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Clonal dispersion and pathogenic potential of multidrug-resistant *Aeromonas* spp. isolated from *Oncorhynchus mykiss* with hemorrhagic septicemia

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Abstract

This study was important to improve proper biosecurity measures and controlling the spread of *Aeromonas* to prevent future outbreaks. This research sought to determine whether virulent *Aeromans* species were present in morbid rainbow trout, their resistance and their genetic relatedness. A total number of 542 tissue lesion specimens were collected from gill, liver, heart and kidneys in morbid domesticated fish in Duhok province, Iraq. The *gyrB* DNA sequence analysis was used to determine the species classification. Drug susceptibility testing was conducted for all isolated strains using disc diffusion technique. The genotyping analysis was carried out using enterobacterial repetitive intergenic consensus-polymerase chain reaction. Thirty-four isolates were found and they were classified into three species (*Aeromonas veronii, Aeromonas sorbia,* and *Aeromonas allosaccharophila)*, where *A. veronii* stand as one of the most prevalent species. The most frequently affected organ by *Aeromonas* was the gills among four different organs. The detection frequencies of the virulence genes *aerolysin, outer membrane protein, glycerophospholipid-cholesterol acyltransferase, elastase, flagella, serine protease, cytotonic heat*-labile, and *hemolysin* were 100%, 100%, 79.41%, 64.70%, 76.47%, 67.64%, 70.58%, and 41.17, respectively. None of the strains possessed all of the virulence markers. All isolates were completely resistant to ceftazidime, amoxicillin and doxycycline. All isolates were found to be multi-drug-resistant. Regardless of the nearest geographic source area of samples and the same *Aeromonas* species, there was a high genetic diversity. The results of this study could help farmers and researchers make informed decisions about measures of biosecurity and proper therapeutic drugs to apply to prevent current outbreaks and prevent them from recurring again.

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Introduction

The predisposition of farmed fish to opportunistic infections, such as *Aeromonas* infection, is influenced by a number of factors, including a substantial amount of "stock" as well as improper methods of farming.[1] The "*Aeromonas* genus" that are more commonly implicated in fish diseases are *A. hydrophila, A. dhakensis, A. jandaei, A. sobria, and A. veronii.*[2,3]

Hemolysin, aerolysin and cytotonic enterotoxins are just some of the toxins produced by *Aeromonas* that can be damaging to its hosts.[4] It is therefore essential to discover the genes that confer virulence in *Aeromonas* in order to assess its pathogenicity potential and creating appropriate measures.[5] However, it should be added that it is difficult to properly determine the pathogen leading an outbreak when it occurs due to the complicated nature of *Aeromonas* genus' morphological and genetic features, which might result in cases of incorrect identification.[6] Numerous phylogenetic investigations were conducted to determine the *Aeromonas* species, utilizing the sequence markers to find the "*rpoD, 16s rRNA*, and *gyrB*" genes.[7,8]

The tracking of the infectious spread of diseases is important to prevent further spreads of pathogens.[9] Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) can allow for the rapid assessment of genetic diversity among bacterial strains. This can be done by analyzing the presence or absence of ERIC sequences. Researchers can create a profile or fingerprint for each strain.[10]

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In order to maintain fish health, it is necessary to continuously monitor patterns of antimicrobial resistance and the frequency of virulence genes among *Aeromonas* species. The present investigation also tried to figure out the genetic relationships among the isolated strains in order to locate the source of the infection.

Materials and Methods

Fish sampling and study area description. In Duhok province, Iraq, seven rainbow trout (*Oncorhynchus mykiss*) ponds were sampled from two independent farms (four ponds from Sheladize area: 1, 2, 3, and 4; and three ponds from Amedi districts: ponds 5, 6, and 7). Each farm was located in a distinct state of Duhok to give a wide sampling dispersion while also maximizing the recovery and diversity of isolated *Aeromonas* species. For Sheladeze's farm, the fish fingerlings were gated from Poland, and for Amedi's farm, the fingerlings were gated from Iran. A total number of 200 morbid or with clinical signs rainbow trout were collected. Each freshly dead fish was placed on a sterile plastic bag with a label and brought to the Duhok Research Centre at the University of Duhok. The sample took place between August 2021 and September 2022.

