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REGULAR ARTICLE

ANTIBIOTICS RESISTANCE AND PUTATIVE VIRULENCE FACTORS OF *AEROMONAS HYDROPHILA* ISOLATED FROM ESTUARY

Olumide Adedokun Odeyemi [1], Ahmad Asmat [1], Gires Usup [2]*

Address: [1] School of Biosciences and Biotechnology, Faculty of Science and Technology, [2] School of Environment and Natural Resources, Faculty of Science and Technology, National University of Malaysia, UKM Bangi, Malaysia.

*Corresponding author: oluodeyemi@gmail.com

ABSTRACT

This study aim to investigate antibiotics resistance profile and putative virulence factors of *Aeromonas hydrophila* isolated from estuary. Bacteria used for this study were isolated from water and sediment samples obtained from Sungai Melayu, Johor, Malaysia. Serially diluted 100 µL water and 1g sediment were inoculated on modified Rimler - Shott (mRS) agar. Colonies with distinct cultural characteristics were picked for further studies. Isolates were tested for biofilm productions, protease enzyme and antibiotics resistance profile using agar well diffusion method against 10 commercial antibiotics. Congo Red Agar (CRA), Microplate and Standard Tube (ST) methods were used for assessment of biofilm formation among the isolates while Skim Milk Agar was used for protease production. Sw.KMJ 3 and Sw.KMJ 9 produced black crystalline colonies on CRA. Six of the isolates were biofilm producers in ST method. Result of Microplate method, helped in grouping the isolates into weak (n = 8), moderate (n = 3) and strong producers (n = 4) at 540 nm wavelength. All the isolates were classified as weak $OD_c < OD_i < 0.1$, moderate $ODi = 0.1 < 0.12$ and strong producers $OD_i > 0.12$ respectively at 540 nm wavelength. Antibiotics susceptibility test also revealed that all the isolates were resistant to between 6 and 10 antibiotics. Two isolates each were resistant to 6 (60 %), 7 (70 %) and 9 (90 %) antibiotics respectively. Eight of the isolates showed resistance to 8 (80 %) antibiotics while only isolate

Sw.KMJ-7 showed resistance to all the tested antibiotics. Sw.KMJ-3, Sw.KMJ-8 and Sw.KMJ-9 produced protease enzyme on SMA. The isolates were also found to be resistant to both antibiotics and heavy metals.

Key words: Antimicrobial, Inhibition, Biofilm production, Virulence factor

INTRODUCTION

Aeromonas species are ubiquitous microorganisms found in both aquatic and environmental habitats such as estuary, sediment, sea water , sea grass, sea weed, waste and used water, food and drinki ng water (**Abbott *et al.,* 2003; Matyar *et al.,* 2007; Martinez-Mucia *et al.,* 2008**). They are Gram negative, short rod shape, oxidase and catalase positive, motile, facultative anaerobes, resistant to 0/129 vibriostatic agent and non spore forming. Nineteen species of the genus have been identified till date (**Alperi *et al.,* 2010).** Matyar *and co workers in* 2007 grouped *Aeromonas* spp. into pychrophiles (non-motile) and mesophiles (motile), (**Matyar et al., 2007**). Motile group includes *Aeromonas hydrophila, A. sobria* and *A. caviae*. Non-motile group mainly consist of *A. salmonicida*, mostly fish pathogen. Among these species, *A. hydrophila* is the most studied due to its presence in food (**Radu *et al.,* 2003**), water (**Asmat and Gires, 2002**), estuary (**Odeyemi *et al.,* 2012**), antibiotic resistance and it ability to cause infections in human and animals (**Evangelista-Barreto *et al.,* 2010**). *A. hydrophila* has been identified as causative agent of human diseases such as septicemia, meningitis, wound infections as a result of exposure to contaminated marine environment and diarrhea (**Evangelista-Barreto *et al.,* 2010; Messi *et al.,* 2003**).

