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Occurrence of Multiple Antibiotic Resistance among Children Diarrhoeal Isolates of *Aeromonas hydrophila*

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Abstract: Of the samples examined, 25.7% of children diarrhoeal samples harbored *Aeromonas* spp. Among them 16.7% were *A. hydrophila*. Though all the isolates were resistant to all β-lactam antibiotics (except carbapenem) and non β-lactam antibiotics tested and further studied for the presence of plasmid in all the strains. The majority of isolates were resistant to clarithromycin and vancomycin. Thirty one (77.5%) of the 40 isolates harbored plasmids, with sizes ranging from 2.6 to 15.2 kb. These results indicate that multiple antibiotic resistant and genetically diverse aeromonads are easily available among the children diarrhoeal samples collected from the study area. It indicates that the practice of frequent application of therapeutics for the treatment of diseases might have enhanced occurrence of drug resistance and carrying the resistant plasmids.

Key words: *Aeromonas hydrophila*, gastroenteritis, β-lactam, non β-lactam antibiotics, plasmids

INTRODUCTION

Aeromonas spp. comprises mesophilic, motile organisms, which are distributed universally in fresh water, estuarine, coastal water bodies and food samples. They are associated with wide spectrum of infection in human diseases especially diarrhoea in children, aged individuals and immunocompromised patients (Gracey *et al*., 1982; Agger *et al*., 1985). Among *Aeromonas* species, *A. hydrophila* is widely studied and most commonly involved in causing human infections (Agger *et al*., 1985; Ananthan and Alavandi, 1999). Clinical and environmental strains secrete many extracellular products, such as haemolysins, enterotoxins, aerolysin, haemagglutinins and protease (Kuhn *et al*., 1997). Occurrence of *A. hydrophila* from water and food sources, are widely increasing the resistance to various antimicrobial agents and chlorine in potable water, which presents a significant threat to public health (Thayumanavan *et al*., 2003). About 6.5 to 13% of the diarrhoeal cases of south India have been attributed to *Aeromonas* (Alavandi and Ananthan, 2003) and the frequency of diarrhoea is more in children than adults. It compels the necessity to investigate the incidence of pathogenic *Aeromonas* spp.

The strains of *A. hydrophila* were highly susceptible to tetracycline, chloramphenicol, polymyxin-B, gentamicin and trimethoprim-sulfamethoxazole (Ramteke *et al*., 1993). Increased incidence of multiple antibiotic resistant *Aeromonas* isolated from clinical and environmental sources have been reported worldwide and they may pose a serious problem in chemotherapy (Pettibone *et al*., 1996).

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The present work was aimed to evaluate *in vitro* susceptibilities for wide range of antimicrobial agents, against the strains of *A. hydrophila* isolated from diarrhoeal samples of children. During the treatment of infections with multi drug resistant bacterial pathogens is a serious problem faced by the clinicians. This may also help to know the emergence of drug resistant *A. hydrophila* and regular up-taking of antibiotic resistant pattern will be useful for formulate the hospital policy.

MATERIALS AND METHODS

Bacterial Strain

A total of 239 stool samples were collected from children, who had affected with acute diarrhoea in the hospitals. The samples were procured just before starting treatment with chemotherapy from hospitals located in Coimbatore, Tirupur, Kovilpalayam and Pattukottai of South India, during the period of January 2003 to December 2003. The stool samples were processed in Alkaline Peptone Water (APW) (Cat. No. M6181, HiMedia, India) for pre-enrichment and then cultured on starch ampicillin agar medium and Rimler-Shotts medium (Cat. No. M576, HiMedia, India) and Rippey Cabelli Agar medium (Cat. No. M859, HiMedia, India). The presumptive identification was performed by oxidase reaction, fermentation of glucose, presence of lysine, arginine decarboxylation and absence of ornithine decarboxylase, proposed by Cowan and Steel (1970). Kaper's multi test medium was used for the confirmation of *A. hydrophila* (Kaper *et al*., 1979). Further, the strains were identified on the basis of the restriction fragment length polymorphism patterns (RFLP) obtained by the 16s rDNA following the method described by Borrel *et al*. (1997) and Figueras *et al*. (2000).

Statistical Analysis

The χ^2 and Fisher's exact tests were used to compare the diarrhoeagenic *A. hydrophila* between the four sampling stations in all the months. A p-value <0.05 was considered as significant. The statistical analysis was performed using SPSS 10.0 for Windows XP.

