



Molecular identification, histopathological analysis and immunohistochemical characterization of non-pigmented *Aeromonas salmonicida* subsp. *salmonicida* in *Mugil carinatus* (Valenciennes, 1836)

Asmaa K. Al-Mokaddem^a, Dalia A. Abdel-moneam^b, Reham A. Ibrahim^c, Mona Saleh^d, Mohamed Shaalan^{a,*,1}

^a Department of Pathology, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt

^b Department of Aquatic Animal Medicine and Management, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt

^c Microbiology Lab, Marine Environmental Division, National Institute of Oceanography and Fisheries (NIOF), Egypt

^d Clinical Division of Fish Medicine, University of Veterinary Medicine Vienna, 1210 Vienna, Austria

ARTICLE INFO

Keywords:

Aeromonas salmonicida
Mugil carinatus
Immunohistochemistry
DNA sequencing
Suez gulf

ABSTRACT

Aeromonas salmonicida was isolated for the first time from *Mugil carinatus* fish sampled from Suez Gulf, Suez governorate, Egypt. Fish samples were identified bacteriologically, molecularly and histopathologically. Biochemical identification of *A. salmonicida* was performed using API 20 NE kits. The universal 16 S rRNA gene of *Aeromonas* species was used for the molecular identification of retrieved isolates by producing amplicons at 461 bp. Serine protease (*ser*) and aerolysin-like protein (*act*) virulent genes were detected in retrieved isolates at 211 bp and 232 bp, respectively. DNA sequence analysis showed high identity with *A. salmonicida* subsp. *salmonicida* strains listed in the GenBank database. Histopathological analysis revealed localization of basophilic bacterial colonies in liver and kidney. Congestion, hepatocellular necrosis and renal tubular necrosis were the marked pathological lesions in liver and kidney tissues of infected fish. An immunohistochemistry technique was used for confirming the presence of *A. salmonicida* antigen. Immune positive antigen antibody reaction was observed as brown coloration in liver parenchyma and kidney sections of *M. carinatus*.

1. Introduction

The family Aeromonadaceae comprises a group of ubiquitous opportunistic bacteria, including *Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas caviae*, *Aeromonas veronii* as well as *Aeromonas salmonicida*, which has been reported to be the main causative agent of fish septicemic bacterial disease in various aquatic habitats (Austin and Austin, 2007).

A. salmonicida was first reported in salmonid fish showing abnormal skin lesions (Emmerich and Weibel, 1894). Since then, it has been isolated from wild and cultured freshwater and marine fish worldwide such as tilapia (*Oreochromis* spp.) in Oman (Alghabshi et al., 2018), farm-raised *Brachymystax lenok* (Li et al., 2014), Largemouth bronze gudgeon (*Coreius guichenoti*) (Meng et al., 2020), and crucian carp *Carassius auratus* in China (Lian et al., 2020), walking catfish (*Clarias batrachus*) in India (Thomas et al., 2013), European perch (*Perca*

fluviatilis L.) in Lithuania (Skrodenytė-Arbačiauskienė et al., 2012), and sea bass (*Dicentrarchus labrax*) in Turkey and Spain (Karatas et al., 2005; Fernández-Álvarez et al., 2016). However, it has never been reported to infect *Mugilidae* species in the wild.

A. salmonicida is categorized as either typical strains (pigment producing) including *A. salmonicida* subsp. *salmonicida* or atypical strains (non-pigment-producing) including *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *smithia*, and *A. salmonicida* subsp. *pectinolytica* (Gudmundsdóttir et al., 2003; Menanteau-Ledouble et al., 2016). However, recent literature reported the isolation of non-pigmented *A. salmonicida* subsp. *salmonicida* from Atlantic salmon in Norway and cultured crucian carp in China (Koppang et al., 2000; Lian et al., 2020).

Extensive ulcerative and hemorrhagic skin lesions that lead to high morbidity and mortality are characteristics of furunculosis, the disease caused by *A. salmonicida* subsp. *salmonicida*. The disease is associated

* Corresponding author.

E-mail address: mohamedibrahim@cu.edu.eg (M. Shaalan).

¹ 0000-0002-0741-954

with severe economic losses in the aquaculture sector (Shaalan et al., 2018a).

Mullets belonging to the Mugilidae family including *Mugil cephalus*, *Mugil carinatus*, *Liza ramada*, and *Liza aurata*, are widely distributed in the Gulf of Suez and Suez Canal marine waters; mullets are ranked 3rd in aquaculture production in Egypt (Shaalan et al., 2018b; Mehanna et al., 2019). *M. carinatus* is also referred to as *Liza carinata* or keeled mullet and mainly inhabits the Red Sea; it could reach the Eastern Mediterranean Sea through the Suez Canal (Shakman et al., 2016). *M. carinatus* represents a commercially important component of the total mullet catch at approximately 18% of the Gulf of Suez and Suez Canal fishery production during the last ten years (GAFRD, 2017).

Immunohistochemical techniques can be used study the pathogenesis of bacterial infection via in-situ identification of the bacterial antigens within tissue constituents by means of specific antigen-antibody reactions (Coscelli et al., 2014).

To the best of our knowledge, this is the first report of detection of *A. salmonicida* in *M. carinatus*. The current study aimed to achieve phenotypic characterization, molecular identification, and immunohistochemical detection of *A. salmonicida* isolated from wild *M. carinatus* fish in Egypt.

2. Materials and methods

2.1. Sampling

A total of 60 *M. carinatus* fish were randomly collected from the Suez Gulf, Red Sea at the start of the spring season of 2019. The fish were examined on-site for the presence of any clinically relevant external lesions. Samples were taken for bacteriology, molecular biology, and histopathological analyses. All sampling procedures were conducted according to regulations of institutional animal use and care committee (IACUC), faculty of veterinary medicine, Cairo University.

2.2. Microbiological examination and phenotyping

The fish surfaces were disinfected with 70% ethyl alcohol. Kidney samples were taken and inoculated in brain heart infusion broth (LabM, Bury, UK) overnight, then streaked on tryptone soy agar (TSA) (LabM, Bury, UK) and blood agar supplemented with 5% sheep blood (LabM, Bury, UK) with the addition of 2% NaCl. Cultured plates were incubated at 29 °C for 48 h (Buller, 2004). The most common morphologically identical colonies were aseptically subcultured and purified, then Gram stained, examined for motility, and subjected to the oxidase and catalase tests. Phenotypic identification of the retrieved isolates was performed using API 20 NE kit (BioMérieux, Marcy-l'Étoile, France). Pure *Aeromonas* stock cultures were cryopreserved in brain heart infusion broth with 15% glycerol at – 20 °C for further analysis.

