Chapter 3.5.1 Aeromoniasis

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Abstract

The genus *Aeromonas* comprises of some of the most important fish bacterial pathogens that affect aquaculture worldwide. Several species other than *A. salmonicida*, the causative agent of furunculosis in salmonids have been responsible for severe losses both in freshwater and marine aquaculture. Aeromonads have a big repertoire of virulence factors that contribute to their pathogenicity. In this chapter, we review the recent developments in the study of those virulence factors, and we describe the pathologies caused in fish focusing on the Mediterranean aquaculture.

Keywords
mesophilic aeromonads, Mediterranean, European seabass, virulence factors, infection

- Chapter starts here -

3.5.1.1 Overview

Aeromonads are Gram-negative, rod-shaped, facultative anaerobic, non-spore forming bacteria (0.3–1.0 x 1.0–3.5 μm), spread worldwide in the aquatic environment (Martin-Carnahan and Joseph, 2005). Most strains are motile by a single polar flagellum but also peritrichous flagella may be observed as well as non-motile strains. They are basically distinguished from Enterobacteria by the oxidase positive reaction and from *Vibrionaceae* by the ability to grow in media not supplemented with salt, being resistant to the vibriostatic agent 2,4-diamino-6,7-diisopropyl-pteridine (O129) and unable to grow on TCBS agar.

Aeromonads can be found in fresh, estuarine and marine waters and are also associated as microbiota or pathogens to cold- and warm- blooded animals (Janda and Abbott 2010). The psychrophilic (optimum growth at 22-25° – no growth at 37°C), non-motile, pigment-producing *A. salmonicida* subsp. *salmonicida* is the typical etiologic agent of furunculosis in salmonids. The rest of the *A. salmonicida* subspecies are considered "atypical" *A. salmonicida* strains related to diseases that affect non-salmonids and warm-water fish (Austin and Austin, 2012; Beaz-Hidalgo and Figueras, 2012; Menanteau-Ledouble *et al.*, 2016). The mesophilic group of species (grow well at 35-37°C) is generally represented by *A. hydrophila*, which is a widely known fish pathogen causing ulcerative, hemorrhagic and septicemic infections mainly in freshwater fish like tilapias, carps, goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*) 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).

In the 2nd Edition of Bergey's Manual of Systematic Bacteriology the genus comprises of 14 species namely: *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 including processes of description of new species, proposals for reclassifications (Beaz-Hidalgo *et al.*, 2013) nominations (Miñana-Galbis *et al.*, 2010) and synonymizations (Huys *et al.*, 2005). To date, among the above mentioned species, *A. hydrophila* includes two subspecies (*hydrophila* and *ranae*) (Huys *et al.*, 2003), *A. salmonicida* five subspecies (*salmonicida, achromogenes, masoucida, pectinolytica* and *smithia*) and *A. veronii* two biovarieties (*veronii* and *sobria*).

New species that have been described in literature are: *A. molluscorum* (Miñana-Galbis *et al.*, 2004), *A. simiae* (Harf-Monteil *et al.*, 2004), *A. bivalvium* (Minana-Galbis, Farfan, Fuste, and Loren, 2007), *A. tecta* (Demarta *et al.*, 2008), *A. piscicola* (Beaz-Hidalgo *et al.*, 2009)*, A. fluvialis* (Alperi *et al.*, 2010b), *A. sanarellii* and *A. taiwanensis* (Alperi *et al.*, 2010a), *A. rivuli* (Figueras *et al.*, 2011), *A. australiensis* (Aravena-Roman et al., 2013), *A. cavernicola* (Martinez-Murcia *et al.*, 2013), *A. dhakensis* (Beaz-Hidalgo *et al.*, 2013), *A. aquatica, A. finlandensis* and *A. lacus* (Beaz-Hidalgo *et al.*, 2015), *A. rivipollensis* (Marti and Balcazar, 2015), *A. lusitana* (Martinez-Murcia *et al.*, 2016), *A. intestinalis, A. enterica, A. crassostreae* and *A. aquatilis* (Figueras *et al.*, 2017) while *A. sharmana* has been excluded from the genus (Martinez-Murcia, Figueras, Saavedra, and Stackebrandt, 2007).

