Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(5): 1791 – 1809 (2025)
www.ejabf.journals.ekb.eg

Tracing the Evolutionary Course of Antimicrobial Resistance Genes Mutations in *Aeromonas* Species from *Mugil cephalus* and *Solea solea*

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ARTICLE INFO

Article History:
Received: Aug. 13, 2025
Accepted: Sep. 20, 2025
Online: Oct. 3, 2025

Keywords:
Aeromonas species,
Antibiotics,
PCR,
Resistant genes,
Sequencing,
Mutation

ABSTRACT

A total of eight isolates of *Aeromonas* species were isolated from 70 *Mugil cephalus* (*M. cephalus*) fish and 30 *Solea solea* (*S. solea*) fish samples collected from Lake Manzala in Port Said Governorate, Egypt, with a prevalence of 8%. The isolated strains were identified biochemically and genetically, and addressed to detect the antibiotic susceptibility profiles. From *M. cephalus* fish, *A. hydrophila* was recorded with the highest (4/70) isolated strains (5.7%), followed by *A. caviae*, *A. sobria* and *A. schaberttii* (1/70), (1/70), and (1/70) strains, with percentages of 1.4, 1.4, and 1.4%, respectively. While *A. veronii* was isolated from *S. solea* fish (1/30) strain (3.3%). The molecular identification of isolated aeromonads by conventional PCR was conducted using *16Sr-RNA* for *Aeromonas* species. Results revealed that all isolates were positive. The genetic determinants revealed 2 acquired antibiotic resistance genes, as bla_{TEM} and *gyrA* were represented in all *Aeromonas* spp. $bla_{CTX\text{-}M}$ gene was detected only in 4 species of *Aeromonas* species (*A. caviae*, *A. sobria*, *A. schabertii* and *A. veronii*), and (*ereA*) gene was detected only in 3 strains of *Aeromonas* species (*A. sobria*, *A. schaberttii* and *A. veronii*). At the genomic level, our study reveals several key mutations in the *gyrA* gene of *A. caviae*, *A. veronii*, and *A. hydrophila*, specifically at positions 83 and 87, which are known to associate with fluoroquinolone resistance. The predicted functional impact of QRDR mutations using PROVEAN revealed deleterious score (below -2.5) in *A. hydrophila* S83L, *A. veronii* D87N and *A. caviae* D87Y. These mutations are known to confer fluoroquinolone resistance, while the prediction score was natural in *A. sobria* E84G (-0.7) associated with unknown significance mutation and *A. schubertii* L82P (-2.5) which is considered a novel mutation, this has not been previously reported in *Aeromonas*, making it an interesting candidate for further studies for its implication in gene function.

INTRODUCTION

Aquaculture is an essential resource for food production, providing the global population with animal-derived protein and alleviating food scarcity caused by overpopulation (**ALgammal *et al.*, 2020**). Egypt ranks as the leading African nation in

aquaculture production, achieving approximately 1.8 million tons of aquatic animal output, including freshwater and marine fish, crustaceans, and shellfish **(Abouelmaatti *et al.*, 2013)**.

Marine life will flourish in seawater **(Algammal *et al.*, 2022a)**. *Aeromonas* species are opportunistic pathogens that affect both fish and humans, causing various diseases **(Yassen *et al.*, 2021)**. In fish, *Aeromonas* spp. cause septicemia, ulcerative diseases, and mortality (**Abdel-Latif & Khafaga, 2020; Sherif *et al*., 2021; Yassen *et al.*, 2021)**.

Among the 14 species of *Aeromonas* that have been described, *A. veronii biovar sobria, A. caviae, A. hydrophila,* and *A. schubertii* have been identified as potential human pathogens **(Radu *et al.*, 2003)**. *A. hydrophila* is recognized as the main pathogen impacting both wild and farmed fish populations, resulting in significant mortality rates within aquaculture and mariculture systems, which incur annual financial losses amounting to millions of dollars **(Ayoub *et al*., 2024)**. *A. veronii* is frequently identified as the etiological agent responsible for hemorrhagic septicemia in marine fish (**Algammal *et al.*, 2022a; Abdellatief *et al.*, 2024)**. Infection with *A. veronii* can lead to food-borne illness in humans, with symptoms including diarrhea, gastroenteritis, and in severe cases, sepsis **(Algammal *et al*., 2022b)**. The bacterium *A. sobria* is a pathogen in aquaculture, infecting various aquatic animals. Human infections can result in conditions ranging from gastroenteritis to life-threatening meningitis, fasciitis, bacteremia, and septic shock **(Liu *et al.*, 2025)**. *A. caviae* has the potential to infect a range of aquatic species (**Xue *et al.*, 2022)**. The prevalence of *A. caviae* infections in various fish species poses a significant risk to the sustainable growth of aquaculture **(Xue *et al.*, 2022**). *A. schubertii* represents a notable opportunistic pathogen that poses significant threats to humans, various mammals, and aquatic animals **(Luo *et al.*, 2024).**

Antibiotic resistance in *Aeromonas* spp. is a growing concern in aquatic environments, particularly in the aquaculture sector **(Algammal *et al.*, 2022b)**. *Aeromonas* spp. demonstrated resistance to multiple antibiotics, complicating the treatment of infections in fish. Antibiotic resistance in *Aeromonas* species was observed against four main groups of antibiotics: tetracyclines β-lactams, aminoglycosides, and quinolones **(Piotrowska & Popowska, 2014)**. Several studies reported high levels of resistance to commonly used antibiotics, including erythromycin, tetracycline, ampicillin, and tetracycline **(Algammal *et al.*, 2022b** ; **Ayoub *et al*., 2024)**.

