

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.707.108>

Determination of Minimum Inhibitory Concentration (MIC), Mutation Frequency and Plasmid Extraction and Transformation of Antibiotic resistant bacterial isolate from Aquatic Environment

Hemanta Pokhrel*, Salini Gopi, Devika Pillai, C.G. Rakesh and K.G. Sneha

Centre for Aquatic Animal Health Management, School of Aquaculture and Biotechnology
Kerala University of Fisheries and Ocean Studies, India

*Corresponding author

ABSTRACT

Keywords

Antimicrobial resistance, Minimum inhibitory concentration (MIC), Mutation, Plasmid transformation, Conjugation

Article Info

Accepted:
08 June 2018
Available Online:
10 July 2018

The present study is done to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium. MIC values are also used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents, mutation frequency the several factors that affect the appearance and spread of acquired antibiotic resistance and plasmid extraction and transformation demonstrates that conjugation and transfer of plasmids, a phenomenon that belongs to the environment and can occur between bacterial strains of human, animal, and fish origins that are unrelated either evolutionarily or ecologically even in the absence of antibiotics. MIC concentration in the study was found to be in the higher side. For maximum number of strains MIC for ampicillin ranged from 2 to 64 μg with maximum at 64 μg shown by *Kluyvera intermedia* and minimum 2 μg shown by various strains like *Acinetobacter johnsonii*, *Edwardsiella spp* and *Pseudomonas alcaligenes*. Mutation frequency was also done in order to determine the rate at which the isolates mutate to acquire resistance for a particular antibiotic. The results indicate that bacteria can mutate and develop resistance to an antibiotic on prolonged exposure. Plasmid extraction and transformation process was also performed in order to understand the process of resistance transformation. One of the major mechanisms of transfer of AMR was found to be plasmid mediated.

Introduction

Antimicrobial resistance (AMR), which is one of the most serious global public health concerns in today's world, has almost reached crisis stage in human medicine. The rapid acceleration of multidrug-resistant bacteria in the past decades has overtaken new drug development and patients and clinicians are

faced with the prospect of untreatable infections. Although much of the problem stems from overuse and misuse of antimicrobial agents in human medicine, large-scale use of antimicrobials in aquaculture and agriculture field for enhanced production, which also contributes to the crisis. Aquaculture and agricultural uses of antibiotics which produce direct or indirect

environmental exposures in a variety of water bodies, which select for resistant microbes and microbial genes. Although various authors also have emphasized the putative negative effects of using antimicrobial agents in fish farms (Cabello, 2006), few studies on antimicrobial resistance in the aquaculture industry have been performed *in situ* (Johnson *et al.*, 2010). However, disease control is an active research field, and alternatives to antibiotic treatments are being explored. The public health hazards related to antimicrobial use in aquaculture include the development and spread of antimicrobial-resistant bacteria and resistance genes and the presence of antimicrobial residues in aquaculture products and the environment.

Antimicrobials are now ubiquitous in the environment. They are used in human medicine by prescription, in over-the-counter preparations, by veterinarians to treat disease in animals, in cleaning products and other consumer products, as pesticides, in aquaculture (fish farming), and in animal agriculture (Levy, 1998). The largest use of antimicrobial agents outside human medicine is in food animals (Swartz, 1989). During the past 30 years, studies have shown that use of antimicrobials in food animal production promotes the development and subsequent dissemination to humans of resistant organisms (NRCCD, 1999). Resistance genes are bred and transferred within environmental reservoirs in which bacteria and antimicrobial agents coexist. Obvious reservoirs include the guts of humans and animals, in which horizontal resistance gene transfer has been documented among pathogenic and commensal species (Shoemaker *et al.*, 1992 and Kuramitsu (2001). Active antibiotics have been identified in water near wastewater treatment plants, animal waste lagoons surface waters, and river sediments (Myer, 2000). This has resulted in speculation that environmental contamination with antibiotics

can augment the selection and dissemination of resistance genes through a wide variety of routes involving animal and nonanimal reservoirs.

Thus this study presents the major lines of evidence documenting the risks to human health of some of the use and misuse of antimicrobials in hospital, aquaculture and in agricultural.

Materials and Methods

Isolation and identification of bacteria

The bacterial colonies that grew on the media were selected, purified and subjected to morphological and biochemical tests for identification. Tests carried out for identification included Gram's stain, catalase, oxidase, coagulase, motility, O-F, indole, gelatin hydrolysis, methyl-red, Voges-Proskauer, ONPG, lysine decarboxylase, citrate utilization, Triple Sugar Iron (TSI) agar, nitrate reduction and fermentation of sugars such as Fructose (Fc 25mg), Melibiose (Me 25mg), Sucrose (Su 25mg), Cellobiose (Ce 25 mg), Xylose (Xy 25mg), Galactose (Ga 25mg), Mannose (Mo 25mg), Trehalose (Te 25mg), Inositol (Is 25mg), Dulcitol (Du 25mg), Dextrose (De 25mg), Mannitol (Mn 25mg), Lactose (La 25mg), Adonitol (Ad 25 mg), Sorbitol (Sb 25 mg), Rhamnose (Rh 25mg), Maltose (Ma 25mg), Raffinose (Rf 25mg), Arabinose (Ar 25mg), Salicin (Sa 25mg), Inulin (In 25mg).