3.5.1.2 Virulence and Pathogenicity

Until today, the pathogenesis of *Aeromonas* spp. has not been fully explained and some phenomena can even be strain-specific. Pathogenic or virulence factors refer to the proteins that participate in the disease-mediating mechanisms. A large variety of virulence factors has been described and their roles in pathogenicity are important. They are involved in locomotion, adhesion, protection against immune system, promotion of host-cell lysis, and interbacterial competition. This group of factors includes toxins, enzymes that cause cell damage, secretion systems, fimbriae, flagella, a capsule, lipopolysaccharides, iron-binding proteins, biofilm-formation proteins and quorum-sensing systems (Beaz-Hidalgo and Figueras, 2013; Janda and Abbott, 2010; Tomás, 2012). Such a variety of factors is observed due to the fact that the aeromonads can be found in multiple infection sites. Some virulence factors are used for local action such as the injection of toxins through the type III or type IV secretion systems, others for distant targets or the extracellular milieu (Leggett *et al.*, 2012; Teunis and Figueras, 2016). The usual procedure to characterize the pathogens' virulence factors is the PCR-based detection (Hossain *et al.*, 2018; Igbinosa and Okoh, 2013; Körkoca *et al.*, 2014; Li *et al.*, 2011; Nawaz *et al.*, 2010; Silva *et al.*, 2017). An evaluation of the method in twenty-seven species of the genus has been published recently (Talagrand-Reboul *et al.*, 2018). Nowadays, the virulence and antigenic profile can be generated from continuously enriched whole-genome-sequencing data.

3.5.1.2.1 Structural virulent components

Adhesins are the structures that play a key role during the colonization in the first steps of the host-bacteria interaction. Aeromonads can express two flagellar systems, the polar and the lateral. Flagella protrude from the cell membrane. The filament is the external part of the flagellum and the basal body is the internal. There are around fifty genes involved in the synthesis of the flagellar proteins, there is a strict transcriptional regulation and they are highly conserved among flagellated species (Liu and Ochman, 2007; Lowry *et al.*, 2014). All motile species bear a single polar flagellum used mainly for swimming (Canals *et al.*, 2006; Tomás, 2012). The multiple lateral flagella are used for swarming motility, adhering better to surfaces and in forming the biofilm (Gavin *et al.*, 2003; Lowry *et al.*, 2014). The key genes to both types of flagella have been studied in the *Aeromonas* species (Beaz-Hidalgo and Figueras, 2013; Gonçalves Pessoa *et al.*, 2019; Janda and Abbott, 2010). In *A. hydrophila*, forty-five genes have been linked to the formation of the polar flagellum and thirty-eight to the lateral flagella (Canals *et al.*, 2006) and their correlation to virulence has been established. Mutations in genes involved in the biosynthesis and motility like the *flgC* and *flgE* (Qin *et al.*, 2016), *flgE, flgN, flhA, fliJ, flmB, lafK*, and *maf-5* (Jiang *et al.*, 2015) reduced the ability of the bacteria to adhere to the host mucus. In addition, flagellar proteins are good potential targets to produce vaccines through reverse vaccinology and the *A. hydrophila*'s *flgK* protein has been tested as a vaccine candidate in catfish (Yeh and Klesius, 2011).

The pili are small structures on the surface of the bacteria that are responsible for cell adhesion. There are four types of pili described in the genus *Aeromonas*. Type IV pili, described as long-wavy surface structures, are mostly associated with virulence (Austin and Austin, 2012; Lowry *et al.*, 2014). The type IV pili comprise the Tap, Flp and Bfp pili. The genes of the Tap cluster code pili-forming and securing subunits along with proteins related to pili maturation (Boyd *et al.*, 2008) and they are generally found in aeromonads (Barnett and Kirov, 1999). The type IV pili of *A. salmonicida* are the Tap pili which are polar and the Flp which are peritrichous. Mutations in these structures made no vast differences in the pathogenicity compared to the wild-type bacteria in *S. salar* infection trials (Boyd *et al.*, 2008). An important gene for the early stages of infection is the *bfp* (bundle-forming pili) or *msh* gene, which shows sequence homology with the mannose-sensitive haemagglutinin (MSHA) protein of the *Vibrio cholera* (Lowry *et al.*, 2014). The bundle-forming pilus is essential for the adherence to solid surfaces and biofilm formation in *A. veronii* bv. *sobria* (Hadi *et al.*, 2012) and in *A. hydrophila* (Qin *et al.*, 2014).

