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Molecular detection and resistance profiling of aminoglycoside-modifying enzymes in *Aeromonas hydrophila* isolated from different clinical cases

Hadeel Turki Hussein

Department of Biology, College of Education, University of Al-Qadisiyah, Iraq. E-mail: edu.bio.posta139@qu.edu.iq

Azhar Noory Hussein

*Corresponding author: Department of Biology, College of Education, University of Al-Qadisiyah, Iraq. E-mail: azhar.almousawi@qu.edu.iq

Abstract

Objective

Aeromonas hydrophila is an emerging opportunistic pathogen that poses a serious public health concern due to its increasing resistance to numerous antibiotics, containing aminoglycosides. This study was designed to detecting the phenotypic and genotypic features of *A. hydrophila* strains obtained from clinical (blood) and environmental samples in Al-Diwaniyah, Iraq, with a focus on mechanisms responsible for aminoglycoside resistance.

Materials and Methods

Among a total of 200 samples screened, 26 isolates were identified as A. hydrophila using classical microbiological techniques along with VITEK 2 Compact identification system. Isolates were investigated for resistance to aminoglycosides (kanamycin, Kna; gentamicin, Gen; amikacin, Ami). The isolates were found to be resistant to the three antibiotics tested, i.e., neomycin, tobramycin and streptomycin (isolates had low to intermediate levels of resistance). The resistance pattern of these isolates suggests an ominous scenario involving both first- and second-line aminoglycosides.

Results

A total of 5 aminoglycoside-modifying enzyme (AME) genes were identified by PCR, which were distributed in the isolates. The most common genes wereaac(3) (80%), aph(3')-Ia (65%) and aph(6)-Id (60%). Other genes, encoding aac(3)-IIa, aac(6')-Ib, aac(3)-IV and ant(4')-IIa were also observed at different frequencies, demonstrating a complex of resistance determinants to the tested aminoglycosides.

Conclusions

Significantly, high-level phenotypic resistance correlated to the concomitant presence of multiple AME genes. Strains carrying 4 or more resistance genes, were pan-resistant to all

aminoglycosides tested (except 1 that was also resistant to another one known plasmid mediated AG-r) Conversely, these findings serve to highlight the importance of regional anti-microbial stewardship and local molecular surveillance programs in assessing the emergence and spread of resistant A. hydrophila clones. The novel finding of the multiple AME genes in clinical as well as environmental isolates is worrisome and suggests that therapeutic failures may occur, underlining transferable resistance determinants between aquatic and clinical systems.

Keywords: aminoglycoside resistance, AME genes, antimicrobial resistance, environmental isolates, PCR

Paper Type: Research Paper.

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Introduction

Aeromonas hydrophila is a virulent, Gram-negative bacterium which has been identified as saprophyte and resident in aquatic environments but highly associated with various diseases in both humans and aquatic animals (Pablos et al. In aquaculture, it is only recognized as the cause of hemorrhagic septicemia, an important systemic infection in fish conspiring to serious economic losses worldwide. It can cause gastroenteritis, wound infections, septicemia and urinary tract infections in humans particularly the Immunocompromised Citizens. Its ecological versatility allows it to live in freshwater, estuarine and wastewater systems. This capacity to survive in a variety of environments coupled with high rates of detecting A. hydrophila is an important concern due to its wide spread increase of resistance against antimicrobial agents, that makes it a candidate for public health care globally (Sleman & Farj, 2024; Abo-Shama et al., 2025). Contemporary therapeutic challenge of A. hydrophila is resistance to aminoglycosides which has begun over the last few years. Aminoglycosides are primarily used as first-line antibiotics for infections caused by Gram-negatives —limited only to infections of the urinary and respiratory tracts. Mechanism of action: They bind to the 30S ribosomal subunit because of which the protein

synthesis in bacteria is disrupted. Yet as they have spread globally, the efficacy of these drugs has consistently diminished because of the dissemination of aminoglycoside-modifying enzymes (AMEs), which render them inactive by modifying their antibiotic structure through acetylation, phosphorylation or adenylation (Zhang et al., 2023; Thacharodi and Lamont, 2022). These resistance enzymes are characterized by three major superfamilies; N- acetyltransferases (AAC), O-phosphotransferases (APH) and O-nucleotidyltransferases (ANT) which facilitate various enzymatic modifications that results in weakened antibiotic binding (Sun, 2025). In A. hydrophila, AME genes exist and distribute differently as to environmental impact of geographical areas. Type of AME genes was obscure, nonetheless, the aac(6')-Ib and aph(3')-Ia were recognized in various continents from human samples while ant (3")-Ia has tackled as most opaque announced gene in clinical and environmental isolates. Newly described resistance genes such as aac(6′)-Va, with exceptionally high catalytic efficiency against ribostamycin and other aminoglycosides, have been discovered more recently, thereby heightening anxiety about therapeutically related non-response (Zhang et al.localctx 22023). These resistance determinants are frequently carried by mobile genetic elements including plasmids, integrons and transposons favoring gene dissemination between bacteria such that the oblique expansion of resistance genes is enhanced (Jacobs & Chenia, 2007; Marti & Balcázar, 2012). Multidrug-resistant Aeromonas hydrophila has been frequently documented in aquaculture environments across various Iraqi provinces, including Basra, Duhok, and Salah Al-Din. Strains isolated from these areas have demonstrated resistance to key antimicrobial agents, particularly gentamicin, imipenem, and several other aminoglycosides. (Othman et al., 2017; Al-Haider et al., 2019). The concerning development is supposedly caused by overuse and misuse of antibiotics in aquaculture particularly the rampant administration of antibiotics into fish feed. These practices exert a selective pressure which favours the survival and spread of resistant bacterial strains (Weli & Al-Rasheed, 2020). In order to address and control the antibiotic resistance in A. hydrophila, it is necessary to understand the genetic mechanism of its resistance behaviors in details. Conventional microbiology and biochemical methods do not always achieve the necessary specificity and sensitivity for accurate bacteriophage diagnosis, particularly where mixed infections are encountered or from complex environmental reservoirs (Ahsani et al., 2010; Mohammadabadi et al., 2004; Khabiri et al., 2025). Molecular diagnostic methods, especially polymerase chain reaction (PCR) are indispensable for fast and accurate detection of antimicrobial resistance genes (Mohammadabadi et al. 2011; Khabiri et al. 2023; Mohammadabadi et al., 2025). PCR represents a molecular technique that dates back to the early developments in genetic research, initially designed to enable the amplification of specific gene sequences within a matter of hours (Shahdadnejad et al. 2016; Mohammadabadi et al. 2024), outperforming classical culture-based

