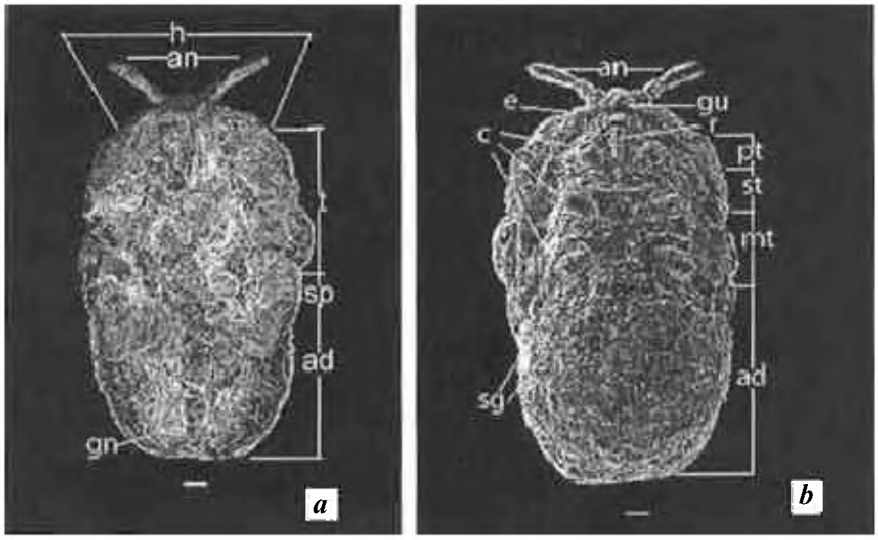


**Figure 2.** SEM micrograph of fossil bug from late Quaternary of Netal, Uttaranchal, India. Bar represents 100 µm. ***a***, Dorsal view; ***b***, Ventral view. Notations as in text.

It provides an important link in the evolution of insects. The morphological characters of the reported bug, such as small body, short antennae comprising three segments, three-segmented, backwardly directed rostrum with thorax being the largest among the three parts of the insect body, are not yet known in a living or fossil insect family. It also throws light on the biodiversity of the late Quaternary about which our knowledge is still poor.

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# Abdominal dropsy disease in major carps of Meghalaya: Isolation and characterization of *Aeromonas hydrophila*

Diseases in intensive freshwater aquaculture have assumed great importance in India due to economic loss observed in recent years. Infections due to *Aeromonas* are common and pose a threat to the development of the aquaculture enterprise. *Aeromonas hydrophila*, a ubiquitous organism present in the aquatic environment causes diseases in fish under stress[1,2]. There are reports of isolation of *A. hydrophila* from dropsy-infected common carp, *Cyprinus carpio* from Meghalaya[3]. Dropsy outbreaks have taken heavy toll in the neighbouring state of Assam[4,5] and also in Andaman[6,7]. All the outbreaks reported earlier were diagnosed based on symptoms, isolation and identification of the causative agent. Isolation and identification of Aeromonads from environmental sources pose serious challenges to microbiologists because of the large variety of strains present in water and the imprecision of conventional detection and identification methods[8]. Diagnosis based on isolation and identification of pathogen is not only time-consuming and tedious, but also can be misleading, specially in case of mesophilic and motile aeromonads. The present correspondence reports the involvement of *A. hydrophila* in abdominal dropsy disease by isolation, identification and detection of aerolysin and haemolysin genes as genetic markers of virulence determinants by multiplex polymerase chain reaction (mPCR).

Dropsy-infected *Catla catla* and *Cirrhinus mrigala* showing distended abdomen, loose scales, deep ulcers on the dorsal surface and extensive haemorrhages on the ventral part were collected from freshwater pond. The pond was stocked with catla, mrigal, silver carp and grass carp according to the standard ratio in a pond size of 0.2 h with 2.5 m water level. The disease was reported during August, when the temperature and humidity ranged between 25 and 30°C, and 80 and 90% respectively. The disease symptoms were noticed in 5–10% of the total population, among which catla and mrigal weighing approximately 500 g were mostly infected and suffered 5% mortality. Postmortem of catla (2) and mrigal (5) revealed degeneration of the lamellar structure of the gills, congested liver, distended intestines and fluid in the abdomen.

For isolation of *A. hydrophila*, samples from gills, heart blood, liver and abdominal fluid from both catla and mrigal were aseptically inoculated onto Ampicillin Dextrin Agar (ADA, Difco, USA) and *Aeromonas*-selective agar (ASA, Hi Media, Mumbai), and incubated at 24°C for 48 h.

