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Genetic diversity, virulence attributes and antimicrobial resistance of *Aeromonas hydrophila* isolated from fishes and water in Puducherry

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Abstract

Aeromonas hydrophila is one of the emerging foodborne pathogens that are autochthonous to aquatic ecosystems. The present investigation was undertaken to study the occurrence, virulence properties and antimicrobial drug resistance pattern of *A. hydrophila* in fishes (n = 100) and water (n = 100) in Puducherry. About 15 *Aeromonas* spp. isolates were obtained by standard bacteriological methods which were presumptively confirmed by morphological and biochemical tests. All the 15 isolates (100%) were confirmed as *A. hydrophila* by PCR targeting species specific 16S rRNA gene. Eight out of fifteen isolates (53.33%) produced β -haemolysis. Also 6 isolates (40%) were positive for gelatinase activity. Molecular detection of virulence genes revealed that haemolysin (*hly*) gene and cytotoxic enterotoxin (*act*) gene was present in 5 (33.33%) and 3 isolates (20%) respectively. Antimicrobial sensitivity test showed that all the isolates were resistant to co-trimoxazole (100%) and penicillin G (100%) and sensitive to gentamicin (100%). Multidrug resistant was found in 46.67% of isolates (n = 7) as they showed resistance against more than 3 antimicrobial classes tested. Molecular detection of antimicrobial resistance genes showed that *tetA* gene and *bla_{TEM}* gene was present in 3 (20%) and 4 isolates (26.67%) respectively. Molecular typing by ERIC-PCR revealed grouping of isolates into four different clusters and two isolates remained outgroup. A wide genetic diversity of isolates irrespective of their source of isolation was noticed and no particular clone was predominant. The study documented the presence of multidrug resistant, virulent *Aeromonas hydrophila* among fishes and its environment (water) which is of public health concern.

Keywords: *Aeromonas hydrophila*, antimicrobial resistance, fish, ERIC-PCR, one health

1. Introduction

Foods of animal origin is considered superior mainly because of its high-quality protein with higher biological value. Fish is an excellent nutritious medium for the microbes to grow, due to high moisture, minerals, accessory growth factors and a favourable pH. The genus *Aeromonas* comprises 36 species of which at least 19 are considered emerging pathogens to humans, causing a broad spectrum of infections [1]. The most relevant clinical species are *A. caviae*, *A. veronii*, *A. hydrophila* and *A. dhakensis* which are also found in a wide variety of fresh produce, meat and dairy products [2]. These foods play an important role in the dissemination of the potentially pathogenic *Aeromonas* to humans. The important fish pathogens like *A. salmonicida* and *A. hydrophila* particularly affect salmonids, causing ulcers, hemorrhages, furunculosis, and septicemias [3]. *A. hydrophila* cause cellulitis, abscess, wound infection, necrotizing fasciitis, myonecrosis, pneumonia, empyema, septicemia, septic arthritis, osteomyelitis, endocarditis, meningitis, gastroenteritis, appendicitis, peritonitis, acute suppurative cholangitis and corneal ulcer in humans [4, 5]. *Aeromonas* spp. has been reported to secrete exotoxins (hemolysins, cytotoxins and enterotoxins), hemagglutinins, adhesions and several hydrolytic enzymes [6]. Many of the studies also reported the distribution of virulence genes among the isolates [7]. Global concern over the use and abuse of antimicrobials/antibiotics in animal husbandry-aquaculture and the parallel emergence of drug-resistant microbes are increasing every day. Based on the above discourse, the present investigation was undertaken to study the occurrence of one of the most relevant clinical species, *A. hydrophila* in fishes and water in Puducherry with reference to their virulence potential and antimicrobial resistance.

2. Materials and Methods

A total of 50 marine fishes, 50 freshwater fishes, 50 marine water and 50 fresh water samples were collected from different sites in Puducherry. Indian oil sardines (*Sardinella longiceps*) and Indian grey mullets (*Mugil cephalus*) were considered for the study among the marine fishes and Catla (*Catla catla*) and Rohu (*Labeo rohita*) were considered for the study among the freshwater fishes since they top the list of marine fish landings and inland fish landings in Puducherry (Handbook on Fisheries Statistics-2020, GOI) [8]. The samples were aseptically collected and transported to the Department of Veterinary Public Health and Epidemiology (VPE), Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry for further processing.

