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Molecular Detection of Esbl Genes in Antibiotic Resistant *Aeromonas hydrophila* Isolates

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Over the last few years, the interest in *Aeromonas* infection in India has gone beyond the boundaries due to the increase of diseases which is responsible for watery diarrhea. Most reports have described gastroenteritis, septicemia, meningitis, pneumonia, or surgical wound infections, primarily in immune-compromised patients[1]. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness costing a financial burden to families and greater risk of death. This bacterium produces a large number of extracellular virulence factors that are closely associated with specific diseases. It is known that *Aeromonas* spp possess different chromosomal β-lactamase genes and most of the antibiotics have been rendered futile against this environmental pathogen thus forcing mankind to device methods for control and treatment against them by locating new drug targets. In this study, a total of 30 stool samples were collected from patients in an around hospitals in south Chennai with diarrhoea. A large number of *A. hydrophila* colonies were isolated and their antibiotic susceptibility pattern was analyzed. Amp C beta-lactamase is Ambler class C enzymes that confer resistance to extended spectrum cephalosporins and their detection is crucial, since the phenotypic tests are not standardized leading to ambiguity in interpretation of results. PCR technique detects antibiotic resistance to more than one antibiotic resistant genes thus facilitating to identify the pervasiveness of extended spectrum β-lactamase (ESBL) producing genes such as CTX-M, TEM and SHV in this study.

Key words: *Aeromonas hydrophila,* Antibiotic resistance, CTX-M, TEM, SHV.

Among the etiological agents of bacterial fish diseases, the motile Aeromonas group, and especially *Aeromonas hydrophila*, is considered to be ubiquitous in most aquatic environments[2]. The genus comprises a group of Gram-negative, facultative anaerobic bacteria that are pathogenic for aquatic and terrestrial animals and have also been increasingly being recognized as human pathogens[3,4]. Gastroenteritis is one of the most vital diseases it can cause in immune susceptible humans and especially in young children[5]. Although the pathogenesis of Aeromonas infections remains poorly understood, several studies have demonstrated that strains of *A. hydrophila* produce lectins and adhesins which enable adherence to epithelial surfaces and gut mucosa [6]. This bacterium is linked with two types of gastroenteritis (i) rice-water diarrhea (ii) dysentery with blood and mucus while the latter is most severe. Acute gastrointestinal disease in children usually resolves within 7 days, and it is characterized by watery diarrhoea (100%), fever (70%) and vomiting (30%). Transmission among children in daycare centers[7], nursing homes[8], and patients in intensive care[9] have been reported.

Aeromonads produce multiple virulence factors typically associated with gastrointestinal disease in other bacteria, but the direct relationship between most of these virulence factors and gastrointestinal disease has not been proven *Aeromonas* spp are characteristically resistant to ampicillin (94.9%), with variable resistance to

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cephalexin (76.3%), trimethoprim (37.3%), tetracycline (11.9%), cefuroxime (5.1%), and ceftazidime (1.7%). Nearly all Aeromonads are susceptible to quinolones, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, sparfloxacin, moxifloxacin and gatifloxacin[10]. Role of antibiotics and various methods of resistance mechanism are shown in table 1.

Antibiotic resistance has been classified by the World Health Organization as one of the three major public health threats of the 21st century[11]. Extended spectrum β-lactamases (ESBLs) are definedas β-lactamases capable of hydrolyzing cephalosporins with an oxyimino side chain. The majority of ESBLs contains a serine at the active site and belongs to Ambler's molecular class A. Class A enzymes are characterized by an active-site serine and the preferential hydrolysis of penicillins. Class A β-lactamases include enzymes such as CTX-M, TEM-1, SHV-1, and the penicillinase[12]. The incidence of ESBL producing Aeromonas strains among clinical isolates has been progressively increasing over few decades resulting in limitation of therapeutic options[13]. Characteristically, they derive from genes for TEM-1, TEM-2, CTX-M, OXA or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. Thus extends the spectrum of β-lactam antibiotics are susceptible to hydrolysis by these enzymes. Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited. The application of PCR based molecular detection is a gold standard due to its accurateness and rapid detection which is irrevocable for diagnosing antibiotic resistance. Hence, this study was undertaken (i) to find out the prevalence of TEM, CTX-M and SHV genes in stool samples (ii) to determine its antibiotic resistant pattern and the prevalence of these genes.