The molecular determination of most inherited antibiotic resistance genes must be regularly assessed to prevent antibiotic-resistant strains that can affect community health **(Algammal *et al.*, 2022b)**. The genetic characterization of *Aeromonas* spp., especially *A. hydrophila*, confirmed resistance genes bla_{TEM} and *tetA* **(Sitting *et al.*, 2024)**.

Quinolone resistance in *Aeromonas* species is mainly linked to mutations within the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes **(Goñi-Urriza *et al.*, 2002; Alcaide *et al.*, 2010)**. The most common mutation occurs at codon

83 of *gyrA*, resulting in a Ser-83→Ile substitution **(Goñi-Urriza *et al.*, 2002; Alcaide *et al.*, 2010)**.

This study aimed to molecular detect mutation events in antibacterial resistance genes among *Aeromonas* species.

MATERIALS AND METHODS

1. Sampling

One hundred (70 *M. cephalus*, 90±5 g. body weight and 30 *S. solea*, 40±5 g.bw) from Lake Manzala in Port-Said Governorate, Egypt. All fish were collected with clinical signs, transferred immediately to the laboratories of the Animal Health Research Institute (AHRI), Port-Said branch, in aerator bags for bacteriological examinations. Clinically, naturally infected fish were examined for abnormalities, and necropsy was performed to detect post-mortem lesions **(Austin & Austin, 2016)**.

1. Bacteriological examination and isolation

Fish samples were subjected to bacteriological examination undergoing sterile conditions, following the procedures detailed by **Austin and Austin (2016)**. Sterile loop from different gills, spleen, kidney, liver, and external lesions if detected, were streaked on tryptic soy agar (TSA; Eir Pharm, CONDA, (Spain), afterward incubated aerobically at 37°C for 24hrs. Suspected colonies were streaked on *Aeromonas* agar base (Liofichem, Italy) with ampicillin supplement, incubation for 24hrs at 37°C. Identification of all isolates was accomplished through an assessment of culture morphology, Gram stain reaction, motility, and biochemical profiles (Voges Proskauer, methyl red, indole, catalase, oxidase, citrate utilization, urease, H2S production, sugar fermentation tests), as stated by **Austin and Austin (2016)**. Pure colonies were preserved at −80°C in tryptic soy broth (TSB) with 10% glycerol for further examination.

2. Antimicrobial susceptibility testing

The purified *Aeromonas* isolates were characterized for antibiotic susceptibility on Mueller–Hinton agar (HIMEDIA, India) plates by the disc diffusion method **(Quinn *et al.*, 2002)** utilizing 16 various antibiotic discs (lincomycin MY10µg, colistin CT 10 mcg, amoxicillin AMX 20µg, amikacin AK30 mcg, nitrofurantoin F 300 mcg, ampicillin AMP 10 mcg, nalidixic acid NA 30µg, tetracycline TE 30µg, oxytetracycline OT 30mcg, oxolinic OA 2µg, erythromycin E 15mcg, danofloxacin DFX 5µg, ciprofloxacin CIP 5mcg, gentamicin CN 10µg, ceftriaxone CRO 30µg and trimethoprim/sulphamthoxazole (SXT) 25µg. The findings were analyzed according to the Clinical and Laboratory Standards Institute **(CLSI, 2024)**.

3. Molecular detection of resistance genes

Aeromonas isolates were inoculated in TSB and maintained at 37°C for 24 hours. DNA extraction from samples was conducted utilizing the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), incorporating modifications to the manufacturer's

guidelines. Two hundred microliters of lysis buffer was inocupated at 56°C for 10 minutes. Following incubation, 200µl of 100% ethanol was introduced to the lysate. The sample was subsequently washed and centrifuged in accordance with the manufacturer's guidelines. Nucleic acid was eluted with 100µl of elution buffer. PCR amplification was carried out in a total volume of 25µl, consisting of 12.5µl Emerald Amp Max PCR Master Mix (Takara, Japan), 1.0µl of each primer (20 pmol), 4.5µl nuclease-free water, and 6µl of DNA template. Reactions were processed in an Applied Biosystems 2720 thermal cycler. Amplification products were resolved by electrophoresis on a 1.5% agarose gel (AppliChem, Germany) in 1x TBE buffer at 5V/ cm. For analysis, 20µl of each product was loaded onto the gel. Fragment sizes were determined using GelPilot 100 bp (Qiagen, Germany) and GeneRuler 100 bp (Fermentas, Germany) ladders. Gels were imaged with an Alpha Innotech gel documentation system (Biometra), and the resulting data were analyzed with companion software.

Oligonucleotide Primer: Primers utilized were provided by Metabion (Germany) and are listed in Table (1).

Table 1. *16Sr RNA*, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16Sr RNA	GAAAGGTTGATGCCTAATACGTA CGTGCTGGCAACAAAGGACAG	685	94˚C 10min	94˚C 45sec	55˚C 45sec	72˚C 45sec	72˚C 20min	**Niclsen *et al.*, (2001)**
bla$_{TEM}$	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Colom *et al.*, (2003)**
ereA	GCCGGTGCTCATGAACTTGAG CGACTCTATTCGATCAGAGGC	420	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Nguyen *et al.*, (2009)**
bla$_{CTX-M}$	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Archambault *et al.*, (2006)**
gyrA	AAATCTGCCCGTGTCGTTGGT GCCATACCTACTGCGATACC	344	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Fàbrega *et al.*, (2009)**

DNA sequencing:

The nucleotide sequence of *gyrA* was obtained through PCR product purification utilizing QIAquick PCR Product Extraction Kit. (Qiagen, Valencia). Following the manufacturers' protocols, the sequence reaction was carried out with the BigDye Terminator V3.1 cycle sequencing kit (PerkinElmer), purified using a Centrisep spin column, and run on an Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). The obtained sequences for the five *Aeromonas* strains were deposited in GenBank with the following accession numbers: *A. hydrophila* (PQ067305), *A. veronii* (PQ067306), *A. sobria* (PQ067304), *A. schubertii* (PQ067303), and *A. caviae* (PQ067302).

