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Molecular identification, antibiotic resistance and plasmid profiling of *Aeromonas hydrophila* isolated from diseased fishes of Eastern India

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Abstract

In the present study 14 different strains of *Aeromonas hydrophila* isolated from disease infected freshwater fishes were identified at the molecular level through the sequenced based on 16S rRNA gene and accordingly phylogenetic analysis was carried out. Further all the strains were tested for susceptibility to various antibiotics and screened for the presence of plasmids. All the strains are found to be multi-drug resistance. In the plasmid profiling study only 10 strains harbored plasmid with size ranging from 2.5 to 11.4 kb. These results revealed the 16S rRNA gene as the rapid tool for detection of microorganisms and are helpful for effective disease diagnosis and its treatment. It also provides a base line profile of drug resistance and presence of plasmid among the strains of *A. hydrophila* isolated from freshwater fishes.

Key words: *Aeromonas hydrophila*, 16S rRNA, drug resistance, plasmid

Introduction

The genus *Aeromonas* comprises of a group of bacteria that is widespread in natural habitats such as soil, freshwater, brackishwater, sewage water and waste water. Out of the 14 currently described species of *Aeromonas*, the *Aeromonas hydrophila* has been considered as potential fish pathogens [1]. It poses a serious threat to aquaculture industry as well as to human health. *A. hydrophila* has been implicated in a variety of

systemic and localized diseases in different fish, mammals, reptiles and humans [2]. Although the knowledge in the mechanisms of pathogenesis is poor, but it is known that the microorganisms produce a wide range of virulent factors like hemolysin, proteases, enterotoxin, endotoxin and adhesions which together contribute to overall disease progress in fish [3].

The presence of *A. hydrophila* is an indicator of bad zoohygiene and zootechnical conditions of fish ponds. Reduced quality and quantity of feed, mechanical injuries, parasitosis, and seasonal oscillation in temperature present some of the factors that produce favourable conditions for the proliferation of *A. hydrophila* in fish ponds. This leads to outbreak of diseases in freshwater fishes and commercial economic loses to aquaculture industry. The detection of these groups of microbial pathogens with utmost accuracy is the solution to take effective control measures for the recognization and prevention of the problems. The choice of antibiotics for the treatment should be based on antimicrobial susceptibilities of the organisms. Usually the conventional methods of detection of microbial pathogens relies mainly on morphological, biochemical and physiological criteria which is very cumbersome, time consuming and often gives ambiguous results.

Hence, there is a need for the detection of new methods which will help in the quicker identification of microbes. Several molecular techniques act as major tool for effective and fast identification of microbial diversity in different environment. In recent year 16S rRNA sequence analysis has become a very stable and specific marker for both bacterial identification and delineation of evolutionary relationships [4]. Bacterial 16S rRNA genes generally contain nine hypervariable regions that demonstrate considerable sequence diversity among bacterial species and can be used for species identification [5]. Hypervariable regions are flanked by conserved sequences in most bacteria, enabling PCR amplification of target sequences using universal primer. Plasmid profile determination is a useful and earliest DNA-based method applied to epidemiological studies [6].

The aims of the present study were identification of 14 different strains of *A. hydrophila,* isolated from diseased fishes of freshwater aquaculture system of Eastern India, on the basis of 16S rRNA sequencing and accordingly phylogenetic analysis were carried out. Further the above strains were characterized with respect to antibiotic resistance patterns and plasmid profiling. This study provides a basis for accurate identification of *A. hydrophila* strains, for their epidemiological study and drug sensitivity patterns.

Material and Methods

Bacterial strains

In the present study 14 different strains of *A. hydrophila* maintained in the Fish Health Management Division of Central Institute of Freshwater Aquaculture, Bhubaneswar was used. The strains were isolated from different diseased fishes of Eastern India (Table 1). The preliminary identification of above strains was carried out on the basis of their growth on selective RS-media.