2.1 Isolation and identification of *Aeromonas* spp.

Fish samples were processed to isolate the *Aeromonas* spp. as per the method described by Agarwal *et al.* (2003) [9] with suitable modifications. Ten grams of fish Samples (Gills, intestine, muscle) was homogenized with normal saline solution in a sterile mortar and pestle and volume was made to 100 ml. About 1 ml of the homogenized sample was inoculated in 10 ml Alkaline Peptone Water (APW), mixed thoroughly and incubated at 37 °C for 24 h. A loopful of enriched inoculum from APW was further inoculated on Ampicillin Dextrin Agar (ADA) and incubated at 37 °C for 24 h. Water samples were processed to isolate the *Aeromonas* spp. as per the method described by Rippey *et al.* (1979) [10] with suitable modifications. About 100 ml of water sample was allowed to pass through the membrane filter of size 0.22 µm. After filtration, the filter papers were placed on ADA plates and incubated at 37 °C for 24 h. Yellow coloured colonies 1-2 mm in diameter on ADA were picked up on the brain heart infusion agar slant and stored at 4 °C for further characterization.

2.2 Morphological and biochemical characterization

The confirmatory characteristics used to confirm the isolates as *Aeromonas* spp. were gram negative motile rods, oxidase and catalase positive, glucose and mannitol fermentation positive and ornithine decarboxylase negative [11, 12].

2.3 Molecular confirmation of *Aeromonas* spp. and *A. hydrophila*

The DNA extraction was performed using boiling and snap chilling method as described by Arora *et al.* (2006) [13]. *Aeromonas* spp. isolates identified by biochemical tests were subjected to PCR using genus specific primers targeting 16S rRNA gene (Table 1) to identify *Aeromonas* spp. as described by Wang *et al.* (2003) [14]. On confirmation, the isolates were screened to identify *A. hydrophila* by using species specific primers targeting 16S rRNA gene as described by Trakhna *et al.* (2009) [15] (Table 1). PCR mixture concentration included 4 µl of template DNA, 1 µl of 10 pmole/µl of forward and reverse primer each, 12.5 µl of 2X master mix and 6.5 µl of nuclease free water. PCR assay for both the genes were standardized using the standard strain of *A. hydrophila* subsp. *hydrophila* (MTCC 1739).

2.4 Virulence characterization

2.4.1 Haemolysin assay: The isolates were examined for haemolysin production as described by Khuntia (2011) [16].

The isolates were grown in Tryptone Soya broth for 18-24 h and inoculated on Tryptic Soya Agar (TSA) plates supplemented with 5% defibrinated sheep blood. The plates were incubated at 37 °C up to 72 h and observed for the appearance of clear zone of haemolysis.

2.4.2 Gelatinase test: The isolates were examined for gelatinase production by stab method in nutrient agar supplemented with 12% (w/w) gelatin [16]. The pure cultures of isolates were stabbed individually into media tubes and incubated for 48 h at 37 °C. Thereafter, the tubes were placed under refrigeration for 30 min at 4 °C. The tube that did not solidify after refrigeration were considered positive for gelatinase production.

2.4.3 Molecular screening of virulence genes: PCR was carried out to detect haemolysin (*hly*) and Cytotoxic Enterotoxin (*act*) genes using the primers (Table 1) as described by Gonzalez *et al.* (2002) [17] and Kingombe *et al.* (1999) [18] respectively.

2.5 Screening for antimicrobial resistance

2.5.1 Antimicrobial sensitivity test: All the isolates were subjected to antimicrobial sensitivity test by disc diffusion technique as described by Bauer *et al.* (1966) [19] against a panel of 8 antibiotics which includes Amoxicillin (30 mcg), Ciprofloxacin (10 mcg), Chloramphenicol (25 mcg), Co-trimoxazole (10 mcg), Cefotaxime (10 mcg), Gentamicin (50 mcg) Penicillin G (2 units) and Tetracycline (10 mcg). Briefly the isolates were grown in nutrient broth at 37°C for 16 hrs. Individual broth cultures were smeared on the Mueller-Hinton (MH) agar plates with the help of sterile cotton swabs. The plates were allowed to dry for few minutes. The antibiotic disc were placed on the agar surface within 15 minutes of inoculation of plates and incubated overnight at 37°C. Based on the zone of inhibition, the isolates were graded as sensitive, intermediate and resistant as per CLSI guidelines (CLSI, 2023) [20]. Multi drug resistant isolates were defined as those which depicted resistance to more than 3 classes of antimicrobial tested [21]. Multiple antibiotic resistance (MAR) index was determined for each isolate by using the formula $MAR = A/B$, where “A” represents the number of antibiotics to which the test isolate depicted resistance and “B” represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility [22].