Virulence factors such as aerolysin, haemolysin, cytosine, enterotoxin, proteolytic activity, lipolytic activity, gelatinase, slime production and antimicrobial peptides have been identified in *A. hydrophila* (**Asmat and Gires, 2002; Castro - Escarpulli *et al.,* 2003; Martins *et al.,* 2002; Illanchezian *et al.,* 2010**). These virulence factors are used as survival means, self defense mechanism and establishment of pathogenicity. In a research in 1995, some researchers stated that virulence factors are determinant of bacterial pathogenicity **(Vadivelu *et al.,* 1995**). These are mostly found in bacteria including *Aeromonas* spp. (**Singh *et al.,* 2010**). Aeromonads have been attributed to human infections like *gastroenteritis*, septicemia and wound infections (**Illanchezian *et al.,* 2010).** In 2004, Subashkumar and colleagues**,** stated protease, aerolysin, hemolysin, enterotoxins, lipases, gelatinase and biofilm formation as virulence factors in *Aeromonas* spp. Biofilm is an irreversible growth of

aggregated bacterial micro-colonies on surfaces embedded in extracellular polysaccharide matrix. Biofilm formation results into resistance of bacteria to conventional antibiotics and persistent infections (**Rodney 2008**). Biofilm helps in recycling of minerals (**Brown *et al.*, 1999**).

Some researchers have also reported resistance of *Pseudomonas aeruginosa* isolated from soil to three heavy metals (Pb^{2+}, Cu^{2+} and Zn^{2+}) to be two to 600 times when compared with planktonic cells (**Teitzel & Parsek, 2003; Harrison *et al.*, 2005**). Heavy metals although needed in small amount by all forms of life, yet in high quantity become dangerous to living systems including microorganisms. This is as a result of binding with other cellular components to form complex compounds (**Anne Spain and Elizabeth Alm, 2003**). These metals are found in microbial environment occurring either naturally or through anthropogenic activites (**Adarsh *et al.*, 2007**). **Anne Spain and Elizabeth Alm, (2003)** attributed increase of antibiotics resistance genes to presence of heavy metals in the environment, hence posing threat to human health and environment in general.

This study therefore aims to investigate antibiotic resistance and putative virulence factors of *A. hydrophila* isolated from estuarine environment.

MATERIAL AND METHODS

Bacteria source

Bacteria used for this study were isolated from estuary environment-sediment and water obtained from Sungai Kampung Melayu, Johor, Malaysia. The following physical parameters of water collected in sterile 500 mL Schott bottle were noted: temperature, pH, salinity and dissolved oxygen (DO). *A. hydrophila* strains were isolated using modified Rimler Shott -mRS agar (**Asmat and Gires, 2002**) with the following composition L - lysine 5 g/L, L-ornithine 6.8 g/L, Maltose 3.5 g/L, Sodium thiosulphate 6.8 g/L, L - cystein 0.3 g/L, Ferric ammonium citrate 0.8 g/L, Bile salt 5 g/L, Yeast extract 3 g/L, Bromothymol blue 0.03 g/L and Bacto agar 13 g/L without autoclaving while pH was adjusted to 7 ± 0.2. Stock cultures of isolates were kept at appropriate temperature. For the purpose of this research, the isolates were re activated using modified Rimler Shott -mRS agar and further inoculated on freshly prepared Trypticase Soy Agar (TSA) supplemented with 0.65 %w/v yeast extract (YE) –TSAYE (**Asmat and Gires, 2002**).

The isolates were identified morphologically (Gram stain, motility test and colony morphology) and biochemically as *A. hydrophila* using the following tests: catalase, oxidase, starch hydrolysis, citrate utilization, triple iron, bile esculine, protease production, lysine decarboxylase, arginine decarboxylase, ornithine decarboxylase methyl red, acid production from inositol, arabinose, mannose, mannitol, dulcitol, glucose, raffinose, cellulose, xylose, salicin, sucrose and lactose. All morphologically and biochemically identified isolates were differentiated from *Vibrio* species using 10 µg and 150 µg of 0/129 vibriostatic agent. Bacteria resistant to the agent were noted. Stock cultures and master plate were prepared and stored at - 4 ^{o}C for further study.

