Abstract

Fisheries is an important source of commercial export in India and serves as a means of livelihood for the people at large. In India, West Bengal is a one of the leading producers of fish owing to its optimum agroclimatic conditions and inhabitants' ingrained fondness for fish. Being placed at the foot hills of the great Himalayas, North Bengal holds unique endemism and icthyodiversity which set it as a class apart from rest of the state. Fishes are sometimes reared in small scale in homestead ponds, which forms the alternative sources of income among the rural population. However, fishes are reared without proper farm practices which leads to disease outbreaks with ultimate impact on the productivity.

The genus *Aeromonas* comprises of gram-negative bacteria widely disseminated in the aquatic environments. Mostly the members of these genus are opportunistic pathogens and capable of inducing diseases within a wide host range. Various researchers have reported the presence of these microorganisms in Epizootic-Ulcerative syndrome and haemorrhagic lesions in the fishes in Northern part of West Bengal. The primary goal of the present study comprised of analyzing the prevalence of *Aeromonas* sp. from water samples collected from fish farming environments of three districts of North Bengal. Secondly, the aeromonads were screened for virulence factors and its encoding genes responsible for pathogenic invasion of host. Thirdly, due to its abundance, the isolated aeromonads were evaluated for resistance against commonly used antibiotics and investigated for the underlying resistance genes.

The objectives of the present research were: (i) Isolation of *Aeromonas* spp. from fish farming environments in sub-Himalayan West Bengal. (ii) Biochemical characterization and phylogenetic analysis of bacterial isolates based on 16S rRNA gene sequence. (iii) Study of the virulence properties of the isolates of *Aeromonas* strains. (iii) Antibiotic sensitivity profiling of the bacterial isolates. (iv) Analyzing bacterial DNA for the presence of genetic

determinants of resistance and virulence. (v) Study the mobility of antibiotic resistance coding genes by *in vitro* conjugation.

For fulfilment of the objectives, water samples were collected from ten different small fish farming ponds distributed across three districts (Darjeeling, Jalpaiguri and Coochbehar) of North Bengal. The samples were subjected to microbiological processing which led to the isolation of total 83 putative *Aeromonas* strains in *Aeromonas* isolation medium. All these isolates were further screened by following the modified scheme of biochemical identification known as Aerokey-II which led to the identification of 34 strains as *Aeromonas* sp. The isolates were further confirmed to belong to this genus by PCR methods. The16S rRNA gene sequencing and BLAST similarity search revealed that the strains matched *Aeromonas* with percentage identity of the isolates as > 98%. The gene sequences were subjected to phylogenetic characterization and all the isolates rearranged to four reference strains: *A. veronii* (n=19), *A. hydrophila* (n=7), *A. jandei* (n=5) and *A. caviae* (n=3). The sequences were deposited in NCBI GenBank, and accession numbers were assigned to all the thirty-four isolates, *viz. A. veronii* (MT378391, MT379551, MT379645, MT379646, MT385145, MT380477, MT383121, MT383124, MT384421, MT384337, MT385097, MT396085, MT393933, MT393944, MT396230, MT396438, MT397061, MT397058, MT397063), *A. hydrophila* (MT378381, MT379550, MT379552, MT396436, MT396445, MT396437, MT395673), *A. jandei* (MT378390, MT393937, MT393941, MT393945, MT393942), *A. caviae* (MT393443, MT393930, MT393932).

The pathogenicity in *Aeromonas* is multifactorial and linked to a large number of genes that encode for various toxins and structural elements which aids proliferation within the host. In this study, six important virulence factors, hemolysin, protease, lipase, amylase, DNase and siderophore production were tested in all the 34 identified isolates of *Aeromonas*. Among the tested virulence traits, hemolytic and proteolytic activity was exhibited by 100% of the isolates. Amylase was detected in 70.6% of the isolates and DNase activity was detected in 44.10% of the isolates. Siderophore was

detected in 38.20% of the total isolates and a very small proportion of only 5.80% of isolates showed lipase activity.

