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Assessment of *Aeromonas* species prevalence and virulence in fresh meats around Chennai, India

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ABSTRACT

The present study was conducted to identify various *Aeromonas* species and assess their virulence potential in meat samples, including fish, chicken, mutton, and pork, collected from retail markets and slaughterhouses in and around Chennai. A total of 200 samples (50 each from fish, chicken, mutton, and pork) were analyzed. Isolation and identification of *Aeromonas* species were performed using conventional culture techniques, biochemical tests, and multiplex PCR assays targeting the 16S rRNA**,** *gyr*B**,** and *rpo*B genes. Out of the 200 samples, 95 (47.5%) were confirmed as *Aeromonas* species, comprising 22 (44%) from fish, 18 (36%) from chicken, 24 (48%) from mutton, and 31 (62%) from pork. Among the isolates, *A. veronii* was the most prevalent species with 59 (62.11%) isolates, followed by *A. hydrophila* (20; 21.05%), *A. caviae* (11; 11.58%), and *A. mediae* (5; 5.26%). The virulence genes *hly*A**,** *aer*A**,** *ast***,** *alt*, and *act* were detected in 63 (66.32%) of the 95 isolates by multiplex PCR. Specifically, 85% of *A. hydrophila*, 62.71% of *A. veronii*, 60% of *A. mediae*, and 54.55% of *A. caviae* harbored one or more of these virulence genes. The presence of multiple virulence genes, along with the high prevalence of *Aeromonas* spp. in various meat sources, highlights the potential public health risk associated with the consumption of contaminated meat. This study underscores the need for routine monitoring and hygienic handling practices to limit the spread of pathogenic *Aeromonas* in the food chain.

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1. Introduction

Meat is the most valuable livestock product, which requires proper attention right from animal slaughtering to human consumption to ensure safety. The consumption of meat has been increasing in India over the last decade as it is associated with the quality of life. Global demand for meat is growing: over the past 50 years, meat production has more than tripled. Livestock production plays a key role in augmenting income; mainly, poultry and dairy are the major sectors

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contributing to economic development. Most meats consumed in India are fish, bovine, mutton, goat, pig, and poultry (1).

The global production and consumption of broilers have gradually increased worldwide in recent years, with an estimated projectedproduction of 100 million tons, respectively in the year 2022 (2).

Meat is a prime vehicle for the dissemination of foodborne pathogens to humans worldwide. Microbial meat contaminants can cause food-borne diseases in humans. Almost 16 lakh people get sick due to unsafe food in one day, on average. Around 200 foodborne diseases are primarily caused by unsafe food, with clinical signs ranging from diarrhea to cancer (3).

Among the foodborne pathogens, *Aeromonas* spp. is related to food spoilage and is found distributed in various foods widely, including poultry, fish, shrimp, pork, and others. The food contact surfaces are a potential source of contamination, frequently causing cross-contamination during food processing (4). The mesophilic aeromonads are associated with various human diseases, including gastrointestinal diseases, wound infections, and septicemia (5,6). Among the recognized species of *Aeromonas* (7), a subset of four species is more commonly implicated in human infections *(Aeromonas hydrophila, A. caviae, A. veronii biovar sobria,* and *A. dhakensis)*.

Recently, a lot of interest has been focused on this bacterium because it affects not only aquatic animals, but the impact on financial loss is tremendous (8). In humans, septicemia, skin necrotizing fasciitis, gangrene, gastroenteritis, and diarrhea among travelers are due to unhygienic handling of meat and consumption of contaminated food (9).

A. hydrophila represents the most virulent of these species and produces multifactorial virulence factors, such as aerolysin, which leads to lysis and toxicity of the cells (10,11,12). The potential role of *A. hydrophila* in humans has been reported by many researchers, of which most (>85%) of gastroenteritis cases are attributed to three major *Aeromonas* species, one of being *A. hydrophila* (13).