Sample processing and bacterial isolation. A total number of four distinct specimens were gathered, including gill (n = 200), heart (n = 114), liver (n = 114), and kidney (n = 114) from each fish from seven farms. To avoid contaminating the samples with indigenous flora, each area of the fish lesion was sampled under precise and sterile circumstances. The gills, kidneys, liver and hearts were extracted. Materials from each organ were collected with sterile cotton swabs and each organ was placed in a sterile petri dish and cut open with a sterile surgical blade to reveal the inside lesion. Each blade was changed out in each incision step. Each swab was immediately put onto tubes that contained 10.00 mL of brain-heart infusion broth (HiMedia, Thane, India) and were pre-enriched for 20 hr at 22.00 - 25.00 ˚C. One loopful from each pre-enriched sample was cultured on blood agar (Lab M, Lancashire, United Kingdom) with a previous incubation condition.[11] Colonies with characteristic clear hemolysis were subcultured on MacConkey agar (Lab M, Lancashire, UK). Pale (no lactose fermenter) colonies were recognized as *Aeromonas* by Gram's stain, indole production, oxidase test and urease test positive results and confirmed as a genus *Aeromonas* through the VITEK2 Compact system (Bio-Mérieux, Craponne, France) using the VITEK2 GN ID Card (Gram-Negative Identity Card). Presumptive *Aeromonas* colonies were stored as stock cultures at – 20.00 ˚C in brain-heart infusion broth with 25.00% glycerol. Finally, the *Aeromonas* species differentiation was carried out through PCR amplification and sequence analysis of the *gyrB* gene.[12]

DNA extraction. The DNA sample was extracted following the protocol applied by Ownagh *et al.*,[13] with minor modifications, in which 4 - 5 similar-morphology colonies were chosen and mixed with 500 µL nuclease-free water (Qiagen, Amtsgericht, Germany), then boiled for 12 min and ice-frozen for 5 min, after which it was directly centrifuged. The 400 µL supernatant were stored at – 20.00 ˚C for further analysis. The concentration and purity of extracted DNA were measured by a nanodrop spectrophotometer (2000c; Thermo Scientific, Waltham, USA).

***Aeromonas* species differentiation based on *gyrB* sequence analysis**. Specific primers (forward: 5'-TCCGGC GGTCTGCACGGCGT-3′; reverse: 5' TTGTCCGGGTTGTACTC GTC-3′) were employed to amplify the *gyrB* gene, with the resultant PCR product measuring around 1,124 bp.[14] The PCR reaction involved one cycle of pre-denaturation at 94.00 ˚C for 5 min before going through 35 amplification rounds of denaturation at 94.00 ˚C for 30 sec, annealing at 62.00 ˚C for 30 sec, extension at 72.00 ˚C for 1 min, and final elongation at 72.00 ˚C for 10 min. Sanger sequencing (Macrogen, Seoul, South Korea) was performed using PCR products as templates. Using BioEdit (version 7.2; Ibis Therapeutics, Carlsbad, USA), the raw data were trimmed and cleaned. The species identification was determined by comparing the cleaned sequences in the GenBank database to the appropriate *Aeromonas* species sequences using BLAST online service (www.ncbi.nlm.nih.gov/blst).

