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Research Article

Prevalence of Enterotoxin Genes among Clinical Isolates of *Aeromonas hydrophila*

Ahmed Hameed Ali, Azhar Noory Hussein*

Department of Biology, College of Education, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.

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AHA designed the study and performed lab work. ANH analysed data and wrote the manuscript. Both authors approved the final manuscript.

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Aeromonas hydrophila, Clinical isolates, 16S rRNA, *Act* gene, *Ast* gene, PCR

Abstract | *Aeromonas hydrophila* is a chance pathogen that is linked to numerous human diseases, specifically gastrointestinal illness. The sample of the research was performed at 15 September to 10 December 2025 in hospitals and private laboratories in Al-Diwaniyah, Iraq, to isolate and identify *A. hydrophila*in different clinical samples and to examine the prevalence of the chosen enterotoxin genes by means of molecular techniques. There were 200 clinical samples in total consisting of stool, urine, blood, wound, and burn samples. One percent of the isolates (20) were *A. hydrophila*. The maximal isolating rate was in stool (15%), urine (7.0%), and blood (6.7%), but none of the samples contained any isolates in wound or burn samples. The VITEK 2 Compact system was also used to confirm the identification at the species level alongside PCR amplification of the 16S rRNA gene. Every isolate generated the desired amplicons of around 1500 bp. The screening of PCRs showed that 16 out of 20 isolates (80 percent) had the cytotoxic enterotoxin gene (act, 303 bp) and heat-stable cytotonic enterotoxin gene (ast, 1340 bp). Such results identify stool samples as the predominant source of *A. hydrophila* and show nearly high prevalence of enterotoxin genes in clinical isolates. The research gives the baseline molecular evidence that could be used in future epidemiology surveillance in Iraq.

Novelty Statement | This study investigates the prevalence of enterotoxin genes (act and ast) among clinical isolates of *Aeromonas hydrophila* in Iraq using molecular techniques. It combines conventional, automated (VITEK 2), and PCR-based identification within a single framework. The findings provide baseline molecular data on the virulence potential of local clinical isolates.

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Introduction

Aeromonas hydrophila is a Gram-negative, motile, facultative anaerobic bacterium that is broadly distributed in aquatic habitats, such as freshwater, brackish water, seawater, sewage and food products. In recent 20 years, it has become more and more popular as a new opportunistic pathogen of humans or aquatic animals (Pessoa *et al.*, 2022; Fernández *et al.*, 2020; Albarral *et al.*, 2016; Grim *et al.*, 2013). *A. hydrophila* has been implicated in clinically significant potential mortality in humans with a wide range of clinical manifestations including self-limiting gastroenteritis and severe invasive infections, including bacteremia, necrotizing fasciitis, gas gangrene, and sepsis (Ugarte-Torres *et al.*, 2018; Mohanty *et al.*, 2022; Sakurai *et al.*, 2023; Lin and Lin, 2019).

A. hydrophila is also an important pathogen in aquaculture, where it causes motile *Aeromonas* septicemia in fish species worldwide (Dubey *et al.*, 2021; Zhang *et*

Corresponding author: Azhar Noory Hussein
bio.edu.posta24.39@qu.edu.iq

al., 2019). The zoonotic potential of certain strains has raised public health concerns, particularly in regions with close human contact with aquatic environments (Eid *et al*., 2022).

Conventional *Aeromonas* species identification is based on culture features, biochemical tests and commercial identification. Nevertheless, some studies have shown that phenotypic techniques are often ineffective in isolating between closely related *Aeromonas* species because of similar biochemical patterns (Puthucheary *et al*., 2012; Shin *et al*., 2015; Dubey *et al*., 2021). This has led to a need of molecular methods in order to ensure good identification. Of these, PCR amplification of the *16S rRNA* gene is the most commonly employed technique to confirm genus level of *A. hydrophila* and initial identification of species in a clinical, environmental, and aquaculture environment (Sarkar *et al*., 2012; Shin *et al*., 2015; Sakurai *et al*., 2025).

