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SEQUESTRATION OF *AEROMONAS HYDROPHILA* FOR SERINE PROTEASE GENE DETECTION FROM DIARRHOEAL SAMPLES THROUGH PCR

Sasikumar.C *[1], Sundari.B[2], Mohana.S.J[3]

[1]Department of General Engineering, Jeppiaar Institute of Technology*, Chennai, India.
[2] Department of Biotechnology,A.V.C College, Mayiladuthurai, India
[3] Department of Plant Biotechnology, Gurunanak College, Velachery, Chennai, India.

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Corresponding Author:
C.Sasikumar,
Asst.Professor,
Department of General Engineering,
Jeppiaar Institute of Technology,
Chennai, India.
sasikumarc@jeppiaarinstitute.org

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ABSTRACT

Statement of the problem: Diarrhoea disease is an significant cause of morbidity and mortality in developing countries, predominantly in newborns and elders. Diarrhoea is caused by viral, bacterial, and parasitic taints, as well as food bigotries, response to medicines, and other physiological and immunological disorders (Tianyan song et al., 2004).In the last decades, *Aeromonas* have been progressively acknowledged as relevant etiological agents in gastrointestinal taints, as well as extraintestinal taints, septicemia, Urinary tract taints. **Aim:** To identify the serine protease gene from *Aeromonas hydrophila* from diarrheal sample by using PCR method **Methodology:***Aeromonas hydrophila* is impervious to chlorine, freezing or cold temperatures. *Aeromonas hydrophila* can be ingested through fodder products that have already been crawling with the bacterium, such as sea food, meats and even certain vegetables such as sprouts. The virulence of *Aeromonas* is multi factorial, including adhesions, S-layer, lipopolysaccharides, siderophores and an assortment of exoenzymes and exotoxins, i.e., aerolysin/haemolysin, lipases and proteases.. Strains producing S-layer are more pathogenic for fish, but the role of S-layer in human taints is not clear.Serine protease (22 KDa) was stable at 56^{0}C for 10 minutes, possessed cytotoxic activity and had an LD 50 of 150ng/g fish.) (Rodriguez et al., 2004). Polymerase chain reaction is a sensitive and specific method for identification of virulence gene. **Inference:** Hence for the direct detection of pathogenic *Aeromonas* species isolates, virulence determinants are used as a genetic marker. Thus PCR method is used to spot the contagious gene encoding serine protease using precise primers.

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INTRODUCTION

In developing countries, Diarrhoea disease is an important cause of morbidity and mortality, particularly in newborns and elders. Diarrhea is caused by viral, bacterial, and parasitic taints, as well as food intolerances, reaction to medications, and other physiological and immunological disarrays (Tianyan song et al., 2004). Bacterial taints are responsible for 20-40% of diarrhea ailment, and numerous bacterial species have been frequently ascribed to diarrhea occurrences, including *Campylobacter jejuni, Escherichia coli, Salmonella spp., Vibrio cholera, Yersinia enterocolitica, Aeromonas species*., and *Plesiomonas species.*

In the last decades, *Aeromonas* have been increasingly recognized as relevant etiological agents in gastrointestinal taints, as well as extraintestinal taints, septicemia, Urinary tract taints. Members of genus *Aeromonas* are Gram negative, catalase and oxidase positive facultative anaerobic bacilli (Seetha *et al.*, 2004). The family *Aeromonadaceae* includes over 14 species in the genus *Aeromonas* (Carnahan *et al.*, 1991) with only 6 species being presently acknowledged as human pathogens. Different species of *Aeromonas* are *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii,* Aeromonas *salmonicida, Aeromonas sobria, Aeromonas jandaei,*

Among this *Aeromonas hydrophila, Aeromonas caviae and Aeromonas veronii* biotype sobria are considered of clinical significance (Tianyanson et al., 2004).The *Aeromonas hydrophila* isolated from diarrhoeal sample is a Gram negative rod that has polar flagella, hence it is motile, and it is a facultative anaerobe. It is also oxidase positive and glucose fermenting (Pollard et al., 1990). The bacterium is a straight rod with rounded ends (bacilli to coccibacilli shape) usually from 1 to 3.5μm length and 0.3-1μm in width and has optimal growth at 28^{0}C but can also grow at the extremes from 4^{0}C to 37^{0}C (Pollard et al., 1990). *Aeromonas hydrophila* can digest materials such as gelatin and haemoglobin. It is also very hard to destroy, because it is very enduring bacterium. *Aeromonas*

hydrophila is resistant to chlorine, refrigeration or cold temperatures.