Virulence gene profile. The genes for *cytotonic heat-stable enterotoxin* (*ast*) (F-CGCCATCAACAGCTCGCCCA and R-CGGGCCTCGTTGAGGAAGCG),[4] *elastase* (*ela*) (F-ACACGG TCAAGGAGATCAAC and R-CGCTGGTGTTGGCCAGCAGG),[4] *glycerophospholipid-cholesterol acyltransferase* (*gcat*) (F-CTCCTGGAATCCCAAGTATCAG, R-GGCAGGTTGAACAGCAG TATCT),[11] *serine protease* (*ahpA*) (F-GGCAACGACCTCAA CCTCTG, R-CGAGCCTGGACGCACATT),[15] *hemolysin* (*hly*) (F-TCCAGCAGCAGGTAGTCCG, R-CCTCAACGTCAACCGCAAG),[15] *lipase* (*lip*) (F-CGCCGCATCTGTCGTGTT, R-TGGCATCCTTC TGCTCCTG),[15] *outer membrane protein* (*omp*) (F-TGGGCAT CAGCGACACTA, R-CAGCCATACCTTTGCTTACC),[15] *aerolysin* (*aer*) (F-CCTATGGCCTGAGCGAGAAG and R-CCAGTTCCAG TCCCACCACT),[16] *cytotonic heat-labile* (*alt*) (F-TGCTGG GCCTGCGTCTGGCGG, R-AGGAACTCGTTGACGAAGCAGG),[17] *flagella* (*fla*) (F-TCCAACCGTYTGACCTC, R-GMYTGGTTGC GRATGGT),[18] *cytotoxic heat-labile enterotoxin* (*act*) (F-ATCGTCAGCGACAGCTTCTT, R-CTCATCCCTTGGCTTGTT GT),[19"] were amplified for every single isolate using monoplex PCR.

The DNA fingerprinting and diversity analysis. The ERIC-PCR was employed to distinguish the related strains using primer sequences (ERIC1: 5'-ATGTAAGCTCCTGGG GATTCAC-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGC G-3') recently adopted from a previous study.[20] The PCR program was followed by Taha.[10] Subsequently, a software analysis using GelJ (version 2.0; https://source-forge.net/projects/gelj/) was done for creating a dendrogram.[21]

Strains exhibiting a correlation higher than 90.00% were allocated to a singular genotype.[22] The isolates were categorized according to the diversity of species and the source of isolation, specifically whether they originated from the same or different geographic locations.

Antibiotic-resistant testing. The Kirby-Bauer disc diffusion method for susceptibility testing was done on every isolate of *Aeromonas*. Ceftazidime (CAZ; 30.00 µg), amoxicillin (AMX; 35.00 µg), ticarcillin (TIC; 75.00 µg), ciprofloxacin (CIP; 5.00 µg), nitrofurantoin (NIT; 300 µg), oxytetracycline (OXY; 30.00 µg), norfloxacin (NOR; 30.00 µg), aztreonam (ATM; 30.00 µg) doxycycline (DOX; 10.00 µg) florfenicol (FLO; 30.00 µg), gentamicin (GEN; 10.00 µg), and trimethoprim/sulfamethoxazole (SXT; 25.00 µg) from Bioanalyse (Ankara, Turkey) were the selected drugs that were chosen to provide the best therapeutic choice for treating this bacterium in both human and veterinary settings. Following the Clinical Laboratory Standards Institute's criteria, zones of inhibition were evaluated and the results were explained in terms of bacteria that were sensitive (S) as well as resistant (R) to each selected antibiotic.[23] Every strain that was resistant to at least three distinct antibiotics was classified as having a multi-antibiotic resistance (MAR) feature.

Results

Based on cultural properties and some biochemical tests, non-lactose fermenters (pale colonies) on Mac-Conkey agar, beta-hemolytic colonies on blood agar, oxidase positive and Gram-negative, 34 strains were assumed to be *Aeromonas* spp. Then, VITEK2 Compact system was used to confirm the identification and the result showed that all of these strains were *Aeromonas* spp.

Species differentiation based on *gyrB* gene sequencing. *Aeromonas* isolates were precisely identified as *A. veronii, A. sorbia*, and *A. allosaccharophila* with 99.00 - 100% resemblance to the strains in the gene bank with the following accession numbers: OQ472288–OQ472319, OP713767 and OQ590003.