Sequence analysis and mutation tracking:

Both nucleotide sequences and their corresponding predicted amino acid sequences were aligned and compared with those of *Aeromonas* strains and with sequences available in published sources (http://www.ncbi.nlm.nih.gov/Genbank/). BLAST analyses (Basic Local Alignment Search Tool) **(Altschul *et al.*, 1990)** were conducted utilizing accessible online tools: (http://www.ncbi.nlm.nih.gov), selected and retrieved *gyr*A nucleotide sequences of some reference strains corresponding to our sequenced strains available in the GenBank

databases with the following accession numbers: *A. hydrophilia* (NC_008570.1), *A. veronii* (NZ_CP044060.1), *A. sobria* (NZ_CDBW01000041), *A. schubertii* (NZ_LPUO01000034) and *A. caviae* (NZ_JAPQMO010000048.1).

Multiple Sequence Alignment (MSA)**:** Each strain sequence was aligned with its corresponding reference utilizing Clustal Omega **(Sievers *et al.*, 2011)** on the EBI server (https://www.ebi.ac.uk/Tools/msa/clustalo/). Single-nucleotide polymorphisms (SNPs) were identified using SNP-sites **(Page *et al.*, 2016)** (https://github.com/sanger-pathogens/snp-sites).

The aligned nucleotide sequences were translated into protein sequences employing the ExPASy Translate Tool (https://web.expasy.org/translate/).

The protein sequences were realigned using Clustal Omega, focusing on the Quinolone Resistance-Determining Region (QRDR), particularly codons 83, 87, and 92 **(Cattoir *et al.*, 2008)**.

Functional impact prediction

The amino acid substitutions' potential functional impact was predicted using PROVEAN (Protein Variation Effect Analyzer) according to **Choi and Chan (2015)** (http://provean.jcvi.org/).

4. Biosafety measures

This study implemented biosafety protocols in accordance with pathogen safety data sheets**:** Infectious substances *Aeromonas* species, Pathogen Regulation Directorate **(Public Health Agency of Canada, 2019)**.

RESULTS

1. Clinical and postmortem observations

Fish showed hemorrhagic septicemia, which is represented by the presence of surface lesions and hemorrhage at the anal opening. Abdominal distention, the postmortem examination indicated enlargement and congestion of spleen and liver (Fig. 1).

Fig. 1. Infected *M. cephilus* with *Aeromonas* spp. showing abdominal acities, redness at pelvic fins (a and b), hemorrhagic spots on the body surface at caudal area (b), enlargement and congestion of internal organs (c). Dark skin *S. solea* (d), redness at the abdomen with a red spot (e).

2. Phenotypic characteristics and prevalence of *Aeromonas* spp.

Phenotypic analysis of *Aeromonas* spp. colonies on TSA medium emerged round, convex, white, creamy, and opaque dark green colonies with a dark center on *Aeromonas* agar base. Gram-negative bacilli, *Aeromonas* spp. produced oxidase, catalase, Indole, VP, and citrate positive, motile, while the isolates were negative for H_2S and urea hydrolysis. The commonness of *Aeromonas* spp. within the examined fish was 8% (8/100). From *Mugil cephalus* fish (7 isolates): *A. hydrophil*a was 5.7% (4/70), *A. caviae* 1.4% (1/70), *A. sobria* 1.4% (1/70), and *A. Schabertii* 1.4% (1/70). From *S. solea* fish, one isolate: *A. veronii* 3.3% (1/30).

3. Antimicrobial sensitivity results

The antimicrobial susceptibility tests indicated that the *A. hydrophila* isolates obtained exhibited notable resistance to several antimicrobial agents, such as lincomycin, colistin, amoxicillin, ampicillin, tetracycline, and oxytetracycline (100% for each), as well as erythromycin (75% for each). The retrieved *A. caviae* isolates showed significant resistance to lincomycin, colistin, amoxicillin, ampicillin, tetracycline, oxytetracycline, Danofloxacin, and nalidixic acid (100% for each), as well as erythromycin (50%). The retrieved *A. sobria* isolates showed significant resistance to lincomycin, amoxicillin, ampicillin, tetracycline, oxytetracycline, and erythromycin (100% for each), as well as colistin (50%). The retrieved *A. schabertii* isolate showed significant resistance to lincomycin, amoxicillin, ampicillin, colistin, tetracycline, danofloxacin, and erythromycin. The retrieved *A. veronii* isolate exhibited strong resistance to lincomycin, ampicillin, colistin, tetracycline, danofloxacin, and nalidixic acid (Fig. 2).