Determination of virulence factors

Screening for proteolytic enzyme

Presence of protease enzymes from producer bacteria was carried out using Skim milk agar (**Wery *et al.*, 2003**) with the following composition (Skim milk powder 10 g/L – Sunlac, New Zealand; yeast extract 1 g/L and Bacto agar 13 g/L). Other components were firstly prepared and autoclaved at 121 ^{o}C for 15 minutes and allowed to cool to 55 ^{o}C before adding sterilely prepared skim milk solution. Both mixture was swirled gently and allowed to mix thoroughly before pouring into sterile Petri dishes. Producer bacteria were spot inoculated into the plates and incubated at 30 ^{o}C. Plates were observed after 18 - 24 hours of incubation for possible clear zones.

Slime production - Congo Red Agar Method

Presumptive isolates were inoculated on Congo Red Agar (CRA) as described by **Yazdani *et al.*, 2006; Illanchezian *et al*., 2010** and **Hassan *et al.*, 2011**. Briefly, 37 g/L Brain Heart Infusion Broth, 50 g/L of Sucrose, 10 g/L of Bacto agar and 8 g/L Congo red were used. However, Congo red was prepared as a separate concentrated and autoclaved solution before adding to warm autoclaved BHA, sucrose and agar constituents (**Freeman *et al.*, 1989**). Spot inoculated CRA plates were incubated at 30 ^{o}C for 18 - 24 hours. Plates were then observed for dry crystalline black colonies for slime producers and red colonies indicating non slime producers (**Yazdani *et al.*, 2006).**

Biofilm production assay - Standard Tube Method (STM)

The method of **Christensen *et al.*, (1989**) for used for quantitative analysis of the isolates for biofilm production. Briefly, a colony of each Aeromonas spp was inoculated into 10 mL of Trypticase Soy Broth (TSB) supplemented with 1 % w/v of glucose (**Hassan *et al.*, 2011**). Tubes were then incubated at 30 ^{o}C for 18 – 24 hours. Tube contents (sessile cells) were decanted and washed thrice with 1 × phosphate buffer saline (PBS). The tubes were drained and dried by inversion. 1 % w/v of Safranin was used to stain the dried tubes for 5 minutes (**Yazdani *et al*., 2006**). Presence of adherent stained film was taken as positive result. However, adherent stained film at liquid and air interface was disregarded as positive result.

Biofilm production assay - Microplate Method (MPM)

Quantitatively, biofilm formation among the isolates was evaluated using micro plate method described by **Pfaller *et al*., (1988**). A 10 minutes UV sterilized 96 wells tissue culture plate was used to grow the bacteria for 18 – 24 hours at 30 ^{o}C. 20 µL cell suspension from overnight TSB culture was used to inoculate the wells containing 200 µL TSB to give a dilution of 1: 100. This was performed in triplicate. Uninoculated TSB was used as negative control while *A. hydrophila* ATCC 7699 was used as positive control respectively. After incubation, free cells were removed using 1000 µL pipette (Eppendorf) and then washed thrice with 1 × PBS. Plates were stained with 1 % Safaranin for 5 minutes before draining (**Turkyilmaz and Esklizmitliler, 2006)**. Microplate reader (Bio Rad 680 model) was used to read the plates at 540 nm wavelength. Average optical density (OD) of each triplicate result was taken including positive and negative controls. Isolates were grouped according to modified methods of (**Turkyilmaz and Esklizmitliler, 2006; Cevahir *et al.*, 2008; Tahmourespour *et al.*, 2010** and **Hassan *et al.*, 2011**) as non biofilm producer (ODi < ODc); weak ($OD_c < OD_i < 0.1$); moderate ($OD_i = 0.1 < 0.12$) and strong producers ($OD_i > 0.12$).

Motility test

Modified method of **Kirov *et al.*, 2009** was used for this test. Swarm agar (1 % w/v tryptone, 0.5 % w/v NaCl and 0.6 % w/v Bacto agar) and swim agar (1 % w/v tryptone, 0.5 % w/v NaCl and 0.25 % w/v Bacto agar) were used. Isolates were spot inoculated at the centre and incubated overnight at 30 ^{o}C. Plates with bacterial growth spreading from the spot of inoculation to the edge of the plate were noted as positive.

