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SCREENING OF VIRULENCE AND TOXIN GENES IN *AEROMONAS HYDROPHILA*

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ABSTRACT

Aim and Objectives: To detect the virulence and toxin genes of *Aeromonas hydrophila* by performing PCR technique

Materials & Methods: The present study was performed to detect the virulence and toxin genes of *Aeromonas hydrophila* by performing PCR technique. The genomic DNA of *Aeromonas hydrophila* was isolated and used as template DNA for PCR. Prior to performing PCR with the selected isolates, each primer set was tested with MTCC control strain no. 646 to confirm the production of an amplicon of predicted size.

Results: PCR was performed to detect the virulence and toxin genes (haemolysin, aerolysin and serine protease). Amplification of genes took place and bands were observed at level 597 bp, 416 bp and 211 bp for genes (hlyA, aerA and ser) respectively. The present study report describes PCR technique which detects the presence or absence of haemolysin, aerolysin and serine protease genes in *Aeromonas hydrophila* by using a pair of primers for each gene.

Conclusion: It is concluded from our study that screening of specific virulence and toxin genes by using PCR technique proved to be an effective way for the detection, identification, differentiation, characterization and to know the distribution of virulence factors in *Aeromonas hydrophila*.

KEYWORDS : *Aeromonas hydrophila*, haemolysin, aerolysin, serine protease, PCR.

Introduction

Aeromonas hydrophila is an opportunistic bacterial pathogen. It is a gram negative, motile, straight rods with rounded ends (bacilli to coccibacilli shape), facultative anaerobic, non-spore forming, oxidase positive, glucose- fermenting bacterium which belongs to Aeromonadaceae family (Eirna-liza, Saad, Hassim, & Karim, 2016). They usually grow from 0.3 to 1.0 µm diameter and 1.0 to 3.5 µm in length. This bacterium is motile by polar flagella. *Aeromonas hydrophila* are heterotrophic organisms(Rasmussen-Ivey, Figueras, McGarey, & Liles, 2016). *Aeromonas hydrophila* was isolated from diseased cold- and warm- blooded animals for over 100 years and from humans since the early 1950s (Abdelhamed et al., 2017a). *Aeromonas hydrophila* is resistant to chlorine and refrigeration or cold temperatures (Vijayakumar et al., 2017).

The degree or intensity of pathogenicity of an organism as indicated by case fatality rates or ability to invade host tissues and cause disease or the relative ability of a micro-organism to cause disease by overcoming body defences ; being extremely poisonous is called virulence (Baldissera et al., 2016). Most environmental isolates of *Aeromonas hydrophila* and related *Aeromonads* secrete many extracellular products, some of which, such as areolysin, haemolysin, enterotoxin, protease and cytolytic enterotoxin are considered as virulence factors in pathogenesis (Abdelhamed et al., 2016). The occurrence of genes encoding haemolytic, cytotoxic and enterotoxin activities may contribute to diarrhoeal related virulence and that the expression of peritrichous flagella encoded by the *laf* gene cluster enhances eukaryotic cells adherence and invasiveness, and also the ability to form biofilms *in vitro* (Dong et al., 2017). Moreover, the presence of a type III secretion system can play a role in the virulence of motile

Aeromonas by delivering a range of proteins (including toxins) into the host cell (Ghatak et al., 2016). In addition, evidence of plasmid - encoded expression by *Aeromonas* isolates of cytotoxins similar to Shiga-like toxins of verotoxigenic Escherichia coli has been observed.