Many *Aeromonas* spp. can grow in raw and cooked food under a modified atmosphere at refrigerated temperatures (14). High prevalence of *Aeromonas* spp. may be possible in slaughterhouses located in India or food markets due to poor sanitation and temperature menace (15,16,17). Prevalence of *Aeromonas* spp. of 22% was reported in raw meat samples, with 26%, 24%, and 19% prevalence among chevon, chicken, and milk samples, respectively (18).

Aeromonads are motile by a single polar flagellum, catalase-positive, oxidase-positive rods, which ferment glucose. It is neither salt (<5%) nor acid (min. pH ~ 6.0) tolerant and grows optimally at around 28°C. It can grow at cold temperatures, reportedly as low as -0.1°C for some strains. Ampicillin dextrin agar (ADA) media is used for the isolation of aeromonads from fish and chicken. Hemolytic types could be identified by streaking the bacterial isolates on blood agar (19). In addition to aerolysin, which induces β-hemolysis, complete lysis of erythrocytes, β-hemolysins, another type of enzyme with hemolytic activity, can also be evaluated by molecular methods (20).

The 16S rRNA gene in mPCR analysis has been reported as a fast and effective way to identify and confirm *Aeromonas* species (21,22). With multiplex PCR

assay, the presence of *A. caviae* (26.39 %), *A. hydrophila* (20.83 %), and *A. veronii* (8.34 %) was reported (23).

Determination of bacterial virulence factors is a key aspect of understanding bacterial pathogenicity. Pathogenic bacteria to cause disease in susceptible hosts via the activity of multiple virulence factors that act individually or in combination, and pathogenic bacteria produce many substances that are toxic to host cells either directly or indirectly (24).

Regarding virulence factors, they are capable of producing the cytotoxic enterotoxins (*alt* and *ast* genes, and *act* gene), aerolysins (*aer*A gene), and hemolysins (*hly*A gene) (5,25).

22 *Aeromonas hydrophila* isolates were screened by author El-Bahar (8) for the presence of four virulence genes; sixteen of the isolates (72.72%) were positive for the aerolysin gene (*aer*); 4 (18.18%) harboured the cytotoxic enterotoxin gene (*act*); and 2 (9.09%) carried the *hemolysin* A gene (*hyl*A), while the cytotoxic heat-stable enterotoxin gene (*ast*) was absent from all the tested isolates.

The present study aimed to ascertain the prevalence status and virulence potential of *Aeromonas* isolates and characterize them genotypically by multiplex PCR assay for species identification and for targeted virulent genes.

2. Materials and Methods

2.1. Study area and sampling

Sampling of animal products was carried out from month of January to September 2023. 50 samples from Fish, chicken, mutton, and pork meat were collected from retail markets and slaughterhouses around Chennai. A total of 200 samples were collected under refrigerated conditions and transported immediately to the Department of Veterinary Public Health Laboratory, MadrasMadras Veterinary College, Chennai, for further analysis.

2.2. Isolation and identification of *Aeromonas* species

The Food and Drug Administration-Bacteriological Analytical Manual (FDA-BAM) protocol was followed for the isolation and identification of *Aeromonas* species. The conventional method of isolation was performed by initial inoculation in nutrient broth, followed by selective plating on Ampicillin Dextrin Agar (ADA). *Aeromonas* species exhibited characteristic yellowish green colonies and were picked and further characterized by Gram's staining and biochemical tests, viz., catalase, oxidase, citrate, TSI agar inoculation, growth on Mac conkey, and hemolytic pattern on 10% sheep blood agar observed. DNA extraction kit (Qiagen) was used in this study for the extraction of pure and high-concentration genomic DNA. The presumptive isolates were confirmed by Multiplex PCR, targeting the 16S rRNA for genus-specific identification, and species-specific primers were used targeting *the gyr*B and *rpo*B genes (Table 1).

2.3. Detection of virulence gene

The presence of five virulent genes, *hly*A, *aer*A, *ast*, *alt*, and *act,* was detected by multiplex PCR using standardized cycling conditions with suitable primers (Table 2).

