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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 damsela*, 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 anguillicaudatus*) 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°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 °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. *masoucidal/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°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 H₂S 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°C while the psychrophilic *A. salmonicida* presents optimum growth at 22-25° and does not grow at 37°C.

Aeromonads are Gram-negative, facultative anaerobic, short rods (0.3–1.0 x 1.0–3.5 μ m). They generally appear as single cells but they also form short chains. Most strains 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 μ g) 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'- GGTTCTTTTCGCCTTTCCT -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 σ^{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	TTGTCCGGGTTGTA CTGTC
rpoD	rpoD 70F	280–323	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGG GNACNGT
	rpoD 70Fs	280-302	ACGACTGACCCGGTACGCATGTA
	rpoD 70Fs1	740-757	GTCAATTCGCGCTGATGC
	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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Photos



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

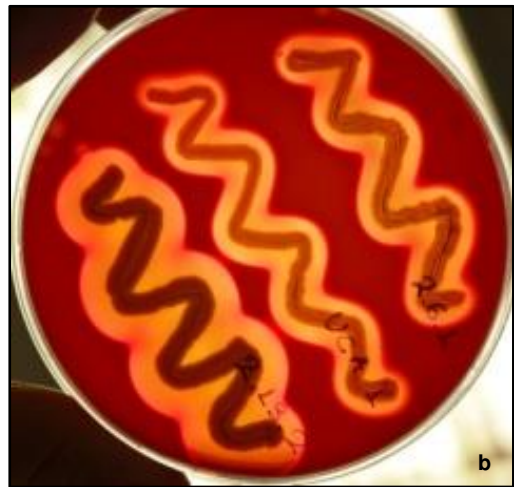
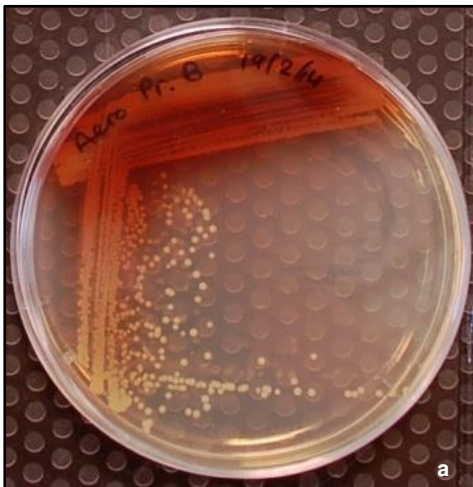


Fig. 12.2. a) Brown pigment secreted from *A. veronii* bv. *sobria*; b) Haemolytic activity of *A. veronii* bv. *sobria* using fish blood