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Molecular Detection of Virulence Genes in *Aeromonas hydrophila* Isolated from Market Milk

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ABSTRACT

Virulence gene profiling of *Aeromonas* species can be conducted using PCR to target various virulence factors, including endotoxins, enterotoxins, adhesins, cytotoxins, hemolysins, lipases, and proteases. A polymerase chain reaction (PCR) assay was employed for the detection of *Aeromonas hydrophila* (*A. hydrophila*) in raw market milk samples. A total of 350 milk samples were collected from different markets in Udaipur during the period from June 2025 to September 2025. PCR amplification using species-specific primers targeting the *gyrB* gene produced specific amplicons of 1100 bp, confirming the presence of *A. hydrophila*. The primers used in the study successfully amplified the corresponding target sequences of *gyrB* gene. Furthermore, several virulence-associated genes were amplified to assess the pathogenic potential of the isolates. The virulence genes targeted included *aer*, *ast*, *alt* and *act*. PCR amplification of these virulence genes yielded specific bands of 252 bp, 212 bp, 442 bp, and 232 bp, respectively. Out of the 12 *A. hydrophila* isolates, 11, 6, 9 and 8 isolates were found to be positive for *aer* (91.66%), *ast* (50%), *alt* (75%), and *act* (66.66%) genes, respectively. As raw milk can act as a vehicle for transmission of pathogenic strains to humans, its contamination represents a significant public health risk. The presence of virulence genes among the recovered isolates of *A. hydrophila* in this study highlights the pathogenic potential of milk-borne microorganisms, emphasizing the need for continuous surveillance through molecular approaches to strengthen food safety management and prevent future outbreaks.

HIGHLIGHTS

- The confirmation of *Aeromonas hydrophila* in raw market milk samples collected from Udaipur.
- Virulence gene profiling revealed a high prevalence of pathogenic determinants among isolates.
- The occurrence of virulent *A. hydrophila* in raw milk highlights the importance of continuous molecular surveillance for effective food safety management.

Keywords: *Aeromonas hydrophila*, PCR, virulence genes, milk, Udaipur

The genus Aeromonas has undergone several taxonomic and nomenclatural revisions over time. Initially classified within the family Vibrionaceae alongside the genera Vibrio, Photobacterium, and Plesiomonas, advances in molecular and phylogenetic analyses later resulted in its reclassification into the family Aeromonadaceae, which comprises mesophilic and psychrotrophic groups. *Aeromonas* species are widely distributed in aquatic environments and are increasingly recognized as emerging foodborne and opportunistic pathogens affecting both humans and animals, particularly fish. In humans, these bacteria are most commonly associated with gastroenteritis, although they are also implicated in several extra-

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intestinal infections. While contaminated water remains the principal route of transmission, foodborne exposure has emerged as an important public health concern (Teodoro *et al.,* 2022). In India, Aeromonas-associated gastroenteritis is frequently reported, and studies from Kolkata have demonstrated the enterotoxic potential of *Aeromonas* species and their prevalence in food sources such as meat, milk, and fish (Manna *et al.,* 2013).

Several species of *Aeromonas* are considered significant human pathogens capable of causing a broad spectrum of diseases, including primary and secondary septicemia, particularly among immunocompromised individuals. They are also associated with severe wound infections, including those occurring during therapeutic leech therapy, as well as infections in otherwise healthy individuals. In addition, *Aeromonas* species have been linked to meningitis, peritonitis, respiratory tract infections, and infections involving bones, joints, and eyes (Janda and Abbott, 1998; Fernandez-Escartin and Garcıa, 2001). These bacteria are believed to contribute significantly to the globally recognized seasonal illness known as "summer diarrhea", which predominantly affects travelers, children under five years of age, and elderly individuals. The pathogenicity of *Aeromonas* species is attributed to the production of multiple virulence factors, including flagella, adhesins, elastase, hemolysins, aerolysins, phospholipases, enterotoxins, and lipases, which facilitate colonization, invasion, and tissue damage in the host (Carusi *et al.,* 2024).

