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Incidence of *Aeromonas* species isolated from Diarrhoea patients and water samples from coastal districts of Odisha, India

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ABSTRACT

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A comprehensive study was performed to examine the incidence of different *Aeromonas* species, their drug sensitivity patterns and virulence genes isolated from diarrhoea patients and environmental water samples between September, 2006 to August, 2007 in Odisha. The rectal swab and water samples were bacteriologically analyzed using standard techniques; detection of toxic and clonality were done by PCR and RAPD-PCR Assay respectively. Out of 280 diarrhoea samples and 100 water samples, 20.2% from diarrhoea patients and 62.5% from water samples were positive for *Aeromonas* spp. *A. hydrophila* (50.9%) was isolated as dominant pathogen followed by *A. caviae* (27.2%) and *A. sobria* (21.8%). The common resistance profile of three different *Aeromonas* species were piperacillin, kanamycin, bacitracin, rifampicin, erythromycin, sulfadiazine, roxithromycin, lomefloxacin, ciprofloxacin, ampicillin, furazolidone, cefuroxime, and amoxycillin. On blood agar plates 52.7% of *Aeromonas* spp. showed β - hemolysis and production of *hly* A gene as revealed by PCR assay. RAPD PCR assay revealed that the *Aeromonas* spp. were quite similar in clonality. As diarrhoea is the most promising health problem in Odisha, this type of study should be extended for longer period in different parts of the state.

Introduction

Aeromonas species are ubiquitous water borne organisms and cause gastrointestinal infection in humans (Horneman *et al.*, 2007). *Aeromonas* species comprises of mesophilic motile and psychrophilic nonmotile gram negative bacterium. Three distinctive gastroenteritis are caused by *Aeromonas* spp. (a) acute watery diarrhoea, (b) dysentery and (c) sub acute or chronic

diarrhoea (Janda *et al.*, 1998). Acute diarrhoeal diseases constitute an important cause of morbidity and mortality throughout the world, particularly among the infants and children in developing countries (Rao *et al.*, 1965). According to the World Health Organization (WHO), diarrhoeal diseases are among the ten major causes of death among young children in eleven countries of Africa, Asia and South America. The

species principally associated with gastroenteritis are *A. caviae*, *A. hydrophila* and *A. veronii* bio vars *sobria* (Joseph *et al.*, 1996). The outbreak of cholera has been reported in Odisha from time to time. The major etiological agents are different strains of *E. coli*, *Shigella* spp. and *V. cholerae*, which has been reported by different researchers (Chhotray *et al.*, 2002, Palet *et al.*, 2000, Albert *et al.*, 1995). So far, no study has been conducted on *Aeromonas* related diarrhoea in Odisha. So, the present study has been envisaged to know the prevalence of *Aeromonas* spp. related to gastroenteritis by isolation, characterization and genetic analysis isolated from indoor diarrhoea patients and water samples from the coastal districts of Odisha.

Materials and Methods

Bacterial culture analysis

The rectal swabs were collected from hospitalized diarrhoea patients before the administration of antibiotics in Cary Blair's transport medium between September, 2006-August, 2007. The samples were immediately incubated in double strength APW and GN broth for 24 hrs. Then the samples were streaked into the MacConkey, TCBS agar and HEA plates (HiMedia, Mumbai). The samples from water and diarrhoea patients also were streaked into *Aeromonas* isolation agar and Rimler Shott's medium. The hemolytic activity of *Aeromonas* species were done in Blood agar (HiMedia, Mumbai) plates by taking 5% sheep blood. Blood agar plates were incubated in aerobic and anaerobic conditions at 37°C for 24 hrs. The degree of hemolysis was measured for species detection. Significant colonies were picked up for gram staining and finally biochemical test was done for confirmation. Identification of strains was done based on

colony morphology, motility test and biochemical tests like urease, TSI, manitol motility, indole, citrate utilization, oxidase, methyl red, voges-proskauer, ONPG, KIA chain, oxidation-fermentation, starch hydrolysis, sugar fermentation, deaminase, decarboxylase, gelatinase, etc (HiMedia, Mumbai) (Pal *et al.*, 2000).