Colonies characteristic of *Aeromonas* (round, elevated, mucoid and green-coloured colonies on ASA and yellow and opaque colonies on ADA media) were picked up from all the plates and purified on 10% sheep blood agar. The β-haemolytic colonies were found to be motile and thick, rod-shaped, Gram-negative bacteria. The isolates were oxidase, catalase and Vogues Proskauer-positive, produced indole, liquefied gelatin, utilized citrate and reduced nitrate to nitrite. All the isolates fermented arabinose, salicin, galactose, lactose, xylose, sucrose, mannose and maltose sugars, and hydrolysed arginine and esculin. Based on cultural, morphological and biochemical tests, all the seven isolates were identified as *A. hydrophila* sub sp. *hydrophila*[9].

To determine the virulence of all the isolates, pathogenicity test was carried out in two numbers each of *C. catla* and *C. mrigala* (≃ 250 g) at a concentration of $1.5 \times 10^9$ CFU/0.2 ml/fish by intraperitoneal (I/P) inoculation. Clinical manifestation of the disease was observed first in *C. mrigala* and then in *C. catla* 72 h post-inoculation, followed by death after 4 and 6–8 days respectively. *A. hydrophila* was re-isolated in pure form from the experimental fish, which satisfied the criteria of Koch's postulates. Isolation and identification of *A. hydrophila* from dropsy-infected catla, mrigal and rohu (*Labeo rohita*) using identification scheme based on biochemical characteristics were reported earlier[3–7]. Apart from dropsy infection, there are reports of isolation of *A. hydrophila* from septicaemic infection of cultured carps[10,11]; from ulcerative infection of catla[12]; acute septicaemic condition of rohu and catla[13], and haemolytic bleeding disease of *L. rohita*[14].

The isolates were found to be sensitive to gentamicin and chlortetracycline, resistant to ampicillin, chloramphenicol and erythromycin and showed varied resistance to cloxacillin, when tested by *in vitro* antibiotic sensitivity assay[15]. There are reports of *A. hydrophila* strains showing resistance to penicillin, ampicillin, cloxacillin, amoxycillin and carbencillin[16], gentamicin, streptomycin, tetracycline, penicillin and neomycin[17], and more than 60% antibiotic resistance level[18]. Two types of multiple drug resistance (MDR) patterns, AmCmEr and AmCmCxEr were observed among the isolates. Though there is no practice of using antibiotics either in aquaculture or agriculture in Meghalaya, the bacterial isolates exhibited high degree of MDR. Similar patterns of high natural resistance of *A. hydrophila* to different antibiotics in fish from geographically remote waters, were reported[19]. The serious consequences of MDR could be in terms of failure to control disease outbreak in fish farms and also transfer of drug resistance from fish to man through the food chain.

The soluble whole-cell proteins of seven *A. hydrophila* isolates obtained after boiling in sample buffer for 10 min, were separated by SDS–PAGE in a 5–12.5% gradient gel[20]. Two different polypeptide profiles were observed in the molecular weight range of 12.5–205 kDa. Interestingly, the two isolates, Ah-1 and Ah-5 (lanes 1 and 5, Figure 1) having common polypeptide bands pattern produced mild indole after 48 h of incubation. The rest of the isolates showed different polypeptide pattern having 28–30 bands. The major differences between the two patterns were in the very high molecular weight polypeptides (above 130 kDa) and around 43 kDa (Figure 1). The difference in the whole-cell protein profile of *A. hydrophila* was also observed by other workers[7,21].

All *A. hydrophila* isolates screened (6 out of 7) showed single large plasmid of molecular size 22.5 ± 1.0 kb, when extracted by alkaline lysis method[22]. However, isolate number Ah-6 (lane 6; Figure 2) had an additional plasmid of approximately 8.1 ± 0.2 kb size. Dendrogram of overlaid graphs arising from plasmid profiles of all the isolates revealed simi-

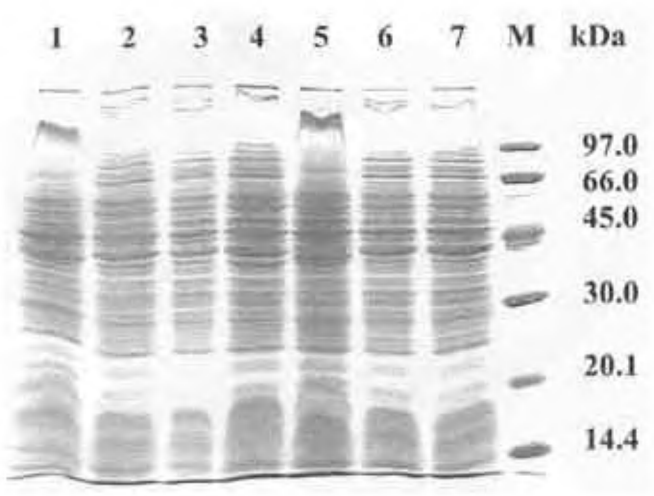


**Figure 1.** Whole-cell polypeptide profiles of *Aeromonas hydrophila* strains (lanes 1–7, Ah1–Ah7) isolated from dropsy-infected fish: M, Molecular marker.