The outer membrane proteins (OMPs) also have virulent features as they participate in crucial physiological processes of the bacterium. In the outer layer of the bacterial cell many types of proteins are found: A-proteins, porins, protein transporters and receptors for nutrients. The VapA protein, or surface layer protein, is the most abundant (Ebanks *et al.*, 2005). It is shown that proteins of the outer layer of the cell act as adhesins in the host cell (Lowry *et al.*, 2014). OMPs, like the PepO protein which is a metalloendopeptidase, are shown to contribute to the formation of ulcers (Abolghait *et al.*, 2010). Also, they play a role in the induction of inflammatory response (Romero *et al.*, 2016). OMPs are vaccine candidates and subunit vaccines based on those proteins can provide protection against aeromonads (Khushiramani *et al.*, 2012; Vazquez-Juarez *et al.*, 2005).

As another component of the outer membrane, the lipopolysaccharide (LPS) constitutes trigger molecules for the immune system of the host (Beaz-Hidalgo and Figueras, 2013). Three domains make up the LPS, the O-antigen (O-polysaccharide), the core oligosaccharide and the lipid A. The O-antigen is particularly involved in the inflammatory induction and defense of the bacteria against serum complement killing and phagocytosis (Tomás, 2012). The composition of this polymer has one to six repeating units of sugars, which explains the variability of the serogroups (Lowry *et al.*, 2014; Tomás, 2012). The structural and chemical variants in the O-antigen within the genus have been suspected to play a role in the virulence of the strains (Turska-Szewczuk *et al.*, 2010).

The outer membrane is coated by the capsule (CPS), a highly hydrated structure composed of polysaccharides. The composition of the CPS may vary among species (Lowry *et al.*, 2014) and the polysaccharide structure depends on the culturing method (Tomás, 2012). It functions as a first-line shield against immunological response and it is crucial for the survival of the bacteria. It provides protection against complement activation, phagocytosis, as well as covering of the wall components (Merino and Tomás, 2015). The genes in *Aeromonas hydrophila* that contribute to the formation of the structure are grouped together in a cluster (Aguilar *et al.*, 1999; Zhang *et al.*, 2002).

The aeromonads produce a biofilm, that contains microorganisms within an extracellular polymer structure consisted of proteins, polysaccharides and DNA molecules (Dunne, 2002). Biofilm formation gives the bacterial population and polymicrobic communities a surviving advantage through acquisition of nutrients (Dunne, 2002), regulation of the cell density (Janda and Abbott 2010), protection against antimicrobial molecules and participating in the early stages of infection (Dias *et al.*, 2018; Igbinosa *et al.*, 2017). The flagellar genes, polar and lateral are definitely involved with biofilm formation (Gavín *et al.*, 2002; Kirov *et al.*, 2004; Santos *et al.*, 2011). The pili have been also linked with biofilm in *Aeromonas* spp. (Hadi *et al.*, 2012; Qin *et al.*, 2014).

3.5.1.2.2 Extracellular Products

Aeromonads secrete exotoxins and extracellular proteins such as aerolysins, hemolysins, amylases, chitinases, elastases, gelatinases and proteases (Austin and Austin, 2012; Igbinosa and Okoh, 2013; Janda and Abbott, 2010). A lot of attention has been devoted mainly to the hemolytic enzymes and related genes (*aerA, act, hlyA*) as they cause the main clinical signs of the disease. Aerolysin causes total osmotic lysis of blood cells as a cytotoxic pore-forming enzyme and it is one of the most prominent virulence factors of aeromonads closely linked to the pathogenesis of the disease (Ran *et al.*, 2018). The rest of the secreted proteins are cytotonic enterotoxins (*alt, ast*), that morphologically change but do not cause target-cell death, lipases (*lip, gcat, lipH3, pla, alp-1*), serine protease (*ser*), nuclease (*exu*), elastase (*ahyB*) and Shiga-toxins (*stx1, stx2*) (Talagrand-Reboul *et al.*, 2018).

