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ORIGINAL RESEARCH

Detection of Hemolytic Activity of *Aeromonas* sp Isolated from Water Samples of Coastal Area, Kochi

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ABSTRACT
Aeromonas is a Gram negative rod shaped bacteria that causes gastroenteritis, wound infections and sometimes respiratory diseases. They are frequently isolated from fishes and from water. The aim of this study is to isolate and identify *Aeromonas* sp from different water samples in and around Kochi. Out 16 different water samples collected of which 7 showed positive for presence of *Aeromonas sp.* Starch ampicillin agar was used for *Aeromonas* isolation and the colonies were identified phenotypically and genotypically through biochemical characterization and 16S rRNA amplification respectively. Further hemolytic activity of *Aeromonas* sp isolates were performed on 5% blood agar plates in which 3 samples showed hemolytic activity. The *hyl* gene responsible for hemolysis in *Aeromonas* sp were detected using PCR. These findings show that the *Aeromoas* sp species isolated from water samples are enterotoxigenic.

.**KEY WORDS:** *Aeromonas, Hemolysis, hyl gene.*

INTRODUCTION

Aeromonas sp is a microorganism widely distributed in nature: in water, soil, food. It is also part of the normal bacterial flora of many animals. As an opportunistic microorganism it is a secondary biological agent that contributes to the occurrence of a fish disease and its deterioration (Yu., *et al* 2004). *A. hydrophila* is a Gram-negative aerobic and facultative anaerobic, oxidase-positive motile bacterium that dwells in aquatic environments and in gastrointestinal tracts of healthy fish. Significant mortalities due to *Aeromonas hydrophila* infection were recorded in the South and South-East Asia farmed fish.

In the study of Musa et al stated that bacterial isolates from sick freshwater ornamental fish from aquarium shops in Terengganu-Malaysia consisted of mostly *A. hydrophila* (60%). The bacterium causes diverse pathologic conditions such as dermal ulceration, rotting of the tails, fin haemorrhagic, septiceamia, red sores, exophthalmia, erythrodermatitis and scale protrusion especially for common carp *Cyprinus carpio*. Chronic infections could lead to ulceration, inflammation, and dermal lesions with focal haemorrhages Cipriano and during acute septicaemia, the liver and kidney are the common target organs.

Ribosomes are a part of the translational machinery of a cell and rRNA plays a vital for cellular growth, function and survival. Consequently, the primary, secondary and tertiary structures of rRNA molecules have been conserved during

evolution (Gutell., 1992). 16S rRNA analysis have stated the primary structure of rRNAs and comparative oligonucleotide consists of highly conserved regions interspersed by regions of moderate to low homology within related species (Gopo *et al.,* 1988) Direct sequencing of the 16S rRNA gene is generally accepted as a stable and specific marker for bacterial identification (Marchandin *et al.,* 2003 and Woese., 2003). The 16S rRNA gene is often organized as a multigene family, with the copy number ranging from 1 to 15 rRNA (rrn) operons in the bacterial genomes (Coenye and Vandamme., 2003).

Usually the maximum size of the 16S rRNA is 1500 bp. Despite the conserved nature of rRNAs, they vary in size and in the organization of the spacer as well as variable regions within the rRNA. However, the size of the 16S rRNA, sequence conservation during evolution confirmed its importance. Classification of bacteria through this technique also been proven that this may be effectively useful for phylogenetic identification at various levels. To believe the usefulness of this fingerprinting technique *Aeromonas* sp. was chosen.

Hemolysis is the breakdown of red blood cells that results in the release of hemoglobin to the surrounding medium. The hemolysis test is done on blood agar medium.

In blood agar three kinds of hemolysis may be seen, they are Alpha hemolysis: It is the greenish discoloration that is seen around the bacterial colony when grown in blood agar. Alpha hemolysis represents partial hemolysis of the hemoglobin in red blood cells. Beta hemolysis: It represents complete breakdown of the hemoglobin in red blood cells. In this case a clear zone can be seen around the colony. Gamma hemolysis: it represents the lack of hemolysis around the bacterial colony

RESULTS

The aim of the present study was to develop a rapid detection method for *Aeromonas* sp. in water samples using amplification of 16S rRNA gene. The drug resistance pattern and hemolytic activity of the isolates obtained from water were also investigated. The isolates were subjected to screening of antibacterial activity of various medicinal plant extracts.