MATERIALS AND METHODS

Sample Collection

Stool samples from 30 patients with diarrhoea and a control were collected from various laboratories and hospitals in south Chennai. Nearly 68.2% of patients were mustered from less than 15 years old. For hospitalized patients, clinical history was obtained from the notes and by visiting the patients. Most of the subjects were outpatients for whom their pediatrician or consulting doctor had requested a culture for acute diarrhoea.

Isolation of Aeromonas hydrophila

To isolate *Aeromonas hydrophila*, stool samples were enriched in Alkaline peptone water (APW) and then streaked on Rimler-Shotts (RS) Medium [14]. The isolates were preliminarily grouped according to colony morphology and subjected to biochemical tests. The type strain of *Aeromonas hydrophila* MTCC 646 obtained from Institute of Microbial technology, Chandigarh was included in the phenotypic characterization.

Microbial and Biochemical tests and Antibiotic test screening (Kirby Bauer's method)

The isolates from the RS Medium plates were subjected for biochemical parameters for the confirmation of *Aeromonas hydrophila.* Microbial and Biochemical parameters such as Gram staining, Catalase, Oxidase and Motility tests were performed (Table 2). Phenotypic characterization tests such as IMViC, Indole, Methyl red, Voges-proskaur, Citrate utilization, Nitrate reduction, Triple sugar iron (TSI) agar, Urease, Carbohydrate fermentation, Gelatinase, ONPG, LOA and Phenylalanine deaminase were also performed (Table 3).

Pure cultures were inoculated in 4-5 mL of nutrient broth and incubated at 37ºC for 24 hrs. The diluted inoculums were swabbed on the surface of Muller Hinton agar plate by swab method. Antibiotic discs were placed on the surface at 37°C in the incubator and the diameter of the zones were measured and compared with the performance standards for antimicrobial disc susceptibility tests[15] and interpreted if they were sensitive, resistant or intermediate. All the strains of *Aeromonas hydrophila* (n= 13) were subjected to 21 commonly used antibiotics and their concentrations as shown in Table 4.

Antibiotic susceptibility was determined by Disc Diffusion method[16]. Multiple antibiotic resistances (MAR) index[17] was calculated by the formula[18].

MAR Index = y/nx.

Where, y= Total number of resistance scored; n = number of isolates; x = Total number of antibiotics tested.

Screening ESBL genes

The genomic DNA was extracted from the isolated strains using standard phenol: chloroform

method[19]. Internal regions of 700, 1100 and 200bp from CTXM, TEM and SHV respectively were amplified using PCR. Amplification was carried out in a 20µl reaction set up containing 0.3µM of each primer, 0.2mM deoxy nucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 100ng of template DNA sample and 1 U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisted of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (45 s at 60°C), and extension (1 min at 72°C), with a final extension(10 min at 72°C).

RESULTS

A total of 13 isolate of *Aeromonas hydrophila* were collected 30 patients with acute diarrhoea.*Aeromonas hydrophila* was absent in control patients. The highest rate of *Aeromonas hydrophila* isolation was seen in the age group 11 to 15 and it was around 30.8% and 6 to 10 years is 29% and a minimum in the age group 21 to 25 and 46 to 50 (0.9%). The prevalence of *Aeromonas hydrophila* associated diarrhoea was not much significant in the age group of 26 and above. Plausible, *Aeromonas hydrophila* isolates were preliminary characterized as Gram negative motile, catalase and oxidase positive with haemolysis in blood agar (Table 2).

The isolation and biochemical studies resulted in the characterization of *Aeromonas hydrophila*. The biochemical results in table 3 indicate that the 13 isolated strains belong to *Aeromonas hydrophila.* Multiple drug resistance was observed with remarkable variation in susceptibility as indicated in table 4. The MAR index was calculated and the isolates showed less than 0.2 indicating that they are not much exposed to antibiotics (Table 5). The presence of CTX-M (520bp), TEM (1100bp) and SHV (200bp) genes were confirmed using PCR technique.