3.5.1.2.3 Secretion Systems

Out of the six described secretion systems in the Gram negative bacteria, four are found in aeromonads, the type II, III, IV and VI (Beaz-Hidalgo and Figueras, 2013). The type II secretion system (T2SS) is commonly found in the *Aeromonas* spp. and promotes the secretion of toxins and enzymes through the cell envelop. It is thought to be involved in the induction of cell lysis (Sandkvist, 2001). It contributes to the assemblage and transport in the outer membrane of the type IV pili (Marden, 2016). It is formed mainly out of the *ExeA-ExeB* complex and the secretin *ExeD* (Vanderlinde *et al.*, 2014).

The type III secretion system (T3SS) is the system more closely connected to the virulence of the aeromonads. It is considered a nano-injection system being assembled and regulated by approximately forty proteins. It transports proteins inside the cytoplasm of the target cells and is a vital system of Gram-negative bacteria for inducing infection (Chacón *et al.*, 2004; Diepold and Armitage, 2015; Löwer and Schneider, 2009; Pang *et al.*, 2015). There is a large prevalence of the T3SS in both environmental and clinical strains of the species *A. veronii* (Silver and Graf, 2009). *A. veronii* strains appeared to have the highest cytotoxic activity compared to *A. caviae* and *A. hydrophila* (Krzymińska *et al.*, 2012). Moreover, in a symbiotic relationship with a host, the T3SS increases the fitness of the bacteria, helping the microorganism to avoid phagocytosis during colonization of the digestive tract (Puhar and Sansonetti, 2014). A genetically manipulated *A. veronii* with a non-functional T3SS showed both lower pathogenicity and was not successful as a symbiont, due to failure to colonize the epithelium (Silver *et al.*, 2007).

The type VI secretion system (T6SS) causes intraspecies toxicity and tilts the balance in the microbiome. It is a tool for controlling host responses and outcompeting other bacteria of the gut (Logan *et al.*, 2018). The genes of the T6SS may or may not be always present in the environmental or pathogenic strains of the *A. veronii* and this appears to be the case also for *A. sobria* (Gauthier *et al.*, 2017). Even if there is not a T6SS gene cluster there are multiple homologous copies of the *Hcp* and *VrgG*, which are effectors and structural elements of the secretion system (Russell *et al.*, 2014). These proteins influence the motility, the protease production and the biofilm formation (Sha *et al.*, 2013).

3.5.1.2.4 Iron acquisition mechanisms

The availability of iron in the host environment in a form that bacteria can readily use for their metabolism is limited, therefore it is extremely important for the bacteria to develop mechanisms for efficient iron acquisition and for the host to block them (Wilson *et al.*, 2016). Siderophores are small units that turn ferric ions to ferrous and are involved in their transport (Sah and Singh, 2015). The siderophores produced by aeromonads most of the times are the enterobactin and amonabactin (Janda and Abbott 2010). Nevertheless, not only siderophores bear iron-bind affinity but also heme receptors for heme utilization. The presence and form of the two systems vary among the strains (Najimi *et al.*, 2008b; Najimi *et al.*, 2009), both are very important but heme utilization genes seem to be more widely distributed in the genomes of aeromonads (Maltz *et*

al., 2015). There are ferric uptake regulators, siderophore receptors, small RNAs, two-component systems and surface signaling that contribute to the regulation of the iron uptake (Braun, 2004; Ebanks et al., 2004).