Antimicrobial Susceptibility

A. hydrophila (n = 40) strains were examined for their resistance to 42 antibiotics (β-lactam and non β-lactam) (HiMedia, India). The resistance pattern was determined by the disc diffusion method of Bauer *et al*. (1966), using Mueller Hinton agar medium (Cat. No. M1084, HiMedia, India). The plates were incubated for 24 h at 37°C, the size of inhibition zones were measured and classified as resistant, intermediate and sensitive according to manufacturer's instruction. The intermediate strains were also scored as resistant.

MAR Indexing

The MAR index, when applied to a single isolate is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed (Krumperman, 1983). MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 considered the origination of strain from animals in which antibiotics are seldom or never used.

Detection of Plasmids

Plasmid DNA was extracted, followed by electrophoresis, essentially as described by Sambrook *et al*. (1989). The approximate molecular mass of each plasmid was determined by comparison with plasmids of known standard molecular mass (Cat # MBD 22, Genei, India).

RESULTS AND DISCUSSION

In recent years, *Aeromonas* spp. have been recognized with increasing frequency as a cause of gastroenteritis in children and adults. Several investigators have determined the asymptomatic carrier rate to assess the role of *Aeromonas* species in diarrhoeal diseases (Janda, 1991; Janda and Abbott, 1998; Joseph and Carnahan, 2000; Alavandi and Ananthan, 2003; Sinha *et al.,* 2004). Significant number of diarrhoeal samples (25.7%) ($p<0.05$) were found to harbor *Aeromonas* species with a highest (47.2%) at Coimbatore and followed by Pattukottai (26.4%), Kovilpalayam (20.8%) and Tirupur (21.4%). Among them 16.7% of *A. hydrophila* were noticed in the above-mentioned four places (Table 1). Higher percentage of incidence (17.6%) was observed from the samples collected from Coimbatore, followed by 16.6, 15.1 and 14.3% in Kovilpalayam, Pattukottai and Tirupur, respectively, where the diarrhoeal stool samples were found to harbor in a significantly higher number of *A. hydrophila* in all the sampling areas ($p<0.05$).

The samples were also found with other enteropathogens (*Vibrio*, *Salmonella*, *Shigella*, *Proteus*, *Escherichia* and *Staphylococcus*) and *Pseudomonas* and *Proteus* (data not shown). But the present investigation was mainly focused on the incidence of *A. hydrophila*. In Coimbatore, 17.6% of incidence was higher than the findings observed in Chennai, Mumbai and Goa, India, with 4.7, 1.4 and 7.7%, respectively (Alavandi and Ananthan, 2003). Numerous cases of incidence of *Aeromonas* from patients with diarrhoea and the bacterium isolated from stool of healthy persons have been reported (Janda and Abbott, 1998; Albert *et al*., 2000; Joseph and Carnahan, 2000). To our knowledge this is the first study describing the *Aeromonas* in clinical aspects in this study area.

All the (n = 40) *A. hydrophila* strains were tested against 42 (17 β-lactam and 25 non β-lactam) antimicrobial agents. Resistance of *A. hydrophila* isolates towards β-lactam antibiotics are given in Table 2. The study showed the existence of multiple antibiotic resistant strains of *A. hydrophila*. Most of the isolates were resistance towards β-lactam antibiotics, such as penicillins and cephalosporins. It was observed that 22.5% of the *A. hydrophila* isolates showed resistance towards carbapenem (imipenem). Bravo *et al*. (2003) stated that the genus *Aeromonas* has been considered to be dependant on chromosome-mediated β-lactamases. First, second and third generation cephalosporins were found to be more active against *A. hydrophila*. The similar findings have been reported by several authors (Ko *et al*., 1996; Nwosu and Ladapo, 1999; Joseph and Carnahan, 2000). Most of the strains were resistant towards cephalexin, cephaloridine, oxacillin, amoxycillin, amoxycillin plus clavulanic acid (amoxyclav) and cefoperazone. As a result, there was increasing isolation of drug resistant *A. hydrophila* and taxonomic inconsistency present in diarrhoeal sources (Carnahan *et al*., 1991). Generally, β-lactam group of antibiotics are not prescribed for the treatment of diarrhoea. Our study also indicate that most of the β-lactam antimicrobial agents were ineffective to *A. hydrophila*, it is not recommended for treatment of *Aeromonas* infection in human. In contrast Ko *et al*. (1998) reported the β-lactam agents could be used for the treatment of invasive *A. hydrophila* infections.

