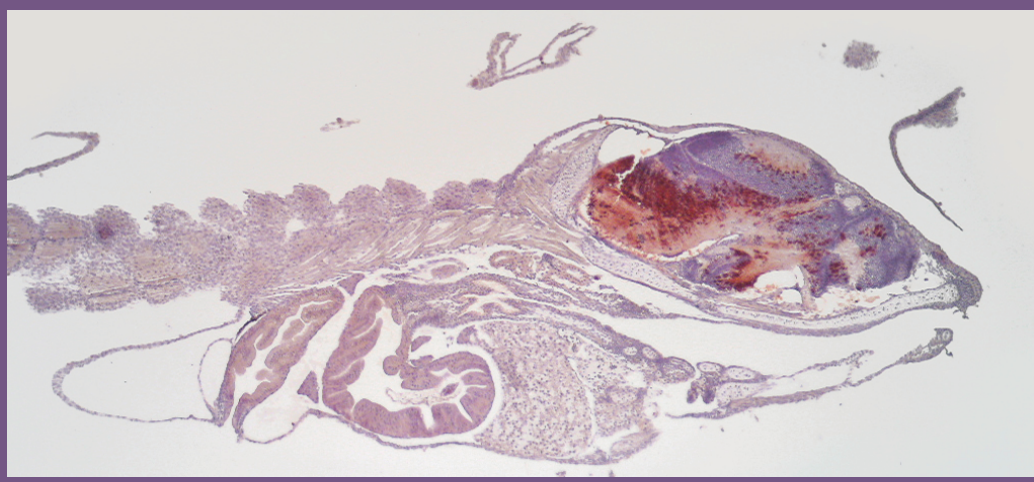

OPTIONS méditerranéennes

SERIES B: Studies and Research
2020 - Number 75

CIHEAM

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This project has received funding from the *European Union's Horizon 2020 research and innovation programme* under grant agreement No 727315.

OPTIONS
méditerranéennes

Head of publication: Plácido Plaza

2020 | Series B: Studies and Research | Number 75

Centre International de Hautes Etudes Agronomiques Méditerranéennes
International Centre for Advanced Mediterranean Agronomic Studies

L'édition technique, la maquette et la mise en page de ce numéro d'Options Méditerranéennes ont été réalisées par l'Atelier d'Édition de l'IAM de Zaragoza (CIHEAM)

Technical editing, layout and formatting of this edition of Options Méditerranéennes was carried out by the Editorial Board of MAI Zaragoza (CIHEAM)

Crédits des photos de couverture / *Cover photo credits*:
Tobia Pretto (Istituto Zooprofilattico Sperimentale delle Venezie)
Tirage / *Copy number*: 400 ex.
Printer: INO Reproducciones, S.A.
Pol. Malpica, calle E, 32-39
(INBISA II, Nave 35)
50016 Zaragoza-Spain
Dep. Legal: Z-2893-91

Fiche bibliographique / *Cataloguing data*:

Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture. S. Zrnčić (ed). Zaragoza: CIHEAM 2020, 174 p. (*Options Méditerranéennes*, Series B: Studies and Research, No. 75)

Catalogue des numéros d'Options Méditerranéennes sur / *Catalogue of Options Méditerranéennes issues on*:
www.ciheam.org/publications

ISSN: 1016 -1228 – ISBN: 978-2-85352-596-1

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Foreword

MedAID (Mediterranean Aquaculture Integrated Development) is a European H2020 Research and Innovation Project approved in 2017, with the main objective of increasing the overall competitiveness and sustainability of the whole value chain of the Mediterranean marine aquaculture sector by improving its technical and business performance and by shifting to a sustainable market-oriented approach with a higher social and consumer perception. In the field of disease control and welfare (Work package 4), MedAID aims to provide essential components for a better health and welfare management system for the Mediterranean marine aquaculture industry both at an overall and at a company level. The development and dissemination of up-to-date diagnostic manuals and media platforms will allow an efficient networking between stakeholders and experts, and a diagnostic harmonisation at Mediterranean level.

Although many publications exist on the diagnostics of aquatic animal diseases, there is a lack of reference diagnostic methodologies for seabass and seabream, the main species produced in Mediterranean marine fish farming. This gap is considered an obstacle for further development of the industry and for designing and coordinating harmonised health management strategies in the different regions of the basin. A possible reason for this constraint may be that seabass and seabream are not listed as susceptible host species for the notifiable pathogens listed in the current EU legislation, or in the list of diseases notifiable to the OIE, which generates a lack of approved diagnostic methods. Although not notifiable, only viral encephalopathy and retinopathy, due its importance, is included in the OIE Diagnostic Manual for Aquatic Animal Diseases.

Thus, a "Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture" was conceived as up-to-date guidelines providing the standardised methods enabling a harmonised approach to the health challenges due to viral and bacterial pathogens in the farming of seabass and seabream. The parasitic diseases are omitted in this document to avoid overlapping with the H2020 project ParaFishControl, dealing solely with diagnostic procedures for parasitic diseases.

In Europe and Mediterranean countries there are many research and diagnostic laboratories, consultants and practitioners involved in diagnostic activities of seabass and seabream. Their existence and known willingness for cooperation have made this publication possible, which besides being an output from the MedAID project, has counted on the support of the European Association of Fish Pathologists (EAFP). Experts that participate in MedAID and others willing to collaborate have contributed to the different chapters of the Manual. We take this opportunity to express our gratitude to them.

Finally, we specifically wish to thank the Editor of the publication, General Secretary of EAFP, Dr. Snježana Zrnčić of Croatian Veterinary Institute, Zagreb (HVI). This Manual would not have been possible without the coordination, and exhaustive and meticulous work she has carried out.

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Abbreviations

ADH	Arginine dihydrolase
ADR	European Agreement concerning the International Carriage of Dangerous Goods by Road
AFLP	Amplified Fragment Length Polymorphism
AIA	Actinomycete isolation agar
API	Analytical Profile Index
ASK	Atlantic salmon kindney
BA	Blood agar
BF-2	Bluegill fins cell line
BFNNV	Barfin Flounder Nervous Necrosis Virus
BHI	Brain heart infusion agar
BLAST	Basic Local Alignment Search Tool
BNF	Buffered- neutral formalin
BSA	Bovine serum albumin
CD	Council Directive
CFU	Colony forming unit
CHAB	Cysteine enriched blood agar
CHSE	Chinook salmon embrio
CIT	Citrate
CLSI	Clinical and Laboratory Standards Institute
CPE	Cytopathic effect
CAMHB	Cation supplemented Mueller Hinton broth
Ct	Cycle threshold
CV	Coefficient of variation
DEPC water	Diethyl pyrocarbonate - used to inactivate RNase enzymes in water and on laboratory utensils
DMHA	Diluted Mueller Hinton agar
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotid triphosphate
E-11	Clone of SSN-1 cell line
EAFP	European Association of Fish Pathologists
EAS	European Aquaculture Society
EATIP	European Aquaculture Technology Innovative Platform
EC	European Community
ECOFFinder	Epidemiologial cut off values for MICs
EEC	European Economic Community
EPC	*Epithelioma papulosum cyprinid* cell line
EPCs	Extra cellular products
ERIC-PCR	Enterobacterial repetitive intergenic consensus
EU	European Union
EURL	European Union Reference Laboratory
FAO-GFCM	Food and Agriculture Organisation-General Fisheries Commission for Mediterranean
FAT	Fluorescent antibody technique
FCS	Fetal Calf Serum
FHM	Fathead minnow cell line
FMM	*Flexibacter maritimum* media

FreeCalc - EpiTools	Epidemiological calculators
GF-1	Orange spotted grouper fin cell line
GMO	Genetically modified microorganism
H2020	Horizon 2020- EU Research and Innovation programme
IATA	International Air Transport Authority
ICAO	International Civil Aviation Organisation
ID	Identity
IEC	International Electrotehnical Commission
IF	Immunofluorescence
IFAT	Indirect fluorescent antibody techniques
IHC	Imunohistochemistry
IHNV	Infectious heamatopoietic necrosis virus
IPNV	Infectious pancreatic necrosis virus
ISO	International Organization for Standardization
ISO/IEC 17025	General requirements for the competence of testing and calibration laboratories
ISR	Intergenic spacer region
IU	International Units
LCD	Lymphocystic disease
LCDV	Lymphocystic disease virus
LDC	Lysin decarboxylase
LIMS	Laboratory information management system
LPS	Lipopolysaccharide
MA	Marine agar
Mabs	Monoclonal antibodies
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MARLAP	Multi-Agency Radiological Laboratory Analytical Protocols
MB	Marine Broth
MEM	Minimal Essential Medium
MGG	May Grünwald Giemsa
MHA	Mueller Hinton Agar
MIC	Minimal Inhibitory Concentration
MIRUs	Mycobacterial Interspersed Repetitive Units
MLSA	Multi locus sequence analysis
NA	Nucleic acid
NCBI	National Centre for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
NGS	Next Generation Sequencing
NNV	Nervous necrosis virus
NPV	Negative predictive value
NRI	Normalised Resistance Interpretation
NTB	Non tuberculous mycobacteria
ODC	Ornithine decarboxylase
OIE	World Organisation for Animal Health
OMPs	Outer membrane proteins
ONPG	β galactosidase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PCR ELISA	Polymerase Chain Reaction Enzyme Linked Immunoabsorbent Assay

PCR-RFLP	Polymerase chain reaction - restriction fragment length polymorphism
Phdd	*Photobacterium damselae* subsp. *damsela*
Phdp	*Photobacterium damselae* subsp. *piscicida*
PMCV	Piscine myocarditis virus
PPV	Positive predictive value
PRV	Piscine Orthoreovirus (subtypes 1,2 and 3)
qPCR	Real time polymerase chain reaction
RAL 555	May Grünwald Giemsa staining
RAPD	Random Amplification of Polymorphic DNA
RDP	Ribosomal Database Project
REP-PCR	Repetative element palindromic PCR
RGNNV	Red Grouper Nervous Necrosis virus
RNA	Ribonucleic acid
RSIV	Red Sea bream irido virus
RT	Room temperature
RT PCR	Reverse transcription polymerase chain reaction
RT-PCR-EHA	Reverse transcriptase polymerase chain reaction-enzyme hybridisation assay
RTG-2	Rainbow trout gonads cell line
Se	Sensitivity
SJNNV	Stripped Jack Nervous Necrosis virus
SOP	Standard operating procedure
SOR	Sorbitol
Sp	Specificity
SSN-1	Stripped snakehead cell lines
SUC	Saccharose
SVA	Swedish National Veterinary Institute
TBST	Tris Buffered Saline with Tween
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
$TCID_{50}$	Tissue culture infective dose, the amount of pathogenic agent that will produce pathological change in 50% of cell cultures inoculated
TDA	Tryptophan deaminase
TPNNV	Tiger Puffer Nervous Necrosis Virus
Tris-HCl	Tris (hydroxymethyl)aminomethane-HCl
TSA	Tripton Soy Agar
TSB	Tripton Soy Broth
UN 2814	Infectious substances affecting humans
UN 2900	Infectious substances affecting animals
UN 3245	Label for transportation of GMO organisms or microorganisms
UN 3373	Human or animal materials that are being transported only for the purpose of diagnosis or investigation
URE	Urease
USA	United States of America
VBNC	Viable but not culturable
VER	Viral encephalopathy and retinopathy syn. VNN
VetMIC	MIC based system for antimicrobial sensitivity testing of bacteria
VHSV	Viral haemorrhagic septicaemia virus
VNN	Viral Nervous Necrosis syn. VER
VNTR	Variable Tandem Repeats

VP	Voges Proskauer test
VTM	Viral transport media
WHO	World Health Organisation
WinEpi	Working in epidemiology. An online epidemiological tool
WP	Work package

Introduction

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A number of examples in recent history have shown how “diseases shape aquaculture”. Relevant examples to be mentioned are the outbreak of infectious salmon anemia in Chile in 2007 and the Faroe Islands in 2000; viral haemorrhagic septicaemia in Denmark in the 1980s and its eradication campaign which followed in 2009 and the occurrence of white spot syndrome virus in Southeast Asia in 1994.

The MedAID project aims to improve key performance indicators of Mediterranean mariculture and envisage the concept of healthy fish for sustainable production, considering health and welfare as prerequisites for sustainable and profitable aquaculture in the Mediterranean area. Recent reports of the different regional events discussing Mediterranean marine aquaculture, such as the European Aquaculture Society (EAS) in 2014 and the European Association of Fish Pathologists (EAFP) in 2015, showed that the industry needs healthier fish throughout the whole production cycle. Therefore diseases are an overall priority for the development and improvement of the Mediterranean aquaculture sector (European Aquaculture Technology Innovative Platform, EATiP 2014; Vendramin *et al.,* 2016.

The current European legislative framework for control of aquatic animal diseases refers primarily to Council Directive 2006/88. This directive covers the whole aquaculture production giving general guidelines on disease control and providing some specific provisions for certain diseases.

European seabass and gilthead seabream are not currently listed as susceptible species to any listed disease and thereby are not targeted by specific provisions for the listed diseases. Nevertheless, the Directive on at least 3 occasions describes the opportunity for Member States to design and implement *ad hoc* measures for specific diseases considered relevant for national aquaculture.

Starting from pre-requisition 30, CD 2006/88 envisages the implementation of the national programme and additional guarantees for disease not subject to Community measures.

“For diseases not subject to Community measures, but which are of local importance, the aquaculture industry should, with the assistance of the competent authorities of the Member States, take more responsibility for preventing the introduction of or controlling such diseases through self-regulation and the development of ‘codes of practice’. However, it may be necessary for the Member States to implement certain national measures. Such national measures should be justified, necessary and proportionate to the goals to be achieved. Furthermore, they should not affect the trade between the Member States unless this is necessary in order to prevent the introduction of or to control the disease, and should be approved and regularly reviewed at the Community level. Pending the establishment of such measures under this Directive, the additional guarantees granted in Commission Decision 2004/453/EC of 29 April 2004 implementing Council Directive 91/67/EEC as regards measures against certain diseases in aquaculture animals (3) should remain in force”.

Article 10 of this Directive broadly includes all aquaculture production and delegates the establishment of a risk-based animal health surveillance scheme to each Member State.

"Animal health surveillance scheme

1. The Member States shall ensure that a risk-based animal health surveillance scheme is applied in all farms and mollusc farming areas, as appropriate for the type of production.

2. The risk-based animal health surveillance scheme referred to in paragraph 1 shall aim at the detection of: (a) any increased mortality in all farms and mollusc farming areas as appropriate for the type of production; (b) the diseases listed in Part II of Annex IV, in farms and mollusc farming areas where species susceptible to those diseases are present". Finally, article 43 specifically address

"Provisions for limiting the impact of diseases not listed in Part II of Annex IV

1. Where a disease not listed in Part II of Annex IV constitutes a significant risk for the animal health situation of aquaculture or wild aquatic animals in a Member State, the Member State concerned may take measures to prevent the introduction of or to control that disease. Member States shall ensure that these measures do not exceed the limits of what is appropriate and necessary to prevent the introduction of or to control the disease.

2. Member States shall notify to the Commission any measures referred to in paragraph 1 that may affect trade between Member States. Those measures shall be subject to approval in accordance with the procedure referred to in Article 62(2).

3. Approval referred to in paragraph 2 shall only be granted where the establishment of intra-Community trade restrictions is necessary to prevent the introduction of or to control the disease, and shall take into account the provisions laid down in Chapters II, III, IV and V".

The possibility to apply specific national regulations to prevent the introduction, or to control the spread of diseases that, despite not being included in the EU legislation as such, constitute a significant risk for the health of aquatic animals in a Member State, is a criteria which is also included in the new Animal Health Law, which will be implemented starting from December 2019. (Article 171 Regulation (EU) 2016/429)

To the best of the authors' knowledge, no national surveillance programmes have been implemented to date for these fish species (Vendramin *et al*., 2016).

Many relevant players are involved in the health management of seabass and seabream in the Mediterranean, including research and diagnostic laboratories, private testing laboratories, consultants and practitioners involved in diagnostic activities, national reference laboratories and official veterinary officers.

In the current circumstances, each player usually has a narrow focus either for the area of interest or for a specialty in terms of diagnostics (either bacteriology or virology or parasitology). There is a need for a centre to receive, collect, compile and analyse all information in order to have clear figures of production trends, the impact of diseases and so forth. This gap is an obstacle for further development of the industry and for designing and coordinating harmonized strategies in the different regions of the basin.

MedAID (WP4, health management and diseases and fish welfare) is endeavouring to provide tools and common strategies for the prevention and diagnosis of major diseases by creating an operative and collaborative platform at Mediterranean level. This platform will produce codes of good practice and harmonized standards for integrated health management through the establishment of a network of laboratories capable of obtaining a proper diagnosis in case of known pathogens and support in case of emerging or aetiologically unsolved diseases.

Currently, technical and economic constraints have driven the aquaculture industry to prioritize production rather than disease control and efforts from stakeholders in health management are striving to minimize the incidence of disease. One of the key elements of the health

management strategy is fast, reliable, validated and efficient diagnostic techniques capable of a timely detection of the health threat.

The "Diagnostic manual for the main pathogens in European seabass and gilthead seabream aquaculture" is conceived as an instrument to provide up-to-date guidelines and standardized methods enabling the harmonized approach to the health challenges due to viral and bacterial pathogens in seabass and seabream farming.

Parasitic diseases are currently addressed by ParaFishControl, another H2020 project, to take advantage of the synergies between the two projects.

The list of relevant diseases was agreed during the meeting of the WP4 partners held in Zagreb, on 8th and 9th November 2018. The selection was based on the result of the survey questionnaires conducted among experts during the past few years by the EU Reference Laboratory (EURL) for fish diseases, the report of the MedAID questionnaire output on the prevalence of diseases and their impact on production as well as the national presentations during the FAO-GFCM workshop on animal health and risk analysis in finfish aquaculture, Larnaca, Cyprus, 3rd and 4th October 2018. The approach adopted is to describe in detail the existing diagnostic and standardized procedures, suggest improvements or enable the introduction of new methods for diagnosing the specific disease. A specific focus of MedAID has been the viral nervous necrosis for which the diagnostic capacity of European laboratories has been further evaluated through Inter laboratory proficiency testing (Toffan *et al.,* 2018)

The Manual is designed as follows:

- Part I "Sampling procedures" provides guidelines and instructions for: a) on-farm sampling for targeted surveillance in order to certify freedom from a specific disease; b) diagnosis in case of mortalities; c) analysis of mortalities caused by unknown aetiology; d) packing and shipping of samples; e) laboratory receiving the samples.
- Part II "General requirements for the laboratory methods" provides detailed information about the organization, equipment needed and management of a diagnostic laboratory with a focus on specific techniques (bacteriology, virology, molecular methods) which will enable the establishment of a reliable and competent diagnostic unit.
- Part III "Viral diseases with impact to the Mediterranean fish farming" deals with general principles of viral disease management and acquaints the reader in detail with all steps in setting up the diagnostics for VNN.
- Part IV "Bacterial diseases with impact on sea bass and sea bream farming" informs about several most important bacterial diseases affecting seabass and seabream farming describes the aetiological agents and available validated methods of screening, isolation, identification and confirmation of these pathogens.
- Part V "Mortality caused by unknown aetiology" describes the diagnostic procedure in the case of disease outbreaks which may not be attributed to any known causative agents.
- Part VI "Interpretation and reporting of results" gives instructions on how to communicate to the stakeholders the results of diagnostic procedures applied.
- Part VII "Annexes" which includes summary sheets of main diseases, list of contacts and a template for submission form.

The Manual will provide useful contact information to the OIE and EU reference laboratories as well as contacts of MedAID partners dealing with European seabass and gilthead seabream health.

It is hoped that this Manual will assist the public and private diagnostic laboratories, consultants and on-farm health managers in setting up a harmonized approach to the diagnostics of fish

diseases throughout the Mediterranean basin. It is opted to be a basis for collaboration, harmonization and transparency to prevent infectious diseases. Such an achievement will mirror the concepts in the Scandinavian countries where collaborative health management programmes between industry-authorities-research have been necessary for the control and eradication of infectious diseases.

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Part I. Sampling procedures

1. Guidelines for on-farm sampling for targeted surveillance to certify disease freedom, diagnosis in case of mortalities and for analysis of mortalities caused by unknown aetiology

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1.1. Introduction

Sampling on the farm is an important first step for assessing the disease situation within a group of animals, a farm, a region or a country. Although it might be possible to sample and analyse all animals in question for a small population like a group of broodstock of some hundred fish, this is not achievable or cost-effective for populations with tens of thousands of individuals. A sampling strategy designed according to the purpose of any particular investigation is therefore fundamental for gathering the information we are aiming for, and to ensure that the conclusion about the population drawn from the samples is statistically valid. Depending on the purpose, sampling can be performed randomly (probability sampling) in order to obtain samples that give representative information about a population or a subpopulation, or sampling can be non-random or purposive (non-probability sampling) in order to increase the probability of finding the condition of interest such as the detection of disease.

A thorough description of sampling principles and sampling applications is given in the survey toolbox for aquatic animal diseases by Cameron (2002).

1.2. Sampling elements

Sampling requires a good knowledge of the population to be sampled, either on a national scale for nation-wide surveillance programmes or at farm level when the on-farm disease/infection

status is of interest. Therefore, in order to perform proper sampling, the following principles must be taken into consideration:

- Characteristics (of the population of interest) that influence the disease status e.g. reared species, number of fish, cages/tanks, stocking density, disease history, the disease/infection status, farm location, epizootiology data of the region
- Units to be sampled
- Disease characteristics
- Test characteristics (sensitivity (Se) and specificity (Sp))

Sampling procedures also relate to the particular target fluid and/or organ(s) to be sampled from the chosen individuals and on how these biopsies should be handled and the tissues transported. These are discussed in the relevant sections dealing with the particular diseases/pathogens.

1.3. Sampling for diagnostic confirmation and disease detection purposes

When a disease outbreak is under investigation, purposive sampling of target fish, which are most likely to test positive for the infectious pathogen, or disease under investigation, are sampled. Such target fish can be freshly dead fish, moribund fish, or fish that exhibit disease symptoms, or simply behave differently than the rest of the group. The probability of detection depends on the number of samples collected, the prevalence of the pathogen in the population, and the diagnostic test sensitivity and specificity. A larger number of samples is usually required when moribund fish, or fish with overt symptoms are rare and the prevalence of the pathogen is expected to be low, especially during the early stage of an infection. Such cases require diagnostic tests with high sensitivity.

1.4. Sampling to certify the disease-free status

When the aim is to demonstrate freedom from a specific pathogen, it is important to sample the fish that are most likely to carry the pathogen. Such fish may be freshly dead or moribund or showing signs of disease that may be connected to the pathogen in question. In addition, it is also important to identify the most susceptible age group and perform sampling when environmental conditions promote infection by the particular pathogen (e.g. water temperature).

1.5. Sample size calculation and examples

A larger sample size reduces sampling error and increases the likelihood that the sample accurately reflects the population of interest. The minimum required number of samples that need to be collected for analyses depends on a number of factors and the final sample number is often a decision based on a balance of:

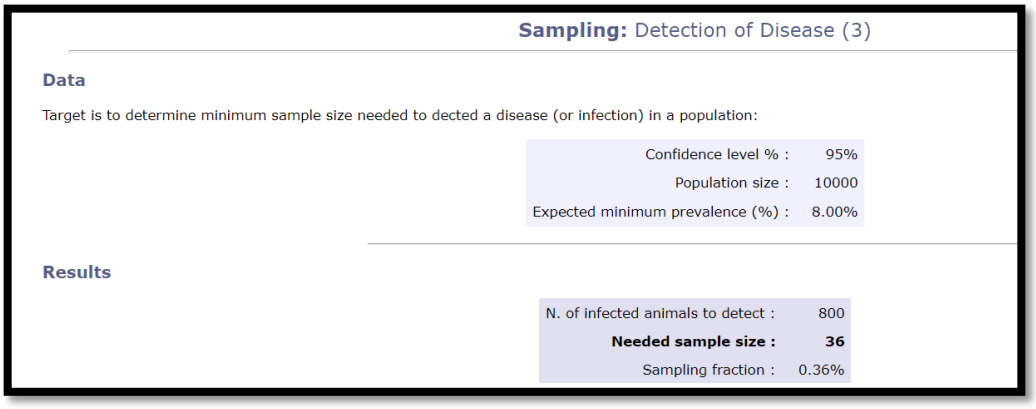
- the required degree of confidence of the results (i.e. consequences of missed cases or false positive cases),
- the sensitivity and specificity of the diagnostic test,
- available resources (economic, personnel, laboratory capacity).

Online tools (i.e. WinEpi, FreeCalc - EpiTools) are available to perform sample size calculation for different sampling purposes. Examples using the WinEpi tool (http://winepi.net/winepi2) are shown below (de Blas and Muniesa 2010). These examples have been modified in accordance with the course on Application of Epidemiology in Aquatic Animal Health in Zaragoza (Spain),

25th February – 1st March 2019, as part of MedAID project (http://www.medaid-h2020.eu/index.php/event/advanced-couse-on-application-of-epidemiology-in-aquatic-animal-health/).

Case 1. Sample size calculation for disease detection

In a sea cage with 10,000 seabass, the veterinary services want to check if the nervous necrosis virus (NNV) is present in the population. If present, the assumption is that the prevalence would be at least 8%. The aim is also to be 95% certain that the sampling would give a correct answer (confidence level of 95%). Using the WinEpi tool, the minimum number of fish required for detecting NNV in this situation is 36, given that the diagnostic test is 100% sensitive and specific and the fish are randomly sampled. Thirty-six fish are 0.36% of the total population (sampling fraction). It must be kept in mind that most tests are not perfect (Sp=Se=100%), so 36 is the lowest number that should be sampled.



Sampling: Detection of Disease (3)

Data

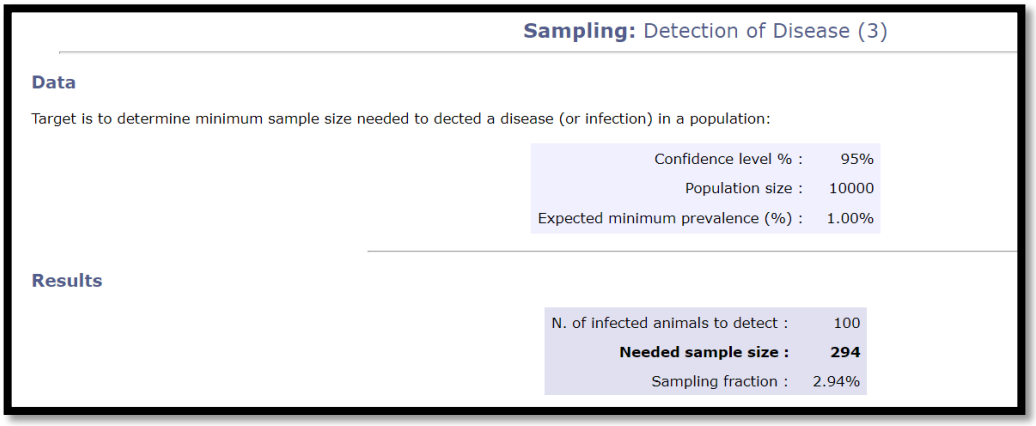
Target is to determine minimum sample size needed to dected a disease (or infection) in a population:

Confidence level % :	95%
Population size :	10000
Expected minimum prevalence (%) :	8.00%

Results

N. of infected animals to detect :	800
Needed sample size :	**36**
Sampling fraction :	0.36%

Then, for the same size population they want to initiate an early detection programme to detect the infection at an early stage, at an expected prevalence of 1% and with 95% confidence level. Using the WinEpi tool, the minimum number of fish required to sample is 294 under the same assumptions as above. A larger sample size is needed to detect a possible infection at a lower prevalence.



Sampling: Detection of Disease (3)

Data

Target is to determine minimum sample size needed to dected a disease (or infection) in a population:

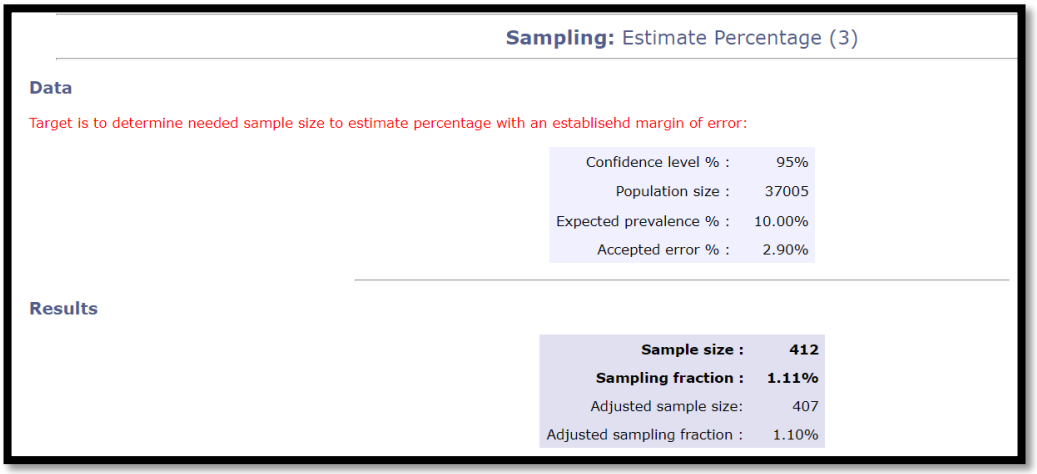
Confidence level % :	95%
Population size :	10000
Expected minimum prevalence (%) :	1.00%

Results

N. of infected animals to detect :	100
Needed sample size :	**294**
Sampling fraction :	2.94%

Case 2. Sample size calculation for prevalence estimation

The veterinary services want to estimate the prevalence of vibriosis in a cage with 37,000 seabream, assuming an expected prevalence of 10% with a precision of 2.9% (10% +/-2.9%), and a confidence level of 95%. Using WinEpi to calculate the sample size for estimation of prevalence, the sample size required is 412.



Sampling: Estimate Percentage (3)

Data

Target is to determine needed sample size to estimate percentage with an establisehd margin of error:

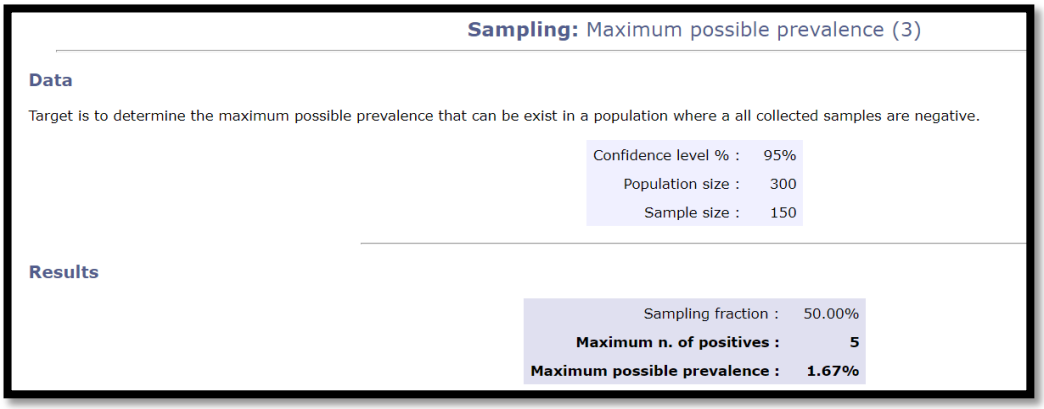
Confidence level % :	95%
Population size :	37005
Expected prevalence % :	10.00%
Accepted error % :	2.90%

Results

Sample size :	**412**
Sampling fraction :	**1.11%**
Adjusted sample size:	407
Adjusted sampling fraction :	1.10%

Case 3. Sample size to calculate the maximum possible prevalence

A fish health veterinarian suspects that a hatchery of 300 gilthead seabream might have been infected with *Vibrio alginolyticus.* Half of the population is inspected, and all are found to be negative. Based on information from half the population, we want to know what the maximum possible prevalence is if the population is still infected with 95% level of confidence. Using WinEpi, and the 150 negative samples, the maximum possible prevalence would be 1.7 % (5 positive fish).



Sampling: Maximum possible prevalence (3)

Data

Target is to determine the maximum possible prevalence that can be exist in a population where a all collected samples are negative.

Confidence level % :	95%
Population size :	300
Sample size :	150

Results

Sampling fraction :	50.00%
Maximum n. of positives :	**5**
Maximum possible prevalence :	**1.67%**

1.6. Sampling examples given by OIE and EU

OIE (2018) has provided a table on how to interpret the test results from sampled fish given specific test criteria (Table1). In the example of testing 330 fish using a design prevalence of 5% (Table 1, in bold), we can expect as many as 23 fish to test false positive when the Sp=95%. This means that there is a 95% confidence that the prevalence in the population is 5% or less given that all 23 are confirmed negative.

In many cases we do not know the Se and/or Sp. For demonstrating freedom (or a maximum prevalence), all positives should therefore be confirmed true or false positives.

Table 1.1. Examples of how to interpret test results at a given design prevalence of 5% (OIE, 2018)

Design prevalence (%)	Sensitivity (%)	Specificity (%)	Sample size (no. of fish)	Maximum number of expected false positives
2	100	100	149	0
2	100	95	1671	98
2	95	100	157	0
2	95	95	1854	108
5	100	100	59	0
5	100	95	330	23
5	95	100	62	0
5	95	95	351	24
10	100	100	29	0
10	100	95	105	9
10	95	100	30	0
10	95	95	109	9

EU (2015) has laid down rules for sampling by Member States in connection with the disease status of the Member States, or zones or compartments thereof for the non-exotic aquatic animal diseases (Table 2). These rules also define sampling procedures for surveillance over time, which is not a part of this manual.

Table 1.2. Screening for confirming disease status according to EU legislation

Design prevalence	Number of fish	Frequency	Confidence Interval
2%	150	Once a year	95%
5%	75	Once a year for two years	95%
10%	30	Once a year for four years	95%

1.7. Some reflections on sampling and sampling size

A tailored sampling strategy is an important criterion to achieve a reliable conclusion about the disease status in a population. The sampling procedure applied should therefore always accompany the result report. By focusing on the subpopulation of fish at risk of having the infection, one can increase the prevalence in the sampled population and increase the probability of finding positive fish.

One sampling is, however, just a snapshot at the time of sampling. To maintain knowledge of the disease status it is important to have proper information about biosecurity and disease history (risk of disease introduction), and have frequent samplings as shown in the examples in Table 2.

References

Anon, 2015. Commission Implementing Decision (EU) 2015/1554 of 11 September 2015 laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods. *Official Journal of the European Union L* 247, 1-62.