2.3. Molecular identification, virulence genes detection, and partial 16 S rRNA gene sequencing

DNA extraction from purified bacterial colonies was performed using PrepMan Ultra Reagent (Applied Biosystems, Massachusetts) according to the manufacturer instructions. The PCR reaction was prepared on ice

in 0.2 ml thin-walled PCR tubes containing the following: 25 µl of 2X ViRed Taq master mix (Vivantis, Malaysia), which included Taq DNA polymerase, reaction buffer, dNTPs, MgCl₂, inert red dye, and stabilizers needed for routine DNA amplification, and 2 µM of each primer (10 pmol), 4 µl of template DNA, and 17 µl of PCR-grade water to adjust the final volume to 50 µl.

The universal 16 S rRNA gene of *Aeromonas* species was used for the identification of retrieved isolates. The serine protease (*ser*) and aerolysin-like protein (*act*) genes were used to determine the virulence potential of isolated *A. salmonicida* (Table 1). Electrophoresis of amplified products was performed using 1% agarose (w/v) with Tris-acetate EDTA and ethidium bromide staining, then visualized under UV light to show the resulting bands for the 16 S rRNA gene at 461 bp. The amplified bands were sequenced using the Genetic Analyzer 3500 sequencer (Applied Biosystems), then the sequences were blasted with the National Center for Biotechnology Information (NCBI) database and registered on GenBank using BankIt.

2.4. Phylogenetic analysis

The obtained sequences were used for multiple alignments using the CLUSTALW program of the BIOEDIT version 7.2.5. The phylogenetic tree was constructed using the Neighbor-Joining approach with 1000 bootstrap resampling (Tamura et al., 2004) using MEGA X software (Kumar et al., 2018).

2.5. Histopathology and immunohistochemistry (IHC) of *A. salmonicida*

Liver and kidney samples were collected and kept in 10% neutral buffered formalin. After fixation, tissue samples were processed routinely by passage in different grades of alcohol, cleared in xylene, and embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin for light microscopy (Bancroft, 2013). Tissue slides were examined using an Olympus BX43 light microscope and images were captured using an Olympus DP-27 camera.

For immunohistochemistry, anti-*A. salmonicida* hyperimmune serum was raised in rabbits as described by Rakib et al. (2018). In brief, rabbits were subcutaneously injected with killed bacteria with Freund's complete adjuvant. After two weeks, three booster doses were given without adjuvant. Finally, blood samples were collected and centrifugated for serum separation. The serum purification protocol by precipitation using ammonium sulfate was performed as described by Page and Thorpe (1996). Purified IgG was used as the primary antibody at a dilution of 1:1000 in PBS. For the immunohistochemistry, the protocol was conducted according to Mesalam et al. (2021). Heat-induced antigen retrieval step was performed in microwave oven for 15 min using Tris-EDTA buffer (10 mM Tris-base, 1 mM EDTA solution, 0.05% Tween 20, pH 9). Then, tissue slides were washed with phosphate buffered saline (PBS). Endogenous peroxidase blocking was performed by adding 3 drops of H₂O₂ 3% (Bio-SB, CA, USA) to tissue sections and incubated for 10 min. Tissue slides were incubated with the primary antibodies (rabbit anti-*Aeromonas*, 1:1000 in PBS) overnight in a refrigerator, followed by washing with PBS and incubation with goat anti-rabbit HRP-labeled secondary antibodies (Abcam, Cambridge, UK) for 2 h at room temperature. Finally, a DAB substrate kit (Abcam, Cambridge, UK)

Table 1
Primer sequences used for detection of *A. salmonicida*.

Gene	Primer sequence	Product size (bp)	Annealing temp. (°C)	Reference
<i>Aeromonas</i> universal 16 S rRNA	CGACGATCCCTAGCTGGTCT GCCTTCGCCACCGGTAT	461 bp	65 °C	Abdel-moneam et al. (2021)
Serine protease (<i>ser</i>)	ACGGAGTGCCTCTCTCTACTCCAG CCGTTTCATCACACCGTTGTAGTCG	211 bp	64 °C	Nam and Joh (2007)
Aerolysin-like protein (<i>act</i>)	AGAAGGTGACCACCAAGAACA AACTGACATCGGCCTTGAAGTC	232 bp	55 °C	Sen and Rodgers (2004)

was used for substrate detection. Same procedure was applied on fixed tissues from *Aeromonas salmonicida*-free fish as a negative control.

3. Results

3.1. Clinical signs and postmortem lesions

No external clinical lesions were reported, except for mouth hemorrhages, which were observed in some collected fish. (Fig. 1A–B).

3.2. Microbiological examination and phenotyping

The identified purified colonies of *A. salmonicida* were retrieved from the TSA supplemented with 2% NaCl after 2 days of culturing with no pigment production. On blood agar, the purified colonies produced a beta hemolytic reaction (Fig. 2). Phenotypically, *A. salmonicida* colonies appear as gram-negative short rods. No bacterial motility was detected. The oxidase and catalase test results were positive. Arginine dihydrolase (ADH), nitrate reduction, gelatin liquefaction, and aesculin hydrolysis tests were positive, while urea production and citrate assimilation tests were negative when using the API 20 NE Kits with identification code 5555744 (Table 2).

3.3. Molecular identification, virulence genes detection and partial 16 S rRNA gene sequencing

For the *Aeromonas* universal 16 S rRNA gene, positive amplicons were successfully produced at 461 bp (Fig. 3A). Virulence genes detection through identification of serine protease (*ser*) and aerolysin-like protein (*act*), showed amplicons at 211 bp and 232 bp, respectively (Fig. 3B). The sequence analysis and NCBI blast showed that isolated bacteria matches with *A. salmonicida* strains (GenBank accession nos. MT576565.1 and MT525253.1) by 99.76% and *A. salmonicida* subsp. *salmonicida* strain by 99.52% (GenBank accession no. CP052034.1) listed on the GenBank database. The obtained sequences were published as *A. salmonicida* on GenBank under accession numbers MW741720 and MW741722. The phylogenetic analysis of *A. salmonicida* 16 S rRNA sequences were grouped with their identical sequences using Neighbor-Joining method (Fig. 4).