Although 16S rRNA-based identification has been widely used, the high similarity in sequence between the species of *Aeromonas* and intragenomic heterogeneity inherently limit the method of identifying these genomes at the species level (Shin *et al*., 2015; Sakurai *et al*., 2025; Puthucheary *et al*., 2012). To address these shortcomings, some further molecular methods have been developed, including housekeeping genes (e.g., gyrB, rpoB) sequencing and multiplex species-specific PCR have also been applied in recent years (Shin *et al*., 2015; Sakurai *et al*., 2025). However, 16S rRNA PCR has continued to be the foundation of molecular identification in most laboratories, especially in resource-restrained environments.

A wide range of virulence factors, such as enterotoxins, hemolysins, aerolysins, lipases, proteases, and others, in addition to extracellular enzymes have been largely credited with the pathogenicity of *A. hydrophila*. These include heat-labile cytotonic enterotoxin (enterotoxin genes), act, heat-stable cytotonic enterotoxin (ast), and alt (heat-labile cytotonic enterotoxin) which are considered to be the key agents of gastrointestinal disease and tissue injury (Albarral *et al*., 2016; Grim *et al*., 2013; Zhang *et al*., 2019). As has been shown by a variety of studies, those strains, which contain multiple enterotoxin and virulence genes, are more pathogenic, their LD 0 value is lower in experimental infections, and the histopathology is more severe (Zhang *et al*., 2019; Mansour *et al*., 2019b; Hu *et al*., 2025; Kwon *et al*., 2019).

Molecular detection of enterotoxin-coding genes in *A. hydrophila* has been widely reported both in fish and environmental and clinical isolates worldwide, with an apparent high level of genetic diversity and variability of virulence profiles (Eid *et al*., 2022; Kwon *et al*., 2019; Raji *et al*., 2019; Dubey *et al*., 2021). Conversely, Iraqi data are limited, and the majority of the published studies are devoted to aquaculture and vaccine development instead of molecular characterization of virulence genes of clinical and environmental isolates (Shnawa *et al*., 2019). Due to the ecological resemblance between Iraq and adjacent areas where *A. hydrophila*-related illnesses have been reported, more in-depth analysis of the molecular characteristics is required in order to uncover the distribution and virulence of the local isolates.

Despite the recognized clinical importance of *Aeromonas hydrophila*, molecular epidemiological data regarding virulence genes among clinical isolates in Iraq remain limited. Therefore, the present study aimed to isolate and identify *A. hydrophila* from various clinical specimens using conventional, automated (VITEK 2 Compact), and molecular methods, and to determine the prevalence of selected enterotoxin genes in order to better understand the virulence potential of local clinical strains.

Materials and Methods

Clinical samples collection

The study was cross-sectional and carried out in Al-Diwaniyah Teaching Hospital and sampled private medical laboratories in Al-Diwaniyah, Iraq between 15 September and 10 December 2025. The Ethics Committee of the College of Education, the University of Al-Qadisiyah, reviewed the study protocol and granted its approval (Approval No: 35/2025). Informed consent of all the participants or their legal guardians was obtained in written form before sample collection.

Two hundred clinical specimens were taken on patients with suspected bacterial infection. The specimens were stool (n= 100), urine (n= 57), blood (n= 15), wound swabs (n= 15), and burn swabs (n= 13). Simple demographic data like age, sex where available were recorded. The disproportionality of the types of specimens is due to the common exercise of clinical sampling at the time of the study and stool samples are more commonly ordered because of suspected gastrointestinal infections.

Phenotypic identification and isolation of Aeromonas hydrophila

The standard bacteriological tests were done on every clinical sample. Sool samples were first inoculated in alkaline peptone water and incubated at 37°C 6-8 h then streaked on Macconkey agar and Blood agar plate. The samples were swabs of urine, blood, wounds, and burns directly inoculated on MacConkey agar and Blood agar, and not enriched. Incubation All the plates were incubated aerobically at 37 C for 18 24 h.