Cameron A., 2002. Survey Toolbox for Aquatic Animal Diseases. A Practical Manual and Software Package. ACIAR Monograph No. 94, 375p. ISBN 1 86320 350 8.

de Blas I., Muniesa A., 2010. WinEpi 2.0: Working in Epidemiology. World Wide Web electronic publication: http://winepi.net/winepi2

OIE, 2018. Aquatic Animal Health Code. Chapter 1.4. Article 1.4.8. pp 18-20.

2. Packing and shipping of materials for laboratory analysis with guide to shipping biological materials

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2.1. Introduction

Accurate diagnosis demands properly collected and prepared samples for submission to the diagnostic laboratory. Fish must be alive when collected and fish showing signs of the disease in question are preferable and should be collected with minimal stress. It is a large advantage for the laboratory to start the examination with live samples. However, it is hardly possible to

keep seabass and seabream alive during transportation except in the situation when the laboratory is in close vicinity of the farm. If fish cannot be maintained alive, samples should be properly packed and shipped to the diagnostic laboratory as soon as possible after sampling. Dead fish are not suitable for disease diagnosis because they decompose rapidly after death and very often saprophytic bacteria overgrow the bacterial pathogen. Parasites also require a live host and even viruses survive in dead fish for a limited time.

2.2. Transport of fresh dead samples

Fish showing the signs of the disease should be collected alive from the population using a net or trap. The most suitable specimens for diagnostic purposes are fish with pronounced symptoms or moribund fish. They should be placed in a separate plastic bag and sealed. Each separate sample should be placed inside a larger, strong plastic bag and sealed ("double bagging"). The double bagged samples should be placed in a cooling box or insulated styrofoam box with sufficient freezer blocks (not ice cubes, which could melt and produce liquid inside the package, unless thermally sealed in strong plastic bags) to prevent the temperature inside the box from rising above 10^{o}C during transport. Each sample should be labelled with a permanent ink marker. Any accompanying document should be placed in a separate, small plastic, leak-proof envelope placed inside the transport box. The document should contain all data about environmental and rearing conditions. A full record of environmental data, culture conditions (stocking, feeding), observations regarding fish behaviour, mortality patterns as well as the name and address and other contact details of the sender should be provided, either in writing or by e-mail sent to the laboratory in advance (Annex 3).

Sufficient inert material (e.g. used paper, paper threads, or plastic wrapping) should ensure that the samples do not move inside the box during transport.

Shipment should be delivered via fast overnight courier service.

2.3. Transport of material for histological examination

When it is not possible to send whole diseased fish to the laboratory for histology, organs already fixed in the appropriate fixative solution (most often buffered neutralized formalin BNF 10% 1:20 v/v) may be sent in tightly capped plastic bottles or tubes containing fixative. The container should be large enough to prevent squeezing of tissue pieces inside the tube or bottle. The diagnostician must be able to detect tissue changes indicative of the disease and not be confused by the post mortem decomposition process. This requires carefully-fixed tissue samples immediately after the death of the animal. To safeguard against spillage, the containers should be wrapped in plenty of absorbing material and placed in sealed plastic bags before placing them in an outer envelope or carton box. The outer package should be strong and made of stiff cardboard, polystyrene etc. Enough inert material (e.g. used paper, paper threads, or plastic wrapping) should ensure that the samples do not move about inside the box during transport.

2.4. Transport of samples for virological examination

For the diagnosis of viral diseases, sampling is sometimes performed on the farm and small portions of selected organs (i.e. anterior kidney, spleen, heart, gills and brain) are placed in plastic tubes containing transport medium. The tubes containing transport media (cell cultivation media with added foetal bovine serum, antibiotics, fungicides and buffer) may be provided by diagnostic laboratories a few days prior to sampling. The tubes containing tissue samples should be wrapped in plastic and sent with freezer blocks in a cool box to maintain the temperature below 10^{o}C without freezing. Generally, organs from ten fish can be pooled in one

tube, bearing in mind that the volume of transport medium should be twice that of the organs. However, in some cases it is recommended to test individual fish samples. It is advisable to ask the laboratory staff for specific advice before pooling samples. Samples for virology should reach the diagnostic laboratory within 24-48 hours after sampling. A written record form should accompany samples, or be e-mailed to the laboratory.

Where practical difficulties arise (e.g. bad weather conditions, holidays, laboratory problems) which make it impossible to process the samples within 48 hours post collection, it is acceptable to freeze the tissue specimens in transport medium at -20°C or below and carry out virological examination within 14 days. The tissue samples, however, must be frozen and thawed only once before the examination.

2.5. Transport of the samples for molecular analysis

Molecular techniques are widely used for the detection of many fish pathogens. Samples selected for nucleic acid-based diagnostic tests should be handled and packaged with care to minimize the cross-contamination among the sample degradation. Use of separate containers (plastic sample bags or bottles) and immersion of instruments in bleach when target tissues or organs are dissected should minimize sample cross-contamination. Target tissue should be cut to less than 0.5 cm in one dimension and submerged in the preservative solution: RNAlater™ for RNA viruses and RNAlater™ or ethanol 95% for DNA viruses. The ratio between samples and fixative should be 1:5 w/v. Small organs can be immersed whole in the solution while bigger organs are cut to fulfil required proportions. Manufacturers' instructions should be followed for the correct use of RNAlater™.

A water-resistant label, with the appropriate data filled out, should be placed in each package or container for each sample set. The major advantage of using RNAlater™ is that samples can be shipped at room temperature if the shipment lasts for less than 1 week. The samples preserved in this way may be stored up to 25°C for 1 week, at 4°C for one month, at or at –20°C indefinitely.

2.6. Guide to shipping biological materials

The transport of biological materials is subject to stringent requirements based on national and international legislation. It is governed by ADR (European Agreement concerning the International Carriage of Dangerous Goods by Road). The packaging, labelling and dispatch of biological samples are regulated by the UN and described in the International Air Transport Authority (IATA) and the International Civil Aviation Organisation (ICAO) regulations. Under this regulation, specimens are classified into diagnostic specimens and infectious substances.

All biological materials must be properly packaged and checked in as luggage or transported via courier (carrying biological materials on board an aircraft is prohibited).

The appropriate steps to ship biological materials include:

- Classification
- Packaging
- Labelling
- Documentation

It is important to define the classification correctly as it determines how the goods should be packaged and labelled.

Biological materials fall into the following categories:

- Infectious substances
  - Category A infectious/potentially infectious substances
  - Category B infectious substances
- Diagnostic specimens
- Biological products

2.6.1. Infectious substances

Infectious substances are those known to contain, or are reasonably expected to contain, pathogens. Pathogens are defined as microorganisms or recombinant microorganisms that are known or are reasonably expected to cause infectious disease to humans or animals. However, they are not subject to the provisions of shipping if they are unlikely to cause human or animal disease. Infectious substances are subject to the regulations only if they are capable of spreading disease when exposure to them occurs.

2.6.1.1. Category A infectious substances

Category A infectious substances are capable of causing permanent disability, life-threatening or fatal disease to humans or animals when exposure to them occurs. Category A infectious substances have two shipping names: “Infectious substances, affecting humans” (UN 2814) or “Infectious substances, affecting animals” (UN 2900). The fish pathogens do not generally belong to this category.

2.6.1.2. Category B infectious substances

Category B infectious substances are infectious but do not meet the criteria for Category A. Category B infectious substances have the proper shipping name “Biological Substance, Category B” and the identification number UN 3373. Fish pathogens (virus and bacteria) fall into this category.

2.6.1.2.1. Packaging

Category B infectious substances must be triple packaged and compliant with IATA Packing Instruction 650. The maximum quantity for a primary receptacle is 500 ml or 500g and outer packaging must not contain more than 4L. or 4 kg.

2.6.1.2.2. Labelling

The outer container of all Category B infectious substance packages must display the following on two opposite sides:

- Sender’s name and address
- Recipient’s name and address
- The words “Biological Substance, Category B”
- UN 3373 label
- Class 9 label, including UN 1845, and net weight if packaged with dry ice

2.6.2. Diagnostic specimens

Any human or animal material including, but not limited to, excreta, secretions, blood and its components, tissue and tissue fluids, being transported for the diagnostic or investigational purpose but excluding live infected animals. Fish, organs, swabs belong to this category.

Diagnostic specimens must be assigned to UN3373 unless the source patient or animal has or may have a serious human or animal disease, which can be readily transmitted from one individual to another, directly or indirectly and for which effective treatment and preventable measures are not usually available, in which case they must be assigned to UN2814 or UN 2900.

2.6.2.1. Packaging

Diagnostic and clinical specimens must be triple packaged and compliant with IATA Packing Instruction 650 detailed in (Fig. 1). The maximum quantity for a primary receptacle is 500 ml or 500g and outer packaging must not contain more than 4 l or 4 kg.

2.6.2.2. Labelling

The outer container of all diagnostic/clinical specimen packages must display the following on two opposite sides:

- Sender's name and address
- Recipient's name and address
- The words "Biological Substance, Category B"
- UN 3373 label
- Class 9 label, including UN 1845, and net weight if packed with dry ice

2.6.3. Biological products

These are products derived from living organisms that are manufactured and distributed in accordance with the requirements of national governmental authorities which may have special licensing requirements, and are used either for prevention, treatment or diagnosis of disease in human or animals or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines and diagnostic products. Biological products transported for final packaging, distribution, or uses by medical professionals are not subject to shipping regulations. Biological products that do not meet these requirements must be assigned to UN 2814, UN 2900, or UN 3373, as appropriate.

2.6.3.1. Packaging biological products

Potentially hazardous biological materials must be packaged to withstand content leakage, shocks, temperature changes, pressure changes, and other conditions that can occur during transport. When ordering, specify what category of materials will be shipped: infectious substances, diagnostic specimens, dry ice, ice packs, etc. All biological materials must be triple packaged.

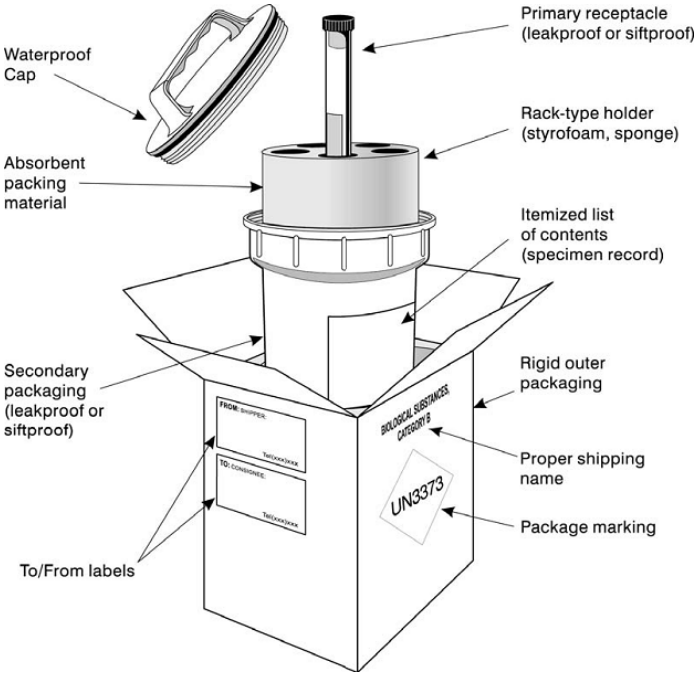

Fig. 2.1: Packaging of biological products

2.6.4. Shipping papers

Documentation required by transporter or operator must always be accessible without opening the package.

A Shipper's Declaration for Dangerous Goods must be completed when shipping a Category A infectious substance or a genetically modified organism or micro-organism assigned to UN 3245. A declaration is not required for shipments in which dry ice is the only hazardous material. A declaration is not required for Category B infectious substances or diagnostic/clinical specimens assigned to UN 3373. All shippers' declarations must be in English, typed, and printed in colour with red hatchings bordering the document. Three copies must be presented to the courier with a fourth copy retained by the shipper for at least 375 days. All shippers' declarations must conform to the standardized format provided by the courier company used for transport.

Considering that proper packaging and labeling are mandatory and have to comply with international regulations, it is always advisable to contact the chosen courier company beforehand to check the correctness of the procedure.

A specialized courier company should be used to transport Category substances (i.e. World Courier, PHSE or another company to which you are accustomed).

References

IATA, 2019. Infectious Substances Shipping Guidelines 15th Edition.
IATA, 2019. Dangerous Good Regulation 60th Edition.
WHO, 2015. A Guide for Shippers of Infectious Substances
https://www.who.int/ihr/infectious_substances/en/

3. Guidelines for the laboratory receiving the samples

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3.1. Scope

The aim of the Chapter is to provide a standardized approach for sample receiving operations in order to ensure high sample integrity and the validity of the analytical results. It provides guidance on laboratory sample reception and surveying, inspecting, documenting, and assigning laboratory tracking identifiers (IDs).

3.2. General considerations

Before the samples are received, communication between laboratory personnel and farm staff in the field allows the parties to coordinate activities, schedules, and sample reception. Sample reception and inspection activities need to be done in a timely manner to allow the laboratory and field personnel to resolve any problems (e.g., insufficient material collected, lack of field preservation, etc.) with the samples received by the laboratory as soon as is practical. Effective communications between field personnel and the laboratory not only facilitates problem resolution but also prevents unnecessary delays in the analytical process. The information

about the client, points of contact, number of samples, and types of analyses can be entered into the laboratory information management system (LIMS) to facilitate communication between the parties.

Laboratory sample reception occurs when a package containing samples is accepted. Sample inspection starts by checking the physical integrity of the package and samples, confirming the identity of the sample, confirming field preservation (if necessary), and recording and communicating the presence of hazardous materials. Laboratory sample tracking is a process that starts with logging in the sample and assigning a unique laboratory tracking identifier (numbers and/or letters) to be used to account for the sample through analyses, storage, and shipment.

3.3. Sample reception

All samples should be properly collected, identified and correctly transported from sites to the receiving laboratory in a transportation box or an individual biohazard plastic bag. Samples submitted for analysis must be accompanied by an Analysis Request Form, duly filled (Annex 3). Upon receipt of the sample, initial steps should be taken:

- Document the date and time of sample receipt.
- Assign an accession number to be used as sample identification in the laboratory.
- Verify that the sample identification on the Analysis Request Form matches the identification on the sample.
- Examine the sample visually to evaluate acceptability.
- Review the Analysis Request Form for suitability of the collected biological material.
- Determine the suitability, with respect to the test(s) ordered, to the transport conditions, including the following:
    - Transport medium or preservative for the sample,
    - Temperature of the sample upon receipt,
    - Length of time between sample collection and receipt,
    - Transport container integrity, i.e. no leaks or cracks.

3.4. Opening the package and inspection of the sample

After breaking the Official Seal (if present) and opening the package, the analyst removes and inspects the physical appearance of the sample. Using the laboratory's procedure, the analyst documents any discernible abnormalities, discrepancies, and problems such as the following:

- Discrepancies between the sample received from the sample described in the collection sheet.
- Broken paper seals without initials or date in the designated area.
- Records failing to describe the type of analysis requested, and samples inappropriate for the sample analysis requested.

3.4.1. Sample identity confirmation

Visual inspection is a means to confirm that the correct sample has been received. Verification of the sample identity is a simple process where the appearance, sample label, and records are compared. The sample must be properly labelled and must include:

1. The sample's unique identifier matching the Analysis Request Form,

2. If appropriate, the date and time of sample collection, and

3. Any additional information relevant and necessary for a specific test.

3.4.2. Verification of the analysis request form

Documents accompanying the samples should be reviewed upon receipt at the laboratory. Accurate identification details on laboratory samples are of utmost importance. Samples must be correctly labelled and request details (analysis request forms) have to be completed to the required standard. Sample and request details must be compatible. The form should contain the following information:

a. The identity of the fish farm (name, address, phone and e-mail),

b. Affected population data including location, species, size, age, source, data collection of sampling and time since introduction,

c. Data on the disease, including the morbidity and mortality rates (number of dead fish, onset date…), the duration, the clinical signs of disease, the behaviour of affected fish, any abnormalities found at necropsy and whether similar problems have occurred previously, and if so whether they were treated successfully or not.

d. Environmental data (salinity, temperature, pH and oxygen levels), the presence of harmful algae or other pollutants, and recent weather pattern such as storms, lightning etc.

e. Management data such as stocking rate, type of food, any medications used or vaccination performed any recent management changes, and stress factors present.

f. The telephone number and email address of person authorizing request for analysis.

g. Invoicing data of the owner (in case of charged examinations).

3.4.3. Sample integrity

Samples should be received or placed in a specified storage area in which environmental conditions are monitored and recorded.

The sample must be:

1. Collected in the correct, intact, container, device or non-expired transport media.

2. Transported under the correct conditions.

3. Processed/handled according to approved laboratory procedure.

4. In sufficient quantity to perform testing.

5. Received within acceptable time limitation (specific criteria to be determined by each laboratory).

3.4.4. Laboratory records

All the information that has any particular relevance to the materials and the analysis performed on them must be documented in a systematic manner at any point during its transit through the laboratory. Records must allow a test material to be traced back to its arrival and any information that arrived with it. Records must be retained and protected from misuse, loss or deterioration for an agreed time.

3.5. Storage conditions

The analyst that receives a sample for analysis must ensure that it is kept under proper storage conditions in accordance with the demands of the diagnostic procedures. Samples must be properly stored to ensure pathogen viability (frozen, refrigerated, or ambient temperatures). Samples should be stored away from all standards, reagents, food and other potentially contaminating sources. Samples should be stored in such a manner as to prevent cross contamination.

3.6. Laboratory sample tracking

Sample tracking should ensure that analytical results are reported for the correct sample. It is a process by which the location and status of a sample can be identified and documented at any moment. When the samples are received by the laboratory they are usually prepared for different analyses. In such cases the samples must be aliquoted. The minimal laboratory tracking process consists of providing a receipt of the received samples as well as the documentation of the sample storage (location, amount, date and time). The procedure for accomplishing the above mentioned varies from laboratory to laboratory, but the exact details of performing the operations of sample tracking should be documented in adopted SOP.

Laboratory sample IDs should be assigned to each sample in accordance with the laboratory SOPs. Each sample should receive a unique sample ID by which it can be logged, scheduled for analysis, tracked, and disposed of. Information to be recorded during sample log-in should include the field sample identification number, laboratory sample ID, date and time of sample collection on site and reception by the laboratory, the method of shipment, the analyses requested, the number and type of each sample, the quality control requirements, any special instructions, and other information relevant to the analysis.

3.7. Possible problems causing sample rejection

Specimens delivered to the receiving laboratory are rejected for the following reasons:

- Specimen without a batch /cage identification label or unlabeled.
- Specimen label that does not match the label on the attached Analysis Request Form or is mislabeled.
- Request form or Label with insufficient information.
- Request form without physician’s stamp and/or signature.
- Request form with no mark on the required test.
- Specimen placed in an unsuitable container.
- General or unspecific testing mentioned in the request form.
- Leaking or contaminated container.
- Request form received without specimens and vice versa.
- Test requested is not available.
- More than a single sample in one bag/tube.

A rejected specimen should be documented in the rejection sheet or electronically, explaining the reason for rejection. All available information related to the specimen, date and time of rejection, the signature of the laboratory staff and the action taken should be mentioned.

References

Heil N. (Ed) 2009. National Wild Fish Health Survey – Laboratory Procedure Manual. 5.0. Edition. US Fish and Wild Life Service. Warm Springs, GA.

MARLAP., 2004. Manual Volume II: Chapter 11, Sample Receipt, Inspection and Tracking. Pp 1-13.

U.S. Nuclear Regulatory Commission (NRC), 1998a. Procedures for Receiving and Opening Packages. 10 CFR Part 20.

Part II. General requirements for the laboratory methods

4. Laboratory requirements for viral diseases

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4.1. Introduction

Laboratories play a critical role in the surveillance, diagnosis and monitoring of diseases in general and in particular for those of viral aetiology. Establishment of the virology laboratory performing a reliable diagnosis is a prerequisite for effective disease management. The choice of the system used in a certain laboratory to confirm or rule out a viral infection would primarily depend on the facilities and resources available and the level of knowledge available for the specific virus under study.

Generally, in virology the following diagnostic methods can be used:

(1) Isolation and identification of viruses

(2) Detection of viral nucleic acid and sequencing

(3) Detection of viral antigens

(4) Detection of virus-specific antibodies.

Virtually all these techniques can be applied to fish virology, but in practice, only the first two methods are widely available, have been sufficiently validated and consequently are recognized by the scientific community.

With reference to detecting fish virus antigens or antibodies, a few laboratory assays have been developed and described (mainly ELISA tests) therefore, they are used mainly for research purposes.

4.2. General management

Veterinary laboratories must be managed under a quality assurance system according to international standards (i.e. ISO/IEC 17025) and ideally should also be accredited by a recognized accreditation body.

The laboratory should ensure that its procedures are robust, reliable and repeatable. The quality standards require that each diagnostic test used in the laboratory should be validated. Standard material must be used for positive and negative controls and participation in proficiency tests (when available) is always recommended. A one way organization is recommended.

Useful information about the general management of veterinary diagnostic laboratory and test validations can be found in the following links:

http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_quality_management.htm

http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_validation_diagnostics_assays.htm

4.3. The minimum requirement for the virology laboratory

For laboratories running cell isolation, the following minimum requirements are necessary:

Two rooms/units (one “clean room” for cell maintenance and one “diagnostic room” for sample processing and inoculation) equipped with:

- 1 biological safety cabinet
- 1 optical microscope (at least one equipped with fluorescence)
- 1 or more thermostatic chamber (temperature range 15-25°C)
- Pipetting systems of different volumes
- 1 refrigerated centrifuge
- 1 or more fridge/freezer
- 1 balance
- 1 pH meter

Separate cleaning room equipped with autoclave is also necessary.

Liquid nitrogen container may be necessary for stocking cells.

4.4. The minimum requirement for the molecular biology laboratory

For laboratories running a molecular diagnostic, the following minimum requirements are necessary:

At least two separate rooms for genetic material extraction and analysis and master mix preparation. Each room should be equipped with:

- 1 safety/chemical cabinet,
- 1 or more fridge/freezer,
- 1 electrophoresis system,
- 1 magnetic stirrer and vortex,
- 1 or more thermal cycler / real-time PCR platform,
- Gel documentation system,
- 1 refrigerated centrifuge,
- 1 or more microcentrifuge,
- Pipetting systems of different volumes,
- 1 balance,

- 1 or more microcentrifuge,
- 1 pH meter,
- Sequencing platform (optional).

Additional equipment includes icemaker, water bath, spectrometer, microwave oven and ultra pure water production system, ultra low freezer (-80°C).

Separate cleaning room equipped with autoclave is also necessary.

Additional information can be found at the following link:

http://apps.searo.who.int/PDS_DOCS/B4249.pdf

4.5. Biosafety requirements

Fish virus can be classified as notifiable and/or causing World Organisation for Animal Health (OIE) listed diseases according to the criteria set out in Chapter 1.2 of the OIE Aquatic Animal Health Code. The majority of fish pathogens, however, are not classified under a biohazard ranking system. This makes difficult to decide an appropriate biocontainment level for laboratory work and transport. Luckily, only a few fish bacteria and no viruses have the ability to produce a zoonotic disease. With reference to fish viruses, no zoonotic potential has ever been reported. Therefore a risk analysis is generally considered not necessary and for this reason, biosafety level 1 is generally accepted for laboratories working with fish viruses. Only in specific cases (activities with GMO cell or GMO viruses) may there be additional biosafety requirements. For the minimum biosafety requirements please consult the WHO and OIE websites:

https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

http://apps.who.int/medicinedocs/documents/s22409en/s22409en.pdf?ua=1

http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.04_BIOSAFETY_BIOSECURITY.pdf

5. Laboratory requirements for bacterial diseases

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5.1. Introduction

In disease management, bacterial analyses are required to identify and implement curative or preventive solutions. Isolation of the pathogenic agent(s) and their *in vitro* sensitivity profile to antibacterials need to be provided fast in order to implement early treatment. The identification of the bacterial agent by different methods will allow the application or development of preventive approaches (vaccination strategies, batch certification, epidemiology and surveillance) in the long-term.

Depending on the production system and the life stage of the sampled fish, identification of bacteria for microbiota management may be required or bacterial strain identification for diagnosis and/or differential diagnosis may be needed. Bacterial disease diagnosis is still based on conventional bacteriological methods that can be performed in small laboratories, but recent modern analytical methods have been applied in bacteriology opening new fields of investigation (microbiota analysis, mass spectrometry, sequencing, and more recently infra-red) but these require heavy investments and therefore platforms and laboratory networks.

Different analytical methods are used for bacterial disease diagnosis and bacteria identification:

Different analytical methods are used for bacterial diagnostics:

- Subculture and strain isolation on artificial media.
- *In vitro* susceptibility testing to antimicrobials.
- Detection of the antigen by serum agglutination tests.
- Strain identification by biochemical tests.
- Strain identification by mass spectrometry and infra-red.
- Detection of bacterial nucleic acid using molecular methods.
- Whole genome sequencing and typing of the bacteria.

The first three methods are widely applied for presumptive diagnostics and treatment recommendation. Nowadays, identification of bacteria by mass spectrometry is substituting biochemical profile identification in routine procedures, being faster and accurate. It requires, however, a validated dataset of reference pathogens to be able to correctly identify the

pathogens in the diagnostic samples. The latest methods of identification are mainly applied for antigen selection in vaccination strategies and for research purposes.

5.2. General management requirement

The general management requirements needed for bacteriology laboratories are the same as those reported for virology laboratories.

Additional information is available in the manual prepared by (Sutton and Singer, 2011).

5.3. The minimum requirement for bacteriological laboratory

A bacterial laboratory should consist of a central room for bacterial analysis with an area for sample reception and a cleaning room, as well as a separate area for media preparation. The one-way organization is recommended. The minimum material requirement should include:

- 1 light microscope (x400 – X1000 immersion),
- 2 thermostatic chambers at a temperature of 20-25°C,
- 1 burner,
- 1 antibiotic disc dispenser and a spectrometer for inoculum density,
- 1 fridge and an autoclave,
- 1 low freezer (-80°C) for bacterial strain conservation and
- 1 microwave oven to melt media.

For media preparation, a balance, a pH meter and a heating magnetic plate will be needed. A biological safety cabinet is not mandatory but it is essential. Basic media for fish bacterial pathogen detection include TSA – Marine agar – TCBS – Blood agar and Mueller-Hinton 2. Additional media may be required for fastidious bacteria such as transport and isolation media for *Tenacibaculum*. Similarly, broth media may be necessary to reactivate some fastidious bacterial strains.

Additional equipment is required for quantitative bacteriology including pipetting systems of different volumes, sterile seawater, an agitator and a bacterial colony counter.

5.4. Biosafety requirements

Few bacterial fish pathogens are zoonotic but for most of them, the zoonotic diseases they might cause are rarely severe. However, a zoonotic infection caused by *Mycobacterium* spp. can be difficult and take a long time to treat in humans. Some human cases of infection with *Vibrio* strains such as *Vibrio vulnificus* or *Vibrio cholerae* have been reported to be harmful to immunocompromised individuals.

For these reasons, even if a biosafety level 1 is generally accepted for laboratories working with fish bacterial pathogens, it should be born in mind that a zoonotic risk remains and that adequate biosafety procedures should be maintained.

Additional information on biosafety is available on the OIE and WHO websites.

References

Sutton S. and Singer S., 2011. Microbiological Best Laboratory Practices, USP Value and Recent Changes to a Guidance of Quality Laboratory Practices. *American Pharmaceutical Review,*14(4); 41-47.

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Part III. Viral diseases with impact on Mediterranean fish farming

6. Viral Encephalopathy and Retinopathy/Viral Nervous Necrosis (VER/VNN)

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6.1. Introduction

Among the disease outbreaks caused by infectious agents at animal farming facilities, those triggered by viruses are the most challenging to manage, and fish are no exceptions to this rule. Luckily, in the Mediterranean region, only a few viral diseases threaten the marine fish industry. Without any doubt, the most significant in terms of severity, economic impact and spread, is viral nervous necrosis (VNN, syn. viral encephalopathy and retinopathy - VER) which will be widely described in the following chapter. Another relevant disease, affecting mainly seabream (*Sparus aurata*), is lymphocystis disease (LCD) caused by a member of the Iridoviridae family, the LCDV. However, due to the transient nature of this disease and its limited impact on affected fish, it will not be included and described in the present diagnostic manual.

Viral agents other than NNV and LCDV have been described in marine fish species, such as birnaviruses, alphaviruses and aquareovirus, but they are reported only sporadically and their impact on marine aquaculture is unknown and presumably limited. More attention should be paid to exotic viruses, in particular to the red seabream iridovirus (RSIV) and related virusesbelonging to the Megalocytivirus genus, still undetected in the Mediterranean basin but a potential threat to aquaculture if introduced by the natural or anthropogenic route.

Fig. 6.1. a) Appearance of experimentally NNV infected European seabass. There are dead fish on the bottom of the tank, while live fish show impaired swimming capacity with some of them floating on the water surface with the typical "sickle" position; b) Hyperinsufflation of swim bladder in infected European sea bass; c) Different head lesions. From the left: mild congestion (left fish), congestion and erosion of the head skin (middle fish), necrosis of the nostril (right fish); d) hyperaemia of the brain in diseased seabass.

6.2. Aetiology of VNN

The causative agent of VER/VNN is the nervous necrosis virus (NNV), a *Betanodavirus* that belongs to the *Nodaviridae* family. Betanodaviruses are naked particles of 28 nm in diameter, which contain a segmented genome composed of two single-stranded, positive-sense RNA molecules named RNA1 and RNA2. These two segments encode the RNA-dependent RNA polymerase (RdRp) and the capsid protein (Cp), respectively. A subgenomic transcript (RNA3) synthesized from the RNA1 3' terminus during the acute phase of the disease produces a non-structural protein called B2 inhibiting the cell's RNA silencing mechanisms.

At present, *Betanodaviruses* are classified into four species, RGNNV, SJNNV, BFNNV, TPNNV and two types of reassortants, namely RGNNV/SJNNV and SJNNV/RGNNV. Three serotypes are described (A, B and C) partially correlating with the genotype. Reassortants fall in the same serotypes as their RNA1 parental species (Panzarin *et al*., 2016). Each genotype has a different optimum temperature range of replication, explaining the occurrence of outbreaks and geographical distribution (Costa and Thompson, 2016; Toffan *et al*., 2016; Doan *et al*., 2017). Barring the exception of BFNNV and TPNNV species, the other genotypes are all present in the Mediterranean Basin.

Betanodavirus replicates only in the brain, spinal cord and retina, where it causes the necrosis of the nervous cells, and consequently the typical abnormal swimming behaviour. Clinical signs include: apathy alternated to swirling and spinning movements, swim bladder hyperinflation, and blindness. Congestion and erosion of the head and nose, darkening and anorexia are also often present. Mortality can be as high as 100% in larvae, while in older fish is generally less severe. Stressors (i.e. feeding, sorting, netting, etc.) can increase the severity of the clinical signs and cumulative mortality. Survivors remain persistently infected and can transmit the disease to healthy fish for long time.

Table 6.1. NNV genotype serological classification and optimum growth temperature (*Modified from OIE Manual of Diagnostic Tests for Aquatic Animals and Panzarin et al., 2016)*

Genotype	Serotype	Optimum growth temperature
RGNNV	C	25-30°C
SJNNV	A	20-25°C
BFNNV	B	15-20°C
TPNNV	B	20°C
RGNNV/SJNNV	A	25-30°C
SJNNV/RGNNV	C	20-25°C

6.3. Host range

Among the fish species farmed in the Mediterranean basin, the European seabass (*Dicentrarchus labrax*) is certainly the most severely affected by VNN. European seabass is particularly susceptible to RGNNV, which is the most widely spread betanodaviral species in the Mediterranean countries. RGNNV is also highly virulent for groupers (*Epinephelus* spp.), and for this reason, it represents both an economic and ecological threat. It is noteworthy that in recent years, the emergence of the reassortant RGNNV/SJNNV has also caused recurrent outbreaks in larval stage gilthead seabream (Toffan *et al*., 2017), which was initially believed to be a species resistant to VNN. Flatfish (turbot, sole) are also very susceptible to NNV. Overall, betanodavirus has been detected in more than 160 fish species and several molluscs, both as susceptible host as well as carrier animals. Therefore, given the broad host range of

betanodavirus, all fish species can be considered susceptible to infection and monitoring and diagnosis should be performed accordingly.

6.4. Collection and shipment of fish samples for detection of NNV

Upon arrival at the laboratory, samples should be delivered in leak-proof containers and labelled properly. Please refer to Chapter 2.2. for specific instructions on biological substance shipping.

Specimens submitted to the laboratory may include:

- Fish carcasses (adult/market size specimens collected in a pool of 5; juveniles in pools of 10 specimens; pooled larvae at least 0.1 gr. of material).
- Organs (brains and/or eyes from adult/market size specimens collected in a pool of 5; brains and/or eyes from juveniles in pools of 10).
- Broodstock (always collected singly).
- Serum samples (collected singly).
- Live prey (Artemia and rotifer at least 0.1 g of material).
- Other (cell culture supernatant at least 0.5 ml, water samples and algae in suitable amounts depending on the scope).

The central nervous system (CNS) and the retina are the target organs for betanodavirus. Therefore, the analysis of tissue samples other than CNS (i.e. spleen, kidney, gills, blood etc.) is advisable for research purposes only.

When clinical signs and mortality are present, sampling of 5-10 diseased fish is sufficient to confirm the diagnosis of VNN (targeted surveillance). In the absence of a clinical outbreak, statistically significant numbers of fish should be collected (see Chapter 2.1.5.) in order to consider the batch as NNV-free (active surveillance). When looking for potential carriers, fish should be tested singly. On the other hand, the presence of betanodavirus should be excluded every time that an increase in mortality is observed, especially in larvae and juveniles (passive surveillance). As an additional biosecurity measure, it is recommended to test every new fish batch produced (in case of hatcheries) or introduced (from another farm), irrespective of species, origin and absence of mortality/clinical signs. Finally, because the NNV can be transmitted vertically, analysis of samples from broodstock might be required. Due to the low viral load in carrier fish, gonads and/or reproductive fluids should never be pooled. Furthermore, for broodstock, it would be advisable to combine molecular tests of reproductive fluids with serum samples for detection of antibodies (in those species where serological assays are available). Alternatively, mixed fertilized ova from the collection basket, produced by several fish in a broodstock tank, can also be analysed for preventive diagnosis. However, they are not considered a good target, mainly because of the difficulty in homogenizing this matrix and releasing the virus, which is present at very low concentrations. For a better and reliable diagnosis, it is recommended to test other target samples too (i.e. entire larvae, brains/eyes/heads) in order to increase the accuracy of the analytical result.