Isolation of *Aeromonas* from water sample

Out of 15 samples 7 showed yellow to honey colored positive colonies for *Aeromonas* in SAA medium

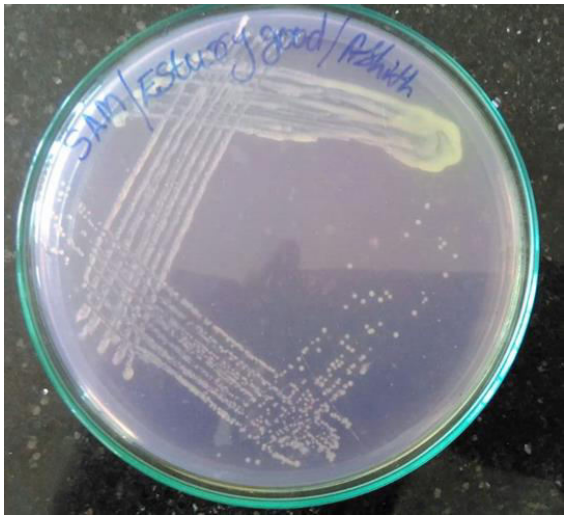

Figure 1: *Aeromonas* sp isolated on SAA

Gram's staining

Pink colored rod shaped bacteria were observed under oil immersion objective. Thus the presence of Gram negative rods were confirmed.

Motility Test

Actively motile rod shaped bacteria were seen when then the edge of the drop was focused.

Biochemical Tests

Biochemical test were performed and showed results for *Aeromonas*

Amplification of 16S rRNA gene

The presumptive Aeromonas isolates were further confirmed on the basis of amplification of signature region 16S rRNA (1050bp) using genus specific primers. Out of 15 samples 7 showed presence of *Aeromonas* sp

Detection of *hyl* gene

All the isolates were screened for presence of hyl gene which is of 550 bp. All the 7 isolate exhibited the presence of *hyl* gene with clear band at 550bp()

Hemolytic test

Out of 7 samples 1 showed alpha hemolysis, 2 showed beta hemolysis and 4 showed Gamma hemolytic activity. The distribution of hemolytic activity in *Aeromonas* sp is shown in fig