Genomic DNA Extraction

Genomic DNA of *A. hydrophila* strains were extracted using HiPurA^{TM} Bacterial and Yeast Genomic DNA Purification Spin Kit (HiMedia). Overnight bacterial culture of *A. hydrophila* strains (1.5 ml) were taken in different microcentrifuge tubes and centrifuged at 10,000 rpm for 1 min. The supernatant were discarded and the pellets were resuspended in 180 µl of Lysis solution AL. RNase A Solution of 20 µl were added to all the tubes followed by incubation for 2 min at room temperature. Proteinase K of 20 µl were then added, mixed properly and incubated for 30 min at 55^{o}C in a water bath. It was then followed by addition of 200 µl of Lysis Solution C1, vortex thoroughly and incubated at 55^{o}C for 30 min. Then 200 µl of ethanol (95-100 %) were added to all the lysate and were transferred to HiElute Miniprep Spin Column and centrifuged at 10,000 rpm for 1 min. The flow through liquid was discarded. Then 500 µl of Prewash Solution PWB was added all the column and centrifuged at 10,000 rpm for 1 min, followed by addition of 500 µl of Wash Solution WS to the column and centrifuged at 10,000 rpm for 3 min. Then the spin column were placed in a new collection tube, Elution Buffer ET of 200 µl was added to all the columns, incubated for 1 min at room temperature and centrifuged at 10,000 rpm to elute the DNA of *A. hydrophila* strains. The concentrations of genomic DNA were checked by using spectrophotometer (BioRAD-Smart Spec 3000).

Amplification and sequencing of 16S rRNA gene

The 16S rRNA gene of all the strains of *A. hydrophila* as well as reference strain ATCC 49140 was amplified by using the universal primer as described Martinez-Murcia et al. [7]. The primers were obtained from Bangalore Genei with following sequence forward primer (5'-AAGAGTTTGATCCTGGCTCAG-3') and reverse primer- (5'-GGTTACCTTGTTACGACTT- 3'). The master mix of reaction volume 25 µl was prepared by adding 2.5 µl of 10x assay buffer, 0.5 µl of Mgcl2, 1 µl of dNTPs (10 mM), 1 µl of each primer (10 pmol/µl), 0.3 µl of Taq polymerase (5U) and sterilized milliQ distilled water. Genomic DNA of 2 µl (50 ng DNA/reaction) of each strain was added separately to each tube. The PCR was performed in a thermal cycler (Minicycler, MJResearch) under the following conditions: initial denaturation at 95°C for 5 min, then 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by a final extension of 10 min at 72°C. The PCR amplified products, of expected size of 1.5 kbp were analyzed through 1.2 % agarose gel containing ethidium bromide (0.1 µg/ml), with 1 kb DNA ladder (Banglore Genei, India) as standard molecular weight marker. The gel was visualized under gel documentation system (Gel Doc-IT^{TM} Imaging System). The amplified PCR products were purified by using Hi-Pura^{TM} PCR purification kit and then sent for sequencing to Eurofin laboratories Pvt. Ltd, Bangalore.

Sequence analysis and construction of phylogenetic tree

After sequencing, database searches were conducted with the BLAST (Basic Local Alignment Sequencing Tool) algorithm provided by NCBI (National Center for Biotechnology Information). For phylogenetic analysis a total of 25 sequences of 16S rRNA gene, including the present strains, reference strain and other related taxa,

obtained from Gene bank database were aligned using multiple-sequence alignment software CLUSTAL W.

The phylogenetic tree was constructed by Neighbor-Joining method [8] using MEGA-4 software [9]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of taxa analyzed and the percentage of replicate trees in which the associated taxa were clustered together are shown next to the branches.