Aeromonas species are Gram negative facultative anaerobic bacteria that can be isolated from many sources such as food, drinking water, sewage, environmental water and human clinical specimens (Janda and Abott , 1998)

Aeromonas hydrophila can be ingested through food products that have already been infected with the bacterium, such as sea food, meats and even certain vegetables such as sprouts. Substantial evidence points to Aeromonads as causative agents of sporadic diarrhea, dysentery and extra intestinal taints that may be life-threatening (Janda & Abott, 1998).The degree of pathogenicity of *Aeromonas* is multi factorial, including adhesions, S-layer, lipopolysaccharides, siderophores and an group of exoenzymes and exotoxins, i.e., aerolysin/haemolysin, lipases and proteases.

Aeromonas produce an array of filamentous structure, including short rigid and long wavy pilli, polar and lateral flagella. Short rigid pilli are similar to those of Escherichia coli type I pilli, while long wavy pilli belongs to class of type II bundle developing pilli (BFP) (Kirov & Sanderson, 1996).

Removal of pilli or neutralization of attachment sites by homologous antibody action limits or defeats adherence properties in all culture systems (Iwanaga & Hokama, 1992; Kirov et al., 1999).Tap pilli were distinct pilli. It has a molecular mass of 17 KDa. It facilitates attachment to enterocytes and enhances colonization. While BFP have a molecular mass of 19-23 KDa. It facilitates adherence to erythrocytes (Kirov et al., 2009). Polar flagellum helps swimming motility in liquid while lateral flagella in solid matrix.

Capsule production has been reported in *Aeromonas hydrophila* (Martinez et al., 1995). But the function of capsule material is vague. It is presumed to repel complement bustle and perhaps improve adherence (Merino et al., 1996). S-layer is a paracrystalline structure made up of identical protein subunits that are translated across the cell membrane and assemble on the cellwall surface via a group effort with O-polysaccharide. Strains producing S-layer are more pathogenic for fish, but the role of S-layer in human taints is not clear.

Aeromonas hydrophila is able to produce various extracellular toxins or enzymes such as haemolysins (Lallier et al., 1984) cytotoxin (Dontal & Haddow, 1978), Enteroxin (Chakraborty et al.,1984) and protease (Denis & Viellet 1984).

It has been suggested that proteolytic enzymes produced by *Aeromonas* spp. play an important role in invasiveness and establishment of taints by overcoming initial host defenses and by providing nutrients for cell proliferation (Hsu et al.,1986; Leung 1988).

There are many reports describing the number and nature of proteases found in culture supernatants of *Aeromonas hydrophila*. Some of them have reported finding the temperature stable metalloprotease (TSMP) (Allan et al.,1981) Other reports have described the temperature stable metalloprotease (TSMP) to be one of the two proteases formed, the other being temperature-labile serine protease. In one study it was examined that 47 *Aeromonas hydrophila* strains and 29 other *Aeromonas* for protease production under uniform cultural conditions. They found three distinct types of proteases in extracellular yields, a temperature labile serine protease (Octavio Rivero etal.,1990).

Aeromonas hydrophila is not as pathogenic to humans as it is fish and amphibians. One of the diseases it can cause in human is gastroenteritis. This disease can disturb anyone, but it ensues most in fledgling children and people who have compromised immune systems or growth complications. This bacterium is linked to two types of gastroenteritis.

Serine protease (22 KDa) was stable at 56^{0}C for 10 minutes, possessed cytotoxic activity and had an LD 50 of 150ng/g fish.) (Rodriguez et al., 2004). Serine protease or serine endopeptidases are proteases (enzymes that cut peptide bonds in proteins) in which one of the aminoacids at the active site is serine. Serine protease is known to activate toxins such as aerolysin and Glycerophospholipid Cholestrol acyl transferase (GCAT). This process is called quorum sensing (Whitby et al., 1992).

. Glycerophospholipid cholesterol acyl transferase (GCAT) and Aerolysin both are activated by serine protease.

Polymerase chain reaction is a sensitive and specific method for identification of virulence gene. Hence for the direct detection of pathogenic *Aeromonas* species isolates, virulence determinants are used as a genetic marker. Thus PCR method is used to detect the virulent gene encoding serine protease using specific primers.