techniques (Nakamura et al., 2011; Arora & Poddar, 2020; Golden et al., 2020). WGS and more advanced techniques enhance our ability to identify new resistance genes, enabling us to track their epidemiological spread (Armstrong et al., 2024; Mustafa et al., 2025). Accordingly, the present study was aimed to study the molecular detection and prevalence of AME genes among A. hydrophila isolates from aquaculture sources in Iraq. This study aims to fill this knowledge gap in antibiotic resistance among bacterial pathogens of the aquatic environment by combining molecular diagnosis with antimicrobial susceptibility profiling and contribute to the global fight against antimicrobial resistance (AMR) in aquaculture.

Materials and methods

Sample collection: A total of 200 samples including clinical and aquatic isolates were collected from DG province, Iraq throughout the period from October 2023 till January 2024 for genetic studies. The specimens comprised wound swabs, freshwater fish tissues, and water samples. All samples were transported anaerobically in sterile containers at 4 °C and processed within 24 hours of collection applying standard microbiological methods (Eid et al., 2022).

Isolation and identification of *Aeromonas hydrophila:* Samples were pre-enriched in alkaline peptone water and incubated at 28 °C for 18–24 hours. Subcultures were streaked onto Rimler-Shotts agar and Aeromonas selective agar (Oxoid, UK) supplemented with ampicillin (10 µg/mL). Colonies exhibiting typical morphology were selected and examined microscopically applying Gram staining. Subsequent biochemical experiments included oxidase, catalase, citrate utilization, indole production, and glucose fermentation, as described by Rahman et al. (2021). Final identification was affirmed applying the VITEK 2 Compact system (bioMérieux, France), following the protocol of Abo-Shama et al. (2025).

Antimicrobial susceptibility testing: The antimicrobial susceptibility of the bacterial isolates was assessed using the disk diffusion technique on Mueller–Hinton agar (HiMedia, India), conducted in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2021). The panel of aminoglycoside antibiotics tested in this study comprised gentamicin (10 µg), kanamycin (30 µg), amikacin (30 µg), tobramycin (10 µg), neomycin (30 µg), and streptomycin (10 µg). Following incubation at 37 °C for a period ranging from 18 to 24 hours, inhibition zone diameters were measured and interpreted based on the breakpoint values provided by CLSI, as reported by Fauzi et al. (2021).

DNA extraction: For genomic DNA isolation, overnight cultures grown in nutrient broth were processed using the boiling lysis method. The collected bacterial pellets were resuspended in 200 µL of TE buffer and subjected to thermal treatment at 95 °C for 10 minutes. After

centrifugation at 10,000 rpm for 5 minutes, the supernatant containing the genomic material was carefully transferred and used as the DNA template. The extracted DNA's concentration and purity were evaluated using a NanoDrop spectrophotometer, following the procedure described by Leanovich et al. (2025).

PCR amplification of aminoglycoside resistance genes: Using standard PCR, aminoglycoside resistance genes were amplified for detection of specific resistance types including aac(3)-IV, aac(3)-IIa, aph(6)-Ia, aph(3')-Ib, acc(3), aac(6')-Ib and ant(4')-IIa. The primers required for this are designed by NCBI's Primer-BLAST so that they will bind only to the correct DNA sequences with no non-specific or unintentional results. The PCR amplification was carried out in a reaction mixture of 25 µL, containing 12.5 µL of GoTaq® Green Master Mix (Promega, USA), 1 µL each primer, 2 µL bacterial DNA and nuclease-free water to complete the volume. The PCR machine was then preprogrammed to start by initially heating at 94 ° C for 5 min in order to open the DNA strands. Next, it went through 35 cycles of heating the DNA to 94°C for 30 seconds (to break up the strands), cooling it to a temperature between 52-58°C (depending on the primer) for 30 seconds (so that primers can attach), then back up to 72°C for an additional minute and half so the DNA could replicate. Finally, the machine was maintained at 72 °C for 7 min to complete with any ongoing DNA synthesis. Then the PCR products were loaded onto a 1.5% agarose gel and ran in a TBE buffer solution at 100 volts for approximately one hour. The gel was then stained with ethidium bromide (0.5 µg/mL) and visualized using UV light for DNA bands. A 100 base-pair DNA ladder (Thermo Scientific™) was loaded in one lane to obtain the sizes of the amplified gene fragments. The primer sequences, their product band sizes and annealing temperatures are shown in Table 1.