3.5.1.2.5 Quorum Sensing (QS)

Inter-bacterial communication occurs through chemical signaling that induces gene expression. The genes involved in quorum sensing molecules in aeromonads are considered to be the N-acylated homoserine lactones (AHLs) that belong in the metallo-β-lactamase superfamily. The reaction to the QS signaling favor the survival of the cell in cases of changes of the environment or intercellular antagonism. QS has been reported to play a key role in many bacterial physiological mechanisms such as conjugation, biofilm formation, exotoxin expression etc. (Beaz-Hidalgo and Figueras, 2013). In aeromonads there are mainly short acyl side chain AHLs and the signaling involves the trigger of the LuxR- LuxI system, an interacting homologue (Jangid et al., 2007). Quorum Sensing Inhibitors (QSIs) can be used as an alternative method to battle pathogens. This practice can be more environmentally friendly and healthier for the food consumption. (Kalia et al., 2019). For example, curcumin liposomes have been reported to block AHL production in A. sobria, interacting with LuxI and resulting in altering the virulence profile of the bacteria (Ding et al., 2017).

3.5.1.3 Aeromonads in the Mediterranean Marine Aquaculture

The biggest Mediterranean aquaculture facilities are located in Greece, Turkey, Spain, France, Italy, Cyprus, Malta and Egypt (Barazi-Yeroulanos, 2010). European seabass (Dicentrarchus labrax) and gilthead seabream (Sparus aurata) are the most economically important species in the area, both farmed almost exclusively in marine waters (FAO, 2018a, 2018b). Other important farmed fish species are: the European eel (Anguilla anguilla), the meagre (Argyrosomus regius), the flathead grey mullet (Mugil cephalus) and mullets in general (Barazi-Yeroulanos, 2010; FAO, 2018b). In Spain, the aquaculture sector is also active in the Atlantic Ocean producing mainly cold-water species like turbot (Scophthalmus maximus) and Atlantic salmon (Salmon salar) but also gilthead seabream. Furthermore, in the low salinity environment of the Black Sea, Turkey produces both cold- and warm- water species such as European seabass, turbot, rainbow trout and Atlantic salmon.

Major bacterial pathologies in the Mediterranean mariculture affecting both the traditional and the newly-introduced fish species are generally caused by Vibrio anguillarum and other Vibrio spp. (V. alginolyticus, V. ordalii, V. harveyi etc.) and Photobacterium damselae subsp. piscicida. Other common pathogens implicated in infections are Tenacibaculum maritimum, Mycobacterium marinum, Pseudomonas spp., Streptococcus iniae, Lactococcus garvieae etc (Colorni, 2004; Rigos and Katharios, 2009; Ghittino et al., 2003; Öztürk and Altinok, 2014; Rodgers and Furones, 1998; Toranzo et al., 2005; Vendramin et al., 2016). Aeromonad infections are not common in Mediterranean mariculture. As described previously, aeromonads are frequently found in coastal marine and brackish waters as well as in sediment (Dumontet et al., 2000; Sechi et al., 2002), and have been reported as part of the gut microbiota and/or isolated from tissues of apparently healthy marine fish (Floris et al., 2013; Pedonese et al., 2012; Scarano et al., 2018; Toranzo et al., 1993).

Moreover, even in cases that Aeromonas spp. have been isolated from diseased fish, most of published reports provide no data to support the pathogenicity of the isolates. In numerous studies, aeromonads have been isolated during routine microbial screenings mainly on farmed, diseased gilthead seabream and European seabass and sporadically from sharpsnout seabream (Diplodus puntazzo), common pandora (Pagellus erythrinus), common dentex (Dentex dentex), groupers (Epinephelus spp.), turbot, horse mackerel (Trachurus mediterraneus), meagre and flathead grey mullet (Athanassopoulou et al., 1999; Balebona et al., 1998; Colorni et al., 1981; Martino et al., 2011; Öztürk and Altinok, 2014; Soliman et al., 2011; Uzun and Ogut, 2015; Yardımcı and Timur, 2015; Yiagnisis and Athanassopoulou, 2011). In most cases they are found in low frequencies and/or in mixed infections with predominant marine pathogens of the genera Vibrio, Photobacterium, Pseudomonas, and Tenacibaculum.