2.5.2 Molecular detection of antimicrobial genes: PCR was carried out to detect the presence of *tetA* and *bla_{TEM}* genes using the primers (Table 1) as described by Schmidt *et al.* (2001) [23] and Rasheed *et al.* (1997) [24] respectively.

2.6 Heat map analysis

OriginPro software was used to generate a heatmap supported by hierarchical clustering (dendrogram) on binary data from different sources (fish/water) to visualize the overall distribution pattern of antimicrobial resistance (AMR) and virulence genes in *A. hydrophila* isolates.

2.7 Molecular typing by ERIC PCR

ERIC-PCR was performed to determine the clonal relationship and genetic diversity of *Aeromonas* spp. isolates from different sources as described by Versalovic *et al.* (1991) [25] with slight modifications. PCR was performed

under steps and conditions described in Table 1 with the PCR mixture concentration 4 µl of template DNA, 1 µl of 50 pmole/µl of forward and reverse primer each, 12.5 µl of 2X master mix and 6.5 µl of nuclease free water. Dendrogram was constructed using the unweighted pair group method with average linkages (UPGMA) using PyElph ver. 1.4. software.

3. Results and Discussion

3.1 Identification of *A. hydrophila*

Aeromonads are present in the aquatic environment which is said to be their natural habitat and they are sporadically transmitted to humans from foods of animal origin, seafood and vegetables. They are responsible for Gastroenteritis in humans along with other severe illnesses, such as systemic infections, which is less frequent and normally associated with immunosuppressed patients. In the present study, out of 100 fish samples, 8 samples (8%) were positive for *Aeromonas* spp. comprising of 5 from marine fish samples (10%) and 3 from fresh water fish samples (6%). Similarly, out of 100 water samples, 7 samples (7%) were positive for *Aeromonas* spp. comprising 4 from marine water samples (8%) and 3 from fresh water samples (6%). All the positive isolates produced yellow colour colonies of 1-2 mm in diameter on ADA. The overall occurrence of *Aeromonas* spp. isolated from fish and water samples was 7.5%. All the obtained isolates when examined for their morphological and biochemical characters, revealed that they were gram negative, motile rods, catalase and oxidase positive. Further all the isolates were negative for ornithine decarboxylase activity and were able to ferment mannitol and glucose. Molecular confirmation of genus *Aeromonas* by targeting the 16S rRNA gene (356 bp) confirmed all the 15 biochemically positive isolates were as *Aeromonas* spp. (Figure 1). Further, species level identification of *A. hydrophila* was done by targeting the 16S rRNA gene (103 bp) (Figure 2). It was found that all the 15 confirmed *Aeromonas* spp. isolates were belonging to *A. hydrophila* species.

Development of DNA-based molecular techniques in identifying bacterial species has ignited a great influence on prokaryotes identification based on 16S rRNA gene analysis [26]. Moreover, the 16S rRNA gene classification has been widely accepted and used for the detection and delineation of *A. hydrophila*. Previous studies demonstrated that more than 70% of genomic similarities were discovered in organisms that shared 97% of their 16S rRNA sequences [27, 28]. The present study proves the usefulness of PCR targeting 16S rRNA gene for quick identification and confirmation of *Aeromonas* spp. both at genus and species level.

3.2 Virulence attributes

The members of the genus *Aeromonas* possess many virulence factors which plays an important role in inflicting diseases in man and animals [7]. Overall, 53.3% (n = 8) of isolates were showing haemolytic activity in TSA with 5% sheep blood. Source wise, 60% of marine fish isolates (Indian oil sardine-2, Indian grey mullet-1), 50% of marine water isolates, 66.67% of fresh water fish isolates (Catla-2, Rohu-0) and 33.33% of fresh water isolates were haemolytic. Haemolysins are exotoxin protein produced by bacteria and the lytic activities of haemolysins on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anaemia [29]. In the

present study, haemolytic strains were found in fish and water samples indicating both can act as a source of pathogenic *Aeromonas* to humans and animals. Similar reports of haemolytic *A. hydrophila* were demonstrated by Illanchezhian *et al.* (2010) [30] and Amsaveni *et al.* (2014) [31]. Further, 40% (n = 6) of isolates exhibited gelatinase activity. Source wise, 40% of marine fish isolates (Indian oil sardine-1, Indian grey mullet-1), 50% of marine water isolates, 33.33% of fresh water fish isolates (Catla-1, Rohu-0) and 33.33% of fresh water isolates showed gelatinase activity. Gelatinase is known for its contribution to biofilm formation and is also thought to contribute to virulence through degradation of a broad range of host substrates, including collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a [32].