The selection of presumptive *Aeromonas* colonies used was founded on characteristic morphology whereby pale, non-lactose-fermenting colonies appeared on MacConkey

agar and 6-hemolytic colonies appeared on Blood agar. Repeated sub-culturing was used to obtain pure cultures. The identification was done by preliminary identification by Gram staining, oxidase test, catalase test and motility test. Presumptive isolates of *A. hydrophila* were grams-negative, oxidase positive and motile rods.

Identification using VITEK 2 compact system

To ensure accurate species-level identification, all presumptive *Aeromonas* isolates were further analyzed using the VITEK 2 Compact system (bioMérieux, France) according to the manufacturer's instructions. Bacterial suspensions were prepared in sterile saline to a turbidity equivalent to 0.5 McFarland standard and inoculated into GN identification cards. The system automatically analyzed biochemical reactions and provided species identification along with probability percentages. All twenty isolates were confirmed as *A. hydrophila*, with confidence values ranging from 90% to 99%.

Genomic DNA extraction

The confirmed isolates were subjected to genomic DNA extraction using a commercial DNA extraction kit (Genomic DNA Mini Kit for Bacteria, Geneaid, Taiwan) as per the instructions given by the manufacturer. In brief, the cells of the overnight bacterial cultures were centrifuged to pellet them and lysed with the help of the lysis buffer and proteinase K provided. The binding, washing and elution processes were conducted with the lysates. The DNA was purified and the pure solution eluted in nuclease-free water and kept at -20 °C until PCR assays.

Molecular identification using 16S rRNA gene

PCR amplification of the 16S rRNA gene was performed using universal primers described by Frank *et al*. (2008). The primer sequences were:

Forward primer (27F): 5′-AGAGTTTGATCCTGGCTCAG-3′

Reverse primer (1492R): 5′-TACGACTTAACCCCAATCG-3′.

PCR reactions were carried out using AccuPower® PCR Premix (Bioneer, Korea). Each 50 µL reaction mixture contained 25 µL of PCR premix, 5 µL of template DNA, 3 µL of each primer, and 14 µL of nuclease-free water.

Amplification was performed under the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec; with a final extension at 72°C for 7 min. A confirmed *A. hydrophila* isolate was used as a positive control, and nuclease-free water served as a negative control.

Detection of enterotoxin genes

The presence of virulence-associated enterotoxin genes was investigated by PCR amplification using primers designed in this study. Target gene sequences were retrieved from the GenBank database, and conserved regions were selected for primer design using Primer3 software. Primer specificity was verified in silico using NCBI BLAST analysis to ensure selective amplification of the target genes.

The primer sequences and expected amplicon sizes were as follows:

Cytotoxic enterotoxin gene (act)

Forward primer: 5′-AAGCATCTGGATCGGCAGTC-3′

Reverse primer: 5′-AGGCTCCAGCAGGAATGTCT-3′

Expected amplicon size: 303 bp

Heat-stable cytotonic enterotoxin gene (ast)

Forward primer: 5′-TATCGCACCTATGTCGGCAC-3′

Reverse primer: 5′-CAGGACTTTTTCACCGCAGC-3′

Expected amplicon size: 1340 bp

PCR reactions were carried out in a total volume of 50 µL using AccuPower® PCR Premix (Bioneer, Korea), containing 25 µL of premix, 5 µL of template DNA, 3 µL of each primer, and 14 µL of nuclease-free water.

Thermal cycling conditions included initial denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for the enterotoxin gene and 63°C for the heat-stable cytotonic enterotoxin gene for 30 seconds, extension at 72°C for 45 seconds; and a final extension at 72°C for 7 minutes.

A confirmed *A. hydrophila* strain harboring the target genes was used as a positive control, and nuclease-free water served as a negative control in each PCR assay.

Agarose gel electrophoresis

The products of PCR were examined with the help of electrophoresis on the agarose gel of 1.5 percent in 1XTBE buffer. The gel was stained using ethidium bromide (0.5 µg/mL). Each run contained a molecular size marker, a 100 bp DNA lane. The time used in electrophoresis was 90100 V during 4560 minutes. DNA bands were observed in the ultra-violet light condition through a gel documentation system and photographed as a record to be taken.