Antimicrobial Susceptibility

Antibiotic susceptibility analysis was performed by modified Kirby Bauer Disk diffusion technique (Bauer *et al.*, 1966) with commercially available antibiotic disc (HiMedia-Mumbai). Characterization of stains as susceptible, intermediately resistant or resistant were done as based on the size of the inhibition zone according to the manufacturer's instruction which matched the interpretive criteria recommended by WHO. Antibiotics used in this study were piperacillin (Pc, 100µg), kanamycin (K, 10µg), gentamicin (G, 10µg), bacitracin (B, 10µg), rifampicin (R, 30µg), trimethoprim (Tr, 30µg), cefotaxime (CTX, 30 µg), erythromycin (E, 15µg), sulfadiazine (SD, 25 µg), roxithromycin (RXT, 15 µg), lomefloxacin (LOM, 10 µg), azithromycin (AZM, 15 µg), cefuroxime (Cu, 30µg), doxycycline(dx,30 µg), tetracycline (T, 30µg), chloramphenicol (C, 30µg), ampicillin (A, 10µg), nalidixic acid (Na, 30µg), furazolidone (Fr, 50µg), norfloxacin (Nx, 10µg), ciprofloxacin (Cf, 5µg), co-trimoxazole (Co, 25µg), amikacin (Ak, 30µg), streptomycin (S, 10µg) and amoxicillin (Am,10 µg).

PCR assay

The PCR assay was done for the detection of *hly A* gene of *Aeromonas* spp. isolated from water and stool samples. The DNA was isolated from culture grown in Luria Bertani (LB) broth for overnight by boiling in a

water bath for 10 min and instant cooling on ice. The forward primer sequence (F: 5'-GAGACTGCAGATGCCAAAAGTCAATC GTTGCGCAATC-3') and the reverse primer sequence (R: 3' - GAGAGAATTCTTAGTGCAAATCAAAT TGAACCCCTTTCACCAA-5') were used in this assay (3B Black Bio Biotech India Ltd). The reaction mixture was prepared with preparation of master mix by adding adequate water, buffer, dNTP, *hlyA* gene, primer 1 and 2 and *Taq* DNA polymerase. The thermal cycle condition for this assay was 94°C for 4 min, initial denaturation; 94°C for 1 min, denaturation; 65°C for 1.5 min, annealing; 72°C for 2.5 min, extension; for 30 cycles, 72°C for 7 min, final extension. The PCR product will be electrophorated and the specific DNA bands was visualized under UV illumination (Sinha *et al.*, 2004).

RAPD- PCR assay

RAPD-PCR assay of *Aeromonas* species were done by taking random primer 1281 (5' AAGGCGCAAC-3') (3B Black Bio Biotech India Ltd) in a 25 micro liter reaction mixture to calculate the genetic similarity between the strains. The template DNA was prepared similarly as PCR assay. Amplification was done with appropriate vol. of 2.5 µl of 10X amplification buffer, 2.5 µl each of DNTPs, 1.0 µl of each primer, 0.3 µl of *Taq* DNA polymerase and 2 µl of template DNA. The reaction volume was adjusted to 25 µl using sterile triple distilled water. The cycling program was initial denaturation at 95°C for 30 sec and final extension at 72°C for 10 min; cycling conditions were 94°C for 1 min, 36 °C for 1 min and 72 °C for 2 min, continued for 45 cycles. After completion, 8 micro liter aliquots of products were done for electrophoresis in 1.2% agarose gel containing 1 microgram ethidium bromide

and visualized under UV light (Sinha *et al.*, 2004). Genetic similarity of *Aeromonas* species was calculated using the formula $N_{AB}=2S_{AB}/S_A+S_B$; in which N_{AB} =Genetic similarity between two strains A&B, S_{AB} =No. of amplified bands shared common between A&B and S_A & S_B = Total no. of amplified bands present in strains A and B respectively.

Results and Discussion

Bacteriological analysis

A total of two hundred and eighty rectal swabs and 100 water samples collected during the study period were bacteriological analyzed using standard techniques. The bacteriological analysis of stool samples revealed 44.3% were culture positive of which 20.2% were *Aeromonas* spp., followed by other pathogens as 31.5% of *V. cholerae*, *E. coli* -43.5%, *Shigella* spp. - 4.8% (Table-1). The distribution of the bacterial pathogens from different water samples were as, *Aeromonas* spp., *V. cholerae* non O1 and non O139 and *E. coli* as shown in table 2. Out of 100 samples 40% were isolated from ponds, 37% from well, 12% from lake and 11% from canal water respectively (Table-2). Out of 100 water samples, 30 (62.5%) *Aeromonas* spp. was isolated, out of which 17 isolates were *A. hydrophila*, 8 were *A. caviae* and 5 were *A. sobria* respectively.