A number of genetic determinants encoding virulence factors have been reported [7]. Among these profuse number of genes reported for aeromonads, two genes [Haemolysin (*hly*) and aerolysin-related cytotoxic enterotoxin (*act*)] were selected due to their widespread application [33]. Findings of our study showed that *hly* gene (597 bp) was present in 33.33% (n = 5) of the isolates including 50% (n = 4) in fish isolates and 14.28% (n = 1) in water isolates (Figure 3). Further *act* gene (232 bp) was present in 20% (n = 3) of the isolates including 25% (n = 2) in fish isolates and 14.28% (n = 1) in water isolates (Figure 4). The presence of these virulence genes in the study suggests the potential pathogenicity of *A. hydrophila* isolates in the study area, highlighting the risk of foodborne infections.

3.3 Antimicrobial resistance

Microbial resistance to antibiotics is partially as a result of bacterial dynamism in adapting to its environment as well as increasing use and misuse, of existing antibiotics in agriculture, human and veterinary medicine [34]. All the fifteen isolates when subjected to antimicrobial sensitivity test by disc diffusion technique showed resistant to cotrimoxazole (100%) and penicillin G (100%) while sensitive to gentamicin (100%). Varying levels of anti-microbial resistance was shown against ciprofloxacin (66.67%), tetracycline (53.33%), amoxicillin (53.33%), chloramphenicol (20%) and cefotaxime (20%). In the present study, 46.67% of the isolates were multidrug resistant and MAR index of all the 15 isolates were above 0.2 ranging from 0.38 to 0.75. Resistance to ciprofloxacin was noticed among the isolates, which is being used to treat aeromonadal infections in humans [35]. Presence of multidrug resistant isolates and isolates with MAR index above 0.2 reflects that all the isolates were likely to be from high-risk source of contamination [22].

Antimicrobials are used in the aquaculture industry by using different methods such as feed medication, bioencapsulation, immersion baths, dipping and flushing [36]. Findings of our study showed that *tetA* gene (956 bp) was present in 20% (n = 3) of the *A. hydrophila* isolates (Figure 5). Among the fish isolates, 25% (n = 2) and among water isolates 14.3% (n = 1) harboured *tetA* gene. Similarly, *bla_{TEM}* gene (867 bp) was present in 26.67% (n = 4) of the isolates with 25% (n = 1) from fish isolates and 28.5% (n = 3) from water isolates (Figure 6). The findings of our study indicate that antimicrobial resistance genes were present in both marine and fresh water environments with a higher percentage of prevalence in marine environment (66.67%)

than the fresh water environment (16.67%). Thus, the aquatic environment can serve both as a natural reservoir of antibiotic resistance and a key conduit for the spread of antimicrobial resistance traits through fishes which is of major public health concern.

3.4 Heat map analysis

Heat map analysis (Figure 7) showed the grouping of isolates into mainly two clusters (A and B). Cluster A consists of four isolates (FW 24, MW 32, MW 05, MF 14) derived from marine fishes, marine water and fresh water sources. Cluster B comprises of a major group of isolates belonging to all four sources. Cluster B was further delineated into two sub-clusters: cluster B1 and cluster B2. Cluster B1 consists of 3 isolates (FF 03, FF 39, MF 07) derived from fresh water fish and marine fish samples. Cluster B2 consists of 8 isolates (MF 33, MW 40, MF 22, FW 45, FW 08, FF 33, MW 35, MF 02) derived from fishes and water of both marine and fresh water origin. Five isolates derived from different sources (FW 45, FW 08, FF 33, MW 35, MF 02) harboured neither AMR genes nor virulence genes. Hence, it is essential to identify and determine their transmission routes to understand the possible dissemination of AMR. Further 3 isolates MW 32, MW 05 and MF 14 harboured both AMR and virulence genes which can be a potential hazard to the public health. Hence, it is essential to establish a proper control strategy to prevent the dissemination of AMR and virulence genes and

development of evidence-based and effective One Health interventions that can ultimately reduce the burden of AMR in humans.