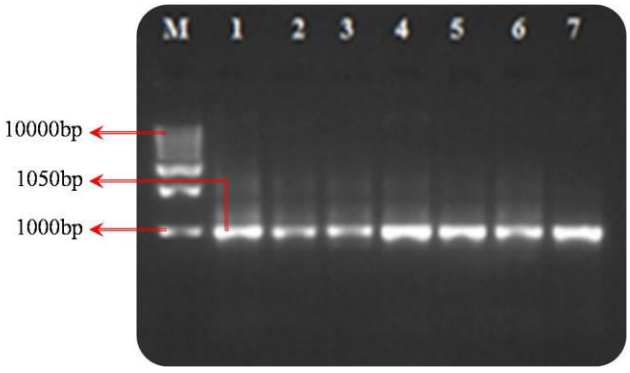

Figure 2: Detection of Aeromonas by 16S rRNA method

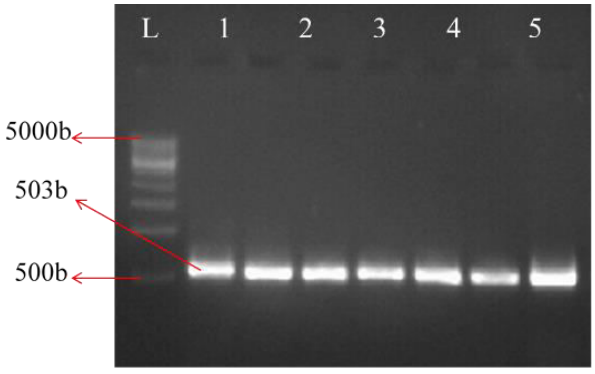

Figure 3: Detection of *hyl* gene

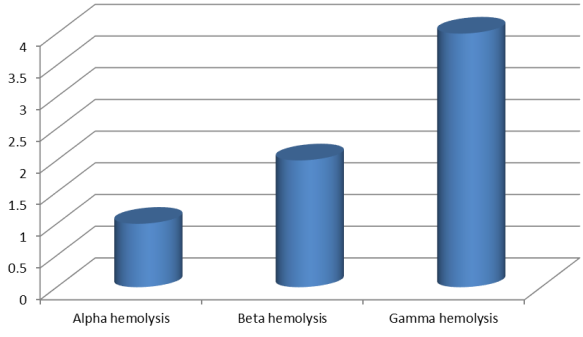

Figure 4: Graph representing type of hemolysis obtained on blood agar

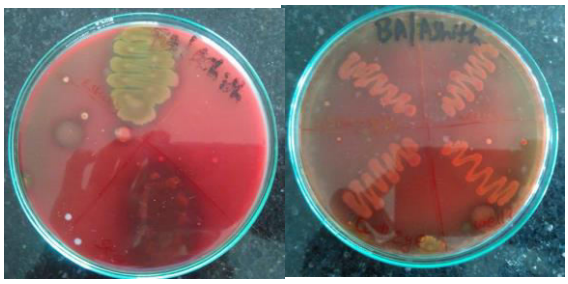

Figure 5: Alpha, Beta and Gamma hemolysis

DISCUSSION

Water is an important factor to sustain life. The different forms of water available for human use may sometimes be contaminated by pathogenic microorganisms. The consumption of such water can lead to serious diseases. *Aeromonas* which is an emerging pathogen may be seen in water sources. The increase in the biomass of *Aeromonas* in water indicate high pollution. The presence of multidrug resistant *Aeromonas sp.* from environmental sources like water is a problem to be seen with much importance. The multidrug resistant bacteria is very hard to treat since it shows resistance to most of the commonly employed antibiotics. The presence of virulence genes like *hyl* which is responsible for hemolysis makes it an even dangerous pathogen. These multidrug resistant *Aeromonas* may show susceptibility to some plant extracts. Thus the use of the plant extract comes useful as the bacteria is resistant to antibiotics.

In the study, *Aeromonas* sp. were identified by conventional and genotypic methods. The primary identification of *Aeromonas* sp in water samples were identified by culturing the sample in starch ampicillin medium and the presence of *Aeromonas* was identified by the appearance of honey colored colonies after incubation. Further identification was done by Gram staining. *Aeromonas* is a Gram negative rod shaped bacteria. The organism showed high motility in the hanging drop slide thus confirming the *Aeromonas* as a motile bacteria.

Then it was subjected to various biochemical tests and the *Aeromonas* showed positive results for methyl red, citrate, mannitol motility, catalase and oxidase tests. It also fermented glucose and mannitol in sugar fermentation test.

Primary identification of isolates as members of the genus *Aeromonas* is relatively simple. Many laboratories should be able to assign the mesophiles to one of the classical complexes (*A. hydrophila*, *A. caviae*, and *A. sobria*), but identification to phenospecies or genospecies level through biochemical testing can be problematic because of taxonomic complexities within the genus (Millership., 1996). As yet, there are few published immunological or molecular methods for detecting mesophilic *Aeromonas* spp. in water compared with the number currently available for *Escherichia coli* and other members of the Enterobacteriaceae. A polymerase chain reaction (PCR) procedure based on 16S rRNA (Khan & Cerniglia., 1997) has been successfully used for the detection of *A. caviae* and *A. trota* in seafood and water samples, and PCR amplification of 16S rDNA sequences has been used to identify environmental isolates of *Aeromonas* (Dorsch *et al*., 1994).

