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Antibiotic Sensitivity and Characterization of Aeromonas Hydrophila Isolated from Water Samples

KEYWORDS: Aeromonas hydrophila, antibiotic sensitivity, aerolysin gene.

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ABSTRACT Total 40 Aeromonas hydrophila were isolated from water and sediment samples and 20 isolates were selected on the behalf of biochemical test for antibiotic sensitivity with nine antibiotics such as Ampicillin, Novobiocin, Nitrofurazone, Cephalothin, Gentamicin, Oxytetracycline, Ciprofloxacin, Chloramphenicol and Co-trimaxazole. All the isolates were resistant to Cephalothin, Ampicillin, Novobiocin and Nitrofurazone, and sensitive to Gentamicin, Co-trimaxazole, Chloramphenicol and Ciprofloxacin. Out of these, 24 isolates (60%) produced ♌-hemolysin (aerolysin) on sheep blood agar, whereas 40% of isolates produced ♋-hemolysin.

1. INTRODUCTION

Opportunistic and zoonotically important A. hydrophila is Gram negative, motile, rod shaped bacterium which is associated with hemorrhagic septicemia in cold-blooded animals, fishes, reptiles, amphibians (Austin and Austin, 1999). It also causes soft tissue infection and diarrhea in human (Barghouthi et al., 1989).

At present, most widely used methods of controlling motile aeromonads contamination water and sediment are the use of antimicrobial drugs. Because there is no suitable vaccine available to control such an economically important disease the appropriate antimicrobial therapy should be taken in consideration.

Most environmental and human isolates of motile aeromonads secrete extracellular enzymes such as proteases, DNase, RNase, elastase, lecithinase, amylase, lipase, gelatinase, chitinase (Pemberton et al., 1997) and cytotoxic/ cytolytic enterotoxins (Chopra et al., 1993) and three hemolysins (Hanes and Chandler, 1993). In this manuscript we have described the characterization by biochemical tests and antibiotic sensitivity used for genuine chemotherapy of A. hydrophila isolates. This study also demonstrates the phenotypic production of aerolysin (β-hemolysin) in A. hydrophila.

2. MATERIALS AND METHODS

2.1. Sampling

Water sample were collected from Allahabad sangam in a sterile tube, while the sediment samples from same site were collected in a sterile polythene bag and kept on ice.

2.2. Isolation and identification of *A. hydrophila*

Water (10 ml) and sediment 10 gm was mixed in 90 ml alkaline peptone water. Homogenates of these samples were serially diluted up to 10^{-6} in sterile normal saline (0.85% NaCl) solutions and 100 µl aliquots of each dilution were plated on Starch Ampicillin Agar (Ampicillin 10mg/L) (Palumbo et. al., 1985) using spread plate technique in duplicate. These plates were incubated at 37^{0}C overnight. Yellow and amylolytic colonies (Kidd and Pemberton, 2002), 2-5mm in diameter were subjected to biochemical assay as per the method of Barrow and Feltham (1992). Aero-key given by Joseph and Carnahan (1994) was used for identification of *A. hydrophila*

2.3. Antibiotic sensitivity

Antibiotic sensitivity test was done for the isolated cultures as per the method described by Bauer et al (1966). *A. hydrophila* was tested for sensitivity pattern against different antibiotics by disc diffusion method on Mueller Hinton agar. Zone of inhibition was recorded after 24 hrs of incubation at 37^{0}C when a lawn of bacteria was visible against a clear zone of inhibition around the discs. Nine antibiotics were selected for this study such as Ampicillin (10mcg), Novobiocin (30mcg), Nitrofurazone (100mcg), Cephalothin (30mcg), Gentamicin (10mcg), Oxytetracycline (30mcg), Ciprofloxacin (5mcg), Chloramphenicol (30mcg) and Co-trimaxazole (1.25/23.75mcg).

2.4. Hemolysin Assay

Isolates of *A. hydrophila* were tested for β hemolytic activity on Nutrient agar (Hi media) supplemented with 5% rabbit erythrocytes. The isolates were streaked on sheep blood agar plates and incubation at 37^{0}C for 24 h.