Table 1. Primers used in the mPCR method for *Aeromonas* species identification

S. No.	TARGET ORGANISM and GENE	PRIMER SEQUENCE (5'-3')	SIZE (bp)
1.	***Aeromonas Universal* - 16S rRNA**	F - CGACGATCCCTAGCTGGTCT R - GCCTTCGCCACCGGTAT	461
2.	***A. hydrophila* - *gyrB***	F - AGTCTGCCGCCAGTGGC F - CRCCCATCGCCTGTTCG	144
3.	***A. veronii*– *rpoB***	F -CGTGCCGGCTTTGAAGTC R – GATCACGTACTTGCCTTCTTCAATA	224
4.	***A. mediae* - *gyrB***	F – GGCCAAGCGTCTGCGT R - CGCCCTCGTAGCAGAAGTGA	99
5.	***A. caviae* - *gyrB***	F - TGCTGCTGACCATCCGC R - GGTGCCTGCGGCTCG	70

*Primers designed for this research study using NCBI- Primer BLAST

Table 2. Primers used for detecting virulence genes of *Aeromonas* species

S. No.	Target gene	Primer Sequence (5'-3')	Size (bp)	References
1.	***aer*A**	F: CAAGAACAAGTTCAAGTGGCCA R: ACGAAGGTGTGGTTCCAGT	309	(27)
2.	***alt***	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	
3.	***ast***	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	328	(4)
4.	***act***	F: TACACGACAGCGCGAATTTT R: GAGTTCGGTTTCCCCTACCA	449	Designed for this study using NCBI-Primer BLAST
5.	***hly*A**	F: TATGTCAGTGCGCAGGATACC R: GGTCCGTACGCTCACATTCT	219	

2.4. Statistical analysis

Statistical analysis was performed using SPSS software (Version 25.0).

The prevalence of virulence genes and antibiotic resistance patterns was analyzed using Chi-square tests, and significance was set at $p<0.05$.

The differences in prevalence among the different meat types were assessed using the Chi-square test to determine if the observed frequencies of positive isolates were significantly different from expected frequencies. A p-value of <0.05 was considered statistically significant. The percentage occurrence of each species was calculated. A one-way ANOVA was conducted to compare the mean prevalence of different species across the meat types, with post-hoc Tukey's test to identify specific group differences.

The frequency of each virulence gene was calculated, and the data were analyzed using logistic regression to assess the association between species and the presence of virulence genes. Odds ratios (OR) with 95% confidence intervals (CI) were calculated to evaluate the strength of the association. The distribution of multiple virulence genes was assessed using a contingency table, and the Chi-square test was applied to determine if there was a significant association between the number of virulence genes and the species of *Aeromonas*.

3. Results

3.1. Identification of *Aeromonas* species

A total of 200 meat samples from fish, chicken, mutton, and pork were screened for the presence of *Aeromonas* species. Of these, 95 presumptive *Aeromonas* isolates were obtained based on colony morphology on selective media and biochemical tests. The distribution of isolates was as follows: fish (13), chicken (9), mutton (7), and pork (28) (Table 3). Presumptive identification was based on characteristic yellowish-green, translucent colony morphology on Ampicillin Dextrin Agar (ADA).

Biochemical identification followed the Bacteriological Analytical Manual (29). All isolates were positive for catalase, oxidase, citrate utilization, and motility. However, variable results were noted for growth on MacConkey agar, hemolysis on sheep blood agar, and fermentation patterns in Triple Sugar Iron (TSI) agar. Based on biochemical profiling, isolates were identified as *A. hydrophila*, *A. caviae*, *A. veronii* biogroup sobria, and *Aeromonas* spp.

3.2. Species-wise biochemical behaviour

- *hydrophila*: TSI showed acid butt and alkaline slant with H_2S; 14/20 was hemolytic; all showed pale lactose-fermenting colonies on MacConkey agar.
- *A. veronii*: Acid butt and alkaline slant without H_2S; 29/59 were hemolytic; 25 showed lactose fermentation while 34 showed no growth on MacConkey agar.
- *A. mediae*: Acid butt and acid slant without H_2S; 2/5 hemolytic; no growth on MacConkey agar.
- *A. caviae*: 2 showed acid butt and acid slant, 9 showed acid butt and alkaline slant; 2 were hemolytic; all formed pale lactose-fermenting colonies.