The different species of *Aeromonas* isolated (*A. hydrophila*, *A. caviae* and *A. sobria*) (Table-3) were different from each other in lysine decarboxylase, esculin hydrolysis, and nitrate test, production of hydrogen-sulphide and in fermentation of different sugars. *A. hydrophila* used L-histidine, L-arabinose, L-arginine and salicin as sole carbon source. They hydrolyzed esculine, grew in KCN medium, fermented salicin, and produced gas from glucose and H₂S

from cysteine. *A. caviae* strains used L-histidine, L-arabinose and salicin as sole carbon source. They hydrolyzed esculine, grew in KCN medium, fermented salicin, did not produce gas and acetoin from glucose or H₂S from cysteine. *A. sobria* did not use L-histidine, L-arabinose, L-arginine and salicin as sole carbon source. They did not hydrolyze esculine, do not ferment salicin. They produced gas from glucose and produced H₂S from cysteine.

Antibiogram profile

The different *Aeromonas* species (*A. hydrophila*, *A. caviae* and *A. sobria*) were multidrug resistant. They were sensitive to gentamicin, trimethoprim, azithromycin, tetracycline, doxycycline, chloramphenicol, nalidixic acid, norfloxacin, co-trimoxazole, amikacin and streptomycin; but were resistant to a number of antibiotics such as piperacillin, kanamycin, bacitracin, rifampicin, erythromycin, sulfadiazine, roxithromycin, lomefloxacin, ciprofloxacin, ampicillin, furazolidone, cefuroxime, and amoxicillin (Table-4).

PCR assay result

Twenty nine (52.7%) strains of *Aeromonas* spp. showed β -hemolysis on blood agar plates. The degree of hemolysis between different strains of *Aeromonas* spp. varied from 1.17 to 1.57; in *A. caviae* from 1.18 to 1.46 in *A. sobria* from 1.23 to 1.35 respectively. Among the *Aeromonas* spp.; 13 strains of *A. hydrophila*, 8 strains of *A. caviae* and 8 strains of *A. sobria* showed β -hemolysis (table-3) and the results were correlated with the presence of *hly A* (702bp) gene as detected in PCR assay (Fig- 1).

RAPD-PCR Assay

RAPD-PCR Assay of *Aeromonas* spp. (Fig: 2) produced 4 to 8 amplified fragments of

molecular weight varied from 181 to 2111 bp respectively. The strains of *A. hydrophila* produced 4 to 5 amplified bands of molecular weight varied from 181 to 2472 bp in *A. caviae* strains produced 5 amplified bands of molecular weight 181 to 2512 bp and in *A. sobria* strains produced 8 amplified fragments of molecular weight varied from 281 to 4572 bp respectively. The amplified fragments as 2472, 1493, 833, 820, 679, 658, 598, 544 and 181 bp were common within the different strains of *Aeromonas* spp. The genetic similarity value of the strains of *Aeromonas* spp. varied from 0.308 to 1.0; similarity value between the strains of *A. hydrophila* and *A. caviae* varied from 0.4 to 1.00 between strains of *A. hydrophila* and *A. sobria* varied from 0.308 to 0.462 respectively. The genetic similarity between the strains of *A. caviae* and *A. sobria* were varied from 0.308 to 0.462. Thus it is concluded that the strains of three *Aeromonas* spp. were quite similar as observed in RAPD-PCR assay, through the above strains were isolated from different sources. The phenotypic and genotypic characters, different toxic gene of *Aeromonas* spp. isolated from environmental and clinical sources were quite similar which caused diarrhoea, dysentery and gastroenteritis in human beings.

The role of *Aeromonas* spp. has been recognized for some time (Janda *et al.*, 1998), but only during the past three decades their role in a variety of human illness has been documented. The role of *Aeromonas* spp. in bacterial gastroenteritis is not yet clearly understood owing to a paucity of long term studies (Janda *et al.*, 1998) and the inability to differentiate between pathogenic and nonpathogenic strains. The present study was envisaged to determine the existence of different *Aeromonas* spp. isolated from water and diarrhoea patients.