Antibiotic resistance pattern

Antibiotic susceptibility of *A. hydrophila* isolates and indicator bacteria was carried out using Mueller-Hinton agar (MHA, Merck) following manufacturer's instruction by agar disc diffusion method firstly described by **Bauer *et al.* (1966**). Each isolate was aseptically streaked on MHA using sterile swab. The following antibiotics discs were then placed on the surface of the solidified Agar and allowed to diffuse into the agar for 10 -15 minutes before incubating at 30 ^{o}C for 18 - 24 hours : Vancomycin 5 µg, Polymycin B 300IU, Ciprofloxacin 5 µg, Tetracycline 15 µg, Neomycin 30 µg, Novobiocin 30 µg, Ampicillin 10 µg, Kanamycin, Oxytetracycline and Bacitracin 2IU. Multidrug resistance (resistance to more ≥ 3 antibiotics tested) according to **Oteo *et al.*, (2005**) was noted. Result was interpreted as sensitive – inhibition zone ≥ 18mm, intermediate – inhibition zone 13 - 17 mm and resistance – inhibition zone < 13 mm (**Okonko *et al.*, 2009).**

Multi Antibiotic Resistance index (MARI)

This was carried out as described by **Matyar *et al.*, (2007)** with slight modification. MARI = resistant antibiotics ÷ total antibiotics tested. MARI values > 0.2 indicate existence of isolate(s) from high – risk contaminated source with frequency use of antibiotics (s) while values ≤ 0.2 show bacteria from source with less antibiotics usage (**Krumperman 1985**).

Resistance to Heavy Metals

Resistance of the isolates to heavy metals was carried out using modified method of **Sharma and Thapaliya, (2009).** The following Merk manufactured metal ions Cobalt (Co^{2+}),

Cadmium (Cd^{2+}), Chromium (Cr^{3+}), Zinc (Zn^{2+}), Mercury (Hg^{2+}), Copper (Cu^{2+}), Manganese (Mn^{2+}), and Nickel (Ni) were added as $CoCl_2.6H_2O$, $CdSO_4.8H_2O$, $Cr(NO_3).9H_2O$, $ZnSO_4.7H_2O$, $HgCl_2$, $CuSO_4$, $MnSO_4.H_2O$ and $NiSO_4.6H_2O$ salts with the following concentrations 50 µg/mL, 150 µg/mL, 300 µg/mL, 450 µg/mL, 600 µg/mL and 750 µg/mL respectively for all the metals. The heavy metals were added to TSA while isolates were streaked on each plate and then incubated at 30 ^oC for 24 hours. Plates were observed for visible growth at 24 and 48 hours of incubation respectively. Minimum Inhibitory Concentration (MIC) for the bacteria was hence noted as lowest concentration that inhibited growth of the bacteria (**Akinbowale *et al.*, 2007**).

RESULTS AND DISCUSSION

Table 1 Biofilm production

	Biofilm production and optical densities	
Isolates	**OD_i ± SD**	**Biofilm production status**
Sw.KMJ-1	0.076±0.01	weak producer
Sw.KMJ-2	0.065±0.05	weak producer
Sw.KMJ-3	0.120±0.03	strong producer
Sw.KMJ-4	0.065±0.003	weak producer
Sw.KMJ-5	0.095±0.02	weak producer
Sw.KMJ-6	0.119±0.02	moderate producer
Sw.KMJ-7	0.098±0.005	weak producer
Sw.KMJ-8	0.060±0.005	weak producer
Sw.KMJ-9	0.146±0.01	strong producer
Sw.KMJ-10	0.107±0.009	moderate producer
Sd.KMJ-11	0.090±0.01	weak producer
Sd.KMJ-12	0.141±0.02	strong producer
Sd.KMJ-13	0.113±0.01	moderate producer
Sd.KMJ-14	0.165±0.04	strong producer
Sd.KMJ-1 5	0.063±0.006	weak producer
Control	0.058±0.002	

Legend: Non biofilm producer (ODi < ODc); weak ($OD_c < OD_i < 0.1$); moderate ($OD_i = 0.1 < 0.12$) and strong producers ($OD_i > 0.12$).