6.4.1. Sample preservation for viral isolation in cell culture

Specimens must be frozen (-20°C or lower) and subsequently shipped on dry ice. Alternatively, specimens must be placed in viral transport medium (VTM) and shipped frozen or refrigerated. VTM could be a cell culture medium with 10% fetal calf serum (FCS) and 1% antibiotics/antimycotics. The combination of 10,000 IU/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 µg/ml amphotericin B is commonly adopted, but other antibiotics/antimycotics of proven efficiency may be used as well.

6.4.2. Sample preservation for molecular diagnosis and typing

Specimens must be placed in sealed tubes with RNA stabilization solution (e.g. RNAlater®)[1] (1:5-w/v) and shipped at room temperature. Cell culture supernatants and tissue homogenates can be spotted in FTA®[2] cards and shipped at room temperature. Alternatively, refrigerated or frozen samples can also be used for molecular diagnosis, but the cold chain must be preserved during delivery.

6.4.3. Sample preservation for serological diagnosis

Anticoagulants should not be used when collecting blood. Sera (without blood clots) must be placed in sealed tubes and shipped frozen or refrigerated. Whole blood should never be sent directly to the laboratory because haemolysis of the red blood cells occurs releasing haemolysis products in the serum.

6.4.4. Samples preservation for histology and immunohistochemistry

Specimens should be placed in sealed containers with 4% buffered formalin and shipped at room temperature.

6.5. Diagnostic procedures for NNV

Since it is quite common that different betanodavirus species circulate in the same geographic region, the capability of detecting all viral species, as well as their correct identification is of utmost importance to provide accurate and reliable laboratory results. Therefore, as a first step in the diagnostic process, a molecular protocol capable of detecting all known betanodaviral species must be used. Real-time RT-PCR is preferable to conventional or nested PCR, due to better performances in terms of sensitivity, specificity and turn-around times. Upon positive results, virus isolation should be used as a confirmatory analysis, especially for the first detection in a certain region or in a new fish species. In these cases, genotyping of the NNV strain detected is also essential to gain information on viral phenotype, as different betanodaviruses show diverse pathogenicity, host tropism and temperature sensitivity.

[1] Invitrogen RNA*later*® Stabilization Solution is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. It minimizes the need to immediately process tissue samples or to freeze samples in liquid nitrogen for later processing. Tissue pieces can be harvested and submerged in RNA*later* solution for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation.

[2] FTA® Card, Whatman™ is a paper matrix laced with a proprietary mixture of chemicals that lyse cells and stabilize nucleic acids on contact for long term storage at room temperature.

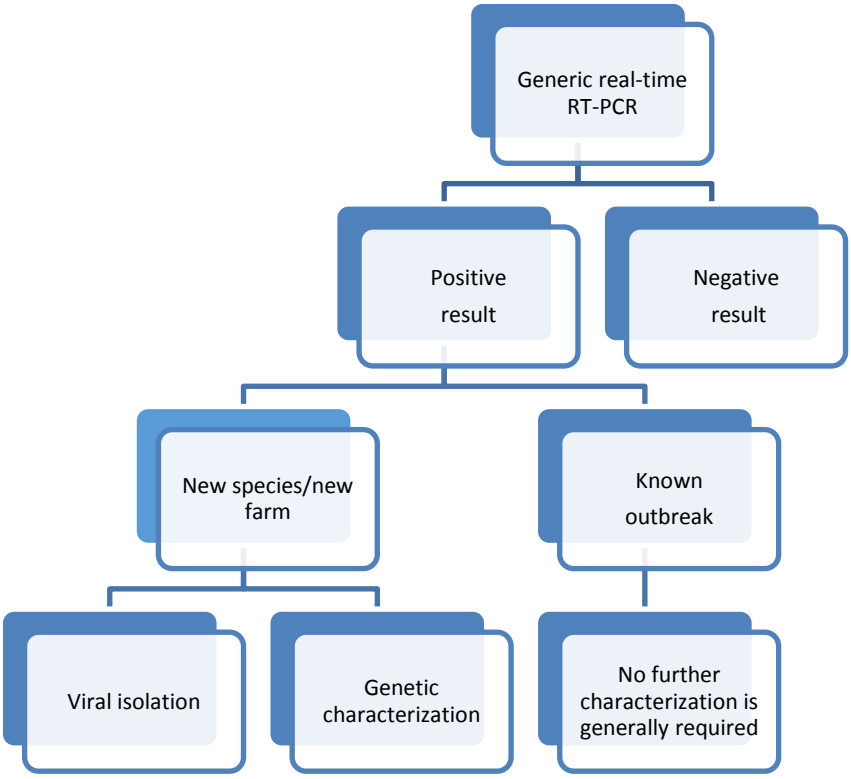

Fig. 6.2. Workflow for detection of NNV.

6.5.1. Preparation of samples for examination

Tissue samples collected from fish carcasses must be homogenized either by mechanical blender or with mortar and pestle, and subsequently resuspended in fresh VTM to a final dilution of 1:3 w/v. The homogenate is subsequently clarified by centrifugation at 2-5°C for 2 minutes at 2000-4000 x *g*. For molecular diagnosis, clarified supernatant can be directly processed, according to the commercial kit used for RNA purification. In the case of samples stored in RNA stabilization solution (e.g. RNAlater®), organs must be removed from the solution and processed as described above. For virus isolation, supernatant must be treated overnight at 4°C (or for 4 hours at 15°C) with 1% antibiotics/antimycotics. The combination of 10,000 IU/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 µg/ml amphotericin B is recommended, but other antibiotics/antimycotics of proven efficacy may be used as well. The antibiotic treatment aims at preventing bacterial contamination and makes filtration steps with membrane filters unnecessary. Betanodavirus, in fact, is a “sticky” virus and filtration may cause loss of viral particles and should be avoided.

6.5.2. Real-time RT-PCR

Total RNA can be isolated using commercial kits based on RNA affinity spin columns, according to the manufacturer’s instructions. To preserve RNA integrity, it is recommended to store purified RNA for subsequent use at -80°C by adding RNAse Inhibitor. For molecular diagnosis, two generic protocols capable of detecting the four betanodavirus species are recommended.

These assays have been extensively validated and are used by many diagnostic laboratories (data from the 2nd International proficiency test for NNV). Adequate controls must be always included in every analytical session. The real-time PCR performances can vary depending on the reagents and the platform used. It is therefore recommended to adapt the protocols to the reagents and equipment available at the laboratory, verify the performances and establish a diagnostic cut-off.

6.5.2.1. One-step real-time PCR targeting RNA1 (Baud *et al*., 2015):

Oligonucleotides

Oligonucleotide	Sequence 5' → 3'	Position in RNA1 (GenBank reference sequence JN189865)
oPVP154 (For)	TCCAAGCCGGTCCTAGTCAA	2717-2736
oPVP155 (Rev)	CACGAACGTKCGCATCTCGT	2865-2884
tqPVP16 (Probe)	Cy5-CGATCGATCAGCACCTSGTC-BHQ2	2772-2791

Reaction mix

Use QuantiTect Probe RT-PCR Kit (Qiagen)

Reagent	Final concentration/volume
oPVP154 (For)	600 nM
oPVP155 (Rev)	600 nM
tqPVP16 (Probe)	400 nM
2X RT-PCR Master Mix	1X
Enzyme mix	0,25 □l
RNA template	5 □l
DEPC Water	To 25 □l

Thermal profile

RT	Denaturation	Denaturation	Annealing/Extension
50°C	95°C	94°C	60°C
30 min	15 min	15 sec	60 sec
		40 cycles	

Technical performances

Analytical sensitivity: 100 copies of plasmid DNA with 95% confidence, $10^{2.5}$-$10^{2.85}$ $TCID_{50}$/ml.

Analytical specificity: capable of detecting RGNNV, SJNNV, BFNNV, TPNNV, RGNNV/SJNNV, SJNNV/RGNNV; negative results when testing VHSV, IHNV and IPNV.

Repeatability: 0,05-1,1% CV

Reproducibility: 0,43-1,78% CV

Robustness: 0,31-0,6% CV

6.5.2.2. Two-step real-time PCR targeting RNA2 (Panzarin *et al*., 2010):

Oligonucleotides

Oligonucleotide	Sequence 5' → 3'	Position in RNA2 (GenBank reference sequence JN189992)
RNA2 FOR	CAACTGACARCGAHCACAC	418-436
RNA2 REV	CCCACCAYTTGGCVAC	471-486
RNA2 probe	6FAM-TYCARGCRACTCGTGGTGCVG-BHQ1	448-468

Reaction mix for RT
Use High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

Reagent	Final concentration/volume
10X RT Buffer	1X
10X RT Random Primers	1X
25X dNTP Mix	1X
MultiScribe Reverse Transcriptase 50U/µl	2,5U
RNA template	15 µl
DEPC Water	To 30 µl

Thermal profile for RT

Pre-incubation	RT
25°C	37°C
10 min	120 min

Reaction mix for real-time PCR

Use LightCycler TaqMan Master (Roche)

Reagent	Final concentration/volume
RNA2 FOR	900 nM
RNA2 REV	900 nM
RNA2 probe	750 nM
5X Reaction Mix	1X
cDNA template	5 µl
DEPC Water	To 20 µl

Thermal profile

Denaturation	Denaturation	Annealing	Extension
95°C	95°C	58°C	72°C
10 min	10 sec	35 sec	1 sec
	45 cycles		

Technical performances

Analytical sensitivity: 300 copies/µl of in vitro transcribed RNA, 10 $TCID_{50}$/ml

Analytical specificity: capable of detecting RGNNV, SJNNV, BFNNV (including AhNNV and AcNNV), TPNNV, RGNNV/SJNNV, SJNNV/RGNNV; negative results when testing *A. hydrophila*, *P. damselae* subsp. *damselae*, *V. anguillarum*, VHSV

Repeatability: CV 0.02-2.87%

Reproducibility: CV 1.1-3.48%

N.B: this assay can be adapted from two-step to one-step protocol, but a great loss of sensitivity may occur and therefore this modification is not advisable.

6.5.3. Sequencing

In order to genetically characterize betanodaviruses and identify possible re-assortment events, it is necessary to sequence both genomic segments.

The primers herein reported for partial sequencing allow performing a preliminary but informative characterization of the virus (Bovo *et al*., 2011). The protocol is intended for application on viral isolates, however, good results can also be obtained from diagnostic samples stored in an RNA stabilizing solution, and yielding less than 20-25 threshold cycle by real-time PCR.

Oligonucleotides

Target	Primer	Sequence 5' → 3'	Amplicon size (bp)	Position in the reference sequence (GenBank acc. no for RNA1: JN189865; GenBank acc. no for RNA2: JN189992)
RNA1	VNNV5	GTTGAGGATTATCGCCAACG	478	178-197
	VNNV8	CAGCAACACGGTAGTG		640-655
RNA1	For 521	ACGTGGACATGCATGAGTTG	630	521-540
	VNNV6	ACCGGCGAACAGTATCTGAC		1131-1150
RNA2	VNNV1	ACACTGGAGTTTGAAATTCA	605	342-361
	VNNV2	GTCTTGTTGAAGTTGTCCCA		927-946

Reaction mix

Use QIAGEN OneStep RT-PCR Kit (Qiagen)

Reagent	Final concentration/volume
Primer For	400 nM
Primer Rev	400 nM
dNTP Mix 10 mM each	0.4 mM
Qiagen OneStep RT-PCR Buffer 5X	1X
Qiagen OneStep RT-PCR Enzyme Mix	1 µl
RNA template	5 µl
DEPC Water	To 25 µl

Thermal profile

RT	Denaturation	Denaturation	Annealing	Extension	Final extension
50°C	95°C	94°C	55°C	72°C	72°C
30 min	15 min	40 sec	40 sec	70 sec	10 min
		40 cycles			

Other RT-PCR kits of proven efficacy and amplification profiles might be used as an alternative. Purity and size of PCR products must be evaluated by gel electrophoresis.

For a more complete characterization of betanodavirus strains, the complete sequence of RNA1 and RNA2 genetic segments should be obtained (protocol available upon request).

6.5.4. Virological examination

6.5.4.1. Cell cultures and media

The best cell line for NNV isolation is SSN-1 (Frerichs *et al*., 1996). This cell line has been specifically designed to show clear cytopathic effects (CPE) when infected with betanodavirus. However also the E-11 cell line (Iwamoto *et al*., 2000), actually a clone of SSN-1, can be adapted to this task. The latter is more stable and resistant therefore is easier to multiply compared to the progenitor cell line. However, if the virus is not present in a high amount, CPE can be transient in E-11 and therefore be difficult to see. For a diagnostic reason, no other cell line should be used. Both cell lines prefer L-15 medium (unless adapted to other cell culture medium) and primary plastic.

Cells have to be prepared 24h prior to infection and incubated with medium supplemented with 10% FBS and antibiotics at 25°C for the very first hours. Only when they are almost confluent can they be moved to the lower incubation temperature.

Multiplication ratio can vary from 1:2 to 1:4 according to the cell line.

Susceptibility of cells must be checked periodically (see chapter below).

6.5.4.2. Incubation and inoculation of cell monolayers

Inoculate the antibiotic treated tissue suspension at two different dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in a final dilution of tissue material in the cell culture medium of 1:10–100.

Each 100 µl dilution should be inoculated into at least 2 cm^2 actively replicating cell culture monolayers. Both the normal and adsorption method may be used.

If the adsorption method is adopted, allow the inoculum to adsorb on the drained monolayers for 1 hour at 20°C. After the adsorption period, add the new medium without FBS supplement. If the normal method is adopted, the culture medium needs to be changed with a new one without FBS, before adding the inoculum.

Incubate at 20° (BFNNV – SJNNV - SJNNV/RGNNV) or at 25°C (RGNNV- RGNNV/SJNNV) according to the origin of the sample and the genotype expected (Panzarin 2014).

N.B: Optimum temperature for cold water betanodavirus is considered 15 °C, however, cells may suffer at this temperature.

Follow the course of infection by regular microscopic examination at ×40–100 magnification every 2-3 days for 10 days.

If the cytopathic effect (CPE) appears, identification procedures must be undertaken (see below). If no CPE occurs after the primary incubation period (10 days), subcultivation must be performed on fresh cultures, using a similar cell growing area to that of the primary culture.

CPE in SSN-1 or E-11 cells is characterized by thin or rounded, refractive, granular cells with large vacuoles, and partial or complete disintegration of the monolayer.

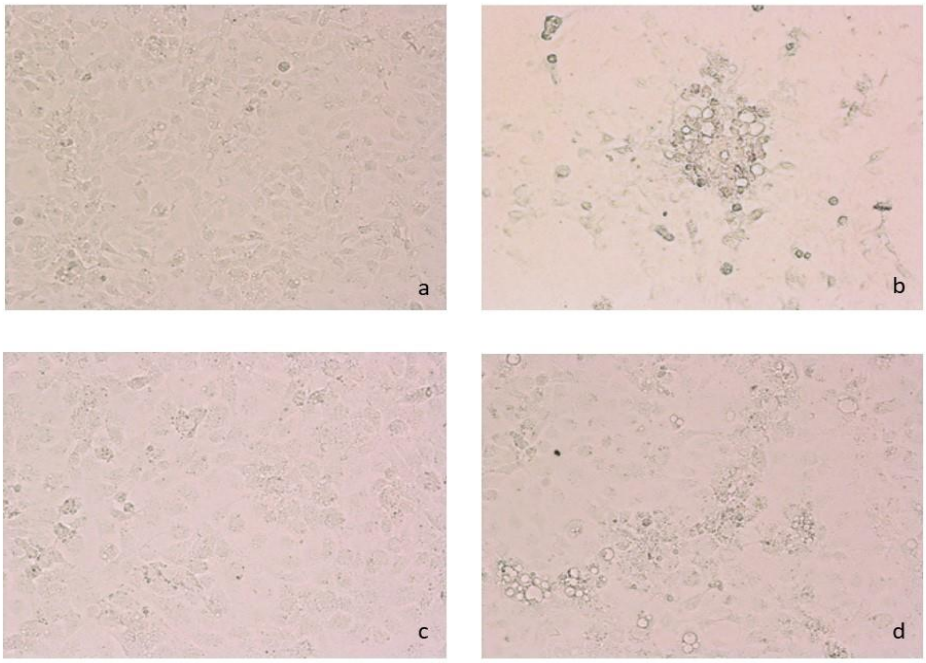

Fig. 6.3. a) Normal cell monolayer of SSN-1 and c) E-11 cell lines, respectively; b) Cytopathic effect of NNV at day 4 post-infection of SSN-1 and d) E-11 cell lines, respectively. Vacuolations are clearly visible. The observation made under light microscope Zeiss at 20 x

6.5.4.3. Subcultivation procedures

Collect aliquots (10%) of cell culture medium from all inoculated monolayers. Inoculate those aliquots constituting the primary culture into wells with the new cell monolayers, as described above (well-to-well subcultivation). Incubate and monitor as described above for a further 10 days.

If no CPE occurs during this period, collect an aliquot of cell culture supernatant and subject to generic real-time RT-PCR. If molecular analysis yields negative results the test may be considered negative.

If molecular analysis detects a positive signal a third passage can be performed in order to further multiply the virus and allow CPE to appear.

If CPE appears, identification procedures must be undertaken (see below).

6.5.4.4. Procedure for titration to verify the susceptibility of the cell cultures to infection

A batch of NNV in low cell culture passage numbers should be used. This virus must be propagated in cell culture flasks of SSN-1 or E-11 cells as described before. At total CPE, the virus is harvested by centrifugation of cell culture supernatant at 2000 x g for 15 minutes and stored in 1 ml cryotubes. The virus shall be kept at -80 °C.

One week after freezing, three replicate vials with the virus are thawed under cold water and titrated on the cell lines. Titres are calculated according to the Reed and Muench formula. The average of the titre of these 3 vials is the reference value for cell susceptibility evaluation.

At least every six months, or if it is suspected that the susceptibility of a cell line has decreased, 1 new vial of the same NNV batch is thawed and titrated. Titration by endpoint dilution should include at least six replicates at each dilution step. The titre is then calculated as before and the value obtained is compared with the initial titre. If the titre decreases by a factor of 2 logs or more, compared with the initial titre, the cell line should no longer be used for surveillance purposes and a new cell line should be recovered from nitrogen or obtained by an approved source.

6.5.5. Virus identification

Virus identification can be performed with different laboratory techniques:

- Real-time RT-PCR/RT-PCR and if necessary sequencing
- Indirect fluorescent antibody test.

6.5.6. Indirect fluorescent antibody test

- Prepare monolayers of susceptible cells (E-11 or SSN-1) directly in 2 cm^2 wells of primary cell culture plastic plates or on cover-slips or chamber slides in order to achieve around 80-90% confluency, which is usually reached within 24 hours of incubation at 25°C.
- Inoculate 100µl of the viral suspensions to be identified using at least two tenfold dilutions.
- Incubate at 20°C or 25°C (according to the NNV strain) for 48–72 hours.
- Remove the culture medium and fix with cold 80% acetone for 10-30 minutes at room temperature.
- Rinse three times with PBS-Tween 0.05% (PBST).
- Allow the cell monolayers to air-dry.
- Add the cell monolayers with a drop (around 200-500 µl) of the primary antibody (i.e. rabbit anti-betanodavirus immune serum) and incubate for 30 minutes at 37°C in a humid chamber.[3]
- Rinse three times with PBST.
- Allow the cell monolayers to air-dry.

[3] N.B. The primary antibody must be diluted according to the manufacturer's instructions. Different antibodies according to the NNV serotype suspected or detected by molecular techniques should be used.

- Add the cell monolayers with a drop (around 200-500 µl) of the primary antibody with commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody) and incubate for 30 minutes at 37°C in a humid chamber.
- Rinse three times with PBST.
- Examine the treated cell monolayers directly on plates, or mount the cover-slips using 50% glycerol-PBS solution, prior to microscopic observation.
- All immunofluorence runs should include one positive and one negative control well.

Brilliant fluorescent cells scattered on the monolayer are visible in positive samples. The fluorescent signal is cytoplasmic with the unmarked nucleus clearly visible.

Always use negative control wells, where uninfected cells only are present.

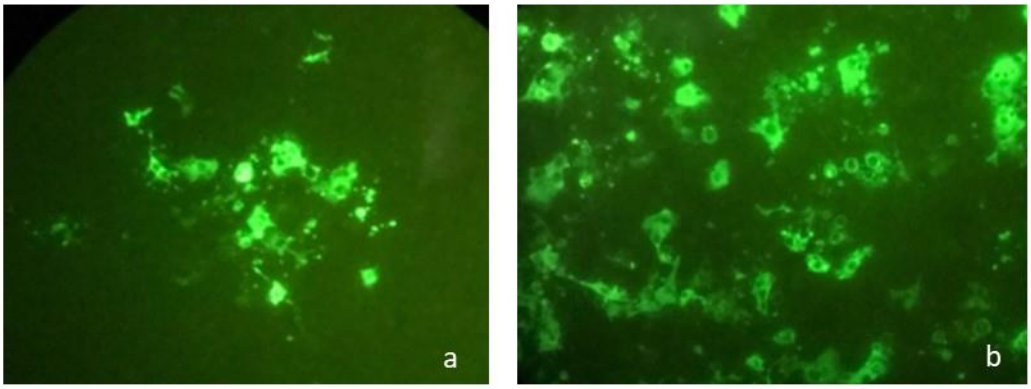

Fig. 6.4. Positive IF reaction on infected SSN-1 cell monolayer 3 days post-infection. a) Single positive focus, and b) spread positive cells. Observation made under fluorescence microscope Zeiss Axioskop at 20 x.

6.5.7. Histology

Buffered formalin-fixed samples should be processed according to standard histological techniques. Lesions occur only in the nervous tissues: retina, brain and spinal cord. Typical lesions are characterized by multiple intracytoplasmatic vacuolations appearing as empty areas of 5-10 µm in diameter, clearly separated from the surrounding areas, mainly present in the grey matter. Pyknosis, karyorrhexis, neuronal degeneration and inflammatory infiltration have been described in all the nervous tissues of the infected fish. Mild to severe congestions of the blood vessels evolving also to haemorrhages in the encephalic parenchyma and meninges are frequently observed. In the retina, vacuoles are generally evident in the outer and inner nuclear layers, as well as in the ganglion cell layer. Similarly, vacuoles can be observed in the optic nerve as well as in the spinal cord. These lesions are by far more prominent in diseased larvae and juveniles while in older symptomatic fish they are sometimes very rare and difficult to detect.

In clinically healthy fish, vacuoles in the brain and especially in the retina can be due to artefacts in particular processing problems; therefore it is always suggested to confirm the presence of the NNV antigen by IHC.

6.5.8. Immunohistochemistry IHC (avidin-biotin-peroxidase technique)

- Dewax sections (3 µm thick) with xylene (2 passages for 10 minutes each) and ethanol 100° (2 passages for 5 minutes each).
- Hydrate tissue sections in a decreasing ethanol series: 95°, 70°, 50°, distilled water (5 minutes each).
- Rinse with Tris Buffered Saline with Tween 20 (TBTS) for 5 minutes.
- Incubate sections with 0.1% trypsin (Porcine Trypsin, Sigma) in distilled water, for 30 minutes at 37°C in a humid chamber.
- Rinse twice with TBST for 5 minutes each time.
- Block endogenous peroxidase by incubating the slides for 10 minutes with anti peroxidase solution (Dako) at room temperature (RT).
- Rinse twice with TBST for 5 minutes each time.
- Incubate sections with ready to use normal horse serum (Vector Laboratories) for 20 minutes at RT.
- Rinse in TBTS for 5 minutes.
- Incubate sections with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) diluted in antibody diluent (Dako) for 60 minutes at RT[4].
- Rinse three times in TBST for 5 minutes each.
- Incubate with the secondary biotinylated antibodies (i.e. goat anti-rabbit immunoglobulins in 2.5% BSA) for 30 minutes at RT.
- Rinse two times in TBST for 5 minutes.
- Incubate with avidin/biotin-based peroxidase system (Vectastain ABC HRP - Vector Laboratories) for 30 minutes at RT.
- Rinse twice in TBST for 5 minutes.
- Incubate with chromogen substrate 3-amino-9-ethylcarbazole (AEC), prepared just before use, for 20 minutes at RT.
- Rinse with distilled water for 5 minutes.
- Counterstain with Harris' hematoxylin for 30 seconds.
- Mount sections in glycerol gelatine.
- All immunohistochemical runs should include one positive and one negative control section.

IHC should be always performed in case of NNV suspicion even in the absence of evident lesion. It has been described that immunoprecipitate can be visualized largely before the apparition of vacuoles or even in absence of them (Toffan *et al*., 2017; Valencia *et al.,* 2019; Mladineo, 2003). Positive samples will present red immunoprecipitates in nervous tissues mainly surrounding vacuoles but also following the shape of apparently normal neurons. Consider that pale diffuse red stain of tissues is not considered as specific immunolabelling (background) and depends on the quality and dilution of the antibodies used. Always use a negative control slide.

[4] N.B. The primary antibody must be diluted according to the manufacturer's instructions. Different antibodies according to the NNV serotype suspected or detected by molecular techniques should be used.

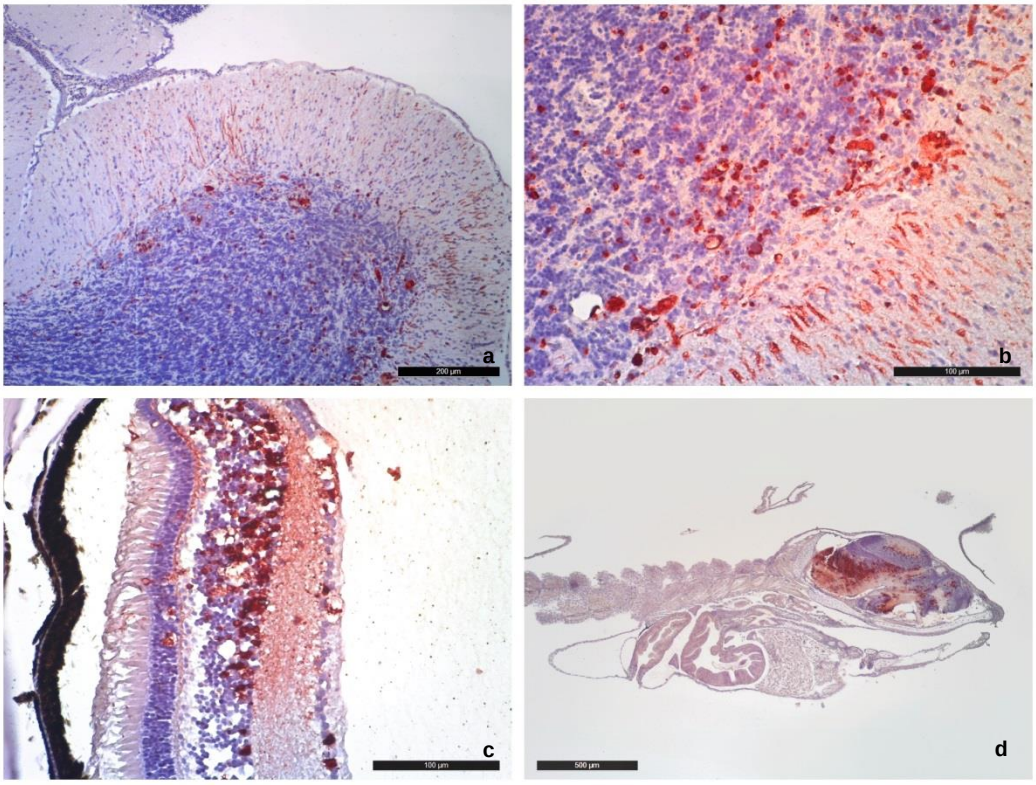

Fig. 6.5. a) and b) NNV infected seabass cerebellum showing strong immunoprecipitates in neurons soma and dendrites (10x and 25x); c) NNV infected seabass retina showing vacuolations in inner nuclear layer and in ganglion cell layer and spread immunoprecipitates mainly localized in inner and outer nuclear layer and in ganglion cell layer (25x); d) Widespread immunoprecipitates in the brain of 16 day post-hatching seabream larvae RGNNV/SJNNV infected.

6.5.9. Conservation of samples

Samples in RNA stabilization solutions can be stored indefinitely at -20°C.

Organs can be conserved at -80 C for months and isolated virus for many years. The viability of the virus decreases with time; however, betanodaviruses are quite resistant agents and therefore virus can be retrieved from positive samples after several years of storage.

Viral isolates can be stored indefinitely at -80°C.

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Part IV. Bacterial diseases with impact on sea bass and sea bream farming

7. Introduction to bacterial diseases

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7.1. Introduction

The marine environment favours the survival of bacteria outside their host and potentiates bacterial diseases to become a major obstacle to aquaculture (Pridgeon and Klesius, 2012). However, only a few of the bacteria normally inhabiting the Mediterranean marine environment are associated with disease outbreaks (Pujalte *et al*., 2003). Among pathogenic bacteria, a few of them constitute primary pathogens while many others are opportunists colonizing and causing disease to already compromised hosts (Austin and Austin, 1999) thus making the distinction between primary and opportunistic pathogens difficult. A disease outbreak is not necessarily caused by a single bacterial species but may involve synergistic interactions between two or more taxa.

Among the bacterial pathogens in farmed European seabass and gilthead seabream, the most frequent, harmful and economically most important are bacteria from the genus Vibrio namely *Vibrio anguillarum* and *Photobacterium damselae* subsp. *piscicida.* In recent years, there has been an increasing frequency of disease incidence caused by the emerging bacterium pathogen *Vibrio harveyi*, previously mostly known as a pathogen of crustaceans, molluscs and fish in subtropical areas (Mancuso, 2014).

Together with vibriosis and photobacteriosis, *Tenacibaculum* spp. infections (tenacibaculosis, previously known as flexibacteriosis) are considered among the most important diseases for European seabass.

Furthermore, *Aeromonas veronii* bv *sobria,* an opportunistic pathogen of fish both in freshwater and in the marine environment as well as members of the genus *Mycobacterium* increasingly gain importance for the Mediterranean aquaculture industry.

7.2. PCR amplification and sequencing of the 16S rRNA gene

For many bacteria, ascertaining the genus or species is virtually impossible without using molecular methods. One of the most common techniques is PCR amplification and sequencing of the 16S rRNA gene followed by a comparison of the sequence obtained with a large database (e.g. nucleotide BLAST using NCBI database or RDP database). There are many possible combinations of primers that can be used for this, depending on the fragment size and

phylogenetic group. One of the better approaches is to amplify a large fragment in order to have more data for BLAST search. For this, 16 nucleotides long reverse primer 1492R 5' – TACCTTGTTACGACTT – 3' (Frank *et al*., 2008) can be used as a universal primer, and if higher temperatures are required, the expanded, 22-nt long version of the primer 5' – TACGGYTACCTTGTTACGACTT – 3' may be used (Newby *et al*., 2004). However, as regards the forward primer, the analysis is more complicated, because the region closer to the beginning of the gene is much less conserved between different groups of bacteria.

Table 7.1. Examples of different forward primers based on the group of bacteria to be detected

Primer binding site sequence[1]	Phylogenetic group(s) containing the binding site sequence
AGAGTTTGATCCTGGCTCAG	Most Bacteria
**********A********	Many Bacteria, especially enteric bacteria
**********T*********	Actinobacteria, some Proteobacteria
A****T***T****	Chlamydiales
******C**********	Atopobium and chloroplasts
****************T**	Borrelia spp.
**********TA********	Campylobacterales and Sphingomonadales
G*C***T*********	Bifidobacteriales
G********	Thermotogales and Planctomycetales

[1]*Sequence variations are shown as differences from the first (most common) sequence.*

A suggested example of a wide spectrum screening method for bacteria from aquatic environments:

Forward Primer	**27FYM**	5'–AGAGTTTGATYMTGGCTCAG–3'
Reverse Primer	**1492R**	5'–TACGGYTACCTTGTTACGACTT– 3'

Termal profile (using Qiagen HotStarTaq Plus Master Mix Kit):

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	94°C	49°C	72°C	72°C
15 min	1 min	30 sec	2 min	10 min
		40 cycles		

Reaction mix

Reagent	Quantity
Water (molecular biology grade)	6 µl
Master mix 10X	10 µl
10 µM primer 27FYM	1 µl
10 µM primer 1492R	1 µl
DNA samples	2 µl
Total volume	20 µl

Currently, there are many commercial kits available for the extraction and purification of DNA, both from cultures and from tissue samples. A common mistake that can happen when extracting the DNA from tissue samples is shortening or even skipping the homogenization step. Since tissue samples are usually small (~25 mg) and some of the bacteria are intracellular, tissues have to be homogenized using tissue homogenizers, and thoroughly lysed before proceeding with magnetic or spin column purification.

7.3. *In vitro* susceptibility testing

Although disease management is not in the scope of this document, due to the fact that fish farmers want to know, as soon as possible, how to manage bacterial disease outbreaks, all diagnosticians are faced with the challenge of advising on a suitable antibacterial treatment. Antibacterial substances are usually mixed in feed and the appropriate choice of substance based on susceptibility testing is of utmost importance to minimize possible hazards to aquatic ecosystems and the development of resistant bacterial strains.

Veterinarians and other aquatic animal health professionals are in charge of prudent and responsible use of antimicrobials in aquatic animals. They are obliged to carry out the diagnostic procedures including clinical and postmortem assessment of the aquatic animal(s), bacteriology with culture and sensitivity testing and other laboratory tests to arrive at the most definitive diagnosis prior to initiating treatment with an antimicrobial agent. F or this reason the main principles of sensitivity testing that have to be an obligatory part of bacteriological diagnostic procedure (OIE, 2018) will be addressed.

There are two groups of methods available to generate an *in vitro* measure of susceptibility; one includes measuring the minimum concentration required to inhibit bacterial activity related to cell propagation (MIC) and is measured in µg/ml. Another method relies on the measurement of the inhibition zones produced by discs containing the antimicrobial agent and measures susceptibility in mm (Disc diffusion test, also known as the Kirby-Bauer test).

For disc diffusion tests at least 3 colonies of tested bacterial strains are harvested from the agar media and suspended in sterile physiological saline solution adjusted to 1-2 x 10^8 CFU (Alderman and Smith, 2001). Inoculum is streaked onto Mueller-Hinton agar (MHA) supplemented with 1% NaCl and discs containing antibiotics in a certain concentration are placed onto the agar. The plates are incubated at 20 to 25^{0}C, read after 24 to 48 hours by measuring the inhibition zone around each different antibiotic disc in mm (Smith and Egan, 2018). The results are interpreted based on the epidemiological cut-off values as sensitive (Wild type, WT) or resistant (Non-wild type, NWT) using NRI (Normalized Resistance Interpretation); http://www.bioscand.se/nri/ (Smith, 2017) available online.