Virulence profiling of *Aeromonas* species can be effectively performed using PCR-based detection of genes encoding toxins, adhesins, hemolysins, lipases, and proteases. Among these, exotoxins constitute the major virulence determinants, including hemolysin (*hlyA*), aerolysin (*aerA*), cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), and cytotonic heat-stable enterotoxin (*ast*) (Roges *et al.,* 2020). As Gram-negative bacteria with increasing clinical and environmental significance, Aeromonas species warrant continuous surveillance and effective control measures (Selim *et al.,* 2025). Moreover, the genus occupies a critical position within the One Health framework and may play an important role in the dissemination of antimicrobial resistance in the environment (Bojar *et al.*, 2025). Considering the growing public health importance of *A. hydrophila* in raw market milk, the present investigation was undertaken to detect virulence genes among *A. hydrophila* isolates.

MATERIALS AND METHODS

A total of 350 pooled raw milk samples were randomly collected from retail dairies across different areas of Udaipur city, Rajasthan from June 2025 to September 2025. The samples which were found to be positive for *A. hydrophila* based on cultural identification, Gram's staining, motility characteristics and biochemical confirmation were subjected to molecular characterization based on the detection of virulence genes.

Detection of virulence genes in *A. hydrophila* isolates

Isolation of DNA from pure test culture was undertaken by using the Nucleo-pore gDNA fungal/bacterial mini kit (Genetix Biotech Asia Pvt. Ltd, India). The primers used for the molecular characterization of *A. hydrophila* isolates targeting the species-specific gene (*gyr*B) and virulence genes (*aer, ast, alt* and *act)* are described in Table 1.

Table 1: Primers used for molecular characterization of *A. hydrophila* isolates targeting species specific and virulence genes

Sl. No.	Target Gene	Sequence	Product Size (Bp)	Reference
1	*gyr*B	**F**: TCCGGCGGTCTGCACGGCGT	1100	Algammal *et al.* (2020)
		R: TTGTCCGGGTTGTACTCGTC		Xu *et al*. (2023)
2	*Alt*	**F**: TGACCCAGTCCTGG	442	Hu *et al*. (2012)
		R: GGTGATCGATCACC		Mansour *et al*. (2019)
3	*ast*	**F**: CGCCATCAACAGCTCGCCCA	212	Bernabè *et al.* (2023)
		R: CGGGCCTCGTTGAGGAAGCG		Sadiq *et al.* (2024)
4	*aer*	**F**: GCAGAACCCATCTATCCAG	252	Porteen *et al.* (2006)
		R: TTTCTCCGGTAACAGGATTG		Narmada (2022)
5	*act*	**F**:AGAAGGTGACCACCACCAAGAACA	232	Abo-Shama *et al*. (2025)
		R: AACTGACATCGGCCTTGAACTC		Eid *et al*. (2019)

The PCR procedures to screen the species-specific and virulence genes were standardized. Followed by preliminary trials, the reaction mixture was optimized to contain 12.5µl Green Taq PCR master mix, 10 nmol (0.5µl) of each forward and reverse primer, 10.5µl nuclease free water and 1µl of DNA template. The PCR reaction was carried out in a thermal cycler with a pre-heated lid set to 105°C. The cycling conditions for the various primer pairs are detailed in (Table 2).

A 1% agarose gel was prepared in 1X TBE buffer, stained with ethidium bromide, and cast using an acrylic comb. The gel was run in 1X TBE buffer, with wells oriented toward the cathode. PCR products (6 µl) and a 100 bp/1 kb DNA ladder (5 µl) were loaded into the wells, while a negative control was included in the last well. Electrophoresis was performed at 120 V for 60 min, and amplified products were visualized under a UV transilluminator.

RESULTS AND DISCUSSION

All the 12 *A. hydrophila* isolates obtained from 350 raw milk samples were confirmed by using the primers targeting the species-specific (*gyrB*) gene, yielding the product size of 1100 bp, (Fig. 1). Similarly, *gyrB* gene was used to confirm the *A. hydrophila* isolates in earlier studies conducted by Yanez *et al*. (2003), Soler *et al*. (2004) & Nhinh *et al*. (2021). Soler *et al.* (2004) used *gyrB* & *rpo D* gene sequence independently as excellent molecular markers for assessing phylogeny in the genus *Aeromonas*.