Statistical analysis

Statistical analysis was performed using the chi-square test to compare isolation rates among different specimen types. A p-value of less than 0.05 was considered statistically significant.

Results

Isolation rate of Aeromonas hydrophila

Out of 200 clinical specimens examined, 20 isolates (10%) were identified as *Aeromonas hydrophila*, while 180 samples (90%) showed no growth of the organism. Stool samples demonstrated the highest isolation rate (15/100, 15%), followed by urine samples (4/57, 7.0%) and blood samples (1/15, 6.7%). No isolates were recovered from wound (0/15) or burn samples (0/13). Statistical analysis using the chi-square test revealed no statistically significant difference in isolation rates among the different specimen types ($\chi^2 = 6.7$, df = 4, p = 0.15).

Isolation and identification of Aeromonas hydrophila

Morphological identification

Aeromonas hydrophila isolates were first discovered after determining their morphological features of the colonies developing in various culture media. The isolates exhibited a good growth on MacConkey agar, with the colonies turning pale because of failure to ferment lactose as indicated in Figure 1.

Figure 1: Colonial morphology of *A. hydrophila* on MacConkey agar showing pale, non-lactose-fermenting colonies after incubation at 37°C for 24 h.

Identification using VITEK 2 compact system

All presumptive isolates were subjected to identification using the VITEK 2 Compact system. The results showed complete agreement with conventional biochemical testing, and all 20 isolates were confirmed as *A. hydrophila*, with probability values ranging from 90% to 99%.

Molecular confirmation by 16S rRNA PCR

PCR amplification of the 16S rRNA gene produced a clear and specific band of approximately 1500 bp in all analyzed isolates (n= 20). The observed amplicon size corresponded to the expected product size of the bacterial 16S rRNA gene. The uniform amplification pattern across all isolates confirmed successful molecular detection and supported the identification results obtained by the VITEK 2 Compact system (Figure 2).

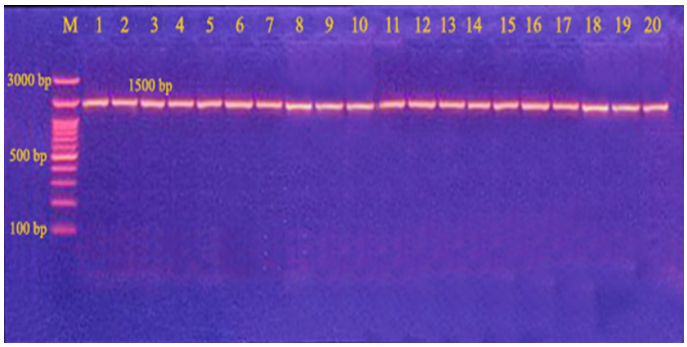

Figure 2: Agarose gel electrophoresis of PCR amplification of the 16S rRNA gene (~1500 bp) in *A. hydrophila* isolates. Lane M: 100 bp DNA ladder; Lanes 1–20: clinical isolates showing the expected amplification product.

Detection of enterotoxin genes

PCR amplification of the cytotoxic enterotoxin gene (act, 303 bp) was detected in 16 out of 20 isolates (80%), while four isolates (Isolates 4, 5, 16, and 20) were negative (Figure 3).

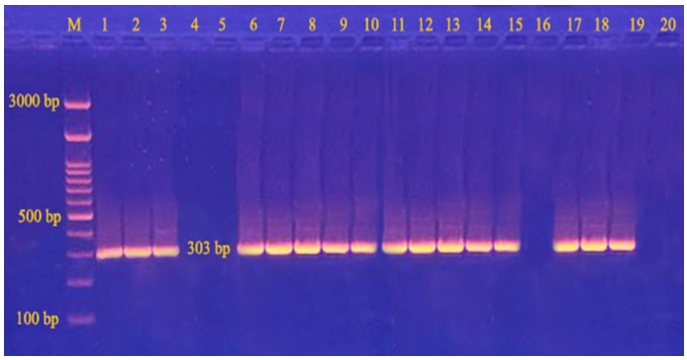

Figure 3: Agarose gel electrophoresis of PCR amplification of the enterotoxin gene (303 bp) in *A. hydrophila* isolates. Lane M: 100 bp DNA ladder; Positive amplification observed in 16 isolates; Isolates 4, 5, 16, and 20 showed no detectable band.