For measuring the MIC, antimicrobials are diluted in the buffer, depending on the substance, and subsequently serially diluted to reach a final concentration of 250 to 0.015 µg/ml (Alderman and Smith 2001). Bacterial cultures are suspended into 0.9% sterile saline at a concentration of 1-2x10^8 CFU/ml and diluted into cation-supplemented Mueller Hinton broth (CSMHB). Microdilution methods are carried out in the 96 well sterile microtiter plates with U shaped bottom with 100 µl of two-fold dilution series of an antibiotic solution in CSMHB with the exception of those wells acting as drug-free controls. Each well except those acting as sterility controls should then receive 100 µl of bacterial suspension. Plates are put in the incubator for appropriate temperature for 72 hours. The highest dilution of the antibiotic at which no growth is visually determined is considered as the MIC. Reference strains such as *E.coli* or *A. salmonicida* should be included in each plate as a quality control organism. The results are interpreted based on the epidemiological cut-off values as sensitive or resistant, using NRI (Normalized Resistance Interpretation); http://www.bioscand.se/nri/ or ECOFFinder clsi.org/standards/micro/ecoffinder/ spreadsheet available online (Smith, 2017). It is a very useful method for the determination of developing resistance of bacteria to antimicrobials, or

when there is a need to study the effectiveness of antimicrobials in the treatment of a bacterial disease.

It is worth mentioning that there are several kits for MIC measuring such as Etest produced by bioMerieux, France (https://www.biomerieux-usa.com/clinical/etest) and VetMIC produced by SVA, Sweden (https://www.sva.se/en/service-and-products/vetmic) or Thermo Scientific™ Sensititre™ (https://assets.thermofisher.com/TFS-Assets/MBD/brochures/Sensititre-Custom-Plates-Product-Overview.pdf).

Finally, it is of utmost importance to underline the necessity of rapid, reliable and highly sensitive diagnostics in effective control and treatment of bacterial diseases.

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8. *Vibrio anguillarum*

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8.1 Aetiology of Vibrio anguillarum

Vibrio anguillarum, a facultative anaerobic, fermentative, curved rod and Gram-negative bacterium is the aetiological agent of the so-called “classical vibriosis” affecting many brackish water and marine Mediterranean species; mainly European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), sole (*Solea* spp.), sea mullet (*Mugil spp.*), turbot (*Scopthalmus maximus*) and Europan eel – (*Anguilla anguilla*) (Austin and Austin, 2013). The disease is typical haemorrhagic septicaemia characterized by petechiae and suffusions above all in the skin and fins, but also in internal organs (liver, kidney, intestine, gonads, brain) and gills. Darkening, anorexia and lethargy are usually the first symptoms of the disease while exophthalmos and abnormal swimming may be also present. Sudden changes in water temperature and environmental conditions, as well as any kind of stress, can influence the onset and spread of the disease. *V. anguillarum* serotype O1 and O2 are also considered the most virulent strains, causing high mortality, above all in fingerlings and juveniles.

8.2. Sampling for diagnostic procedures for *V. anguillarum*

8.2.1 Sampling

Decomposed/putrefied or frozen fish must be avoided, so the sampling procedure is very important for a good bacteriological investigation.

8.2.2. Preparation and shipment of samples from fish

Live sick (moribund) fish or freshly dead (fish that died less than 2-3 hrs before) must be collected and sent refrigerated to the laboratory as soon as possible and no later than 1 day after (Midtlyng *et al.,* 2000). If possible, for example, when fingerlings are affected, living fish may be sent inside a double plastic bag (one part water and two parts air/oxygen). At best, the fish must be sampled before the start of antimicrobial treatment in order to avoid false-negative results.

Samples may also be submitted as swabs from skin or internal organs and transported in Amies transport medium at a temperature lower than 10°C, not frozen.

8.3. Diagnostic procedures for *V. anguillarum*

8.3.1. Primary cultivation of bacteria (Choice of media and isolation of strain)

Vibrio anguillarum can easily grow on Blood agar (BA), Tryptone soya agar (TSA) + 2 % NaCl or Marine agar (MA). Tryptone soya broth (TSB) + 1.5-2 % NaCl or Marine broth (MB) can be useful if antimicrobial treatment has commenced in the past few days or carried out during the last 15-20 days or in case healthy carriers are sought.

8.3.1.1. Swabs

Swabs can be inoculated directly to the appropriate culture media (see above).

8.3.1.2. Sampling from fish

If fish are bigger than 4-5 cm, the abdominal wall may be removed aseptically during necropsy and sterile cotton-tipped swabs (or sterile disposable inoculating loops) used to sample the tissue and perform uniform streaking of agar plates (BA, MA or TSA + 2 % NaCl) or inoculate broth medium (TSB or MB). Head kidney, liver and spleen are usually suitable target organs to be sampled. In addition, material from the edge of a skin lesion or from haemorrhagic eyes may be collected.

When the fish are smaller, or in the case of larvae, the animals must be repeatedly washed three to four times with sterile distilled or normal saline water, macerated with flame sterilized scissors and then used to inoculate the appropriate media (see above). Thiosulfate citrate bile salt (TCBS) agar or Vibrio Chromagar can help avoid overgrowth of invading bacteria.

8.3.2. Isolation and growth conditions

The medium shall be incubated at 25 ± 2°C for 2-4 days. If a bacteriological incubator is not available, agar plates and broths may be kept at room temperature (18°C < T < 37°C). Culture plates must be examined every day, any suspect colonies selected and subcultured to BA and

TCBS to obtain pure growth cultures (secondary plates). Negative plates should not be discarded prior to completing 3 days of incubation.

8.3.3. Screening of pure cultures

Suspect colonies can be selected from secondary BA plates (greyish-whitish, 1-2 mm, circular and usually beta-haemolytic in BA after 48 hrs) in order to perform primary identification tests (microscope observation, Gram staining, oxidase and vibriostat test).

Microscope observation: a single colony from the secondary BA plates is picked and mixed with a drop of sterile distilled water on a disinfected glass slide and observed at 400 x in phase contrast. *Vibrio anguillarum* bacteria appear singly or in pairs, are slightly bent motile rods without spores.

Gram stain: commercial reagents are available to stain bacteria. *Vibrio anguillarum* is a Gram-negative bacterium appearing as a straight and/or curved rod.

Oxidase test: *V. anguillarum* is oxidase positive. When a single colony is picked and streaked on a commercial strip, a purple/blue colour is obtained within 1 minute.

Vibriostatic test: Vibrio discs O/129 (10 µg and 150 µg) are placed on BA plates inoculated with a pure bacterial inoculum prepared to a density of Mc Farland tube 1. *Vibrio spp*. are usually sensitive to both the vibriostat concentrations while *Aeromonas* spp. are resistant.

Presumptive identification: So far only a suspicion of Vibriosis is evident (*Vibrio* spp. are Gram-negative, curved rods, oxidase and vibriostatic positive). Correct identification of *Vibrio anguillarum* requires one or more of the following tests to be performed.

8.3.4. Identification of the strain

Biochemical tests that involve carbohydrate fermentation or enzymatic hydrolysis can be performed with specific reagents produced at the laboratory or purchased as commercial kits, such as the API20E strips BioMerieux® or the Biolog® system. All these methods are not conclusive because of phenotypic diversities due to different bacterium strains, or environmental conditions. Sometimes the test outcome is dependent on optimal NaCl or other seawater salt concentrations. The biochemical profile index provided by the manufacturer is used as a guide. It has to be cross-checked against the database listed at least in two different books/papers in order to avoid misidentifications.

Biochemical results: *V. anguillarum* has an optimum growth at 20-25°C, is fermentative, oxidase, ADH, indole, β-galactosidase positive, while lysine and ornithine decarboxylase, urease, and H_2S negative. It ferments glucose, maltose, mannitol, sucrose, sorbitol. Citrate can be variable. The API20E kits should be used with caution: 1.5-2 % NaCl should be added in the inoculating fluid and the reactions on the strips must be read after 24 and 48 hours of incubation. The most probable API20E identification codes for *V. anguillarum* are: 304452456, 304572557, 304652456, 304752456, 304752476, 304752557, 304752657, 324472757, 324562757, 324632657, 324752457, 3, 24752557, 324752656, 324752657, 324752756, 324752757, 324752777, 324712677, 324772656 (Buller 2014).

Serotyping: Based on the detection of the heat-stable somatic O antigen, *V. anguillarum* isolates have been divided to date into 10 serotypes, but only serotype O1 and O2 are considered pathogenic, while the others comprise environmental isolates (Sorensen and Larsen, 1986). Specific antisera may be produced according to the Sorensen and Larsen protocol or purchased from private companies (e.g Bionor®). A colony can be mixed with one drop of antiserum to perform slide agglutination testing: when positive, whitish granular sand will appear within 1 minute.

8.3.5. Mass spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) is a special mass spectrometry tool able to identify bacteria, yeasts and fungi according to their different ribosomal protein composition. Some *Vibrio* species are included in the system's database, but *V. anguillarum* is not. Prior to employing this method, the database must be complemented subsequent to analysing many *V. anguillarum* reference strains. This pathogen is well identified in the MALDI-TOF database as genera and species, but not as different serotypes.

8.3.6. Molecular methods

Molecular methods can be useful to confirm the presumptive biochemical identification of *V. anguillarum*. Sequencing the 16S rRNA gene is not recommended, as many *Vibrio* species are similar, while Multilocus Sequence Analysis – MLSA and DNA-DNA hybridization are more specific (Pascual *et al.,* 2010), but specialized equipment and training are required. Analysis using end-point PCR for amplification of the pyrH gene is nowadays a good molecular technique (Sawabe *et al.,* 2017). Primer sequences, as well as genetic profiles submitted to databases (e.g. GenBank®), are available.

End-point PCR for the *pyrH* gene, following (Sawabe *et al*., 2007; Pascual *et al*., 2010)

Forward primer	**pyrH80F**	5'- GATCGTATGGCTCAAGAAC-3'
Reverse Primer	**pyrH530R**	5'-TAGGCATTTTGTGGTCACG-3'

PCR mix contains the following reagents

Reagent	Quantity
Water (molecular biology grade)	34.20µl
10X Buffer (-MgCl2)	5 µl
50mM MgCl2	1.5 µl
10 mM dNTPs	4 µl
10 µM primer pyrH80F	2 µl
10 µM primer pyrH530	2 µl
Platinum Taq polymerase 5 U/µl	0.3 µl
DNA samples	1 µl
Total volume	50 µl

Thermal profile

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	94°C	53°C	72°C	72°C
2 min	1 min	2 min 15 sec	75 sec	7 min
		40 cycles		

Expected amplified product is 449 bp long.

The species-specific PCR protocol capable of discriminating *V. anguillarum* from 25 species of the Vibrio genus including *V. ordalii* is the one targeting the amiB gene, which encodes N-acetylmuramoyl-L-alanine amidase. By comparing available amiB sequences from different *Vibrio* species, (Hong *et al.,* 2007) have identified a variable region in the amiB gene, and designed specific primers van-ami8 and van-ami417:

Forward primer	**van-ami8**	5'-ACAT CATCCATTTGTTAC-3'
Reverse Primer	**van-ami417**	5'-CCTTATCACTATCCAAATTG-3'

As usual, the exact PCR conditions depend on the DNA concentration in the isolate, and the type of polymerase used, so what follows is an example using Qiagen HotStarTaq or similar polymerase with DNA extracted from bacterial culture using commercial kit.

PCR mix contains the following reagents

Reagent	Quantity
RNA/DNA free water	6 µl
Master mix 10X	10 µl
10 µM primer pyrH80F	1 µl
10 µM primer pyrH530	1 µl
DNA samples	2 µl
Total volume	20 µl

Thermal profile (using Qiagen HotStarTaq Plus Master Mix Kit)

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	95°C	56°C	72°C	72°C
15 min	30''	30''	30''	10 min
		25 cycles		

8.3.7. *In vitro* susceptibility testing

Kirby Bauer Disk diffusion test can be effectively performed on Mueller-Hinton agar supplemented with 2% NaCl evaluated at 25°C after 24 h (CLSI 2011). Minimum Inhibitory Concentration can be performed following the CLSI protocol (CLSI 2014) applying a suspension of 18-24 h young culture in cation-adjusted Mueller Hinton broth (CAMHB) with a final concentration of 5×10^5 cfu/ml. Incubation is best performed at 22°C for 24 h.

Vibrio anguillarum is naturally resistant to amoxicillin and ampicillin. Currently, there is no significant resistance to commercially used antibiotics (Oxytetracycline, potentiated sulphonamides, flumequine, florfenicol).

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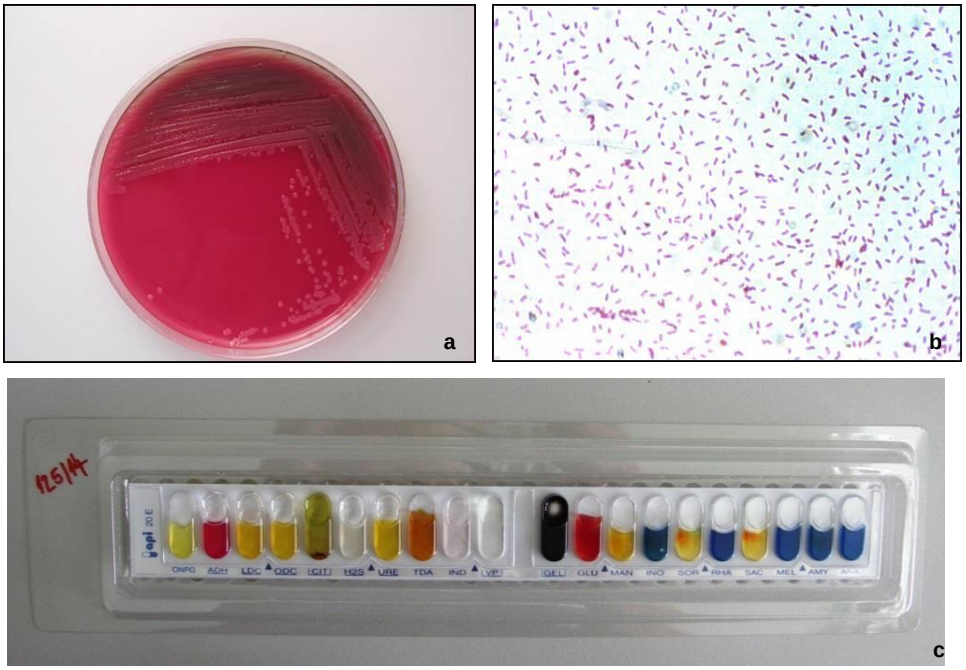

Fig. *8.1*. a) *Vibrio anguillarum* growth on blood agar with greyish-whitish, 1-2 mm, circular colonies; b) Gram stained smears of pure colony reveals Gram-negative straight and/or curved rods; c) most frequent *V. anugillarum* results of API®20E™ after 24 h.

Fig. 8.2. a) Hemorrhages on the mouth, operculum fin base and fins in subacute form of vibriosis caused by *V. anguillarum*; b) Exophthalmos in subacute vibriosis; c) Hemorrhages on the liver and intestines.

9. *Vibrio harveyi* group

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9.1. Introduction

Vibrio harveyi and related species are referred to as the *Harveyi* clade (Sawabe *et al*., 2007; Sawabe *et al*., 2013). There is no definitive consensus on which species comprise the *Harveyi* clade, and up to 11 species have been included in this clade following MLSA analysis: *V. harveyi, V. alginolyticus, V. campbellii, V. mytili, V. natriegens, V. parahaemolyticus, V. rotiferianus, V. azureus* (Yoshizawa *et al*., 2009), *V. sagamiensis* (Yoshizawa *et al*., 2010), *V. owensii* (Cano-Gómez *et al*., 2010), *V. jasicida* (Yoshizawa *et al*., 2012). Members of this clade share a high degree of genetic and phenotypic similarity and are commonly found in marine and estuarine water and sediments or as commensal, opportunistic or primary pathogens of marine species. Among vibrios of the *Harveyi* clade, four species (*V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii)* known as the *V. harveyi* group, are well-known pathogenic agents in marine reared fish, crustaceans and shellfish, being responsible for high mortality rates in commercial farms worldwide (Cano-Gómez *et al*., 2011).

9.2. Aetiology of *V. harveyi*

*Vibrio harvey*i (Johnson and Shunk, 1936) is a Gram-negative bacterium of the family *Vibrionaceae* (Gammaproteobacteria) that has been recognized as an emerging pathogen for global marine aquaculture (Austin and Zhang, 2006). It has been reported with increasing frequency in finfish species reared in subtropical regions (groupers, barramundi, flatfish, pompano), in sharks and in gastropods (abalone), and it is a well-known problem in the husbandry of post-larvae stages of penaeid shrimps (luminous vibriosis). In Europe and particularly in the Mediterranean basin this microorganism is gaining importance as a primary pathogen of European seabass (*Dicentrarchus labrax*) and flatfish (*Solea* spp.).

Type strains of the previously recognized species *Vibrio carchariae* (Grimes *et al.,* 1985) and *Vibrio trachuri* (Iwamoto *et al*., 1995) were determined to be synonyms of *V. harveyi* based on molecular studies (Pedersen *et al*., 1998; Thompson *et al*., 2002). *V. harveyi* is found in a free-living state in aquatic environments and as a part of the normal flora of marine animals (Makemson and Hermosa, 1999). *V. harveyi* isolates from the environment or invertebrate hosts (crustacean) are frequently characterized by luminescence.

V. harveyi causes, in the majority of teleost fish, cutaneous, ophthalmic or gastroenteric infections that frequently generalize in septicaemia (Austin and Zhang, 2006). Cutaneous lesions appear as erosions, haemorrhaging at the base of the fins, ulcerations or necrotic vesicles of the dermis. Ocular lesions encompass keratitis, corneal opacities or panophthalmitis frequently related to secondary infection after traumatic or parasitic lesions (Pakingking *et al*., 2018; Minami *et al*., 2016). Gastrointestinal infections show serous or serous-catarrhal enteritis with marked dilatation of the intestinal lumen (proximal tract) and accumulation of yellowish exudate (Lee *et al*., 2002; Yii *et al*., 1997). *V. harveyi* has been isolated in sharks from cutaneous ulcers and in septicemic forms characterized by vasculitis (Grimes *et al*., 1985).

In *D. labrax*, *V. harveyi* has been isolated during mortality outbreaks characterized by lethargy, anorexia and ataxia, occurring mainly during the grow-out phase (40-160 g) and in juveniles housed in hatcheries. Affected specimens showed cutaneous or ophthalmic lesions, enteric inflammation and encephalic congestion.

9.3. Sampling

9.3.1. Preparation and shipment of samples from fish

Symptomatic or moribund fish or recently dead specimens (less than 2-3 h) should be preferred and sent refrigerated to the laboratory as soon as possible (no later than 24-36 h). If possible, for example when fingerlings are affected, live fish should be sent inside a double plastic bag (one part water and two parts air/oxygen). When feasible, collect the animals before the administration of any antimicrobial treatment in order to avoid false-negative results.

V. harveyi can be isolated from swabbing from skin ulcers or internal organs (head kidney), swabs embedded in Amies transport medium and maintained at a temperature lower than 10°C (not frozen).

All materials should be placed in leak-proof containers and precisely labelled. Please refer to Chapter 2.2. for specific instructions on shipping biological substances.

9.4. Diagnostic procedures for *V. harveyi*

9.4.1. Primary cultivation of bacteria

V. harveyi can be retrieved by standard bacteriological sampling from head kidney, spleen, brain, cutaneous lesions or ocular lesions.

If submitted specimens are smaller than 3-4 cm (juveniles or larvae), rinse the animals with three or four washes of sterile saline solution, mince the fish with sterilized blades and with a sterile loop inoculate the appropriate media.

V. harveyi can be easily isolated in blood agar (BA), tryptone soya agar (TSA) supplemented with 2% NaCl, marine agar or in liquid mediums (TSB 2% NaCl or Marine broth) maintained at 22-25°C. Colonies can be observed after 24-48 hours. Direct isolation in a selective and differential medium like TCBS is a viable option.

9.4.2. Screening of pure cultures

Macroscopically *V. harveyi* colonies do not show any discriminant features on BA, except luminescence (for some isolates) when observed in the dark; they generally appear as greyish-white, slightly translucent, non swarming colonies. Haemolytic activity (α or β-haemolysis) can be observed on BA (≈50% of isolates) after 24-48 h. Luminescence is relatively infrequent in isolates from fish (10% of isolates) but, if present, is easier to observe in colonies cultivated on BA than TSA 2%NaCl.

V. harveyi isolates from finfish typically metabolize sucrose and appear yellow on TCBS agar, while their coloration may vary on CHROMagar Vibrio™. Most frequently isolates appear bicolour (lilac and white): colonies at high densities appear pale lilac or rose, while isolated colonies appear white. Other colorations are uniform pale rose or pale lilac or milky white (Pretto, 2018).

V. harveyi is a Gram-negative short rod, slightly curved, pleomorphic, facultative anaerobic, motile by single polar flagella with dimensions that vary according to author: 1.0-1.6 × 0.5-0.7 µm (Buller, 2014); 1.9-3.0 × 1.2-1.4 µm (Shen *et al.,* 2017), 1.6-2.2 × 1.0-1.1 µm (Tu *et al*., 2017). It is capable of oxidative and fermentative metabolism, oxidase and catalase positive. *V. harveyi* is halophilic and grows at NaCl concentration between 1-7%, temperature between 10-40°C; no growth is observed at 42°C. It can swarm on TSA 2% NaCl (≈50% of isolates) or marine salt agar but not on BA, TCBS, MacConkey agar (Buller, 2014) or CHROMagar Vibrio™. *Vibrio harveyi* is sensitive to vibriostat agent O/129 (2, 4- diamino-6, 7-di-iso-propylpteridine phosphate) at high concentrations (150 µg), whereas results may vary at low concentrations (10 µg) (Buller, 2014).

9.4.3. Identification of the strain

9.4.3.1. API

API®20E™ (bioMérieux) test gives an effective identification for *V. harveyi*; it should be performed with inoculum at 0.5 McFarland (bacteria suspended in a solution with 2% NaCl) maintained at 25°C and evaluated after 24 and 48 hours. The most discriminating tests for *V. harveyi* isolated from teleost fish are related to aminoacid metabolism (ADH -; LDC +; ODC +), citrate (CIT+), hydrogen sulphide (H2S -), β-galactosidase (ONPG -), tryptophan deaminase (TDA -), indole (IND +), Voges-Proskauer test (VP -) and gelatinase (GEL +, 80%). Citrate metabolism and gelatinase may appear later (after 48-72 h). Sugar metabolism may vary for sorbitol (SOR +; 60%) and sucrose (SAC +; 95%) and appears positive for glucose, mannose and amygdalin and negative for inositol, rhamnose, melibiose and arabinose (Pretto, 2018).

Urease test appears frequently negative (URE -; 95%), whereas positive results are obtained with Christensen agar slant.

The most frequent API®20E™ code for *V. harveyi* isolates in *D. labrax* are 4346525, 4346125, 4344125; less frequently: 4356525, 4354525, 4346105, 4344525, 4246525, 4344125 (Pretto 2018).

The interpretation of the API®20E™ (bioMérieux) results via Apiweb™ does not effectively identify *V. harveyi*, because the bioMérieux database does not contain a profile for this species in the API 20E system. Consequently, the identification of codes obtained from *V. harveyi* strains is assigned to *Vibrio alginolyticus*, which is the most phenotypically similar species between the Vibrionaceae of the database.

9.4.3.2. Mass spectrometry

The identification of isolates belonging to the species *V. harveyi* can be effectively obtained through MALDI-TOF analyses, generally with good identification scores. It is important to evaluate the presence of all the species considered belonging to the *Harveyi* clade in the database of the instrument and if necessary integrate the database with reference strains of the species missing or improve the database with more than one isolate for each species.

9.4.3.3. PCR

Molecular analysis can be performed on isolated bacterial colonies. DNA extraction is performed on a single isolate (clonal growth after 24 h in BA or TSA 2%NaCl) collected with a loop and mixed in a suitable amount with the lysis buffer of the DNA extraction kit selected, following the manufacturer's instructions. Extracted DNA should be standardized at 100 ng/µl.

Identification of *V. harveyi* can be performed by diagnostic laboratories with two different approaches:

(i) Amplification of the housekeeping gene uridine monophosphate kinase (*pyrH*) followed by sequencing and phylogenetic analysis in order to confirm the identification of the isolate at the species level through the comparison with the sequences available in the literature.

(ii) Amplification of the *toxR* gene in its hypervariable region by *V. harveyi* species-specific primers (Pang *et al*., 2006).

(i): end-point PCR for the *pyrH* gene, following (Sawabe *et al.,* 2007; Pascual *et al.,* 2010).

Forward primer	**pyrH80F**	5'- GATCGTATGGCTCAAGAAC-3'
Reverse primer	**pyrH530R**	5'-TAGGCATTTTGTGGTCACG-3'

PCR mix contains the following reagents:

Reagent	Quantity
Water (molecular biology grade)	34.20µl
10X Buffer (-MgCl2)	5 µl
50mM MgCl2	1.5 µl
10 mM dNTPs	4 µl
10 µM primer pyrH80F	2 µl
10 µM primer pyrH530	2 µl
Platinum Taq polymerase 5 U/µl	0.3 µl
DNA samples	1 µl
Total volume	50 µl

Thermal profile:

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	94°C	53°C	72°C	72°C
2 min	1 min	2 min 15 sec	75 sec	7 min
		40 cycles		

Expected amplified product is 449 bp long.

(ii): end-point PCR *V. harveyi*, species-specific (*toxR* gene) following (Pang *et al*., 2006).

Forward primer	**toxRF1**	5' - GAAGCAGCACTCACCGAT-3'
Reverse primer	**toxRR1**	5'- GGTGAAGACTCATCAGCA-3'

PCR mix contains the following reagents:

Reagent	Quantity
Water (molecular biology grade)	34.20 µl
10X Buffer (-MgCl2)	5 µl
50mM MgCl2	1.5 µl
10 mM dNTPs	4 µl
10 µM primer toxRF1	2 µl
10 µM primer toxRR1	2 µl
Platinum Taq polymerase 5 U/µl	0.3 µl
DNA samples	1 µl
Total volume	50 µl

Thermal profile (Pretto, 2018), modified from (Pang *et al*., 2006):

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
94°C	94°C	61°C	72°C	72°C
1 min	1 min	1 min 15	1 min	10 min
		30 cycles		

Expected amplified product is 382 bp long.

9.4.4. *In vitro* susceptibility testing

The Kirby Bauer Disk diffusion test can be effectively performed on Mueller Hinton agar supplemented with 2% NaCl evaluated at 25°C after 24 h. Minimum Inhibitory Concentration can be performed following the CLSI protocol (CLSI, 2014) applying a suspension of 18-24 h young culture in cation-adjusted Mueller Hinton broth (CAMHB) with a final concentration of 5×10^5 ufc/ml. Incubation is best performed at 22°C for 24 h.

V. harveyi isolated from farmed *D. labrax,* collected in the Mediterranean basin, evidenced a limited number of antibiotic resistance. Susceptibility to florfenicol, tetracycline, flumequine and sulfamethoxazole+trimethoprim has been observed in the majority of isolates (Pretto, 2018). Resistance is observed to ampicillin and colistin.

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Photos

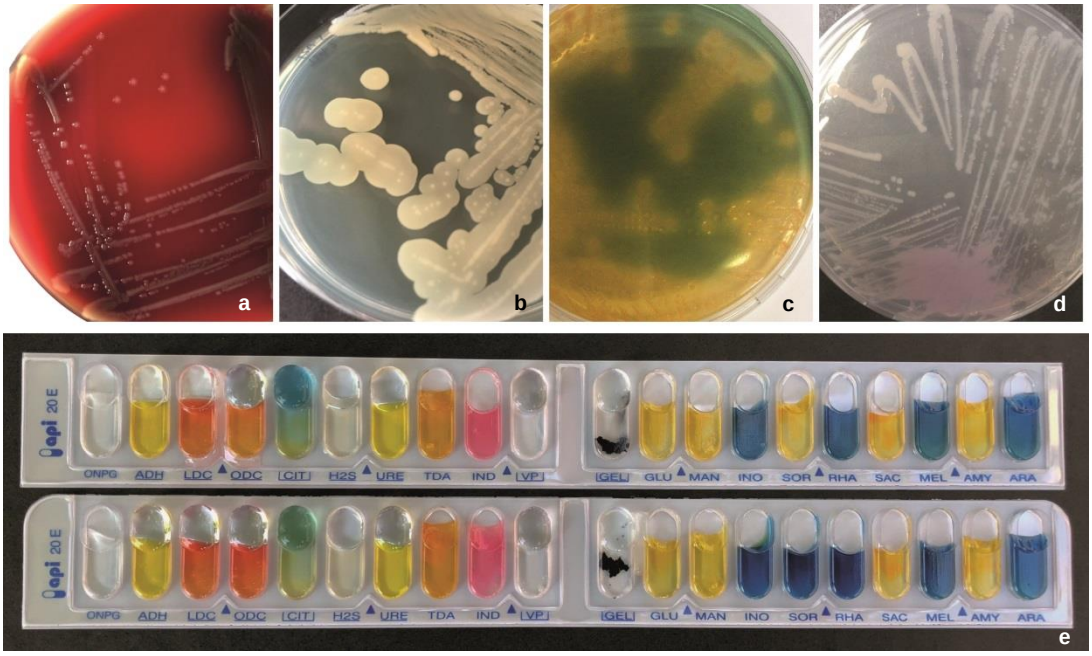

Fig. 9.1. a) *V. harveyi* growth on blood agar with greyish slightly translucent colonies; b) colonies of *V. harveyi* on TSA 2%NaCl may show some swarming; c) yellow colonies of *V. harveyi* on TCBS; d) bicolour growth on CHROMagar Vibrio™, with pink and white colonies; e) most frequent *V. harveyi* results of API®20E™ after 24 h; gelatinase and citrate should be evaluated after 48 h.

Fig. 9.2. a) Cutaneous erosions and ulcers in *D. labrax* juveniles (arrows); b) meningeal and encephalic congestion of blood vessels in *D. labrax* juvenile; c) serous-catarrhal enteritis with marked dilatation of the intestinal lumen (arrows).

10. *Photobacterium damsela* subsp. *piscicida*

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10.1. Aetiology of pasteurellosis (photobacteriosis)

Photobacterium damselae subsp*. piscicida* (former name: *Pasteurella piscicida* (Gauthier *et al.,* 1995; Jansen and Surgalla, 1968) causes a disease of importance resulting in serious losses among cultured fish species in Europe, such as gilthead seabream (*Sparus aurata*), red porgy (*Pagrus pagrus*), red seabream (*Pagrus major*), European seabass (*Dicentrarchus labrax*), meagre (*Argyrosomus regius*) and sole (*Solea* spp.). Gilthead seabream and European seabass are suffering most of the economic losses under aquaculture conditions in Europe including countries such as France, Italy, Malta, Spain, Portugal and Greece (Toranzo *et al.,* 1991; Baudin-Laurencin *et al*., 1991; Ceschia *et al.,* 1991; Baptista *et al*., 1996; Bakopoulos *et al.,* 1997; Zorilla *et al*., 1999).

The disease is also of importance in Turkey (Candan *et al*., 1996; Korun and Timur, 2005) as

well as Japan (Kusuda and Yamaoka, 1972; Koike *et al.,* 1975) and the USA, where it was first isolated from white perch (*Roccus americanus*) by Janssen and Surgalla, 1968.

Photobacterium damselae subspecies *damsela* (*Ph.d.d.*) *and Photobacterium damselae* subspecies *piscicida* (*Ph.d.p*) belong to the thermotolerant group of the genus *Photobacterium* and are genotypically homogeneous subspecies of *Photobacterium damselae*, based on small-subunit rRNA sequencing and DNA:DNA hybridization (Romalde, 2002). The pathogenic subspecies *piscicida* (*Ph.d.p.*) is phenotypically and serologically homogeneous and is the only non-flagellated member of the genus.

Amplified Fragment Length Polymorphism (AFLP) can help epizootiological and taxonomic studies of the highly homogeneous subspecies *Photobacterium damselae* subspecies *piscicida* (Kvitt *et al*., 2002).The combination of PCR direct amplification of a 16S rRNA gene sequence and AFLP in a 2-step procedure grouped *Ph.d.p.* isolates from different geographic regions into distinct clusters on the basis of AFLP intraspecific polymorphisms. The Japanese isolates of *Ph.d.p*. were distinguished from the Mediterranean/European isolates from Italy, Spain, Greece and Israel at a cut-off value of 83% similarity. Further subclustering of the Med/European isolates at a cut-off value of 97% discriminated the Italian, Spanish and Greek isolates from the Israeli isolates.

10.2. Clinical diagnosis

Pasteurellosis presents itself in the hatcheries as hyperacute or acute septicaemia. Seabream larvae, juveniles or fry are often found dead in large numbers on the bottom of the tank with only a few darker fish swimming sluggishly and off-balance near the surface, often showing nervous convulsions (bacterial encephalopathy) prior to death (Abu-Elala *et al*., 2015).

During acute cases of photobacteriosis, fish exhibit only a few pathological signs. Usually, there are no alarming signs with fish behaving and feeding normally before the disease strikes; hence, many among the dead fish carry amounts of feed in their stomach and gut. Convulsive erratic swimming prior to death often comprise the only clinical signs of the acute outbreaks, where internal lesions are often absent at necropsy. Anorexia, lethargy, darkening and ulceration of the skin follow shortly afterwards.

As the disease progresses the gills become pale with excessive mucous secretions with congested inflamed patches and often focal necroses next to congested areas. Lip, opercula skin and lower jaw inflammation and necrotic skin patches on the body flanks, dorsal area and tail become common. The fins, mainly pelvic, dorsal and caudal may be eroded. Skin and fin erosions are covered with mucous, thus the lesions appear in the water as white patches. Overall, there is no haemorrhagic appearance. The liver is most often inflamed and congested; the spleen is enlarged (splenomegaly) and the kidney pale and oedematous. The intestine carries a moderate quantity of fluid and some whitish mucous clots. The swim bladder is not distended, thus, the majority of dead fish have sunk to the bottom of the tank or cage.

In the more chronic form of the disease typical pseudotuberculi develop mainly in the spleen and/or kidney parenchyma. They comprise creamy-white granulomatous nodules, composed of masses of bacterial cells, epithelial cells, fibroblasts, phagocytes and necrotic cell debris. These have led to the descriptive name "pseudotuberculosis". Bacteria accumulate in phagocytes, capillaries and interstitial spaces (Andreoni and Magnani, 2014) and bacteraemia is pronounced.The gill, skin and fin epithelial lesions are suggestive of their susceptibility to bacterial exotoxins as the bacteria gain entrance into the fish body during horizontal transmission.

