ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Virulence determinants, drug and metal resistance of clinical and environmental Aeromonas species

U. Indra, M. Sureshkumar, B. Laksman Kumar and G. Vivekanandhan*

PG and Research Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore-641029, India.

Manuscript Info

Manuscript History:

Received: 22 February 2015
Final Accepted: 25 March 2015
Published Online: April 2015

Key words:

Aeromonas hydrophila, Virulence determinant, drug resistance, secretion system, metal resistance

Corresponding Author

U. Indra

Abstract

Seventy nine clinical and environmental samples were screened for the presence of Aeromonas species and a total of twenty seven isolates were recovered. Various virulent determinants were screened which showed the presence of Type 3 Secretion System genes (aexT and ascU) and Type 6 Secretion System regulatory protein gene hcp2. Antibiotic susceptibility test revealed resistance to amoxicillin, aztreonam and cephalothin and 21 different patterns of resistance was observed. Screening of TEM1 for β lactamases showed that 18 isolates carried the respective gene. Over 25 % isolates produced β haemolysis on 5 % sheep blood agar medium. The Minimum Inhibitory Concentration (MIC) values for silver nitrate were determined to be 250μM and for copper sulphate was 6mM. Resistance genes for silver and copper like silP and copA was screened and determined that 12 isolates and one isolate were positive for each, respectively. Sequencing of 16S rRNA gene (IAH22) showed the isolate was Aeromonas hydrophila. Thus the Aeromonas sp. is considered to be an emerging pathogen and identified as a high-risk carrier.

Copy Right, IJAR, 2015,. All rights reserved

INTRODUCTION

Aeromonas sp. is a pathogenic organism which has been frequently isolated from many sources including fresh water and estuaries. It is receiving increasing attention because of its association with human disease and food borne infections (18). They are often associated with hemorrhagic septicemia in cold blooded animals including fish, prawn, reptiles and amphibians. The virulence determinants like surface molecules, extracellular enzymes, adhesins and various toxins colonize bacteria, gain entry, establish themselves, replicate, cause damage in host tissues, evade the host defense system, and spread, eventually kill the host (23). Infection results in various diseases including urinary tract infection, peritonitis, gastroenteritis (19) septicemia, meningitis and localized wound infections in susceptible hosts, such as immunocomporomised, infants and the elderly (4). Aeromonas sp. has been frequently associated with traveller's diarrhoea (29). It has been placed on the Environmental Protection Agency's (EPA) "Contaminant Candidate List" (8).

Secretion in bacterial species means the transport of effector molecules such as proteins, enzymes or toxins from across the bacterial cell to its exterior (7). Of several secretion systems, Type 3 Secretion System (T3SS) and Type 6 Secretion System (T6SS) were found to play a major role in Aeromonas pathogenesis.

The Type 3 secretion apparatus (T3SS) is a key factor for the virulence of the pathogen and is formed of 20-30 different proteins (1). ADP-ribosyltransferase toxin (AexT) is a bifunctional toxin, with NH2-terminal domain having GTPase-activating protein, while the COOH-terminal domains have ADPRT activity (20). T6SS components are encoded within the gene clusters that vary in organization. Components that have been partially characterized include, lcmF homologue, ATPase ClpV, a regulatory FHA domain protein and the secreted VgrG and Hcp protein (4). A. hydrophila also express extracellular haemolysins (13) i.e., they form oligomers on erythrocyte membranes.

Multiple drug resistance occurs more in A. hydrophila than other species of Aeromonas. The isolates from humans and animals are more resistant to antibiotics (21). Bacterial antibiotic and heavy metal resistance determinants commonly reside on plasmids (26). Extended-Spectrum Beta-Lactamases (ESBLs) are enzymes that can be produced by bacteria making them resistant to cephalosporins. β-lactam resistance in Aeromonas involves three chromosomally mediated enzymes: a cephalosporinase, a penicillinase, and a carbapenemase (27). As like multidrug resistance, Aeromonas sp. also possesses resistance to heavy metals like silver and copper. These heavy metals were used as antimicrobial agent in curing various wound and skin diseases. Genes responsible for both silver and copper resistance may be encoded by the chromosome or by plasmids. Hence, this study consists of determining the major virulence factors that are functional in Aeromonas sp.

METHODS

Isolation and presumptive identification of Aeromonas sp.

Water samples from pond, lake and well and clinical samples like blood and pus were processed for the isolation of Aeromonas sp. Water samples (1ml) and the clinical samples were streaked on starch ampicillin agar plates (SAA) (14) supplemented with 10µg ampicillin incubated at 37 ^{o}C for 18-24 hours. Positive samples from SAA was inoculated in Kaper's multitest medium (9) and incubated at 37 ^{o}C for 18-24 h. Presumptively identified Aeromonas isolates were subjected to a series of biochemical tests, motility, catalase and H_2S production (25).

Genotypic identification of the isolates

DNA was extracted using CTAB/NaCl method and visualized after electrophoresis in a 1% agarose gel stained with ethidium bromide (0.5 mg/ml). The DNA was quantified and absorbance was taken at A_{260}. Further the organism was confirmed by 16S rRNA gene (1050bp) amplification using specific primers, 16S rRNA F-5′ CAGAAGAAGCACCGGCTAAC 3′ and 16S rRNA R-3′ TTACCTTATTACGACTTCAC 5′ with initial denaturation at 95°C for 5min, 30 cycles of denaturation at 94°C for 30sec, annealing at 52 °C for 30sec and extension at 72°C for 1min, and final extension at 75°C for 5min. PCR of 15µl reaction was carried out using 7µl of 2X master mix (Fermentas, USA), 3µl of nuclease free water, 1.5µl of each forward and reverse primers (10pM) and 2µl of template DNA (50ng).

Haemolytic activity

The isolates were streaked on the blood agar base supplemented with 5% sheep blood and incubated over night at 37 ^{o}C. The lysis of RBC results in the formation of clear zone around the colony and is considered to be positive.