3.4. Histopathology and immunohistochemistry

Microscopic examination of liver sections of affected fish showed marked congestion (Fig. 5a) with hepatocellular vacuolation suggesting microvesicular steatosis in addition to extensive multifocal hepatocellular necrosis was noted (Fig. 5b), associated with the presence of blue-stained bacterial colonies within the hepatic parenchyma (Fig. 5c). IHC showed the presence of *A. salmonicida* antigen antibody response within the hepatic parenchyma (Fig. 5d).

Kidney sections exhibited congestion of renal blood vessels (Fig. 6a), and scattered hemorrhages (Fig. 5b). The brown bacterial colonies were

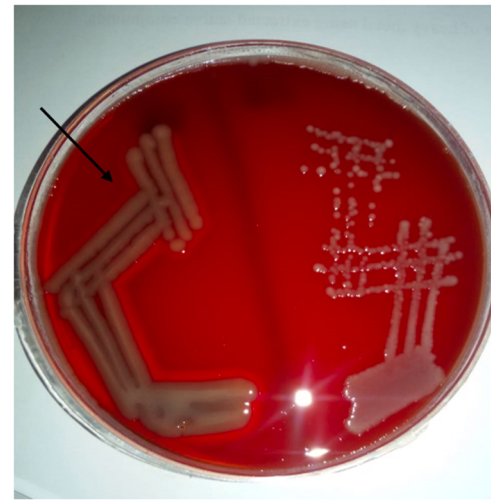


Fig. 2. Beta Hemolytic zone (on the left) by *Aeromonas salmonicida* (arrow) versus other non-hemolytic bacteria (on the right) on blood agar plate.

detected in the renal tissue by IHC (Fig. 6c,d). Negative control tissue sections from liver and kidney showed no immunoreactivity.

4. Discussion

A. salmonicida are important bacteria affecting a variety of salmonid and non-salmonid fish species (Bartkova et al., 2017). *A. salmonicida* subsp. *salmonicida* is incriminated as the causative agent for furunculosis in salmonid fish and is defined as the typical *A. salmonicida* (Cipriano and Bullock, 2001). However, there was no evidence that this bacterium could be isolated from *M. carinatus*.

In past decades, few studies have reported the isolation of *A. salmonicida* from sub-clinically infected fish with no external lesions; this may be because the majority of scientists neglect to examine apparently healthy fish and other environmental samples that may harbor *A. salmonicida* (Alghabshi et al., 2018).

In our study, no external skin lesions appeared on the infected *M. carinatus*. Similarly, no external skin lesions appeared on Atlantic salmon, turbot, European perch (*Perca fluviatilis* L.), various Omani fish species, and abalone (*Haliotis mariae*) infected with *A. salmonicida* (Koppang et al., 2000; Coscelli et al., 2014; Skrodenytė-Arbačiauskienė et al., 2010; Alghabshi et al., 2018). This may be attributed to the ability of infected fish to silently carry and transmit the disease, by vertical or horizontal dissemination of the pathogen (Austin and Austin, 2007; Bartkova et al., 2017) and from fresh to sea water without showing any typical signs of the disease (Karatas et al., 2005; Fernández-Álvarez et al., 2016). The clinical signs only appear in stressed or immunocompromised fish. Besides, the sub-acute form of the disease also has a slow onset of symptoms (Dallaire-Dufresne et al., 2014).

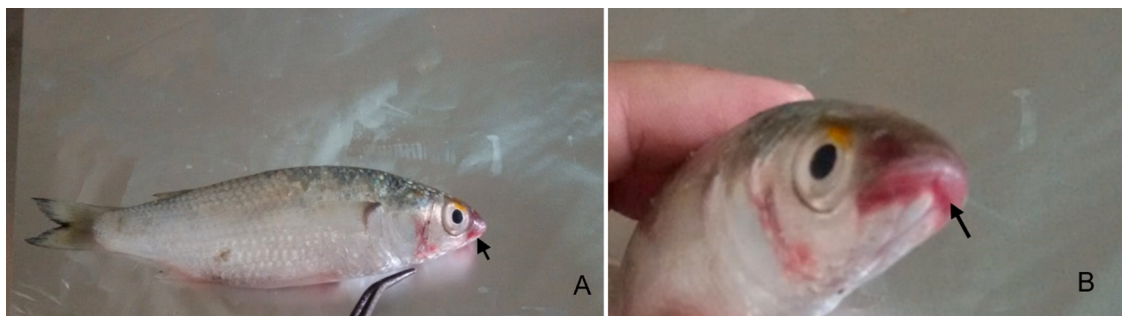


Fig. 1. *Mugil carinatus* fish showing mouth hemorrhages.

Table 2

API 20 NE kit results for isolated *A. salmonicida* and non-pigmented *A. salmonicida*. *Biochemical data were obtained from Buller, 2004. V, variable; -ve, negative reaction; +ve, positive reaction; NO₃, nitrate; TRP, indole; Glu, glucose fermentation; ADH, arginine dihydrolase; Ure, urease; Esc, aesculin; Gel, gelatin hydrolysis; Pnpg, p-nitrophenyl-b-D-galactopyranoside; Glu, glucose assimilation; Ara, L-arabinose assimilation; Mne, mannose assimilation; Man, mannitol assimilation; Nag, N-acetylglucosamine; Mal, maltose assimilation; Gnt, gluconate assimilation; Cap, caprate assimilation; Adi, adipate assimilation; Mlt, malate assimilation; Cit, citrate assimilation; Pac, phenylacetate.

Phenotypic characteristics	Non-pigmented <i>A. salmonicida</i> *	Isolated <i>A. salmonicida</i>
Gram stain	-VE	-VE
Oxidase	+VE	+VE
Catalase	+VE	+VE
Motility	-VE	-VE
Pigment production	+/-	-VE
NO ₃	+VE	+VE
TRP	-VE	-VE
Glu fermentation	+VE	+VE
ADH	+VE	+VE
Urea	-VE	-VE
Esc	V	+VE
Gel	+VE	+VE
Pnpg	-VE	-VE
Glu assimilation	+VE	+VE
Ara	+VE	+VE
Mne	V	-VE
Man	+V	+VE
Nag	+VE	+VE
Mal	+V	+VE
Gnt	+VE	+VE
Cap	-VE	-VE
Adi	-VE	-VE
Mlt	+VE	+VE
Cit	-VE	-VE
Pac	-VE	-VE

Morphologically, the cultivation of *A. salmonicida* on blood agar produces a beta hemolytic reaction (Austin et al., 1998; Fernández-Álvarez et al., 2016), while cultivation on TSA agar at 29 °C revealed the production of non-pigmented pinpointed colonies (Hirvelä-Koski, 2005; Tewari et al., 2014; Huys, 2014; Alghabshi et al., 2018). The use of pigment production as a diagnostic confirmation of *A. salmonicida* subspecies must be re-assessed, as several members of the aquatic aeromonads and pseudomonads can produce similar pigments (Altmann et al., 1992; Cipriano and Bullock, 2001).