To avoid misidentification, all the isolates were confirmed on the basis of 16S rRNA gene amplification using a genus specific primer. Aeromonas sp isolated from skin, gills and gut of fresh water fish were identified by different biochemical test and confirmed by genus specific 16S rDNA primers (Roy *et al*., 2013b)

7 0ut of the 15 samples tested showed positive for *Aeromonas*. The present study revealed 46.6% of the water samples were contaminated with *Aeromonas* which may create health problems to humans. High prevalence of *Aeromonas* sp. is largely due to poor personal hygiene practices and environmental sanitation, lack of supply of safe water, ignorance of health-promotion practices etc. Pollution and cross contamination levels are very high in many developing countries like India due to lack of infrastructure. Also the sewage contamination of the seawater plays an important role in the higher incidence of virulent *Aeromonas* sp. Alzainy., (2011). Reports from Australia (Burke et al., 1984a; 1984b) have suggested that there may be a connection between cases of *Aeromonas*-associated diarrhoea and the numbers of *Aeromonas* in the drinking-water. In later studies following increases in numbers of aeromonads in treated water in the Netherlands, some of the strains isolated demonstrated strong cytotoxic properties (van der Kooij., 1988).

The presence of *hyl* gene which is responsible for haemolytic activity was also confirmed using PCR and 51.5% of the isolates were positive for *hyl* gene (550 bp) and among metagenomic samples 42% showed the presence of *hyl* gene. A possible explanation for the difference in *hyl* gene percentage and β-haemolytic activity might be due to the fact that gene is present but expression is low Reshma *et al*.,(2015) . *Aeromonas* sp. isolated from environmental and shellfish samples revealed that out of 38 isolates, 20 showed the presence of *hly*A gene (Yousr *et al*., 2007). Yogananth *et al*. (2009) also confirmed the haemolytic activity of *A. hydrophila* isolated from fish using the amplification of *hyl* gene. Escarpulli *et al*. (2003) in their work reported that 96% of *Aeromonas* sp. isolated from frozen fish from local markets of Mexico city carried haemolysin gene. Fatlawy and Ammar., (2013) detected 80% *hyl* gene in *A. hydrophila* isolated from stool samples.

The *hyl* gene has been observed to be responsible for disease occurrence, especially diarrhea, which is confirmed by some studies that some bacteria cause diarrhea by production of enterotoxins or by invasion of the gastrointestinal epithelium (Janda and Abott., 2010). Reshma *et al*., (2015) found that the presence of *hcp*2 gene and its expression was observed in 2 isolates whereas 42% of isolates showed the presence of *hyl* gene and among them 23% of *Aeromonas* sp. showed *hyl* gene expression. It does not mean that the remaining 19% of the isolates are non-pathogenic, they might turn pathogenic and express the virulence protein once they come across a specific host/substrate.

Haemolysins are exotoxin protein produced by bacteria and the lytic activities of haemolysins on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anaemia (Griffiths *et al*., 1988). Haemolytic proteins are commonly isolated from pathogenic bacteria, and β-haemolysins are one of the important bacterial virulence factors (Erova *et al*., 2007). In the present study about hemolysis on blood agar 14.3% showed alpha hemolysis, 28.6% showed beta hemolysis and 57.1% showed gamma hemolysis. Rahim *et al*. (2004) reported β-haemolytic activity by *A. hydrophila* around the wells on sheep blood agar plate. The production of haemolytic toxins has been regarded as strong evidence of

pathogenic potential in Aeromonads and the property of haemolysis on blood agar is directly related to enteropathogenecity (Jandaand Abbott, 2010). Ghenghesh *et al*., (2001) reported that 40% of *Aeromonas* sp. isolated from well water showed haemolytic activity. Thayumanavan *et al*., (2007) performed haemolytic activity for *A. hydrophila* isolates and reported that 84.9% of the isolates were β-haemolysin producers.

In a study conducted by Illanchezian *et al*. (2010) in Chennai, Tamil Nadu, it was found that 43.8% of *Aeromonas* isolated from 5 major fish markets, exhibited β-haemolysis. From this it is clear that the environmental aeromonads also possess pathogenic potential and may be a potential contaminant of water supplies, fishes and widespread in seafood. According to Chacón *et al*., (2003) haemolytic activity of clinical isolates is significantly more frequent than that of environmental isolates. According to Abbott *et al*., (2003), the increased activity of clinical isolates is due to horizontal transfer of haemolysin genes present in haemolytic *Aeromonas* to non-haemolytic species was recorded. Tsai and Chen., (1996) reported that about 87.5 and 100% of the strains of *A. hydrophila* isolated from fish and prawns of Taiwan exhibited haemolytic activity.