Biofilm production

The three methods used in this study are CRA, STM and MPM respectively. Congo Red Agar was used for preliminary screening of the isolates for slime production. After 18 – 24 hours incubation at 30 ^{o}C, Sw.KMJ-3 and Sw.KMJ-9 were positive for slime production as a result of formation of black consistent crystalline colonies (Figure 1). All the isolates were further studied for biofilm production using STM and MPM respectively (Table 1). Six of the isolates produced biofilm in Standard Tube Method. However, MPM helped in grouping all the isolates into weak, moderate and strong biofilm producers. Eight (53.3 %) of the isolates were weak producers with observed optical density greater than the optical density for the control (ODi < ODc < 0.1), 3 (20 %) moderate producers (OD = 0.1 < 0.12) and 4 (26.6 %) are strong biofilm producers (OD > 0.12).

Table 2 Antibiogram

	Antibiotics resistance profile
Isolate	**Antibiotics**
Sw.KMJ-1	Amp, Bac, Kan, Neo, Oxt, Pb, Rl, Sul
Sw.KMJ-2	Amp, Bac, Cip, Pb, , Rl, Su, Te,Van
Sw.KMJ-3	Amp, Bac, Kan, Neo, Oxt, Pb, Rl, Su
Sw.KMJ-4	Kan, Neo, Pb, Oxt, Rl, Su, Van
Sw.KMJ-5	Amp, Bac, Cip, Oxt, Pb, Rl, Su, Te
Sw.KMJ-6	Bac, Neo, Oxt, Pb, Rl, Su, Te
Sw.KMJ-7	Amp, Bac, Cip, Kan, Neo, Oxt, Pb, Rl, Su, Te
Sw.KMJ-8	Bac, Cip, Pb, Rl, Su, Te
Sw.KMJ-9	Amp, Bac, Nov, Oxt, Pb, Rl
Sd.KMJ-10	Amp, Bac, Kan, Oxt, Pb, Rl, Te, Van,
Sd.KMJ-11	Amp, Bac, Kan, Nb, Oxt, Pb, Rl, Te
Sd.KMJ-12	Amp, Bac, Kan, Nov, Oxt, Pb, Rl, Su, Te
Sd.KMJ-13	Amp, Bac, Nov, Oxt, Pb, Rl, Te,Van
Sd.KMJ-14	Amp, Bac, Kan, Oxt, Pb, Rl, Su, Te
Sd.KMJ-15	Amp, Bac, Kan, Neo, Oxt, Pb, Rl, Su, Te

Legend: Amp = Ampicillin; Bac = Bacitracin; Kan = Kanamycin; Cip = Ciprofloxacin; Pb = Polymycin B; Neo = Neomycin; Nov = Novobiocin; Su = ; Te = Tetracycline ; Van = Vancomycin; Rl = ; Oxt = Oxytetracycline

Antibiotics Resistance

Results obtained from the antibiotic susceptibility test reveal that all the isolates were resistant to between 6 and 10 antibiotics. Two isolates each were resistant to 6 (50 %) and 7 (58.3 %) and 9 (75 %) antibiotics respectively. Eight *Aeromonas* species were resistant to 8(66.6 %) antibiotics and lastly 1 isolate (Sw.KMJ-7) was resistant to 10 antibiotics as seen in Table 2.

Table 3 Motility test assay

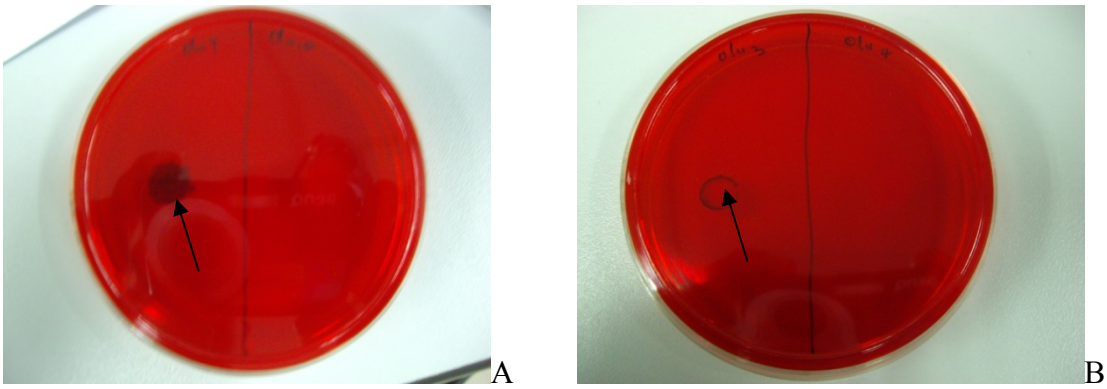
Isolates	Swarming agar	Swimming agar
Sw.KMJ-1	-	-
Sw.KMJ-2	+	+
Sw.KMJ-3	+	+
Sw.KMJ-4	+	+
Sw.KMJ-5	+	+
Sw.KMJ-6	+	+
Sw.KMJ-7	+	+
Sw.KMJ-8	-	+
Sw.KMJ-9	-	+
Sd.KMJ-10	+	+
Sd.KMJ-11	+	+
Sd.KMJ-12	+	+
Sd.KMJ-13	+	+
Sd.KMJ-14	+	+
Sd.KMJ-15	-	-