Table 1. Primer Sequences, Product Sizes, and Annealing Temperatures

Gene	Primer Sequence (5′–3′)	Product Size (bp)
aac(3)-IV	F- 5`-GGTGCCGACCTTCAGTTACA-3` R- 5`- GACAGGTCGCGCACATAGTA-3`	402
aac(3)-IIa	F- 5`- CAAGATAAAGTCGCGGCACG-3` R- 5` CACGACTGTTTCGGTCAGGA-3`	339
aph(6)-Ia	F- 5`- ACTCCTGCAATCGTCAAGGG-3` R- 5`-GGATCTATCACCAGCCAGCC- 3`	464
aph(3')-Ib	F- 5`- ATCGCGTATTTCGTCTCGCT-3` R- 5`- GGAGAAAACTCACCGAGGCA-3`	292
acc (3)	F- 5`- TCACCTCGGGGCAAGCCACACC-3` R- 5`- TTGGTGAATCGCATTCTGAC -3`	183
aac(6')-Ib	F-5`-CATATCGTCGAGTGGTGGGG-3` R- 5`- CTTGGTTCCCAAGCCTTTGC-3`	264
ant(4')-IIa	F-5`-ATGACTTGCCTTCCACTCCG-3` R- 5`- CAAACCGCTTACCCACCTCT-3`	388

Gel electrophoresis: PCR products were analyzed applying 1% agarose gel electrophoresis. Gels were prepared in 1× TBE buffer and stained with ethidium bromide (0.5 µg/mL). Electrophoresis was carried out at 100 V and 80 mA for 60 minutes applying the Mini-Sub Cell GT system (Bio-Rad, USA). A DNA marker ranging from 100 to 3000 bp (Promega, USA) was included to estimate the sizes of amplified products. DNA bands were visualized under a UV transilluminator, and gel images were captured for documentation and further analysis (Plattner et al., 2020; Woo et al., 2022).

Results

Sample collection: A total of 200 clinical specimens were gathered from patients aged 7 to 35 years, either hospitalized at Al-Diwaniyah Hospital or attending private clinics between December 1, 2024, and March 3, 2025. Following cultivation on numerous selective and differential media, 26 samples (13%) yielded positive bacterial growth, while 174 samples (87%) showed no detectable microbial growth. Among the culture-positive samples, diarrheal specimens exhibited the highest recovery rate (14.5%), followed by urine (17.5%), blood (9.1%), and wound swabs (5.6%). Notably, no bacterial growth was observed in samples achieved from burn wounds.

Isolation and identification-Morphological identification: Preliminary identification of *Aeromonas hydrophila* was based on colony morphology on selective and differential media. On Chromagar Orientation, colonies appeared mucoid, pink, and diffuse with indistinct margins due to β-glucuronidase activity. On MacConkey agar, the isolates formed non-lactose fermenting, pale colonies with smooth, elevated surfaces. On blood agar, colonies were creamy to grayish, circular, and demonstrated β-hemolysis, indicative of hemolysin production. On nutrient agar, the bacteria generated medium to large, white-to-creamy colonies with a faint, mold-like or non-distinct odor.

Microscopic diagnosis: Gram staining revealed short, rod-shaped, Gram-negative bacilli with rounded ends, arranged singly or in pairs. These microscopic characteristics are consistent with those described by Abo-Shama et al. (2025).

Biochemical characterization: Biochemical assays affirmed the phenotypic identity of the isolates. All isolates were Gram-negative and tested positive for oxidase, catalase, indole, methyl red, esculin hydrolysis, citrate utilization, and glucose fermentation. A summary of the biochemical characteristics applied for identification is prepared in Table 2.

Identification by VITEK 2 compact: Phenotypic characterization of the isolates was further affirmed applying the VITEK 2 Compact automated identification system (bioMérieux, France). All 26 isolates were accurately identified as *Aeromonas hydrophila*, showing 100% concordance

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with the initial morphological and biochemical identification results. This strong agreement supports the diagnostic reliability of the combined identification approaches.

Table 2. Biochemical characteristics applied for the identification of *Aeromonas hydrophila* isolates

Test	Result
Gram Stain	Negative
Oxidase	Positive
Catalase	Positive
Indole	Positive
Methyl Red	Positive
Esculin Hydrolysis	Positive
Citrate Utilization	Positive
Sugar Fermentation	Positive

Aminoglycoside Antibiotic Resistance in *A. hydrophila:* The antibiotic susceptibility of *A. hydrophila* isolates to aminoglycoside antibiotics was evaluateed applying the disk diffusion method. Interpretation of prevention zone diameters was carried out based on the Clinical and Laboratory Standards Institute (CLSI, 2021) guidelines. Kanamycin resistance was the most prevalent, observed in 92.3% of isolates. Resistance to gentamicin (53.8%) and amikacin (26.9%) followed. Lower resistance rates were observed for neomycin (23.1%), tobramycin (15.4%), and streptomycin (11.5%). These results raise concern over the reduced effectiveness of different commonly applied aminoglycosides in the treatment of *A. hydrophila* infections. A summary of the resistance and susceptibility profiles for each aminoglycoside antibiotic is shown in Table 3.

Table 3. Antimicrobial resistance and susceptibility patterns of *Aeromonas hydrophila* isolates to selected aminoglycoside antibiotics

Antibiotic	Resistant Isolates (n)	Resistant (%)	Sensitive Isolates (n)	Sensitive (%)
Gentamicin	14	53.8%	12	46.2%
Tobramycin	4	15.4%	22	84.6%
Streptomycin	3	11.5%	23	88.5%
Kanamycin	24	92.3%	2	7.7%
Amikacin	7	26.9%	19	73.1%
Neomycin	6	23.1%	20	76.9%

Molecular detection of aminoglycoside resistance genes: To investigate the genetic basis of aminoglycoside resistance in *Aeromonas hydrophila*, a conventional PCR assay was carried out targeting six aminoglycoside-modifying enzyme (AME) genes across 20 clinical isolates. The selected genes included aac(3), aph(3')-Ia, aph(6)-Id, aac(3)-IIa, aac(6')-Ib, and ant(4')-IIa.