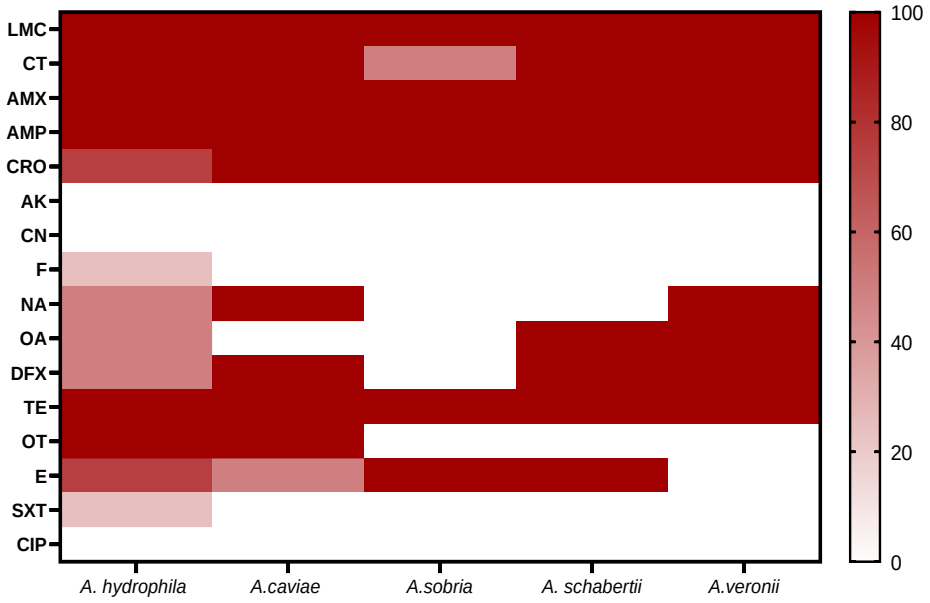

Fig. 2. Heap map illustrating the results of antimicrobial sensitivity

4. Dissemination of antimicrobial resistance genes

The PCR indicated that the bla_{TEM} and *gyrA* genes (100%, each) in all *Aeromonas* spp. followed by $bla_{CTX\text{-}M}$ gene (80%, each) in *A. caviae*, *A. sobria*, *A. schabertii* and *A. veronii* isolates, and succeeded by *ereA* gene (60%) in *A. sobria*, *A. schabertii* and *A. veronii* isolates (Figs. 3, 4).