Biochemically, the API 20 NE results identified the isolated *A. salmonicida* as *A. salmonicida* subsp. *salmonicida*, even though it was not a pigment-producing strain. This is because it fulfills all the biochemical criteria that differentiate it from atypical *A. salmonicida* subsp. *achromogenes* strains: glucose fermentation, ADH production, aesculin hydrolysis, and N-acetylglucosamine, maltose, gluconate, and malate assimilation (Wiklund et al., 1993; Karatas et al., 2005; Hirvelä-Koski, 2005; Lim and Hong, 2020).

Non-pigment-producing *A. salmonicida* subsp. *salmonicida* was previously isolated from brown trout (*Salmo trutta m. lacustris*) and sea trout (*S. trutta m. trutta*) in Finland, Atlantic salmon (*Salmo salar* L.) in Norway and crucian carp (*Carassius auratus*) in China (Wiklund et al., 1993; Koppang et al., 2000; Lian et al., 2020).

The lack of pigment production in *A. salmonicida* subsp. *salmonicida* strains may be related to a mutation or deletion in genes controlling melanin pigment production (Wiklund et al., 1994; Koppang et al., 2000), disturbance of the melanin production pathway (Sanchez-Amat et al., 1998), or even an increase in water temperature (>32 °C) (Hirvelä-Koski, 2005) or incubation temperature (>22 °C) (Cipriano and Bullock, 2001). In addition, this may be correlated with subculturing multiple times. The loss of pigmentation in *A. salmonicida* strain noted in the current study is mainly attributed to the warm water temperature in the Suez Gulf, which ranges from 21.91 °C to 29.22 °C (Mahmoud et al., 2020).

Although in most prokaryotic organisms, the ability for pigment production is highly correlated to virulence, non-pigmented strains were found to cause higher mortality rates in experimentally infected Atlantic salmon (Koppang et al., 2000). This indicates that the absence of

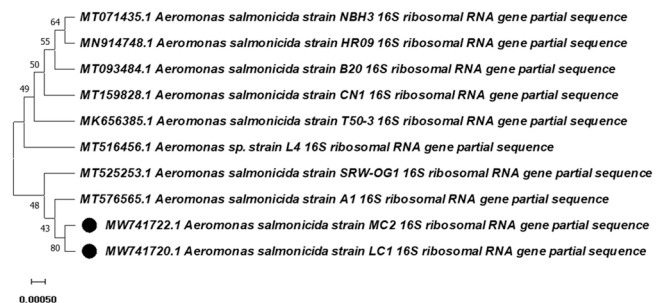


Fig. 4. Neighbour joining phylogenetic tree of 16S-rRNA gene sequences of isolated *Aeromonas salmonicida* and other closely related sequences. The Evolutionary analysis was performed with MEGA version X.

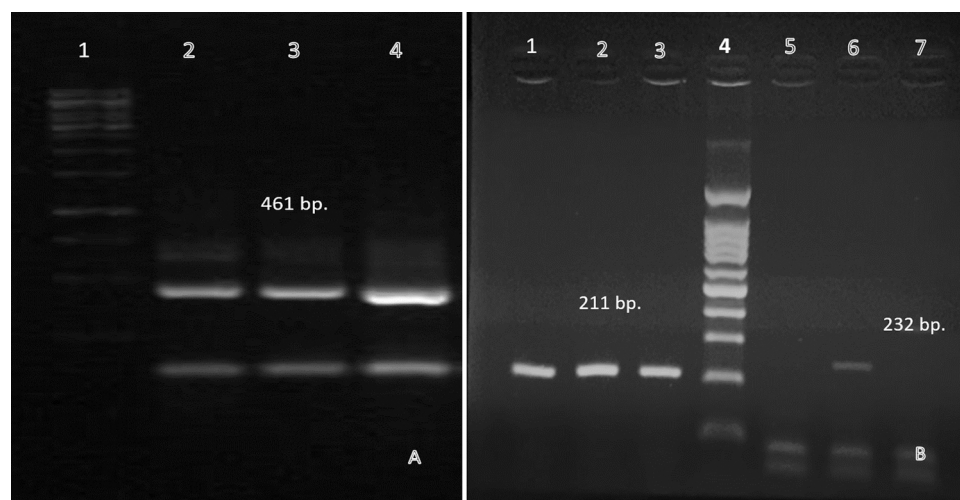


Fig. 3. Agarose gel electrophoresis of *A. salmonicida*; A. Lane 1 ladder, lane 2, 3&4 showed the amplified bands of *Aeromonas* universal 16SrRNA gene at 461 bp.; B. Lane 1, 2 & 3 *Serine protease (ser)* amplicons at 211 bp, lane 4 ladder, lane 6 *Aerolysin-like proteins (act)* amplicons at 232 bp.