Prevalence of *Aeromonas* strain in fish farms and organs. The prevalence of 34 isolates was dispersed on 7 fish farms in which 4 (11.76%) *Aeromonas* sp. were isolated from each of farms 1, 3 and 4, while, 6 (17.64%) *Aeromonas* strains were isolated from each of farms 2 and 7. Also, 5 (14.70%) *Aeromonas* spp. were isolated from farms 5 and 6. The detection rates of *A. veronii* mostly occurred in the gill (64.70%; n = 22), while the detection rates of *A. veronii* in the liver were 14.70% (n = 5), in the kidney were 8.82% (n = 3) and in the heart were 5.88% (n = 2). The detection rates of *A. allo-saccharophila* and *A. sorbia* were only found in the gill at 100% (n = 1 each; Table 1).

The virulence profile of the isolated strains. Table 2 displays the virulence gene characteristics of the recovered *Aeromonas* strains. Overall, *Aeromonas* isolates (n = 34). Recognition levels of genes for virulence *aer, omp, gcat, ela, fla, ahpa, alt*, and *hly* were 100%, 100%, 79.41%, 64.70%, 76.47%, 67.64%, 70.58%, and 41.17% respectively. All 34 strains carried both *aer* and *OMP.* Neither of the isolates contained all of the virulence genes (*ast, lip,* and *act* genes were not found in all strains). A total number of 20 virulence profiles were distributed among the 34 isolates. Each isolate carried at least three genes. There were 2/34 (5.88%) isolates that had eight genes. In contrast, 12/34 (35.29%) isolates carried seven genes. All (14/34; 41.17%) of the isolates contained seven or more genes, all of which belonged to *A. veronii*. Likewise, three to eight virulence genes were found in 32 of 34 (94.11%) *A. veronii* strains. While only one isolate of *A. allo-saccharophila* harbored five genes: *aer, ahpa, omp, ela*, and *gcat* (1/34; 2.94%), nevertheless, one isolate of *A. sobria* (1/34; 2.94%) had six virulence genes: *aer, omp, ahpa, alt, gcat,* and *hlyA*.

Susceptibility testing of the tested antibiotic. The outcomes are presented in Table 3. All isolates were completely resistant to CAZ, AMX, and DOX (100%; 34/34). All strains were lacked the resistance trait against CIP. The majority of *Aeromonas* strains remained susceptible to OXY, NOR, ATM, and SXT, with resistance rates of 11.76% (4/34), 2.94% (1/34), 5.88% (2/34), and 5.88% (2/34), respectively. All isolates were found to be multi-drug resistant. The thirty-four isolates were classified into 17 resistant categories. Each isolate possessed at least four resistance traits. There were 10 resistant traits carried by (1/34; 2.94%) isolate, eight resistant traits carried by (1/34; 2.94%) isolates, seven resistant traits carried by (7/34; 20.58%) isolates, six resistant traits carried by

Table 1. Distribution of different *Aeromonas* species according to fish farms in the study area and fish organs.

Geographic areas	*Aeromas* species and fish organ			Total No. (%)
	Veronii	*Sorbia*	*allosaccharophila*	
Farm1 (n = 4)	4(100%): 3 gills, 1 liver	-	-	4 (11.76)
Farm2 (n = 6)	6(100%): 4 gills, 2 livers	-	-	6(17.64)
Farm3 (n = 4)	4(100%): 2 gills, 1 heart,1 kidney	-	-	4 (11.76)
Farm4 (n = 4)	4(100%): 3 gills, 1 heart	-	-	4 (11.76)
Farm5 (n = 5)	5(100%): 3 gills, 1 liver, 1 kidney	-	-	5(14.70)
Farm6 (n = 5)	4(80.00%): 4gills	-	-	5(14.70)
Farm7 (n = 6)	5 (83.33%): 3gills, 1 kidney, 1 liver	1(16.66%): 1 gill	1(20.00%): 1 gill	6(17.64)
Total	32(94.11%) 22 gills, 2 heart, 5 livers, 3 kidneys	1(2.94%)	1 (2.94%)	34 (100)