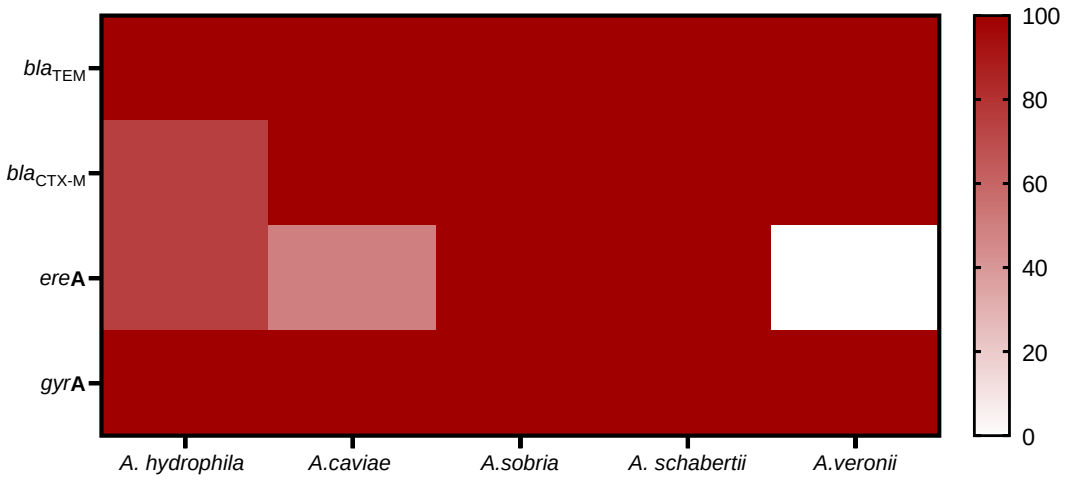

Fig. 3. Heatmap illustrating the distribution of resistance genes

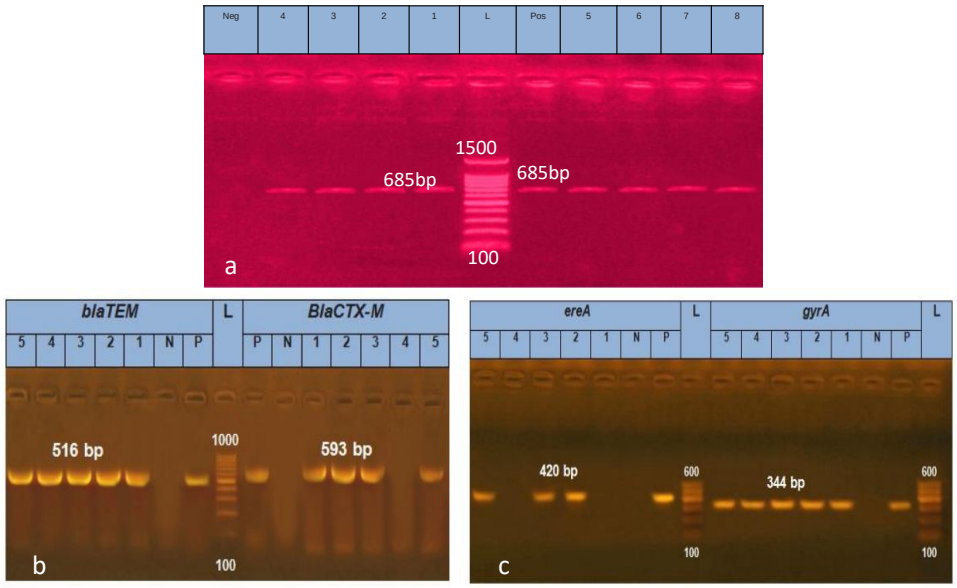

Fig. 4. Agarose gel electrophoresis showing:
a-*Aeromonas* spp. 16Sr-RNA gene. L:100-1500 bp ladder) + control (Positive, Negative) + different strains of *Aeromonas* spp. (16Sr -RNA gene derived products at 685 bp).
b- *Aeromonas* AMR gene *bla*TEM. L:100-1000 bp ladder) + control (P: positive, N: Negative) + different strains of *Aeromonas* spp. (*bla*TEM gene products at 516 bp). *Aeromonas* AMR gene *bla*CTX-M. (*bla*CTX-M gene products at 593 bp).
c- *Aeromonas* AMR gene *ereA*. L: 100 – 600 bp ladder) + control (Positive, Negative) + different strains of *Aeromonas* spp. (*ereA* gene derived products at 420bp). *Aeromonas* AMR gene *gyrA*. (*gyrA* gene products at 344 bp).

5. Correlation analysis results

Remarkable positive correlations were noted between antibiotics e.g., DFX, and NA (r = 0.41), DFX and OA (r = 0.25). Remarkable positive correlations were recorded between antibiotics and their resistance genes, e.g., CRO and *bla*$_{CTX-M}$ (r=1), and E and *ereA* (r=1), (Fig. 5).

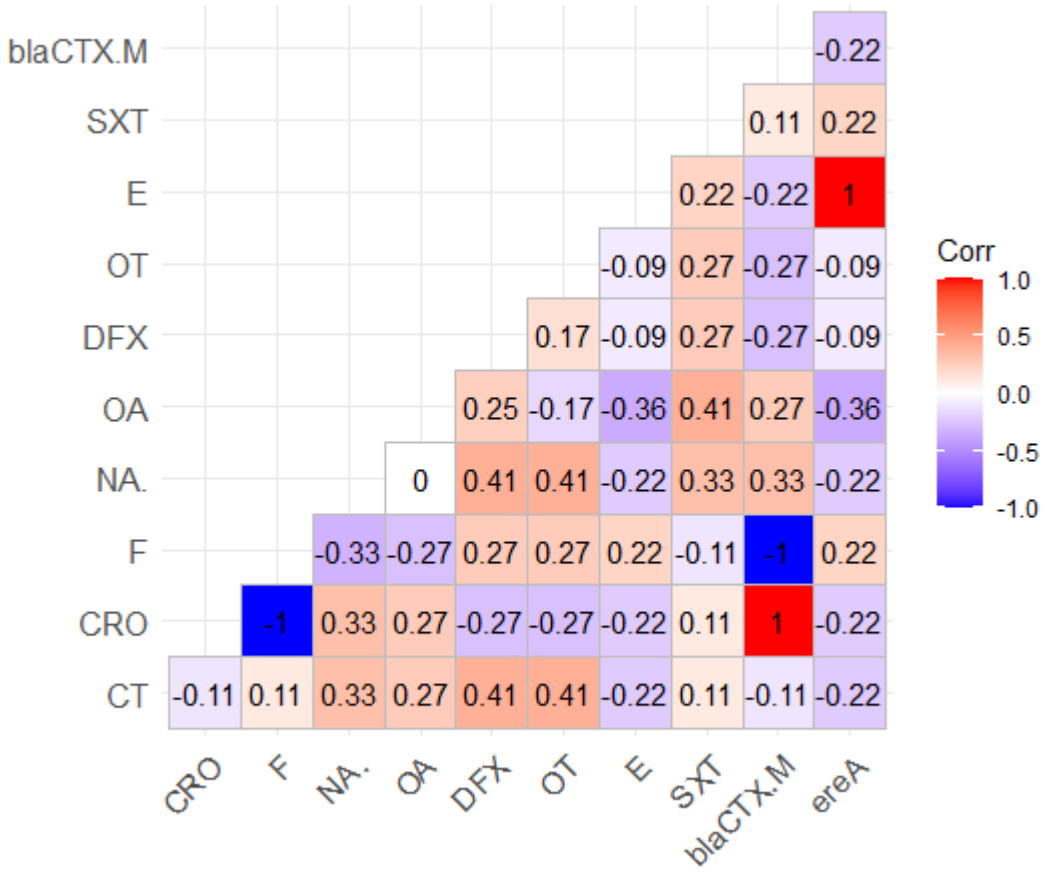

Fig. 5. Correlation analysis of antibiotics and resistance genes of *Aeromonas* spps. resistance genes

6. Sequencing of *gyr*A

At the genomic level our study reveals many key mutations in *gyrA* gene in *Aeromonas* spp. of *A. veronii*, *A. hydrophila*, and *A. caviae* in positions 83 and 87 that are known to associate with fluroquinolones resistance, as outlined in Table (2). The Predicted Functional Impact of QRDR Mutations using proven revealed deleterious score (below -2.5) in *A. hydrophila* S83L, *A. veronii* D87N and *A. caviae* D87Y. These mutations are known to confer fluoroquinolone resistance, while the prediction score was natural in *A. sobria* E84G (-0.7), associated with unknown significance mutation and *A. schubertii* L82P (-2.5) which is considered a novel mutation.