Table.1 Incidence of *Aeromonas* spp. isolated from stool samples

Sl.No.	Hospitals	Total samples	Culture +ve	Culture –ve	<i>Aeromonas</i> spp.	<i>V. cholerae</i>	<i>E. coli</i>	<i>Shigella</i> spp.
1.	BBSR & Cuttack	58 (20.7)	21(16.9)	37	6	2	12	1
2.	Puri	222 (79.3)	103(83.1)	119	19	37	42	5
	Total	280	124(44.3)	156 (55.7)	25(20.2)	39(31.5)	54(43.5)	6(4.8)

Table.2 Isolation of *Aeromonas* spp. from different water sources

Sl. No.	Water Sources	Total Samples	Culture +ve	Culture –ve	<i>Aeromonas</i> spp.	<i>V. cholera</i>	<i>E. coli</i>
1.	Pond	40	20	20	15	1	3
2.	Well	37	18	19	10	3	5
3.	Lake	12	5	7	3	0	2
4.	Canal	11	5	6	2	1	1
	total	100	48	52	30(62.5)	5(10.4)	11(22.9)

Table.3 Total percentage of hemolysis and hlyA gene from stool and water samples.

Types of <i>Aeromonas</i>	Stool samples	Water samples	Hemolysis		<i>hlyA</i> gene	
			Stool	Water	Stool	Water
<i>A. hydrophyla</i>	11(44%)	17(56.7)	4	9	4	9
<i>A. caviae</i>	7(28%)	8(26.7)	2	6	2	6
<i>A. sobria</i>	7(28%)	5(16.7)	4	4	4	4
Total	25	30	10(40%)	19(63.3%)	10(40%)	19(63.3%)

Table.4 Resistance profiles (%) of *Aeromonas* spp. isolates from clinical and environmental samples.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
<i>A. hydrophyla</i>	S	100	100	20	80	80	60	60	80	80	80	80	80	80	80	40	100	100	100	60	80	100	80	80	40	100
	W	94.1	82.4	35.3	94.1	56.6	11.8	64.7	88.2	94.1	88.2	56.6	23.5	88.2	58.8	27.7	94.1	58.8	94.1	94.1	64.7	70.6	64.7	76.5	29.4	100
<i>A. caviae</i>	S	100	100	0	66.7	66.7	33.3	66.7	66.7	66.7	66.7	66.7	66.7	66.7	33.3	33.3	100	100	100	66.7	66.7	66.7	0	66.7	33.3	66.7
	W	83.3	100	0	83.3	66.7	0	33.3	66.7	83.3	66.7	0	0	66.7	50	0	100	0	100	0	50	83.3	66.7	83.3	50	88.3
<i>A. sobria</i>	S	100	66.7	0	66.7	66.7	0	0	66.7	66.7	66.7	66.7	0	66.7	33.3	33.3	100	33.3	100	0	66.7	0	0	0	33.3	100
	W	75	100	0	75	50	0	25	75	100	75	75	50	75	25	0	100	50	100	0	75	50	75	50	0	75

S: Rectal swab, W: Water samples;

A-piperacillin (Pc); B- kanamycin (K); C- gentamicin (G); D- bacitracin (B); E-rifampicin (R); F-trimethoprim (Tr); G-cefotaxime (CTX); H- erythromycin (E); I-sulfadiazine (SD); J-roxithromycin (RXT); K-lomefloxacin (LOM); L- azithromycin (AZM); M- ciprofloxacin (Cf); N- tetracycline (T), O- chloramphenicol (C); P- ampicillin (A); Q- nalidixic acid (Na); R- furazolidone (Fr), S- norfloxacin (Nx), T- cefuroxime (Cu); U- co-trimoxazole (Co); V- amikacin (Ak); W-doxycycline (Dx); X- streptomycin (S) and Y- amoxicillin (Am).