Table 3. Number of *Aeromonas* species isolated from various meat samples

	FISH	CHICKEN	MUTTON	PORK	TOTAL
A. hydrophila	10	1	5	4	20 (21.05%)
A. veronii	3	17	17	22	59 (62.11%)
A. mediae	2	-	1	2	5 (5.26%)
A. caviae	7	-	1	3	11 (11.58%)
TOTAL (out of 200)	22 (44%)	18 (36%)	24 (48%)	31 (62%)	**95 (47.5%)**

Table 4. *Aeromonas* species-wise virulence gene presence and their percent occurrence

Details of the virulence gene in various *Aeromonas* species	*A. hydrophila*	*A. veronnii*	*A. mediae*	*A. caviae*	Percent occurrence of the Virulence gene
No. of positive isolates	20	59	5	11	95
Isolates with the virulent gene	17	37	3	6	63 (66.32%)
*hly*A	14	28	2	2	46 (48.42%)
*aer*A	8	6	1	3	18 (18.95%)
ast	2	6	3	2	13 (13.68%)
alt	6	13	1	4	24 (25.26%)
act	11	14	2	4	31(32.63%)

3.3 Molecular confirmation

Multiplex PCR was performed to confirm all 95 presumptive *Aeromonas* isolates. Species-specific primers targeting 16S rRNA (461 bp), *rpo*B (224 bp for *A. veronii*), and *gyr*B (144 bp for *A. hydrophila*, 70 bp for *A. caviae*, and 99 bp for *A. mediae*) were employed. All isolates displayed expected amplicons, confirming species identity. Gel electrophoresis (1.7%) and documentation substantiated the results.

3.4. Virulence gene detection

A multiplex PCR assay was employed to detect five key virulence genes—*hly*A (219 bp), *aer*A (309 bp), *ast* (328 bp), *alt* (442 bp), and *act* (449 bp)—among the 95 confirmed *Aeromonas* isolates. These genes are known to encode hemolysins and enterotoxins, which play a crucial role in the pathogenicity of *Aeromonas* species.

Out of the total isolates, 63 (66.32%) were found to harbor at least one of the targeted virulence genes, indicating a high potential for pathogenicity (Table 4**)**. The gene *hly*A, encoding hemolysin, was the most frequently detected, present in 46 isolates (48.42%). This was followed by *act* in 31 isolates (32.63%), *alt* in 24 isolates (25.26%), *aer*A in 18 isolates (18.95%), and *ast* in 13 isolates (13.68%).

The relatively high prevalence of *hly*A, *act*, and *alt* genes suggests that many of these isolates possess cytotoxic and enterotoxic capabilities, reinforcing their potential threat to food safety and public health. The diversity in virulence gene profiles among isolates further emphasizes the necessity of molecular characterization for assessing the health risks posed by *Aeromonas* species in meat products.

3.5. Multiple virulence genes

Among the 95 *Aeromonas* isolates analyzed, 23 strains (24.21%) were found to harbor more than one virulence gene, underscoring their multifactorial pathogenic potential. Species-wise distribution of multiple gene carriage revealed the following patterns:

- *A. hydrophila*: 8 out of 20 isolates (40%) possessed multiple virulence genes
- *A. veronii*: 10 out of 59 isolates (16.95%)
- *A. mediae*: 2 out of 5 isolates (40%)
- *A. caviae*: 3 out of 11 isolates (27.27%)

The relatively high proportion of isolates carrying multiple virulence determinants, particularly among *A. hydrophila* and *A. mediae*, indicates their enhanced pathogenic potential. This observation supports earlier studies suggesting that combinations of hemolysin and enterotoxin genes may contribute synergistically to the virulence of *Aeromonas* strains. Such genetic configurations pose a greater risk to consumers, especially in undercooked or contaminated meat products.