METHODOLOGY

a) Sample Collection

A total of 35 stool samples were collected from all age groups of people who has affected with diarrhea and admitted in the hospitals in Chennai. The information was obtained from each patient regarding age sex and duration of disease. The age of the patients ranges from 1-42 years. The density of *Aeromonas* in the diarrhoeal sample is less and in order to enrich the organism, the diarrhoeal samples were inoculated in alkaline peptone water, 1 ml of diarrhoeal samples were inoculated in 9 ml of Alkaline peptone water (Rall et al., 1998). The inoculated tubes were incubated at 37°C for 18 hours.

b)Composition of Alkaline Peptone Water

Peptone -10 g
Sodium chloride -10 g
Distilled water -1000ml
pH -8.4

After incubation at 37°C for 18 hours in alkaline peptone water, 1 ml of the diarrhoeal sample is taken and serially diluted. From different dilutions .0.1ml of the diluted sample is transferred in Starch Ampicillin Agar plate (SAA)(Palumbo et al.,1985) and spreaded evenly by using the L-rod (spread plate technique).The inoculated SAA plates were incubated at 37°C for 24 hours. Yellow to honey coloured 3-5 mm diameter sized, amylase positive colonies (those having a clean zone surrounding the colonies) were scored as presumptive *Aeromonas hydrophila* (Palumbo et al., 1985) and were subcultured into the nutrient agar slants and stored at 4°C for further studies. The pure cultures were presumptively identified as *Aeromonas hydrophila* using the Kaper's multitest medium (Kapers et al., 1979). Appearance of alkaline slant and acid butt after 24 hours incubation at 37 ^{o}C was considered presumptive *Aeromonas hydrophila.*

c) Composition of Starch Ampicillin Agar

Peptone - 0.1g
Sodium chloride - 1.5 g
Beef extract - 0.1g

Starch - 1g
Agar - 2 g
Ampicillin - 100µl
Phenol red - 0.0024 g
Distilled water - 100 ml
pH - 8

d) Composition of Kapers Multi Test Medium

Peptone - 5 g
Yeast extract - 3 g
L-ornithine hydrochloride - 5 g
Mannitol - 1 g
Inositol - 10 g
Sodium thiosulphate - 0.4 g
Ferric ammonium citrate - 0.5 g
Agar - 20 g
Bromocresol purple - 0.02 g
Distilled water - 1000 ml
pH - 6.7

e) Presumptive Test for Identification of *Aeromonas hydrophila.*

Gram Staining

i) Material Required

Crystal violet
Crystal violet : 2 g
Ethyl alcohol : 120 ml
Ammonium oxalate : 0.8 g
Grams iodine
Potassium iodide : 2 g
Iodine : 1 g
Distilled water : 300 ml
Decolouriser (95% ethanol)
1% safranine
Safranine :1 g
Distilled water : 100 ml

ii) Procedure

A thin smear of the isolated culture was made in a clean glass slide. The smear is air dried and then heat fixed. Crystal violet was added on the smear and it was allowed to stain for 1 minute. The slide was washed and Grams iodine solution was added over the smear and allowed to stain for 1 minute. The slide was washed and absolute alcohol (decolouriser) was added drop by drop till the smear was completely decolourised. The slide was immediately washed after decolourisation. The safranine solution was auxiliary and acceptable to stain was 1 minute. Then the smear was washed in tap water, air dried, and viewed in light microscope.

f) Motility Test

Hanging Drop Method

One drop of broth culture of isolated culture was placed on a clean cover slip. The Vaseline was pasted to the four corners of the cover slip the cavity slide was placed on the cover lip so that the cavities of the slide do not disturb the drop that was placed on the cover slip. The set up was turned upside down so that the drop containing the culture hangs through the cover slip. The edge of the drop was focused in the microscope under 45X objective. Motile organism move actively towards the edge of the drop.

g)Catalase Test

i) Material Required

3% Hydrogen peroxide
24 hours broth culture of isolated culture

ii) Procedure

One drop of the culture broth was kept over the slide one drop of the hydrogen peroxide solution was added to the culture drop. Effervescence or bubbles obtained indicates that the organisms produce catalase and hence it was catalase positive.

h) Oxidase Test

i) Material Required

- N N tetra methyl paraphenylene diamine dihydrochloride impregnated filter paper.
- Isolated culture

ii) Procedure

With the help of a clean glass rod or plastic applicator stick a colony from the culture isolated was picked and rubbed over the filter paper. The colour change (purple) was observed which indicated that the isolated organism was oxidase positive.

i)Identification of *Aeromonas hydrophila* by Biochemical Test

Indole Test

i) Material Required

Tryptone broth
Peptone - 0.5 g
Sodium chloride - 0.5 g
Distilled water - 50 ml
24 hours broth culture of isolated *Aeromonas hydrophila*
Kovac's reagent
Paradimethyl aminobenzaldehyde - 5 g
Amyl alcohol - 75 ml
Concentrated hydrochloric acid - 25 ml

ii) Procedure

The isolated culture was inoculated in tryptone broth and incubated at 37^{0}C for 24 hours. After incubation 200 µl Kovacs reagent was added. The formation of red ring indicates indole positive.