Determination of multiple antibiotic resistance

Kirby-Bauer modified disc diffusion technique was used to examine the antimicrobial susceptibility of the isolates (3). Aeromonas strains were tested against the 17 antibiotic discs (HiMedia, India) by swabbing onto Muller Hinton agar (HiMedia, India) plates and allowed to stand for 5 min. After 30 minutes of pre-diffusion time, the plates were incubated at 37 ^{o}C for 18-24 h. The diameter of the inhibition zone was measured after incubation and compared with the interpretative chart and multiple antibiotic resistance (MAR) index was calculated (10).

Minimum inhibitory concentration (MIC)

One molar silver nitrate ($AgNO_3$) (Sigma, USA) and copper sulphate ($CuSO_4.5H_2O$) (Sigma, USA) stock was prepared and stored in dark at room temperature. Overnight culture of Aeromonas sp. was diluted up to 10^{-8} dilution in sterile PBS solution. Dilutions of 10^{-6}, 10^{-7} and 10^{-8} were inoculated as spots (5µl) on the surface of two sets of Luria burtani agar plates, one supplemented with different concentrations of silver nitrate (0µM, 10µM, 30µM, 50µM, 100µM, 150µM, 200µM and 250µM) and the other with different concentrations of copper sulphate (0mM, 1mM, 2mM, 3mM, 4mM, 5mM, 6mM and 7mM). Plates were incubated at 37 ^{o}C for 24 h and were observed for growth.

Screening for the presence of virulence and resistant genes in Aeromonas sp.

PCR of 15µl reaction as given above was carried out for screening of the virulence genes like aexT and ascU (T3SS), hcp2 and vgrG (T6SS) and resistant genes like TEM1 (antibiotic resistance), silP and copA (metal resistance) using specific primers. The primers and the conditions used are given in table I and II. The amplicons were set to electrophoresis with different concentrations of agarose gel (based on the base pairs of the gene) (low EEO grade, HiMedia, India) supplemented with ethidium bromide (0.5mg/ml) for 45 min at 50 V in Tris-acetate-EDTA buffer. The DNA bands were visualized using gel documentation system (Biorad, Italy) and the images were photographed.

RESULTS

Table I: Primers used in this study

S.No	Primers	Primer Sequence	Base pairs
1	aexT	F-5′ TCAAACCTCGACCGAGGTGA 3′ R-5′ CCTCAGGACTGCTTTCGCTA 3′	503
2	ascU	F-5′ TGGTGATCGCCATCGCCGA 3′ R-5′ GACGGCGCTTGCTCTTGAT 3′	779
3	hcp2	F-5′ TCATAGATCTAATGCCAACTCCATGTTATTCAG 3′ R-5′ GGTCCTCGAGTTAGGCCTCGATCGGCGCGCG 3′	519
4	vgrG1	F-5′ TCATACGCGTATGGCAGACAGCACAGG 3′ R-5′ GGTCGCTAGCTTATAATACGGAAACCTC 3	2232
5	TEM1	F-5′ GACAGTTACCAATGCTTAATCA 3′ R-5′ TTGGGTGCACGAGTGGGTTA 3′	719
6	silP	F5′- AGTGCAACACAACAAC-3′ R5′- ACTTTCTCTGCACGGA-3′	1200
7	copA	F5′- CTTTACGGACTTTTACCCGCC-3′ R5′- GCGGCGGCCGCCTTTGGGAAGTTGAAAAC-3′	1300

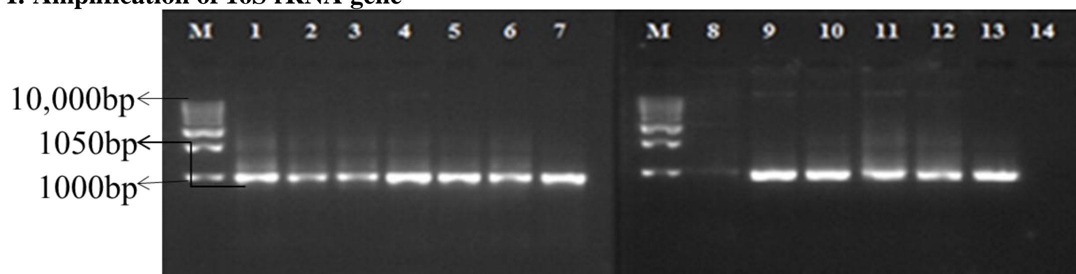
Table II: PCR conditions for amplification of various genes

S.No.	Genes amplified	PCR conditions					
		Initial Denatur. (°C)	Denaturation (°C)	Annealing (°C)	Extension (°C)	Final extension (°C)	No. of Cycles
1	aexT	95° /5min	94°/30sec	55 °/30sec	72°/1min	75°/5min	30
2	ascU	94° /5min	95° /1min	45.8°/30sec	72°/1.15min	72°/5min	30
3	hcp2	95° /5min	94°/30sec	59.5°/30sec	72°/1min	75°/5min	30
4	vgrG	95°/5min	94°/30sec	56-66.5°/30s	72°/1min	72°/5min	30
5	TEM1	95°/5min	94°/30sec	57.4°/1min	72°/1min	72°/5min	29
6	silP	96°/4min	96°/20sec	54.2°/20sec	72°/2min	72°/5min	35
7	copA	95° /5min	95° /1min	50.7°/40sec	72°/1min	72°/10min	35

Isolation and identification of Aeromonas sp.

A total of 79 samples from both clinical (9 samples) and environmental (70 samples) sources were processed and 27 samples (5 clinical and 22 water) were found to be positive for Aeromonas sp. On SAA the organism showed yellow to honey coloured colonies and in Kaper's multitest medium it resulted in the formation of a yellow coloured butt. The isolates were positive for motility, oxidase, catalase, indole and methyl red whereas it exhibited negative results for VP, citrate utilization and H_2S production. Further, the isolates were subjected to 16S rRNA gene amplification which confirmed the isolates to be Aeromonas sp. with the expected amplicon size of 1050bp (Fig I).