Table 2. Virulence gene profile of 34 *Aeromonas* species from rainbow trout *(Oncorhynchus mykiss*) with hemorrhagic septicemia

Profile	*aer* (100%)	*omp* (100%)	*gcat* (79.41%)	*ela* (64.70%)	*fla* (76.47%)	*ahpa* (67.64%)	*alt* (70.58%)	*hlyA* (41.17%)	No. of carried genes	No. of isolates	species
1	+	+	+	+	+	+	+	+	8.00	2.00	*A. veronii*
2	+	+	-	+	+	+	+	+	7.00	2.00	*A. veronii*
3	+	+	+	+	+	+	+	-	7.00	6.00	*A.veronii*
4	+	+	+	+	+	+	+	-	7.00	2.00	*A. veronii*
5	+	+	+	+	+	+	-	+	7.00	1.00	*A. veronii*
6	+	+	+	+	+	-	+	+	7.00	1.00	*A. veronii*
7	+	+	+	+	-	+	-	+	6.00	1.00	*A. veronii*
8	+	+	+	+	+	-	+	-	6.00	1.00	*A. veronii*
9	+	+	+	-	+	+	+	-	6.00	1.00	*A. veronii*
10	+	+	+	+	+	-	+	-	6.00	1.00	*A. veronii*
11	+	+	+	-	-	+	+	+	6.00	1.00	*A. sobria*
12	+	+	-	-	+	+	+	+	6.00	1.00	*A. veronii*
13	+	+	+	-	+	-	+	+	6.00	4.00	*A. veronii*
14	+	+	+	+	-	+	-	-	5.00	2.00	*A. veronii, A. allosaccharophila*
15	+	+	+	-	-	+	+	-	5.00	1.00	*A. veronii*
16	+	+	+	+	-	+	-	-	5.00	2.00	*A. veronii*
17	+	+	-	+	+	-	+	-	5.00	1.00	*A. veronii*
18	+	+	+	-	+	-	-	-	4.00	2.00	*A. veronii*
19	+	+	-	-	+	+	-	-	4.00	1.00	*A. veronii*
20	+	+	-	-	-	-	-	+	3.00	1.00	*A. veronii*

Table 3. Antibiotic-resistant profile of 34 *Aeromonas* strain isolated from *Oncorhynchus mykiss* suffering from hemorrhagic septicemia

Profile	CAZ (100%)	DOX (100%)	AMX (100%)	TIC (94.11%)	CIP (0.00%)	NIT (64.70%)	OXY (11.76%)	NOR (2.94%)	ATM (5.88%)	FLO (38.23%)	GEN (73.52%)	SXT (5.88%)	No. of resistant traits	No. of isolates	Species
1	R	R	R	R	S	R	R	R	R	R	R	S	10.00	1.00	*A. sobria*
2	R	R	R	R	S	R	R	S	S	R	R	S	8.00	1.00	*A. veronii*
3	R	R	R	R	S	R	S	S	S	S	R	R	7.00	1.00	*A. allosaccharophila*
4	R	R	R	R	S	R	S	S	S	R	R	S	7.00	2.00	*A. veronii*
5	R	R	R	R	S	R	R	S	S	S	R	S	7.00	2.00	*A. veronii*
6	R	R	R	R	S	R	S	S	S	R	R	S	7.00	2.00	*A.veronii*
7	R	R	R	R	S	R	S	S	S	S	R	S	6.00	6.00	*A. veronii*
8	R	R	R	R	S	S	S	S	S	R	R	S	6.00	2.00	*A.veronii,*
9	R	R	R	S	S	R	S	S	S	R	R	S	6.00	2.00	*A. veronii*
10	R	R	R	R	S	R	S	S	S	R	S	S	6.00	1.00	*A. veronii*
11	R	R	R	R	S	S	S	S	R	S	R	S	6.00	1.00	*A. veronii*
12	R	R	R	R	S	S	S	S	S	S	R	S	5.00	4.00	*A. veronii*
13	R	R	R	R	S	R	S	S	S	S	S	S	5.00	4.00	*A. veronii*
14	R	R	R	R	S	S	S	S	S	R	S	S	5.00	2.00	*A.veronii*
15	R	R	R	R	S	S	S	S	S	S	S	R	5.00	1.00	*A. veronii*
16	R	R	R	R	S	S	S	S	S	S	R	S	5.00	1.00	*A. veronii*
17	R	R	R	R	S	S	S	S	S	S	S	S	4.00	1.00	*A. veronii*