The hemolytic activity of the isolates in the present study points to the potential pathogenic significance of *Aeromonas* sp. Since majority of the isolates in the present study showed hemolytic activity, this phenotype could be attributed to the activity of a product of *hyl* gene/hemolysin gene.

MATERIALS AND METHODS

Description of study area

Kochi is a developing city which is part of Ernakulam district in the state of Kerala. The area is mostly surrounded by water. The water used for human consumption mainly comes from the purified river water and the water from the wells in households are also used for drinking purposes. The samples were collected from many water sources in and around Kochi. Samples were collected from varied water sources like wells, lakes, public distribution waters, sea etc.

Collection and transportation of samples

The water samples were collected in a pre-sterilized container and sealed, the samples were then transported to the laboratory without any delay. The samples were processed without any delay to prevent growth of contaminating microorganisms. Special care was taken during collection of sample to avoid collection of unwanted materials like rotting debris, soil etc.

Serial dilution and plating

The water samples collected from various sources were serial diluted. Set of 7 tubes with 9ml water were taken and sterilized. Marked the tubes from 10^{-1} to 10^{-7}. 1ml of the sample was transferred to test tube labelled 10^{-1} using a sterile pipette and mixed well. Transferred 1ml from first tube to the second and mixed well. Repeat the same step until all seven tubes are done. Discard 1ml from the seventh tube. All the samples were serial diluted in the same way. It is made sure that separate sterile test tubes were used for each dilution. 0.1ml of aliquot was transferred on to the prepared starch ampicillin agar plates and spread using a sterile L rod. The dilutions 10^{-5}, 10^{-6}, 10^{-7} were taken for all the samples. The plates were then incubated at 37^{o}C for 24hours.

Isolation of presumptive *Aeromonas* sp

After incubation, honey colored colonies were selected from starch ampicillin agar which is a selective media for presumptive identification of *Aeromonas*. The selected colonies were purified using quadrant streaking on nutrient agar plates and pure cultures of the Aeromonas were maintained on nutrient agar plates for further studies.

Gram's staining

Gram's staining classifies bacteria in to two Gram positive and Gram negative. It is used as a preliminary method for identification of *Aeromonas* spp.

A clean glass slide was taken and a thin smear of the isolates were made using a drop of sterile water. The smear was air dried and heat fixed. This smear was flooded with crystal violet and kept for a minute. It was then washed on running tap water and Gram's iodine was added as mordant. Kept for a minute. It was again washed with running tap water and 2 to 3 drops of Grams decolorizer (95% ethyl alcohol) was added. Slide was immediately washed and air dried. Safranin was added and kept for a minute.

It was again washed and dried. The slide was observed through microscope.

Motility test (Hanging drop)

It is a test use to differentiate between motile and non-motile bacteria. The motile bacilli exhibits active motility in the edge of the drop.

A loop full of culture was inoculated in to sterilized nutrient broth tubes and incubated at 37^0 C for 2-4 hours. After incubation a drop of broth culture was placed on a clean, dry cover slip using inoculation loop. Vaseline was applied to the four corners of the cover slip and the slide was placed over it. Inverted the slide and observed under 40 X object. Edge was focused to observe