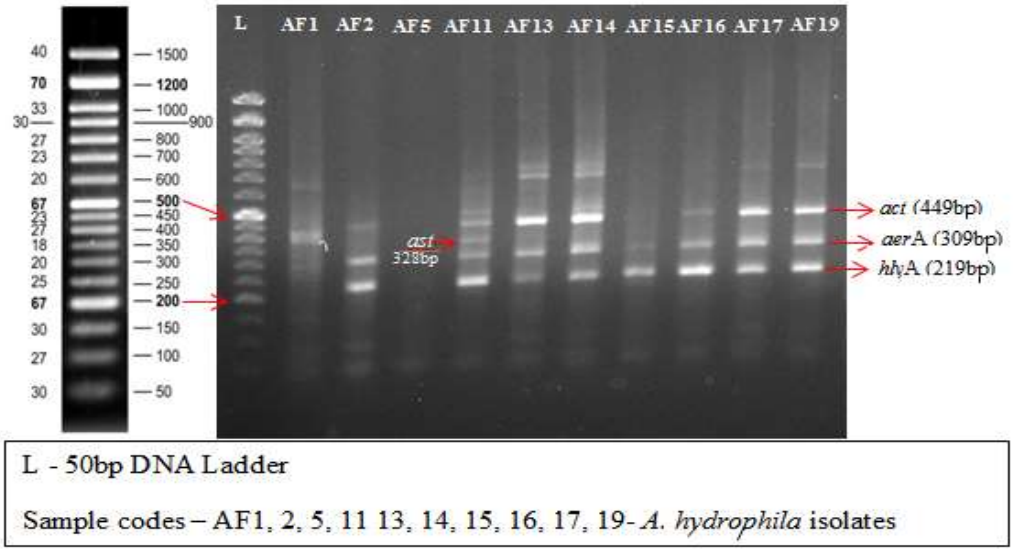

Figure 1. Four meat sample isolates were analyzed by mPCR for *Aeromonas* species wise virulence genes identifications. Each primer set was designed to be specific for the particular virulence genes and to have a unique amplicon size when analyzed by agarose gel (1.7%) electrophoresis. Lane 1, 50-bp DNA marker; lane 2 to Lane 11, virulence genes in *A. hydrophila* isolates.