CAZ: ceftazidime, AMX: amoxicillin, TIC: ticarcillin, CIP: ciprofloxacin, NIT: nitrofurantoin, OXY: oxytetracycline, NOR: norfloxacin, ATM: aztronam, DOX: doxycycline, FLO: florfenicol, GEN: gentamicin, and SXT: trimethoprim/sulfamethoxazole. R: resistant, S: susceptible.

(12/34; 35.29%) isolates, five resistant traits carried by (12/34; 35.29%) isolates, and four resistant traits carried by (1/34; 2.94%) isolates. When compared to other species, *A. sobria* had an extreme resistance profile (resistant to ten drugs; Table 3).

ERIC-PCR molecular typing. The ERIC-PCR fingerprinting analysis yielded that the genetic similarity among the 34 *Aeromonas* strain ranged from 55.00 to 96.00%. Thirty-three unique single genotypes were identified out of 34 strains. Nevertheless, it was observed that two strains of *A. veronii*, derived from separate ponds (2 and 3) within the same geographical vicinity, were determined to share the same genotype (genotype 2). Despite originating from the same geographical region (the same farm) and belonging to the same *Aeromonas* species, the remaining strains (n = 32; 94.11%) were classified as a singular genotype due to the notable genetic variation observed among these three *Aeromonas* species (Fig. 1).

Discussion

Our understanding of *Aeromonas* identification at the species level has improved as a result of the use of sequencing analysis of the *gyrB* gene in this study. Based on some investigations, the *Aeromonas* could not be identified at the species level by conventional bio-chemical indicators (API systems or VITEK compact systems). This is partly due to the fact that there are more than 18 species of *Aeromonas* and that there is now no precise biochemical or phenotypic scheme to distinguish among them.[12,24] As a result, we employed the VITEK approach to identify the *Aeromonas* at the genus level and the *gyrB* gene sequence analysis to distinguish between species.

A higher isolation rate of different *Aeromonas* species was seen. This could be attributed to the human contribution of bacterial transmission in aquatic environments.[25] Another important note seen during sampling