Genotypic identification of isolates

Isolation of DNA

A single bacterial colony was inoculated in 5 mL of Luria-Bertani broth and incubated overnight at 37 °C in a shaker incubator. Overnight cultures (1.5 mL) were transferred to microfuge tubes and centrifuged at 8,000 rpm for 5 min. Supernatant was removed and cells were washed with 400 µL STE buffer and centrifuged for 8000 rpm for 5 min. The pellets were resuspended in 200 µL TE buffer and 100 µL Tris- saturated phenol (Rankem., India), followed by a vortex mixing for 60 sec and centrifuged at 13,000 rpm for 5 min at 4 °C to separate the aqueous phase and organic phase. The 160 µL upper aqueous phase was transferred to a clean microfuge tube and 40 µL TE was added to make 200 µL and mixed with 100 µL chloroform (Rankem., India) and centrifuged for 5 min at 13000 rpm at 4 °C. The lysate was purified by chloroform extraction until a white interface was no longer present. This step was repeated for 2 - 3 times. To the upper aqueous phase, 40 µL TE and 5 µL RNase were added and incubated at 37 °C for 10 min to digest the RNA. Then, 100 µL chloroform was added to the tube, mixed well and centrifuged for 5min at 13,000 rpm at 4 °C. The upper aqueous phases which contain purified DNA was transferred to clean microfuge tube and stored at -20 °C.

DNA quantification and purity

Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. DNA of 20 µL was diluted in 1980 µL of Tris- EDTA buffer and absorbance was taken after setting the zero with blank at A260 and A280. Based on OD value, the concentration in µg ml-1 in the sample was calculated and multiplied by dilution factor. The final concentration of DNA was µg ml-1 in the sample, which shows the quality of DNA.

The ratio of the absorbance at 260 and 280nm (A260/280) is used to assess the purity of nucleic acids. If the ratio of A260/A280 is 1.8, it shows the presence of pure DNA and if it is less than 1.8 and more than 1.8 it shows the contamination with RNA and protein, respectively (Hoisington et al., 1994).

Preparation of agarose gel

Agarose gel (1%) was prepared (low EEO grade, HiMedia, India) using 1X Tris Acetic acid EDTA (TAE) buffer. Agarose (0.25 g) was mixed in 25 mL of 1X TAE, boiled to melt the agarose and was allowed to cool. At bearable temperature ethidium bromide (final conc. of 0.5 mg/mL) was added, mixed well and poured into the gel casting platform with well former. The gel was allowed to polymerize at room temperature.

Electrophoresis of genomic DNA

Following amplification, the PCR products were subjected to horizontal agarose gel electrophoresis through 1.5% agarose gel (low EEO grade, HiMedia, India) supplemented with ethidium bromide (final conc. of 0.5 mg/mL). DNA ladder (1 µL) was mixed with 3 µL of bromophenol blue and 3 µL of water and was loaded. The 10 µL of sample was mixed with 3 µL of bromophenol blue and was loaded into the wells of the gel carefully using sterile micropipette tip. The electrophoresis was carried out for 45 minutes at 50 V in Tris-acetate-EDTA buffer. The DNA bands were visualized using gel documentation system (Gel doc 2000, BioRad, Italy).

16S rRNA PCR amplification

Based on morphological and biochemical characteristics a number of colonies representing all recovered aeromonads in this study were chosen for identification based on 16S rDNA analysis. DNA extraction was first evaluated by the amplification of the 16S rRNA gene using the gene sequence universal primers: forward primer (F-5'-CAGAAGAAGCACCGGCTAAC-3') and R- 5' TTACCTTATTACGACTTCAC-3'

Detection of virulence *hyl* genes by PCR

PCR was carried out in all the test isolates for screening of virulent gene responsible for hemolysis using genus specific primers of 550bp with initial denaturation at 95^0C for 5 minute, 35 cycles of denaturation at 94^0C for 30 seconds, annealing at 62^0C for 30 sec

and extension at 72^{0}C at 2 minute and final extension at 72^{0}C for 1 minute.

Table 1: Primers used in this study

S.No.	Gene	Primer Sequence	Size (bp)
1	hyl	F- 5′ TCAGGACACCAAGGACATGA 3′ R-5′ GGTGCCGAAGAAGTCGTTGA 3′	550

Hemolysis test

Blood agar medium was prepared and sterilized. The medium was poured on to plates and allowed to solidify. The isolates were steaked on to blood agar plates using a sterile loop. The plates were incubated at 37^{0}C for 24 hours. After incubation the plates were observed for hemolysis pattern around the colonies.

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