10.3. Epizootiology of pasteurellosis (photobacteriosis)

The bacteria spread via infected phagocytes, mainly macrophages and the spread is rapid with lethal effects after a few days of infection (Barnes and Ellis, 2004). The disease is hard to eradicate by antibiotic treatments due to the intracellular location of the bacterium, but also due to transferable genetic elements (R plasmids) carrying genes of resistance to many antibiotics (Andreoni and Magnani, 2014). The intracellular location seems to protect from circulating antibodies after vaccination against the disease and may explain the low RPS conferred by vaccination as well as the short duration of immunity post-vaccination. Carriers persist on farms and may show disease under stressful conditions (personal observations). In addition, surviving fish from natural or experimental infection are not protected during subsequent re-challenge (Barnes and Ellis, 2004). Iron acquisition from its host, by means of iron-binding siderophores, increase virulence and the cytotoxic extracellular products (ECPs) damage the infected cells with the consequent release of the bacterium and the invasion of adjacent cells (Andreoni and Magnani, 2014).

A variety of marine fish are natural hosts of the pathogen (Romalde, 2002), but the exact behaviour of the organism outside the host is unknown. *Ph.d.p*. strains are able to survive in culturable state in sea water and sediment for only 6 to 12 days (Magarinos *et al*., 1994), but virulent *Ph.d.p.* cells can enter a "viable but not culturable" (VBNC) state in response to environmental stresses, such as starvation, antibiotic exposure, or low temperature, that allows the cell to enter a state of dormancy and survive until conditions allow resuscitation and reinitiation of infection (Magarinos *et al*., 1994; Oliver, 2010; Pinto *et al*., 2015).

The ability to enter a VBNC state is also common for the survival of virulent strains of the subspecies *damsela*. *Ph.d.d.* maintains infectivity in sea water and sediments for at least 1 year (Fouz *et al*., 1998). Hence, water and sediments can also act as reservoirs for virulent *Ph.d.d.* strains.

Pasteurellosis, or rather photobacteriosis, is a temperature-dependent septicaemic disease. Outbreaks occur when the water temperature rises above 18^{o}C (Korun and Timur, 2005). Below this temperature fish may be subclinical carriers harbouring the pathogen for long periods (Romalde, 2002). In grow-out facilities, pasteurellosis outbreaks occur from late spring or early summer until late autumn, while sea water temperature is maintained above 20°C. In hatcheries, where warm borehole water (>18°C) is in use, photobacteriosis is a major threat all year round.

There is no fish species-specific characteristic lesion other than the differences in sensitivity of age classes among species (Andreoni and Magnani, 2014).

Seabream is susceptible when very young or around the weaning stage of juveniles and remains very sensitive until the size of 6g. Its sensitivity gradually decreases from then onwards, thus, for this species, photobacteriosis is mostly a problem in the hatchery/nursery and during the first months in the grow-out facilities, especially when the transfer to cages coincides with the warm season.

Seabass is susceptible to pasteurellosis beyond the size of 1g (nursery stage onwards). The disease causes the highest mortalities in caged seabass between 5g and 40g. Thus, for bass, photobacteriosis is mostly a problem during the first summer and autumn in the grow-out facilities. Nevertheless, despite the gradual lowering of losses as the seabass grow, they remain considerably susceptible until harvest.

10.4. Sampling

10.4.1. Preparation and shipment of samples from fish

Marginalized, lethargic, moribund fish showing pronounced external lesions or behavioural abnormalities, but not dead specimens, should be collected and sent refrigerated to the laboratory for delivery within 12-24h (fast courier). It is best to collect the fish prior to administering any antibacterial treatment.

Samples must be shipped according to the procedure described in Chapter 2.2.

Freshly dead fish, target organs (e.g. spleen, liver, kidney), fish eggs, live prey organisms, or even tank and filter sediments may be submitted for PCR examination to confirm the presence of the pathogen. These may be placed in screw-cap tubes of appropriate size. The samples may be fixed in RNA later (1:5 v/v), or simply kept deep-frozen at below -20^{o}C until dispatch. If dispatch is planned on the same day, the samples may simply be kept refrigerated.

When running PCR for diagnostic purposes, the nucleic acid (NA) extraction step prior to its targeted amplification procedure is of utmost importance. The pooling of target tissues most likely to be infected by the pathogen (spleen, kidney, liver, brain), or whole specimens, when very small, must not be excessive and over-dilute the pathogen if assumed present in a small fraction of the pooled tissues.

At the laboratory, meticulous homogenization of the tissues ensuring cell lysis is important and the use of tissue lysing machines is recommended instead of grinding the tissues manually by tube and pestle.

10.5. Diagnostic procedures

10.5.1. Overview

Apart from history, clinical symptoms and necropsy findings confirming the tissue lesions of the target organs (e.g. gills, kidney, spleen), additional diagnostic procedures may be employed in the field, such as quick Giemsa-stained spleen imprints or blood smears (characteristic bacteraemia). Isolation and identification may then follow.

A rapid fluorescent antibody (FA) technique, specific for *Ph.d.p*., has been tested for early detection of the pathogen in fish farm waters prior to and subsequent to disease outbreaks among cultured fish (Mancuso *et al*., 2013). Such a technique might prove useful to detect the "viable but not culturable" (VBNC) states of the bacterium early (Fouz *et al.,* 1998; Magarinos *et al*., 1994; Pinto *et al.,* 2015) in water and sediments prompting prophylactic actions or aiding environmental surveys. This technique has shown experimentally that *Ph.d.p.* can be detected in water 20 days after the end of mortalities, proving that the pathogen is present in asymptomatic fish and is released into sea water for some time after an outbreak. The fluorescent antibody technique could be developed to distinguish inactive, active or damaged bacterial cell physiological states (Caruso *et al*., 2003).

10.5.2. Primary cultivation of bacteria

Photobacterium damselae subspecies *piscicida* can be isolated by standard bacteriological sampling from trunk kidney, spleen, liver and/or brain and seeding on agar plates (usually TSA, blood or BHI agar). The brain is a suitable target tissue when fish are small, e.g. 2cm or less, after rinsing well with sterile saline solution.

Ph.d.p. grows well on standard Tryptone Soy Agar (TSA) with no need to supplement NaCl, since commercially available substrates contain 0.5% salt. Characteristic colonies appear after

about 24-36 hours of incubation at 22-28°C. These practical observations are in contrast to what is being described in the literature cited, where it is mentioned that the bacterium needs 1-2% NaCl supplementation of the substrates and takes 2-4 days of incubation to form its distinctive colonies (Romalde, 2002).

Photobacterium damselae subsp*. piscicida -Ph.d.p.*is a halophilic Gram-negative bacterium, appearing as a non-motile rod of 0.5 x 1.5 µm in size with bipolar staining and is pleomorphic in older cultures. Usually, bacterial cells shorten with age and acquire an ellipsoid shape. This "dwarfing" is compatible with cells entering the VBNC state (Magarinos *et al.,* 1994; Olivier, 2009).

10.5.3. Screening of pure cultures

Macroscopically *Ph.d.p.* colonies on the TSA medium are of characteristic morphology. They are smaller than or can measure up to 0.5mm in diameter. They are whitish (semi-translucent with irregular margins, like dewdrops, if observed under the light magnification of a stereoscope), somewhat viscous and adhere well to the substrate.

10.5.4. Identification of the strain

10.5.4.1. API

The interpretation of the API20E™ (bioMérieux) results via Apiweb™ does not identify *Photobacterium damselae* subsp*. piscicida,* because its profile is not contained in the bioMérieux code index. Nevertheless, this system is useful for the presumptive diagnosis of the pathogen, which produces the profile 2005004 almost invariably (Romalde, 2002). The phenotypic homogeneity of *Ph.d.p*. allows the use of this miniaturized system for its identification. Infrequently, however, the profile 0005004 has been produced (personal observation). Hence, this test should best be combined with other phenotypic characteristics, such as non-motility, negative urease test, no growth on thiosulfate citrate bile salts-sucrose (TCBS) agar as well as additional diagnostic methods, such as sero-agglutination and molecular tests.

Fig. 10.1. Biomerieux API 20E micro-tube test strip biochemical profile 2005004 identifying *Photobacterium damselae* subsp. *piscicida.* The typical score obtained on API 20E for *Ph.d.p.*

10.5.4.2. Slide agglutination

Photobacterium damselae subspecies *piscicida -Ph.d.p*. is serologically homogeneous, hence, serotypes have not been established (Bakopoulos *et al*., 1997). The antigenic uniformity of lipopolysaccharide (LPS) profiles and outer membrane proteins (OMPs) encouraged the development of serological techniques for its detection and identification.

Commercial slide agglutination and latex agglutination test kits (Mono-Pp by Bionor AS), utilizing specific antiserum with polyclonal antibodies, may confirm the identification of the bacterium isolated on culture (Romalde *et al*., 1995). No cross-reaction with other bacterial groups has been reported.

Nonetheless, in order to recognize minute serological differences among strains of disparate geographical regions, monoclonal instead of polyclonal antibodies (MAbs), that are specific against particular immunogens, may be utilized to identify *Ph.d.p.* intraspecific variations (Bakopoulos *et al*., 1997). Thus, for example, antigenic differences between Japanese and European strains have been revealed.

10.5.4.3. Mass spectrometry

Photobacterium damselae subspecies *damsela* and *piscicida* have important epizootiological and virulence differences. MALDI-TOF Mass Spectra biotyper analysis may correctly identify the species and discriminate the subspecies (Perez-Sancho *et al*., 2016) based on five differential peaks (m/z 4183 and 8367 for subsp. *damsela* and 4197, 8397 and 8856 for subsp. *piscicida*) using a genetic algorithm (ClinProTools software). This approach could be integrated into the workflow of laboratories possessing MALDI based tools for bacteria identification.

10.5.4.4. PCR

PCR may be utilized for both the confirmation of photobacteriosis and the screening for latent carriers among apparently healthy specimens. Several efforts to select appropriate PCR primer sets to discriminate between the closely related *Photobacterium damselae* subspecies, *Ph.d.p*. and *Ph.d.d*. have been published.

Developed PCR-based diagnostic methodologies are either multiplex, that is, they utilize sequences of different genes in addition to the 16S rRNA (e.g. the gene 1A coding for a penicillin-binding protein or the ureC gene, which is absent from *Ph.d.p.*), or are combined with other molecular techniques, such as AFLP or RFLP, or with plating the *Photobacteria spp.* on TCBS where only *Ph.d.d*. grows producing green colonies (Amagliani *et al*., 2009; Andreoni and Magnani, 2014; Essam *et al*., 2016, Osorio *et al*., 2000; Rajan *et al*., 2003; Zappulli *et al.,* 2005). Primer combinations are important in order to cope with the genotypic homogeneity between the *Photobacterium damselae* subspecies *damsela* and *piscicida*. Lately, a single-step real-time PCR assay, based on a bamB gene sequence (the gene responsible for the outer cell membrane protein assembly factor bamB) has proven sensitive and specific to discriminate between subspecies and quantify the existing genome copy numbers of the bacterium in infected fish tissue samples (Rajan *et al.,* 2005).

In order to bypass the need for prior isolation of the bacterium in pure culture and to overcome the 16S rRNA gene homogeneity between the *Ph.d*. subspecies, A PCR-RFLP method has been documented based on novel primer pairs designed on non-conserved sites of two genomic regions of several *Ph.d.p*. strains. These primers have been constructed subsequent to cloning and sequencing selected RAPD fragments and were found to be highly specific to *P. damselae* (Zappulli *et al*., 2005). In a second step, *Ph.d*. subspecies identification could be effected by restriction analysis of the PCR amplified products, which showed a unique digestion profile for all *Photobacterium damselae* subsp. *piscicida* strains tested. A distinctive RFLP pattern for *Ph.d.p*. allows the detection of this subspecies when *Ph.d.d*. is also present in the sample. This two-step method may be implemented directly on infected fish tissues, either from moribund fish or asymptomatic carriers.

Farm samples for PCR testing may include suspect fish, fertilized eggs, live prey, larvae/juveniles/fryor even sediments in order to reveal sub-clinical infection as well as moribund fish in order to confirm the diagnosis in case of overt disease. For this purpose, standardised, easy to use commercial qPCR kits have been made available from a number of companies.

The commercial PCR kits are designed for the in vitro quantitative detection of *Ph.d.p*. genomes and are designed to have the broadest detection profile possible, whilst remaining specific to the target bacterium genome. The primers and fluorogenic probe sequences in these kits are

proprietary and covered by patents and are advertized to have 100% homology with a broad range of *Ph.d.p*. sequences based on bioinformatics analyses.The qPCR kits provide copy number standard curves for the quantification of the amplified products and internal extraction template (DNA or RNA) controls for the quality of the nucleic acid (NA) extraction in order to eliminate false-negative results.

Subsequent to tissue lysing, NA extraction procedures with associated reagents are commercially available and most of them utilize spin column or magnetic bead technologies applied according to stepwise instructions.

Example of thermal cycles programmed into the PCR thermal cycler according to a commercial qPCR amplification kit for *Photobacterium damselae* subspecies *piscicida*, suggesting 50 thermal cycles:

Polymerase activation	Denaturation	Annealing - Extension - data collection
95°C	95°C	60°C
2 min	10 sec	60 sec
	50 cycles	

Example reaction mix and final volume in each well/micro-tube:

Mastermix	10 µl
Primer/probe mix	1 µl
RNAse/DNAse free water	4 µl
Reaction mix volume	15 µl
DNA template (sample NA extract)	5 µl
Final volume	20 µl

Primer sequences are proprietary and not disclosed.

However, laboratories may choose to develop their own protocol to detect *Ph.d.p.*, rather than use the commercial kits. Carraro *et al.* (2018) designed a highly sensitive real-time PCR assay for simultaneous detection and quantification of *P. damselae*subsp. *piscicida* and *P. damselae* subsp. *damsela* that was tested for specificity and sensitivity on laboratory-generated samples as well as on experimentally infected seabream tissue samples.

This assay targets a partial sequence of the bamB gene for amplification using specific primers PhPisc.B (For and Rev). Two single nucleotide polymorphisms in the target amplicon region determine two distinctive qPCR dissociation curves, so melting curve (dissociation) analysis can distinguish between *Ph.d.p. -Ph.d.d*.

Primers

Oligonucleotide	Sequence 5' → 3'
PhPisc B (For)	TGCTGGTGGTGTATTCTGGG
PhPisc B (Rev)	AACAGGTGTCGCATCAACGT

This assay can be performed using any colour-based chemistry and instrument that supports melting curve analysis. An example follows using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on LightCycler 480 System (Roche):

Reaction mix

Reagent	Final concentration/volume
PhPisc B (For)	10 uM
PhPisc B (Rev)	10 uM
Platinum SYBR Green Super-Mix-UDG	1X
DNA template	2.5
Water (molecular grade)	To 10 µl
Total volume	10 µl

Thermal profile

UDG incubation	Polymerase activation	Denaturation	Annealing/Extension	Dissociation
50°C	95°C	95°C	60°C	From 40°C to 95°C
2 min	2 min	10 sec	60 sec	at 4.4°C/s
			45 cycles	

Ph.d.p. strains should be characterized by melting temperature (Tm) of 83.3–84°C while *Ph.d.d.* strains should be characterized by a Tm of 84.3–84.9°C making the two subspecies distinguishable.

10.5.5. *In vitro* susceptibility testing

The most commonly used in vitro method to assess bacterium susceptibility to antimicrobials is the disc diffusion method (CLSI, 2011; Puttaswamy *et al.,* 2018). Although *Ph.d.p.* isolates may be distinguished from each other according to their antimicrobial susceptibility and even provide clues of their geographical origin (Bakopoulos *et al*., 1995; Thyssen and Olivier, 2001), it is evident in everyday practice that their sensitivity profile changes dynamically, depending on the degree of exposure of the bacterium to particular antibiotics/chemotherapeutics in the field (Smith, 2008). For example, regular use of a particular antibiotic on a farm renders it ineffective after about 3-4 treatment cycles.

By disc diffusion testing on TSA or MH agar plates (antibiogram), the pathogen is most often found sensitive to oxytetracycline, flumequine, oxolinic acid, florfenicol and potentiated sulphonamides (trimethoprim + sulfadiazine) and frequently resistant to ampicillin, amoxicillin, erythromycin. At times, however, amoxicillin shows potency.

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Photos

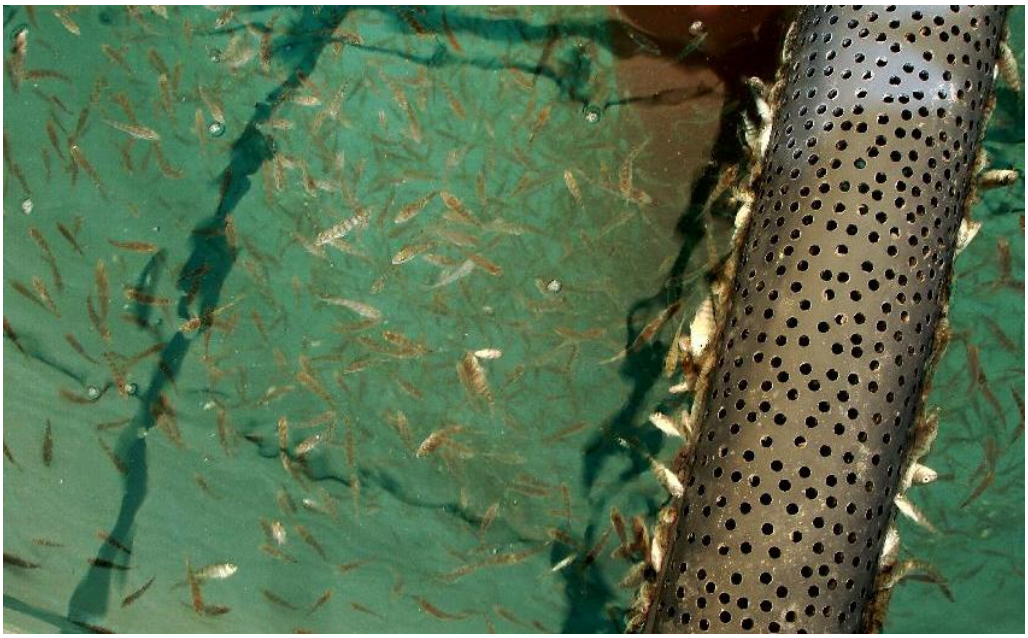

Fig. 10.2. Seabream fry (1 g) in nursery dying at the surface of their tank.

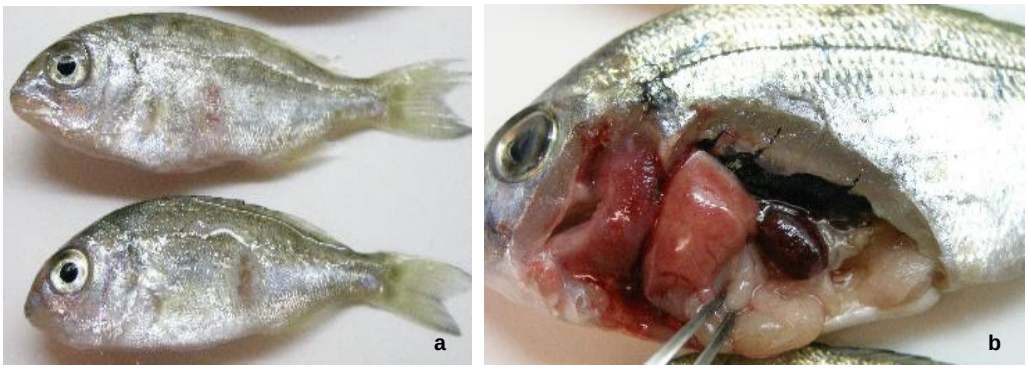

Fig. 10.3. a) Seabream fry (1 g) in nursery suffering photobacteriosis with skin lesions on the flanks; b) Mild liver congestion but gross spleenomegaly in bigger seabream (7 g).

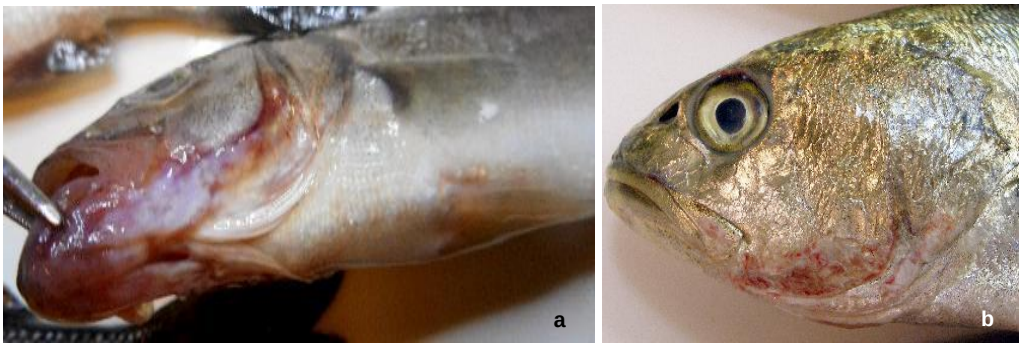

Fig. 10.4. a) Seabass weighing 30 g suffering photobacteriosis with external inflammatory lesions around the head epithelia, mainly lower jaw and opercula; b) Operculum skin with haemorrhagic inflammation on a meagre (200 g) infected by *Ph.d.p*.

Fig. 10.5. a) Seabass (50 g) with pale gills with large focal necrotic lesions; b) Inflamed and necrotic gill lamellae under light magnification 25 x.

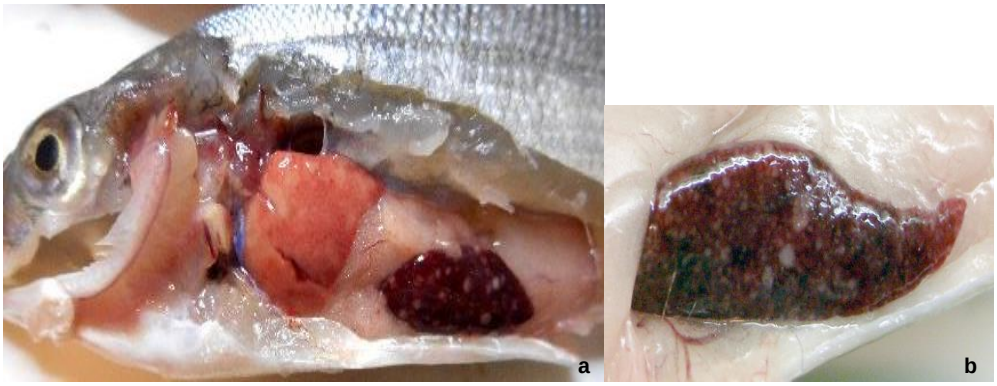

Fig. 10.6. a) Seabass (40 g) with pale gills, liver inflammation and gross splenomegaly with pseudotubercules; b) Close-up photo on seabass spleen with abundant pseudotubercules in the parenchyma.

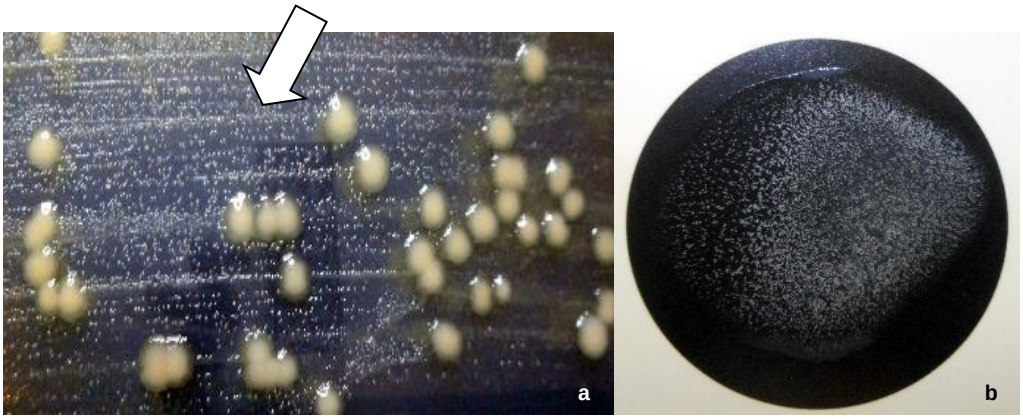

Fig. 10.7. a) Characteristic pin-point *Ph.d.p.* colonies on TSA agar, photographed against a black background for better contrast, after 24h incubation at room temperature (23oC). The larger 1.5mm colonies were produced by *Vibrio* spp., -mixed infection- but serve as a good comparison; b) Positive seroagglutination rapid test (Bionor Mono -Pp) confirming the pathogen.

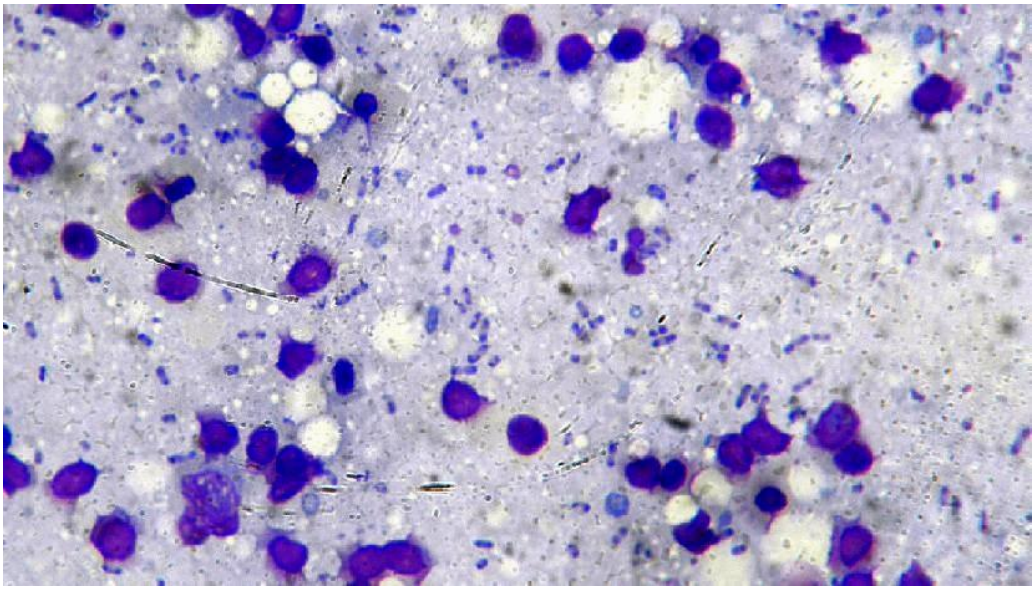

Fig. 10.8. Smear of homogenized splenic parenchyma from 70 day old seabream juveniles at the weaning section of a hatchery, fixed and stained with Giemsa. *Photobacteria* depicting characteristic biopolar staining were observed under 1000x magnification.

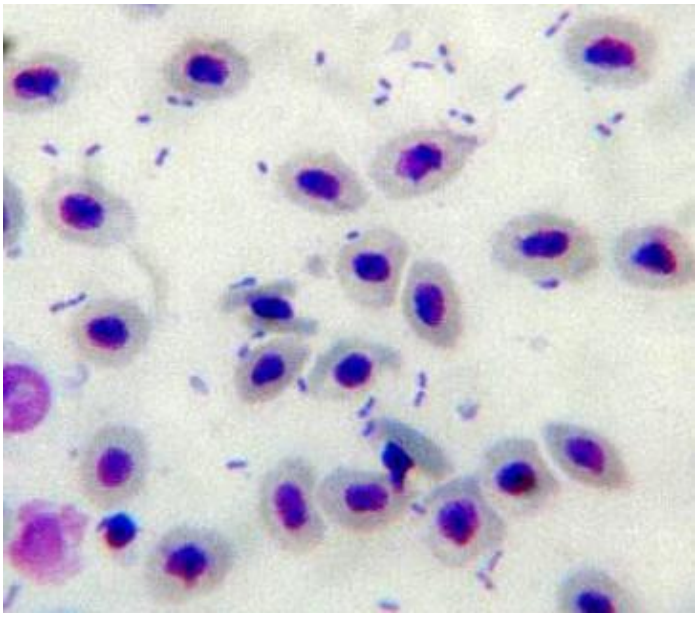

Fig. 10.9. Typical bacteraemia observed microscopically on Giemsa stained blood smear from seabass (17 g) suffering photobacteriosis.

11. *Tenacibaculum* group

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11.1. Aetiology of *Tenacibaculum* group

Tenacibaculosis, also known under several names, such as "black path necrosis", "saltwater columnaris disease", "marine flexibacteriosis", "eroded mouth syndrome" and "gliding bacterial disease of sea fish" (Santos *et al*., 1999; Toranzo *et al*., 2005) is caused by *Tenacibaculum* spp., an opportunistic Gram-negative filamentous bacteria, 0.4-0.5 µm in diameter and 1.5-30 µm long, occasionally up to 100 µm long. Most of the isolates show gliding motility on wet surfaces produce catalase and oxidase activity and cells can produce a yellow pigment, which is mainly zeaxanthin (Wakabayashi, 1986; Avendaño-Herrera *et al*., 2006; Suzuki, 2015).

The most described species to be considered a major fish pathogen worldwide is *Tenacibaculum maritimum* which causes high mortalities and important economic losses in a wide range of wild and cultured marine fish species including seabass, seabream, sole and turbot (Toranzo *et al*., 2005; Avendaño-Herrera *et al*., 2006). Other *Tenacibaculum* species described associated to fish diseases in the Mediterranean are: *T. soleae* from diseased sole (*Solea senegalensis*) (Piñeiro-Vidal *et al*., 2008a) and European seabass (*Dicentarchus labrax*)

(Castro *et al*., 2014), *T. gallaicum* from a holding tank for turbot (*Psetta maxima*) (Piñeiro-Vidal *et al*., 2008b) and sole (*S. senegalensis*), *T. dicentrarchi* from skin lesions of European seabass (*Dicentarchus labrax*) (Piñeiro-Vidal *et al*., 2012) and *T. discolor* from cultured sole (*S. senegalensis*) (Piñeiro-Vidal *et al*., 2008b).

Clinical signs of tenacibaculosis caused by *T. maritimum* are characterized by gross lesions on the body surface. This depends on the fish species and age and generally includes eroded mouth, haemorrhagic and necrotic lesions on the skin, frayed fins and tail rot and in some cases also necrosis on the gills and eyes (Toranzo *et al*., 2005; Piñeiro-Vidal *et al*., 2007). Deeper ulcerative lesions on the body sides or close to the dorsal fin have been reported in cases of *T. discolor* infection (Le Breton *et al*., 2018).

11.2. Sampling

11.2.1. Preparation and shipment of samples from fish

The genus *Tenacibaculum* can be isolated from marine samples, particularly from the surface of marine organisms, external lesions or kidney lesions by streaking directly onto agar plates (Suzuki *et al*., 2015). *T. maritimum* can be prepared for storage or transport by culturing at 20 to 25°C for 48 to 72h onto *Flexibacter maritimum* medium (FMM) or Marine agar (MA) medium (Avendaño-Herrera *et al.,* 2006).

Cultures could be then shipped on ice or temporarily preserved for several weeks refrigerated at 4°C. For longer storage, cultures can be kept in broth containing 10% glycerol at -80°C or in the gas phase of liquid nitrogen. Lyophilization may give satisfactory results (Suzuki *et al*., 2015).

Specific transport medium has been developed for skin lesion swab preservation during transport to the diagnostic laboratory (Moalic *et al.,* 2018).

11.2.2. Live fish

As these bacteria are quite sensitive and easily destroyed during transportation, live affected fish should be sent alive to the laboratory for analysis in containers half-filled with water and oxygen. Occasionally, scrapings of the lesions from dead fresh fish with typical lesions submitted for diagnostic revealed presence of *Tenacibaculum* but it is not possible to cultivate them on specific media (either specific culture media such as FMS or isolation media for *Tenacibaculum*). Bacteria were either dead or not reverifiable. In order to enable isolation of bacteria, either live moribund fish or fish and swabs from the lesions in specific transport media are requested to be submitted for diagnostic purposes.

11.3. Diagnostic procedures for the *Tenacibaculum* group

Diagnosis of tenacibaculosis is usually based on the isolation of the causative agent followed by morphological, biochemical, analysis of their microbial susceptibility profiles and serological characterization (Fernandez-Alvarez and Santos, 2018).

11.3.1. Presumptive diagnosis from fresh samples

Presumptive diagnosis is usually made by microscopic observation (400 x or 1000 x magnification) of the bacteria on fresh smears from scrapes the lesion or after Gram staining or MGG (May-Greenwald Giemsa) staining of the slides, using quick coloration kits (Gram stain/ RAL 555, RAL Diagnostic, Martillac, France).

11.3.2. Primary cultivation of bacteria (choice of media and isolation of strain)

Isolation of *Tenacibaculm* spp. from fish tissues is difficult due to the slow growth of the bacteria and overgrowth by other bacteria in tissue samples. The commercially available Marine agar (MA, 2216E; Difco Laboratory, Detroit, MI, USA) and *Flexibacter maritimum* medium (FMM) (Laboratorios Conda, Madrid, Spain) are considered the most efficacious media for the primary isolation of this gliding bacteria (Fernandez-Alvarez and Santos 2018). A selective medium for *T. maritimum* recovery following transportation has been elaborated to limit the growth of contaminants and to ensure good growth of the bacterial colonies (Moalic *et al*., 2018). This medium enables the growth of different species of the *Tenacibaculum* genus.

T. maritimum is an obligate marine microorganism which does not grow on media prepared by just adding NaCl. It must be cultured in oligothrophic media elaborated with seawater (30-100% strength seawater) (Wakabayashi *et al.,* 1986. In MA (or broth) this species grows rapidly however it is difficult to recognize the typical long filamentous *T. maritimum* colonies (Toranzo, 2015). FMM is considered the most appropriate medium for the isolation of *T. maritimum* from fish tissue (Pazos *et al*., 1996). Although when using FMM this species shows slow growth and low density, it allows a better recognition of its typical flat, pale-yellow colonies with irregular uneven edges that adhere to the medium (Toranzo, 2015). In adverse conditions these non-sporulating *Flavobacteriaceae* can produce a spheroplast-like form**,** which makes diagnosis by direct microscopic observation more difficult.

11.3.3. Screening of pure cultures

Tenacibaculum spp. belongs to a group of *Flavobacteriaceae* including pathogenic strains for marine fish. *T. marinum* is the most common representing almost 50% of the strains reported in clinical cases in the Mediterranean area (Le Breton *et al*., 2018). All species are Gram-negative filamentous bacteria and rod-shaped, positive for catalase, oxidase and degradation of casein, non sporulating and producing a spheroplast-like form under adverse conditions.

Depending on the strains, macroscopically, on soft media, colonies appear pale to bright yellow with typical irization. Colony shapes may vary from uneven regular circles to irregular with spreading edges. For most pathogenic strains, growth will be achieved within a range of temperature from 15°C to 34°C.