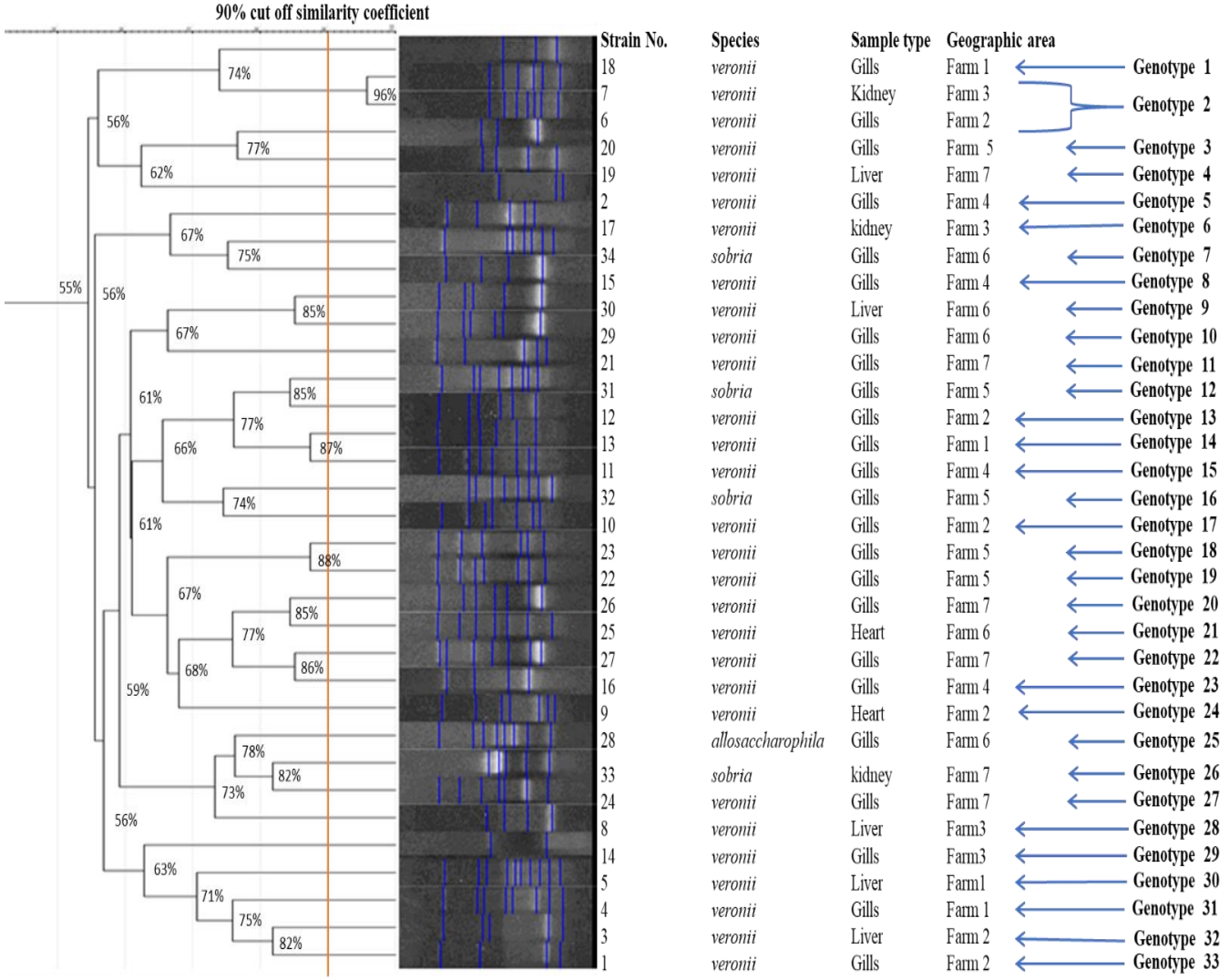

Fig. 1. The enterobacterial repetitive intergenic consensus-polymerase chain reaction dendrogram displays the banding patterns of the *Aeromonas* strains isolated from hemorrhagic septicemia cases in rainbow trout (*Oncorhynchus mykiss*).

was that different fish types from the neighboring river were taken and mixed with salmon without taking any consideration of this risky contamination factor, which in turn could be another source of *Aeromonas* contamination to the farms.[26]

In aquatic ecosystems, *Aeromonas* species are thought of as pathogenic opportunistic organisms. Therefore, any changes in water parameters could result in immunological stress situations that would make fish more susceptible to *Aeromonad* infection.[27]

Aeromonas veronii was the most frequent *Aeromonas* species seen in this study. This might be a sign of an emerging infection by *A. veronii* in fish farms in Duhok, Iraq. The species dominance could be explained by the fact that it is a mesophilic species, meaning it thrives best in an intermediary climate.[2] For growth, salmon require cold water temperatures.[28] On the other hand, *A. allosaccharophila* was isolated for the first time from the fish farm in Iraq. This might be due to transmission with the fingerlings that have been imported from the source country.[29,30]

On the other hand, the detection rates of *Aeromonas* species were mostly seen in gills. This could be due to the fact that the gill is an external organ of fish and has direct contact with the water environment and the possibility of infection in this organ with this bacterium is high.[31]

In order to confirm their potential pathogenicity, the present investigation examined the virulence gene harboring status of distinct *Aeromonas* strains. The results showed that the majority of them contained seven or more genes, all of which belonged to *A. veronii*. A study indicated that the greater the number of virulence genes possessed by the strain, the lower the median lethal dose and the greater the pathogenicity[32] indicating that the epidemic caused by these strains may be the consequence of a synergistic combination of these virulence factors, resulting in the severity of infection. As a result, we were able to confirm that the number of virulence genes carried by *A. veronii* might be positively associated with pathogenicity.