Fig I: Amplification of 16S rRNA gene



1050bp amplicon

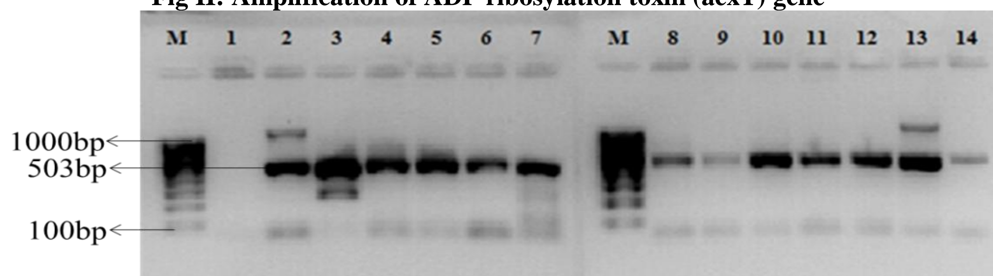
Lane M – Marker 1000bp-10,000bp

Lane 1 to 42 - Distinct bands indicate amplification of 16S rRNA of *A. hydrophila* isolates

Prevalence of virulence and drug resistance gene

Screening for T3SS virulent genes aexT (ADP- ribosylation toxin, 503bp) and ascU (regulatory gene, 779bp) revealed 88.88% and 18.51% of the isolates encoding these genes (Fig II and III) whereas screening for T6SS virulent genes hcp2 (haemolysin coregulated protein, 519bp) and vgrG1 (valine-glycine repeat G, 2232bp) revealed 18.51% and 0% of the isolates encoding the gene respectively (Fig IV).

Fig II: Amplification of ADP ribosylation toxin (aexT) gene

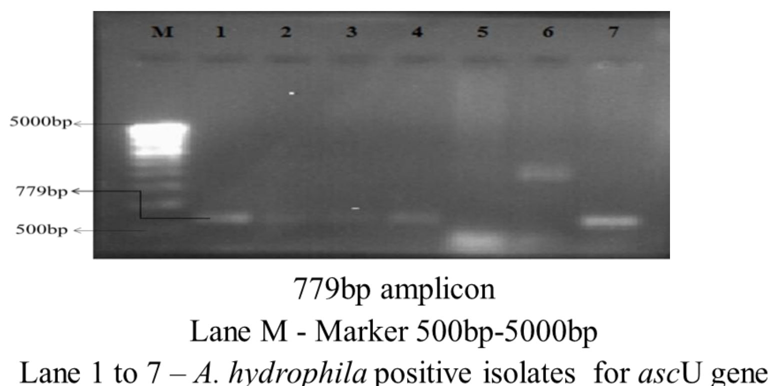


503bp amplicon

Lane M – Marker 100bp-1000bp

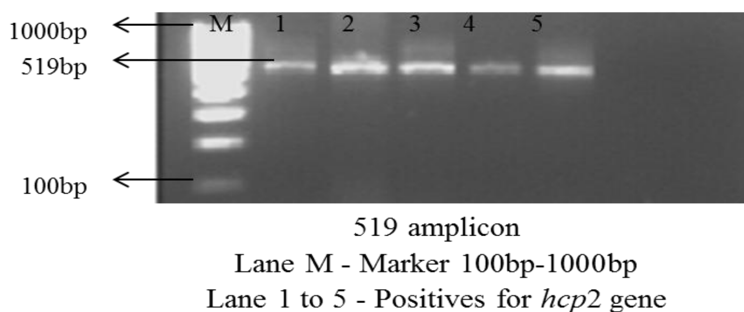
Lane 1 to 28 - *A. hydrophila* isolates IAH 1 – IAH 28

Fig III: Amplification of ascU-T3SS regulatory gene



779bp amplicon
Lane M - Marker 500bp-5000bp
Lane 1 to 7 – *A. hydrophila* positive isolates for *asc*U gene

Fig IV: Amplification of haemolysin co-regulated protein (hcp2) gene



519 amplicon
Lane M - Marker 100bp-1000bp
Lane 1 to 5 - Positives for *hcp*2 gene

Haemolytic activity

Among the isolates 33 % were α haemolytic with partial haemolysis i.e. a clear greenish zone, 26 % were β haemolytic with the clear zone and 41 % were γ haemolytic with no zone.

Antibiotic resistance

Antibiotic susceptibility test showed that all the 27 isolates were resistant to amoxicillin, aztreonam and cephalothin followed by piperacillin (96.29%), and least resistance was shown towards gentamicin (44.44%) and ciprofloxacin (37.03%) (Table III). The isolates exhibited 21 different types of resistance patterns showing resistance to a minimum of 4 antibiotics (Table IV). Over, 60 % of the isolates exhibited MAR index value above 0.8 followed by 17.85% between 0.6 and 0.8, 14.28% between 0.4 and 0.6. Two isolates exhibited a MAR index value of below 0.4. PCR amplification of antibiotic resistant gene TEM1 (719bp) was performed which resulted in amplification of 67% of the isolates (Fig V).