3.5 Molecular typing by ERIC PCR

The clonal variability among the isolates were studied using ERIC PCR, in which the appeared patterns were evaluated for the phylogenetic closeness with the help of Pyelph software. In the present study, ERIC-PCR fingerprinting of 15 *A. hydrophila* isolates revealed 15 different ERIC types forming four different clusters (Figure 8, 9). Cluster 1 consisted of one fresh water isolate (FW 08), one fresh water fish isolate (FF 39) and one marine water isolate (MW 05). Cluster 2 consisted of one fresh water isolate (FW 24), one marine water fish isolate (MW 32) and two marine fish isolate (MF 22, MF 33). Similarly, Cluster 3 comprises of one fresh water isolate (FW 45), one fresh water fish isolate (FF 33), one marine water isolate (MW 40) and one marine fish isolate (MF 07). Cluster 4 consisted of one fresh water fish isolate (FF 03) and one marine water isolate (MW 25). Two isolates from marine fishes remained as an outgroup from other clusters (MF 02, MF 14). A wide genetic diversity of isolates irrespective of their source of isolation was noticed and no particular clone was predominant. However, ERIC PCR could help in the development of sensible control measures to prevent the spread of this ichthyo-zoonotic pathogen.

Table 1. Oligonucleotide sequences used in this study

Gene	Primer sequence (5' → 3')	Cyclic condition	No. of cycles	Amplicon size (bp)
16S rRNA (<i>Aeromonas</i> spp.)	F- GGGAGTGCCTTCGGGAATCAGA R - TCACCGCAACATTCTGATTTG	Initial denaturation - 95 °C/5 min; Denaturation - 95 °C/0.5 min; Annealing - 59 °C/0.5 min; Extension - 72 °C/0.5 min; Final Extension - 72 °C/7 min	50	356
16S rRNA (<i>A. hydrophila</i>)	F - GGCCTTGCGGATTGTATAT R - GTGGCGGATCATCTTCTCAGA	Initial denaturation - 94 °C/2 min; Denaturation - 94 °C/0.5 min; Annealing - 56 °C/0.5 min; Extension - 72 °C/0.5 min; Final Extension - 72 °C/10 min;	35	103
<i>hly</i>	F - GGCCGGTGGCCCGAAGATACGGG R - GGCGGCGCCGGACGAGACGGG	Initial denaturation - 95 °C/5 min; Denaturation - 95 °C/2 min; Annealing - 55 °C - 1 min; Extension - 72 °C/1 min; Final Extension - 72 °C/7 min	30	597
<i>act</i>	F - AGAAGGTGACCACCAAGAACA R - AACTGACATCGGCCTTGAACCTC	Initial denaturation - 94 °C/5 min; Denaturation - 94 °C/1 min; Annealing - 55 °C/1 min; Extension - 72 °C/1 min; Final Extension - 72 °C/7 min	30	232
<i>tetA</i>	F - GTAATTCTGAGCACTGTTCGC R - CTGCCTGGACAACATTGCTT	Initial denaturation - 95 °C/5 min; Denaturation - 95 °C/0.5 min; Annealing - 62 °C/0.5 min; Extension - 72 °C/45 sec; Final Extension - 72 °C/7 min	23	956
<i>bla_{TEM}</i>	F - ATGAGTATTCAACATTTCCG R - CTGACAGTTACCAATGCTTA	Initial denaturation - 96 °C/5 min; Denaturation - 96 °C/1 min; Annealing - 58 °C/1 min; Extension - 72 °C/1 min; Final Extension - 72 °C - 10 min	35	867
ERIC PCR	ERIC 1R - ATGTAAGCTCCTGGGGATTAC ERIC 2 - AAGTAAGTGACTGGGGTGAGCG	Initial denaturation - 95 °C/5 min; Denaturation - 95 °C - 1 min; Annealing - 50 °C - 1 min; Extension - 72 °C - 4 min; Final Extension - 72 °C - 7 min	30	-

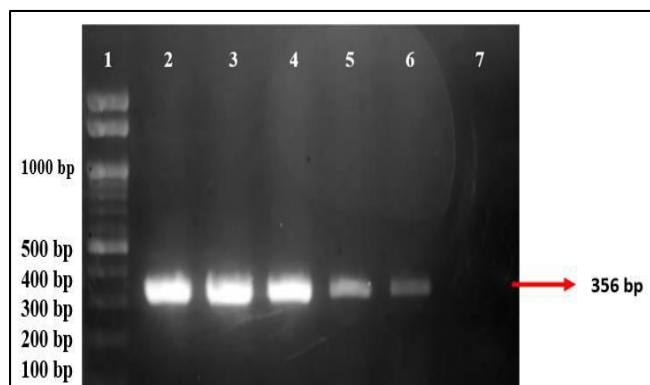


Fig 1: PCR amplification of 16S rRNA gene of *Aeromonas* spp.