The pore-forming aerolysin-related *aer* gene was detected in all (100%) tested *Aeromonas* strains, and this data was compatible with the results of hemolysin production in blood agar as all isolates showed hemolytic activity on blood agar at the primary isolation step, whereas, the *hlyA* gene was detected in only 14 isolates. Because both of these genes were equally important for *Aeromonas* virulence (hemolysis), their deletion resulted in a marked decrease in the pathogenic potential of the bacterium.[33]

All *Aeromonas* strains were found to be completely resistant to CAZ, AMX and DOX. Since these antimicrobial drugs are employed in both aquatic and human clinical preventative and therapeutic efforts, this finding definitely demonstrated the possibility of selection resistant mechanisms in these settings.[34] Ceftazidime was never used in aquatic environments, either therapeutically or preventatively, although *Aeromonas* species frequently possess chromosomal β-lactam-resistant genes has been investigated.[35] This resistance may have developed due to prior exposure in human medicine because this antibiotic was extensively used in humans to treat serious infections by gram-negative bacteria that are resistant to antibiotics.[36] This resistance may have been spread to aquatic habitats by human effluent (there were tourist sites located near these fish ponds) or rivers with beta-lactam-resistant genes[37] or due to the presence of residual antibiotics in the water supplies used by these fish farms resulting in pollution of aquatic ecosystems.[38]

Amoxicillin and tetracyclines, including DOX, are the two most often prescribed categories of antibiotics in fisheries owing to their inexpensive cost and incredible efficacy as broad-spectrum antibiotics for both the treatment and prevention of infections.[39] As a result, resistance to this drug was expected as a result of selective pressure. Although none of the strains showed positive results for CIP resistance, recent research indicates that *Aeromonas* species from aquatic environments may have acquired CIP resistance.[40]

An alarming level of multi-antibiotic resistance in the present study suggested that a highly probable source of contaminants was where the *Aeromonas* strains were derived from and that different antibiotic categories might have been used as a preventative measure in these farms to improve fisheries or to decrease the chance of the occurrence of infections[41], or it could be attributed to the dissemination of microbes that were resistant to antibiotics from animal waste used as fertilizer.[42]

This study showed that *A. sobria* exhibited a higher resistance profile when compared to other species. This could be attributed to the greater capacity of this species for the acquisition of resistant plasmids carrying multi-resistant genes from the surrounding microflora in the environment than the other species.[43]

In terms of strain diversity and similarity, the majority of them were clustered in a single genotype, regardless of the same geographic source location or *Aeromonas* species. The significant amount of genetic variation suggests that multiple clones of a single species of *Aeromonas* are circulating in these fish farms, which might originate in various geographic spots (anthropogenic effects and human wastewaters).[44] Other reasons for this diversity might be due to animal or bird manure[45,46], as there is no such restricted border line surrounding these farms. Additionally, the entrance of different *Aeromonas* strains from the surrounding environment to these farmed ponds might come with the flooding.[47] All of the above-mentioned factors may collectively be responsible for the higher genetic diversity of strains in this study.

Aeromonas classification based on *gyrB* gene sequence comparison was successfully approved to be a reliable diagnostic approach, which is crucial to avoid incorrect identification. As a result, fisheries can benefit from the study findings, and researchers can take immediate biosecurity precautions using the appropriate therapeutic or preventative medications to treat existing outbreaks or avoid future outbreaks.

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Conflict of interest

The authors declare that there is no conflict of interest that affects the publication of this work.

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