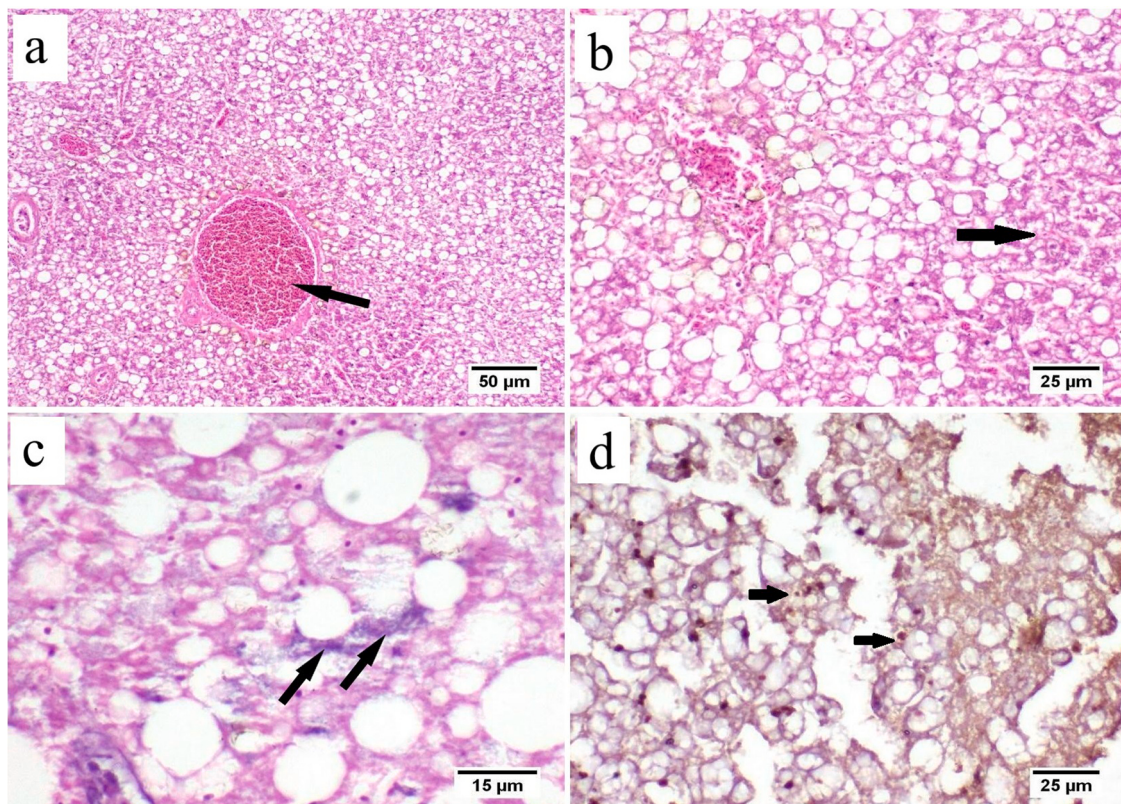


Fig. 5. Photomicrograph of liver from *Aeromonas salmonicida* infected *Mugil carinatus*, (a-c) H&E stained and (d) Immune staining with *A. salmonicida* antibody. (a) congestion of hepatic blood vessel (arrow), (b) marked and diffuse hepatocellular vacuolations mainly fatty change, with presence of multi focal hepatocellular necrosis (arrow), (c) bacterial colonies (arrows) within liver parenchyma, (d) positive immune staining due to presence of *A. salmonicida* in hepatic tissue (arrows).

pigment production has no effect on the infection potential or virulence ability of *A. salmonicida*.

Molecular identification and 16 S rRNA gene sequencing are adopted to confirm the membership of the isolated strain within the family *Aeromonadaceae* (Han et al., 2011; Benagli et al., 2012; Jin et al. (2019)), the high interspecies similarity of the 16SrRNA gene sequence makes their taxonomical identification complex (Ormen et al., 2005; Abd El Latif et al., 2019). However, sequence analysis still a powerful tool for the accurate detection of *Aeromonas* to subspecies level. (Skrodenytė-Arbaciauskienė et al., 2012).

The sequencing analysis of the isolated strains revealed that they show similarity to *A. salmonicida* (99.76%) and *A. salmonicida* subsp. *salmonicida* (99.52%) with a high percentage identity, confirming the phenotypic identification from the API 20 NE diagnostic kit. Furthermore, the phylogenetic analysis confirmed the identities of *A. salmonicida* isolates with most relevant *A. salmonicida* strains.

Several virulence factors have been inherited among different *Aeromonas* species, such as lipopolysaccharide, iron-regulated outer membrane proteins, and extracellular products such as aerolysin (*aer*), lipases, collagenase (*acg*), elastase (*ela*), cytotoxic enterotoxins (*act*, *ast*, *alt*), and proteases (*ser*) (Sen and Rodgers, 2004; Ottaviani et al., 2011; Alghabshi et al., 2018).

The serine protease gene (*ser*) was found to be involved in bacterial multiplication and the development of furunculosis by liquefying fish muscles to produce furuncles (Dallaire-Dufresne et al., 2014). Moreover, it has the ability to activate other toxins such as aerolysin and GCAT (glycerophospholipid cholesterol acyltransferase), which is secreted by *A. salmonicida* in the process referred to as quorum sensing (Cascon et al., 2000; Nam and Joh, 2007). The *ser* gene-positive amplicons were previously detected at 211 bp from diseased trout, king soldier bream, and white-spotted rabbit fish (Nam and Joh, 2007; Alghabshi et al., 2018).

The aerolysin-like protein (*act*) gene was previously detected at 232 bp in *A. salmonicida* strains isolated from salmonid fish, king soldier bream, tilapia, and white-spotted rabbit fish (Lim and Hong, 2020; Alghabshi et al., 2018). There is a direct relationship between the presence of the *act* gene and the beta hemolysis induced on blood agar (Hoel et al., 2017). In our study, the presence of the *ser* and *act* virulence genes in the isolated *A. salmonicida* strain, without appearance of any external lesions, may be correlated with the bacterial load in the host tissue or surrounding environment, the innate resistance of infected fish (Ottaviani et al., 2011) or the variation in fish species susceptibility to *A. salmonicida*.

Liver sections of affected fish showed extensive hepatocellular necrosis associated with the presence of blue-stained bacterial colonies. Moreover, IHC confirmed the presence of *A. salmonicida* antigen within the hepatic parenchyma. The visualization of the bacterial antigen associated with liver necrotic tissues supports a strong relationship between lesions and the presence of bacteria. Tissue necrosis likely resulted from the direct damage caused by the action of toxins and enzymes secreted by *A. salmonicida* (Vanden Bergh et al., 2013). Similarly, diffuse liver necrosis, degenerative changes and vacuolations were demonstrated in the diseased walking catfish, *Clarias batrachus*; goldfish and European perch, *Perca fluviatilis* naturally infected with *A. salmonicida* (Thomas et al., 2013; Rupp et al., 2019; Jin et al., 2020; Lian et al., 2020).