DISCUSSION

The purpose of this study was to determine whether *Aeromonas hydrophila* could be considered as a causative agent of diarrhoea in our geographical area – South Chennai. As Cumberbatcnh *et al*[20] suggested, we can assume that the isolation of aeromonads from human faeces can possibly mirror the ingestion of water or food contaminated with this organism. Members of the genus *Aeromonas* are known as important waterborne pathogens of animals and humans[21]. *A.hydrophila*is associated with both diarrhoeal and extra-intestinal infections in human diseases[3, 22].

In this study, *A. hydrophila* was found in large numbers in stool specimens from patients with diarrhea while it was absent in the control sample. In contradiction, Echeverria *et al*.[23] found *A. hydrophila* in normal subjects nearly as frequently as in patients with gastroenteritis. These divergent results may be related to geographic location, season of collection, and to the microbial media used for isolation. The sensitivity and specificity varied when different media were compared by Von Graevenitz and Bucher[24].

Kaper'smultitest medium[25] proved to be a very useful test in screening the suspected colonies. All bacterial isolates from diarrhoeal samples exhibited the typical aeromonad morphological characteristics such as mucoid yellow coloured colonies on RS Medium plates. All 13 isolates were found to be gram-negative, oxidase-positive, rod-shaped bacteria with colony morphology of round, 2-3 mm in diameter. This agrees with the findings of many reports including Rimler and Shotts who obtained yellow colonies

Table 1. Role of antibiotics in antibiotic resistance

Antibiotic	Method of resistance
Chloramphenicol	reduced uptake into cell
Tetracycline	active efflux from the cell
B-lactams,Erythromycin, Lincomycin	eliminates or reduces binding of antibiotic to cell target
B-lactams,Aminoglycosides, Chloramphenicol	enzymatic cleavage or modification to inactivate antibiotic molecule
Sulfonamides, Trimethoprim	metabolic bypass of inhibited reaction

Table 2. Preliminary Investigation of *Aeromonashydrophila* isolates

Preliminary tests	Result
Gram staining	Gram negative rods
Catalase	+
Oxidase	+
Motility	Motile
Haemolysis on blood agar	Haemolytic

when was inoculated on to RS Media[14] and these type of colonies indicating maltose fermentation. Hazen *et al*[26] who stated that RS Media was 94% efficient for isolation of *Aeromonas hydrophila* and Hsu *et al*[27] who noted that all 127 strains of *A. hydrophila* tested produced yellow colonies on the same. Mucoid yellow colonies resembling *Aeromonas* were sub-cultured on TSI agar showed typical reaction and were recorded for all their biochemical characteristics (Table 3).

The isolates obtained were subjected to antibiotic resistance analysis using antibiotic discs and were categorized based upon the diameter of the zone. The variation in the drug resistance may be related to the source of *A. hydrophila* and the

Table 3. Biochemical tests for the identification of *Aeromonashydrophila*

S.No	Tests	Result
1.	Indole	+
2.	Methyl red	+
3.	VogesProskauer	+
4.	Citrate	+
5.	Nitrate reduction	+
6.	Triple sugar Iron agar	K/A,H_2S^-,G^+
7.	Urease	-
8.	Carbohydrate fermentation	
	i. Arabinose	-
	ii. cellobiose	-
	iii. Fructose	AG
	iv. Glucose	AG
	v. Maltose	AG
	vi. Mannitol	-
	vii. Mannose	AG
	viii. Raffinose	-
	ix. Sorbitol	-
	x. Trehalose	AG
9.	Gelatinase	+
10.	ONPG	+
11.	LAO	++-
12	Phenylalanine deaminase	++

Table 4. Antibiotic susceptibility data of *Aeromonashydrophila* isolates collected from diarrhoeal samples