3.5.1.4 Aeromonas as a primary pathogen in the Mediterranean Sea

Infections caused by aeromonads are affecting mainly the European seabass. Two incidents of mesophilic aeromonad species have been reported in the Aegean Sea, Greece. In the first case, A. hydrophila caused morbidity and low daily mortality (0.5-1%) to European seabass (150 and 330 g) and sharpsnout seabream (~45 g) (Doukas et al., 1998). Clinical signs of disease included erythema and swelling of the anus, hemorrhagic spots on the skin and internally enlargement of the organs, haemorrhages and ecchymoses. The two species were isolated from different fish farms and A. hydrophila was detected in water samples in the sharpsnout seabream farm. The disease outbreak was generally attributed to environmental and management stressors.

In the second case, A. veronii bv. sobria affected European seabass (250-400 g), leaving unaffected gilthead seabream fish reared in neighboring cages (Smyrli et al., 2017). The disease first appeared in 2008 affecting a single fish farm in central Greece, observed mainly in bigger fish approaching the commercial size (>200 g in weight). Daily mortality was also low (0.5%) but cumulative mortality reached 20% in a 4-month period. The affected fish were usually lethargic with no appetite

while later they appeared icteric. Internally, multiple abscesses were mostly found in the spleen and the kidney of affected fish (Figure 3.5.1.1). Histologically, extensive necrosis may be found in various organs (Figure 3.5.1.2). The pathogenicity of the strains isolated from the outbreak was verified by virulence tests causing 100% mortality in bath challenged (10^5 cfu/ml) seabass within 7-10 days (Smyrli *et al.*, 2017). Since then, more farms even in distant areas have been found affected while the disease expanded also to younger fish with weight lower than 50 g (Smyrli *et al.*, 2019). 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 disease outbreaks occur during the warm months of the year, when water temperature is over 21°C.

Figure 3.5.1.1 European seabass infected by *Aeromonas veronii* bv. *sobria*. Note the enlarged spleen bearing multiple abscesses, typical of the disease (Photo courtesy of Maria Smyrli).
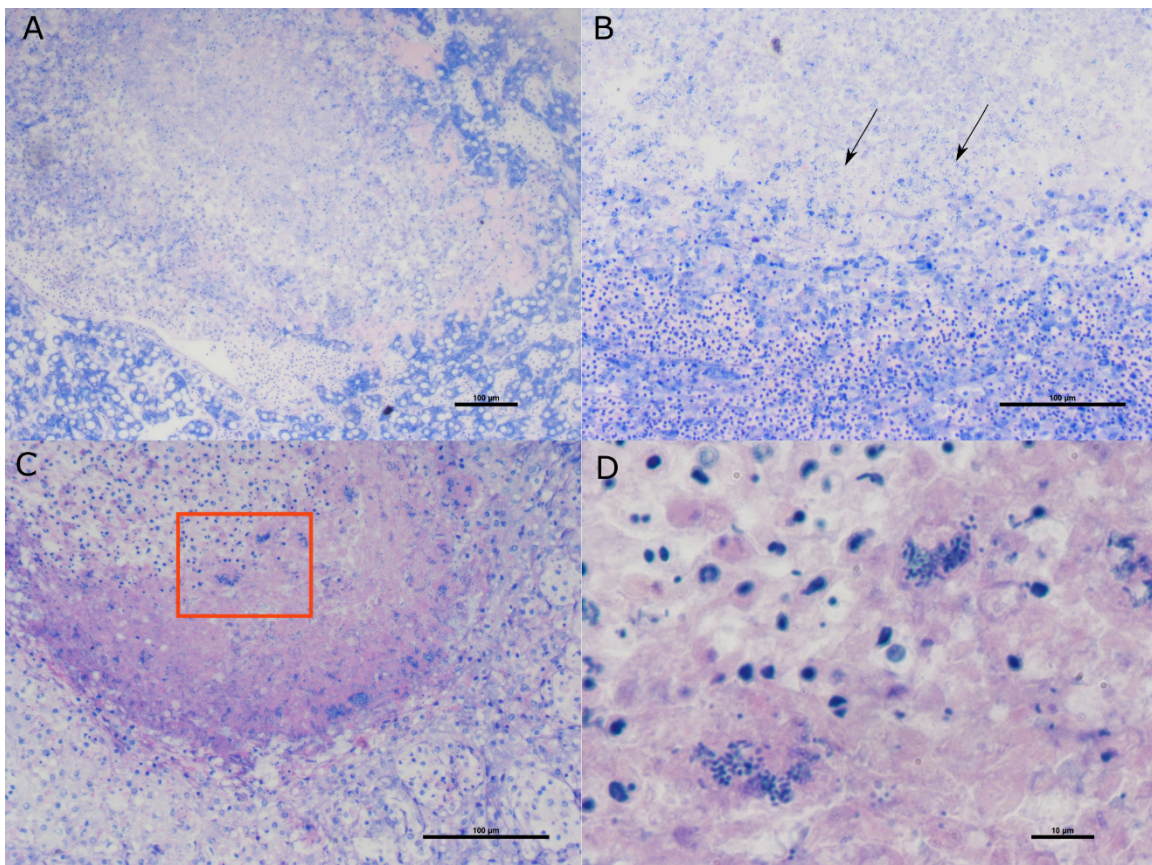
Figure 3.5.1.2 European seabass infected by *Aeromonas veronii* bv *sobria*. A. Extensive necrosis in the liver. B. Abscess in the spleen (arrows point to bacterial cells). C. Caseous necrosis in the spleen of the fish. Note the bacterial colonies (inset). D. Higher magnification of the inset showing bacterial colonies (Photo courtesy of Dr. Pantelis Katharios).