A microbial product or component that injures another cell or organism is called toxin. Often the term refers to a poisonous protein, but toxins may be lipids or other substance. Aerolysin is cytolytic extra cellular water soluble protein of *Aeromonas hydrophila*, secreted during active growth phase(Pang et al., 2017). During invasion, the aerolysin is inserted into lipid bilayer of host red blood cell and destroys its membrane permeability barrier, thereby causing haemolysis (Nagar, Bandekar, & Shashidhar, 2016). It can be detected by the presence of zone of haemolysis on blood agar. *Aeromonas hydrophila* is very toxic to many organisms because of its structure. When it enters the body of fish, amphibians, or humans, it travels via the bloodstream to the first available organ. It produces areolysin cytotoxic enterotoxin (Act) which is one of the major virulence factors(Abdelhamed et al., 2017b). Its toxin is produced and secreted by the cell from a type II secretion system. The toxin binds to high affinity receptors and undergoes oligomerization to form a heptameric pore -forming complex which allows passage of small molecules in the plasma membrane, resulting in permeab ilization of the cell, cell death and eventually tissue destruction (Zhang et al., 2017a).

Aeromonas hydrophila is also known as an opportunistic pathogenic bacterium, meaning they only infect hosts with weakened immune responses(Du et al., 2016). Other virulence functions include a surface layer which inhibits complement - mediated killing, type IV pili for attachment, and a set of extracellular proteases which can cause tissue damage(Ghatak et al., 2016). Though *Aeromonas hydrophila* is considered a pathogenic bacterium, scientists have not been able to prove that it is the actual cause of some of the diseases it is associated with. It is believed that this bacterium aids in the infection of diseases, but do not cause the diseases themselves (Hamid, Ahmad, & Usup, 2016).

Polymerase chain reaction (PCR) technique was used to assay for the detection of specific genes in the genomes of the *Aeromonas* spp. isolated from environmental and shellfish sources, particularly aero and hlyA genes, responsible for aerolysin and hemolysin toxins production in this genus (Abbasi, Khansari-Nejad, Abtahi, Akbari, & Ghaznavi-Rad, 2016). The method described here may be a useful detection tool to assist in further investigation of aero and hlyA genes in the genus *Aeromonas*, especially for food microbiologist (Zhang et al., 2017b). Synthetic oligonucleotide primers were used in a polymerase chain reaction (PCR) technique to detect the gene for aerolysin in strains of *Aeromonas hydrophila* and to screen for identical genes in *A. caviae, A. sobria, and A. veronii* isolated from patients with diarrheal disease (Rama et al., 2016).

Materials and Methods

Sample collection: A total of 40 food and water samples were collected from various places in Chennai. The samples collected were as follows. Fish (n=7), Prawn (n=4), Crab (n=2), Cow milk (n=4), Carrot (n=3), Water (n=6), Mutton (n=3), Chicken (n=3), Goat milk (n=3) Onion (n=2), Tomato (n=3).The samples were collected in sterile polythene bags and were kept in ice box and were transported to the laboratory. The time interval between the collection and the transport was observed to be 2 hours.

Bacteriological isolation: The samples were first washed to remove the surface contaminants. The samples were cut and then crushed and ground with sterile distilled water in mortar and pestle and used for serial dilution. The samples were inoculated into alkaline peptone water and were incubated overnight at 37°C for 24 hours. The samples were serially diluted using sterile distilled water. The samples were diluted with different dilution such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.

Isolation in Starch Ampicillin agar: Starch ampicillin agar (HiMedia, India) was prepared and sterilized. At bearable warmth, Ampicillin was added and plated. After incubation at 37oC for 24 hours in alkaline peptone water 1 ml of the samples were taken and serially diluted. From different dilutions 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}. 0.1ml of the diluted samples was transferred in Starch Ampicillin Agar plates (Palumbo *et al.*, 1985) and spreaded evenly by using the L-rod (spread plate technique).The inoculated SA agar plates were incubated at 37oC for 24 hours.

Presumptive identification:
Isolation in Rimler - Shotts medium: RS medium (HiMedia, India) which is a selective media was prepared and boiled. At bearable warmth, novobiocin was added and plated. Colonies from SA agar were taken and streaked onto RS medium and the plates were incubated at 37°C for 24 hours. Morphology of the colonies and the colour changes were noted.

Confirmation test:
Inoculation in Kaper's Multitest medium: Kaper's multitest medium was prepared and sterilizezd for confirmation test. Yellow to honey coloured colonies from the RS medium were taken and stabbed and streaked onto Kaper's multitest media tubes. The tubes were incubated at 37°C for 24 hours.