Fig.1 Detection of hly A(720bp) gene of *Aeromonas* isolates. Lane 1, 100bp ladder; Lane 2-3, *A. hydrophyla* strains ; Lane 4-5, *A. caviae* strains; Lane 6, *A. sobria* strains

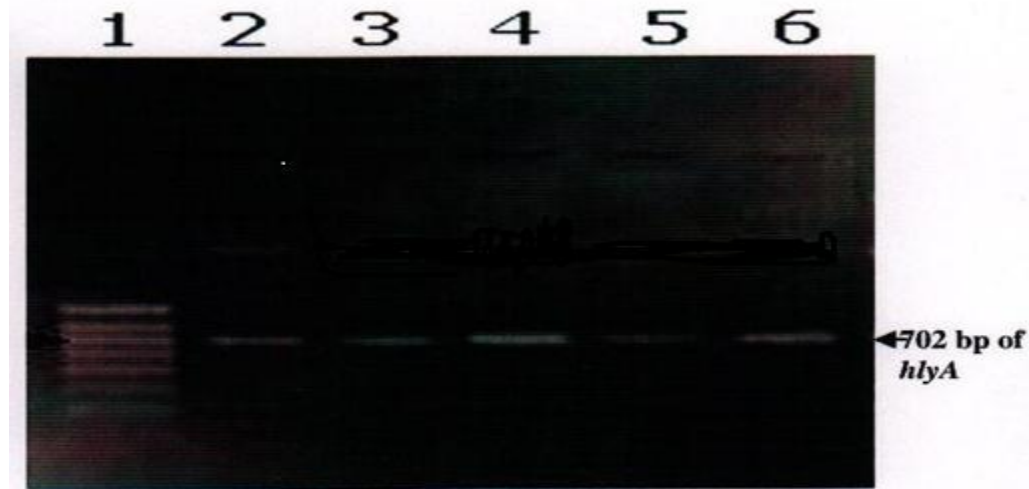
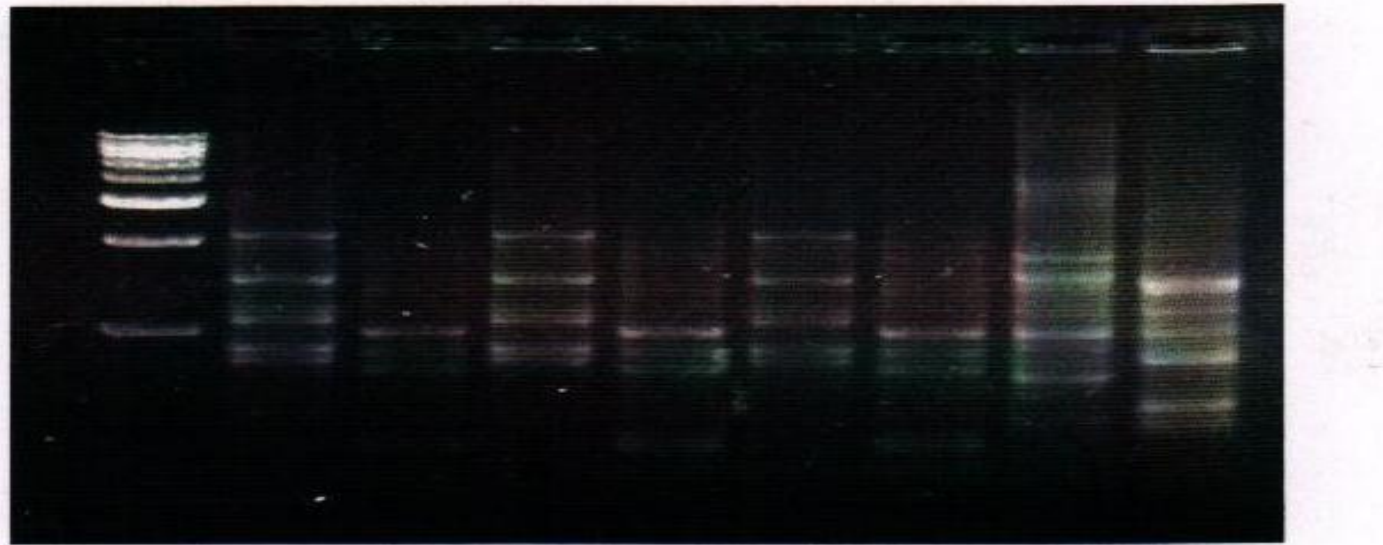


Fig.2 RAPD PCR of *Aeromonas* spp. using primer 1281. Lane 1, 1KB DNA ladder; Lane 2-3, *A. hydrophila* strains; Lane 6-7, *A. caviae* strains; Lane 8-9, *A. sobria* strains.