Depending on the species, cells size is approximately 2 - 30 µm long x 0.5 µm wide. They can grow in a range of 5.9 to 8.6 pH in a salinity range of 20 to 30 ppt, preferably in presence of 30ppt to full-strength seawater (Wakabayashi *et al.,* 1986; Hansen *et al.,* 1992; Suzuki *et al.,* 2001; Pazos *et al*., 1996; Moalic *et al.,* 2018).

11.3.4. Identification of the strain

11.3.4.1. API

Phenotypic homogeneity within *Tenacibaculum* species associated to fish has facilitated their identification based on their biochemical profile using miniaturized systems such as API ZYM and API 50CH (Fernandez-Alvarez and Santos, 2018).

When using API ZYM gallery it is difficult to distinguish between *T. maritimum* isolates since they are all quite homogenous, with a characteristic profile with positive results on the first 11 enzymatic reactions. All enzymes related to the metabolism of carbohydrates are absent (Avendaño-Herrera *et al*., 2004; 2005b; 2006; Toranzo, 2015). These miniaturized systems are sufficient to identify *Tenacibaculum* at genus level but not at species level or to distinguish isolates.

11.3.4.2. Mass spectrometry (MALDI-TOF)

Recently, the application of a novel proteomic fingerprinting approach based matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) coupled with Mass-UP software (López-Fernández *et al*., 2015) has proven to be a useful and reliable tool for the identification and classification of isolates of *Tenacibaculum* at the species level (Fernández-Alvarez *et al.,* 2017). MALDI-TOF-MS technique was able to identify, regardless of the culture media (FMM or MA) used, eight genus-specific peaks for *Tenacibaculum* and at least one species-specific peak in *T. maritimum*, *T. soleae*, *T. dicentrarchi* and *T. ovolyticum*. However, mass spectra for *T. discolor* and *T. gallaicum* were very similar and no species-specific peaks could be detected (Fernández-Alvarez *et al*., 2017).

The stability of the MALDI-TOF spectra found even under different cultivation conditions is because protein mass fingerprinting mainly represents conserved ribosomal proteins together with several housekeeping and structural proteins which are abundant and relatively independent of the culture or external conditions (Welker, 2011; Singhal *et al*., 2015).

This research suggests that the MALDI-TOF-MS technique could be a good complementary approach to 16S rRNA sequencing and have an even higher discriminating potential than analysis of the 16S rRNA gene (Fernández-Alvarez *et al.,* 2017).

11.3.4.3. PCR

Species specific PCRs targeting a particular fragment of 16S rRNA gene have been designed for the following *Tenacibaculum* spp.:

T. maritimum		
Forward primer	**MAR1**	5-AATGGCATCGTTTTAAA-3’
Reverse primer	**MAR2**	5´-CGCTCTCTGTTGCCAGA-3´
Expected amplified product is 1088 bp (Toyama *et al*., 1996)		
Forward primer	**Mar 1**	5’-TGTAGCTTGCTACAGATGA-3’
Reverse primer	**Mar 2**	5’- AAATACCTACTCGTAGGTACG-3’
Expected amplified product is 400bp (Bader and Shotts 1998)- able to detect pure and mixed cultures of *T. maritimum*		

T. solae		
Forward primer	**Sol-Fw**	5’-TGCTAATATGTGGCATCACAA-3’
Reverse primer	**Sol-Rv**	5’-CAACCCATAGGGCAGTCATC-3’
Expected amplified product is 248 bp - able to detect pure and mixed cultures of *T. solae* (Garcia-González *et al*., 2011).		
Forward primer	**G47F**	5′-ATGCTAATATGTGGCATCAC-3′
Reverse primer	**G47R**	5′-CGTAATTCGTAATTAACTTTGT-3′
Designed at the 5′ region of the 16S gene and of the ISR respectively flanking a 1555 bp fragment. Able to successfully identify pure and mixed cultures of *T. soleae* and from tissues of infected fish (López *et al*., 2011).		

T. dicentrarchi		
Forward primer	**Tenadi Fw**	5’-ATACTGACGCTGAGGGAC-3’
Reverse primer	**Tenadi Rv**	5-TGTCCGAAGAAAACTCTATCTCT-3’
Expected amplified product is 284 bp product. Able to successfully identify pure and mixed cultures of *T. dicentrarchi* and from tissues of infected fish (Avendaño-Herrera *et al*., 2017).		

Simple PCR methods are only capable of detecting acute infections. However, when the pathogen is present in low numbers, such as in asymptomatic or carrier fish, PCR has low sensitivity (Cepeda and Santos, 2002; Avendaño-Herrera *et al*., 2006).

Nested-PCR methods to detect *T. maritimum* have been developed and proven to be more sensitive (Cepeda and Santos, 2002; Cepeda *et al*., 2003; Avendaño-Herrera *et al*., 2004 b, c) however, they are also more expensive and time-consuming since they require two rounds of PCR (Fernández-Alvarez and Santos, 2018).

Other PCR-based methods are: 1) PCR-enzyme linked immunoabsorbent assay (PCR-ELISA) (Wilson *et al*., 2002), 2) reverse transcriptase polymerase chain reaction-enzyme hybridization assay (RT-PCR-EHA) (Wilson and Carson, 2003), 3) DNA microarray probe (Warsen *et al*., 2014). These assays have been able to detect *T. maritimum* from pure cultures but their effectiveness to separate different *Tenacibaculum* species and to detect the pathogen from infected fish tissues have not yet been examined (Fernández-Alvarez and Santos, 2018). Recently, a real-time PCR method, targeting a 164bp fragment of the 16S rRNA gene, has been developed for the detection and quantification of *T. maritumum* in fish and seawater samples (Fernández-Alvarez *et al*., 2019).

11.3.4.4. Typing of the bacteria

Serological typing methods

The identification of serotypes and antigenic characterization are key for diagnostics and epidemiology studies towards a successful vaccine development (Fernández-Alvarez and Santos, 2018).

Three different host-specific major O-serotypes have been identified in *T. maritimum* causing mortalities in cultured marine fish: serotype O1 include strains isolated from gilthead seabream, serotype O2 isolated from turbot, serotype O1 and O3 from sole and serotype O3 and recently also O2 from salmon (Avendaño-Herrera *et al*., 2004; 2005c; Le Breton, 2019).

Other *Tenacibaculum* spp. pathogenic to fish also show antigenic heterogeneity. Lack of cross-reaction between *T. maritimum*, *T. soleae* and *T. discolor* and the existence of at least two serotypes (O1 and O2) in *T. soleae* strains and one in *T. discolor* (serotype O1) have recently been confirmed (Fernández-Alvarez *et al.,* 2018).

Molecular typing methods

Several methods have been developed for the identification and genotyping of bacteria. Ribotyping enables discrimination at the species and sub-species level through DNA fragmentation of *T. maritimum* strains into five different rRNA gene restriction patterns (P1 to P5). These restriction patterns correspond to the four serotypes described for the species. It has a good reproducibility but it is not able to discriminate the strains based on the host source or geographic isolation (Pazos, 1997).

RAPD-PCR is a rapid and easy technique, which not only reveals patterns coinciding with the O-serotypes of *T. maritimum* but also enables the strain to be separated into different groups according to the host species. RAPD-PCR permits *T. maritimum* to be discriminated from *T. discolor*, *T. gallaicum* and *T. soleae* (Piñeiro-Vidal, 2008). The major constraint of the RAPD-PCR technique is that it is difficult to reproduce patterns in different laboratories and compare isolates tested on different days (Fernández-Alvarez and Santos, 2018).

ERIC-PCR and REP-PCR although they display very clear distinct genetic profiles for the different *Tenacibaculum* species and strains from the same species, do not provide correlation between the genetic profiles and the serotypes, host or geographical location of isolation (Fernández-Alvarez *et al*., 2018).

Multi-locus sequence analysis (MLSA) combines PCR and automated DNA sequencing and successfully allows the genetic differentiation of *Tenacibaculum* species, being able to clearly discriminate between *T. maritimum*, *T. gallaicum*, *T. soleae*, *T. discolor*, *T. dicentrarchi* and *T. ovolyticum* (Habib *et al*., 2014). It seems a reliable tool for epidemiological studies and monitoring tenacibaculosis in marine environments (Fernández-Alvarez and Santos, 2018).

11.3.5. In vitro susceptibility testing

For in *in vitro* antimicrobial susceptibility testing two methods have been recommended: 1) using FMM agar and broth and diluting 0.3% Mueller-Hinton Agar (DMHA) (Avendaño-Herrera *et al*., 2005a) or 2) using broth prepared with natural or artificial seawater with or without supplementation with 5% fetal calf serum (CLSI, 2006).

In vitro studies on the susceptibility of *T. maritimum* to various chemotherapeutic agents indicate that strains isolated from different host species and geographical regions exhibit a similar pattern, with susceptibility to penicillins, erythromycin, tetracyclines, trimethoprim, potentiated sulfonamides and fluoroquinolones, and resistance to colistin, kanamycin, neomycin and the quinolones, oxolinic acid and flumequine (Soltani *et al.,* 1995, Avendaño-Herrera *et al.,* 2004, 2005a). However, field results might be different even when the isolated bacteria are highly sensitive (*in vitro*) to a chemotherapeutant used for treating the condition (Cepeda and Santos, 2002).

T. gallaicum, *T. discolor, T. soleae* and *T. dicentrarchi* also show to be susceptible to amoxicillin, florfenicol and oxytetracycline and resistant to oxolinic acid. Resistance to flumequine, enrofloxacin, oxytetracycline and trimethoprim-sulfamethoxazole has also been detected in some strains of *T. gallaicum*, *T. discolor* and *T. soleae* (Avendaño-Herrera *et al*., 2008).

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Photos

Fig. 11.1. ***Tenacibaculum maritimum*** **lesion in** ***D. labrax*****. a) Infected fish in the cage showing extensive skin lesions with whitish to yellowish mucus ; b) lesions on the trunk ; c) gill focused lesions and hemorrhages ; d) lesion with hemorrhages on the dorsal part of the head.**

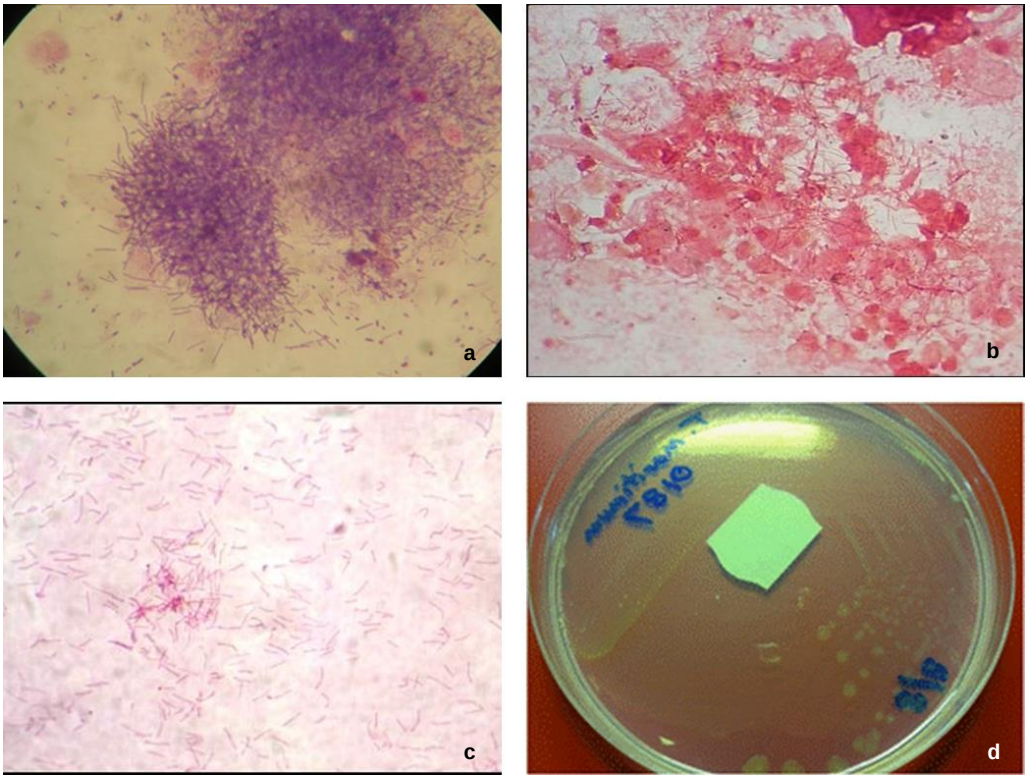

Fig. 11.2. a) and b) Fresh smear Gram staining (RAL555 staining) 400 x microscopic observation of *Tenacibaculum* sp. from scraping of skin lesions; c) stained smears obtained from pure colonies; d) colonies grown on the FMM agar.

12. *Aeromonas* spp.

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12.1. Aetiology of *Aeromonas spp*.

According to the last edition of Bergey's Manual of Systematic Bacteriology (2nd edition 2005) (Martin-Carnahan and Joseph, 2005) the genus Aeromonas comprises at least 14 named species: *A. hydrophila, A. allosaccharophila, A. bestiarum, A. caviae, A. encheleia, A. eucrenophila, A. jandaei, A. media, A. popoffii, A. salmonicida, A. schubertii, A. sobria, A. trota, A. veronii* and two unnamed *Aeromonas* spp. (DHG 11 and G501).

The taxonomy of the genus is still evolving and numerous new species have been described to date based on clinical and environmental samples (i. e. Figueras *et al.,* 2016). Also, numerous reclassifications, nominations and synonymization have been proposed since then. The current taxonomic status of the 14 species described above also includes two subspecies of A. *hydrophila* (*hydrophila* and *ranae*) (Huys *et al.,* 2003), five subspecies of *A. salmonicida* (*salmonicida, achromogenes, masoucida, pectinolytica* and *smithia*) and two biovarieties of *A. veronii* (*veronii* and *sobria*).

The psychrophilic, non-motile, pigment-producing *A. salmonicida* subsp. *salmonicida* is the typical aetiological agent of furunculosis in salmonids. The rest of the *A. salmonicida* subspecies are considered "atypical" *A. salmonicida* strains and are related to diseases that affect non-salmonids and warm-water fish in general (Menanteau-Ledouble *et al.,* 2016; Austin and Austin, 2012). The mesophilic species *A. hydrophila* is a widely known fish pathogen

causing ulcerative, haemorrhagic and septicaemic infections mainly in freshwater fish like tilapias, carps, goldfish, rainbow trout etc. Other species causing infections in fish are *A. bestiarum, A. caviae, A. jandaei, A. piscicola, A. schubertii, A. sobria* and *A. veronii* bv. *sobria* (Austin and Austin, 2012).

12.2. Infections and pathology of *Aeromonas spp*.

In the Mediterranean Sea, aeromonads have been isolated from coastal marine, brackish waters and sediment; they are considered part of the gut microbiota of fish and can be isolated from tissues of apparently healthy marine fish (Scarano *et al.,* 2018; Pedonese *et al*., 2012; Dumontet *et al*., 2000; Toranzo *et al.,* 1993; Martinez *et al*., 2010; Floris *et al*., 2013). In numerous cases of diseased farmed fish e.g. European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), sharp snout seabream (*Diplodus puntazzo),* common pandora *(Pagellus erythrinus),* common dentex *(Dentex dentex)* etc., aeromonads have been isolated generally in low frequency and usually in mixed infections with other marine pathogens such as *Vibrio* spp., *Pseudomonas* spp., *Photobacterium damselae,* and *Tenacibaculum marinum* (Yiagnisis and Athanassopoulou, 2011; Athanassopoulou *et al*., 1999; Yardımcı and Timur, 2015; Colorni *et al*., 1981; Balebona *et al*., 1998; Zorrilla *et al*., 2003; Öztürk and Altınok, 2014; Martino *et al.,* 2011).

In European seabass, pathogenic aeromonads are *A. hydrophila, A. veronii* bv. *sobria* and *A. salmonicida,* which have been reported in both juvenile and grown fish. In the Aegean Sea, *A. hydrophila* caused morbidity and low daily mortalities (0.5-1%) in *D. labrax* (150 and 330 g) and *D. puntazzo* (45 g) as well (Doukas *et al*., 1998). Clinical signs of disease included erythema and swelling of the anus, haemorrhagic spots on the skin and internally, enlargement of the organs, haemorrhages and ecchymosis.

Aeromonas veronii bv *sobria* is an opportunistic pathogen of fish both in freshwater and in the marine environment, which increasingly gains importance for the aquaculture industry. Outbreaks accompanied by significant losses have been reported in loach (*Misgurnus anguillicaudatu*) farmed in China (Zhu *et al*., 2016) and in African catfish (*Clarias gariepinus*), rajputi (*Puntius gonionotus*), rui (*Labeo rohita*), catla (*Catla catla*), and shole *(Channa striatus*) farmed in Bangladesh (Rahman *et al*., 2002). Furthermore, *A. veronii* bv *sobria* has also been reported to cause disease in ornamental fishes (Sreedharan *et al*., 2013). The pathogenicity of *A. veronii* bv. *sobria* is attributed to several virulence factors encoded by genes related to T3SS, T6SS T4p, etc. (Barnett *et al.,* 1997; Nawaz *et al*., 2010; Silver and Graf, 2009; Kirov and Sanderson, 1996; Zhu *et al*., 2016).

This pathogen has become extremely problematic during the past few years for the culture of European seabass in Greece. The disease in farmed seabass has been described and the pathogens partially characterized (Smyrli *et al*., 2017). The disease outbreaks occur during the warm months of the year when water temperature is over 21^{o}C. Affected fish are usually lethargic with no appetite and in progressed stages of the disease, they have an icteric appearance due to the highly haemolytic nature of the pathogen as well as extensive liver damage. Internally, multiple abscesses are usually found in the spleen, liver and kidney of affected fish (Fig. 12.1). The disease first appeared in 2008 affecting a single fish farm in Central Greece, but since then more farms in the same but also distant areas in Greece are affected. In the beginning, the disease was mainly found in bigger fish reaching commercial size (>200g in weight), but lately, it also affects younger fish with weights lower than 50g. Cumulative mortality can be as high as 80% if it is not treated with antibiotics and it is a major concern for the producers in the affected areas.

The same species, *A. veronii* bv. *sobria*, was also reported in the Black Sea as the most prevalent in diseased seabass exhibiting darkening, exophthalmia, erratic swimming, abdominal swelling and ulcerative lesions on operculum and mouth. In this case, the pathogen was

isolated alone or in mixed infections with *Ph. damselae* subsp. *damselae* and *Vibrio* spp. and the study did not present pathogenicity data (Uzun and Ogut, 2015). The water temperature ranged between 20-26 ^{o}C.

Petechial haemorrhages externally, and white lesions on the internal organs and enlargement of the spleen were observed in *D. labrax* infected from *A. salmonicida* subsp. *masoucida*/ *achromogenes* reared in the Black Sea (Karatas *et al.,* 2005). Cumulative mortality of 5-6 g and 100 g fish reached 20% during the outbreak (2 months).

In the Mediterranean coast of Spain, *A. salmonicida* subsp. *salmonicida* caused 3.8% mortality during two disease outbreaks between May-June in juvenile *D. labrax* (9 g) (Fernández-Álvarez *et al*., 2016). Initially, fish were asymptomatic but progressively, ulcerative lesions appeared in the skin and muscle. Internally the only clinical sign recorded was enlargement of the spleen.

In another case, in Gran Canaria (Atlantic), *A. salmonicida* subsp. *salmonicida* caused a hyperacute disease in *S. aurata* (1 g) after transportation of the fish to the ongrowing facility (Real *et al*., 1994). Moribund fish exhibited pale gills, dark coloration of the skin and petechial haemorrhages on mouth and gills. Mortality reached 6-7% in the first 3 days.

12.3. Sampling

12.3.1. Preparation and shipment of samples from fish

Fish presenting clinical signs of disease, moribund or freshly dead should be sampled and examined as soon as possible after collection (ideally within 24 h after collection). Fish samples should be stored in ice until processing. Fish treated with antimicrobial agents should be avoided because of possible false negative results.

12.4. Diagnostic procedures for *Aeromonas spp*.

The kidney and subsequently the spleen and liver are generally suggested for internal sampling and skin ulcers, when present, for external sampling. Whitish nodules that are frequently recorded on the organs of infected fish should also be preferred as they may include bacteria aggregates.

For smaller fish (juvenile/ larvae etc.), washing of the whole fish with sterile saline is proposed before the sampling. In that case, sampling from the kidney can also be achieved with a vertical section of the whole body with a sterile blade just behind the head. Subsequently, bacteriological sampling from the kidney can be achieved with a sterile loop.

At least ten fish should be sampled in order to detect the aeromonads in an apparently healthy population while ten fish should be enough in case of selective sampling of diseased fish (Noga, 2010).

Aeromonads grow well on common culture media such as TSA, BHI and Blood agar supplemented with 0.5-2% NaCl after 24-48h incubation at 22-25^{o}C. Selective media like Aeromonas isolation agar (AIA) supplemented with ampicillin is also recommended. Media supplemented with higher salt concentrations (4-6% NaCl) can be used in order to discriminate after the initial isolation the presumptive aeromonad isolates. TCBS agar can also be informative if combined with gradient NaCl media and after the check for growth on AIA.

12.4.1. Screening of pure cultures

Colony morphology may not be very informative. Aeromonads generally form smooth, circular, shiny and convex colonies of 2-3 mm diameter after 24-48 h incubation. Rough colonies or with

buttery texture may be observed, either translucent or opaque. Coloration is generally greyish-white to buff. Colony morphology may vary among strains of the same species.

Some strains produce brown pigment (pyomelanin) diffused on the agar medium (e.g. TSA) after 24-72 h of incubation. This phenotypic characteristic may be expressed by *A. salmonicida* but also *A. media, A. hydrophila*, *A. bestiarum, A. eucrenophila* and *A. veronii* may present this character.

On sheep blood agar, β-haemolysis can be observed after 24-48 h incubation. Haemolysis is higher when using fish blood (Fig. 12.2). On AIA they appear green as they generally do not metabolise xylose. Depending on the strain and species, production of H_2S would be manifested by the black colour on the top of the colony. Aeromonads are expected to grow significantly less or not at all in media supplemented with NaCl concentrations over 4%. Aeromonads, generally do not grow on TCBS but many isolates do (yellow or green colour) so this phenotype should not be used alone. Generally, if growth occurs, it should be less than when observed in optimum conditions or general media.

Mesophilic species grow well in temperatures up to 35-37^{o}C while the psychrophilic *A. salmonicida* presents optimum growth at 22-25^{o} and does not grow at 37^{o}C.

Aeromonads are Gram-negative, facultative anaerobic, short rods (0.3–1.0 x 1.0–3.5 μm). They generally appear as single cells but they also form short chains. Most stains are motile by a single polar flagellum. Peritrichous or lateral flagella may be observed less frequently, as well as non-motile strains. They are oxidase and catalase positive and generally resistant to the vibriostatic agent O/129 (2, 4- diamino-6, 7-di-iso-propylpteridine phosphate). Nevertheless, some strains of *A. eucrenophila* and *A. veronii* bv. *veronii* are sensitive to high concentrations (150 μg) of the agent. Other metabolic traits include ability to metabolize glucose and trehalose, reduction of nitrate, β-galactosidase activity, inability to hydrolyze urea, inability to ferment inositol and to produce acid from amygdalin etc. (Martin-Carnahan and Joseph, 2005; Abbott *et al*., 2003).

12.4.2. Identification of the strain

12.4.2.1. Biochemical identification

Phenotypic tests often fail to identify aeromonads to species level and miniaturized commercial identification systems such as API 20E, API 20NE and BIOLOG GENIII Microplate present constraints in the identification of fish pathogens and aeromonads specifically (Austin, 2011; Santos *et al*., 1993; Beaz and Jos, 2012). Thus, phenotypic tests are proposed for identification at genus level. Subsequently, key biochemical reactions such as ornithine decarboxylase reaction could be used to differentiate between, for instance, the biovarieties of *A. veronii* such as bv. *veronii* or bv. *sobria*.

12.4.2.2. Molecular detection and identification

Strains that grew on AIA, grew better in low salinity media, and/or were identified as *Aeromonas* spp. with biochemical tests, are proposed to be included in the molecular analysis.

Bacterial DNA can be extracted from an overnight culture obtained from a single colony on a fresh plate (24-48 h). After centrifugation and washing the bacterial pellet in sterile saline, DNA extraction can be performed with commercial kits or with the boiling method.

Detection of aeromonads can be achieved by PCR amplification of the 16S-23S rRNA intergenic spacer regions (ISR) of rRNA operons (Kong *et al*., 1999).

Amplification of the IGS-23S of the ISR according to (Kong *et al*., 1999):

Forward primer	**Aero-F**	5'- GGAAACTTCTTGGCGAAAAC -3'
Reverse Primer	**Aero-R**	5'- GGTTCTTTTCGCCTTTCCCT -3'

The following PCR conditions are recommended:

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
94°C	94°C	60°C	72°C	72°C
2 min	1 min	1 min	1 min	3 min
		35 cycles		

The length of the expected amplified product is 550 bp.

Identification of aeromonads to genus level can be achieved by PCR amplification of the extracellular lipase Glycerophospholipid-cholesterol acyltransferase (GCAT) gene (Chacon *et al*., 2002).

Amplification of the GCAT gene according to (Chacon *et al*., 2002):

Forward primer	**GCAT-F**	5'- CTCCTGGAATCCCAAGTATCAG -3'
Reverse Primer	**GCAT-R**	5'- GGCAGGTTGAACAGCAGTATCT -3'

The following PCR conditions are recommended:

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	94°C	65°C	72°C	72°C
3 min	1 min	1 min	1 min	5 min
		35 cycles		

The length of the expected amplified product is 237 bp.

Identification of aeromonads to species level can be achieved by sequencing housekeeping genes such as gyrB encoding the B-subunit of DNA gyrase and rpoD encoding s^{70} factor which confers promoter-specific transcription initiation on RNA polymerase (Soler *et al.,* 2004; Yanez *et al*., 2003; Beaz and Jos, 2012).

	Primer	Position	Sequence 5' – 3'
gyrB	gyrB 3F	334–354	TCCGGCGGTCTGCACGGCGT
	gyrB 9Rs	980–960	CCTTGACCGAAATGACCGCC
	gyrB 7F	792–812	GGGGTCTACTGCTTCACCAA
	gyrB 9R	979–959	ACCTTGACGGAGATAACGGC
	gyrB 14R	1464–1444	TTGTCCGGGTTGTACTCGTC
rpoD	rpoD 70F	280–323	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGG GNACNGT
	rpoD 70Fs	280-302	ACGACTGACCCGGTACGCATGTA
	rpoD 70Fs1	740-757	GTCAATTCCGCCTGATGC
	rpoD 70R	1139-1096	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCY TTYTT
	rpoD 70Rs	1139-1117	ATAGAAATAACCAGACGTAAGTT
	rpoD 70Rs1	800-782	ATCATCTCGCGCATGTTGT

The following PCR conditions are recommended for gyrB according to (Yanez *et al.*, 2003):

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
94°C 3 min	94°C 30''	55°C 30''	72°C 1 min	72°C 10 min
		35 cycles		

The length of the expected amplified product is 1100 bp.

The following conditions for touch-down PCR are recommended for rpoD according to (Soler *et al*., 2004):

- Initial denaturation at 95°C for 5 min,
- 2 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1 min,
- 2 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min,
- 2 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min,
- 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min.

Final extension step is not described but one cycle at 72°C for 5-10 min should work. The length of the expected amplified product is 820 bp.

12.4.3. *In vitro* susceptibility testing

The CLSI documents (CLSI 2006; CLSI 2014; CLSI 2016) should be considered. Generally, the interpretation criteria and breakpoints for aeromonads are relatively set by *Enterobacteriaceae*. Aeromonads are resistant to ampicillin, penicillin, carbenicillin, and ticarcillin and susceptible to trimethoprim-sulfamethoxazole, second and third generation cephalosporins, aminoglycosides, tetracyclines, quinolones and carbapenems (Martin-Carnahan and Joseph, 2005; Scarano *et al.,* 2018; Lamy *et al*., 2012; Kämpfer *et al*., 1999; Baron *et al*., 2017; Aravena-Roman *et al*., 2012).

From the species isolated from seabass, gilthead seabream and sharp snout seabream in the cases mentioned above, *A. hydrophila* was found to be susceptible to flumequine and oxytetracycline tested by the disk diffusion method (Doukas *et al*., 1998) and using the same method *A. veronii* bv. *sobria* was resistant to ampicillin and susceptible to all commercial antibiotics (tetracycline, oxytetracycline, oxolinic acid, flumequine, florfenicol and sulphamethoxazole/ trimethoprim) (Smyrli *et al.,* 2017). With the same method, *A. salmonicida* subsp. masoucida/ achromogenes was also found to be susceptible to trimethoprim, flumequine, oxytetracycline, oxolinic acid and to most of the antibiotics tested and showed resistance to sulfonamides, amoxicillin-clavulanic acid, ampicillin and ampicillin-sulbactam (Karatas *et al*., 2005). Tested with the agar diffusion method *A. salmonicida* subsp. *salmonicida* was found to be susceptible to all tested antibiotics (ampicillin, amoxicillin, flumequine, enrofloxacin, florfenicol, trimethoprim-sulfamethoxazole, oxolinic acid and pteridine) except for oxytetracycline (Fernández-Álvarez *et al*., 2016). Finally, the same species isolated from seabream was susceptible to most of the tested antibiotics (oxytetracycline, trimethoprim, polymyxin B, kanamycin, doxycycline, nitrofurantoin, tribricin, erythromycin, gentamicin, neomycin and cefotaxime) showing resistance to novobiocin, streptomycin, sulphonamides and penicillin (Real *et al*., 1994).

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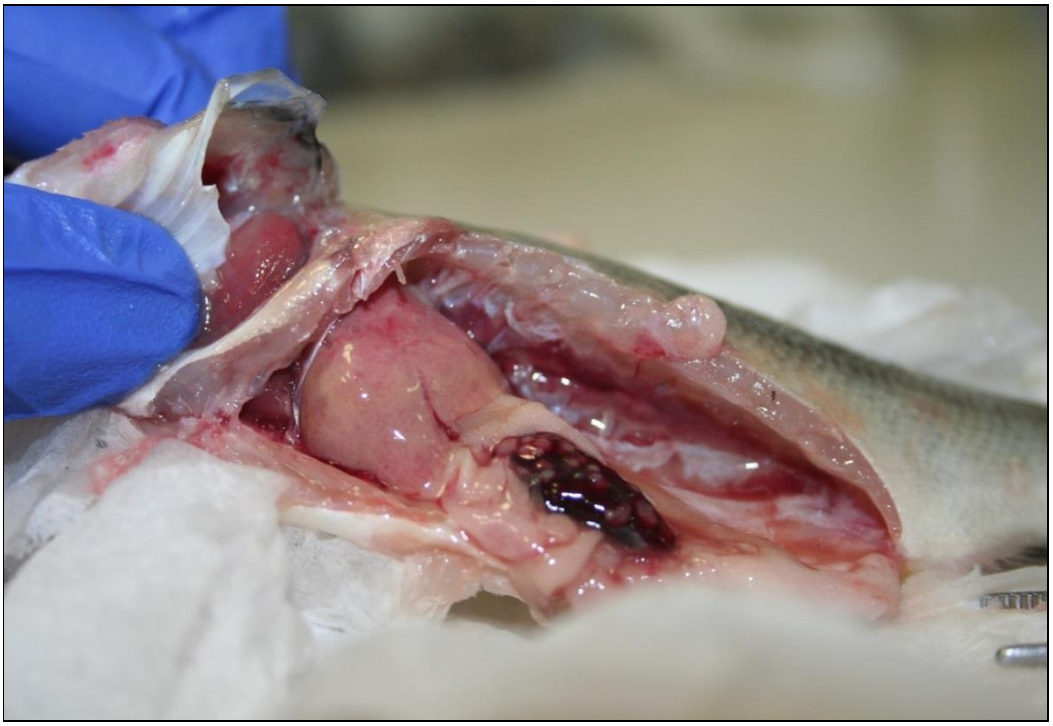

Fig. 12.1. Enlarged spleen with multiple abscesses, typical clinical picture of *A. veronii*-affected European seabass

Fig. 12.2. a) Brown pigment secreted from *A. veronii* bv. *sobria*; b) Haemolytic activity of *A. veronii* bv. *sobria* using fish blood

13. *Mycobacterium* group

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13.1. Aetiology of *Mycobacterium* group

Fish mycobacteriosis, also known as "piscine tuberculosis" is usually a chronic progressive disease caused by several species of the genus *Mycobacterium* (Jacobs *et al*., 2009) even if, on some occasions, septicaemias were reported in sturgeons infected by *Mycobacterium pseudoshotsi*. It is caused by a ubiquitous acid-fast-bacilli identified as non-tuberculous mycobacteria (NTB). The main NTB species affecting fish are *Mycobacterium marinum, M. fortuitum* and *M. chelonae* which can be classified into 1) slow grower NTB mycobacteria such as *M. marinum* and 2) rapid grower NTB mycobacteria such as *M. fortuitum* and *M. chelonae* (Novotny *et al*., 2004; Hashish *et al*., 2018). *Mycobacterium marinum* is the most important fish pathogen, representing a significant threat to seabass culture in the Mediterranean (Toranzo *et al*., 2005).

All *Mycobacterium* spp. infecting fish are also able to infect humans, resulting in local granulomatous inflammation usually at the extremities such as hands and fingers (Jacobs *et al*., 2009; Aubry *et al*., 2017).

Mycobacterium spp. are Gram-positive bacteria, aerobic, acid-fast, with the shape of non-motile rods, 0.2-0.6 µm in diameter and 1-10 µm long (Gauthier and Rhodes, 2009). The bacteria cell wall has a unique specific composition rich in mycolic acids (3-hydroxy long-chain fatty acids), essential for the survival of this genus (Marrakchi *et al*., 2014).

Piscine mycobacteriosis is typically a chronic progressive disease that may not produce clinical signs or take a long time for clinical signs and mortality to develop. External clinical signs are usually non-specific, such as scale loss and dermal ulceration, ascites, pigmentary changes, lethargy and abnormal behaviour. Internally, fish may exhibit spleen, kidney and liver enlargement, and show characteristic grey/white nodules (granulomas) in these internal organs (Decostere *et al*., 2004; Toranzo *et al*., 2005).

13.2. Sampling

13.2.1. Preparation and shipment of samples from fish

Isolation and culture of bacteria from external surfaces and whole viscera are questionable due to the possible presence of contaminants (Rhodes *et al*., 2004).

Tissue samples from fish are normally homogenized and then plated on appropriate culture media, including Middlebrook 7H10 or Lowenstein-Jensen media to enhance *Mycobacterium* spp. growth (Hashish *et al*., 2018).