Among these, the aac(3) gene was the most prevalent, detected in 16 out of 20 isolates (80%). This gene encodes the enzyme 3-N-acetyltransferase, which confers resistance by acetylating aminoglycosides like gentamicin. PCR amplification of aac(3) yielded a distinct band at 183 base pairs, as showd in Figure 1. The aph(3')-Ia gene was the second most common, detected in 13 isolates (65%). It encodes an aminoglycoside phosphotransferase responsible for resistance to kanamycin and neomycin. PCR results generated a band at 292 bp, as shown in Figure 2. The aph(6)-Id gene, detected in 12 isolates (60%), mediates streptomycin resistance through phosphorylation at the 6′-hydroxyl group. Amplification yielded a clear band at 464 bp (Figure 3). The aac(3)-IIa gene, related to resistance to gentamicin and tobramycin, was found in 9 isolates (45%). Its amplification generated a band at 339 bp (Figure 4). The aac(6')-Ib gene, which contributes to resistance against amikacin, kanamycin, and tobramycin, was identified in 6 isolates (30%). PCR amplification yielded bands at 264 bp (Figure 5). Finally, the ant(4')-IIa gene was the least common, detected in only 3 isolates (15%). It encodes an adenyltransferase that confers resistance to tobramycin and amikacin. PCR products appeared at 388 bp (Figure 6). All isolates harbored at least one AME gene, with a large proportion carrying multiple resistance genes. This widespread genetic distribution aligns with the previously observed phenotypic resistance profiles, suggesting a multifactorial molecular basis for aminoglycoside resistance in *A. hydrophila* clinical isolates.

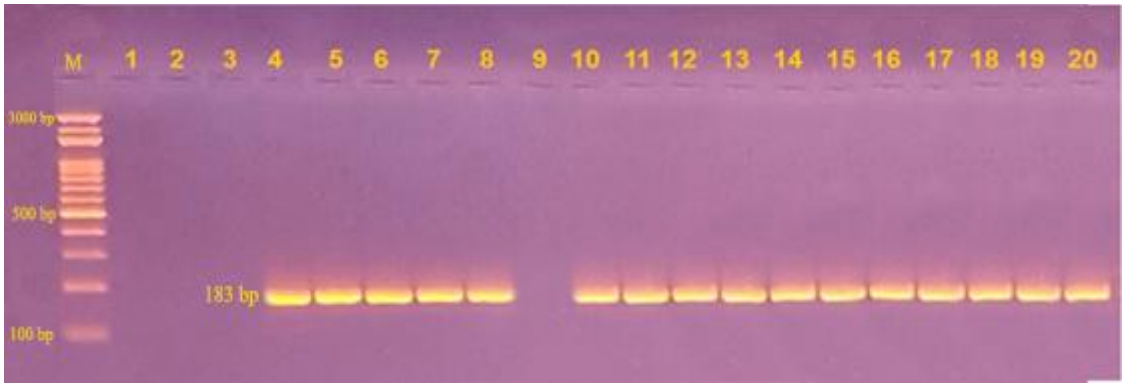

Figure 1. Agarose gel electrophoresis (1% w/v) showing PCR amplification of the aac(3) gene in *A. hydrophila*-positive isolates. Lane M: DNA marker ladder (100–3000 bp). Sixteen positive isolates are shown in lanes 1–20, with a specific band at 183 bp. Electrophoresis was managed at 100 V and 80 mA for one hour

Gene-phenotype correlation: The molecular analysis of 20 *A. hydrophila* isolates revealed a heterogeneous distribution of aminoglycoside-modifying enzyme (AME) genes. All isolates carried at least one AME gene, though the specific combinations and frequencies varied markedly among strains.

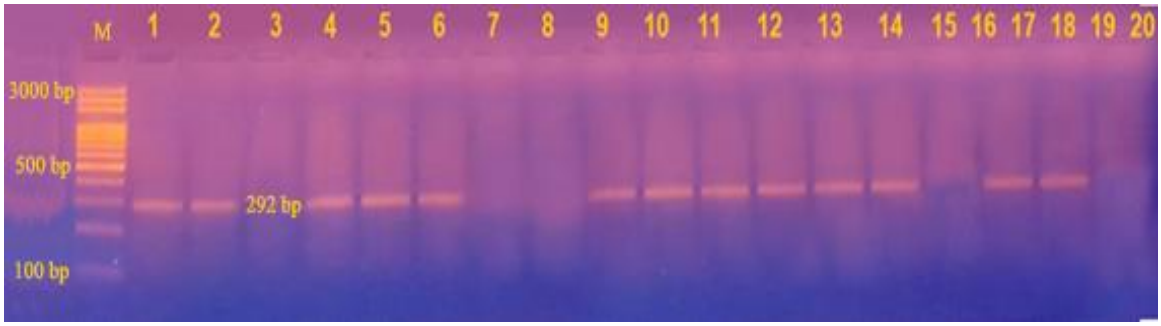

Figure 2. Agarose gel electrophoresis of PCR products for the aph(3')-Ia gene in *A. hydrophila* isolates. Thirteen positive samples demonstrated a distinct band at 292 bp. Lane M: DNA marker ladder (100–3000 bp). Samples were loaded into wells 1–20 and electrophoresed at 100 V and 80 mA for 1 hour