Antibiotic sensitivity tests

The antibiotics susceptibility test was conducted by disc diffusion method as described by Bauer et al. [10], using Mueller Hinton agar (MHA, Himedia). The *A. hydrophila* strains were tested against the following antibiotic disc (Himedia): amikacin (Ak, 10µg), carbenicillin (Cb, 100µg), ciprofloxacin (Cf, 10µg), kanamycin (K, 30µg), nitrofurantoin (Nf, 300µg), streptomycin (S, 10µg), tetracycline (T, 30µg), chloramphenicol (C, 25µg), ampicillin (A, 25 µg), gentamycin (G, 30µg), co-trimoxazole (Co, 25µg), ceftriaxone (Ci, 30µg), cefuroxime (Cu, 30µg), nalidixic acid (Na, 30µg), norfloxacin (Nx, 10µg), bacitracin (B, 10µg), cephalexin (Cp, 30µg), erythromycin (E, 15µg), novobiocin (N, 5µg), amoxycillin (Am, 10µg), cephalothin (Ch, 30µg), oxytetracycline (O, 30µg).

Broth culture of 24 h of all the strains of *A. hydrophila* were prepared freshly using Brain Heart Infusion broth (BHIB, Himedia) and incubated at 37^{o}C. The test bacterial culture were swabbed uniformly on the MHA plates and the standard antibiotics discs (4-5 discs/plates) were placed on the agar surface with the antibiotic disc dispenser. The plate were then incubated at 37^{o}C for 24 h. Characterization of strains as sensitive, intermediate or resistant was based on the size of inhibition zones around each disc according to the interpretive chart of performance standards for antimicrobial disc susceptibility tests, December 1993 (Himedia) [11].

Plasmid profiling

The plasmid DNA of *A. hydrophila* strains was extracted by using HiPurA^{TM} Plasmid DNA Miniprep Purification Kit (Hi-Media). Bacterial cells (1.5 ml) from overnight culture grown in Luria Bertani broth (Himedia) were centrifuged at 13,000 rpm for 1 min. The supernatant were discarded from all tubes and the pellets were resuspended in 250 µl of RNase treated Resuspension buffer (HP1) followed by addition of 250 µl of Lysis solution HP2, mixed thoroughly by gently inverting the tubes for 4-6 times. Then to all the tube 350 µlof Neutralization solution HN3 was added, mixed thoroughly and centrifuged at 13,000 rpm for 10 min to obtain a compact white pellet. The supernatant was transferred from all the tubes to HiElute Miniprep Spin Column and were centrifuged at 13,000 rpm for 1 min. The flow-through was discarded followed by addition of 500 µl Wash solution HPB and then centrifuged at 13,000 rpm for 1 min. Second wash of columns were done by adding 700 µl of diluted Wash solution HPE and centrifuged at 13,000 rpm for 1 min. The column were then transferred to clean collection tubes and then to all tubes 50 µl of Elution buffer ET was added, kept for 1 min at room temperature and were centrifuged at 13,000 rpm for 1 min. Finally the plasmid were eluted and stored at 4^{o}C for further analysis.

The isolated plasmids were electrophoresed on 0.8 % agarose gel containing ethidium bromoide. The approximate molecular mass of each plasmid was determined by comparison with plasmid of known molecular mass of *Escherichia coli* V517 [12].

Results

Over the selective RS-media plates the colonies of the strains used in the present study appeared to be small, rounded and yellowish in colour which helps in preliminary identification of strains as *A. hydrophila*. The isolation sources of these strains are given in Table 1.

In all the 14 strain as well as in reference strain ATCC 49140 the 16S rRNA tends to amplify at 1.5 kbp regions (Fig. 1). For confirmatory identification the sequence analysis of 16S rRNA gene of all the strains suggested to be *A. hydrophila.* The accession numbers of 16S rRNA gene sequence of *A. hydrophila* strains deposited to the gene bank are given in Table 1.