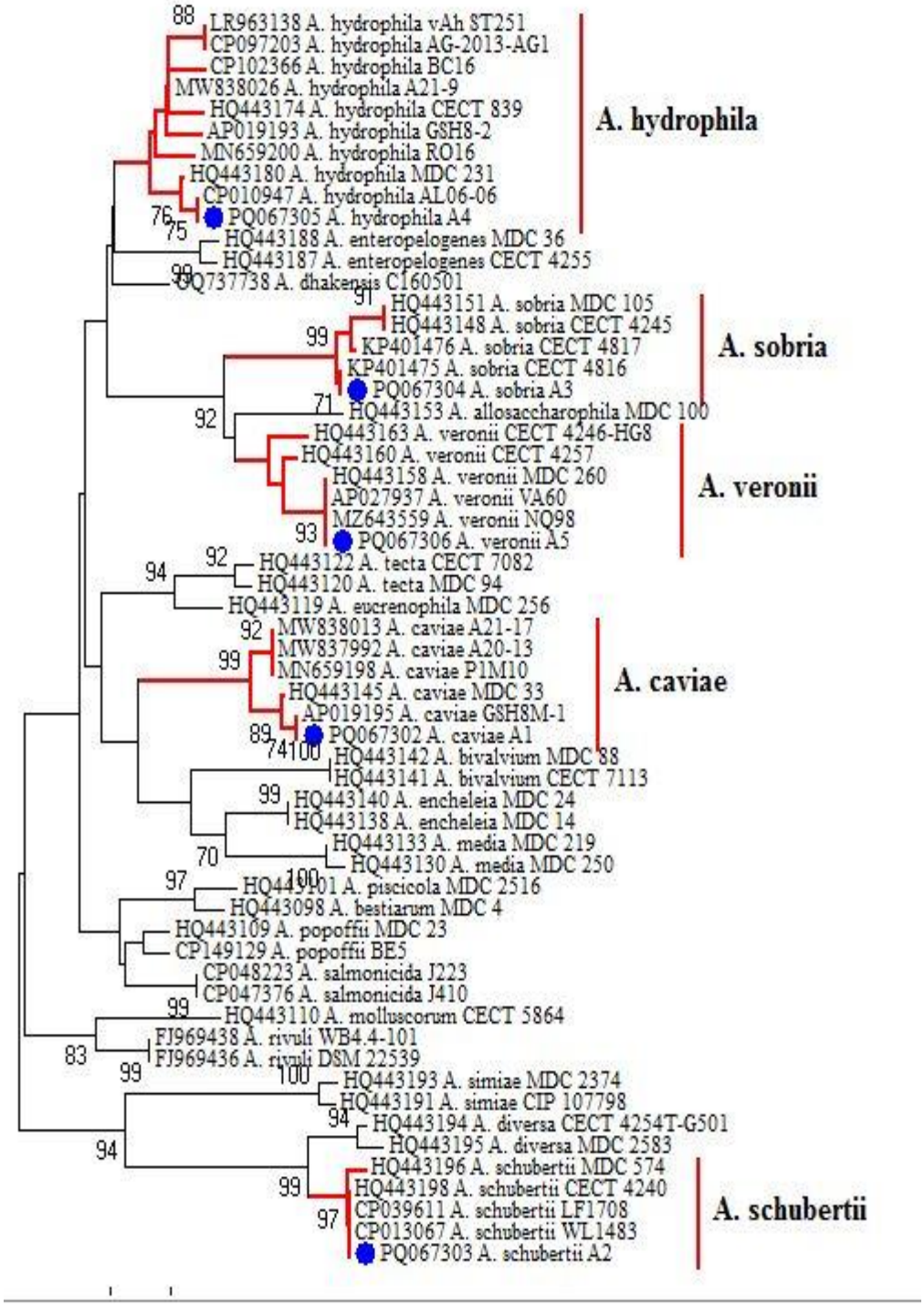

Fig. 6. Phylogenetic tree of *Aeromonas* spp. strains by general Gram- negative *gyrA* illustrating the connections of *Aeromonas* spp. with related species. The neighbor-joining approach was employed to construct a phylogenetic tree.

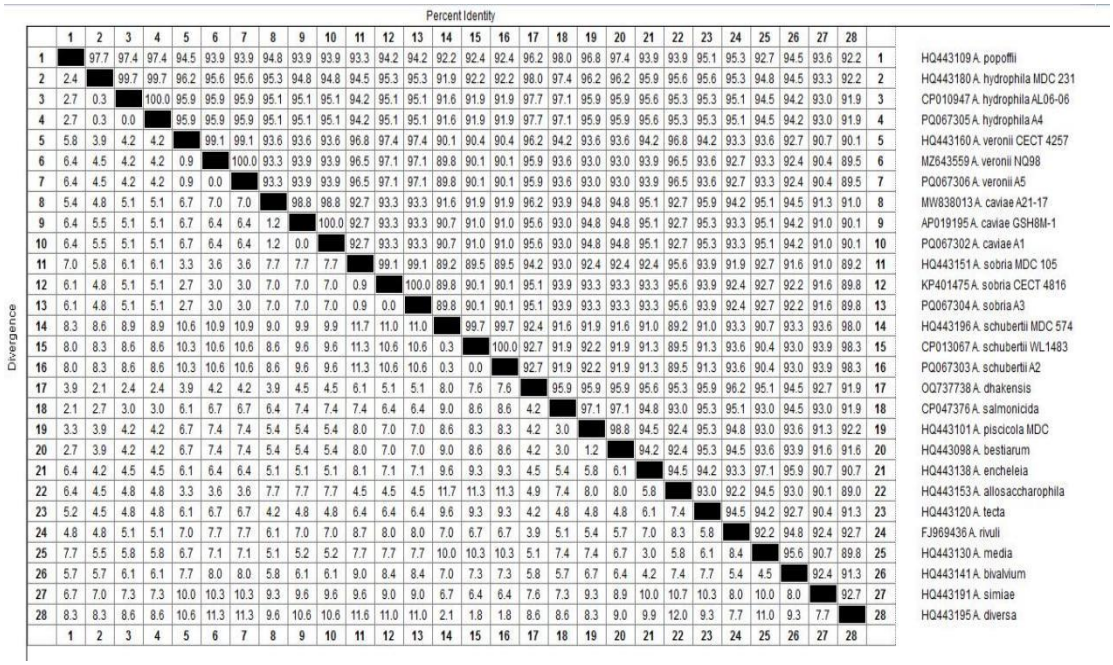

Fig. 7. Nucleotide identity

Table 2. Detecting SNPs in *gyrA* sequences and mutations of *Aeromonas* spp.