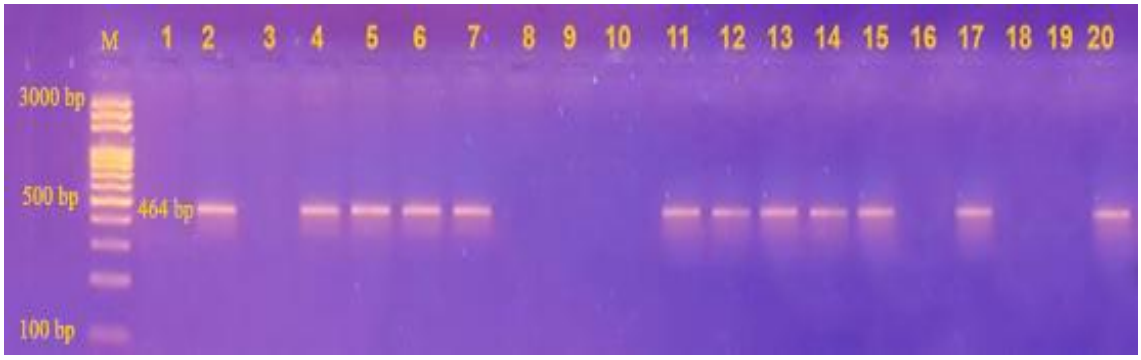

Figure 3. PCR amplification of the aph(6)-Id gene in *A. hydrophila* isolates resolved on a 1% agarose gel. Twelve positive isolates showed the expected band at 464 bp. Lane M: Molecular marker (100–3000 bp). Electrophoresis was managed at 100 V and 80 mA for 60 minutes across wells 1–20

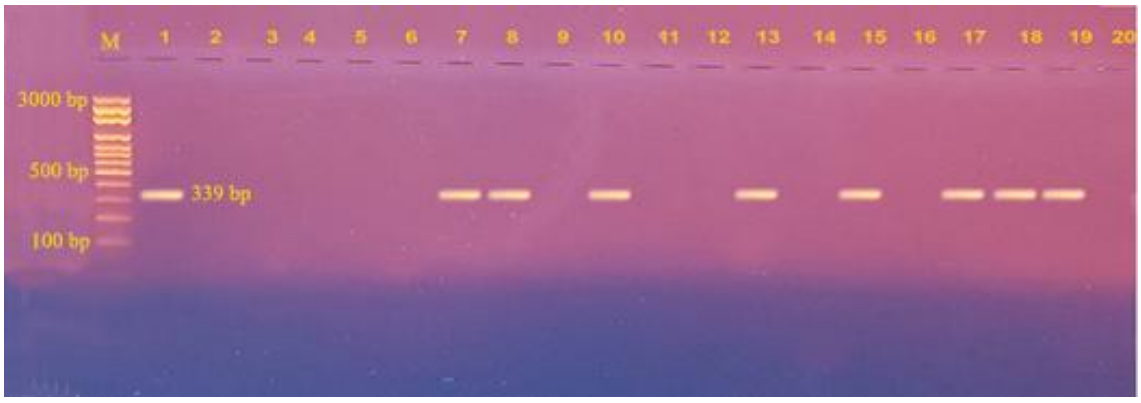

Figure 4. Amplification results for the aac(3)-IIa gene in *A. hydrophila* isolates, visualized on 1% agarose gel. Nine positive samples exhibited a distinct band at 339 bp. Lane M: DNA size marker (100–3000 bp). Gel electrophoresis was carried out at 100 V and 80 mA for 1 hour, with samples loaded into wells 1–20

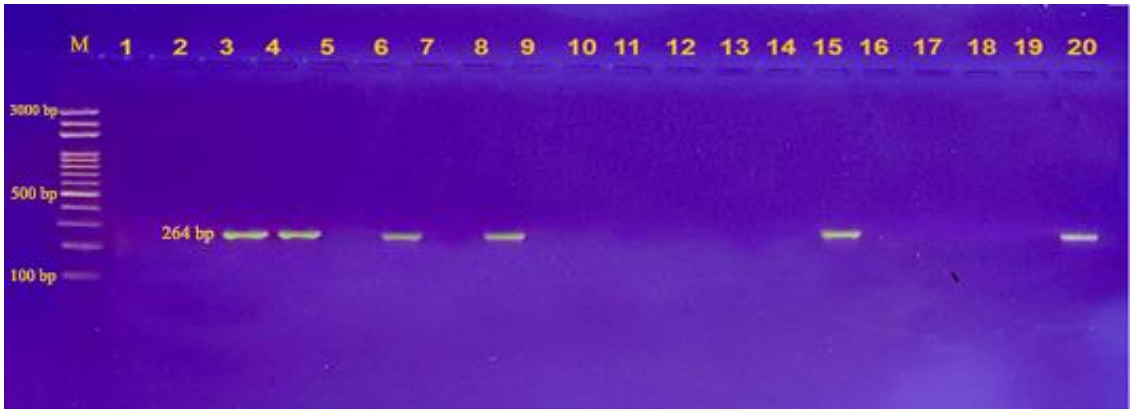

Figure 5. Electrophoretic profile of PCR products targeting the aac(6')-Ib gene in *A. hydrophila* isolates. Six positive isolates demonstrated a specific band at 264 bp. Lane M: Molecular weight ladder (100–3000 bp). Electrophoresis was carried out in wells 1–20 at 100 V and 80 mA for 60 minutes on 1% agarose gel

Figure 6. Agarose gel electrophoresis (1%) showing PCR amplification of the ant(4')-IIa gene in *A. hydrophila* isolates. Three positive samples generated bands at 388 bp. Lane M: DNA marker ladder (100–3000 bp). Electrophoresis was run at 100 V and 80 mA for 1 hour with samples loaded into wells 1–20

The aac(3) gene was the most prevalent, detected in 15 out of 20 isolates (75%), followed by aph(3')-Ia in 13 isolates (65%) and aac(3)-IIa in 11 isolates (55%). Both aac(6')-Ib and aph(6)-Id were shown in 9 isolates each (45%). The least often detected genes were aac(3)-IV, found in 4 isolates (20%), and ant(4')-IIa, detected in 3 isolates (15%). Isolate No. 6 exhibited the highest resistance gene burden, harboring all seven AME genes screened. Isolates 13 and 20 followed closely, each carrying six genes. In contrast, isolate No. 3 exhibited the lowest genetic profile, with only a single resistance gene detected. The complete gene distribution pattern among the tested isolates is summarized in Table 4.