The heat-stable cytotonic enterotoxin gene (ast, 1340 bp) was also identified in 16 isolates (80%), whereas four isolates (Isolates 2, 9, 15, and 19) did not show amplification (Figure 4).

The variation in gene distribution among isolates indicates genetic heterogeneity within the studied *A. hydrophila* population. A detailed distribution of act and ast gene profiles among the clinical isolates is presented in Table 1.

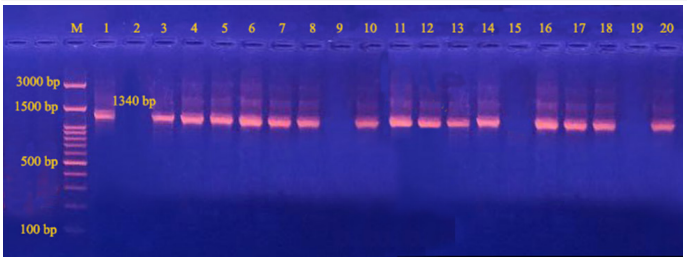

Figure 4: Agarose gel electrophoresis of PCR amplification of the heat-stable cytotonic enterotoxin gene (~1340 bp) in *A. hydrophila* isolates. Lane M: 100 bp DNA ladder; Positive amplification detected in 16 isolates; Isolates 2, 9, 15, and 19 showed no detectable band.

Table 1: Distribution of clinical isolates and detection of *Act* and *Ast* genes.

Isolate ID	Specimentype	*act* gene (303 bp)	*ast* gene (1340 bp)
1	Stool	+	+
2	Stool	+	–
3	Stool	+	+
4	Stool	–	+
5	Stool	–	+
6	Stool	+	+
7	Stool	+	+
8	Stool	+	+
9	Stool	+	–
10	Urine	+	+
11	Urine	+	+
12	Urine	+	+
13	Urine	+	+
14	Blood	+	+
15	Stool	+	–
16	Stool	–	+
17	Stool	+	+
18	Stool	+	+
19	Stool	+	–
20	Stool	–	+

Discussion

The obtained findings suggest that *A. hydrophila*was cultured on a sub-set of the samples examined, and the primary source of isolation was provided by stool samples. This finding can also be compared to the existing literature which reported *A. hydrophila*as one of the major etiological agents of gastroenteritis and diarrheal disease (Pessoa *et al*., 2022; Fernandez, 2019; Lin and Lin, 2019). Other clinical research studies have also reported similar trends, with *Aeromonas* species being commonly identified in gastrointestinal samples, particularly in regions with warm climates and increased environmental exposure to water sources (Ugarte-Torres *et al*., 2018; Sakurai *et al*., 2023).

The amplification of the 16S rRNA PCR is a reliable method for confirming the genus *Aeromonas* using a molecular tool, which has been noted to be effective in many studies in both clinical and aquaculture settings (Sarkar *et al*., 2012; Dubey *et al*., 2021; Shin *et al*., 2015). In the present study, species-level identification was further supported by the VITEK 2 Compact system. These findings are in line with previous reports that demonstrate 16S rRNA PCR is a fast and sensitive method of confirming the identity of *Aeromonas* spp. despite the possibility of phenotypic confusion (Puthucheary *et al*., 2012; Shin *et al*., 2015). Nonetheless, as Shin *et al*. (2015) and Sakurai *et al*. (2025) emphasize, the use of 16S rRNA might be not sufficient to achieve species-level detection, so additional molecular markers would have a beneficial impact on the diagnostic validity.