Among non β-lactam antibiotics tested, netilmicin and bacitracin were found more susceptible to all the isolates followed by norfloxacin (95%) (Table 3). It was also reported that 12.5% of strains were resistant to chloramphenicol. Chloramphenicol resistant *A. hydrophila* is an emerging and high

Table 1: Percentage of incidence of *Aeromonas* spp. and *A. hydrophila*

Station	No. of samples	(%) of incidence	
		Aeromonas spp.	*A. hydrophila*
Coimbatore	148	34.4	17.6
Kovilpalayam	24	20.8	16.6
Pattukottai	53	26.4	15.1
Tirupur	14	21.4	14.3
Total	239	25.7	16.7

Table 2: Antibiotic susceptibility of isolates of *A. hydrophila* to β-lactam antibiotics

	Aeromonas hydrophila (n = 40)		
Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Penicillins			
Amoxycillin	77.5	12.5	10.0
Amoxycillin plus clavulonic acid (Amoxyclav)	77.5	12.5	10.0
Ampicillin	97.5	2.5	0.0
Methicillin	95.0	2.5	2.5
Pipercillin	75.0	10.0	15.0
Ticarcillin	72.5	12.5	15.0
Oxacillin			
Cephalosporins	80.0	10.0	10.0
Cefazoline	67.5	12.5	20.0
Cefadroxil	72.5	10.0	17.5
Cefoperazone	77.5	10.0	12.5
Ceftazidime	72.5	10.0	17.5
Ceftizoxime	65.0	15.0	20.0
Ceftriaxone	60.0	17.5	22.5
Cephalexin	97.5	2.5	0.0
Cephaloridine	85.0	5.0	10.0
Cefotoxime	60.0	20.0	20.0
Carbapenem			
Imipenem	17.5	5.0	77.5

Table 3: Antibiotic susceptibility of isolates of *A. hydrophila* to non β-lactam antibiotics

	Aeromonas hydrophila (n = 40)		
Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Aminoglycosides			
Amikacin	20.0	20.0	60.0
Gentamicin	5.0	10.0	85.0
Kanamycin	70.0	10.0	20.0
Nalidixic acid	42.5	2.5	55.0
Neomycin	62.5	7.5	30.0
Netilmicin	0.0	0.0	100.0
Tobramycin	27.5	7.5	65.0
Macrolides			
Clarithromycin	42.5	22.5	35.0
Erythromycin	22.5	10.0	67.5
Lincosamides			
Lincomycin	75.0	12.5	12.5
Quinolones			
Ciprofloxacin	47.5	17.5	35.0
Norfloxacin	2.5	2.5	95.0
Ofloxacin	10.0	0.0	90.0
Sparfloxacin	15.0	20.0	65.0
Co-trimoxazoles			
Co-trimoxazole	20.0	12.5	67.5
Trimethoprim	37.5	17.5	45.0
Polymyxin			
Polymyxin B	5.0	2.5	92.5
Tetracycline			
Tetracycline	47.5	12.5	40.0
Glycopeptides			
Vancomycin	50.0	27.5	22.5
Others			
Bacitracin	95.0	5.0	0.0
Chloramphenicol	7.5	5.0	87.5
Lomefloxacin	40.0	12.5	47.5
Novobiocin	40.0	12.5	47.5
Nitrofurantoin	42.5	2.5	55.0
Rifampicin	50.0	30.0	20.0

risk levels among the species (Pettibone *et al*., 1996; Vivekanandhan *et al*., 2002; Radu *et al*., 2003; Subashkumar *et al*., 2006b). Lincosamides resistant (87.5%) *A. hydrophila* were noticed in all the strains, such type of lincosamides resistant *A. hydrophila* were reported in the diarrhoeal strains from Kollkata, India (Sinha *et al*., 2004). In this study, 80 and 60% of the strains were resistant to rifampicin and tetracycline. Chang and Bolton (1987) found that greater percentage of Asian isolates of *A. hydrophila* were resistant to tetracycline and rifampicin than Australian isolates.

Increasing ciprofloxacin resistance (60%) observed in all the strains with those of Sinha *et al*. (2004) who found ciprofloxacin resistant *A. hydrophila* have been 22.4%. Most of the *A. hydrophila* strains were exhibited lesser sensitivity to non β-lactam antibiotics. Generally, it was observed that the majority of the strains exhibited a multidrug resistant profile. The increased drug resistance is a significant threat to management of *Aeromonas* mediated diarrhoea (Sinha *et al*., 2004).