Table 4. Distribution of aminoglycoside resistance genes among *A. hydrophila* isolates (n = 20). ("+" = gene present; "−" = gene absent)

Isolates	*acc(3)*	*aac(6')-Ib*	*aph(3')-Ia*	*acc(3)-IIa*	*ant(4')-IIa*	*aac(3)-IV*	*aph(6)-Id*	Genes
1	_	_	+	+	+	_	_	*acc(3)*
2	_	_	+	_	_	+	+	
3	_	+	_	_	_	_	_	
4	+	+	+	_	_	_	+	
5	+	_	+	_	_	_	+	*aac(6')-Ib*
6	+	+	+	_	_	+	+	
7	+	_	_	+	_	_	+	
8	+	+	_	+	_	_	_	*aph(3')-Ia*
9	_	_	+	_	_	_	_	
10	+	_	+	+	_	_	_	
11	+	_	+	_	_	_	+	*acc(3)-IIa*
12	+	_	+	_	_	+	+	
13	+	_	+	+	_	+	+	
14	+	_	+	_	_	_	+	*ant(4')-IIa*
15	+	_	_	+	+	_	+	
16	+	_	+	_	_	_	_	*aac(3)-IV*
17	+	_	+	+	_	_	+	
18	+	_	_	+	_	_	_	*aph(6)-Id*
19	+	_	_	+	_	_	_	
20	+	+	_	_	+	_	+	

Discussion

The current investigation prepares comprehensive insights into the phenotypic and genotypic characteristics of *A. hydrophila* extracted from clinical samples in Al-Diwaniyah, Iraq. Among 200 clinical specimens, 13% yielded *A. hydrophila*, with the highest isolation rates observed in urine and diarrheal samples. This pattern highlights the opportunistic nature of *A. hydrophila*, exclusively in gastrointestinal and urinary tract infections. The isolates exhibited homogeneous morphological features across diverse culture media. This was consistent because broth cultures derived directly from the original swabs were β-hemolytic on blood agar plates, non-lactose

fermenting on MacConkey agar, and showed characteristic colony coloration on Chromagar. These observations align with previous investigations on *A. hydrophila*, containing Abo-Shama et al. (2025), who announced similar colony morphology and typical Gram-negative, rod-shaped microscopic appearance. Biochemical profiling further affirmed the isolates as *A. hydrophila*, showing positive results for oxidase, catalase, indole experiments, and extensive carbohydrate fermentation. These results highlighted the metabolic versatility of *A. hydrophila* in fermenting diverse sugars. The VITEK 2 Compact automated system demonstrated 100% concordance with conventional morphological and biochemical results, underscoring the accuracy of automated phenotypic identification methods for *A. hydrophila*. The antimicrobial resistance profile of *A. hydrophila* in this investigation is concerning, exclusively regarding aminoglycosides. Kanamycin resistance was notably high, observed in 92.3% of isolates—much higher than previously announced figures. For instance, Leanovich et al. (2025) announced 45% resistance, and Fauzi et al. (2021) found 41% resistance in environmental samples. The 74% resistance announced by Nhinh et al. (2021) approaches our results but stays lower. Gentamicin resistance was also meaningfully elevated at 53.8%, considerably higher than the 9.8% announced by Fauzi et al. (2021) and 20% by Leanovich et al. (2025). Zhang et al. (2023) noted a similar 64% resistance in isolates from aquatic environments, suggesting a potential link between environmental reservoirs and clinical dissemination of resistant *A. hydrophila*. Resistance to amikacin was detected in 26.9% of isolates, lower than the 51.9% announced by Saleh (2021), but substantially higher than the 3% resistance announced by Zhang et al. (2024). Conversely, Semwal et al. (2023) announced that amikacin remained the most effective aminoglycoside, with 84% susceptibility, supporting its use in resistant infections. Other investigations, like Shak et al. (2011), announced reduced amikacin susceptibility in soft tissue infections, along with resistance to tobramycin and cephalosporins. Neomycin resistance was observed in 23.1% of isolates, a figure that ranges between the 1% announced by Zhang et al. (2023) and 74% by Nhinh et al. (2021). Such variability likely reflects differences in antibiotic usage and selective pressure across regions. Neomycin is broadly applied, containing in antiviral agents (Larsen et al., 2024), but its clinical use is restricted in some countries due to sensitization and nephrotoxicity concerns. Resistance rates were lower for tobramycin (15.4%) and streptomycin (11.5%), though these still warrant caution. Streptomycin resistance has been variable, with Eid et al. (2022) reporting 66.67%, while Rahman et al. (2021) observed 20%, reflecting geographic and host-related differences. The relatively lower use of aminoglycosides in Iraq compared to other antibiotic classes might have delayed widespread resistance development. However, current results indicate increasing resistance even to second-line therapies. This trend aligns with Wachino and Arakawa