2.5. DNA isolation *A. hydrophila*

The isolation of genomic DNA from *A. hydrophila* was done as per the method described by Hiney et al (1992) with some modification. A single colony bacterium was inoculated in 5 ml of Nutrient broth (NB) medium and grown at 30°C for overnight. Well-grown culture was harvested by centrifuged at 10,000 rpm for 10 minutes at 4°C. The total 500 µl lysis buffer (100mM Tris pH 8.0, 10mM EDTA pH 8.0, 1.25% NaCl and 0.25%Sucrose) with 10µl proteinase K (10 mg/ ml) was added in the pellet and mix properly incubated at 37^{0}C for 2hrs. Equal amount of phenol: chloroform: isoamyl-alcohol (25:24:1) was added to cell lysate and mixed by the inversion of the tube properly. The suspension was centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to new tube. This step was repeated twice. Then 1/10 volume of sodium acetate (pH 7.0) and two volume of chilled

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ethanol was added to aqueous phase so as to precipitate the DNA and incubated at -20^{0}C for 30 minutes. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes at 20^{0}C. The pellet was washed with 1 ml of 70% ethanol and air dried and dissolved in 50 µl of TE buffer (pH 8.0) and stored at –20^{0}C for further study.

2.6. PCR conditions and amplification of Aerolysin gene

The PCR reaction mixture (50 µl) consists of 10 ng of bacterial genomic DNA, 1.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl. pH 8.3), 200 µM dNTP and 5 pmoles of each primer. Amplification includes initial denaturation at 94^{0}C for 3 min, followed by 30 cycles of denaturation at 94^{0}C for 30 sec, annealing of primers at 52^{0}C for 30 sec and extension at 72^{0}C for 30 sec. A final extension at 72^{0}C for 10 min was used. Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.2% agarose with ethidium bromide run at 8V/cm. The PCR products were visualized under Gel documentation system

3. RESULTS

A total of 40 isolates were identified as *A. hydrophila* by biochemical tests. All isolates of *A. hydrophila* exhibited similar biochemical patterns. All these isolates were Gram negative, motile, rod shaped and gave positive tests for oxidase, catalase, fermentative with gas, indole, arginine, lysine and aesculin.

A total of 20 *A. hydrophila* isolates were selected for the antibiotic sensitivity test. All the isolates were resistant to Cephalothin, Ampicillin, Novobiocin and Nitrofurazone, and sensitive to Gentamicin (80%), Co-trimaxazole (92%), Chloramphenicol and Ciprofloxacin (**Table 1**).

Hemolysin assays were performed for 40 isolates. Out of these, 24 isolates (60%) produced β-hemolysin (aerolysin) on sheep blood agar, whereas 40% of isolates produced β-hemolysin (**Fig. 1**).

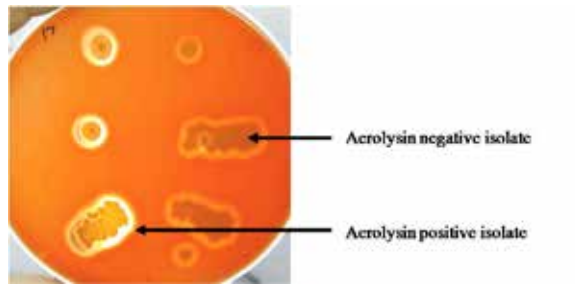

Fig. 5.1: In-vitro demonstration of aerolysin activity of A. hydrophila on 5% rabbit blood agar. Clear zone indicates the positive production of aerolysin and translucent zone indicates negative aerolysin producing A. hydrophila isolates.

Randomaly 10 bacteria were taken and amplified with reported primer of aerolysin gene the size is 326 bp. These PCR products were sequenced and blast and all are approximate 99% similar with aerolysin gene of *Aeromonas hydrophilla* which are already submitted in NCBI Genbank sequences (**Fig.2**).

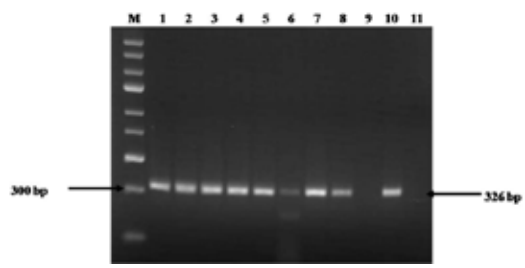

Fig. 5.2: PCR amplification of 326 bp aerolysin gene of A. hydrophila isolates. Lane M: Express DNA 100 bp ladder (Fermentas); Lane 1: AH14; Lane 2: AH15; Lane 3: AH16; Lane 4: AH17; Lane 5: AH20; Lane 6: AH23; Lane 7: AH24; Lane 8: AH25; Lane 9: AH MTCC 1739; Lane 10: AH GFG5 and Lane 11: without template (negative control).

