8. Vibrio anguillarum

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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).

References

Austin B. and Austin D.A., 2013. Bacterial fish pathogens: diseases of farmed and wild fish. Fifth Edition. Springer-Praxis Publishing, Chichester, U.K.

Buller N.B., 2014. Bacteria and fungi from fish and other aquatic animals. A Practical Identification Manual, 2nd edition. CABI Publishing (UK).

CLSI, 2011. Performance standards for antimicrobial susceptibility testing; 21st Informational Supplement M100-S21, Vol 31, No. 1. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI, 2014. Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline. CLSI document VET04-A2. Wayne, PA: Clinical and Laboratory Standards Institute.

Hong G.E., Kim D.G., Bae J.Y., Ahn S.H., Bai S.C., Kong I.S., 2007. Species-specific PCR detection of the pathogen *Vibrio anguillarum*, using the amiB gene, which encodes N-acetylmuramoyl-L-alanine amidase. *FEMS Microbiological Letters*, 269, 201-206.

Midlyng P., Bleie H., Helgason S., Janson E., Larsen J.L., Olesen N.J., Jørgen N., Olsen A.B., Vennerstrøm, P., 2000. Nordic manual for the surveillance and diagnosis of infectious diseases in farmed salmonids. Copenhagen, Nordic Council of Ministers, 100 pp.

Pascual J., Macián M.C., Arahal D.R., Garay E., Pujalte M.J., 2010. Multilocus sequence analysis of the central clade of the genus Vibrio by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes. *International Journal of Systematic and Evolutionary Microbiology*, 60, 154–65. https://doi.org/10.1099/ijs.0.010702-0

Sawabe T., Kita-Tsukamoto K., Thompson F.L., 2007. Inferring the Evolutionary History of *Vibrios* by Means of Multilocus Sequence Analysis. *Journal of Bacteriology*, 189, 7932–7936.

Sorensen U.B. and Larsen J.L., 1986. Serotyping of *Vibrio anguillarum*. *Applied and Environmental Microbiology*, 51, 593-597.

Whitman K.A., Mac Nair N., 2001. Finfish and shellfish Bacteriology Manual. Fish Health Atlantic Veterinary College.

Photos

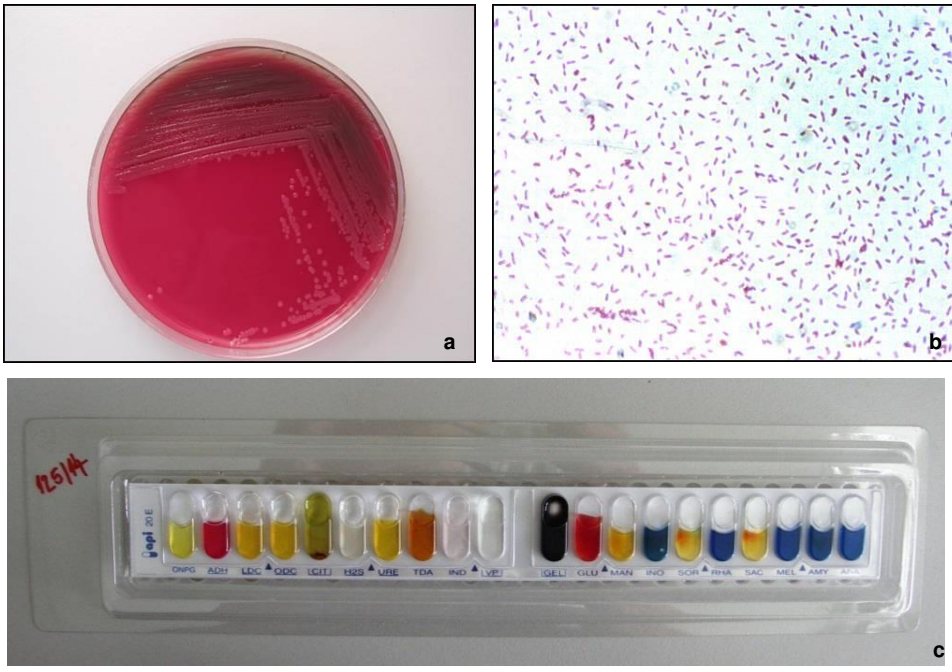

Fig. *8.1.* a) *Vibrio anguillarum* growth on blood agar with greyish-whitish, 1-2 mm, circular colonies; b) Gram stained smears of pure colony reveals Gram-negative straight and/or curved rods; c) most frequent *V. anugillarum* results of API®20E™ after 24 h.

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.