(2020), who emphasized the continued clinical importance of aminoglycosides, exclusively their synergy with β-lactams. Resistance in *A. hydrophila* may also arise through spontaneous mutation, especially under inappropriate dosing, prolonged treatment, or strong selective pressure. Such conditions can lead to chromosomal mutations or acquisition of plasmid-encoded resistance genes. Therefore, stringent antibiotic stewardship and systematic surveillance of antimicrobial resistance in both clinical and environmental contexts are urgently needed. Genotypic analysis revealed diverse aminoglycoside-modifying enzyme (AME) genes among the *A. hydrophila* isolates. These enzymes inactivate aminoglycosides via acetylation, phosphorylation, or adenylation. The predominant gene was *aac(3)*, identified in 80% of isolates, followed by *aph(3')-Ia* (65%) and *aph(6)-Id* (60%). The *aac(3)* gene encodes aminoglycoside 3-N-acetyltransferase, modifying the 3-amino position of aminoglycosides like gentamicin, sisomicin, and fortimicin (Zhang et al., 2022). Its high prevalence is consistent with reports from Onohuean and Nwodo (2023), who documented widespread *aac(3)* distribution among Enterobacteriaceae and non-fermenting Gram-negative bacteria. This suggests *aac(3)* is a major genetic determinant of gentamicin resistance in *A. hydrophila*. The *aph(3')-Ia* gene, encoding aminoglycoside phosphotransferase that modifies the 3′-hydroxyl group (e.g., kanamycin, neomycin), was detected in 65% of isolates. Although Woegerbauer et al. (2014) announced only 13.9% prevalence and Krause et al. (2016) described it in some serogroups, the higher frequency observed here may reflect plasmid-mediated spread within local and European ecological niches. The *aph(6)-Id* gene was exist in 60% of isolates and encodes an enzyme phosphorylating the 6-hydroxyl group of streptomycin. Its association with plasmids and transposons facilitates mobility and conjugational transfer (Nishida & Ono, 2024). Its absence in Chinese lake water isolates (Chen et al., 2024) underscores geographic variation in AME gene patterns. Genes *aac(6')-Ib* (30%) and *aac(3)-IIa* (45%) showed moderate prevalence. The *aac(6')-Ib* gene confers resistance to amikacin, kanamycin, and tobramycin, and its cr variant also affects fluoroquinolones (Lin et al., 2021). Ahmed et al. (2024) and Woo et al. (2022) announced prevalence rates of 100% and 23.5%, respectively. The *aac(3)-IIa* gene, related to gentamicin and tobramycin resistance, matches results from Samadi et al. (2015), who found 37% prevalence in hospital wastewater isolates. Lower prevalence was observed for *aac(3)-IV* (20%) and *ant(4')-IIa* (15%). In spite of lower frequency, these genes have clinical and environmental significance. *aac(3)-IV* was found in 0.7% of clinical bacterial genomes in Plattner et al. (2020), and in 28.3% of wastewater isolates announced by Tourtière et al. (2020) and Obayiuwana and Ibeke (2020), denoting environmental persistence. The *ant(4')-IIa* gene encodes an adenyltransferase modifying tobramycin and amikacin (Zhang et al., 2017), documented globally in Iran, China, and France. The presence of multiple AME genes within single isolates shows the genetic complexity underlying resistance in

A. hydrophila. For example, isolates 6 and 13 harbored up to five AME genes, potentially contributing to their elevated phenotypic resistance. This multilocus genotype structure aligns with Ahmadien et al. (2021), who announced strong correlations between AME gene accumulation and higher minimum inhibitory concentrations, especially for gentamicin and tobramycin. These genes are often located on mobile genetic elements, containing plasmids and integrons (Wang et al., 2022; Coyne et al., 2010), reinforcing the role of horizontal gene transfer (HGT) in their dissemination. Given the ecological versatility of *A. hydrophila*—spanning clinical and aquatic environments, continuous molecular surveillance is essential to monitor and control the emergence of untreatable infections. The correlation between AME gene presence and phenotypic aminoglycoside resistance was meaningful. Each isolate harbored at least one AME gene, reflecting the broad genetic potential for resistance within this population. The *aac(3)* gene was detected in 75% of isolates and is primarily linked to gentamicin resistance, consistent with phenotypic data and previous reports by Zhang et al. (2023). This gene is typically plasmid-borne and prevalent among Enterobacteriaceae. The *aac(6')-Ib* gene, exist in 45% of isolates, confers resistance to tobramycin, kanamycin, and amikacin describing its integration with class 1 integrons. Strains 6 and 13 carrying this gene showed corresponding phenotypic resistance. *aph(3')-Ia*, detected in 65% of isolates, was strongly related to kanamycin and neomycin resistance. This plasmid-encoded gene (Vakulenko and Mobashery, 2003) likely contributes to the higher resistance rates observed. The *aac(3)-IIa* gene was exist in 11 isolates, correlating with co-resistance to gentamicin and tobramycin. Watters et al. (2006) similarly announced its presence on both plasmids and chromosomes of Gram-negative bacteria, consistent with this investigation's genotypic results. Though less common, *ant(4')-IIa* was strongly linked to tobramycin and amikacin resistance in three isolates. Coyne et al. (2010) highlighted its plasmid-mediated clinical relevance in spite of low prevalence. The *aac(3)-IV* gene, detected in 20% of isolates, is known to confer resistance to multiple aminoglycosides, containing gentamicin, tobramycin, and kanamycin, and is often plasmid-associated (Krasowiak et al., 2002).