13.3. Diagnostic procedures for *Mycobacterium* group

Mycobacterium spp. are best visualized in tissue sections (such as smears from spleen and kidney) with the Ziehl-Neelsen acid-fast stain. The “acid-fastness” or resistance of the cell wall to acid-alcohol decolourization after staining with carbol-fuchsin is characteristic of *Mycobacterium* species (Toranzo 2004; Gauthier and Rhodes, 2009). A Quick TB stain kit is available for rapid staining (RAL Diagnostic, Martillac, France).

13.3.1. Primary cultivation of bacteria (choice of media and isolation of strain)

Culture continues to be an important diagnostic method for diagnosis of fish mycobacteriosis, however, in many cases; the isolation of the aetiological agent often fails due to the fastidiousness of the pathogen (Austin and Austin, 2012). Successful isolation has been achieved from homogenates of infected tissue (from kidney, liver or spleen) on standard mycobacterium media such as Petragnani, Löwenstain-Jensen, Middle-brook 7H10 and Dorset egg media. All fish mycobacteria are cultured at 20-30°C. Incubation for 2-30 days is suitable for the fast-growers such as *M. fortuitum* and *M. chelonae,* which typically show growth after seven days. *M. marinum*, a slow grower, requires longer incubation for visible growth and may require months (Frerichs 1993; Decostere *et al*., 2004; Gauthier and Rhodes, 2009).

M. marinum is a strict aerobe and its preferred carbon sources are glycerol, pyruvate and glucose but ethanol can also be used. It grows in all media used for mycobacterial growth such as egg-based, broth or agar-based without any additives or only 2-5% oleic acid-albumin-dextrose-catalase and also ion blood-containing agar. After subcultures, some strains may grow on ordinary media but addition of 2-5% of carbon dioxide in the gas phase above the medium improves its growth. It has an optimal growth temperature of 30°C, whereas small colonies or no growth is observed at 37°C (Aubry *et al*., 2017).

13.3.2. Screening of pure cultures

Culture-based detection and isolation of *Mycobacterium* spp. from skin or gills of fish is complicated by the existence of background microbiota, which competes with the *Mycobacterium* growth on the standard media, therefore, it is recommended to carry out an aseptic necropsy. Plating of tissue homogenate on a non-selective medium such as BHI is recommended in order to detect a non-mycobacterial infection or sample contamination (Rhodes *et al*., 2004; Hashish *et al*., 2018).

Mycobacterium spp. show high hydrophobicity and are very resistant to treatment with both acidic and basic chemicals in addition to benzalkonium chloride and hypochlorite. These substances have been used to assist the isolation of pure cultures of *Mycobacterium* spp. with a high content of microbiota although it can affect negatively the recovery of mycobacteria (Rhodes *et al*., 2004).

13.3.3. *In vitro* susceptibility testing

For *in vitro* susceptibility testing two methods are available: (i) Etest and (ii) agar dilution method. The latter is the method recommended for antibiotic susceptibility testing of *M. marinum*. The agar dilution method can be performed on Mueller-Hinton agar (Difco, Serlabo, Bonneuil sur Marne, France) supplemented with 5% Middlebrook OADC (oleic acid, albumin, dextrose and catalase [OSI, Elancourt, France]) (Aubury *et al*., 2000).

After the *in vitro* testing of 17 antibiotics, *M. marinum* has shown natural multidrug resistance to the anti-tuberculosis drugs: isoniazid, ethambutol, and pyrazinamide. Minocycline, doxycycline, clarithromycin, linezolid, sparfloxacin, moxifloxacin, imipenem, sulfamethoxazole, and amikacin may have moderate activity. Rifampin, rifabutin, tetracyclines (particularly minocycline), amikacin, imipenem, and clarithromycin appeared to be good candidates for testing *in vivo* efficacy (Aubury *et al*., 2000; Aubury *et al*., 2017).

13.3.4. Identification of the strain

For strain identification, the sole utilization of 16S rRNA sequences has proven to be insufficient or too conserved to study the relationship of close related organisms. In *M. marinum,* the combined analysis of the restriction enzyme map of at least two genes (i. e. 16S rRNA and *hsp65*) has proven to be a useful molecular tool for the detection of intraspecific variation (Aubury *et al*., 2000; Ucko *et al*., 2002).

13.3.4.1. API – Biochemical identification

Biochemical identification is based on phenotypic characteristics such as growth rate, colonial morphology, cord formation and pigmentation (Nagwa *et al*., 2000).

In the case of *M. marinum*, colonies are typically smooth, white or beige when the media is kept in the dark and yellow to orange after exposure to light (photochromogenic). Photochromogenecity is due to the active production of beta-carotene mediated by the gene *ctrB* and can be inhibited by chloramphenicol. Biochemically, *M. marinum* does not show nitrate reductase production and cannot grow on a medium containing thiacetazone (Aubury *et al*., 2017).

This traditional method of diagnosis requires that the pathogen needs firstly to be recovered on culture medium and identified by means of a battery of differential biochemical tests. This method, however, often fails to identify *M. marinum* conclusively. Not only may the morphology and biosynthetic capabilities of mycobacteria vary depending on culture conditions but also there are often strain variations that do not quite fit into the typical biochemical profile for the species (Ucko *et al*., 2002). Therefore, the use of other approaches for the identification of

Mycobacterium spp. such as molecular methods or mass spectrometry analysis is being used increasingly (Kaattari *et al*., 2004; Kurokawa *et al*., 2013).

13.3.4.2. Mass spectrometry (MALDI-TOF)

A simple MALDI-TOF MS system called a MALDI Biotyper has been developed for rapid bacterial identification that does not require a highly trained operator (Shitikov *et al*., 2011; Bille *et al*., 2012). The MALDI Biotyper method has high reproducibility generating high-quality spectra, which allows the separation between species closely related to *M. marinum* and also differentiates *Mycobacterium* isolates (Kurokawa *et al*., 2013).

MALDI-TOF MS has been demonstrated to be a rapid and accurate technique which could be an effective diagnostic tool for the identification and differentiation of clinical mycobacterial isolates (Puk *et al*., 2018).

13.3.4.3. PCR

Several DNA-based diagnostic methods have been developed for the identification of *Mycobacterium* spp. in fish, which are particularly useful for its detection from fish tissues when the culture of *Mycobacterium* spp. fails (due to slow and poor growth of some of the species on culture media) (Toranzo *et al*., 2005; Hashish *et al*., 2018).

The small subunit 16S rRNA gene is commonly used for its identification due to the availability of *Mycobacterium* spp. 16S gene sequences in web repositories such as Gene Bank. A PCR has been described in 1993 by Telenti *et al*., targeting *hsp65* sequence present in all *Mycobacterium* species. Exact PCR conditions depend on the DNA concentration in the isolate, and the type of polymerase used, so what follows is an example using GoTaq G2 Hot Start Master Mix (Promega) or similar polymerase with DNA extracted from bacterial culture using commercial kit.

Primers for *hsp65* region

Forward primer	**Tb11**	5'- ACCAACGATGGTGTGTCCAT -3'
Reverse primer	**Tb12**	5'- CTTGTCGAACCGCATACCCT -3'

PCR mix contains the following reagents:

Reagent	Quantity
Water (molecular biology grade)	6µl
Hot Start Master Mix (2x)	10 µl
10 µM primer Tb11	1 µl
10 µM primer Tb12	1 µl
DNA sample	2 µl
Total volume	20 µl

Thermal profile

Initial polymerase activation	Denaturation	Annealing	Extension	Final extension
95°C	94°C	60°C	72°C	72°C
2 min	1 min	1 min	1 min	5 min
		45 cycles		

PCR-RFLP analysis using the 16S rRNA gene as a target has been proven a sensitive and highly specific tool for the identification of *M. marinum*, *M. fortuitum* and *M. chelonae* from fish tissues and blood samples (Talaat *et al*., 1997). PCR-RFLP on two genes, 16S rRNA and *hsp65*, have been able to distinguish a variety of *M. marinum* isolates (Ucko *et al*., 2002).

Due to the high homology between fish isolates, the use of a minimum of two genes for accurate identification is recommended. Other gene targets used for the molecular diagnosis are the: 1) 16S-23S internal transcribed spacer (ITS) 2) 65-k-Da heat shock protein gene (*hsp65*), 3) exported repeated protein gene (*erp*), 4) RNA polymerase B subunit (rpoB) gene (Kaattari *et al*., 2005).

FRET probe assay has shown to have high specificity via melting curve analysis and is able to discriminate and distinguish *M. marinum* from other *Mycobacterium* spp. The FRET assay has two steps. First, PCR with SYBR green (with a detection limit of 10^2 *Mycobacterium* DNA copies) and second, real-time PCR using FRET probes. The kidney is the organ with the strongest detection signal (Salati *et al*., 2010).

Finally, two methods based on PCR coupled with reverse hybridization are currently available commercially for the rapid and accurate identification of *Mycobacterium* spp.: INNOLiPA Mycobacteria v2 (Innogenetics), based on the amplification of the ribosomal gene spacer (16-23S) (Tortoli *et al*., 2001; Tortoli *et al*., 2003) and GenoType Mycobacteria CM/AS (Hain Lifescience), based on the amplification of the 23S rRNA gene (Russo *et al*., 2006).

13.3.5. Typing of the bacteria

13.3.5.1. Genotyping

A novel category of variable tandem repeats (VNTR) called mycobacterial interspersed repetitive units (Mirus) has been applied to *M. marinum* and *M. ulcerans*. The MIRU-VNTR typing is highly reproducible and can be applied directly to clinical samples and allows the intra and inter-specific differentiation among the *M. marinum-M. ulcerans* complex. The genotypes found, however, for *M. marinum* were not clearly related to the geographic origins of the isolates (Stragier *et al*., 2005).

Multilocus Sequence Analysis has been shown that *M. marinum* isolates showed a higher level of intraspecific nucleotide sequence divergence than other closely related species such as *M. ulcerans* (Stinear *et al*., 2000).

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Photos

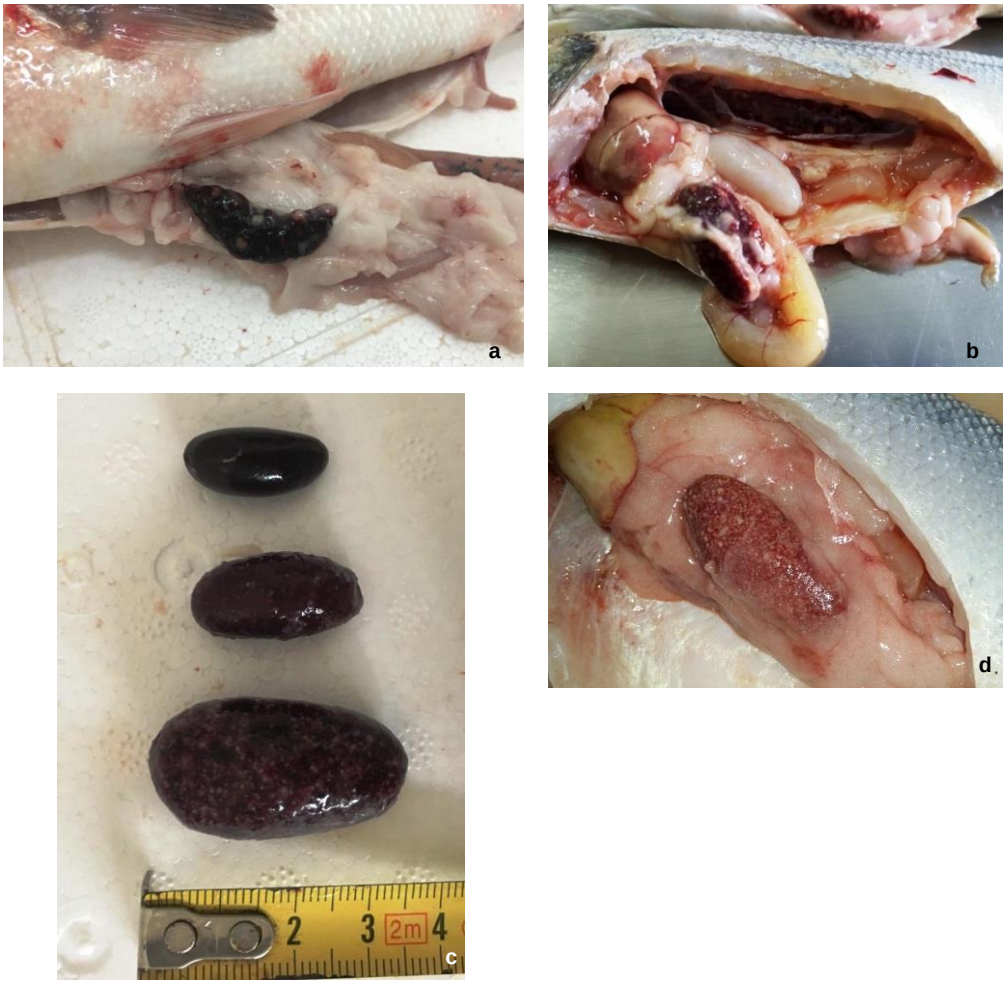

Fig. 13.1. European seabass/granulomatous lesions of the spleen due to *Mycobacterium marinum* (a-d).

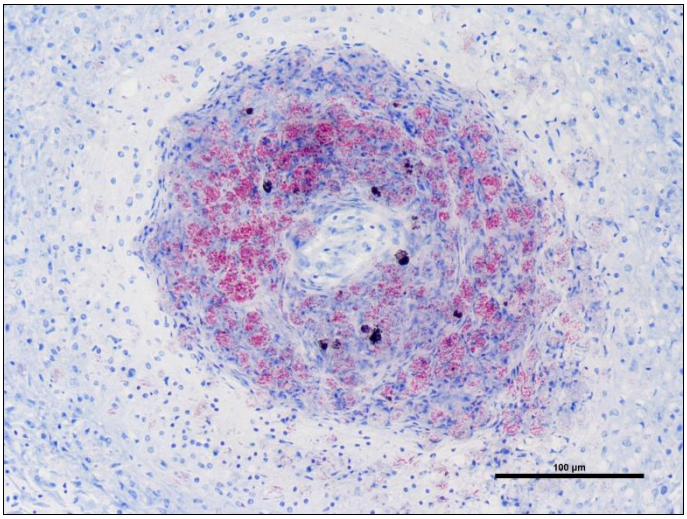

Fig. 13.2. Mycobacterial granuloma in seabass. Resin-embedded (Technovit 7100) stained with Zielh-Neelsen (Photo courtesy of P. Katharios).

Part V. Mortality caused by unknown aetiology

14. Diagnostic procedure in the case of mortality caused by unknown aetiology

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14.1. Introduction

European legislation on aquatic animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals sets criteria to investigate “increased mortality” (EU, 2006; EU, 2008; OIE, 2018). “Increased” means significantly above the level of what is considered to be expected for the farm under the prevailing conditions and is decided in cooperation between the farmer and the competent authority. In accordance with legislation, increased mortality must be investigated to understand the cause, rule out listed pathogens and allow early detection and definition of emerging

disease. An emerging disease is defined by OIE, in "The Aquatic Animal Health Code" (OIE, 2018) as "a disease, other than listed diseases, which has a significant impact on aquatic animal or public health resulting from a change of known pathogenic agent or its spread to a new geographic area or species; or a newly recognized or suspected pathogenic agent." A close collaboration between the diagnostic laboratory, the fish health manager and the fish farm will streamline the process and facilitate solving the issue (Patarnello and Vendramin, 2017). The steps to be considered and taken are explained in the following.

14.2. Case definition

A first important assessment is needed to define whether an infectious agent is suspected or other stressors are primarily involved in the mortality outbreak, such as oxygen failure in the tank, algal bloom around a sea cage farm, toxic leaks etc.

If an infectious aetiology is suspected, it is necessary to provide a specific case definition in order to define the disease outbreak. This will include all anamnestic information related to the disease outbreak including data on the host, the environment, farming practices and preliminary identification of an aetiological agent, if available;

Host: fish species, biological stage (larval, fry, juvenile, brood stock), organ affected, clinical signs, common necropsy findings need to be listed.

Environment: time of the year, water temperature, abnormal meteorological conditions (storms, sea currents etc.) should be recorded.

Farming practices: feeding and stocking procedures, vaccination, antiparasitic treatments etc.

The preliminary characterization of the pathogen is related to the infective agent and can include observation of microscopic parasites in fresh preparation, isolation of bacteria on agar culture, viral isolation on cell culture and thorough histopathological description of the affected organs. For the investigation of specific pathogens please refer to the other chapters in this book and the OIE "Manual of Diagnostic Tests for Aquatic Animals" (OIE, 2019).

14.3. Sampling procedures

Standardized sampling procedures are of paramount importance when investigating a disease outbreak. One possible approach is to sample the same group of fish over time, but it is rarely applied due to the urgent need to implement treatment and control measures to stop the mortality. An alternative approach may be to "capture" different stages of the disease by sampling different groups on the same site.

A standardized approach for sampling, which has been proven effective, consists of collecting fish from three strata (discrete groups that compose the whole farm population):

- Sick fish in an affected production unit (for example a tank or a cage).
- Healthy fish in an affected production unit.
- Healthy fish in a non-affected unit.

When approaching a disease outbreak, it is important to sample at least 5 fish per group, the moribund fish will be selected instead of dead fish in order to avoid post-mortem alterations and proliferation of unspecific organisms in the carcass. When sampling for the investigation of emerging disease, pooling of different specimens should be avoided.

Sampling represents the first phase and a bottleneck in the diagnostic process. It has to be seriously considered, planned with the laboratory and conducted by collecting and preserving material for different analyses. For this reason, it is recommended to collect and store samples

from the same tissue/fish in different ways to allow multiple analyses enabling a precise picture of the disease situation. Ideally, all samples should be collected, fixed and preserved as follows:

- preserved in RNA for molecular analysis later,
- fixed in formalin for histopathology,
- preserved in transport MEM for viral isolation,
- collected by swab for bacteriology,
- maintained frozen for NGS, infectious trial, back up etc.

14.4. Diagnostic procedures

The aim of the diagnostic investigation, specifically in cases of unexplained increased mortality, is to establish a chain of evidence establishing solid links between the clinical signs, necropsy findings, histopathological findings, microbiological tests and specific tests leading to an explanation of the cause of the mortality. In this regard, it is important to keep an open approach and consider that the disease picture observed could be produced by multiple pathogens persisting on the same fish stock. As a general guideline, it is never appropriate to identify a new pathogen relying only on one method and initial identification of pathogens shall be corroborated with different methods.

The following instructions refer to the sampling scheme described above, which includes 5 fish from 3 different strata of a farm, including affected fish in an affected production unit (for example a tank or a cage), healthy fish in an affected production unit and healthy fish in a non-affected unit. The descriptions are based on protocols from the “Manual of Diagnostic Tests for Aquatic Animals” (OIE, 2019).

14.4.1. Histopathology

Histopathological investigation helps to understand the host response to the pathogen, in order to increase the understanding of the effect of the pathogen. Proper sampling is critical to obtain high-quality information from the investigation and avoid artefacts.

Fish larvae, tissue or organs should be fixed in 10% buffered, neutralized formalin for at least 24h.

Each sample has to be preserved in fixative 10 times the volume of the sample.

- For larval stages, with body thickness below 5 mm, the whole fish can be fixed
- For small fish, with body thickness over 5 mm, the abdominal wall and the gill operculum on one side must be removed prior to fixing the whole carcass in formalin.
- For large fish, single organs should be taken out during the necropsy and placed in a container with a fixative. Samples should be of no more than 5 mm thickness and 1-2 square centimetre surface.

The tissue samples should include the area with evident lesions, including the area of transition from normal to affected tissue. Furthermore, independently of the necropsy findings, the following organs and tissues should be sampled: gill, heart, liver, gastrointestinal tract, pancreas, spleen, kidney, an area of the flank including skin-side line-muscle.

14.4.2. Virology

Virus isolation on cell culture can allow detection of viable viral particles, which, if found, is crucial in the diagnostic work. The procedure involves the collection of material, inoculation of cell lines and possibly sub-cultivation.

14.4.2.1. Collection of diagnostic material

Before shipment or transfer to the laboratory, pieces of the organs to be examined must be removed from the fish with sterile dissection instruments and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10 % calf serum and antibiotics. The combination of 200 i.u. penicillin, 200 µg streptomycin, and 200 µg/ml kanamycin can be used, but other antibiotics of proven efficacy may be used as well.

Pieces of organs should be collected in one sterile tube containing a sufficient amount of the transport medium (approximately 10 times the weight of the tissue sample). It is considered acceptable for the analysis to pool the sample from the same stratum. The tissue in each sample should weigh a minimum of 0.5 g.

The material to be used for virological examination depends on fish size:

- Fish below 4 cm (fry) can be used whole. For some viruses, it is recommended to use only the internal organs, therefore consult the OIE Diagnostic Manual (OIE, 2019) for the specific disease under suspicion.
- For fish between 4 cm and 6 cm, the internal organs may be used, after removing the head and caudal fin.
- For larger fish, the kidney, spleen and heart may be used. When a chronic disease is suspected, the brain may be included as well. Other organs can be included depending on which virus is suspected. For example, if there is a suspicion of nodavirus, only the brain should be used.

During transport, the tubes should be placed in insulated containers (for instance, thick-walled polystyrene boxes) together with sufficient ice or cooling elements to ensure chilling of the samples during transportation to the laboratory. Freezing should be avoided. The temperature of a sample during transit should never exceed 10 °C and ice should still be present in the transport box at delivery, or one or more freeze blocks must still be partly or completely frozen.

The virological examination should start as soon as possible and not later than 48 hours after the collection of the samples. Whole fish may be sent to the laboratory when the temperature requirements during transportation can be fulfilled. The fish should be wrapped in absorbent paper and shipped in a plastic bag to avoid cross-contamination. Live fish may be shipped as well. All packaging and labelling must be performed in accordance with national and international transport regulations as appropriate (see Section 2.2).

14.4.2.2. Collection of supplementary diagnostic material

Other fish tissues may be collected as well and prepared for supplementary examinations, according to the agreement with the diagnostic laboratory involved.

14.4.2.3. Freezing in exceptional cases

Where practical difficulties arise (i.e. bad weather conditions, non-working days, laboratory problems, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples, it is acceptable to freeze the tissue specimens in cell culture medium at -20 °C or, preferably, at -80 °C, and carry out virological examination within 14 days. The tissue, however, must be frozen and thawed only once before the examination. Some viruses may not sustain freezing (e.g. IPNV), so always consider what the sample will be examined for, prior to freezing the samples.

14.4.2.4. Cell lines to be included

A comprehensive panel of cell cultures should be included when investigating a new disease outbreak. Cell lines originating from the same species of fish under investigation should be used whenever available. As a minimal requirement, the panel should include Bluegill fry cell line -2 (BF-2) or Rainbow trout gonad cell line - 2 (RTG-2) and either Epithelioma papulosum cyprini (EPC) or Fathead minnow (FHM). The panel may be expanded with striped snakehead (SSN-1) cell line or similar (E-11, GF-1 etc.), and with Atlantic salmon kidney (ASK) and Chinook salmon embryo (CHSE) cell lines. Cells shall be grown at 20 to 30 °C in a suitable medium, namely Eagle's minimum essential medium (MEM) or modifications thereof, with a supplement of 10 % foetal bovine serum and antibiotics in standard concentrations. When the cells are cultivated in closed vials, the medium shall be buffered with bicarbonate. The medium used for cultivation of cells in open units may be buffered with tris (hydroxymethyl)aminomethane-HCl (Tris-HCl) (23 mM) and sodium bicarbonate (6 mM). The pH must be 7.6 ± 0.2. The cell cultures to be used for inoculation with fish tissue material shall be young, normally 1-day old cell culture monolayers where possible; however, a range between 4 to 48 hours old may be accepted. The cells must be actively growing at inoculation.

14.4.2.5. Inoculation of cell cultures

Antibiotic-treated organ suspension shall be inoculated into cell cultures in two dilutions, namely the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in the cell culture medium of 1:100 and 1:1000, respectively, in order to prevent homologous interference. At least two cell lines shall be inoculated as referred above. The ratio between inoculum size and volume of cell culture medium shall be about 1:10. For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture tray, shall be utilized. Cell culture trays shall be used where possible.

14.4.2.6. Incubation of cell cultures

The incubation temperature of cell culture varies in relation to the tolerance of cell cultures, and the optimal growth temperature of the aetiological agent. Inoculated BF-2, EPC, RTG, FHM, ASK and CHSE cell cultures shall be incubated at 15 °C. For SSN-1, E-11 and GF, the temperature of incubation should be 25 °C. Inoculated cell cultures have to be incubated for 7 to 10 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances must be performed to ensure cell susceptibility to virus infection. Inoculated cell cultures must be inspected regularly by microscopy, at least three times a week, for the occurrence of CPE at 40 to 150× magnification. If obvious CPE is observed, virus identification procedures should be initiated immediately. In this case, remaining cell supernatant and cell lysate shall be stored frozen at -20 °C or, preferably, at -80 °C.

14.4.2.7. Subcultivation

If no CPE develops after the primary incubation for 7 to 10 days, subcultivation must be performed on fresh cell cultures utilising a cell area similar to that of the primary culture. Aliquots of medium (supernatant) from all cultures or wells constituting the primary culture shall be pooled according to cell line 7 to 10 days after inoculation. The pools shall then be inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in the point above. Alternatively, aliquots of 10 % of the medium constituting the primary culture shall be inoculated directly into a well with fresh cell culture (namely, well to well subcultivation). The inoculated cultures shall then be incubated for 7 to 10 days at 15 °C (salmonid cell lines) and 25°C for SSN-1 and inspected as described above. If toxic CPE occurs within the first 3 days of incubation, subcultivation shall be performed at that stage, but the cells shall then be incubated for 7 days and subcultivated again

with further incubation for 7 days. When toxic CPE develops after 3 days, the cells shall be passed once and incubated to achieve a total of 14 days from the primary inoculation. There must be no evidence of toxicity in the final 7 days of incubation. If bacterial contamination occurs despite treatment with antibiotics, subcultivation shall be preceded by centrifugation at 2 000 to 4 000× g for 15 to 30 minutes at 2 to 5 °C, or filtration of the supernatant through a 0.45 µm filter or both (low protein-binding membrane). In addition to this, subcultivation shall follow the same procedures as described for toxic CPE in the fourth paragraph of this point. If no CPE occurs, the test may be declared negative.

14.4.3. Bacteriology

The bacteriological examination should be carried out by inoculating on agar plates, samples taken from kidney, brain and from the area where wounds or lesions are present and consistent with the case definition. Sampling and inoculation are done with a sterile loop. The selection of agar plates should at least include blood agar (BA), marine agar (MA) and cysteine heart agar with blood (CHAB).

Agar plates should be incubated at 15 °C and 20 °C for at least 7 days, in an appropriate incubator and away from contamination sources. Each plate must be labelled with the lot, the sample origin, the species and the organ from which the sample was taken. In case of slow-growing bacteria, such as intracellular bacteria, it is appropriate to incubate the plates for longer periods.

Once the microorganism is isolated, it can be characterized and identified by:

- Microscopy and staining. These methods (including among others Gram staining, etc.) give an indication of the shape, morphological features and motility of the bacteria.
- PCR and sequencing. Generic primers for 16 S rRNA sequencing can provide initial and partial identification of newly isolated bacteria.
- MALDI-TOF enables rapid identification of pure bacterial colonies. This method requires a validated database of reference strains to correctly identify the bacteria

14.4.4. Molecular biology

The introduction of NGS (Next Generation Sequencing) and sequence-independent single primer amplification has recently led to the discovery of a number of nucleic acid sequences originating from viruses and other microorganisms which are yet to be classified. A large number of these new microorganisms will have a big impact on the taxonomic order used today, and furthermore, greater focus is required on assessing their biological relevance to farmed animals. Among other fish pathogens recently identified by NGS, are Piscine Myocarditis Virus (PMCV), Piscine Orthoreovirus (PRV subtypes 1,2 and 3) and Salmon gill pox virus.

The NGS platform is based on the immobilization of DNA/RNA onto a solid support, cyclic sequencing reaction through automated fluidic devices and detection of molecular events by imaging. NGS is a technique that is independent of the target sequence, but in order to obtain good coverage of the virus, it is important to analyse a sample that is rich in the viral pathogen with a limited presence of the host genetic material.

At sampling, it is recommended to collect tissues and organs for NGS analysis in case the first series of investigations does not provide conclusive results. For RNA sequencing, organ and tissue samples shall be stored in RNA later at -20°C or, preferably, at -80°C. For DNA sequencing, organ and tissue samples shall be stored frozen at -20 °C or, preferably, at -80 °C. In case an agent is isolated on cell culture, the cell supernatant and cell lysate shall be stored frozen at -20 °C or, preferably, at -80 °C.

14.4.5. Electron microscopy

Electron microscopy techniques can be used to morphologically characterize new pathogens. Electron microscopy has limited analytical sensitivity; therefore, suitable samples for this technique should originate from pathogens isolated on cell culture, or from field samples, where a high concentration of pure pathogen is expected.

14.5. General conclusions

Increased mortality should always be taken seriously and diagnostic investigations should be initiated as soon as possible. The following guidelines, based on this chapter, can be followed to perform a thorough investigation:

- Define the disease case highlighting all anamnestic features in the case description.
- Rule out non-infectious agents such as environmental conditions, toxic agents, etc.
- Rule out known pathogens with a focus on listed ones.
- Secure diagnostic samples from sick fish in an affected production unit (for example a tank or a cage), healthy fish in an affected production unit and healthy fish in a non-affected unit.
- Establish a chain of evidence where solid links are established between the symptomatology, necropsy findings, histopathological findings, microbiological tests and specific tests.
- Store material in different fixatives for future reference in case of inconclusive analyses, if the suspicion of an unknown disease agent is strong.

Keep in mind that it is of paramount importance, in order to conclude an identified pathogen as the cause of the disease and mortality, to corroborate the identification by means of different diagnostic techniques targeting different pathogen components (pathogen genome as well as antigens).

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Part VI. Interpretation and reporting of results

15. Interpretation of diagnostic results in aquatic animal health

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15.1. Assessment of the accuracy of a diagnostic test

Both pathologists and clinicians must always keep in mind that diagnostic tests are not perfect (even Real-Time qPCR test), although we sometimes find it difficult to accept. Once we have assumed this fact, we must reflect on the consequences of possible diagnostic errors in our interpretation of the results. For further information about diagnostic testing, we recommend consulting Dohoo *et al*. (2003), Thrusfield (2007), Gordis (2014), and the OIE manual "*Principles and methods of validation of diagnostic assays for infectious diseases"*.

Classically, the reliability of a diagnostic test has been characterized by assuming the existence of a perfect reference test, known as a **gold standard**, which indicates the true health status of the individuals analysed. So, all the positive individuals for this test are diseased and all the negative results correspond to healthy animals. Actually, this test does not exist, but it serves to understand the concepts that we are going to explain next. An alternative to the gold test is to use two groups of animals with known health status, i.e., a group of animals that have been experimentally infected and have manifested the disease to be diagnosed and another group of disease-free animals (from historically free territories or using Specific Pathogen Free (SPF) animals).

If we were to diagnose a sample with two diagnostic tests: the gold standard and the test to be evaluated, we would obtain a contingency table (Fig. 26), where we would find four possible combinations of diagnostic results. There are the concordant results: true positives, TP (diseased animals diagnosed as positive by the assessed test) and true negatives, TN (healthy animals diagnosed as negative by the assessed test). The problem arises in the discordant results since they are errors committed by the evaluated test: false positives, FP (healthy animals diagnosed as positive by the assessed test) and false negatives, FN (sick animals diagnosed as negative by the assessed test). Therefore, the purpose of designing and interpreting a diagnostic test is to minimize the number of false positives and/or negatives that may appear.

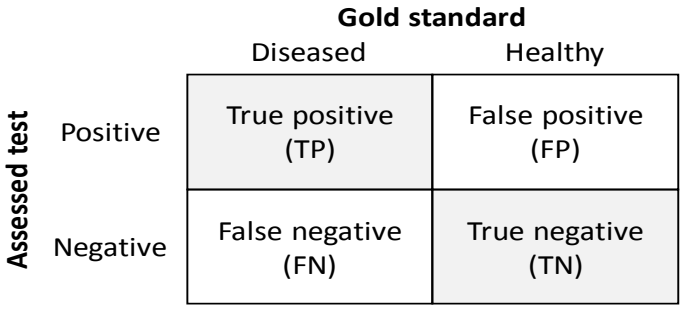

Fig. 15.1. Contingency table for the assessment of a diagnostic test

Considering this contingency table, it is possible to estimate the success rates of a diagnostic test through measures of sensitivity and specificity, respectively; they are independent of the prevalence.

Sensitivity (Se) is defined as the probability of obtaining a positive result when an animal is diseased and corresponds to the following conditional probability formula:

$$Se = p(+|\, D) = TP/(TP + FN)$$

Similarly, **specificity** (Sp) is defined as the probability of obtaining a negative result when an animal is healthy and corresponds to this other conditional probability formula:

$$Sp = p(-|\, H) = TN/(TN + FP)$$

In order to facilitate the understanding of these concepts, we are going to propose an example where 622 trout have been selected, of which 227 are surely diseased and 395 are SPF trout. We wish to assess a diagnostic test which is applied to all trout, obtaining 190 positive results in the group of diseased trout (and therefore 37 negatives) and 303 positive results in the group of healthy trout (and therefore 92 negatives).

If we represent the data of the example to scale, considering the groups created by the gold standard (Fig. 27), we can see that in the group of diseased fish the proportion of successes (TP) is quite high. Something similar happens with the proportion of successes in the group of healthy fish (TN). Therefore, in this example, the sensitivity is 83.70% (=190/227) and the specificity 76.71% (=303/395).

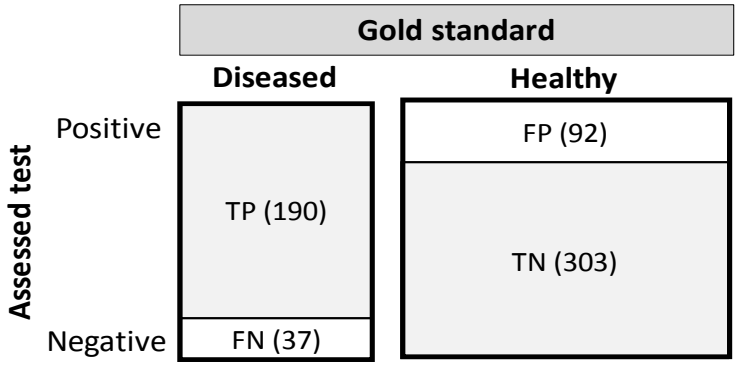

Fig. 15.2. Contingency table for calculation of sensitivity and specificity

As can be seen in (Fig. 26) and in the formulas, the existence of false negatives reduces the sensitivity while false positives affect the specificity. That is to say, when a test has a perfect sensitivity (100%) there will be no false negatives and therefore all negative results will correspond to healthy animals, which would allow us to safely rule out that a negative animal is diseased. While a test with perfect specificity will not have false positives, and all the results obtained will be diseased animals, therefore a positive result will confirm that it is a diseased animal.