The Multiple Antibiotic Resistance (MAR) was calculated and the percentage occurrence of *A. hydrophila* MAR index values are presented in Fig. 1. It was observed that all the strains showed MAR index value of more than 0.2. This clearly indicates that the choice of a MAR index of 0.2 to differentiate between low and high risk source contamination is arbitrary. Indices of between 0.2 and 0.25 are in a range of ambiguity and the samples in this range require careful and specific scrutiny.

Bacteria resistant to β-lactam and non β-lactam antibiotics, may occur by selective pressure or because of antibiotic abuse by humans or over use in animals (White *et al*., 2000). In the present study, it was observed that all the strains isolated from children diarrhoeal samples showed multiple antibiotic resistances. A MAR index value of 0.2 or above is said to be originated from high-risk source of contamination (Krumperman, 1985). In the present study, all the strains showed a MAR index value more than 0.2, which implies that the organism might have been originated from high risk-sources like sewage, animal husbandry waste, biomedical waste, faecal contaminated drinking water. Survival of such MAR *A. hydrophila* strains in the food of animal origin may become contaminated with bacteria of intestinal origin and consequently animals may act as a potential source of resistant bacteria for humans. Multiple antibiotic resistance of *A. hydrophila* has been reported by several researchers throughout the world (De Vicente *et al*., 1990; Davies, 1992; Vivekanandhan *et al*., 2002; Radu *et al*., 2003; Thayumanavan *et al*., 2003; Lakshmanaperumalsamy *et al*., 2005; Subashkumar *et al*., 2006a). The release of such type of organisms through faeces may ultimately pave the way for the contamination of aquatic environments (Lakshmanaperumalsamy *et al*., 2005; Subashkumar *et al*., 2006b). In addition, antibiogram is considered as one of the useful techniques in the characterization of pathogens (Obi *et al*., 2004).

Plasmids were detected in all the isolates and it was ranged from 2.6 to 15.2 kb. The overall prevalence of plasmids was 77.5%. The frequency of occurrence and the detection of small size

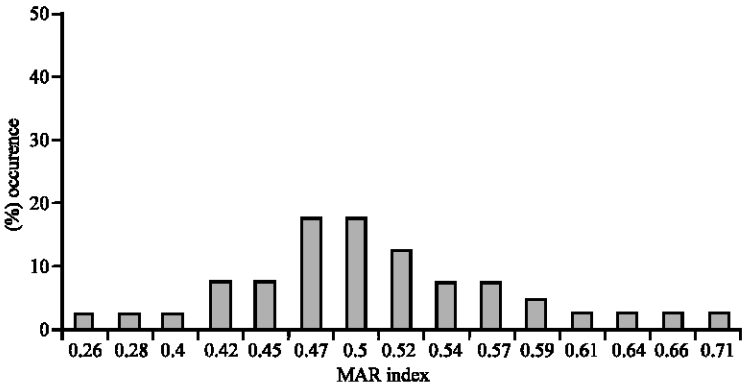

Fig. 1: Percentage occurrence of MAR index value of *A. hydrophila*

plasmids among the *Aeromonas* were in broad agreement with those of other workers who found plasmid prevalence in *Aeromonas* have been 15 to 94%, with most of the isolates harbouring small sized plasmids (Chang and Bolton, 1987; Alabi and Odugbemi, 1990; Vadivellu *et al*., 1995; Chaudhury *et al*., 1996; Brown *et al*., 1997). Chang and Bolton (1987) have suggested that the plasmid-mediated antibiotic resistant is not frequent. Thus the observation that the plasmid containing isolates were devoid of large plasmid and together with the finding of isolates not containing plasmids but being multi-resistant to the antibiotics is of chromosomal origin.

Consequently, the contamination of the environment with bacterial pathogens resistant to various antimicrobial agents is a real threat not only as source of infection but also a source from which R plasmids can easily spread to other pathogens of diverse origin (Linton *et al*., 1996). In the study area, the clinical practitioners are administering tetracycline, gentamycin, amoxycillin, nalidixic acid and cotrimoxazole and streptomycin for the treatment of diarrhoea in children. Increased level of resistances in clinical isolates of *A. hydrophila* to commonly used antibiotics has been observed. Like Gram-negative bacilli, the emergence of resistance among aeromonads will be accelerated by the extensive clinical use of antibiotics (Chaudhury *et al*., 1996). Such high level of multiple drug resistance may arise from selective pressure due to the indiscriminate use of antibiotics. As previously reported, plasmids conferring resistance may also play an important role in linking resistance to more than one drug (Lacey *et al*., 1974). The emergence of resistance among aeromonads may also be increased by the frequent usage of antibiotics. These multiple antibiotic resistance strains from the hospital environments may cause serious health hazards

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