The same species, *A. veronii* bv. *sobria*, was reported as the most prevalent bacterium in diseased European seabass in the Black Sea in water temperatures ranging between 20-26°C (Uzun and Ogut, 2015). The bacterium was isolated alone or in mixed-infections with *Ph. damselae* subsp. *damselae* and *Vibrio* spp. from fish exhibiting darkening, exophthalmia, erratic swimming, abdominal swelling and ulcerative lesions on operculum and mouth. The farms mentioned in the last incident produced also rainbow trout, with the production of the two fish species being diverged seasonally.

Disease outbreaks caused by *A. veronii* bv *sobria* 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 striped snakehead *(Channa striata)* 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). As an opportunistic pathogen of both freshwater and marine fish the species increasingly gains importance for the aquaculture industry.

Psychrophilic aeromonads have also been reported to affect European seabass. In the Black Sea, an atypical infection caused by *A. salmonicida* subsp. *masoucida/achromogenes* affected European seabass (5 and 100 g) (Karatas *et al.*, 2005). Cumulative mortality during the outbreak (2-month period) was as high as 20%. Petechial haemorrhages externally, and white lesions on the internal organs and enlargement of the spleen were observed. Both mesophilic and psychrophilic aeromonads have been recorded in other farmed species in the area such as rainbow trout, Atlantic salmon and turbot (Öztürk and Altinok, 2014).

Recently, a case of furunculosis (*A. salmonicida* subsp. *salmonicida*) in European seabass was described in the Mediterranean coasts of Spain (Fernández-Álvarez *et al.*, 2016). The disease affected juvenile fish (9 g) causing cumulative mortality up to 3.8% in a 2-month period. Initially, fish were asymptomatic but progressively, ulcerative lesions appeared in the skin and muscles. Internally the only clinical sign recorded was enlargement of the spleen. Pathogenicity tests showed that the strain from the seabass was also virulent for turbot and trout, drawing attention to potential risks of transmission between fish species farmed in the same location and/or wild populations. Similarly, pathogenicity tests with "marine" isolates of *A. hydrophila* showed that they were able to cause moderate to high mortality on Nile tilapia (*O. niloticus*) (El-Barbary, 2010; El-Barbary, 2017).

In another case, in Gran Canarias (Atlantic Ocean), *A. salmonicida* subsp. *salmonicida* caused an hyperacute disease to gilthead seabream (1 g) after transportation of the fish to the on-growing facility (Real *et al.*, 1994). Mortality reached to 6-7% the first 3 days. Moribund fish exhibited pale gills, dark coloration of the skin and petechial hemorrhages on mouth and gills. The presence of *A. salmonicida* subsp. *salmonicida* has been repeatedly reported in N/W Spain affecting turbot (Coscelli *et al.*, 2014; Lago *et al.*, 2012; Najimi *et al.*, 2008a; Toranzo and Barja, 1992; Toranzo *et al.*, 2005). Furunculosis was reported to cause cumulative mortality up to 15% in turbot (800 g) within a month (Toranzo and Barja, 1992) while in other cases, pathogenicity data is not available or in the case of granulomatous dermatitis, mortality or other clinical signs of disease or damage in the internal organs were not recorded (Coscelli *et al.*, 2014).