The high prevalence (80%) of act and ast genes observed in the present study suggests that a considerable proportion of the clinical isolates harbor virulence-associated determinants. Similar findings have been reported in clinical and environmental isolates from different geographic regions (Mansour *et al*., 2019a; Zhang *et al*., 2019; Eid *et al*., 2022). Studies involving fish and aquaculture isolates have also documented a high frequency of enterotoxin-related genes (Mansour *et al*., 2019b; Hu *et al*., 2025; Raji *et al*., 2019). Furthermore, previous research has associated the presence of enterotoxin genes with increased virulence potential in *A. hydrophila* (Kwon *et al*., 2019). However, gene detection alone does not confirm expression or clinical severity, and further functional investigations are required to determine their precise role in pathogenesis.

The genetic diversity of the populations of *A. hydrophila* can be explained by the fact that in some of the isolates, the genes of enterotoxins were absent, which is a widely reported phenomenon in the past studies (Albarral *et al*., 2016; Grim *et al*., 2013). This variability could account for the differences that were found in the severity of clinical and disease outcome in infected hosts. Severe soft-tissue infections and necrotizing fasciitis have often reported clinical cases in which strains possessed several types of virulence factors, such as enterotoxins and hemolysins (Ugarte-Torres *et al*., 2018; Mohanty *et al*., 2022; Sakurai *et al*., 2023).

Previous regional and global studies have reported similarities in virulence profiles between aquatic and human isolates of *A. hydrophila* (Dubey *et al*., 2021; Eid *et al*., 2022). Studies involving fish pathogens have also highlighted comparable genetic characteristics (Raji *et al*., 2019). However, the present study focused exclusively

on clinical isolates, and therefore any inference regarding zoonotic transmission remains speculative. In Iraq, available data are largely limited to aquaculture and vaccine-related investigations (Shnawa *et al*., 2019).

Previous studies have reported possible associations between virulence determinants and antimicrobial resistance in *A. hydrophila* isolates, particularly in strains linked to severe infections (Pessoa *et al*., 2022; Sakurai *et al*., 2023; Eid *et al*., 2022). However, antimicrobial susceptibility testing was not included in the present study. Therefore, no conclusions can be drawn regarding resistance patterns among the investigated isolates. Future studies incorporating both virulence profiling and antimicrobial susceptibility testing would provide a more comprehensive understanding of the clinical significance of these strains.

In general, the findings of this research can be compared to world evidence, which shows that *A. hydrophila*is a genetically diverse pathogen with a high proportion of enterotoxin genes that favor its virulence. The 16S rRNA-based PCR, which is used with specific genes of enterotoxins, gives a solid framework of the understanding of the pathogenic potential of clinical isolates and forms the basis of further epidemiological and molecular studies in Iraq.

Study limitations

The present study has several limitations. First, the number of isolates was relatively small, which may limit the generalizability of the findings. Second, species-level identification was based on phenotypic methods, VITEK 2 Compact, and 16S rRNA PCR without sequencing-based confirmation or analysis of additional housekeeping genes. Third, only two virulence genes (act and ast) were investigated, which may not fully represent the complete virulence profile of the isolates. In addition, antimicrobial susceptibility testing was not performed, preventing correlation between virulence determinants and resistance patterns. Finally, the study was conducted within a single geographic region and over a limited time period. Future studies incorporating larger sample sizes, expanded gene panels, sequencing approaches, and antimicrobial profiling are recommended.

Conclusion

The present study demonstrated that *Aeromonas hydrophila* can be isolated from various clinical specimens, with stool samples representing the most frequent source. Species-level identification was supported by the VITEK 2 Compact system and confirmed by 16S rRNA PCR. A high prevalence of act and ast genes was observed among the clinical isolates, indicating the presence of virulence-associated determinants within the studied population. The variation in gene distribution reflects genetic heterogeneity among local strains. These findings provide baseline molecular data on clinical *A. hydrophila* isolates in Iraq and highlight the importance of continued molecular surveillance to better understand their pathogenic potential.

Declarations

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IRB approval

The study protocol was reviewed and approved by the Ethics Committee of the College of Education, University of Al-Qadisiyah, Iraq (Approval No. 35/2025).

Ethical statement

Written informed consent was obtained from all participants or their legal guardians prior to sample collection. All procedures were conducted in accordance with institutional ethical standards.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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