j) Methyl Red and Voges Proskauer Test

i) Material Required

MR VP broth
Peptone - 0.35 g
Dextrose - 0.25 g
Dihydrogen potassium phosphate - 0.25 g
Sodium chloride - 0.25 g
Distilled water - 50 ml
24 hours broth culture of isolated *Aeromonas hydrophila*
Methyl red reagent
Methyl red - 0.1 g
Ethyl alcohol - 300 ml
Distilled water - 200 ml
VP reagent A
Alpha naphthol - 5 g
Ethyl alcohol - 100 ml
VP reagent B
Potassium hydroxide - 40 g
Distilled water - 100 ml

ii) Procedure

The organisms were inoculated into MR/VP broth and incubated at 37^{0}C for 24 hours. The broth was divided into two equal halves and to one 200 µl of MR reagent was added and to the other half 200 µl of VP reagent A was added and allowed to react for 5 minutes and then 200 µl of VP reagent B was added. Red colour formation in both the tubes indicated the isolated culture was MR VP positive.

k)CITRATE TEST

Citrate media

i) Material Required

Sodium chloride - 5 g
Magnesium sulphate - 0.2 g
Ammonium dihydrogen phosphate - 0.1 g

Dipotassium phosphate - 0.1 g
24 hours broth culture of isolated *Aeromonas hydrophila*

ii) Procedure

The isolated organism was inoculated and incubated at 37^{0}C for 24 hours. Change of green coloured citrate media into deep blue colour indicated that the organism is citrate positive.

l) Triple Sugar Ion Agar Test

i) Material Required

TSI agar slant
24 hour isolated culture

ii) Procedure

The isolated organism was inoculated in TSI agar slants and incubated at 37^{0}C for 24 hours. Alkaline slant and acid butt with gas production was observed.

m) Urease Test

Cristensen's urea Agar

i) Material Required

Peptone - 0.1 g
Glucose - 0.1 g
Sodium chloride - 0.5 g
Mono potassium phosphate - 0.2 g
Phenol red (1.2) - 1 ml
Agar - 2 g
pH - 6.8
24 hours broth culture of isolated *Aeromonas hydrophila*

ii) Procedure

The isolated organism were inoculated in the urea agar slant and incubated at 37^{0}C for 24 hours. No colour change indicated that the isolate culture was urease negative organism.

n) LAO Test

i) Material Required

Lysine decorboxylase medium

Peptone -1 g
Sodium chloride - 5 g
Potassium bi phosphate - 3 g
pH - 6.8
Bromocresol purple - 0.016 g
Lysine -1 g
Distilled water - 1000 ml

Arginine dehydrogenase medium

Peptone -1 g
Sodium chloride - 5 g
Potassium bi phosphate - 3 g
pH - 6.8
Bromocresol purple - 0.016 g
Arginine -1 g
Distilled water - 1000 ml

Ornithine decarboxylase medium

Peptone -1 g
Sodium chloride - 5 g
Potassium bi phosphate - 3 g
pH - 6.8
Bromocresol purple - 0.016 g
ornithine -1 g
Distilled water - 1000 ml
24 hours broth culture of isolated *Aeromonas hydrophila*

ii) Procedure

Isolated culture was inoculated on LAO slants and incubated at 37^{0}C for 24 hours. Purple colour indicates positive reaction. No colour change indicated negative reaction. This is one of the confirmatory test for isolation and identification of *Aeromonas hydrophila*.

o)Sugar Fermentation Test:

Organisms utilize various sugars for the growth and carry out sugar fermentation. Sugar fermentation can be visualized by the color change in the indicator bromocresol purple because of acid production. Gas production is visualized by bubble formation inside the Durham's tube.

90 ml of sterile peptone water was prepared and 1 gram of sugar was weighed and dissolved in 10 ml of distilled water. This 10 ml of sugar solutions was added to 90 ml of prepared peptone water. 0.1 ml of bromocresol purple indicator dye was added.

This sugar solution was dispensed in tubes and Durham's tubes were positioned and then sanitized and incubate the tubes at 37ºC for 24 hours. Both acid and gas production were noticed. Realization of yellow color, acid production, gas formation in Durham's tube indicated the positive result and no change in the tube indicated the negative result.