Lane 1 – 100 bp ladder

Lane 2-5 – *Aeromonas* spp. positive field isolates (356 bp)

Lane 6 – *A. hydrophila* subsp. *hydrophila* MTCC 1739 (Positive control)

Lane 7 – No Template Control

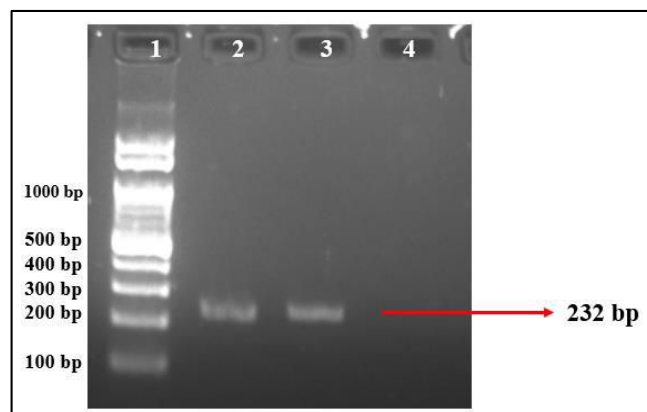


Fig 4: PCR amplification of 'act' gene of *A. hydrophila*

Lane 1 – 100 bp ladder

Lane 2-3 – *A. hydrophila* isolates positive for 'act' gene

Lane 4 – No Template Control

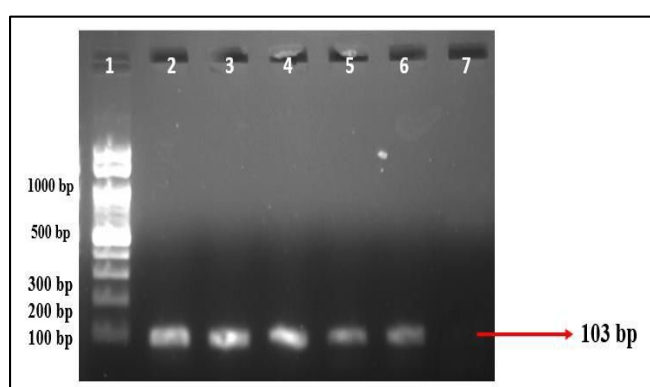


Fig 2: PCR amplification of 16S rRNA gene of *A. hydrophila*

Lane 1 – 100 bp ladder

Lane 2-5 – *A. hydrophila* positive field isolates (103 bp)

Lane 6 – *A. hydrophila* subsp. *hydrophila* MTCC 1739 (Positive control)

