7. Introduction to bacterial diseases

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in

Zrncic S. (ed.).
Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture

Zaragoza : CIHEAM
Options Méditerranéennes : Série B. Etudes et Recherches; n. 75

2020
pages 63-66

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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^{o}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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