15.2. Setting up a diagnostic test

The problem that arises when a diagnostic test is developed is that when we try to improve the sensitivity, the specificity will worsen and vice versa. To explain this fact, we will use the example of an analytical diagnostic test based on quantitative results (for example, an ELISA test). We will apply the test to a group of diseased animals and to another group of healthy animals obtaining a distribution of results similar to the one shown in (Fig. 28). As can be seen, there is an overlap of both distributions which forces us to look for the most suitable cut-off value (or threshold value) to optimize diagnostic reliability, i.e. minimizing false positives and false negatives. Once the cut-off value has been established, all results with values lower than the cut-off value are considered negative and those with higher values are considered positive.

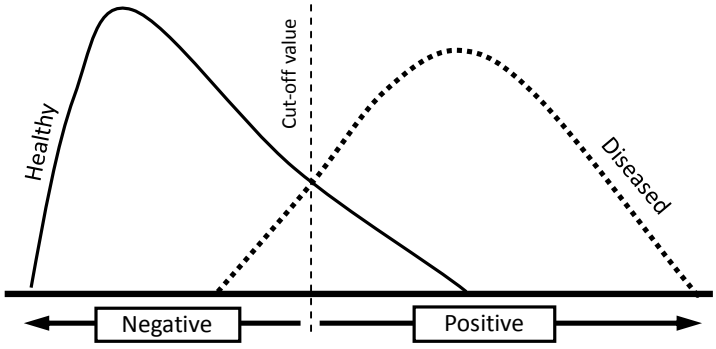

Fig. 15.3. Distribution of results of a diagnostic test stratified by health status

Once the cut-off value is set, we can build the contingency table used to assess a diagnostic test. Diseased animals with values above the cut-off value will be the true positives and healthy animals with values below the cut-off value will be the true negatives. Unfortunately, there will be false positives (the right tail of the distribution of healthy animals) and false negatives (the left tail of the distribution of diseased animals).

If we choose to minimize false negatives (by reducing the cut-off value), we will be able to increase the sensitivity until it becomes perfect (100%) (Fig. 29 a), although at the cost of significantly increasing the number of false positives and therefore worsening the specificity. If, on the other hand, we decide to minimize false positives (by increasing the cut-off value), the specificity will increase until it becomes perfect (Fig. 29 b), but with the corresponding increase in false negatives that leads to a worsening of sensitivity. Normally there is a tendency to maximize sensitivity (minimizing false negatives) at the cost of worsening specificity (increasing false positives). Although there are statistical techniques to identify the value that maximizes both values (using ROC curves).

In some cases, the test results which are under the overlapping area of the curves are considered doubtful, meaning that the test cannot discriminate between false positive and false-negative results.

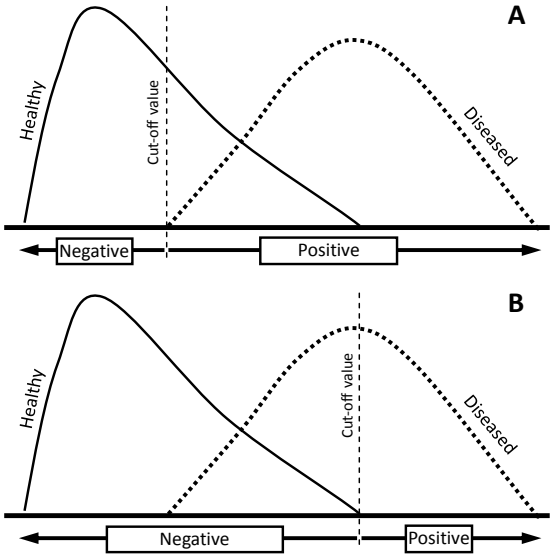

Fig. 15.4. Effect of cut-off value in the diagnostic results and diagnostic accuracy.

15.3. Diagnostic accuracy vs laboratorial accuracy

An interesting aspect that needs to be reflected upon is why these these types of diagnostic errors are made.

In the case of **false negatives**, we must first consider the latency period of the disease (for example, in the case of serological tests, antibodies take a period of time to develop from the moment of infection and during that period they will not be detectable) or the existence of substances that may inhibit the analytical reaction performed.

At the laboratory level, the term “laboratory sensitivity” is used to refer to the detection limit of a diagnostic test. In (Fig. 30) we can see how the pathogen load of an infected fish begins with a low number of pathogens and the amount increments (it usually coincides with the symptomatic phase) and afterwards in the survival animals (convalescent fish) the pathogens disappear or their number decreases significantly.

The performance of a diagnostic test can vary. This means that a simple PCR (Fig. 30 a) can detect, for example, a concentration of at least 10^4 pathogens/g of tissue; however, a qPCR (Fig. 30 b) have a lower detection limit and can give a positive result with concentration of 10^2 pathogens/g. Therefore, the laboratory sensitivity (detection limit) is correlated with the diagnostic sensitivity, since the probability of a negative result is greater with the simple PCR (because the latency period and convalescence periods are longer) and in consequence the sensitivity is lower than in the second case (qPCR) where the probability of negative results in an infected fish is lower, in which case the sensitivity will be higher. This means that low detection limits produce high diagnostic sensitivities.

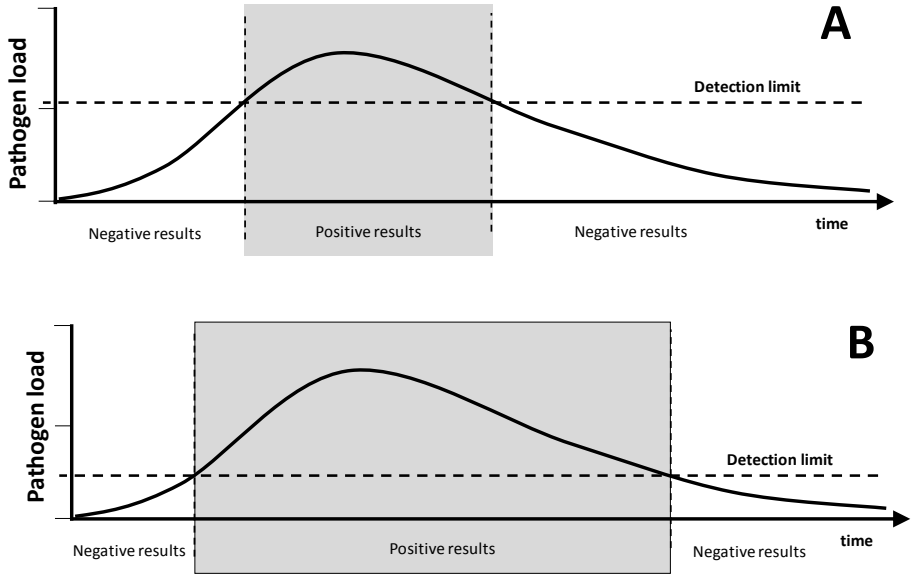

Fig. 15.5. Laboratorial sensitivity vs diagnostic sensitivity

In the case of **false positives** for direct diagnostic tests such as PCR (where the aim is to detect the pathogen) the existence of other organisms with genome fragments identical to the pathogen to be detected should be considered. In the case of indirect diagnosis (where the aim is to find antibodies against the pathogen, such as ELISA) the phenomena of natural immunization (animals recovered from infection) or acquired immunization (vaccinated animals) or serological cross-reactions should be taken into account.

To assess this laboratory specificity, the diagnostic test is tested against several pathogens that we can usually find in the same fish species. For example, the laboratory specificity of a PCR assay for *Aeromonas salmonicida* can be tested with *Aeromonas hydrophila, Vibrio salmonicida, Pseudomonas fluorescens, Shewanella putrefaciens, Yersinia ruckeri*, etc. If these samples do not give positive results, the laboratory specificity of the PCR assay for Aeromonas salmonicida is proven.

15.4. A possible solution to overcome low accuracy of diagnostic test

It is important to keep in mind that a laboratory result gives a punctual indication of the infectious status of an animal linked to a specific time and tissue sampled (i.e blood or organ), as biological activities are highly influenced by internal and external conditions (i.e. water temperature, age, hormonal balance, etc.). It is therefore important to know if the test used is validated for the actual situation, animal and sample tested.

To reduce the problem correlated with false positive or false negative results, the following actions can be undertaken:

- Use a second independent laboratory test, if available, with different sensibility/specificity characteristics in order to confirm the previous results. This should be done according to a specific procedure (described in 1,2,3).

- Change the kind of sample tested (i.e. collect different organs). The optimal organ for testing may vary in time through-out the infection process and between infectious agents. This may be especially important in the early phase of infection.
- Increase the number of samples submitted. This will not influence the result of the individual animals tested, but increase the certainty of the target population being infected or not.
- Repeat the sample a few weeks later. This will allow the disease to progress or the fish to be clear of the cause that resulted in the false positive/doubtful reaction. This is particularly useful when the laboratory test targets antibody level/presence, which evolves quite rapidly.

In conclusion, the correct interpretation of laboratory results requires the knowledge of fish biology and farm history, as well as test performance and pathogen characteristics and epidemiology.

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16. Reporting of results

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16.1 How to correctly report laboratory results

The laboratory is responsible for ensuring that the customer receives the results of laboratory testing correctly, completely, unambiguously, and objectively and timely (AAVLD, 2018). The length of time for laboratories to complete analyses varies. Samples sent to the diagnostic laboratory should have necropsy and basic parasitology results within 24 hours after receipt of the fish. Usually, the results of bacterial isolation and sensitivity testing are completed within 48 to 96 hours in case of bacteria with normal growth patterns. For fastidious bacteria, the time required to produce the report should be prolonged accordingly (Adams and Thompson, 2011). Virological analysis using cell culture inoculation and identification of viruses as well as histopathology may take up to two weeks or more. All mentioned methods involve isolation and cultivation of pathogens from diseased specimens followed by identification. Immunological methods may be used either for identification of the cultured pathogens or for the direct identification of the pathogens in infected tissues. Both direct and indirect fluorescent antibody techniques (FAT and IFAT) are simple methods that can provide the result within several hours. However, diagnostics based on molecular methods are becoming a must as they are more sensitive and specific and provide rapid results. They are useful for the detection of fastidious microorganisms and comprise valuable tools in epidemiological studies. In addition, these techniques are affordable and are becoming cheaper all the time.

When creating the reporting system, the laboratory should be able to prepare the reports at different levels and consider the best way to communicate the results at these levels. The results of the laboratory analysis should be reported clearly and the reports should be simple and easy to understand and targeted to the user. When reporting surveillance results, the laboratory should report results to every party participating in the surveillance as well as those who may need them.

The recording system applied in the laboratory is the prerequisite for correct reporting and transmission of the results to the farmer/client. Moreover, as stated in the introduction, the diagnostic procedure begins with sample selection and collection in the field and continues through sample preparation for shipping, shipment, and receipt of the samples by the laboratory, recording and processing it for diagnosis. All these steps that may influence the result of the diagnostic procedure should be described in the report. Each report should contain the date of sampling, basic environmental and culture conditions during sampling (if relevant for the testing), the shipping condition and the condition of the sample at delivery. The short description of the diagnostic procedures employed for the disease agent determination is followed by the results/diagnosis and any comments.

However, regardless of the intended purpose of the test, a complete and transparent reporting of the steps in the diagnostic procedure and a reference to testing accuracy are essential for the readers to evaluate the validity of the tests as well and to assess the possibility of biases in diagnostic sensitivity and specificity (OIE, 2019).

Each analytical report should consist of the elements quoted in Table 16.1.

Table 16.1. Components of the report on the laboratory results

Mandatory	Optional
A title Name and address of the laboratory Identification of the sample Name and address of the customer Identification of the sample	
Remark on the sampling procedure used by the laboratory or by the client if it is relevant to the quality of the results	*Date of sampling, sample origin which includes the sampling site and the culture unit, reference of sampling plan used if any, details of environmental and culture condition during the sampling, if relevant to the results of testing, identification of the sampling procedure*
Date of the receipt of the sample Evaluation of sample quality upon reception Identification of the test methods employed Date of the testing with start and completion, where it is relevant to the quality of the test The results of the test	
Where appropriate and necessary, interpretation of the test results and opinions	*The basis upon which the opinion and interpretation have been made; in that case, the rationale upon the testing and decision making was performed; presumptive, definitive tests, screening or confirmatory*
The name, function and signature of the person responsible for authorization of the report	

It is important that the reporting format is designed in a manner to include all tests carried out in the process of diagnosis but minimizing the possibility of misinterpretation or misuse. If there is a set of diagnostic procedures with different durations the interim report should be issued to the client, in which case it should be indicated which tests are completed and which are pending. It should be clearly identified as an interim report and upon completion of all tests; a final report should be issued. This final report should contain references to all preceding interim reports.

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Part VII. Annexes

Annex 1. Summary sheets of main diseases

- Summary sheet for Viral Nervous Necrosis – A. Toffan
- Summary sheet for vibriosis caused by *Vibrio anguillarum* – S. Zrnčić
- Summary sheet for vibriosis caused by *Vibrio harveyi* – T. Pretto
- Summary sheet for *photobacteriosis* – P. Varvarigos
- Summary sheet for *Tenacibaculum* infections – A. Le Breton
- Summary sheet for *Aeromonas* spp infections – M. Smyrli and Pantelis Katharios
- Summary sheet for *Mycobacterium* infections – A. Le Breton

Summary sheet for viral nervous necrosis

A. Toffan[1]
[1] Istituto Zooprofilattico Sperimentale delle Venezie,
National Reference Centre for Fish, Molluscs and Crustacean Diseases, Legnaro, Padova, Italy.

Aetiological agent

Betanodavirus

Epidemiology

Host/s (species; age): All Mediterranean farmed fish: seabass *(Dicentrarchus labrax)* is the target species, flatfish (*Solea* spp., *Scophthalmus* spp.) and groupers (*Ephinephelus* spp.) are also very susceptible. Seabream *(Sparus auratus)* and meagre *(Argyrosomus regius)* appear to be affected only during the larval stage. The younger the fish the more sensitive they are.

Morbidity and mortality rates: Depend on age/size class. Larvae up to 100%, juveniles up to 60%, adult fish are more resistant. Survivors remain persistently infected.

Transmission: Horizontal as well as vertical (via infected eggs).

Factors (environmental, others) for disease outbreak: Clinical signs insurgence is temperature-dependent. Outbreaks often occur at seawater temperatures above 20°C.

Clinical signs

Erratic swimming with spinning and swirling movements, swim bladder hyperinflation, congestion and erosion of the skin of the head, eye lesions, darkening.

Samples to be collected for diagnostics

Brains and eyes.

Presumptive diagnostics analysis

Typical clinical signs with the warm water temperature in cages and tanks. Severe congestion of head, brain and meninges in absence of other lesions in internal organs.
In hatcheries, the sudden appearance of high mortality of larvae. Hyperinflation of swim bladder.
Histological observation of congestion, haemorrhages and/or vacuoles in nervous tissues (brain, spinal cord and retina).

Confirmatory diagnostic analysis

Real time RT-PCR, viral isolation, PCR and sequencing.

Abnormal swimming

Congestion and erosion of the head

Summary sheet for vibriosis caused by *Vibrio anguillarum*

S. Zrnčić[1]
[1] Croatian Veterinary Institute, Laboratory for Fish, Molluscs and Crustacean Diseases, Zagreb, Croatia.

Aetiological agent

Vibrio anguillarum

Epidemiology

Hosts: European seabass (*Dicentrarchus labrax)*, gilthead seabream (*Sparus aurata)*, sole (*Solea* spp.), sea mullet (*Mugil spp)*, turbot (*Scopthalmus maximus)* and eel (*Anguilla anguilla).*

Morbidity and mortality rates: Varying depending on age/size, and co-infections; in seabass mortality in the grow-out phase can reach 30% but may increase.

Transmission: Horizontal.

Factors (environmental, others) for disease outbreak: Occurs at seawater temperatures between 13-21^{o}C, but mainly after a sudden increase in temperature in spring or decrease in autumn. In winter months disease is more often in chronic form. The disease may follow stressful conditions.

Clinical signs

Acute form - lethargy, anorexia and darkening of the skin erythema around mouth and vent, on the fin base, edematous skin lesions, opacity in the eyes, exophthalmia; subacute bleeding on the head, operculum, vent, pale gills with haemorrhages; chronically large granulating lesions deep into the muscle, severe anaemia of gills, grey corneal opacity, progressing to ulceration. Autopsy in acute and subacute forms reveals bleeding of the liver, posterior part of the intestine, rarely in the stomach.

Samples to be collected for diagnostics

Moribund whole fish or target organs, such as the spleen, head kidney, brain, cutaneous or ocular lesions.

Presumptive diagnostics analysis

Observation of erythema on the fin base, operculum, head, gills, liver and intestine
Bacterial culture from target tissues on BA, TSA 2%NaCl and Marine Agar produce grey whitish colonies after 24-36 h at 22-25°C. Isolates appear generally yellow on TCBS. Inoculation of API 20E strips with 2%NaCl inoculum produces at 25°C most frequent profiles: 304452456, 304572557, 304652456.

Confirmatory diagnostic analysis

MALDI-TOF, end-point PCR (*amiB* gene) or amplification and phylogenetic analysis of a portion of the *pyrH* gene.

Haemorrhages on the operculum, fin base, fins

Bleeding on the liver and intestine

Summary sheet for vibriosis caused by *Vibrio harveyi*

T. Pretto[1]
[1] Istituto Zooprofilattico Sperimentale delle Venezie,
National Reference Centre for Fish, Molluscs and Crustacean Diseases, Legnaro, Padova, Italy.

Aetiological agent

Vibrio harveyi

Epidemiology

Hosts: seabass (*Dicentrarchus labrax*), sole (*Solea senegalensis, Dicologlossa cuneata*), grouper (*Epinephelus* spp.), common dentex (*Dentex dentex*), greater amberjack (*Seriola dumerili*) and gilthead seabream (*Sparus aurata*). Seabass is sensitive especially during early grow out (40-150 g) and hatchery rearing phase.

Morbidity and mortality rates: Variable, depending on age/size, temperature and co-infections; in seabass mortality in the grow-out phase can reach 10% but may further increase if *Betanodavirus*, other bacterial infection or parasitic infestation is present.

Transmission: Horizontal

Factors (environmental, others) for disease outbreak: Outbreaks often occur at seawater temperatures between 18-27^{0}C, although chronic mortalities are reported at lower temperature (winter season). *V. harveyi* can be frequently isolated in co-occurrence with other infections (*Betanodavirus*, *Photobacterium damselae* spp., *Vibrio* spp., ectoparasites).

Clinical signs

Lethargy, anorexia and ataxia, cutaneous erosion or haemorrhaging at the base of the fins, ophthalmic lesions (keratitis).

Samples to be collected for diagnostics

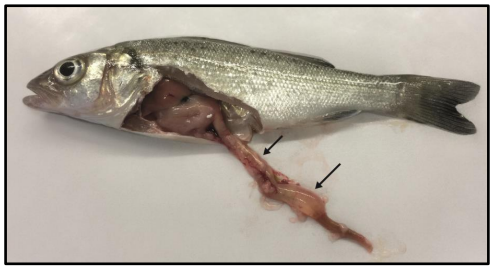
Moribund whole fish or target organs, such as the spleen, trunk kidney, brain, cutaneous or ocular lesions.

Presumptive diagnostics analysis

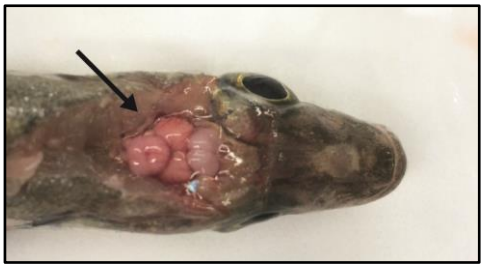
Observation of serous or serous-catarrhal enteritis with marked dilatation of the intestinal lumen, encephalic congestion, cutaneous erosion or ulceration.
Bacterial culture from target tissues on BA, TSA 2%NaCl and marine agar produce colonies after 24-36 h at 22-25°C. Isolates appear generally yellow on TCBS while on CHROMAgar Vibrio may vary between pale lilac, rose or white. Inoculation of API 20E strips with 2%NaCl inoculum produces at 25°C most frequent profiles: 4346525, 4346125, 4344125. Citrate and gelatinase should be read after 48 hours. Some isolates may present luminescence.

Confirmatory diagnostic analysis

MALDI-TOF, end-point PCR (*toxR* gene) or amplification and phylogenetic analysis of a portion of the *pyrH* gene.



Serous-catarrhal enteritis with marked dilatation of the intestinal lumen



Encephalic congestion

Summary sheet for photobacteriosis (ex Pasteurellosis)

P. Varvarigos[1]
[1] Aquahealth Diagnostic Lab, Athens, Greece.

Aetiological agent/s

Photobacterium damselae subspecies *piscicida*

Epidemiology

Host/s (species; age): All Mediterranean farmed fish: seabream *(Sparus auratus)* as well as all other sparids, seabass *(Dicentrarchus labrax)*, meagre *(Argyrosomus regius)*. The younger the fish the more sensitive they are.

Morbidity and mortality rates: Depend on age/size class. Juveniles up to 80%, fry up to 60%, on growers up to 40% when fish are not immunized and are left untreated.

Transmission: Horizontal as well as vertical (via infected eggs).

Factors (environmental, others) for disease outbreak: The disease is temperature-dependent. Outbreaks often occur at seawater temperatures between 18-26^{o}C. Site and cage excessive stocking densities predispose horizontal transmission.

Clinical signs

Sudden high mortality, lethargy and ataxia, skin darkening with eroded, whitish patches, most mortalities sinking to the bottom.

Samples to be collected for diagnostics

Moribund whole fish or target organs, such as the spleen, head kidney, liver, brain.

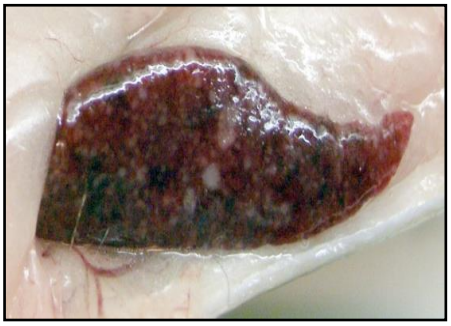
Presumptive diagnostics analysis

History of elevated water temperature and sudden high morality, splenomegaly with or without pseudotuberculi, gill necrosis, liver congestion, bacteraemia evident on Giemsa-stained blood smears or splenic imprints.
Bacterial cultures from target tissues on TSA, BHI, MH, blood agar produce colonies with characteristic morphology in about 36h at 26^{o}C. No growth on TCBS agar. Inoculation of API 20E test strips produces typical profile 2005004.

Confirmatory diagnostic analysis

Seroagglutination of bacterial colonies, MALDI-TOF, PCR

Gill necrosis



Splenomegaly with pseudotuberculi

Summary sheet for *Tenacibaculum* infections

A. Le Breton[1]
[1] VET'EAU, Grenade sur Garonne, France.

Aetiological agent/s

Infections induced by a group of species belonging to the genus *Tenacibaculum* including *T. maritimum, T. discolor, T. dicentrarchi, T. soleae, T. gallaïcum.* Co-infections often described.

Epidemiology

Host/s (species; age): Most Mediterranean aquaculture species including seabass (*Dicentrarchux labrax*), seabream (*Sparus aurata*), Solea (*Solea senegalensis*), Mugilidae. Affect all stages of development with a higher incidence on juvenile stages from weaning to 100gr average.

Morbidity and mortality rates: depend on age, size class, rearing system and the *Tenacibaculum* species involved.

Acute form on fish from 0.5g to 100g: over 50% at weaning and early stages, up to 30% for fry in land based systems, 10 to 20% in pregrowing sea cage units.

Sub-acute form with low mortality rate on larger stages.

Transmission: horizontal transmission from fish to fish or from the environment, especially the biofilm.

Factors (environmental, others) for disease outbreak: Temperature dependent from 14°C to 19°C, higher salinity (>32ppt), low pH in RAS system, water quality parameters (organic load, low redox).

Predisposing zootechnical factors: mechanical lesions, skin parasitic infection, feeding behaviour/aggressivity, mucus erosion.

Co infection with bacterial skin infections: filamentous segmented bacteria, skin vibriosis.

Clinical signs

Whitish to yellowish skin lesions with thick mucus affecting mainly the mouth, the caudal peduncle, or the dorsal fin and body side depending on *Tenacibaculum* species. Can evolve into ulcerative lesions by penetration into the muscular septa (*T. discolor*). Gill-focused necrosis with thick mucus.

Samples to be collected for diagnostics

Scraping from the lesions either for direct microscopic observation or bacterial analysis.
Live fish with clinical symptoms to be sent alive to laboratory as bacteria are quite sensitive or swabs from the lesions with specific transport media.

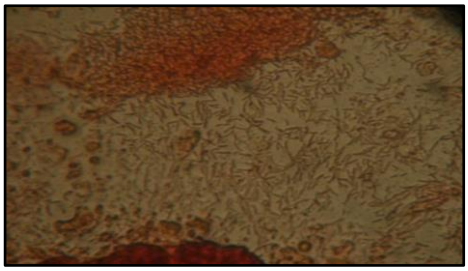
Presumptive diagnostics analysis

Observation of the skin and/or gill typical lesions with thick whitish mucus.
Direct microscopic observation on fresh smears from scraping of the lesion or after staining (gram stain, MGG, methylene blue) (x400 or x1000 immersion).

Confirmatory diagnosis analysis

Isolation and identification of the bacterial strain by mass spectrometry, RAPD-PCR, Sero-typing.

Skin necrosis with whitish mucus



Fresh smear from skin scrapping (400x)

Summary sheet for *Aeromonas* spp infections

M. Smyrli and P. Katharios[1]
[1] Hellenic Centre for Marine Research, Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC), Heraklion Crete, Greece.

Aetiological agent/s

A. veronii bv. *sobria*, *A. hydrophila* and *A. salmonicida*

Epidemiology

Host/s (species; age): European seabass *(Dicentrarchus labrax)* mainly affected; juvenile and grown fish. Also isolated *in mixed infections* from various diseased fish e.g. gilthead seabream (*Sparus aurata*), sharpsnout seabream (*Diplodus puntazzo), common pandora (Pagellus erythrinus) and common dentex (Dentex dentex).*

Morbidity and mortality rates: Variability among cases. Generally, low daily mortality (0.5-1%). Cumulative mortality during outbreaks may reach 20%. Long term, cumulative mortality of affected fish may reach 80%.

Transmission: Horizontal

Factors (environmental, others) for disease outbreak: Outbreaks often occur at seawater temperature > 21^{o}C. Also, stress induced; from sudden deterioration of environmental conditions or transportation of fish.

Clinical signs

Lethargy and loss of appetite. Icteric (yellowish) appearance and internal haemorrhages in the case of *A. veronii* bv. *sobria.* Darkening of fish colouration, haemorrhagic spots and/or ulcerative lesions. Internally, enlargement of the spleen is common among different *Aeromonas* spp. infections. Multiple abscesses on spleen, liver and kidney in fish affected by *A. veronii* bv. *sobria and A. salmonicida* subsp. *masoucida/ achromogenes*.

Samples to be collected for diagnostics

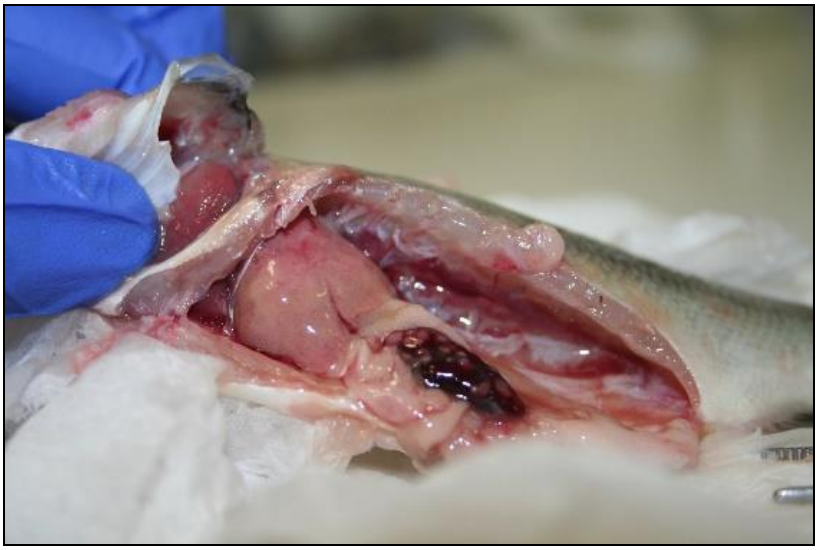
Moribund whole fish. Target organs for microbiology/histology: the kidney and spleen.

Presumptive diagnostics analysis

Disease signs and/or mortality of fish vaccinated for vibriosis and photobacteriosis. History of elevated water temperature or stress factors. Low daily or sudden high mortality. Splenomegaly.
Bacterial cultures fully grown on TSA 2% NaCl at 48h, 25^{o}C. No growth on mediums supplemented with 4% NaCl. Resistance to the vibriostatic agent O/129 and ampicillin. Generally, no or limited growth on TCBS. Biochemical diagnostic kits may identify up to genus level.

Confirmatory diagnostic analysis

PCR (ISR, GCAT, *gyr*B, *rpo*D)



Enlarged spleen with multiple abscesses, *A. veronii*-European seabass

Summary sheet for *Mycobacterium* infections

A. Le Breton[1]
[1] VET'EAU, Grenade sur Garonne, France.

Aetiological agent/s

Infections induced by a group of species belonging to the genus *Mycobacterium* including *Mycobacterium marinum, Mycobacterium chelonae, Mycobacterium fortuitum.*

Epidemiology

Host/s (species; age): Seabass (*Dicentrarchux labrax*), meagre (*Argyrosomus regius*), seabream (*Sparus aurata).* Chronic disease occurring on large size fish.

Morbidity and mortality rates: chronic mortality usually lower than 5-10%.

Transmission: horizontal transmission from fish to fish through skin injuries and digestive track or from the environment, water and related biofilm being the natural reservoir of the pathogen. Vertical transmission is reported in some species but not documented in aquaculture fish.

Factors (environmental, others) for disease outbreak: Temperature dependent above 16°C

Factors influencing the occurrence of skin or intestinal micro lesions.

Zoonotic disease

Clinical signs

Chronic progressive disease. Fish becomes lethargic and melanic with no or weak external clinical signs including scale losses, dermal ulceration or pseudo tumours (meagre), distended abdomen with ascitic liquid. Internally, frequent splenomegaly with development of grey / white granuloma on the spleen, liver and posterior kidney.
Frequent occurrence of asymptomatic carrier.

Samples to be collected for diagnostics

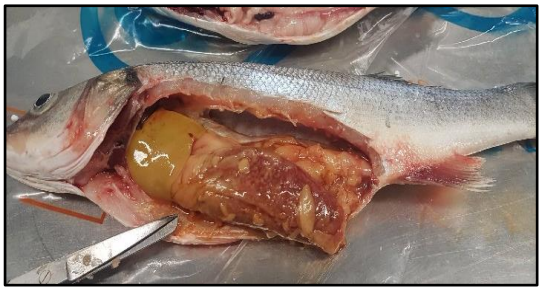
Moribund whole fish or target organs showing macroscopic pathological modifications (spleen, posterior kidney, liver, dermal lesions).

Presumptive diagnostics analysis

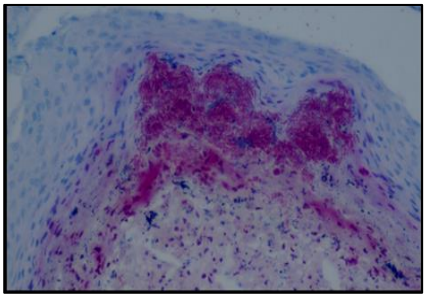
Observation of the tuberculi in target organs and on fresh smears from organs. Detection of the acid fast bacteria on organ prints stained by Zielh-Neelsen acid-fast stain, rapid Quick TB stain or Carbol-fushin stain.

Confirmatory diagnosis analysis

Isolation and identification of the bacterial strain by cultivation on standard *Mycobacterium* media (homogenates of infected tissue). Identification of the strain by mass spectrometry and 16S RNA sequencing.
Detection of the pathogen by PCR.



Splenomegaly in seabas



Acid fast bacteria (x 400 – Ziehl-Nielsen)

Annex 2. Contacts

Contacts of reference laboratories

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Annex 3. Sample request form (Chapter 2.3.)

Company Logo

ANALYTICAL REQUEST FORM

COMPANY INFORMATION

Company Name:	Street address:
Type:	City:
Phone:	Country:
Fax:	State:
Email:	Zip:

SAMPLE DETAILS

Sampling date:				Sampling Contact:			
Type:	Fish specimen ☐	Blood/Plasma ☐	Organs ☐	Other ☐			
Species:		Age:		Weight:		Number:	
Sample preservation	Ambient T°C ☐	Refrigerated ☐	Frozen ☐	RNA later ☐			
Test requested:				Method requested:			

Comments (other relevant information: clinical signs, mortality, treatments...)

.....

.....

.....

.....

Date:

Signature:

(Analysis request forms must be signed and dated to initiate testing)

CIHEAM

Centre International de Hautes Etudes Agronomiques Méditerranéennes

International Centre for Advanced Mediterranean Agronomic Studies

OPTIONS
méditerranéennes

SERIES B: Studies and Research
2020 - Number 75

Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture

Edited by:
Snježana Zrnčić

MedAID (Mediterranean Aquaculture Integrated Development) is a European H2020 Research and Innovation Project that started in 2017. The main objective was to increase the overall competitiveness and sustainability of the whole value chain of the Mediterranean marine aquaculture sector by improving its technical and business performance and by shifting to a sustainable market-oriented approach with a more positive social and consumer perception.

In the field of disease control and welfare MedAID aims to provide essential components for a better health and welfare management system for the Mediterranean marine aquaculture industry overall and at company level. One of the gaps addressed by MedAID was the lack of reference methodologies for disease diagnostics for European seabass and Gilthead seabream, the main species produced in Mediterranean marine fish farming. The present Diagnostic Manual was conceived in the form of up-to-date guidelines providing current methodologies for a harmonised approach to the health challenges due to viral and bacterial pathogens in the farming of seabass and seabream.

CIHEAM ZARAGOZA

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 727315.

ISBN: 978-2-85352-596-1
ISSN: 1016-1228

OPTIONS méditerranéennes