Lane 7 – No Template Control

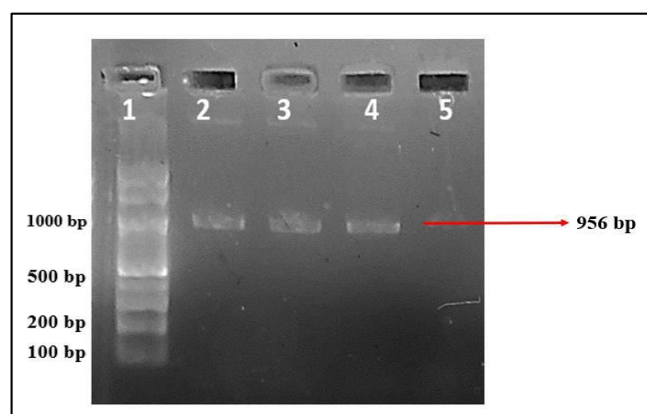


Fig 5: PCR amplification of 'tetA' gene of *A. hydrophila*

Lane 1 – 100 bp ladder

Lane 2-4 – *A. hydrophila* isolates positive for 'tetA' gene

Lane 5 – No Template Control

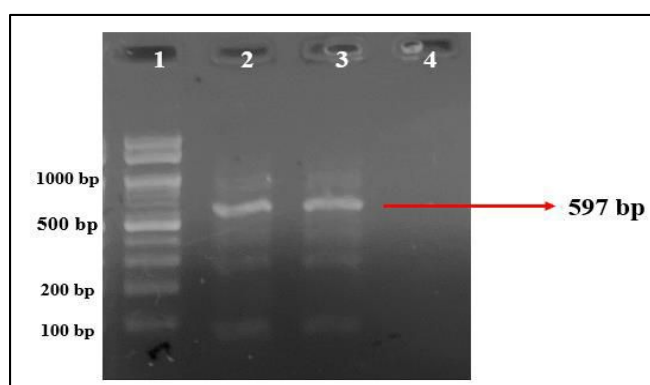


Fig 3: PCR amplification of 'hly' gene of *A. hydrophila*

Lane 1 – 100 bp ladder

Lane 2-3 – *A. hydrophila* isolates positive for 'hly' gene

Lane 4 – No Template Control

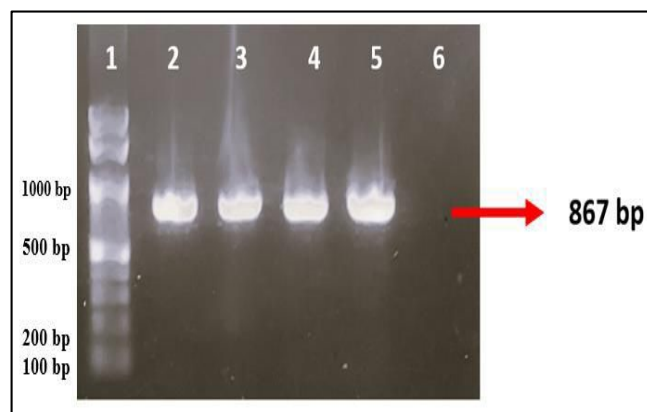


Fig 6: PCR amplification of 'bla_{TEM}' gene of *A. hydrophila*

Lane 1 – 100 bp ladder

Lane 2-3 – *A. hydrophila* isolates positive for 'bla_{TEM}' gene

Lane 4 – No Template Control

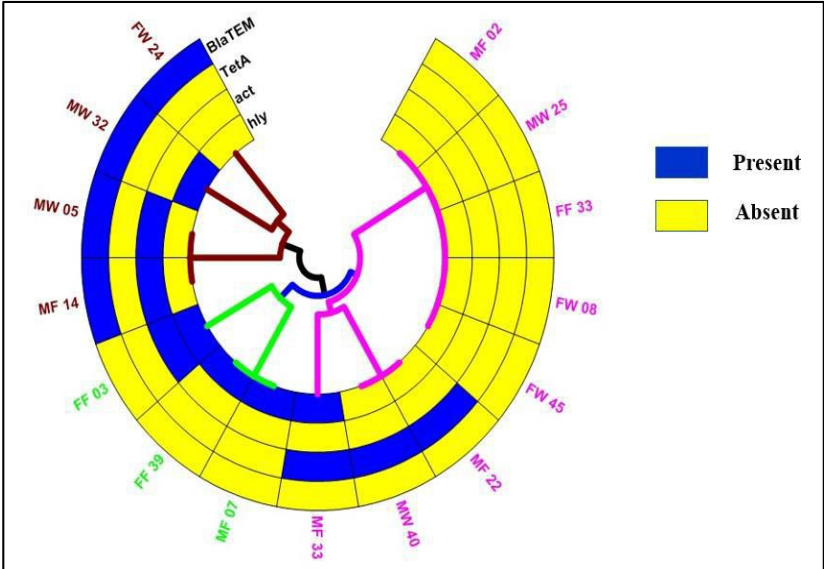


Fig 7: Heat map analysis of antimicrobial resistance and virulence genes

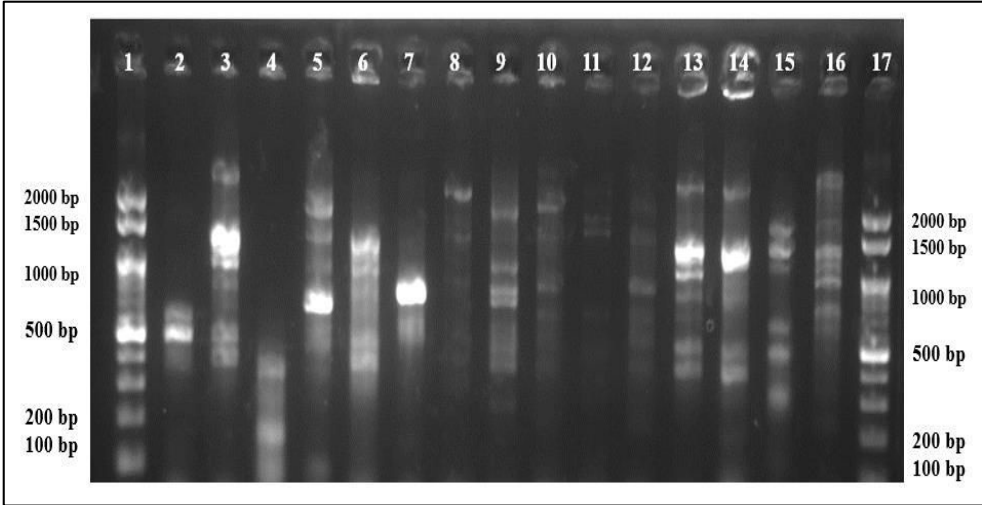


Fig 8: ERIC PCR fingerprints of *A. hydrophila* isolates
Lane 1, 17 – 100 bp ladder
Lane 2-16 – Positive field isolates