Legend: + = positive , - = negative

Motility assay

Results of motility assay show that all the isolates were flagellated except Sw.KMJ-1, Sw.KMJ-8, Sw.KMJ-9 and Sw.KMJ-15. Four isolates (Sw.KMJ-1, Sw.KMJ-8, Sw.KMJ-9 and Sw.KMJ-15 did not displayed swarming cultural characteristics on Swarming agar while only 2 (Sw.KMJ-1 and Sw.KMJ-15.) did not displayed similar characteristics in swimming

agar (Table 3). Correlation between the results of the two media used for motility assay indicates most of the bacteria are flagellated. However, this was not confirmed to either be polar or lateral flagellation.



A B

Figure 1 Slime production on CRA: (A) Sw.KMJ-9 and (B) Sw.KMJ-3 – strong and a weak slime produces.

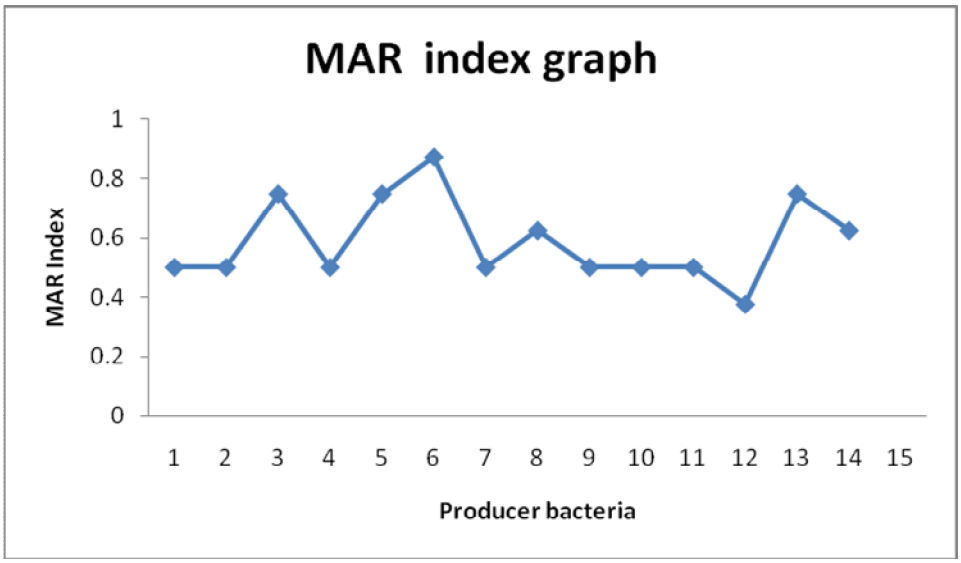


Figure 2 Multi Antibiotics Resistance index

Heavy metals resistance

The isolates tested showed varying degree of resistance to the heavy metals tested. The MIC also varies. For Cadmium (Cd^{2+}) – 13 (300 µg/mL), 1 (50 µg/mL), 1(150 µg/mL); Cobalt (Co^{2+}) – 2 (300 µg/mL), 5 (450 µg/mL), 2 (600 µg/mL), 2 (750 µg/mL) while 4 of the isolates were not inhibited by the highest concentration of the metal tested. Nickel (Ni) – 7 (300 µg/mL), 8 (450 µg/mL), Manganese (Mn^{2+}) could only inhibit the growth of 2 of the isolates at 750 µg/mL while 13 isolates where not inhibited. Mercury (Hg^{2+}) also inhibited