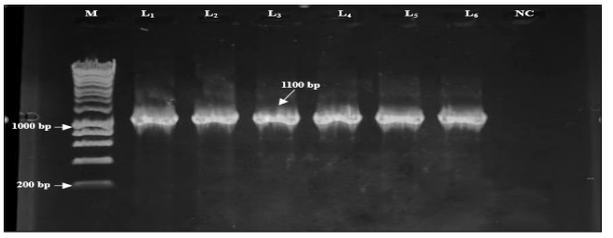

Fig. 1: Agarose gel showing PCR amplified product (1100 bp) for *gyrB* gene. M=1kb DNA ladder, positive samples (L1= 1, L2=2, L3=3, L4=4, L5= 5, L6=6, NC= negative control)

PCR assay for the detection *alt, ast, aer* and *act* genes associated with virulence in *A. hydrophila* were standardized with primers for *alt* (Mansour *et al.,* 2019 & Hu *et al*., 2012); *ast* (Sadiq *et al*., 2024 & Bernabe *et al*., 2023); *aer* (Narmada, 2022 & Porteen *et al.,* 2006) and

Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

Primers	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
gyrB	94ºC	94ºC	62ºC	72ºC	72ºC
	5 min	30 sec	30 sec	1 min	5 min
			Repeated for 30 cycles		
alt	95ºC	94ºC	55ºC	72ºC	72ºC
	5 min	30 sec	40 sec	45 sec	5 min
			Repeated for 30 cycles		
ast	95ºC	94ºC	60ºC	72ºC	72ºC
	5 min	30 sec	30 sec	1 min	5 min
		Repeated for 30 cycles			
aer	94ºC	94ºC	55ºC	72ºC	72ºC
	5 min	30 sec	50 sec	1 min	5 min
			Repeated for 30 cycles		
act	94 ºC	94 ºC	53ºC	72ºC	72ºC
	5 min	30 sec	40 sec	30 sec	5 min
			Repeated for 30 cycles		

act (Abo-Shama *et al*., 2025 & Eid *et al.,* 2019) genes. The PCR assay showed specific amplification products of 442bp, 212bp, 252 bp and 232 bp for *alt, ast, aer* and *act* genes, respectively (Fig. 2 to Fig. 5).

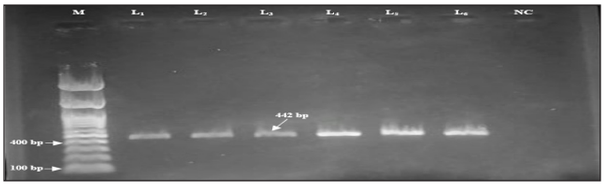

Fig. 2: Agarose gel showing PCR amplified product (442 bp) for *alt* gene. M=100bp DNA ladder, positive samples (L1= 1, L2=2, L3=4, L4=6, L5=7, L6=9, NC= negative control)

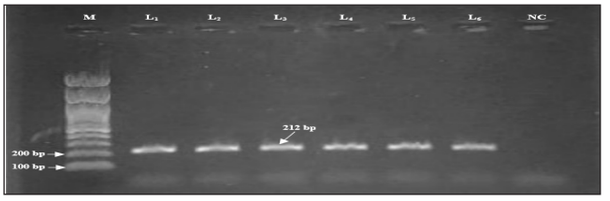

Fig. 3: Agarose gel showing PCR amplified product (212 bp) for *ast* gene. M=100bp DNA ladder, positive samples (L1= 1, L2=3, L3=4, L4=6, L5=7, L6= 11, NC= negative control)