The phylogenetic tree constructed from 16S rRNA sequences of 14 strains of *A. hydrophila* studied and 11 other taxa of *Aeromonas* sp available in the gene bank showed the bacterial species with identical DNA sequences in a given region are present in a single dendrogram (Fig. 2). In general the tree confirms the distant relationship between the *A. hydrophila* used in the present studied with other strains of *A. hydrophila* and other identified *Aeromonas* sp like *A. sorbia, A. veroni, A. punctata*. The dendrogram of relationship indicates that in the first group CAHH1 strain was closely related to P2B5 strain of *A. hydrophila* available at gene bank and in another cluster the presence of *A. hydrophila* strain CAHH10 and CAHH14 isolated from the skin lesion of *Channa marulius* and *Labeo rohita* respectively indicated that they are very similar to one another. A second group was formed by CAHH7, CAHH11 and CAHH12 which are placed in single branch. However CAHH11 and CAHH12 strain exhibit high level of sequence similarity to one another and share a common clade. It may be due to same isolation source i.e. from the skin lesion of *Channa* sp and *C. marulius.* Although the CAHH9, CAHH6 and QDC01 strain of *A. hydrophila* were isolated from three distinct fish species but there was sequence similarity between them and are placed in a single branch. Same case was also detected in another cluster containing *Aeromonas* sp (R1) and two strains of *A. hydrophila* studied CAHH15 and CAHH5. They were isolated from cutaneous hemorrhages of *Clarias gariepineus*, kidney of *L. rohita* and skin lesion of *Channa punctatus* but they are very similar to one another. Similarly the presence of *A. hydrophila* strain CAHH13 and Ah 1 in one cluster as well as the presence of CAHH14 and reference strain ATCC 49140 in another cluster indicates high level of sequence similarity between one another. In another clusters *A. hydrophila* strain CAHH2 share a common clade with CAHH8 strain which indicate the sequence similarity between them.

The antibiotic resistance patterns of *A. hydrophila* strains isolated from various fish samples including reference strain ATCC 49140 were shown in Table 2. All the 14 strains of *A. hydrophila* along with reference strain displayed resistance towards carbenicillin (Cb), chloramphenicol (C), ampicillin (A), cefuroxime (Cu), norfloxacin (Nx), bacitracin (B), cephalexin (Cp), erythromycin (E), oxy-tetracycline (O),

cephalothin (Ch) and amoxicillin (Am). However, all were uniformly susceptible to amikacin (Ak), ciprofloxacin (Cf), gentamycin (G) and nalidixic acid (Na). About 85.5 % of the isolates were found resistant to novobiocin (N) ans 46.2 % of isolates were found resistant towards nitrofurantoin (Nf), streptomycin (S) and cotrimaxazole (Co). The least resistant was noted for tetracycline (20 %) and kanamycin (13.2 %).

The results of plasmid screening are summarized in Table 2. Out of 14 strains of *A. hydrophila* studied 10 strains contained plasmid DNA. The plasmids detected in these strain were diverse, showing differing sizes and differing intensities. The size of plasmid detected with molecular weight ranged from 2.5 kbp to 11.4 kbp.

Discussion

In the present study the presumptive identification of *A. hydrophila* strains used was done through their colony morphology over the RS media. Shotts and Rimler [13] have tested 109 isolates representing 13 genera of bacteria and proposed this differential medium to facilitate diagnosis of *A. hydrophila* infection with 94 % accuracy.

Ribosomal RNA gene sequences play a central role in the study of microbial evolution and ecology. The principle of using bacterial 16S rRNA gene sequence to characterize microorganisms has now gained a wide acceptance [4]. In our study 14 different strains of *A. hydrophila* were identified based on the 16S rRNA sequence analysis. Similar study was carried out by Sahu et al. [14]. They have identified two strains of *A. hydrophila* on the basis of 16S rRNA sequence. Lee et al. [15] characterized 8 strains of *A. hydrophila* from Rainbow trouts in Korea based on 16S rRNA study. Borell et al. [16] identified 76 *Aeromonas* sp isolated from different samples and sources on the basis of restriction pattern of the PCR amplified 16S rRNA gene. The phylogenetic tree constructed helps to differentiate between different species of *Aeromonas* and also provides a distant relationship between various *Aeromonas sp* and among *A. hydrophila* strains. Although various *A. hydrophila* strains were used in the construction of phylogenetic tree along with present strains but they were placed in different cluster. It may be due to different isolation source and from different environment.