Base:

Peptone : 1.0 g
Beef extract : 1.0 g
Sodium chloride : 0.5 g
Distilled water : 100 ml

Carbohydrate solution:

Carbohydrate : 10.0 g
Distilled water : 100 ml

Indicator:

Bromocresol purple : 1.6 g
Ethanol : 100 ml

p)Determination of Protease Activity in *Aeromonas hydrophila*

i) Material Required

Milk agar

Nutrient agar - 2.8 g
Agar - 2 g
Distilled water - 70 ml
The nutrient agar was sterilized in autoclave.
Skimmed milk powder - 1.5 g
Distilled water - 30 ml

Dissolve the skimmed milk powder by boiling.Mix the skimmed milk preparation in nutrient agar while it is in molten stage (60˚C) and poured in agar plates.

ii) Procedure

Inoculate the isolated *Aeromonas hydrophila* culture on Milk agar plate and incubated at 37ºC for 24 – 48 hours. The organism produces protease enzyme so that casein a protein in the milk agar is hydrolysed and zone formation can be seen.

q) Isolation of *Aeromonas hydrophila* Genomic DNA

The stored isolated cultures were inoculated in the fresh Brain heart infusion broth (BHI) which provides the rich turbid growth. The inoculated broth was incubated at 37ºC for 24 – 48 hours. Brain heart infusion broth (BHI) is used for the extraction of the genomic DNA *Aeromonas hydrophila.*

i)Brain Heart infusion broth

Calf brain infusion : 200 gm
Beef heart infusion : 250 gm
Protease peptone : 10gm
Dextrose : 2gm
Disodium phosphate : 250 gm
NaCl : 5gm
Distilled water : 1000 m

ii)DNA Isolation Method

1. Solution A

- TE buffer

Tris : 10.8g
EDTA : 0.92g
Distilled.Water : 1000 mL
pH : 8.0

- 10% SDS

2. Solution B
   Phenol: Chloroform:Isoamyl alcohol (25:24:1)
3. Isopropanol
4. 70% Ethanol
5. TBE buffer
   Tris : 10.8g
   Boric acid : 5.5g
   EDTA : 0.92g
   Distilled.Water : 1000 mL
   pH : 8.3
6. Agarose
7. Gel loading buffer
8. Control DNA

iii) Procedure:

The sub cultures *Aeromonas hydrophila* species in Brain heart infusion broth (BHI) was taken in the centrifuge tube and it was centrifuged at 10000 rpm for 10 minutes.

1. The supernatant was discarded and the pellet obtained after centrifugation is used for DNA isolation.
2. The pellet was then suspended in 300 µl of solution A at room temperature. The solution was vortexed completely.
3. It was then incubated at 60ºC for 20 minutes and then solution B was added, and then solution is completely (vortexing). It was centrifuged at 10,000 rpm for 10 minutes.
4. About 500 µl of the aqueous supernatant solution was collected and add to that equal volume of isopropanol was added into the fresh vials then they are mixed by inverting then the vials where kept for centrifugation at 10,000 rpm for 10 minutes.
5. About 200 µl of ethyl alcohol was added and mixed than by inverting the tube till the white strands of DNA precipitation were seen. It was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded.
6. Then the alcohol was decanted without dislodging the pellet, it was completely air dried to remove the ethyl alcohol smell from the vials.
7. To the final pellet about 20 µl of TE buffer was added and mixed completely by tapping the tube, till the solution settle at the bottom.
8. The isolated DNA was separated and visualized with the help of agarose gel electrophoresis and viewed in the UV transilluminator.

r) Gel Electrophoresis (Sambrook *et al.,* 1989):

Agarose gel electrophoresis is the method used to separate macromolecules based on charge, size and shape. Agarose gel is polysaccharide derivative of agar. The saving property of the gel influence the rate at which a molecule migrates. Smaller molecules move through the pores more easily than larger ones. To, carry out the electrophoresis, samples were placed in an electric field. The field causes the molecules to move negatively charged molecules migrate in the direction of the positive electrode (anode) and positively stimulating ones migrate towards the negative (cathode). As DNA is negatively charged, it moved towards the positive electrode.

i)Procedure:

i) 1% agarose was prepared and dissolved in 1 x TBE buffer.
ii) After cooling 2 µl Ethidium bromide was added in dissolved agarose.
iii) Prepared agarose was poured on to gel boat, wells can made by using the comb.
iv) 20 µl of digested DNA was loaded along with 3 µl of gel loading buffer and Electrophoresis was performed.

s) PCR Detection of Serine Protease Gene (Kiseong *et al.*, 2007)

i) Procedure:

The primer selected for the PCR detection had the following sequence:

PRIMER I: Ser-P 5'-CCG TTC ATC ACA CCG TTG TAG TCG -3' – Serine protease.