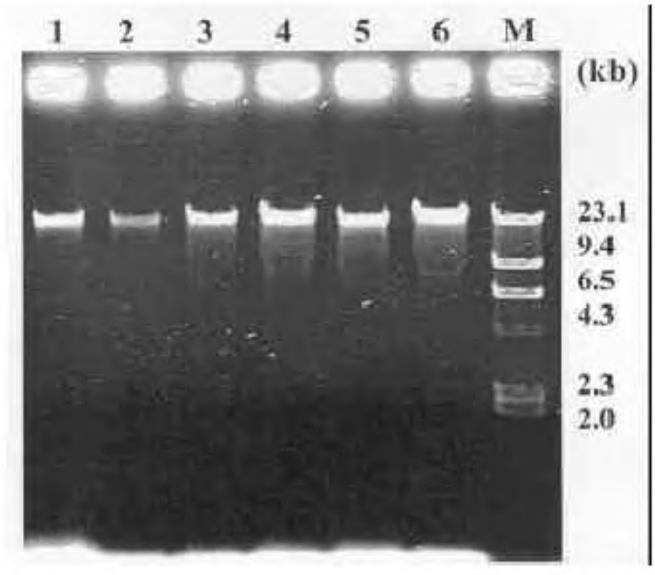


**Figure 2.** Plasmid profile of *A. hydrophila* strains (lanes 1–6, Ah1–Ah6) isolated from dropsy-infected fish: M, marker (kilobases).

**Table 1.** Characteristics of primers used for detection of haemolysin and aerolysin genes of *Aeromonas hydrophila*

| Target gene | Primer code | Primer sequence (5′ to 3′) | Location, amplicon size (bp) | Primer concentration (μM) |
|---|---|---|---|---|
| *ahh1** (*A. hydrophila* extracellular haemolysin gene *ahh1*)[38] | Ahh1F<br>Ahh1R | For: cgagcgcccagaaggtgagtt<br>Rev: gagcggctggatgcggttgt | 961–983<br>1090–1071<br>(130) | 2 |
| *aerA* (*A. hydrophila* aerolysin gene; GenBank Acc. no. M16495) | Ah-aerAF<br>Ah-aerAR | For: atcctattcccgggagtttacg<br>Rev: acgaaggtgtggttccagt | 1323–1344<br>1631–1613<br>(309) | 1.5 |

*From strain ATCC 7966.

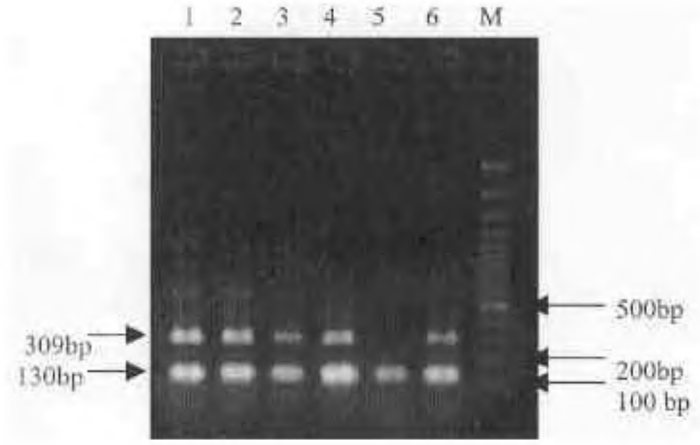


**Figure 3.** Multiplex PCR of *A. hydrophila* toxin genes. Lanes, 1–6, Ah1–Ah6 *A. hydrophila* strains isolated from dropsy-infected fish. M, 100 base-pair ladder.

larity ranging from 92.97 to 98.55% when compared with lane 1 (Ah-1) as reference. Large plasmid (22.5 ± 1.0 kb) observed in the present isolates was also observed in our earlier studies[23], which probably indicated the common distribution of plasmid among *A. hydrophila* strains, irrespective of geographical location. Plasmid-borne control of siderophore and haemolysins production[24] and resistance to tetracyclines[25] in *Aeromonas* species were observed.