Histopathologic picture of the kidney revealed congestion and inflammatory reaction as reported by Lian et al. (2020). Local distribution of *A. salmonicida* in the renal interstitial tissue and tubular epithelium was confirmed by immunohistochemistry. This justifies the marked necrosis of the epithelial lining of the renal tubules (Coscelli et al., 2014; Shaalan et al., 2018a). Likewise, renal tissue sections of farmed rainbow trout (*Oncorhynchus mykiss*) and crucian carp (*Carassius auratus*) naturally infected with *A. salmonicida* showed congestion of renal blood

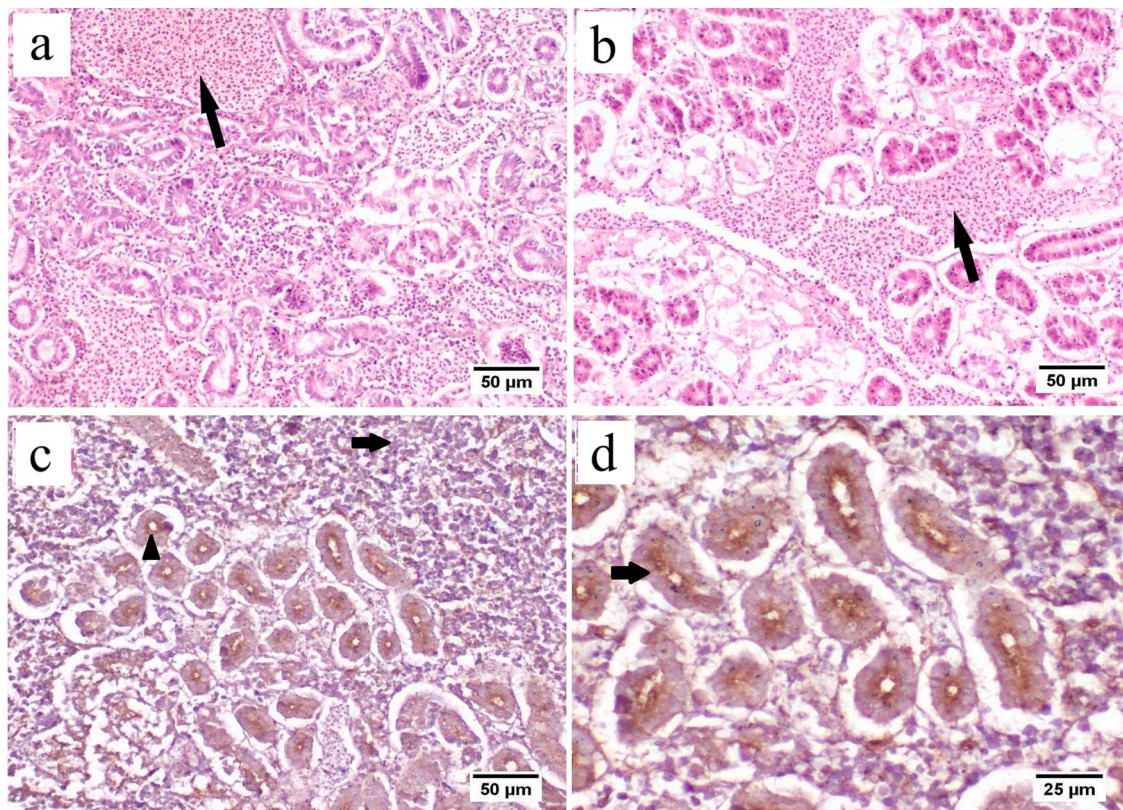


Fig. 6. Photomicrograph of kidney from *Aeromonas salmonicida* infected *Mugil carinatus*, (a-b) H&E stained and (c-d) Immune staining with *A. salmonicida* antibody. (a) Severely dilated and congested renal blood vessel (arrow), (b) Diffuse hemorrhages in the renal interstitial tissue (arrow) and necrosis of some renal tubules, (c) Positive immune staining due to presence of *A. salmonicida* in the renal interstitial tissue (arrow) and tubular epithelium (arrow head), and (d) higher magnification shows positive immune staining of the bacteria in the epithelial lining of renal tubules (arrow).

vessels and diffuse interstitial nephritis (Zepeda-Velázquez et al., 2015; Lian et al., 2020).

In conclusion, this is the first report of *A. salmonicida* detection in *M. carinatus*, these results postulate that fish tissue may harbor the bacteria in a silent way and asymptomatic carriers of *A. salmonicida* could be a source of infection to other susceptible fish species. The results obtained in this study provide a base for further future investigations on the pathogenesis of *A. salmonicida* in Mugilidae and its prevention and control.

Ethics approval

All procedures were carried out according to the regulations of institutional animal use and care committee (IACUC), faculty of veterinary medicine, Cairo University, Egypt.

CRediT authorship contribution statement

Asmaa K. Al-Mokaddem: Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Investigation, Writing final draft. **Dalia A. Abdel-moneam:** Conceptualization, Methodology, Data curation, Writing – original draft preparation, Visualization, Investigation, Writing final draft. **Reham A. Ibrahim:** Methodology. **Mona Saleh:** Writing – review & editing. **Mohamed Shaalan:** Methodology, Data curation, Writing – original draft preparation, Visualization, Investigation, Writing final draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Acknowledgment

This study was funded in part by the University of Veterinary Medicine Vienna, Austria. Mohamed Shaalan was funded by a scholarship from Ministry of Higher Education, Egypt.

References

- Abd El Latif, A.M., Elabd, H., Amin, A., Noor Eldeen, A.I., Shaheen, A.A., 2019. High mortalities caused by *Aeromonas veronii*: identification, pathogenicity, and histopathological studies in *Oreochromis niloticus*. *Aquac. Int.* 27, 1725. <https://doi.org/10.1007/s10499-019-00429-8>.
- Abdel-moneam, D.A., Ibrahim, R.A., Nashaat, M., Shaalan, M., 2021. Multifactorial causes of mass mortality in *Oreochromis niloticus* in Kafr El-Sheikh, Egypt. *Bull. Eur. Ass. Fish. Pathol.* 41 (1), 6–16.
- Alghabshi, A., Austin, B., Crumlish, M., 2018. *Aeromonas salmonicida* isolated from wild and farmed fish and invertebrates in Oman. *Int. Aquat. Res.* 10, 145–152. <https://doi.org/10.1007/s40071-018-0195-4>.
- Altmann, K., Marshall, M., Nicholson, S.E., Hanna, P.J., Gudkovs, N., 1992. Glucose repression of pigment production in atypical isolates of *Aeromonas salmonicida* responsible for goldfish ulcer disease. *Microbios* 72, 215–220.
- Austin, B., Austin, D.A., 2007. *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*. Springer.
- Austin, B., Austin, D.A., Dalsgaard, I., Gudmundsdottir, B.K., Hoie, S., Thornton, J.M., et al., 1998. Characterization of atypical *Aeromonas salmonicida* by different methods. *Syst. Appl. Microbiol.* 21, 50–64. [https://doi.org/10.1016/S0723-2020\(98\)80008-8](https://doi.org/10.1016/S0723-2020(98)80008-8).
- Bancroft, J.D., 2013. *Histochemical Techniques*. Butterworth-Heinemann.
- Bartkova, S., Kokotovic, B., Skall, H.F., Lorenzen, N., Dalsgaard, I., 2017. Detection and quantification of *Aeromonas salmonicida* in fish tissue by real-time PCR. *J. Fish. Dis.* 2017 (40), 231–242. <https://doi.org/10.1111/jfd.12505>.
- Benagli, C., Demarta, A., Caminada, A., Ziegler, D., Petrini, O., Tonolla, M., 2012. Arapid MALDI-TOF MS identification database at genospecies level for clinical and environmental *Aeromonas* strains. *PLoS One* 7 (10), e48441. <https://doi.org/10.1371/journal.pone.0048441>.