Table III: Percentage of antibiotic resistance of A.hydrophila

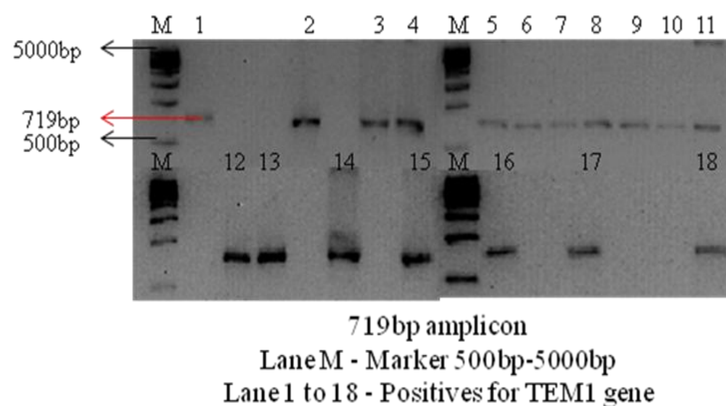
Antibiotics	% of resistance	Antibiotics	% of resistance
Ampicillin	88.88	Gentamicin	44.44
Amoxicillin	100	Neomycin	62.96
Aztreonam	100	Penicillin G	88.88
Carbenicillin	92.59	Piperacillin	96.29
Cefamandole	85.18	Rifampicin	81.48
Cephalothin	100	Streptomycin	81.48
Chloramphenicol	51.85	Tetracyclin	59.25
Ciprofloxacin	37.03	Vancomycin	88.88
Erythromycin	85.18		

Table IV: Drug Resistance patterns of A. hydrophila isolates

No. of resistant antibiotics	No. of pattern s	Resistance Pattern	No. of isolates

4	1	A Ao Ac Ch	1
5	1	Pc Ao Ac Cb Ch	1
7	1	G Pc Ao P Va Ac Ch	1
10	2	A Pc Ao P Va R Ac Cb Ch Cef A E S Pc Ao P Va Ac Cb Ch	2
11	2	A E S Pc Ao P Va Ac Cb Ch Cef E S G Pc Va R Ac Cb N Cf Ch	2
12	1	A E S Pc Ao P Va R Ac Cb Ch Cef	1
13	2	A E S Pc Ao P Va R Ac Cb N Ch Cef A E S Pc Ao P Va R Ac Cb T Ch Cef	2
14	4	A E S Pc Ao P Va R Ac Cb N Ch Cef C A E Pc Ao P Va R Ac Cb T N Ch Cef C A E S Pc Ao P Va R Ac Cb T Ch Cef C A E S Pc Ao P Va R Ac Cb T N Ch Cef	5
15	3	A E S G Pc Ao P Va R Ac Cb T Ch Cef C A E S Pc Ao P Va R Ac Cb T N Ch Cef C A E S Pc Ao P Va R Ac Cb T N Cf Ch Cef	3
16	3	A E S G Pc Ao P Va R Ac Cb T Cf Ch Cef C A E S G Pc Ao P Va R Ac Cb T N Ch Cef C A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef	3
17	1	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	6

Fig V: Amplification of TEM 1- Extended Spectrum β lactamase gene



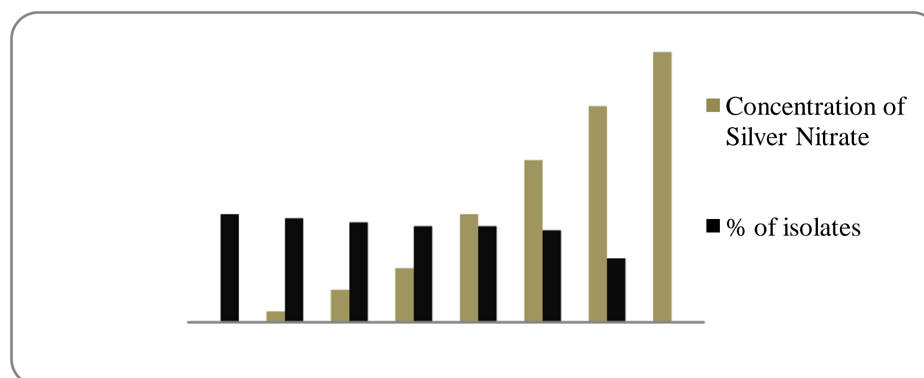
719bp amplicon
Lane M - Marker 500bp-5000bp
Lane 1 to 18 - Positives for TEM1 gene

Minimum Inhibitory Concentration of metals

MIC of silver nitrate

The inhibition of bacterial growth by silver nitrate was observed for different concentrations with varying bacterial load. The LB agar without silver nitrate served as a control. At 10µM concentration, 96.29% of the isolates showed growth and 92.59% in 30µM, 88.88% in 50µM and 100µM, 85.18% in 150µM and 59.27% in 200µM of silver nitrate concentration. At 250µM none of the isolates exhibited growth (Fig VI).

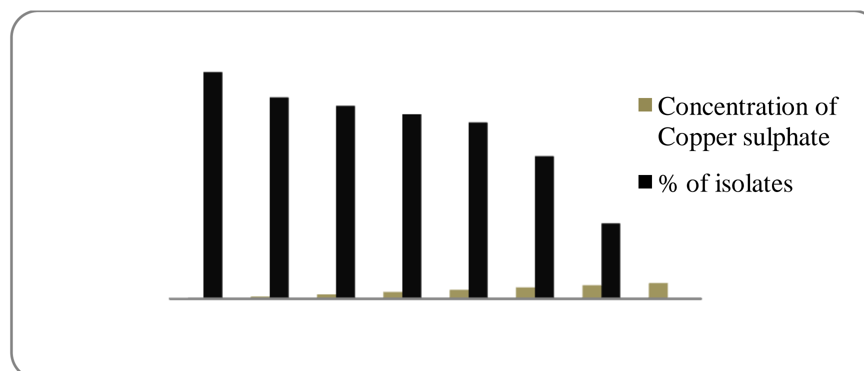
Fig VI: Percentage of isolates showing resistance to different concentrations of silver nitrate