Virulence tests:
In our study the virulence and toxin genes (hlyA, aerA and ser) were screened by virulence tests and PCR technique which has the following pathogenicity..

Determination of haemolytic activity:
Cultures were streaked onto the blood agar and the plates were incubated at 37°C for 24 hours. α-haemolysis , β-haemolysis and γ-haemolysis is observed.

Determination of proteolytic Activity: The isolated cultures were inoculated on Skimmed milk agar plate and incubated at 37°C for 24 – 48 hours. Positive result shows clear zone formation around the bacterial colony as the organism produced protease enzyme which hydrolysed the protein casein present in the milk.

Genomic DNA isolation: Out of the 21 positive isolates of Aeromonas hydrophila 12 isolates of different sources were selected for Genomic DNA Isolation. They are F1, F3, F5, P2, CB1, CB2, CM1, CM3, CT1, CT3, W1 and W4. MTCC control strain no.646 was selected as Control DNA. 1.5ml of broth culture was transferred to 2ml micro centrifuge tubes. The tubes were centrifuged at 10,000rpm for 10 minutes at 4°C.After centrifugation, the supernatant was discarded and the pellet obtained after centrifugation is used for DNA isolation. The pellet was then suspended in 200 µl of solution A at room temperature. The solution was vortexed completely.The tubes were then kept in waterbath at 65°C for 20 minutes and then solution B was added and vortexd. It was centrifuge at 10,000 rpm for 10 minutes. 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 the vials and kept for centrifugation at 10,000 rpm for 10 minutes.About 200 µl of 70% ethyl alcohol was added to the collected pellet and mixed by inverting the tube till the white strands of DNA precipitation are seen. It was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded.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.

Agarose gel electrophoresis: 1% agarose was prepared for 70ml and dissolved in 1 x TBE buffer. After cooling 4 µl Ethidium bromide was added in dissolved agarose.Prepared agarose was poured on to the gel platform and wells can be made by using the comb.20 µl of digested DNA was loaded along with 5 µl of gel loading dye and electrophoresis was performed.After running, disconnect the apparatus and examined nucleic acid bands through UV transilluminator.

Isolates selection for PCR: Seven isolates were selected for subjecting to PCR. They are F1, F3, P2,CB1,CM1,CT3 and W1. Before performing PCR with the selected isolates, each primer set was tested with MTCC control strain no. 646 to confirm the production of an amplicon of predicted size.

PCR detection of haemolysin and aerolysin genes:
PCR analysis: The selected seven isolates F1, F3, P2, CB1, CM1, CT3 and W1 were taken. The primer selected for the PCR detection had the following sequence: Primer 1 : H1 (5'- GGC CGG TGG CCC GAA GAT GCA GC -3') and Primer 2 : H2 (5'- GGC GGC GCC GGA CGA GAC GGG -3') to determine whether a 597 bp hly A gene fragment was present or absent. Primer 1: A1 (5'- GCC TGA GCG AGA AGG T - 3') and Primer 2 : A2 (5'- CAG TCC CAC CCA CTT C -3') to determine whether a 416 bp aer A gene fragment was present or absent.

PCR detection of serine protease gene:
PCR analysis: The selected seven isolates F1, F3, P2, CB1, CM1, CT3 and W1 were taken. The primer selected for the PCR detection had the following sequence. Primer 1 : Ser - F (5'-ACG GAG TGC GTT CTT CCA ACT CCA G -3') and Primer 2 : Ser - R (5'-CCG TTC ATC ACA CCG TTG TAG TCG -3') The sequence information of the gene was confirmed with Genbank database of the National Centre for Biotechnoogy Information (NCBI) by using the BLASTN program (http://www.ncbi.nlm.nih.gov/), Accession number X67043 and AF159142.The primers Ser- F and Ser- R were used to determine whether a 211 bp ser gene fragment was present or absent.