- Buller, N.B., 2004. *Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual*. CABI Publishing, Wallingford, Oxfordshire.
- Cascon, A., Fregenda, J., Allen, M., Yugueros, J., Temprano, A., Hernanz, C., 2000. Cloning, characterization and insertional inactivation of a major extracellular serine protease gene with elastolytic activity for *Aeromonas hydrophila*. *J. Fish. Dis.* 23, 49–59.
- Cipriano, R.C., Bullock, G.L., 2001. Furunculosis and other diseases caused by *Aeromonas salmonicida*. Revision of "Furunculosis and other diseases caused by *Aeromonas salmonicida*," by G. L. Bullock, R. C. Cipriano, and S. F. Snieszko, 1983. National Fish Health Research Laboratory.
- Coscelli, G.A., Bermúdez, R., Losada, A.P., Faílde, L.D., Santos, Y., Quiroga, M.I., 2014. Acute *Aeromonas salmonicida* infection in turbot (*Scophthalmus maximus* L.). Histopathological and immunohistochemical studies. *Aquaculture* 430, 79–85.
- Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A., Charette, S.J., 2014. Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet. Microbiol.* 169, 1–7. <https://doi.org/10.1016/j.vetmic.2014.06.016>.
- Emmerich, R., Weibel, E., 1894. Über eine durch Bakterien erzeugte Seuche unter den Forellen. *Arch. für Hyg. und Bakteriol.* 21, 1–21.
- Fernández-Alvarez, C., Gijón, D., Álvarez, M., Santos, Y., 2016. First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain. *Aquac. Rep.* 4 (2016), 36–41.
- GAFRD, (General Authority for Fish Resources Development)., 2017. Annual report for country fish production, Egypt.
- Gudmundsdóttir, B.K., Hvanndal, Í., Björnsdóttir, B., Wagner, U., 2003. Analysis of exotoxins produced by atypical isolates of *Aeromonas salmonicida*, by enzymatic and serological methods. *J. Fish. Dis.* 26, 15–29.
- Han, H.J., Kim, D.Y., Kim, W.S., Kim, C.S., Jung, S.J., Oh, M.J., Kim, D.H., 2011. Atypical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegelii* Hilgendorf, in Korea. *J. Fish. Dis.* 2011 (34), 47–55. <https://doi.org/10.1111/j.1365-2761.2010.01217.x>.
- Hirvelä-Koski, V., 2005. Fish Pathogens *Aeromonas salmonicida* and *Renibacterium salmoninarum*: Diagnostic And Epidemiological Aspects Faculty of Veterinary Medicine, University of Helsinki, ISSN 1458–6878 ISBN 952–5568–10–5 (Print).
- Hoel, S., Vadstein, O., Jakobsen, A.N., 2017. Species distribution and prevalence of putative virulence factors in mesophilic *Aeromonas* spp. isolated from fresh retail sushi. *Front. Microbiol.* 8, 931. <https://doi.org/10.3389/fmicb.2017.00931>.
- Huys, G., 2014. The family aeromonadaceae. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), *The Prokaryotes*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-38922-1_282.
- Jin, S., Fu, S., Li, R., Dang, H., Gao, D., Ye, S., Jiang, Z., 2020. Identification and histopathological and pathogenicity analysis of *Aeromonas salmonicida* from goldfish (*Carassius auratus*) in North China. *Aquac. Fish.* 5 (1), 36–41.
- Karatas, S., Candan, A., Demircan, D., 2005. Atypical *aeromonas* infection in cultured sea bass (*Dicentrarchus labrax*) in the Black Sea. *The Israeli. J. Aquac. Bamiqeh* 57 (4), 255–263, 2005.
- Koppang, E.O., Fjølstad, M., Melgaard, B., Vigerust, M., Sürum, H., 2000. Non-pigment-producing isolates of *Aeromonas salmonicida* subspecies *salmonicida*: isolation, identification, transmission and pathogenicity in Atlantic salmon, *Salmo salar* L. *J. Fish. Dis.* 23, 39–48.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35 (6), 1547–1549. <https://doi.org/10.1093/molbev/msy096>.
- Li, S., Xu, G., Wang, D., Mou, Z., Lu, T., 2014. Histological studies of the effects of *aeromonas salmonicida* on the tissues in *brachymystax lenok*. *J. Cytol. Histol.* 5 <https://doi.org/10.4172/2157-7099.1000i107>. Issue 61000i107.
- Lian, Z., Bai, J., Hu, X., Lü, A., Sun, J., Guo, Y., Song, Y., 2020. Detection and characterization of *Aeromonas salmonicida* subsp. *salmonicida* infection in crucian carp *Carassius auratus*. *Vet. Res. Commun.* (2020) 44, 61–72. <https://doi.org/10.1007/s11259-020-09773-0>.
- Lim, J., Hong, S., 2020. Characterization of *Aeromonas salmonicida* and *A. sobria* isolated from cultured salmonid fish in Korea and development of a vaccine against furunculosis. *J. Fish. Dis.* 2020 43, 609–620. <https://doi.org/10.1111/jfd.13158>.
- Mahmoud, M.G., El-Khair, E.A., Ebeid, M.H., Mohamed, L.A., Fahmy, M.A., Shaban, K.S., 2020. An assessment on the coastal seawater quality of the Gulf of Suez, Egypt. *J. Environ. Prot.* 11 (1), 34–47.
- Mehanna, S.F., El-Sherbeny, A.S., El-Mor, M., Eid, N.M., 2019. Age, growth and mortality of liza carinata valenciennes, 1836 (Pisces: mugilidae) in bitter Lakes, Suez Canal, Egypt. *Egypt. J. Aquat. Biol. Fish.* 23 (3), 283–290.
- Menanteau-Ledouble, S., Kumar, G., Saleh, M., El-Matbouli, M., 2016. *Aeromonas salmonicida*: updates on an old acquaintance. *Dis. Aquat. Org.* 120: 49–68, 2016 Vol. 120, 49–68. <https://doi.org/10.3354/dao03006>.