PRIMER II: Ser-F 5'-ACG GAG TGC GTT CTT CCA ACT CCA G -3'_Serine protease (**Kiseong et al., 2007**)

1. The mix was prepared in thin walled PCR tubes in a sterile condition. The mixture essentially consists of

Template DNA - 2 µl
Taq buffer with Mg cl_2 - 2µl
Taq DNA polymerase - 0.5µl
Primer I - 1µl
Primer II - 1µl
dNTPs - 1 µl
Molecular grade water - 12.5 µl
Total - 20 µl

2. A brief spin was made to settle down the materials.
3. The tubes were placed on the thermal cycler.
4. PCR was carried out by adjusting the following condition.

Step 1: initial denaturation - 95˚C for 5 minutes
Step 2: denaturation - 94˚C for 1 minute
Step 3: annealing - 64˚C for 30 seconds
Step 4: extension - 72˚C for 1 minute
Step 5: repeated step 2 for 30 cycles
Step 6: final extension - 72˚C for 5 minutes
Step 7: end

5. After the reaction 5µl of loading dye was mixed amplify products and mixed it and load the total 20µl into agarose gel for electrophoresis.

RESULTS AND DISCUSSION

Total of 35 samples were collected to find out the prevalence of *Aeromonas hydrophila.* Out of 35, 30 samples were found to be positive for *Aeromonas hydrophila* and its details were given in table 1 and the biochemical results were given in table 2.

Table:1 Patient Data (Positive result)

Sl No	Patient	Age	Sex	Duration
1	1	8	F	2
2	2	3	F	3
3	3	2	M	4
4	4	10	F	3
5	5	11	F	2
6	6	32	F	7
7	7	13	F	1
8	8	16	M	2
9	9	14	M	3
10	10	11	F	4
11	11	15	F	3
12	12	17	M	3
13	13	26	F	2
14	14	11	M	1
15	15	42	F	3
16	16	10	F	2
17	17	9	F	3
18	18	8	F	4
19	19	7	M	5
20	20	5	M	3
21	21	40	F	2
22	22	13	F	1
23	23	12	M	1
24	24	3	F	3
25	25	9	M	2
26	26	2	F	4
27	27	17	F	5
28	28	30	F	5
29	29	33	F	6
30	30	43	F	7

Table 2 : Identification and Biochemical Analysis of *Aeromonas hydrophila*.

SL. NO	PRELIMINARY TESTS	RESULTS
1	Starch ampicillin agar medium	Yellow color colonies
2	Gram staining	Gram negative
3	Motility	Motile
4	Catalase	positive
5	oxidase	Positive
SL. NO	CONFIRMATION TESTS	RESULTS
6	Kaper's Multi test	Positive
SL. NO	BIOCHEMICAL TESTS	RESULTS
7	Indole test	Positive
8	Methyl Red test	Positive
9	Voges Proskauer test	Positive
10	Urease test	Negative
11	Citrate utilization	Positive
12	Lysine Decarboxylase	Positive
13	Arginine Dihydrolase	Positive
14	Ornithine Decarboxylase	Negative
15	Triple Sugar Iron	Positive
SL. NO	SUGARS	RESULTS
16	Glucose	Acid Gas
17	Sucrose	Acid Gas
18	Mannitol	Acid Gas
19	Maltose	Acid Gas
20	Lactose	Negative