Species	Accession (Query)	Reference	SNP Position	Ref Base	Alt Base	Amino Acid Change	QRDR Mutation	PROVEAN Prediction	Resistance Association
A. hydrophila	**PQ067305**	**NC_008570.1**	**248**	**C**	**T**	**S83L**	**Yes**	**Deleterious (-4.2)**	**Confirmed (Cattoir, *etal*., 2008)**
A. veronii	**PQ067306**	**NZ_CP044060.1**	**260**	**G**	**A**	**D87N**	**Yes**	**Deleterious (-3.8)**	**High-level resistance (Vila,*etal*., 2002)**
A. sobria	**PQ067304**	**NZ_CDBW01000041**	**251**	**A**	**G**	**E84G**	**No**	**Neutral (-0.7)**	**Unknown significance**
A. schubertii	**PQ067303**	**NZ_LPUO01000034**	**245**	**T**	**C**	**L82P**	**No**	**Deleterious (-2.5)**	**Novel mutation**
A. caviae	**PQ067302**	**NZ_JAPQMO010000048.1**	**254**	**C**	**A**	**D87Y**	**Yes**	**Deleterious (-5.1)**	**Rare resistance (Piddock 1999)**

DISCUSSION

Aeromonas species are characterized as opportunistic pathogens capable of inducing immunosuppression and a spectrum of pathological symptoms, especially when host physiology is compromised by global environmental change **(Beaz-Hidalgo & Figueras, 2013)**. As emerging pathogens, *Aeromonas* species are a significant cause of economic loss in aquaculture, capable of instigating epidemic outbreaks that decimate fish stocks intended for commercialization **(Zhang *et al.*, 2020)**.

Infected fish *M. cephulus* and *S. solea* in our research suffered from septicemic lesions and general congestion of internal organs caused by *Aeromonas* spp., from *M. cephalus* fish, *A. hydrophila* was recorded with the highest isolated (4/70) strains with 5.7%, followed by *A. caviae, A. sobria,* and *A. schaberttii* (1/70), (1/70), and (1/70) strains with 1.4, 1.4, and 1.4%, respectively. While *A. veronii* was isolated from *S. solea* fish (1/30) strain (3.3%). All of *A. hydrophila, A. sobria, A. caviae, A. schabertii* and *A. veronii* isolates were recovered from internal organs (liver and kidney) of examined samples. In the study of **Kishk *et al*. (2020)** on *Mugil cephalus*, *A. sobria* was isolated at 44%, in addition to *A. caviae* (28%), *A. hydrophila* (20%), and *A. veronii* (8%). In this context, **Abdelsalam *et al*. (2021)** isolated *A. caviae* (*23.5%*), and *A. veronii* (14.7%) from the moribund Nile tilapia & African catfish from Manzala Lake. While **Sittien *et al.* (2024)** isolated *A. hydrophila* from 31.6% (18/57) of the kidneys, 28.1% (16/57) of the liver of moribund *O. niloticus*. For *A. veronii*, it was isolated at 27.33% from the examined *S. solea* off the Qarun Lake **(Abdellatief *et al.*, 2024)**.

Antibiotic resistance is an increasing concern in aquaculture. *Aeromonas* strains isolated from farmed African catfish (*Clarias gariepinus*) exhibited notable resistance to amoxicillin, ampicillin, and penicillin **(Adah *et al.*, 2024)**. *Aeromonas* spp. isolated from various aquatic sources in Malaysia showed that all isolates (100%) were resistant to sulphamethoxazole/trimethoprim, ampicillin, and novobiocin, while remaining susceptible to gentamicin, tetracycline, kanamycin, and oxytetracycline **(Odeyemi & Ahmad, 2017)**. *Aeromonas* strains isolated from freshwater fish—including sturgeon, carp, salmon, perch, and stickleback—exhibited resistance to ampicillin and amoxicillin (both 100%), imipenem (80%), kanamycin (45%), tetracycline (42.5%), trimethoprim/sulfamethoxazole (22.5%), gentamicin (20%), norfloxacin (15%), and ceftriaxone (12.5%). Notably, 42.5% of these strains showed multidrug resistance **(Leanovich *et al.*, 2025)**. Our finding has slightly different results recorded *A. hydrophila* displayed sensitivity to nitrofurantoin, ciprofloxacin, oxalinic acid and ofloxacin, whereas it exhibited great resistance to tetracycline and penicillin **(Nasser *et al.*, 2022; Thaotumpitak *et al.*, 2023; Sittien *et al.*, 2024)**. On the contrary, the isolates exhibited resistance to oxytetracycline, ampicillin, trimethoprim/sulfamethoxazole, erythromycin, amoxicillin/clavulanic acid, amoxicillin, gentamicin, lincomycin, and tetracycline **(Sarder *et al.*, 2016)**. The drug resistance results of *A. veronii* reveal the resistance to

ampicillin, nalidixic acid, oxanilic acid, and tetracycline **(Abellatief *et al*., 2024)**. **Lazado and Zilberg (2018)** found that *A. veronii* isolate was resistant to norfloxacin, oxytetracycline, and neomycin. In another study, **Abd El Latif *et al*. (2019)** stated that *A. veronii* was sensitive to sulfamethoxazole-trimethoprim and ofloxacin. These findings highlight the importance of choosing antibiotics carefully and using them responsibly in fish farms.