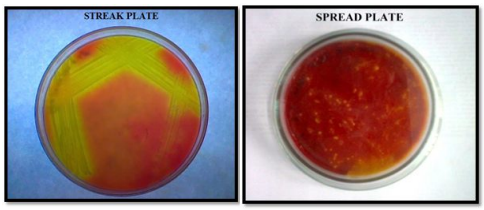
Results

Isolation in Starch Ampicillin agar: 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 sub-cultured into the nutrient agar slants and stored at 4oC for further studies. Refer to Table 1 and Figure 1 for a more detailed description on *Aeromonas hydrophila colony.*

Table 1: Colony on Starch Ampicillin Agar

S.No.	Samples	Colour of colony
1.	Fish	Yellow
2.	Prawn	Yellow
3.	Crab	Yellow
4.	Cow milk	Yellow
5.	Carrot	Yellow
6.	Water	Yellow
7.	Mutton	Yellow
8.	Chicken	Yellow
9.	Goat milk	Colourless
10.	Onion	No growth
11.	Tomato	No growth

Figure 1: *Aeromonas hydrophila* on Starch Ampicillin Agar (SAA)



Presumptive identification:

Isolation in Rimler - Shotts medium: In RS medium plates some samples showed yellow colour colony which confirmed the presence of *Aeromonas hydrophila* and some samples showed green colour colony which confirmed the presence *Aeromonas salmonicida* refer Table 2 and Figure 2 . The yellow colour colonies were convex, circular, regular, smooth and 2mm in size. Gram-negative straight rods with rounded ends (bacilli to coccibacilli shape) were observed by gram staining. Motile cells were observed by hanging drop method.

Table 2: Colony on Rimler - Shotts medium

S. No.	Samples	No. of samples	Samples with Yellow colonies	Samples with Greenish yellow colonies
1.	Fish	7	5	2
2.	Prawn	4	3	1
3.	Crab	2	2	0
4.	Cow	4	4	0
5.	milk	3	3	0
6.	Carrot	6	4	2
7.	Water	3	0	3
8.	Mutton	3	0	3

Figure 2: *Aeromonas hydrophila* on Rimler - Shotts medium(RS)

Confirmation test:

Inoculation on Kaper's Multitest media: After 24 hours of incubation the tubes showed alkaline slant and acid butt along with gas production confirming *Aeromonas hydrophila.* The cultures were confirmed as *Aeromonas hydrophila* using the Kaper's multitest medium (Kaper's et al., 1979). Out of 40 samples collected from different sources 21 samples were found to be positive for *Aeromonas hydrophila.* refer Table 3.

Table 3: Sample coding

S.No.	Samples	Code	No. of positive isolates
1.	Fish	F	5
2.	Prawn	P	3
3.	Crab	CB	2
4.	Cow milk	CM	4
5.	Carrot	CT	3
6.	Water	W	4

Virulence tests: The Table 4 indicates the Virulence test of *Aeromonas hydrophila*

Table 4: Virulence test of *Aeromonas hydrophila*

S. No.	Isolates	Haemolytic activity	Protease activity
1.	F1	β	++
2.	F2	β	+
3	F3	γ	++
4.	F4	α	+
5.	F5	α	+
6.	P1	α	+
7.	P2	β	-
8.	P3	γ	+
9.	CB1	α	++
10.	CB2	α	+
11.	CM1	β	++
12.	CM2	α	+
13.	CM3	α	+
14.	CM4	α	+
15.	CT1	γ	+
16.	Ct2	γ	++
17.	CT3	α	++
18.	W1	β	+
19.	W2	β	+
20.	W3	α	+
21.	W4	β	++

Haemolytic activity: Out of 21 isolates from different sources 48% (10 isolates) showed α-haemolysis, 33% (7 isolates) showed β-haemolysis and 19% (4 isolates) showed γ-haemolysis refer Table 5.

Table 5: Haemolytic activity.