Many factors contribute to the virulence of *A. hydrophila*, such as α-amylase, protease, DNAase, haemagglutination ability, haemolysin, cytotoxin and enterotoxin. Few of the virulence factors were tested using the cell-free culture supernatant after overnight cultivation of the isolates in Brain Heart Infusion broth (BHI). The α-amylase was detected in 1% agarose gel containing 0.4% starch in PBS, and caseinase and gelatinase enzymes were tested separately in nutrient agar media containing 1% sodium caseinate and 0.4% gelatin respectively, by radial diffusion method[26]. Similarly, DNAase was tested using DNAase medium containing 0.2% salmon sperm DNA and 0.001% toludine blue dye[27]. Haemolytic activity was detected by radial diffusion method in TSA medium enriched with 10% calf, rabbit, goat, sheep and chicken blood and haemagglutination test using 0.5% cattle, goat, chicken, rabbit and duck RBC suspension according to the standard procedure[28]. All the seven isolates showed amylase, gelatinase, caseinase and DNAase activities. Besides these invasive virulent factors, isolates produced β-haemolysin (aerolysin) in cattle, goat, sheep, rabbit and chicken blood agar and also haemagglutinated cattle, goat, pig, rabbit and duck RBCs. Presence of these major virulence factors in the isolates and pathogenicity in homologous hosts, amply suggest their virulence potential. Reports on haemolysin[14,29], caseinase, amylase, DNAase, haemagglutinins, cytotoxins, and dermonecrotic factors[6,27], lytic enzymes[30], proteolytic enzymes and haemolysins[13,31,32] of *A. hydrophila* responsible for the death of fish are available.

The haemolysins produced by *A. hydrophila* are divided into two major groups, such as extracellular haemolysin and aerolysin based on immunological studies[33]. An mPCR was done for detecting haemolysin (*ahh1*) as well as aerolysin (*A. hydrophila aerA*) genes as genetic markers for virulence determinants. A single colony from nutrient agar was suspended in 100 μl of HPLC grade water in a 1.5 ml eppendorf tube and boiled for 10 min. The cell debris was removed by centrifugation and supernatant was used as template DNA. The primer pair for Ahh1 designed to amplify 130 bp fragment of the extracellular haemolysin gene (*ahh1*) and the *Ah-aerA* primer amplifying 309 bp fragment of the aeolysin gene (*A. hydrophila aerA*) as described elsewhere[34] (Table 1), were commercially synthesized (GENSET). The amplification reaction was carried out in a final reaction volume of 25 μl containing 12.5 μl of 2 × master mix (4 mM $MgCl_2$ 0.4 mM of each dNTPs, 0.5 units of Taq DNA polymerase, 150 mM Tris-HCl PCR buffer; MBI Fermentas), primers, distilled water and 1.5 μl of template DNA. Amplification was performed in iCycler (BioRad, USA) with thermal cycling parameters: 5 min of initial denaturation at 95°C followed by 35 cycles of 0.5 min at 95°C; annealing for 0.5 min at 59°C and extension of 72°C for 0.5 min. Final extension was at 72°C for 10 min. Amplicons were separated in 1.5% agarose gel (11 × 8 cm) in Tris-acetate EDTA buffer at 70 V for 1 h, stained with ethidium bromide and visualized in gel documentation system. In the present study, 130 bp fragment of haemolysin (*ahhl*) toxin gene was amplified from all the six *A. hydrophila* isolates screened, and all but one (Ah5, lane 5; Figure 3) amplified 309 bp fragment of aerolysin (*Ah-aerA*) gene as expected. None was positive for only aerolysin gene. This is in complete agreement with a previous observation demonstrating that all the *A. hydrophila* isolates possess *ahh1*, while some carried

*Ah-aerA*, but none had *Ah-aerA* alone[34]. Earlier workers observed the presence of both *hlyA* (haemolysin) and *aerA* (aerolysin) genes in all virulent *A. hydrophila* strains[35]. Based on this analogy and the pathogenicity in fish, the isolates Ah1–Ah4 and Ah6 were categorized as highly virulet *A. hydrophila* strains. However, absence of aerolysin gene in isolate Ah5, which was found to be haemolytic as well as pathogenic in experimental hosts, did not fulfil the virulence criterion of presence of both haemolysin and aerolysin genes in all virulent *A. hydrophila* strains. Similarly, observation on presence of haemolysin gene were in all the clinical *A. hydrophila* strains[34] whereas 55% of clinical *A. hydrophila*[34], 79% of *A. hydrophila* stool isolates[36], and 3.3% of *A. hydrophila* strains from healthy fish carrying the aerolysin gene were reported[37].

Considering the clinical signs, postmortem lesions, isolation of *A. hydrophila*, its pathogenicity and demonstration of haemolysin and aerolysin genes by mPCR, the outbreak in fish was diagnosed as dropsy caused by *A. hydrophila*. The study also revealed highly virulent nature of *A. hydrophila* strains due to the presence of multiple virulence factors. The strains harboured a single large common plasmid with the exception of one isolate and differed with respect to protein profiles. The mPCR assay used in this study proved to be a useful tool for the detection of virulent *Aeromonas* genotypes by detecting haemolysin and aerolysin genes as genetic virulence markers.


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