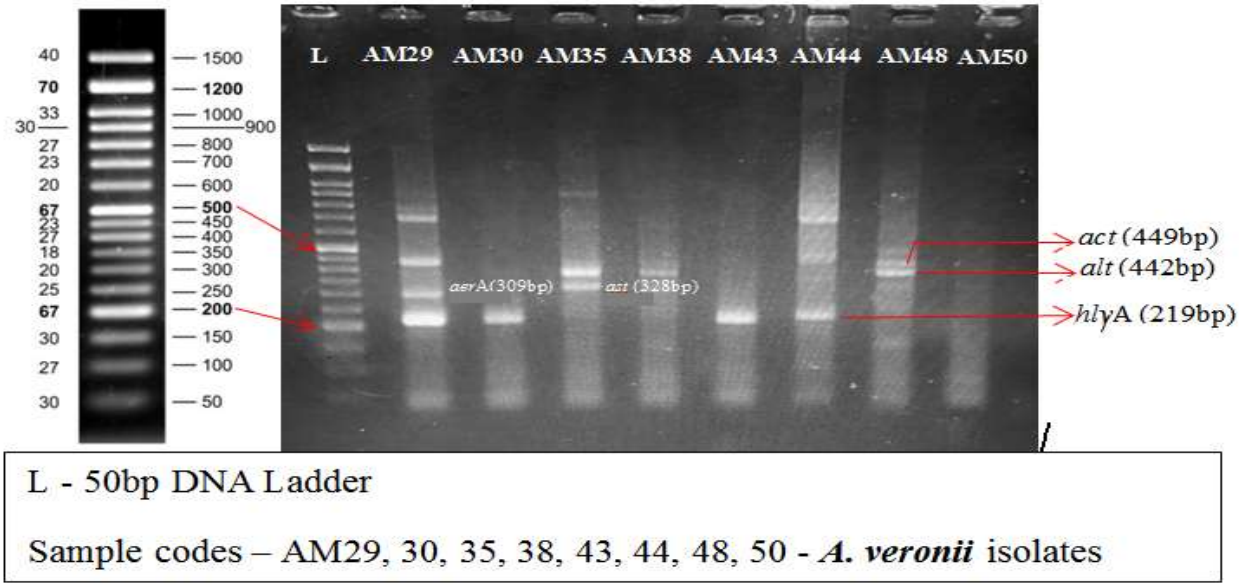

Figure 2. Four meat sample isolates were analyzed by mPCR for *Aeromonas* species-wise virulence gene identifications. Each primer set was designed to be specific for the particular virulence genes and to have a unique amplicon size when analyzed by agarose gel (1.7%) electrophoresis. Lane 1, 50-bp DNA marker; lane 2 to Lane 9, virulence genes in *A. veronii* isolates.

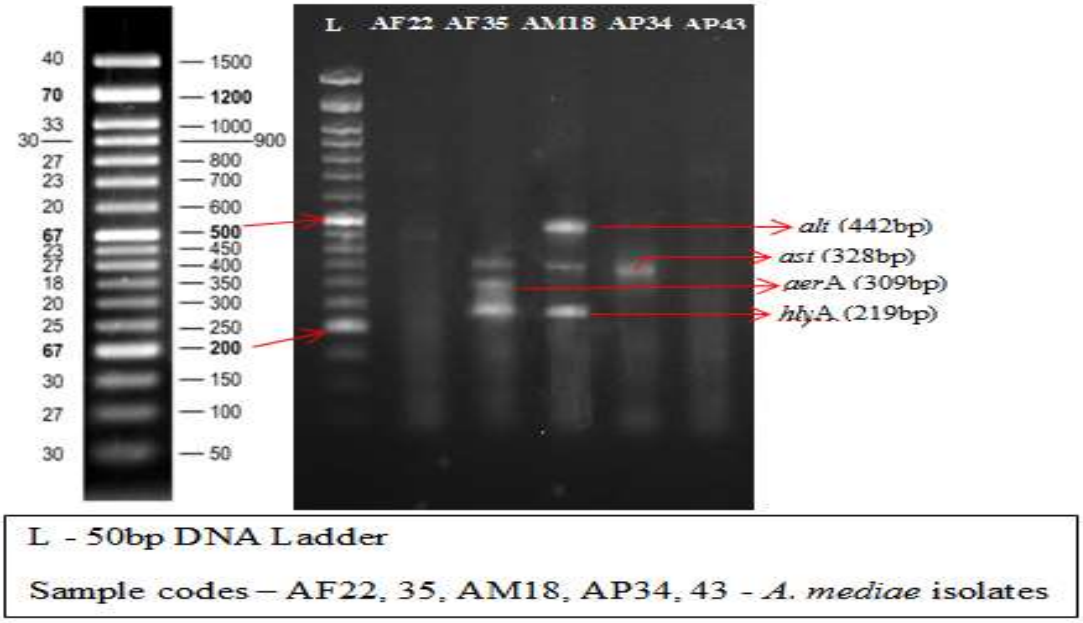

Figure 3. Four meat sample isolates were analyzed by mPCR for *Aeromonas* species-wise virulence genes identifications. Each primer set was designed to be specific for the particular virulence genes and to have a unique amplicon size when analyzed by agarose gel (1.7%) electrophoresis. Lane 1, 50-bp DNA marker; lane 2 to Lane 6, virulence genes in *A. mediae* isolates.