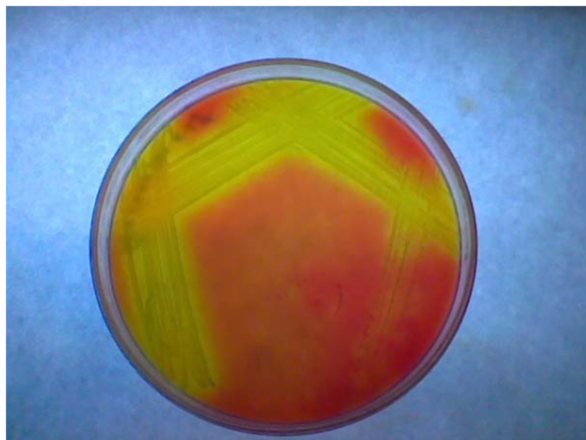


Figure. 1. Starch Ampicillin Agar plate

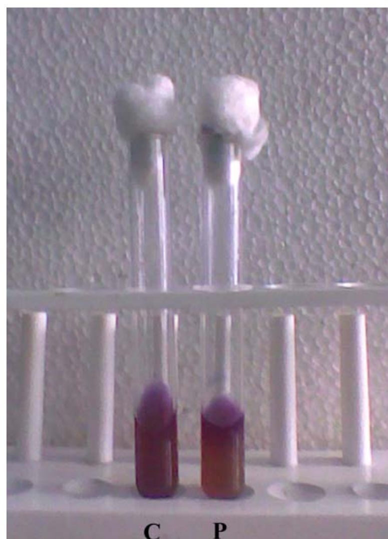


Figure. 2. Kaper's Test

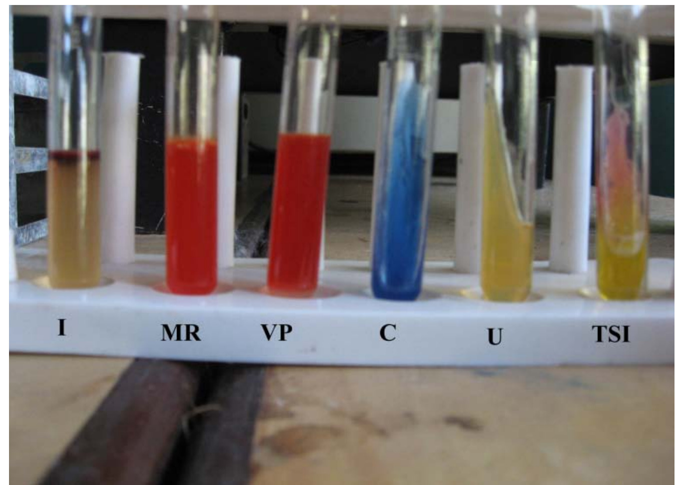


Figure. 3. Biochemical Test

Figure. 4 . LAO Test

PROTEASE ACTIVITY OF *Aeromonas hydrophila*

Out of 30 isolates of *Aeromonas hydrophila* 14 isolates were evaluated for protease production using Milk agar plate and the results were shown in the table 3. The presence of protease was determined by the formation of zone around the colonies

Table 3 : RESULTS FOR PROTEASE ACTIVITY

SL .NO	AEROMONAS ISOLATES	PROTEASE ACTIVITY
		QUALITATIVE ANALYSIS
1	Ah 1	+++
2	Ah 2	++
3	Ah 3	++
4	Ah 4	++
5	Ah 5	++
6	Ah 6	+++
7	Ah 7	++
8	Ah 8	+
9	Ah 9	++
10	Ah 10	+++
11	Ah 11	+
12	Ah 12	++
13	Ah 13	++
14	Ah 14	+++

PREVALENCE OF *Aeromonas hydrophila*.

Prevalence of *Aeromonas hydrophila* according to patient's sex, age and duration of diarrhoea :

Out of 35 diarrheal samples, 30 samples shows the presence of *Aeromonas* species and their details are given in table 1. The overall % incidence was found to be 85.71%.

Among 30 isolates, 30% and 70% cases were isolated from males and females respectively.

From the study it was found that the prevalence level was not detected in the age group of 21-25 years (Table 4). The results clearly showed maximum prevalence level in the age group of 11 to 15 years for males and falls in the range of 6-10 and 11-15 for females (Table 5 & 6).

Table 4: Age-wise % incidence

Age range	Number of samples	% incidence
0 1-05	5	17
06-10	7	24
11-15	8	27
16-20	3	10
21-25	0	0
26-30	2	7
31-35	2	7
35-40	1	4
41-45	2	7

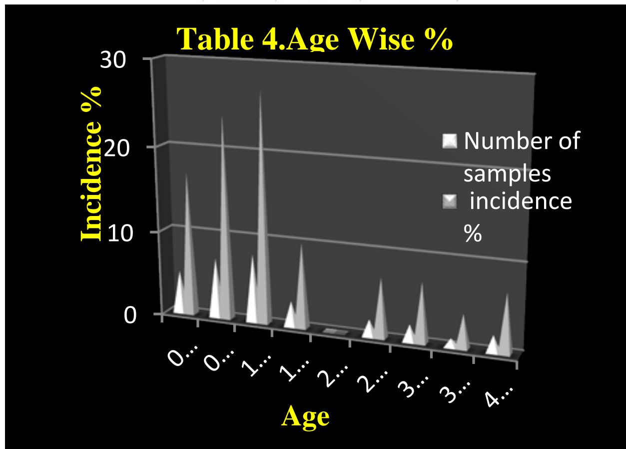


Table 5: Male age-wise % incidence

Age range	Number of samples	% incidence
01-5	2	22.22
06-10	2	22.22
11-15	3	33.33
16-20	2	22.22