Table 1. Antibiotic sensitivity of *A. hydrophila* isolates

Antibiotics along with concentration per disc used (sensitivity in mm)	**Isolates of *A. hydrophila* isolated from water and sediment samples**												
	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**	**A13**
Cephalothin 30µg (18)	R	R	R	R	R	R	R	R	R	R	R	R	R
Gentamicin 50µg **(15)**	R	26	31	24	25	R	32	28	23	24	27	22	R
Ciprofloxacin 10µg (21)	34	30	32	32	34	32	33	33	24	32	30	24	33
Novobiocin 30µg (22)	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline 30µg (19)	R	R	24	20	23	R	R	R	23	22	R	R	19
Co-trimaxazole 25µg (16)	16	R	19	18	20	17	19	21	R	22	20	22	17
Ampicillin 25µg (17)	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol 25µg (18)	29	19	24	24	30	28	18	19	21	25	27	24	30
Nitrofurazone 100µg (26)	R	R	R	R	R	R	R	R	R	R	R	R	R

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Table 1 (Continued)

Antibiotics along with concentration per disc used (sensitivity in mm)	**Isolates of *A. hydrophila* isolated from water and sediment samples**						
	A14	**A15**	**A16**	**A17**	**A18**	**A19**	**A20**
Cephalothin 30µg (18)	R	R	R	R	R	R	R
Gentamicin 50µg **(15)**	25	R	27	26	24	27	29
Ciprofloxacin 10µg (21)	32	36	35	26	25	31	32
Novobiocin 30µg (22)	R	R	R	R	R	R	R
Oxytetracycline 30µg (19)	23	23	R	R	20	R	21
Co-trimaxazole 25µg (16)	16	23	22	24	27	25	20
Ampicillin 25µg (17)	R	R	R	R	R	R	R
Chloramphenicol 25µg (18)	28	18	31	25	24	19	24
Nitrofurazone 100µg (26)	R	R	R	R	R	R	R

4. DISCUSSION

The sampled water did not show any turbidity but still tested positive for *A. hydrophila*. These water uses for washing and bathing for human beings and contribute to spreading these pathogens to newer areas. A total of 36 isolates of *A. hydrophila* were Gram negative, motile, rod shaped and gave positive tests for oxidase, catalase, fermentative with gas, indole, arginine, lysine and aesculin. *A. hydrophila* is known to produce both α-hemolysin and β-hemolysin *in vitro* (Ljungh et al., 1981). Out of 36 isolates tested for the presence of hemolysin, 25 isolates (60%) produced β-hemolysin (aerolysin) on sheep blood agar, whereas 40% of isolates produced α-hemolysin.

Yadav and Kumar (2000) studied *Aeromonas* from diarrheic children, fish, milk as well as ice creams and found 57% isolates producing enterotoxin.

A female (48 years) developed cellulitis, myonecrosis and sepsis after a prick wound in her hand. The bacteria have been isolated and confirmed as *A. hydrophila* (Adamski et al., 2006).

Kao et al (2003) reported the fatal bacteremic pneumonia caused by *A. hydrophila* in a healthy 5-year-old child. The source of infection was not determined and the child died within 4 hours. *A. hydrophila* has been reported as a pathogen of non-traumatic acute osteomyelitis in a cirrhotic patient. A case of liver cirrhosis with *A. hydrophila* infection also presented as acute gastroenteritis and non-traumatic acute osteomyelitis. It has been shown that *A. hydrophila* usually affects immunocompromised subjects such as those with liver cirrhosis (Lee et al., 2003).

Saeki et al (2002) isolated *A. hydrophila* from blood and bilious fluid in female with liver cirrhosis. Minnaganti et al (2000) attributed to *Aeromonas* for diarrhea and soft tissue infections. *A. hydrophila* causes meningitis, endocarditis, peritonitis, hemolytic-uremic syndrome and septicemia in immunocompromised humans.

Widespread antibiotic use has resulted in the rapid spreading of multi- drug resistant pathogens. It is clear that resistance to commonly used antibiotics is emerging rapidly among *Aeromonas* isolates. The apparent resistance of *A. hydrophila* to antibiotics may be a result of the indiscriminate or sub-therapeutic use of the anti-microbials. The present study reports the limited usefulness of Oxytetracycline for the control of *Aeromonas* infections. Although all isolates were sensitive to Chloramphenicol (30mcg), Chloramphenicol is reported to be hazardous to humans causing idiosyncratic and aplastic anemia. It must be remembered that widespread use of antimicrobial is not a substitute for efficient management practice. If possible, alternate methods of disease control (vaccination) should be used to reduce antimicrobial use.

In conclusion, we were able to demonstrate that the toxin aerolysin, which is one the major virulence factor of A. hydrophila, is present in approximately 60% of the isolates from water and sediment samples. Increase in antibiotic resistance level of A. hydrophila may pose a risk for human health.

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