S. No.	Type of haemolysis	Isolates
1.	α- haemolysis	F4,F5,P1,CB1,CB2,CM2, CM3,CM4,CT3,W3
2.	β- haemolysis	F1,F2,P2,CM1,W1,W2,W4
3.	γ- haemolysis	F3,P3,CT1,CT2

Proteolytic activity:

Out of the 21 isolates 33% (7 isolates) showed high protease activity and 62% (13 isolates) showed an intermediate activity and 5% (1 isolate) showed no proteolytic activity refer Table 6.

Table 6: Proteolytic activity.

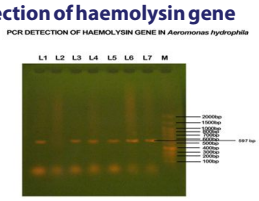
S. No.	Type of proteolysis	Isolates
1.	High activity	F1,F3,CB1,CM1,CT2,CT3,W4
2.	Intermediate activity	F2,F4,F5,P1,P3,CB2,CM2,CM3,CM4,CT1, W1,W2,W3
3.	No activity	P2

Isolation of genomic DNA of *Aeromonas hydrophila:*

The Genomic DNA Isolation was performed for the selected 12 isolates namely, F1, F3, F5, P2, CB1, CB2, CM1, CM3, CT1, CT3, W1 , W4 and the MTCC control strain no.646 which was selected as Control DNA. The genomic DNA was isolated and bands were observed.

PCR detection of haemolysin gene: To detect the presence of haemolysin gene (hlyA) in *Aeromonas hydrophila,* primers were annealed with the template DNA (isolated genomic DNA of *Aeromonas hydrophila*) and caused amplification. The selected seven isolates were subjected to PCR. The isolates were F1, F3, P2, CB1, CM1, CT3 and W1 refer Figure 3. In gene amplification, the isolates F1,P2,CB1,CM1,CT3 and W1were confirmed of this gene with 597 bp using the 2000-100 bp DNA marker whereas in F3 the gene was not amplified as it showed γ-haemolysis on blood agar.

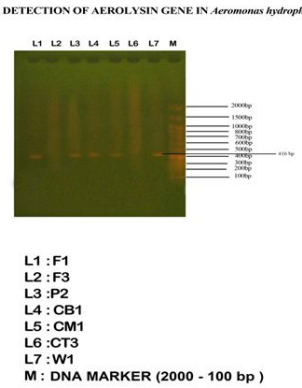
Figure 3: PCR detection of haemolysin gene



L1 : F1
L2 : F3
L3 : P2
L4 : CB1
L5 : CM1
L6 : CT3
L7 : W1
M : DNA MARKER (2000 - 100bp)

PCR detection of aerolysin gene: To detect the presence of aerolysin gene *(aerA)* in *Aeromonas hydrophila,* primers were annealed with the template DNA (isolated genomic DNA of *Aeromonas hydrophila*) and caused amplification. The selected seven isolates were subjected to PCR. The isolates were F1, F3, P2,CB1, CM1,CT3 and W1 refer Figure 4. In gene amplification, the isolates F1,P2,CB1,CM1 and W1were confirmed of this gene with 416 bp using the 2000-100 bp DNA marker whereas in F3 and CT3 the gene was not amplified.

Figure 4: PCR detection of aerolysin gene



PCR detection of serine protease gene:

To detect the presence of serine protease (ser) gene in *Aeromonas hydrophila,* primers were annealed with the template DNA (isolated genomic DNA of *Aeromonas hydrophila*) and caused amplification. Totally seven isolates were subjected to PCR. The isolates were F1,F3, P2,CB1,CM1,CT3 and W1 refer Figure 5. In gene amplification, the isolates F1,F3,CB1,CM1,CT3 and W1were confirmed of this gene with 211 bp using the 2000-100 bp DNA marker whereas in P2 the gene was not amplified as it showed no protease activity on Skimmed milk agar. Table 7 clearly indicates the PCR results of haemolysin , aerolysin and serine protease genes.