growth of 6 bacteria at 600 µg/mL while 9 were not inhibited even at the highest concentration tested. Similarly, Zinc (Zn^{2+}) inhibited growth of 2 bacteria at 750 µg/mL while 13 isolates were not inhibited (Table 4). However, the isolates were inhibited at 600 µg/mL (10) and 750 µg/mL (5) concentrations. It is noteworthy that all tested bacteria did not show any visible growth in all concentration of Zinc tested.

Table 4 Minimum Inhibitory Concentration

	MIC (µg/mL)							
Isolates	Cd	Cr	Co	Ni	Mn	Hg	Zn	Cu
Sw.KMJ-1	300	-	300	450	-	-	-	600
Sw.KMJ-2	300	-	300	300	-	-	-	600
Sw.KMJ-3	50	-	450	450	-	750	-	600
Sw.KMJ-4	300	-	450	300	750	750	-	600
Sw.KMJ-5	300	-	450	300	-	750	-	600
Sw.KMJ-6	300	-	450	300	-	750	-	750
Sw.KMJ-7	300	-	600	300	-	-	-	750
Sw.KMJ-8	300	-	450	300	-	-	-	750
Sw.KMJ-9	300	-	750	300	-	750	-	600
Sd.KMJ-10	300	-	-	450	-	-	-	750
Sd.KMJ-11	300	-	750	450	-	750	-	600
Sd.KMJ-12	150	-	-	450	-	-	-	600
Sd.KMJ-13	300	-	-	450	-	-	-	600
Sd.KMJ-14	300	-	-	450	-	-	750	600
Sd.KMJ-15	300	-	600	450	750	-	750	600

- = shows growth on all the concentrations tested.

Protease production

Only isolates Sw.KMJ-3, Sw.KMJ-8 and Sw.KMJ-9 produced protease enzyme on SMA. Virulence factors have been found to greatly influence bacterial pathogenicity (**Vadivelu *et al,* 1995 and Cevahir *et al.,* 2008**). Among mostly studied virulence factors in *Aeromonas* spp. are protease, lipases, hemolysis, aerolysis flagellation and biofilm production. In a report by **Bagyalakshmi *et al.,* (2009),** occurrence of hemolysin, lipase, protease, gelatinase and

caseinase was established as virulence factors in *Aeromonas* spp. isolated from Bhavani River, Tamil Nadu, India. *A. hydrophila* isolated from both environmental and clinical samples have been described to produce protease and slime (**Subashkumar *et al.*, 2006**). Fifteen presumptive isolates were randomly selected and characterized both phenotypically and genotypically. Morphologically, round, flat, green colonies were selected and Gram stained. Alarming increase in resistance of *Aeromonas* spp. to various antibiotics is of significance to public health (**Palu *et al.*, 2006**). Various research on antibiotics resistance of *Aeromonas* spp. isolated from food (**Rabu *et al.*, 2003**), clinical samples (**Overman and Janda, 1999**), European rivers (**Goni-Urriza *et al.*, 2000**), treated and untreated water, fish gut and fresh water fish (**Vijayabaskar and Somasundaram, 2008**) have been carried out. **Matyar *et al.*, (2007**) revealed in their study on antibiotics resistance of *Aeromonas* spp. isolated from Turkish water that 14.4 % were resistant to tetracycline, 11.3 % chloramphenicol while 7.2 % were resistant to gentamycin.