MIC of copper sulphate

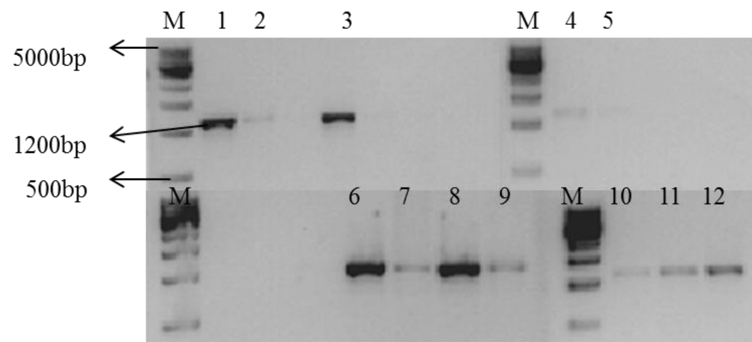
Inhibition of bacterial growth was observed in LB agar medium supplemented with varying concentrations of copper sulphate. Growth was observed in all the isolates at 0mM concentration of copper sulphate. With 1mM copper sulphate concentration 88.88% of the isolates exhibited growth whereas with 2mM, 3mM, 4mM, 5mM and 6mM copper sulphate concentrations 85.18%, 81.48%, 77.77%, 62.96% and 33.33% of isolates exhibited growth. No growth was observed at 7mM copper sulphate concentration (Fig VII).

Fig VII: Percentage of isolates showing resistance to different concentrations of copper sulphate



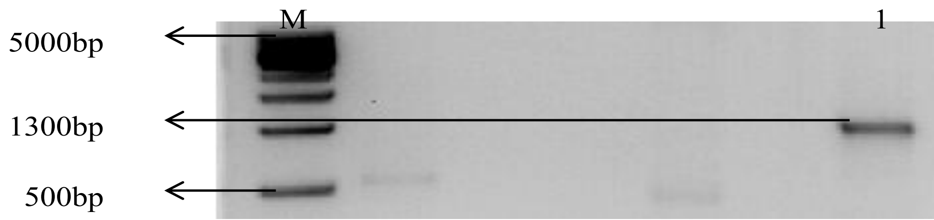
Screening of metal resistant genes like silP (translocation protein, 1200bp) and copA (1300bp) resulted in 44.44% of the isolates carrying the gene for silver resistance and one isolate carrying the gene for copper resistance (Fig VIII and IX). The distribution of various genes among the isolates and the percent of the incidence of various genes were tabulated (Table IX and Table X).

Fig VIII: Amplification of silP gene



1200bp amplicon
Lane M - Marker 500bp-5000bp
Lane 1 to 12 - Positives for *sil*P gene

Fig IX: Amplification of copA gene



1300bp amplicon
Lane M - Marker 500bp-5000bp
Lane 1 - Positive for *cop*A gene

1
2 **Table IX: Distribution of virulence, drug resistance and metal resistance genes in A. hydrophila**

Isolates	Source	T3SS-ascU	T3SS-aexT	T6SS-hcp2	silP	copA	TEM1	Haemolysis
IAH 1	Pond Water	-	-	-	+	-	+	γ
IAH 2	Pond Water	-	+	+	+	-	-	γ
IAH 3	Well Water	-	+	-	-	-	-	γ
IAH 4	Pond Water	-	+	-	+	-	+	α
IAH 5	Well Water	-	+	-	-	-	-	β
IAH 6	Well Water	-	+	-	-	-	+	γ
IAH 7	Well Water	-	+	-	-	-	+	γ
IAH 8	Well Water	-	+	+	+	-	+	α
IAH 9	Well Water	-	+	-	+	-	+	β
IAH 10	Well Water	-	+	-	-	-	+	γ
IAH 11	Well Water	-	+	-	-	-	+	γ
IAH 12	Well Water	-	+	-	-	-	+	γ
IAH 13	Pond Water	-	+	-	-	-	+	β
IAH 14	Well Water	-	+	-	-	-	+	γ
IAH 15	Clinical	+	+	+	-	-	-	β
IAH 16	Pond Water	+	+	-	-	-	+	γ
IAH 17	Pond Water	+	+	+	-	-	+	α
IAH 18	Well Water	+	+	-	+	-	-	α
IAH 19	Well Water	-	+	-	+	-	+	α
IAH 20	Well Water	-	+	-	+	+	-	β
IAH 21	Clinical	+	+	-	+	-	+	α
IAH 22	Clinical	-	-	+	+	-	+	α
IAH 23	Pond Water	-	+	-	+	-	-	β
IAH 24	Pond Water	-	+	-	+	-	+	β
IAH 25	Pond Water	-	+	-	-	-	-	α
IAH 26	Clinical	-	+	-	-	-	-	α
IAH 27	Clinical	-	-	-	-	-	+	γ

3 + Presence of the gene - Absence of the gene

Table X: Percentage of incidence of virulent gene in environmental and clinical isolates

Gene	No. of positive isolates	% in environmental isolates	% in clinical isolates
ascU	5	60	40
aexT	24	87.5	12.5
hcp2	5	60	40
silP	12	83.3	16.6
copA	1	100	0
TEM1	18	83.3	16.6

DISCUSSION

Pathogens employ ingenious mechanisms to establish disease. One of the major challenges is the gastrointestinal diseases in the recent due to increase in the number of probable etiological agents.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy was the most common housekeeping genetic marker which was used for a number of reasons. All the isolates in the present study were confirmed on the basis of 16S rRNA gene amplification using a species specific primer. The expected amplicon size (1050bp) was observed in all the isolates that showed positive for Aeromonas sp. in the presumptive identification tests. The species level identification using phenotypic methods is time consuming and requires a high degree of skill. A PCR of 16S rRNA gene can be used as aid in initial identification of Aeromonas sp (5). Similarly, 16S rRNA PCR was performed for identification of Aeromonas sp. from water samples which resulted in a 1453bp of 16S rRNA product (11).

Among haemolytic proteins commonly isolated from pathogenic bacteria, β-haemolysins are the most important bacterial virulence factors. Haemolysins and related proteins containing cystathionine β synthase (CBS) domains are bacterial toxins that function by assembling identical subunits into a membrane-spanning pore. Almost all enteropathogenic strains of A. hydrophila produce haemolysin and enterotoxin (6).