Further, four important virulence genes *aer/haem* (encoding hemolysin), *aspA* (encoding alkaine serine protease), *ascV* (encoding type 3 secretion system) and *flaA* (encoding polar flagella) were analysed in all the 34 isolates identified as *Aeromonas*. PCR amplification of the genes revealed that 44.11% of the *Aeromonas* isolates carried the *aer/haem* and *flaA* genes. The *ascV* gene was found in 23.5% of the isolates. Only 8.82% of the aeromonads contained the *aspA* gene. The amplification products were cloned and sequenced. The sequences were subjected to similarity search using the BLASTn tool, annotated and submitted to the NCBI database through the BANKIT tool. The virulence genes have the following GenBank accession numbers: *aer/haem* (MT704303-MT704309; MT707932-MT707935; MH607886, MT591426, and MTT813045) *aspA* (MT909568-MT909570); *ascV* (MW001219-MW001222; MH607887-MH607890); and *flaA* (MT942623-MT942626, MT977537- MT977539).

Based on the combination of virulence genes found to be present in the *Aeromonas* isolates, the strains were classified into nine genotypic groups. Most of the *Aeromonas* strains belonged to group G that harboured only the *flaA*$^{+}$ gene. The second common genotype among the isolates was *aer/haem*$^{+}$, *ascV*$^{+}$, *flaA*$^{+}$. This study is the first to report the presence of such virulence genes from aeromonads isolated from this region. Furthermore, a virulent isolate of *Aeromonas* GP3 was able to transfer the *aer/haem* gene to *E. coli* DH5α via conjugation with an efficiency of 0.0394 X10^{-4} transconjugants per recipient cell. The detection of 23 kb plasmids in both donor and transconjugants corroborated to the transfer and gave an insight of the *aer/haem* being plasmid borne.

In order to validate the pathogenicity of the aeromonads six virulent isolates GP3, RB7, BP3, RJB1, MG8 and PP21 from different sampling sites and harboring atleast two virulence traits were injected into *Anabas testudineus*. The most harmful strain was GP3, which also possessed three of the four tested virulence-related genes (*aer/haem*+/ *ascV* +/*flaA*+) and five virulence

features (hemolysin, protease, DNase, siderophore, and amylase). On the other hand, strain PP21 which showed only two pathogenic phenotypes (protease and hemolysin) and the genes *aerA*/*haem*+ and *flaA*+, also showed 100% mortality. Contrarily, RB7, which exhibited four pathogenic phenotypes (hemolysin, protease, DNase, and amylase) and three genes (*aerA*/*haem*+/*ascV*+/*aspA*+), did not induce any mortality. In addition, BP3 was found to be very harmful to fishes despite only carrying one of the four genes under investigation which was *ascV* and exhibited four virulence characteristics. Therefore, in the current study it may be concluded that virulence phenotype expression and fish mortality were unrelated.

The isolated virulent *Aeromonas* sp. was further studied for its cytotoxicity in WRL-68 cell line (human, liver, embryonic). Results of this study revealed that the cell free culture filtrates of the *Aeromonas* strain GP3 were cytotoxic to human liver cells. Microscopic observation of the cells showed visible rounding off and detachment from the surface when compared against control cells, treated with cell free culture filtrate of *Lactobacillus* sp. (non-pathogen) with minor changes in morphology. The cell viability was reduced to 0.48% in GP3 filtrate treated cells as compared to 66% viability in *Lactobacillus* sp. treated cells. Hence the cytotoxic effect of GP3 on human cell lines implicating its pathogenic potential has been well established in this study.

In the current study, the partial nucleotide sequence of hemolysin amplified by PCR from GP3 was found to encode 185 amino acids. The 3D structure of the translated protein was constructed by homology modelling using a reference template 3COM (pro aerolysin of *Aeromonas hydrophila*) showing >90% homology. Further validation was done by PROCHECK software. Such modelling of the protein structure could prove to be beneficial for vaccine development and drug targeting of pathogenic aeromonads.