In the above cases the psychrophilic *A. salmonicida* generally occurred in temperatures ranging between 15-17°C (Fernández-Álvarez *et al.*, 2016; Karatas *et al.*, 2005; Real *et al.*, 1994; Toranzo and Barja, 1992). The mesophilic *A. hydrophila* was reported in decreased temperatures between 13-15°C related to sudden events affecting the water parameters (Doukas *et al.*, 1998), while *A. veronii* bv. *sobria* occurred between 20-26°C (Smyrli *et al.*, 2017; Uzun and Ogut, 2015). The cold-water farmed turbot was only affected by *A. salmonicida* while the eurythermic European seabass was susceptible to mesophilic species as well. Mortality was generally low but cumulative mortality during the outbreaks caused significant losses. Aeromonads were recently included in the list of pathogens with socio-economic impact on seabass and seabream aquaculture in Western Mediterranean area (Vendramin *et al.*, 2016).

3.5.1.5 Aeromonads Isolation and Identification

Aeromonads grow well on common culture media like trypticase soy agar (TSA), brain-heart infusion (BHI) and blood agar supplemented with 0.5-2% NaCl after 24-48h incubation at 22-25°C. Mesophilic species grow well in temperatures up to 35-37°C while the psychrophilic *A. salmonicida* presents optimum at 22-25°C and does not grow at 37°C. Selective media like *Aeromonas* Isolation Agar (AIA), starch-ampicillin agar and MacConkey agar, dextrin agar, all of them supplemented with ampicillin are also recommended for isolation. Media supplemented with higher salt concentrations (4-6% NaCl) can be used to select for possible aeromonad representatives, as they exhibit significantly less or no growth in NaCl concentrations over 4%. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar can also be informative as most strains do not grow or they present limited growth on it. Finally, with infrequent exceptions aeromonads generally appear resistant to the

vibriostatic agent 2,4-diamino-6,7-diisopropyl-pteridine (O129). Phenotypic tests often fail to identify aeromonads to species level and miniaturized commercial identification systems like API20E, API 20NE and BIOLOG GENIII Microplate present constraints in identification of fish pathogens and aeromonads specifically (Austin, 2011; Beaz-Hidalgo and Figueras, 2012; Santos et al., 1993). Thus, the use of phenotypic tests is proposed only for identification in genus level. Also, a DNA probe targeting a fragment of glycerophospholipid-cholesterol acyltransferase *GCAT* gene has been used to identify aeromonads in genus level (Chacon *et al.*, 2002). Finally, sequencing of housekeeping genes is considered essential for identification to species level (Beaz-Hidalgo and Figueras, 2012). The *rpoD* or *gyrB* genes are recommended for fast and reliable identification.

3.5.1.6 Disease Control

The *Aeromonas* spp. infections are usually treated with antibiotics. In the Mediterranean and the Black Sea *Aeromonas* spp. strains isolated from fish farms have been generally found to be susceptible to most of the registered commercially available aquaculture antibiotics (oxytetracycline, sulphamethoxazole/trimethoprim, oxolinic acid, flumequine and florfenicol) while strains exhibiting multiple antibiotic resistance were also detected (Scarano *et al.*, 2018; Smyrli *et al.*, 2017; Uzun and Ogut, 2015). Similar susceptibility and resistance profile was also found in aeromonads from various aquatic sources in Asia (Odeyemi and Ahmad, 2017). Prevention is preferable to treatment, and to this direction, good management practice is of imperative importance. Keeping fish in low densities at good hygiene conditions and lowering stress due to excessive handling will decrease both the likelihood of infection but also its intensity when it occurs. Currently, there are no commercially available vaccines for most of the pathogenic *Aeromonas* species (non-*salmonicida*) and aeromoniasis can be prevented with the use of autogenous vaccines.

3.5.1.7 References

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