In this antibiotic era worldwide, there is growing concern about increased prevalence of antibiotic resistance and it is now generally accepted that the main risk factor for increase in resistance in pathogenic bacteria is due to extensive use of antibiotics and other chemotherapeutants in fish farms either to prevent or cure fish diseases [17]. Odeyemi et al. [18] reported a growing incidence of multidrug-resistant *Aeromonas* sp isolated from clinical and environmental sources. In the present study all the *A. hydrophila* strains isolated from freshwater fishes were found to be multidrug resistance. High resistance of *A. hydrophila* strains towards bacitracin (100 %), erythromycin (100 %), and novobiocin (86.6 %) was similar to the finding of Abulhamad et al. [19] and Son et al. [20]. However Pettibone et al. [21] have not reported any erythromycin resistant strains. The chloramphenicol resistant strains were few among the *A. hydrophila* strains from fish [22] however in the current study all *A. hydrophila* strains isolated from infected fishes were found resistant to

chloramphenicol. In our study a few strains were found resistant to kanamycin (13.2 %). Pettibone et al. [21] have not reported any kanamycin resistant strain whereas the investigation of Ansary et al. [23] supported the existence of kanamycin resistant strains with a frequency of about 38.2 %. Emekdas et al. [24] reported that none of *A. hydrophila* strains from fish and environmental sample was resistant to amikacin, gentamycin and ciprofloxacin, which is similar to our findings. Occurrence of more than 50 % of tetracycline resistant strains of *A. hydrophila* from different sources was also reported by Pettibone et al. [21] but in our study only 20% of *A. hydrophila* strains were found tetracycline resistant. Radu et al. [17] and Borrego et al. [25] reported frequent occurrence of resistance to ampicillin, carbenicillin, cephalothin, erythromycin and streptomycin in association to other antimicrobial agents. Our results are also in agreement with these data.

Such variation in the drug resistant may well be related to source of *A. hydrophila* isolates and the frequency and type of antimicrobial agents prescribed for treatment of *Aeromonas* infections in different geographical areas [20]. Therefore controlled in depth studies are needed to determine the effect of antimicrobial therapy on the microbial ecology of cultured fishes.

In the present study the overall prevalence of plasmids among the *A. hydrophila* strains was 64.2% and their size ranged from 2.5 kbp to 11.4 kbp. The frequency of occurrence and the detection of small size plasmids among the *Aeromonas* sp were in broad agreement with those workers who found plasmid prevalence in *Aeromonas sp* have been between 15 % to 94 % with most of the isolates harboring small size plasmid [26]. However, Redondo et al. [27] isolated and identified 70 strains of *A. hydrophila* from ornamental fish and 100 % of them carried plasmid with molecular weight ranging from 6.6 kbp to 25.7 kbp but none of the plasmid were able to transfer to *E. coli*. Chang and Bolton [28] have suggested that plasmid mediated antibiotic resistant in *Aeromonas* is not frequent. Bacterial antibiotic resistance patterns are usually associated with the presence of large plasmid and their ability for conjugation process. In general plasmid which would be transconjugated usually posses higher molecular weight [17]. Earlier report indicated that R-Plasmid encoding antibiotic resistant in *A. hydrophila* vary in size from 85.6 to 150 kbp [20, 25].

In our study the observation that the plasmid containing *A. hydrophila* strains were devoid of large plasmids and together with the finding that strains not containing plasmid but being multi-resistance to antibiotics tested indicate that resistance to most of these antibiotics is of chromosomal origin. It may be due to mutation in cellular DNA which could modify the antibiotic target site or transport mechanisms causing decrease action of antibiotic on bacterial cell.

Since, specific, sensitive and rapid methods for detecting and identifying pathogenic microorganisms are needed to control bacterial infection in aquaculture and in this regards from our study we concluded that the 16S rRNA can be used as a rapid and diagnostic markers for the identification of *A. hydrophila* strains. This technique can also be useful to highlight the phylogenetically closely related species. As the extensive use of antibiotics leads to the development of antibiotic resistant strains so, restriction of use of drugs in aquaculture sector to control fish diseases will

aid in minimizing the development of drug resistance microorganisms. Thus further works need to carry out to develop effective strategies for the control of this pathogenic microorganism.