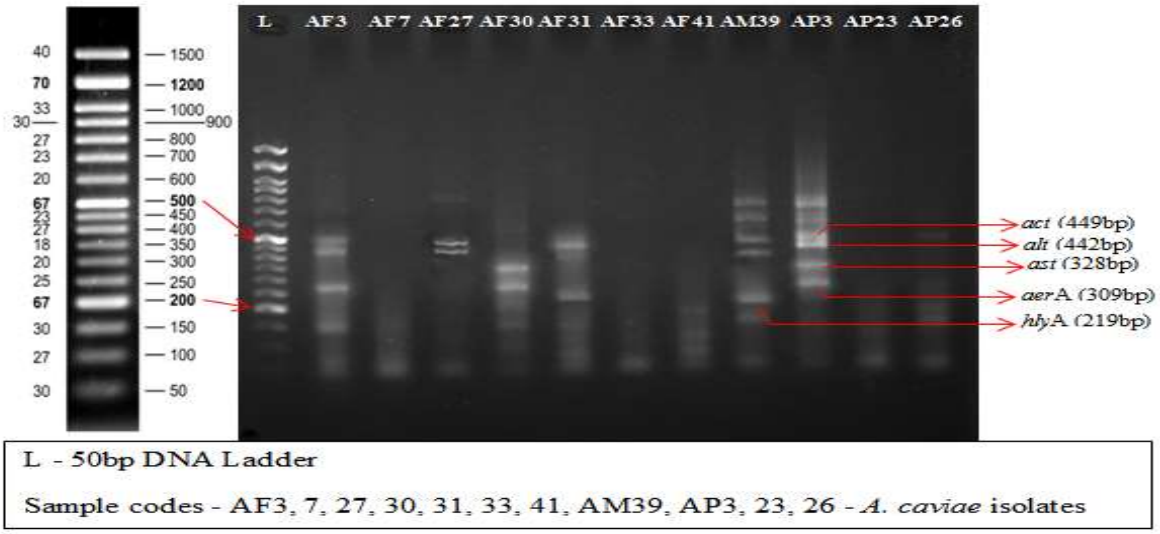

Figure 4. Four meat sample isolates were analyzed by mPCR for *Aeromonas* species-wise virulence genes identifications. Each primer set was designed to be specific for the particular virulence genes and to have a unique amplicon size when analyzed by agarose gel (1.7%) electrophoresis. Lane 1, 50-bp DNA marker; lane 2 to Lane 12, virulence genes in *A. caviae* isolates.

4. Discussion

4.1. *Aeromonas* isolation from meat samples

The present study confirmed the occurrence of *Aeromonas* species in retail meat, especially in pork and mutton, indicating possible substandard hygiene during slaughter and post-processing stages. The highest recovery rates from pork (31/95) and mutton (24/95) samples point to a probable fecal or environmental contamination route, consistent with findings from Gowda et al. (25), who reported frequent isolation of *Aeromonas* spp. from food animals in Indian slaughterhouses.

The use of Ampicillin Dextrin Agar (ADA) facilitated the effective selective isolation of *Aeromonas* spp. Due to its inhibitory effect on other Gram-negative organisms (28). This medium, together with colony morphology, served as a reliable preliminary screening tool, aligning with protocols outlined in the FDA Bacteriological Analytical Manual (28,30).

4.2. Biochemical identification of *Aeromonas* Species

Biochemical characterization of the isolates showed considerable species-level variation, corroborating earlier studies that emphasized the need for polyphasic identification methods (9,30,31). *A. hydrophila* exhibited H_2S production in TSI and consistent lactose fermentation, while *A. veronii* and *A. mediae* demonstrated poor growth or no growth on MacConkey agar, consistent with previous observations (32,33).

The observed hemolytic activity among *Aeromonas* isolates adds another layer of complexity, as hemolysin production has been associated with increased virulence (21,22,34). While 14 out of 20 *A. hydrophila* strains were hemolytic, the variability in hemolysis across species underlines the need for additional virulence testing to accurately assess pathogenic potential (4,21).

4.3. Molecular confirmation by PCR

Multiplex PCR targeting species-specific genes such as 16S rRNA, *rpo*B, and *gyr*B successfully confirmed the identity of all presumptive isolates (29). These molecular markers are widely accepted for phylogenetic resolution and species differentiation within the genus *Aeromonas* (7,9). The use of these housekeeping genes provided enhanced specificity over traditional biochemical tests alone (2,14).