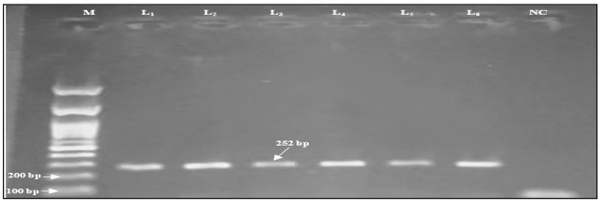

Fig. 4: Agarose gel showing PCR amplified product (252 bp) for *aer* gene. M=100 bp DNA ladder, positive samples (L1= 1, L2=2, L3=3, L4=4, L5=5, L6=6, NC= negative control)

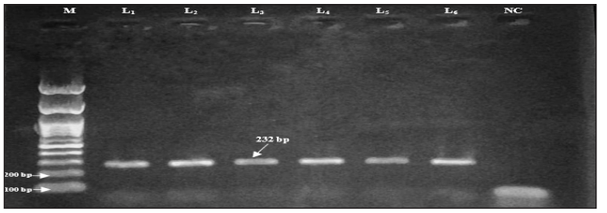

Fig. 5: Agarose gel showing PCR amplified product (232 bp) for *act* gene. M=100bp DNA ladder, positive samples (L1= 1, L2= 2, L3=3, L4= 5, L5=6, L6=8, NC= negative control)

In the current study, out of the 12 *A. hydrophila* isolates 9, 6, 11 and 8 isolates were found to be positive for *alt* (75%), *ast* (50%), *aer* (91.66%) and *act* (66.66%) genes, respectively. The PCR amplified products of *A. hydrophila* isolates revealed band size of 442 bp for *alt*, 212 bp for *ast*, 252 bp for *aer*, and 232 bp for *act* gene by agarose gel electrophoresis. Various virulence genes in *A. hydrophila* were targeted in the present study viz. *aer*, *alt*, *act* & *ast* revealing prevalence as 91.66%, 75%, 66.66% & 50%, respectively. Slightly lower prevalence of *aer* gene was reported by Sughra *et al*. (2022) & Yano *et al*. (2015) as 75% & 74%, respectively. While, lower prevalence rate of *aer* gene in *A. hydrophila* were reported by El-Baz and Hendam (2022), Ayoub *et al*. (2024) & Saleh *et al*. (2021) as 42.1%, 57.9% & 40%, respectively. Yano *et al*. (2015), Saleh *et al.* (2021) & Ayoub *et al.* (2024) reported lower prevalence rate of *alt* gene as 30%, 30% & 26.3%, respectively. While, 100% prevalence of *alt* gene was reported by Rather *et al*. (2014) among *A. hydrophila* isolates.

Pathogenicity in *A. hydrophila* is multifactorial and largely mediated by toxins such as ast, alt, aerolysin-hemolysin, and the act-encoded cytotoxic enterotoxin, which are associated with human gastroenteritis (Balsalobre *et al.*, 2009). The virulence of *A. hydrophila* is closely linked to the production of several virulence factors and its biofilm-forming ability, including serine protease, hemolysin, aerolysin, cytotoxic enterotoxin, and adhesins such as fimbriae and S-layer proteins (Zhou *et al*., 2019). Exotoxins, including the cytotoxic heat-labile enterotoxin (act), cytotonic heat-labile enterotoxin (alt), cytotonic heat-stable enterotoxin (ast), aerolysin/hemolysin, lipase, and phospholipase, play important roles in pathogenicity (Kingombe *et al.,* 2010). Although several PCR methods have been developed for detecting individual cytotoxin and hemolysin genes, a multiplex PCR assay was designed to simultaneously amplify the hemolysin and aerolysin genes of *A. hydrophila* and *A. sobria* (Wang *et al.*, 2003). Variations in the frequency of virulence genes among *A. hydrophila* isolates indicate diversity in their virulence gene profiles.

GENERATIVE AI DECLARATION

The author(s) confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript, and no images were

manipulated using AI. The authors developed and verified all scientific content, data analysis, interpretation of results, and conclusions. The authors take full responsibility for the accuracy, integrity, and originality of the work presented, and no AI tool was listed as an author.

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