Haemolytic activity for A. hydrophila isolates recovered from seafood outlets was performed and reported that 85% of them were haemolysin producers (24). About, 25.92% of isolates revealed β haemolysis indicates that the isolates may be enterotoxigenic. Also from the study it is seen that the isolates from the environmental source shows greater haemolytic activity than clinical sources.

The multiple drug resistance mechanism is attributed to several local selective stresses such as temperature, pH, habitat and frequent exposure to the antibiotic residues. Moreover, horizontal gene transfer of R-Plasmids between the species has been identified as one of the key factors for the spread of drug resistance. In the present study, 100% of isolates showed resistance to Amoxicillin, aztreonam and cephalothin, 96% of isolates showed resistance to piperacillin, 93% to carbenicillin, 89% of resistance towards ampicillin, vancomycin and penicillin, 85% towards cefamandole and erythromycin, 81% towards rifampicin and streptomycin, 63%, 59%, 52% and 44% towards neomycin, tetracycline, chloramphenicol and gentamicin respectively, in contrast the isolates of present study showed only 37% resistance to ciprofloxacin.

All the strains of A. hydrophila isolated from gastrointestinal tract of charal produced β lactamases and were resistant to penicillin. About 20% of the isolates were resistant to ampicillin at 125-250 µg/ml and 80% were resistant at 500-4000 µg/ml (15). A 100% resistance to ampicillin, cephalothin, streptomycin and tetracycline and 50% resistance to gentamicin were recorded with the isolates obtained from stool sample (31). Over 90% of A. hydrophila isolated from seafood outlets showed resistance to rifampicin and gentamicin, whereas all the isolates were sensitive to chloramphenicol and ciprofloxacin (39). A. hydrophila isolated from fish and prawn showed 4.4% resistance to chloramphenicol, 97.3% and 98% of the isolates recovered from fish and prawns, were resistant to erythromycin, 99.6% and 94.1% of resistance towards vancomycin whereas 94.4% and 98% of the isolates were resistant to Neomycin (26).

Even there were few disagreements in the degree of resistance; the results were more or less similar with the earlier reports except for few antibiotics. It was reported that the isolates obtained from prawns were susceptible to chloramphenicol (26) but in the present study 51.85% of isolates recorded its resistance because the origin of isolates was environment and clinical specimens. It was reported that all the isolates were sensitive to ciprofloxacin (39) but in contrast 37.03% of isolates showed resistance to ciprofloxacin. These variations were due to the source of the isolates. Since, the isolates in sea foods and prawns have less chance to expose towards the antibiotic residues but it is not surprise to notice the resistance in clinical and pond water sources. The rate of exposure will be high in these cases. Among 27 isolates, 6 isolates showed higher percentage of resistance pattern towards the antibiotics used. The isolates IAH 15, IAH 16, IAH 20, IAH 21, IAH 22 and IAH 23 showed significant resistance pattern. The MAR index of the test isolates showed that environmental isolates were also equally significant in risk factor as like clinical isolates. Seventeen out of 27 isolates showed the risk value between 0.8 - 1. This shows that the multidrug resistant Aeromonas sp. was predominant in environmental samples as like the isolates obtained from clinical sources.

Extended-spectrum β-lactamases (ESBLs) are rapidly evolving group of β-lactamase enzymes produced by the Gram negative bacteria. These enzymes have been derived from TEM and SHV genes by mutations. Isolates of Klebsiellae obtained from clinical samples and were screened for the TEM and SHV gene which resulted in the amplified product of TEM gene in K. pneumoniae with a fragment size of 717bp. The overall data revealed that TEM gene was present in 19 (20%) of the isolates (12). Similarly screening for the TEM 1 gene was performed in the current study which showed the presence of the gene in 66.66% of the isolates with the expected size of 716bp.

Silver is a broad spectrum antimicrobial agent used for wound management (16). Silver is also used to prevent infection of burns and eye infection. Antibacterial effect of silver nano-particle was performed and was reported that the silver nano-particles were able to reduce the growth of all the isolates of A. hydrophila after 30-90 minutes of exposure at a concentration up to 5µg/ml (22). Silver resistance in the organism was screened in Muller Hinton agar (MHA) containing 50µM, 200 µM, 300 µM and 500 µM $AgNO_3$ (17). Similarly, the present study was carried out with 27 isolates of A. hydrophila and all showed the MIC of silver nitrate at 250 µM. Over 11% of the isolates showed the MIC value at 200µM silver nitrate concentration until 10^{-8} dilution whereas 37% of the isolates showed MIC until 10^{-7} dilution and 11% isolates had its MIC as 200 µM at 10^{-6} dilution alone. This result is almost similar to the study of Pike and slight variation may be due to the environmental issues.

As like silver, copper has antimicrobial property against wide range of microorganism. Copper coated surfaces have antimicrobial property than steel or brass and widely used in the door handles and door fitting and it reduces the hospital borne infections. The current study was performed for the MIC of copper sulphate for various concentrations of 0mM to 7mM. 33.33% of isolates showed resistance to 6mM concentration of copper sulphate. Copper sulphate at 6mM concentration inhibited the growth of the A. hydrophila at 10^{-8} dilution whereas 26% of isolates had MIC value of 6mM until 10^{-7} and 7.4% of isolates showed the MIC value 6mM for at 10^{-6} dilution alone. This difference may be due to the prior exposure of the isolates to metal contaminated environment.

PCR screening for 8 genes, silE, silRS, silP, silCBA and silF was performed and plasmids were isolated from sil positive strains to determine if the genes were present on chromosome. The silver resistance gene was seen in six stains of Enterobacter cloacae (28). Like silver, copper also exhibits resistance against the organisms and hence the screening for the resistant gene was carried out. In the current study the presence of silP and copA resistance gene was also screened which revealed its presence at 1200bp and 1300bp respectively. The silP gene was present in 44.44% of isolates. Among positives 83.3% of occurrence was seen in environmental and 16.6% in clinical isolates. Whereas only one clinical isolate showed the presence of copper resistance gene.