Sequence alignment using NCBI BLASTP program showed that (Figs. 6, 7) *A. hydrophila gyrA* PQ067305 had high genetic similarity OQ737738 (97.7%) with *A. dhakensis*; (97.1%) of *A. salmoncida gyrA* with accession-number CP047376.1 strain from the infected cultured sablefish from Canada. While *A. veronii gyrA* PQ067306 showed identity to (97.1%). KP401475 *A. sobria* CECT 4816; (95.6%) with HQ443153.1 *A. allosaccharophila* strain *gyrA* gene. In the case of *A. sobria gyrA*, PQ067304 showed high similarity (95.6%) to HQ443153.1 *A. allosaccharophila* strain *gyrA* gene. *A. caviae gyrA* gene with accession number PQ067302 showed identity (95.3%) of HQ443120.1 to *A. teca* strain *gyrA* gene from Spain. In respect to *A. Schubertii gyrA* (PQ067303), an identity of 98.3% was shown with (HQ443195.1) *A. diversa* strain *gyrA* from Spain. In this research, we examined mutations in the *gyrA* gene of different isolated *Aeromonas* species with a focus on their role in fluoroquinolone resistance. Our genomic analysis for sequences revealed that vital changes occurred within the quinolone resistance-determining region (QRDR), specifically at codons 83 and 87, which are well-recognized sites for resistance in Gram-negative bacteria, including *Aeromonas*.

The *gyrA* gene of *Aeromonas* isolated strains when compared with retrieved reference sequence revealed many SNPs that resulted in amino acids mutations in positions associated with action of gene function. We detected a mutation in S83L in *A. hydrophila*, D87N in *A. veronii*, and D87Y in *A. caviae*; these mutations have previously involved in a high-level of fluoroquinolones resistance by decreasing the binding affinity of DNA gyrase to the antibiotic molecule **(Vila *et al*., 1994; Ruiz, 2003)**. For *A .hydrophila*, an S83L has been detected in *gyrA* that is well known as a marker for fluoroquinolone resistance according to **Catoir *et al.* (2008)**. This substitution reduces the binding affinity of drugs, which leads to AMR **(Hooper *et al*., 2001)**. The *A. veronii* isolated strain showed a mutation in D87N, correlated with a high level of ciprofloxacin resistance, and the structural studies suggested that this change would lead to drug-DNA interaction disruption, as outlined by **Vila *et al.* (2002)** and **Drlica *et al.* (2009)**. The D87Y mutation is rare in *A. caviae* but has been linked to QRDR in other Gram-negative and *Aeromonas* species according to **Goñi-Urriza *et al.* (2002)**.

PROVEAN predicts a deleterious effect, supporting a resistance phenotype. For *A. sobria*, E84G mutation was recorded which is outside the classical QRDR, but may have an impact in reducing susceptibility according to a prior study **(Goñi-Urriza *et al*., 2002)**. The L82P mutation is novel, but its role in resistance remains unclear. In silico analysis suggests a destabilizing effect on *gyrA*. In *A. schubertii*, the L82P mutation is novel,

while its role in resistance remains unclear. In silico analysis, a destabilizing effect on *gyrA* is assumed.

All three mutations were predicted successfully by PROVEAN to have a deleterious functional effect in gene structure and function (scores below -2.5), strongly supporting their known role in fluoroquinolone resistance. These changes are consistent with previous studies showing that substitutions at these positions reduce DNA gyrase's ability to bind fluoroquinolones, leading to reduced drug efficacy **(Ruiz, 2003; Hooper & Jacoby, 2015; Yang *et al.*, 2021)**.

In contrast, the E84G mutation in *A. sobria* had a neutral PROVEAN score (-0.7) and is of unknown clinical significance. Since codon 84 is close to the QRDR core, it might be a neutral variant or possibly a compensatory change. We also found a novel mutation (L82P) in *A. schubertii* with a deleterious score (-2.5), borderline. This has not been previously reported in *Aeromonas* or other Enterobacteriaceae, making it an interesting candidate for further studies for its implication in gene function.

The combination of established resistance mutations (S83L, D87N, D87Y) and potentially novel changes (E84G, L82P) highlights the evolutionary adaptability of *Aeromonas* in response to antimicrobial pressure. Because fluoroquinolones are considered critically important antimicrobials by the WHO **(WHO, 2019)**, a concern is raised for AMR. Additionally, *Aeromonas* species can act as both opportunistic pathogens and reservoirs of AMR genes.

Our phylogenetic analysis located these strains within distinct, well-supported clades corresponding to their *Aeromonas* species. Importantly, strains carrying the same resistance-associated mutations clustered closely with reference strains from both clinical and aquaculture sources. This pattern suggests possible cloning and hints that aquaculture environments and marine fishes may serve as a reservoir for resistant strains, a finding that aligns with other reports **(Cabello *et al.*, 2013; Miranda *et al.*, 2018)**.

CONCLUSION

Overall, our study enhances the current understanding of QRDR mutations in *Aeromonas* by identifying key *gyrA* mutations in *Aeromonas* spp., with S83L, D87N, and D87Y, and highlights the essential need for integrated genomic surveillance in aquatic ecosystems. Future work should include plasmid analysis and broader genomic screening to link these mutations with actual resistance phenotypes and to assess their potential for horizontal gene transfer in *Aeromonas* species isolated.

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