PCR-based confirmation aligns with the findings of Persson et al. (7), who validated *rpo*B and *gyr*B sequencing as reliable tools for distinguishing closely related *Aeromonas* taxa. The combination of molecular and biochemical tools in the present study thus ensured greater diagnostic accuracy.

4.4. Virulence gene profiling

Out of the 95 confirmed isolates, 63 (66.32%) harbored one or more of the five targeted virulence genes—*hly*A, *aer*A, *ast*, *alt*, and *act*—highlighting the considerable pathogenic potential of meat-derived Aeromonas strains. This prevalence is consistent with findings from El-Bahar et al. (8) and Saleh et al. (35), who reported high virulence gene presence in isolates from fish and tilapia.

The most frequently detected gene was *hly*A (48.42%), followed by *act* (32.63%) and *alt* (25.26%). These genes are known to encode hemolysin and enterotoxins, key factors in cytotoxicity and enterotoxicity (21,23). The high incidence of *hly*A supports previous reports where

hemolysin genes were dominant among virulent *Aeromonas* strains (3,24).

A. hydrophila and *A. veronii* isolates demonstrated the highest frequency of virulence genes, in agreement with earlier studies linking these species to human gastrointestinal infections and extra-intestinal diseases (9,32,36). The relatively lower frequency of *aer*A and *ast* may reflect strain-specific or source-specific variations, as suggested by Yogananth et al. (20) and Yousr et al. (15).

4.5. Occurrence of multiple virulence genes

Nearly a quarter (24.21%) of the isolates harbored more than one virulence gene, with *A. hydrophila* and *A. mediae* being the most frequent carriers. The detection of multiple virulence determinants suggests synergistic interactions among these genes, potentially enhancing the severity of infection (16,35). Similar findings were reported by Ahangarzadeh et al. (37), who noted multifactorial virulence gene profiles in *A. hydrophila* from carp fish.

The presence of combinations such as *hly*A + *act* or *alt* + *aer*A raises concerns about the potential for increased host tissue damage and enterotoxin-mediated illness. Such co-occurrence reinforces the concept that virulence in *Aeromonas* is multifaceted and depends not only on individual genes but also on their interplay (16,34).

This finding is especially significant from a food safety perspective, as undercooked or mishandled meat products carrying multiple virulent Aeromonas strains could act as reservoirs for zoonotic transmission (18,30). It also highlights the public health relevance of meat-borne *Aeromonas* and the importance of molecular surveillance in food microbiology laboratories.

5. Conclusion

In this study, *Aeromonas* species showed the highest occurrence (62%) in pork meat, followed by fish meat. The presence of *Aeromonas* species in food sources was mainly attributed to cross-contamination with contaminated water. Furthermore, the detection of clinically important species such as *A. hydrophila* and *A. caviae* in meats intended for human consumption raised serious concerns regarding public health significance. A major proportion of the isolates (66.32%; 63/95) carried important virulence genes, most of which were commonly associated with *Aeromonas* infections. Notably, *hly*A, *aer*A, and *act* were frequently reported in cases of *Aeromonas*-associated gastroenteritis. This indicated a high prevalence of virulent *Aeromonas* species circulating in the environment. Additionally, 24.21% of the isolates were found to harbor multiple virulence genes. These results highlighted a serious public health concern and emphasized the potential risk of *Aeromonas*-associated foodborne infections due to meat source contamination.

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CRediT authorship contribution statement

- **Conceptualization:** Dharani Manivannan, Sundaram Sureshkannan

- **Methodology:** Dharani Manivannan, Sundaram Sureshkannan
- **Data curation and Formal analysis:** Dharani M.
- **Supervision:** Sundaram Sureshkannan, Porteen Kannan, Narendra Babu Ravindran
- **Writing – original draft:** Dharani Manivannan.
- **Writing – review & editing:** Sundaram Sureshkannan, Porteen Kannan, Narendra Babu Ravindran
- **Project administration:** Sundaram Sureshkannan

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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