Conclusions: This investigation affirms the presence of multidrug-resistant *Aeromonas hydrophila* strains exhibiting high-level resistance to aminoglycosides, exclusively kanamycin and gentamicin. Molecular analysis revealed a high prevalence of aminoglycoside-modifying enzyme (AME) genes, containing *aac(3)* and *aph(3')-Ia*, with strong genotype–phenotype correlations supporting their role in resistance. The coexistence of multiple resistance genes within single isolates suggests the likely involvement of horizontal gene transfer, especially in aquatic environments where gene dissemination occurs rapidly. These results underscore the critical need for molecular surveillance programs, like sequencing isolates before and through

epidemiological monitoring—and stringent control of antibiotic usage to limit the spread of antibiotic-resistant *A. hydrophila* strains in both clinical and environmental settings.

Author contributions

Hadeel Turki Hussein: Conceptualization, sample collection, experimental work, data analysis, manuscript drafting. Azhar Noory Hussein: Supervision, methodology design, manuscript review and editing.

Data availability statement

The datasets generated through and/or analyzed in the current investigation are available from the corresponding author on reasonable request.

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Ethical approval

This investigation was managed in accordance with the ethical standards of the Department of Biology, College of Education, University of Al-Qadisiyah, Iraq, and in compliance with the principles of the Declaration of Helsinki. Ethical approval was granted under reference number 42–11/10/2014. Written informed consent was achieved from all participants or their legal guardians prior to specimen collection.

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Conflict of interest

The authors declare no conflicts of interest related to this research. No financial, personal, or professional relationships exist that could have biased the design, conduct, or reporting of this investigation.

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تشخیص مولکولی و پروفایل مقاومت آنزیم‌های تغییردهنده آمینوگلیکوزید در *Aeromonas hydrophila* جداشده از موارد بالینی مختلف

حسین هدیـل

گروه زیست‌شناسی، دانشکده آموزش، دانشگاه القادسیه، عراق. ایمیل: edu.bio.posta139@qu.edu.iq

حسین ازهر

نویسنده مسئول: گروه زیست‌شناسی، دانشکده آموزش، دانشگاه القادسیه، عراق. ایمیل: azhar.almousawi@qu.edu.iq

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چکیده

هدف: *Aeromonas hydrophila* یک پاتوژن فرصت‌طلب نوظهور است که به دلیل مقاومت روبه‌افزایش آن در برابر آنتی‌بیوتیک‌های مختلف، از جمله آمینوگلیکوزیدها، نگرانی جدی برای بهداشت عمومی ایجاد کرده است. این مطالعه با هدف بررسی ویژگی‌های فنوتیپی و ژنوتیپی ایزوله‌های *A. hydrophila* به‌دست‌آمده از منابع بالینی و محیطی در استان دیوانیه عراق، با تمرکز بر مکانیسم‌های مقاومت به آمینوگلیکوزیدها انجام شد.

مواد و روش‌ها: از میان ۲۰۰ نمونه، ۲۶ ایزوله به‌عنوان *A. hydrophila* با استفاده از روش‌های کلاسیک میکروبیولوژی و سیستم شناسایی VITEK 2 Compact تعیین هویت شدند. ایزوله‌ها از نظر مقاومت به آمینوگلیکوزیدهایی شامل کانامایسین (Kna)، جنتامایسین (Gen) و آمیکاسین (Ami) مورد آزمایش قرار گرفتند. شایان ذکر است که ایزوله‌ها مقاومت کم تا متوسطی در برابر نئومایسین، توبرامایسین و استرپتومایسین نشان دادند. این نتایج نشان‌دهنده الگوی نگران‌کننده‌ای از مقاومت در برابر هر دو گروه آمینوگلیکوزیدهای لاین اول و خط دوم است.

نتایج: در مجموع پنج ژن آنزیم تغییردهنده آمینوگلیکوزید (AME) با روش PCR شناسایی شد که به‌طور گسترده در میان ایزوله‌ها توزیع شده بودند. شایع‌ترین ژن‌ها شامل aac(3) (۸۰٪)، aph(3')-Ia (۶۵٪) و aph(6)-Id (۶۰٪) بودند. سایر ژن‌ها مانند aac(3)-IIa، aac(6')-Ib، aac(3)-IV و ant(4')-IIa نیز با فراوانی‌های متفاوت حضور داشتند که بیانگر شبکه پیچیده‌ای از عوامل ژنتیکی دخیل در مقاومت به آمینوگلیکوزیدها است.

نتیجه‌گیری: ارتباط معناداری بین حضور چندین ژن AME و مقاومت فنوتیپی بالا مشاهده شد. ایزوله‌هایی که چهار ژن مقاومت یا بیشتر را حمل می‌کردند، در برابر همه آمینوگلیکوزیدهای آزمایش‌شده (به‌جز یکی) مقاوم بودند، و حتی همان ایزوله نیز در برابر یک آمینوگلیکوزید دیگر که احتمالاً از طریق انتقال افقی ژن (HGT) منتقل شده بود، مقاومت نشان داد. این یافته‌ها بر ضرورت بهبود مدیریت محلی مصرف آنتی‌بیوتیک و ایجاد برنامه‌های پایش مولکولی برای رصد تکامل و انتشار کلون‌های مقاوم *A. hydrophila* تأکید دارد. شناسایی چندین ژن AME در نمونه‌های بالینی و محیطی نگرانی‌هایی درباره شکست احتمالی درمان و گسترش عوامل مقاومت در هر دو زیست‌بوم آبی و بالینی ایجاد می‌کند.

کلمات کلیدی: مقاومت به آمینوگلیکوزید، ژن‌های AME، مقاومت ضدمیکروبی، ایزوله‌های محیطی، PCR

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