Figure 5: PCR detection of serine protease gene

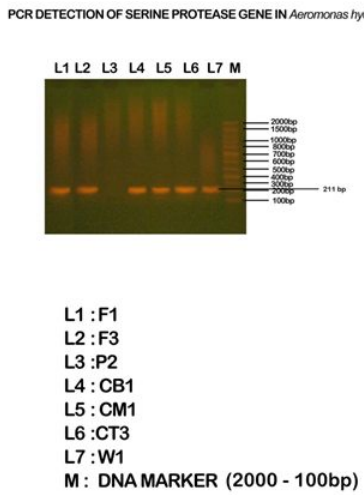


Table 7: PCR results of haemolysin, aerolysin and serine protease genes.

S.No.	Isolates	Haemolysin gene	Aerolysin gene	Serine protease gene
1.	F1	Present	Present	Present
2.	F3	Absent	Absent	Present
3.	P2	Present	Present	Absent
4.	CB1	Present	Present	Present
5.	CM1	Present	Present	Present
6.	CT3	Present	Absent	Present
7.	W1	Present	Present	Present

Distribution of hlyA, aerA and ser genes in *Aeromonas hydrophila* isolates:

PCR detection using primers H1 and H2 showed that 6 isolates (85.71%) were positive for *hlyA* gene and the isolates were haemolytic on blood agar. PCR products of *aerA* gene with A1 and A2 primers were found in 5 isolates (71.43%) and PCR products of ser gene with primers Ser-F and Ser-R were found in 6 isolates (85.71%). Four genotypes in *Aeromonas hydrophila* isolates were found. They were $hlyA^+$ $aerA^+$ ser^+,$hlyA^+$ $aerA^+$ ser^-, $hlyA^+$ $aerA^-$ ser^+, $hlyA^-$ $aerA^-$ ser^+. The genotype $hlyA^+$ $aerA^+$ ser^+ was the most common genotype among the *Aeromonas hydrophila* isolates refer Table 8.

Table 8: Distribution of *hlyA,* aerA and ser genes in *Aeromonas hydrophila* isolates.

S.No.	Genotype	No.of isolates	Isolates
1.	$hlyA^+$ $aerA^+$ ser^+	4	F1,CB1,CM1,W1
2.	$hlyA^+$ $aerA^+$ ser^-	1	P2
3.	$hlyA^+$ $aerA^-$ ser^+	1	CT3
4.	$hlyA^+$ $aerA^-$ ser^+	1	F3

Discussion

The organism Aeromonas hydrophila was isolated through selective media from various food and water samples and identified by Gram's staining, motility test, biochemical tests and presumptive tests. Confirmation test was done by using Kaper's multitest medium (Seethalakshmi et al., 2008).Out of 21 positive isolates of Aeromonas hydrophila 12 isolates from different sources were selected for genomic DNA isolation. The genomic DNA were isolated and bands were observed. The isolated DNA were used as template DNA for the PCR study. From the 12 isolated DNA, 7 isolates were selected from different sources for PCR. Before performing PCR with the selected isolates, each primer set was tested with MTCC control strain no.646 to confirm the production of an amplicon of predicted size. PCR were performed in PCR Thermocycler (MJ Research Model PTC 150 MiniCycler, Watertown, Mass, USA) under the PCR conditions of the genes.

PCR products indicates the presence or absence of the virulence and toxin genes in the isolates by observing the bands at the level 597 bp for haemolysin gene, 416 bp for aerolysin gene and 211 bp for serine protease gene. PCR technique clearly identified the virulent genes as haemolysin gene, aerolysin gene and serine protease in *Aeromonas hydrophila*. Our result are in support in the findings of Christopher *et al*., 1988 ; Aslani *et al*., 2004 and Seethalakshmi *et al.,* 2008 as they studied the haemolysin gene and aerolysin gene and reported the PCR amplified bands at 597 bp and 416 bp respectively and Young *et al.*, 2007 studied the serine protease and reported the PCR amplified bands at 211 bp. The present study results also confirm the same.

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