All the isolates were resistant to between 6 and 10 antibiotics. Two isolates each were resistant to 6 (60 %), 7 (70 %) and 9 (90 %) antibiotics respectively. Eight of the isolates showed resistance to 8 (80 %) antibiotics while only isolate Sw. KMJ-7 showed resistance to all the tested antibiotics (Table 2). According to **Matyar *et al.*, (2007)** and **Krumperman (1985)**, MARI values > 0.2 indicate existence of isolate(s) from high – risk contaminated source with frequency use of antibiotics (s) while values ≤ 0.2 show bacteria from source with less antibiotics usage. This value (MARI > 0.2) shows indiscriminate use of antibiotics among rural dwellers in riverine area of Sungai Melayu, Johor as the value was > 0.2 for the isolates (Figure 2). **Subashkumar *et al.*, (2006)** obtained similar result. They investigated resistance of *A. hydrophila* from clinical isolates obtained from children. 95 % (n = 20) of total isolates (n = 21) have MAR index value greater than 0.2 depicting high level of antibiotic resistance due to either indiscriminate use of antibiotics or horizontal gene transfer. It could also be combination of the two factors. In another related research by **Matyar *et al.*, (2007),** *A. hydrophila* isolated from Turkish water showed MAR index between 0.2 and 0.8 unlike *A. sobria* that have high values. Hence, *Aeromonas* spp. most especially *A. hydrophila* have exhibited high levels of antibiotics resistance in the environment and clinical isolates. **Vadivelu *et al.*, (1995)** stated that protease from bacteria was required to cause disease in host cell as a result of 94 % of tested bacteria were positive for protease production in vitro. However, **Castro – Escarpulli *et al.*, (2003)** stated that protease production was prevalent in

clinical isolates than environmental isolates because protease is required for host colonization. They reported protease as a major virulence enzyme in the isolates.

In this current study, only isolates Sw.KMJ - 3, Sw.KMJ - 8 and Sw.KMJ - 9 exhibited proteolytic activities on Skim Milk Agar (SMA). It could be concluded that some *Aeromonas* spp. from estuary sources although are producers of antimicrobial polypeptides, yet they produced virulence factors such as biofilm production and protease when grown under appropriate conditions of growth. Slime comprises of polysaccharides and proteins (**Hassan *et al.,* 2011**). Biofilm production involves series of biochemical reactions. It is a complex slime matrix surrounding cells. According to **Hassan *et al.,* (2011)** biofilm producing bacteria exhibit resistance to more antibiotics than non producers' especially plank tonic bacteria. Biofilms aid in surface attachment of microorganisms. This provides conditions favorable for the growth of bacteria. Existence of biofilm producing *Aeromonas* spp. poses a serious danger to public health especially to riverside dwellers. Biofilm extracellular polysaccharide (EPS) matrix serves as barrier to incoming antimicrobial agents. According to **Heithoff and Mahan (2004),** biofilm formation involves cell mobility; attachment to either biomaterial or non biomaterial surfaces and maturation of the cells and thereby producing extracellular matrix that protects the micro colonies from environmental influences. Evaluation of biofilm formation among *Aeromonas* spp. isolated from estuarine environment and correlation of biofilm production to antibiotics resistance among the isolates was investigated. The three methods used in this study are CRA, STM and MPM respectively. Congo Red Agar was used for preliminary screening of the isolates for slime production. After 18 – 24 hours incubation at 30 ^{o}C, Sw.KMJ-3 and Sw.KMJ-9 were positive for slime production as a result of formation of black consistent crystalline colonies. All the isolates were further studied for biofilm production using STM and MPM respectively. Six of the isolates produced biofilm in Standard Tube Method. However, MPM helped in grouping all the isolates into weak, moderate and strong biofilm producers. Eight (53.3 %) of the isolates were weak producers with observed optical density greater than the optical density for the control (ODi < ODc < 0.1), 3 (20 %) moderate producers (OD = 0.1 < 0.12) and 4 (26.6 %) are strong biofilm producers (OD > 0.12). Strong biofilm producers were resistant to more antibiotics tested compared to moderate and weak producers. This was in accordance with the study of **Hassan *et al.,* (2011).** They investigated biofilm formation among bacteria isolated from clinical samples in Pakistan and were able to classify the isolates into weak, moderate and strong biofilm producers.

CONCLUSION

The findings of the study indicates that isolated *Aeromonas* species from marine environment posses various virulence factors in – vitro especially resistance of the isolates to both antibiotics and heavy metal. It also reveals that phenotypic evaluation of Aeromonas isolates from marine environment for putative virulence factors can serve as indicator for the presence of pathogenic nature of the bacteria before using molecular confirmatory method. Hence, evaluation of phenotypic virulence factors hence serve as rapid method for detection virulence *A. hydrophila* from marine environment.

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