The present study demonstrated that *Aeromonas* spp. were associated with acute diarrhoea at least in 20.2% of patients. It is difficult to predict the importance of *Aeromonas* in 280 cases, as these patients were co-infected by other pathogens.

Out of 100 samples collected from water and 280 samples from diarrhoea patients, we found 56.7% *A. hydrophila*, 26.7% *A. caviae* and 16.7% *A. sobria* strains from environmental and 44% *A. hydrophila*, 28% *A. caviae* and 28% *A. sobria* strains from clinical samples respectively. The low prevalence of *A. hydrophila* suggested that it might have some etiological significance in the diarrhoea patients.

Examination of the water from the different sources suggested that the organism might have originated from water and that the gastrointestinal tract is a common habitat of human strains, as reported by other workers (Aldová *et al.*, 1966). Wilcox *et al.*, (1992) reported that the prevalence of 4.4% *Aeromonas* spp. in the faeces of pediatric inpatient, Salmonella- 2.2% and Rotavirus- 2.5%. In our study we reported 20.2% of *Aeromonas* spp., followed by other pathogens as 13.5% of *V. cholerae*, *E. coli* - 43.5%, *Shigella* spp. - 4.8%.

We found *A. hydrophila* as the dominant species causing diarrhoea in Odisha. A study from south India has revealed that *A. hydrophila* is the prominent species which co-relates our findings (Komathi *et al.*, 1998). In Bangladesh, *A. trota* was isolated from large number of diarrhoea patients (Albert *et al.*, 2000); however this species was not found in hospitalized diarrhoea cases in Odisha. Ørmen, *et al.*, (2001) revealed the existence of five different phenol species of *Aeromonas* in four springs of Norway as *A. hydrophila* (42%), *A. schobertii* (22%), *A. caviae* (19%), *A. sobria*

(16%) and *A. veronii* bio var *sobria* (1%). Sinha *et al.* (2004) from Kolkata reported that the *Aeromonas* strains revealed different virulence gene combinations by PCR assay.

The dominant combination of enterotoxin genes were *alt* (71.9%), *act* (20.1%) and *hyl A* (28%). The presence of the above three exotoxins in various combinations in different *Aeromonas* strains could increase or decrease the expansion of the specific exotoxin gene and thus dictate the severity of diarrhoea (Sha *et al.*, 2002). In our present study we found 52.7% of the *Aeromonas* strains showed *hyl A* gene; of which 44.8% of *A. hydrophila*, 27.6% of *A. caviae* and 27.6% of *A. sobria* strains respectively. The genetic similarity value of the strains of *Aeromonas* species varied from 0.308 to 1.0. The genetic similarity between the strains of *A. caviae* and *A. sobria* varied from 0.308 to 0.462. The RAPD PCR assay showed that *Aeromonas* strains were genetically heterogeneous and no particular clone was predominant in Odisha. Species of *Aeromonas* are capable of expressing a number of extracellular toxins and enzymes (Howard *et al.*, 1996). Early characterization of the toxins, however, resulted in confusion regarding their number and activities. Clinical and environmental isolates secrete many extracellular products such as hemolysin, enterotoxin, aerolysin, haemagglutinin. Among these toxins, aerolysin is released as a protoxin which is activated by protease released by the bacteria (Hilton *et al.*, 1991, Rodriguez *et al.*, 1992). Species of *Aeromonas* also produce a range of cell-surface and secreted proteases which probably enhance virulence (Howard *et al.*, 1996). Expression of virulence factors including haemolysins and proteases, by aeromonads has been shown to be influenced by environmental temperature (Eley *et al.*, 1993, Mateos *et al.*, 1993).

Despite the association of virulence factors with drinking-water aeromonads, there is increasing evidence that strains isolated from the environment generally belong to different groups of strains associated with gastroenteritis.

In conclusion, the present study clearly indicates that different *Aeromonas* spp. were one of the etiological agents of diarrhoea among hospitalized diarrhoea patients from Odishawhich is the first report from this state. The different *Aeromonas* species were harboring toxic gene like *hly* A and showed multiple drug resistance as observed from this study. So, this type of study should be extended in other parts of the state enrolling more number of water samples and diarrhoea patients together.

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