The PCR method can identify potential pathogenic A. hydrophila strains in < 8 hours. Totally 7 set of primers were used for the identification of various virulence factors, toxin synthesizing machinery, drug resistance and metal resistance gene.

T3SS have been reported in many gram-negative pathogens, including A. hydrophila (30). A study in which an ascU homologue, near one end of the T3SS, was identified in A. hydrophila PPD134/91 by PCR strongly indicated that a T3SS cluster is also present in A. hydrophila. However, analysis of the T3SS gene cluster of A. hydrophila strain AH-1 revealed an ORF found near ascU showing high homology to the P4- family integrase of a variety of bacteria (31). A study on T3SS-secreted effector protein AexT of Aeromonas salmonicida and ascU indicated that they were bifunctional toxins. They obtained a fragment of virulent gene, aexT at about 535bp. aexT gene present in the cytosol of bacteria synthesize ADP dependent ribosylation toxin which will be regulated by ascU gene for translocation of the toxin upon contact of T3SS into host membrane. Totally, 24 out of 27 isolates carried aexT

gene (503bp). Among the positives, 87.5% of the isolates were from environmental and 12.5% was clinical sources. If aexT gene is present and ascU gene is absent, it cannot trigger the pathogenicity in the absence of ascU gene so it will remain non functional / non expressive. As the same, the result reveals that the ascU gene (779bp) encoding for regulation of aexT protein was found to be prevalent in only 5 isolates among which 60% of isolates were of environmental and 40% of isolates were of clinical sources. But we cannot claim that the environmental isolates carrying this gene is pathogenic because it may not be expected that all the time the gene is functional in all the environmental conditions as regulation of this gene is completely based on the availability of appropriate host

Hcp and VgrG monomers are exported into the periplasm through a putative multi-protein complex at the inner membrane. In periplasm, the Hcp monomers form hexameric rings that dock beneath a spike-like VgrG trimer. Additional Hcp rings assemble beneath the VgrG tip to create an elongating tube that pushes through the outer membrane, on contact with the host membrane it punctures the lipid bilayer, VgrG tip detaches and leaves an uncapped Hcp tube which delivers effector protein into the host. The codependency of Hcp and VgrGs for secretion was noted by multiple investigators. In the absence of Hcp, VgrGs cannot reach the extracellular space on top of an extending Hcp pilus, while in the absence of VgrGs, the extending tube cannot pass through the outer membrane (30).The present study revealed the hcp2 gene of about 519bp in 18.51% (60% of environmental and 40% of clinical isolates) of the total isolates tested. No isolates carried the gene for the VgrG protein. The results clearly reveal that the isolates posses the virulent protein gene but the machinery for host infection was lacking. The absence of vgrG gene does not mean that the organism is not pathogenic because the gene might get activated when it comes in contact with its specific host.

Thus, from the present study multidrug resistance, haemolytic activity and presence of virulent factors in the environmental isolates were recorded higher than the clinical isolates. This shows the prevalence of the high-risk carriers of Aeromonas hydrophila in the environment.

ACKNOWLEDGEMENT

Authors are thankful to Secretary, Principal and management of Kongunadu Arts and Science College for providing facilities and encouragement to carry out this research work.

REFERENCES

Arnold, R.; Jehl, A.; Rattei, T. (2010). Targeting effectors: the molecular recognition of Type III secreted proteins. Microbes Infect. 12 (5), 346-58.

Aust, S. D.; Chignell, C. F.; Bray, T. M.; Kalyanaraman, B.; Manson, R. P. (1993). Contemporary issues in toxicology: Free radicals in toxicology. Toxicol. Appl. Pharmacol. 120 (2), 168-178.

Bauer, A. W.; Kirby, W. M. M.; Sherris, J. C.; Turck, M. (1966). Antibiotic susceptibility testing by a standard single disc method. Am. J. Clin. Pathol. 45 (4), 493-496.

Blair, I. S.; McMahon, M. A. S.; McDowell, D. A. (1999). Aeromonas Introduction. Encyclopedia of Food Microbiology. 25-30.

Borell, N.; Acinas, S. G.; Figueras, M. J.; Martinez-Murcia, A. J. (1997). Identification of Aeromonas clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J. Clin. Microbiol. 35 (7), 1671-1674.

Burke, V.; Robinson, J.; Atkinson, H. M.; Dibley, M.; Berry, R. J.; Gracey, M. (1981). Exotoxins of Aeromonas hydrophila. Aust. J. Exp. Biol. Med. Sci. 59 (6), 753-761.

Caron, E.; Crepin, V. F.; Simpson, N.; Knutton, S.; Garmendia, J.; Frankel, G. (2006). Subversion of actin dynamics by EPEC and EHEC. Curr Opin Microbiol. 9 (1), 40-45.

Chauret, C.; Volk, C.; Creason, R.; Jarosh, J.; Robinson, J.; Warnes, C. (2001). Detection of Aeromonas hydrophila in a drinking- water distribution system: a field and pilot study. Can. J. Microbiol. 47 (8), 782-786.

Kaper, J. B.; Seidler, R. J.; Lockman, H.; Colwell, R. R. (1979). Medium for the presumptive identification of Aeromonas hydrophila and Enterobacteriaceae. Appl. Environ. Microbiol. 38 (5), 1023-1026.

Krumpermann, P. H. (1985). Multiple antibiotic indexing of E.coli to identify high-risk sources of faecal contamination of foods. Appl. Environ. Microbiol. 46, 165-170.

Kumar, P.; Ramakritinan, C. M.; Kumaraguru, A. K. (2009). 16S rRNA Based Identification of Aeromonas sp. Kumar by Constructing Phylogenetic Tree and Identification of Regulatory Elements from the Harmful Red Tide Bloom, Gulf of Mannar. IJOO. 3 (1), 29-35.