Antibiotic	(n=13)		
	Resistance (%)	Intermediate(%)	Sensitive%)
Amoxyclav (Ac)	53.27	14.95	31.77
Ampicillin (A)	92.52	6.54	0.93
Bacitracin (B)	64.48	31.77	3.7
Cefatoxine (Cep)	46.72	21.49	31.77
Chloramphenicol (C)	6.54	28.03	65.42
Ciprofloxacin (Cf)	-	-	100
Co-trimaoxazole (Co)	26.16	38.31	35.51
Erythromycin (E)	0.93	28.03	71.02
Gentamycin (G)	3.7	-	96.26
Kanamycin (K)	16.82	44.85	38.31
Methicillin (M)	72.89	22.42	4.67
Nalidixic acid (Na)	9.34	7.47	83.17
Nitrofurantoin (Nf)	18.69	19.62	61.68
Norfloxacin (Nx)	4.67	13.08	82.24
Novobiocin (Nv)	42.99	50.46	6.54
Pefloxacin (Pf)	10.28	39.25	50.46
Polymyxin – B (Pb)	19.62	42.99	36.44
Rifampicin (R)	72.89	11.21	15.88
Tetracycline (T)	13.08	27.10	59.81
Tobramycin (Tb)	8.41	18.69	72.89
Vancomycin (Va)	26.16	26.16	47.66

Table 5. MAR Indexing of the isolates

	Above 0.2	Below 0.2
Frequency	9	4
Perfect	75.7	24.3
Valid Percent	75.7	24.3

frequency of antibiotics prescribed for treating infections in different geographical area[28] which may increase the drug resistance among the strains. High percentage of antimicrobial resistance and emergence of multiple drug resistance among the *A. hydrophila* strains were observed in the present study. The MAR index of the thirteen isolates ranged between 0.14 - 0.52 (Table 5), where 75.7% of the isolates showed a MAR index of more than 0.2 indicating that they might have received high risk exposure to the antibiotics and 24.3 % of the isolates showed a MAR index less than 0.2 indicating that they are not much exposed to antibiotics. MAR index higher than 0.2 has been an indicator of isolates originating from a geographical location where microbial exposure to antibiotics is more[17]. The sensitivity (100%) was attributed to ciprofloxacin followed by gentamycin, nalidixic acid and norfloxacin (96.26%, 83.17% and82.24%). The isolates were highly resistant to Ampicillin, followed by methicillin and rifampicin (92.52%, 72.89% and 72.89%). Resistant to methicillin, which does not correlate with the pervious findings of Motyl *et al*[29], who reported 100% methicillin resistance in strains of human origin. However, Pettibone *et al*[30], observed only 54% of the strains while Kampfer *et al*[31] reported no significant resistance to this antibiotic in both clinical and non-clinical *Aeromonas* isolates. In addition to the previous argument with respect to biochemical tests, antibiotic resistance also demonstrates that geographical location plays a vital role in influencing the level of its resistance. The easiest and most common molecular method used to detect the presence of a β-lactamase belonging to a family of enzymes is PCR with oligonucleotide primers that are specific for β-lactamase gene. Mutants of CTX-M enzymes harboring improved catalytic efficiencies against ceftazidime have recently been observed, suggesting that the enzymes are evolving as a result of ceftazidime selection pressure. The residues implicated in this evolution have never been observed in naturally occurring TEM or SHV ESBLs, suggesting that the CTX-M enzymes probably have a singular evolutionary potential and these enzymes are not very closely related to TEM and SHV[12]. Furthermore, studies has shown that TEM and SHV are 100% resistant to Ceftazidime, Cephotaxime, Ceufuroxime, Cefixime, Cefpodoxime, Amikacin, Ticarcillin and Piperacillin[32].

In this study PCR amplification was performed for CTX–M, TEM and SHV genes. The reactions were subjected with a positive control. From the total isolates 69% of the samples showed prevalence of CTX-M, 53% showed positive for TEM while 61.5% showed prevalence of SHV genes. The presence of ESBL genes show that these genes also play a major role in conferring high levels of antibiotic resistance in the diarrhoeal isolates. In conclusion, we report the presence of CTX-M, TEM and SHV genes in ESBL producing *Aeromonas spp* isolates showing high drug resistance from 30 patients with diarrhoea.

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