- Meng, L., Tong-Tong, L., Yao, J., Qian-Qian, Z., De-Feng, Z., Fu-Tie, Z., Jian-Wei, W., Ai-Hua, L., 2020. Isolation and characterization of *aeromonas salmonicida* subspecies *salmonicida* from largemouth bronze gudgeon (*coreius guichenoti*) cage-cultured in the upper reaches of Yangtze River. *ACTA HYDROBIOLOGICA Sin.* 44, 2020. <https://doi.org/10.7541/2020.018>.
- Mesalam, N.M., Aldhumri, S.A., Gabr, S.A., Ibrahim, M.A., Al-Mokaddem, A.K., Abdel-Moneim, A.M.E., 2021. Putative abrogation impacts of Ajwa seeds on oxidative damage, liver dysfunction and associated complications in rats exposed to carbon tetrachloride. *Mol. Biol. Rep.* 48 (6), 5305–5318.
- Nam, I., Joh, K., 2007. Rapid detection of virulence factors of *aeromonas* isolated from a trout farm by hexaplex-PCR. *The J. Microbiol.* 45 (4), 297–304.
- Ormen, O., Granum, P.E., Lassen, J., Figueras, M.J., 2005. Lack of agreement between biochemical and genetic identification of *Aeromonas* spp. *APMIS Acta Pathol. Microbiol. Immunol. Scand.* 113, 203–207. <https://doi.org/10.1111/j.1600-0463.2005.apm1130308.x>.
- Ottaviani, D., Parlani, C., Citterio, B., Masini, L., Leoni, F., Canonico, C., Pianetti, A., 2011. Putative virulence properties of *Aeromonas* strains isolated from food environmental and clinical sources in Italy: a comparative study. *Int. J. Fd Microbiol.* 144, 538–545.
- Page, M., Thorpe, R., 1996. Purification of IgG using protein A or protein G. In *The Protein Protocols Handbook*. Humana Press, pp. 733–734.
- Rakib, S.M., Hossen, M.N., Rahman, M.A., Sku, S., Akter, S., Rashid, M.M., 2018. Development of anti-*Aeromonas hydrophila* serum from Rabbits. *Res. Agric. Livest. Fish.* 5 (1), 137–145.
- Rupp, M., Pilo, P., Müller, B., Knusel, R., Siebenthal, B.V., Frey, J., Sindilariu, P.D., Schmidt-Posthaus, H., 2019. Systemic infection in European perch with thermoadapted virulent *Aeromonas salmonicida* (*Perca fluviatilis*). *J. Fish. Dis.* 00, 1–7.
- Sanchez-Amat, A., Ruzafa, C., Solano, F., 1998. Comparative tyrosine degradation in *Vibrio cholerae* strains. The strain ATCC 14035 as a prokaryotic melanogenic model of homogenitase-releasing cell. *Comp. Biochem. Physiol. Part B, Biochem. Mol. Biol.* 119, 557–562.
- Sen, K., Rodgers, M., 2004. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. *J. Appl. Microbiol.* 2004 (97), 1077–1086. <https://doi.org/10.1111/j.13652672.2004.02398.x>.
- Shaaan, M., El-Mahdy, M., Theiner, S., Dinhol, N., El-Matbouli, M., Saleh, M., 2018a. Silver nanoparticles: their role as antibacterial agent against *Aeromonas salmonicida* subsp. *salmonicida* in rainbow trout (*Oncorhynchus mykiss*). *Res. Vet. Sci.* 119, 196–204.
- Shaaan, M., El-Mahdy, M., Saleh, M., El-Matbouli, M., 2018b. *Aquaculture in Egypt: insights on the current trends and future perspectives for sustainable development*. *Rev. Fish. Sci. Aquac.* 26 (1), 99–110.
- Shakman, I.A., Etayeb, K.S., Bebe-Abdallah, A.R., 2016. Status of invasive marine species in the Libyan coast. *Rapp. De la Comm. Mar. Méditerranée* 41, 418.
- Skrodenytė-Arbačiauskienė, V., Kazlauskienė, N., Vosylienė, M.Z., Virbickas, T., 2010. Identification of *Aeromonas salmonicida* in European perch from North Lithuanian rivers during mass mortalities in 2008. *Cent. Eur. J. Biol.* 5 (6), 831–838 <https://doi.org/10.2478/s11535-010-0091-4>.
- Skrodenytė-Arbačiauskienė, V., Kazlauskienė, N., Vosylienė, M.Z., Virbickas, T., 2012. *Aeromonas salmonicida* infected fish transfer disease to healthy fish via water. *Cent. Eur. J. Biol.* 7 (5), 878–885. <https://doi.org/10.2478/s11535-012-0066-8>.
- Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. (USA)* 101, 11030–11035. <https://doi.org/10.1073/pnas.0404206101>.
- Tewari, R., Dudeja, M., Nandy, S., KumarDas, A., 2014. Isolation of *Aeromonas salmonicida* from Human blood sample: a case report 2014. *J. Clin. Diagn. Res.* 8 (2), 139–140.
- Thomas, J., Jerobin, J., Seelan, T.S.J., Thanigaivel, S., Vijaya, K.S., Mukherjee, A., Chandrasekaran, N., 2013. Studies on pathogenicity of *Aeromonas salmonicida* in catfish *Clarias batrachus* and control measures by neem nanoemulsion. *Aquaculture* 396–399, 71–75.
- Vanden Bergh, P., Heller, M., Braga-Lagache, S., Frey, J., 2013. The *Aeromonas salmonicida* subsp. *salmonicida* exoproteome: determination of the complete repertoire of Type- Three Secretion System effectors and identification of other virulence factors. *Proteome Sci.* 11, 42–61.
- Wiklund, T., Lönnström, L., Niiranen, H., 1993. *Aeromonas salmonicida* ssp. *salmonicida* lacking pigment production, isolated from farmed salmonids in Finland. *Diseases of aquatic organisms* 15 (3), 219–223.
- Zepeda-Velázquez, A.P., Vega-Sánchez, V., Salgado-Miranda, C., Soriano-Vargas, E., 2015. Histopathological findings in farmed rainbow trout (*Oncorhynchus mykiss*) naturally infected with 3 different *Aeromonas* species. *Can. J. Vet. Res.* 79 (3), 250–254.