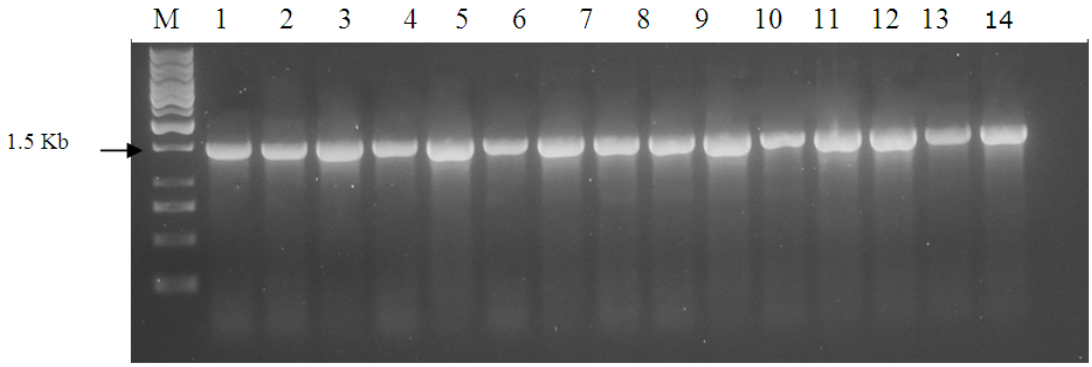

Figure 1: 16S rRNA banding pattern of *A. hydrophila*. Lane M : 1Kb ladder, lane 1: CAHH1, lane 2: CAHH2, lane 3: CAHH4, lane 4: CAHH5, lane 5: CAHH6, lane 6: CAHH7, lane 7: CAHH8, lane 8: CAHH9, lane 9: CAHH10, lane 10: CAHH11, lane 11: CAHH12, lane 12: CAHH13, lane 13: CAHH14, lane 14: CAHH15, lane 15: ATCC 49140