Lal, P.; Kapil, A.; Das, B. K.; Sood, S. (2007). Occurrence of TEM & SHV gene in extended spectrum b-lactamases (ESBLs) producing Klebsiella sp. isolated from a tertiary care hospital. Indian J Med Res. 125, 173-178.

McMahon M. A. (2000). The expression of proteinases and haemolysins by Aeromonas hydrophila under modified atmospheres. J Appl Microbiol. 89 (3), 415-422.

Palumbo, S. A.; Maxino, F.; Williams, A. C.; Buchanan, R. L.; Thayer, D. W. (1985). Starch ampicillin agar for the quantitative detection of Aeromonas hydrophila. Appl. Environ. Microbiol. 50 (4), 1027-1030.

Paniagua G. L.; Monroy, E.; Perches, M.; Negrete, E.; Garcia, O.; Vaca, S. (2006). Antibiotic and Heavy Metal resistance of Aeromonas hydrophila isolated from Charal (Chirostoma humboldtianum, valenciannes, 1835), Hidrobiologica. 16 (001), 75-79.

Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol. Diagn. 6 (4), 313-321.

Pike, R.; Stapleton, P.; Lucas, V.; Roberts, G.; Rowbury, R.; Richards, H.; Mullany, P.; Wilson, M. (2002). Effect of medium composition on the susceptibility of oral streptococci to mercuric chloride. Curr Microbiol. 45 (4), 272-276.

Pitarangsi, C.; Echeverria, P.; Whitmire, R.; Tirapat, C.; Formal, S.; Dammin, G. J.; Tingtalapong, M. (1982). Enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides: prevalence among individuals with and without diarrhea in Thailand. Infect. Immun. 35 (2), 666-673.

Rogo, L. D.; Attah, A.; Bawa, E.; Aliyu, A. M.; Aliyu, M. S.; Gaiya, A. Z. (2009). Antimicrobial susceptibility pattern of Aeromonas hydrophila among patients presented with diarrhea attending two teaching hospitals in Northern, Nigeria. Bajopas. 2 (1), 91-95.

Sierra, J. C.; Suarez, G.; Sha, J.; Baze, W. B.; Foltz, S. M.; Chopra, A. K. (2010). Unraveling the mechanism of action of a new type III secretion system effector AexU from Aeromonas hydrophila. Microb Pathog. 49 (3), 122-134.

Sinha. S.; Shimada, T.; Ramamurthy, T.; Bhattacharya, S. K.; Yamasaki, S.; Takeda, Y.; Balakrish Nair, G. (2004). Prevalence, serotype distribution, antibiotic susceptibility and genetic profiles of mesophilic Aeromonas species isolated from hospitalized diarrhoeal cases in Kolkata, India. J Med Microbiol. 53 (6), 527–534.

Soltani, M.; Ghodratnema, M.; Ahari, H.; Ebrahimzadeh, M.; Atee, H. A.; Dastmalchi, F.; Rahmanya, J. (2009). The inhibitory effect of silver nanoparticles on the bacterial fish pathogens Streptococcus iniae, Lactococcus garvieae, Yersinia ruckeri and Aeromonas hydrophila. Int.J.Vet.Res. 3 (2), 137-142.

Suarez, G.; Sierra, J. C.; Sha, J.; Wang, S.; Erova, T. E.; Fadl, A. A.; Foltz, S. M.; Horneman, A. J.; Chopra, A. K. (2008). Molecular characterization of a functional type VI secretion system from a clinical isolate of Aeromonas hydrophila. Microb Pathog. 44 (4), 344-361.

Thayumanavan, Tha.; Subashkumar, R.; Vivekanandhan, G.; Savithamani, K.; Lakshmanaperumalsamy, P. (2007). Haemolytic and Multidrug Resistant Aeromonas hydrophila Cross Contamination in Retail Seafood Outlets of Coimbatore, South India. Am. J. Food Technol. 2 (2), 87-94.

Vivekanandhan, G.; Hatha, A. A. M.; Lakshmanaperumalsamy, P. (2005). Prevalence of Aeromonas hydrophila in fish and prawns from the seafood market of Coimbatore, South India. Food Microbiol. 22 (1), 133-137.

Vivekanandhan, G.; Savithamani, K.; Hatha, A. A. M.; Lakshmanaperumalswamy, P. (2002). Antibiotic resistance of A. hydrophila isolated from marketed from fish and prawn of South India. Int J Food Microbiol. 76 (1-2), 165-168.

Walsh, T. R.; Payne, D. J.; MacGowan, A. P.; Bennett, P. M. (1995). A clinical isolate of Aeromonas sobria with three chromosomally mediated inducible β-lactamases: a cephalosporinase, a penicillinase and a third enzyme, displaying carbapenemase activity. J. Antimicrob. Chemother. 35 (2), 271-279.

Woods, E. J.; Cochrane, C. A.; Percival, S. L. (2009). Prevalence of silver resistance genes in bacteria isolated from human and horse wounds. Vet Microbiol. 138 (3-4), 325-329.

Yamada, S.; Matsushita, S.; Dejsirilert, S.; Kudh, Y. (1997). Incidence and clinical symptoms of Aeromonas associated traveller's diarrhoea in Tokyo. Epidemiol. Infect. 119 (2), 121–126.

Yu, H. B.; Srinivasa Rao, P. S.; Lee, H. C.; Vilches, S.; Merino, S.; Tomas, J. M.; Leung, K. Y. (2004). A type III secretion system is required for Aeromonas hydrophila AH-1 pathogenesis. Infect. Immun. 72 (3), 1248-1256.

Yu, H. B.; Zhang, Y. L.; Lau, Y. L.; Yao, F.; Vilches, S.; Merino, S.; Tomas, J. M.; Howard, S. P.; Leung, K. Y. (2005). Identification and Characterization of Putative Virulence Genes and Gene Clusters in Aeromonas hydrophila PPD134/91. Appl. Environ. Microbiol. 71 (8), 4469-4477.