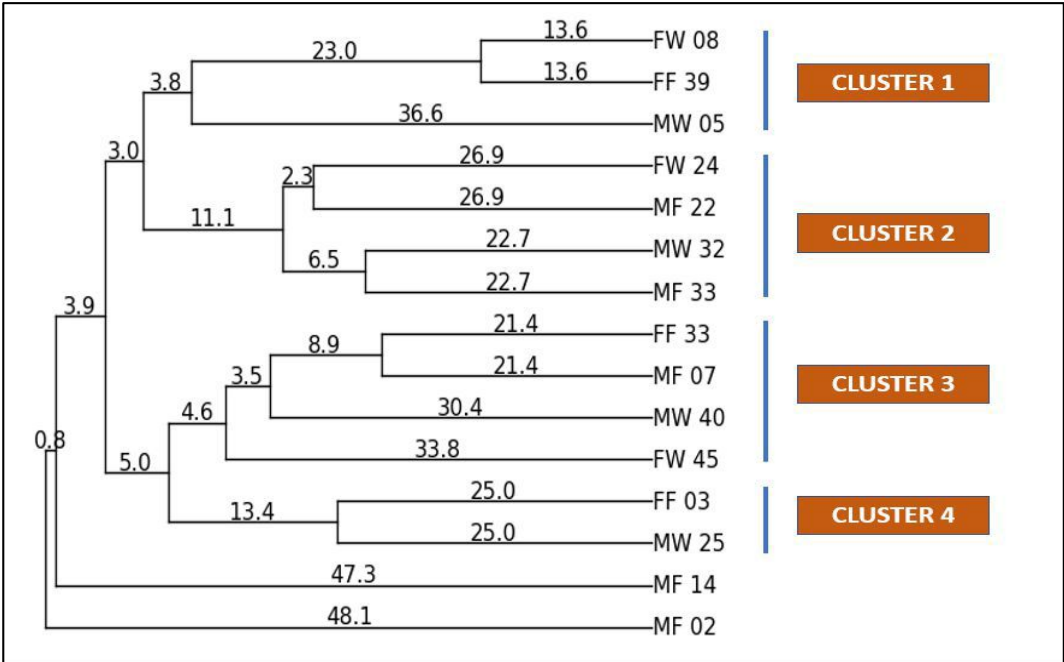


Fig 9: Dendrogram showing ERIC profile of *A. hydrophila* isolates

4. Conclusion

The study documented the presence of multidrug resistant, virulent *Aeromonas* spp. among fishes and its environment (water) which is of public health concern. Therefore, the surveillance of this potential contaminant is crucial for sustenance of public health along with the use of specific media for the recovery of *A. hydrophila* under clinical settings. Further, strengthening of surveillance for antimicrobial consumption and enhanced understanding about the transmission risk of antimicrobial resistance across the aquatic animal-environment-human interface, with targeted policies and regulations promoting antimicrobial stewardship and antimicrobial efficacy is required. Also, comprehensive enteric disease surveillance strategies, prevention, and education are essential for meeting the challenges in the years ahead.

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