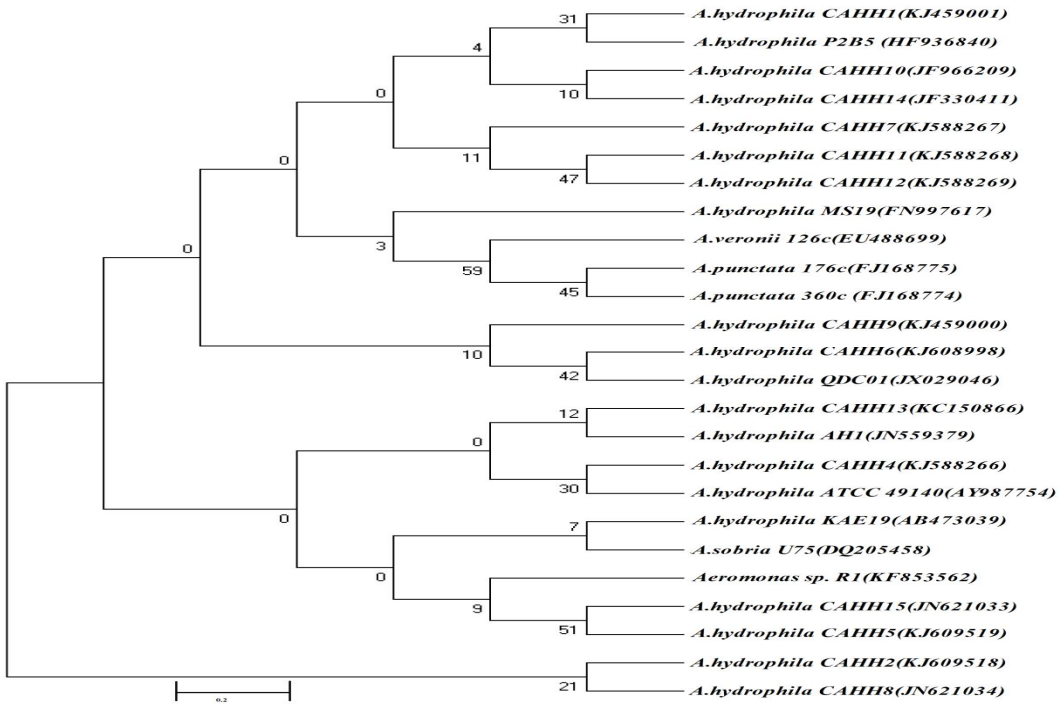

Figure. 2: Phylogenetic tree based on the 16S rRNA sequences. The bootstrap values (%) were shown besides the clades, accession numbers were written besides the name of type strain and scale bar represented distant value.

Table 1: *A. hydrophila* strains isolated from different diseased fish of Eastern India with Accession number

Bacterial strain No.	Host	Organ of Isolation	Place of Isolation	Accession Number
CAHH1	*C. mirgala*	Skin lesion	CIFA pond	KJ459001
CAHH2	*C. punctatus*	Skin lesion	Commercial farm Puri	KJ609518
CAHH4	*C. punctatus*	Liver	Commercial farm Puri	KJ588266
CAHH5	*C. punctatus*	Skin lesion	Commercial farm Puri	KJ609519
CAHH6	*C. batrachus*	Skin lesion	CIFA pond	KJ608998
CAHH7	*C. auratus*	Kidney	CIFA Aquarium	KJ588267
CAHH8	*C. auratus*	Intestine	CIFA Aquarium	JN621034
CAHH9	*C. punctatus*	Kidney	Commercial farm Puri	KJ459000
CAHH10	*C. marulius*	Skin lesion	Andhra pradesh	JF966209
CAHH11	*Channa sp*	Skin lesion	CIFA pond	KJ588268
CAHH12	*C. marulius*	Skin lesion	Andhra pradesh	KJ588269
CAHH13	*L. rohita*	Skin lesion	CIFA pond	KC150866
CAHH14	*L. rohita*	Skin lesion	CIFA pond	JF330411
CAHH15	*L. rohita*	Kidney	CIFA pond	JN621033

Table 2: Antibiotic resistance and plasmid profiles of the *A. hydrophila* strains

Bacterial Strain	Antimicrobial resistance	Plasmid size (kb)
CAHH1	Cb, Nf, S, T, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	2.7, 6.2, 9.3,11.4
CAHH2	Cb, S, C, A, Cu, Nx, B, Cp, E, Ch, O, Am	5.6, 9.3, 11.2
CAHH4	Cb, Nf, S, T, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	-
CAHH5	Cb, K, S, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	5.6
CAHH6	Cb, Nf, S, C, A, Ci, Cu, Nx, B, Cp, E, Ch, O, Am	2.5, 5.6, 9.3, 11.4
CAHH7	Cb, C, A, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	-
CAHH8	Cb, C, A, Co, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	5.6
CAHH9	Cb, Nf, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	2.5, 5.6, 9.3
CAHH10	Cb, C, A, Co, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	5.6, 9.4, 10.2
CAHH11	Cb, Nf, C, A, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	2.7, 5.8, 9.4, 11.4
CAHH12	Cb, S, C, A, Co, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	-
CAHH13	Cb, C, A, Co, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	-
CAHH14	Cb, K, Nf, S, T, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	5.6, 9.4
CAHH15	Cb, C, A, Cu, Nx, B, Cp, E, N, Ch, O, Am	7.8
ATCC 49140	Cb, C, A, Co, Ci, Cu, Nx, B, Cp, E, N, Ch, O, Am	

Note: Cb- Carbenicillin, Nf- Nitrofurantoin, S- Streptomycin, T- Tetracycline, C- Chloramphenicol, A-Ampicillin, Cu- Cefuroxime, Nx- Norfloxacin, B- Bacitracin, Cp- Cephalexin, E- Erythromycin, N- Novobiocin, Ch- Cephalothin, O- Oxy-tetracycline, Am- Amoxycillin, K- Kanamycin, Ci- Ceftrioxone, Co- Cotrimaxazole

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