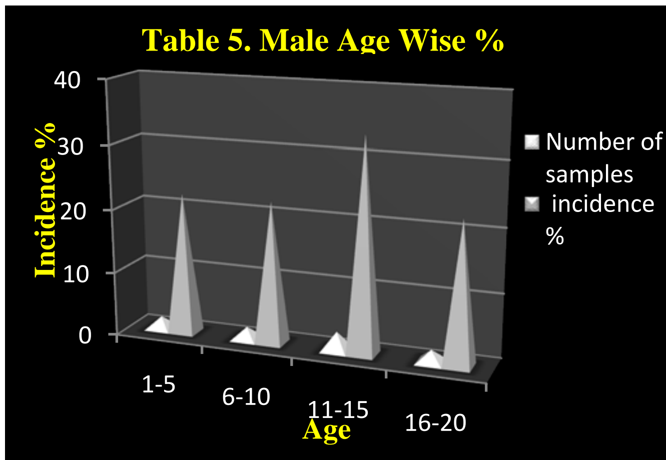
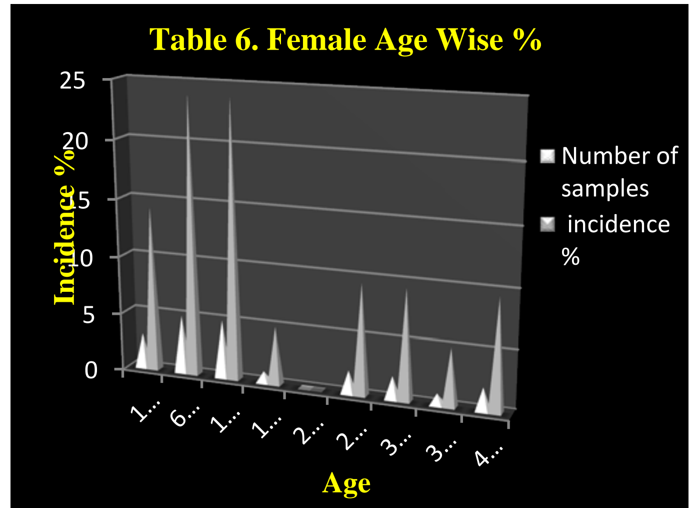


Table 6: Female age-wise % incidence

Age range	Number of samples	% incidence
01-5	3	14.28
6-10	5	24
11-15	5	24
16-20	1	5
21-25	0	0
26-30	2	9.52
31-35	2	9.52
35-40	1	5
41-45	2	9.52



PCR DETECTION OF SERINE PROTEASE GENE IN THE GENOME OF *Aeromonas hydrophila*

PCR technique clearly identified the virulent gene as serine protease gene in *Aeromonas hydrophila.* The genomic DNA of the samples was extracted and bands were observed by performing agarose gel electrophoresis.

The isolated genomic DNA was used as template in the PCR study. From the 30 samples 12 samples were selected based on the protease activity results and PCR was performed in Thermocycler (MJ Research PTC-150 minicycler) using PCR conditions.

When PCR was performed, results clearly indicate that all isolated organisms contained serine protease gene. All the amplified products produced a band at the level of 211 bp. Young and Kiseong (2007) studied the serine protease and reported the PCR amplified band at 211 bp. The results of the present study also confirm the same (Young et al., 2007).

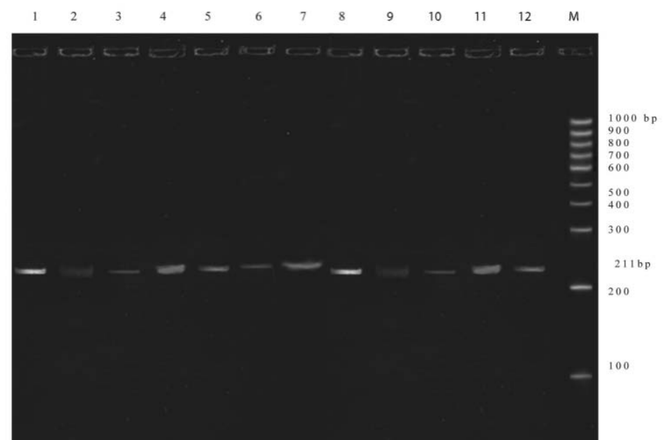


Figure.5.PCR Detection of Serine Protease (Ser gene) in *Aeromonas hydrophila*

CONCLUSION

The diarrheal samples were collected from different patients and inoculated into different test media and biochemical tests were performed.30 samples collected from different patients were studied. The genomic DNA of *Aeromonas hydrophila* was isolated and then PCR was performed to detect the serine protease gene.

PCR detection may prove to be an important tool for the detection, identification, differentiation, and distribution of virulence markers. This method will give microbiologists an substitute way to understand

pathogenicity in *Aeromonas* spp. and their distribution in different sources of diarrhoeal sample.

The present report describes PCR method that detects serine protease genes in *Aeromonas hydrophila* by using a pair of primers for a serine protease gene the most known virulence gene of *Aeromonas hydrophila*.

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