With the rise of diseases which hampers the fish productivity, numerous antibiotics are being used both as therapeutics and prophylactics that often leads to resistance among the bacterial populations. In this study resistance of the 34 isolates against 20 commonly used antibiotics for treatment of

diseases were assessed. In the current study 100% resistance was observed against penicillin and ampicillin followed by imipenem (85.29%), streptomycin (58.82%), cefoperazone (52.94%) and trimethoprim (50%). All the tested aeromonads exhibited resistance to three or more antibiotics. Intermediate level of resistance was observed against cefepime and cefuroxime (38.24%) and cefepime (23.53%). Maximum level of sensitivity was observed against chloramphenicol (91.18%) followed by norfloxacin (82.35%). Very high multiple antibiotic resistance (MAR) indices ranging between 0.15–0.7 in 94.1% of the isolates were observed which indicated that the aeromonads in fish farming environments have been exposed to antimicrobials. A MAR index > 0.2 indicates an environment with continuous exposure to antibiotics. A particular *Aeromonas* strain MG8 showing resistance against 14 antibiotics was found. Two strains, MG3 and HP6 showed resistance to 11 antibiotics, while strain PP19 showed resistance to 10 antibiotics.

Six significant clusters (C1-C6) were found using the Wards minimum distance approach in the hierarchical cluster analysis based on the antibiotic susceptibility phenotype represented as zone widths and sample sites of the 34 *Aeromonas* strains. The strains from different locations grouped to some extent under similar cluster depending on their levels of antibiotic resistance, like, all the Shivmandir isolates with low average MAR index value grouped in C2. However, variations in antibiotic resistance phenotype within a particular location was found to be the predominant feature. Therefore, it was concluded that resistance in aeromonads is irrespective of the location of sampling and the isolates had strain specific resistance pattern.

In the current study, genetic elements encoding antibiotic resistance known as integrons were screened for their presence in all 34 isolates. class I integrons (*intI*) were detected in 38.23% of the isolates with sizes ranging from 0.65 kb-2.5 kb. The integrons of 1.4 kb detected in FP2, MG8 and HP6 were cloned and sequenced. The NCBI accession numbers are MT424748, OP610544 and OP745416 respectively. The 1400 bp integron cassette of FP2 revealed the insertion of resistance gene cassettes of *aadA4* and *qacE* providing resistance against aminoglycosides and quaternary ammonium

compounds respectively. The integron cassette of MG8 with a size of 1400 bp had the resistance gene casettes *dhfrA1* and *aadA1* providing resistance against sulfonamides and aminoglycosides respectively. The integron size of 1400 bp in isolate HP6 possessed *dhfrA* showing resistance against sulfonamides and a hypothetical protein of unknown function. Similarly, tetracycline resistance gene *tetE* encoding an efflux pump was detected in one isolate PP23 and the GenBank accession no. is given as OP745417.

Plasmids of sizes ranging between 1.6kb – 23 kb were present in a few isolates (PP7, RB5, GP1, MG9 and BP5) and all these plasmids were transferable to *E. coli* Dh5α by *in-vitro* conjugation experiments. Four isolates were able to transfer their 23 kb plasmid to *E. coli* Dh5α and the resistance markers of cefepime, cefuroxime, ampicillin, and oxytetracycline resistance could be traced in the recipients as well. Similarly, an isolate BP5 was capable of transferring 4.3kb and 1.6kb plasmids to the *E. coli* DH5α via conjugation. Conjugational transfer frequencies of 0.15×10^{-7} - 0.76×10^{-6} were obtained.

In conclusion, the current study reports the prevalence of *Aeromonas* sp. in small fish farming environments. The major findings of this study were the detection of virulence and antibiotic resistance in aeromonads along with underlying genes from three districts of West Bengal. The study has given a current scenario of the level of contamination of the fish farms by opportunistic pathogens like *Aeromonas* sp. The study may be helpful in spreading awareness among the fish farmers to adopt proper practices and avoid the disease outbreaks. Also, a clear picture regarding the level of antibiotic resistance in fish farms gives a picture about misuse of antibiotics. The data could be utilized to educate small scale farmers regarding the controlled use of drugs and antibiotics in fish culture.