Minimum Inhibitory Concentration determination (MIC)

The minimum inhibitory concentration (MICs) of the different antibiotics was also determined by the standard agar diffusion test (Bauer *et al.*, 1996) on Muller-Hinton agar for different bacterial isolates. 18 different Hi comb Minimum inhibitory concentration strips

(MICs strips Hi media) which were used in the study were Ampicillin (AMP 240-0.01 μ g), Amikacin (AK 256-0.001 μ g), Azithromycin (AZM 128-0.0001 μ g), Carbenicillin (CB 512-0.01 μ g), Cloramphenicol (C 240-0.001 μ g), Co-trimoxazole (COT 240-0.001 μ g), Ciprofloxacin (CIP 240-0.001 μ g), Cefepime (CPM 240-0.001 μ g), Erythromycin (E 240-0.001 μ g), Gentamicin (GEN 240-0.001 μ g), Kanamycin (K 240-0.001 μ g), Methicillin (MET 240-0.001 μ g), Nalidixic acid (NA 240-0.001 μ g), Nitrofurantion (NIT 240-0.001 μ g), Polymyxin-B (PB 240-0.001 μ g), Streptomycin (S 240-0.001 μ g), Tetracycline (TE 240-0.001 μ g) and Vancomycin (VA 256-0.001 μ g).

The plates were prepared and bacterial culture was spread on the plates as described in the previous section on antibiotic susceptibility test. Hi comb MIC strips (Hi media, Mumbai) were placed on the agar media with the MIC scale facing upward with the help of a sterile forceps. The plates were then incubated at room temperature for 12 to 15 h and were observed. Zones of growth inhibition were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) standard (CLSI, 2009).

Mutation frequency

Two vancomycin sensitive strain, viz., *Klebsiella oxytoca* and *Enterobacter aerogenes* isolated from water and sediments samples from the sampling stations were used for determining the mutants resistant to vancomycin. Mutation frequency was estimated by the method of Barnes *et al.* (1991).

Two conical flasks, each containing 100ml of drug free Tryptone soya broth (TSB) were inoculated with vancomycin sensitive strains *Klebsiella oxytoca* and *Enterobacter aerogenes*. The inoculated broth was shaken

overnight at room temperature. The resulting cultures were then centrifuged at 10000g for 8 min. The supernatant was decanted and the pellet was resuspended in 2 ml of freshly prepared TSB to obtain a cell concentration of about 10^{10} cfu ml⁻¹. The culture was then diluted for four- folds i.e. 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} in pre-autoclaved aliquots. The diluted cultures were then spread properly and aseptically on TSA plates containing antibiotics at 5, 10, 15, 20 times the MIC. The inoculated plates were incubated at 22^oC and examined daily for 7 days for the presence of any resistant colonies. Resistant colonies which appeared on the plate were again subcultured onto fresh TSA plate containing an antibiotic. The number of colonies on the plates was counted and mutation frequency was calculated (Lee *et al.*, 2014).

Plasmid extraction

Plasmids were isolated from all the isolates by the method of (Elkin *et al.*, 2001) with modification. 5 ml of LB broth was inoculated with a single colony and grown overnight at 37^oC. Pellet was obtained by centrifuging the colony at 8000rpm at 4^oC. Pellet was suspended in 150 μ l of GTE buffer (50mM glucose, 25mM Tris HCl, 10mM EDTA (pH 8.0)], vortexed well and kept at room temperature for 5 min. Then 150 μ l of lysis solution (0.2M NaOH and 1%SDS) was added and kept at room temperature for 5 min for lysis of cells. To the lysate, 150 μ l of neutralization buffer (5M potassium acetate; pH 4.5) was added and kept on ice for 10 min, followed by centrifugation at 8000rpm for 10 min. The supernatant was transferred to a fresh tube and precipitated with 0.7 volume of isopropanol and kept at RT for 10 min. The tubes were centrifuged at 14000 rpm for 20 min to pellet the plasmids. Pellet was dried and resuspended in 50 μ l TE buffer. 5 μ l of plasmid DNA was then mixed with 1 μ l of 6X loading dye and subjected to electrophoresis in

1% agarose gel. The bands were visualized in a gel documentation system (Biorad, USA). The molecular weight of plasmids was determined by comparing with *Escherichia coli* V517 plasmids.

Preparation of *E coli* HB101 competent cells

Single colony of *E coli* HB101 was inoculated into 10 ml of LB broth and grown overnight at 37°C with constant shaking at 150 rpm. The following day, 1 ml of overnight culture was transferred into 100 ml of LB broth and incubated for 90-120 min with vigorous shaking at 200 rpm at 37°C. Optical density at 600 nm (OD₆₀₀) was measured after every 30 min. When OD₆₀₀ reached 0.4-0.5, culture was chilled on ice, transferred to pre-chilled 2ml polypropylene tubes and centrifuged at 4000 × g for 10 min using cooling centrifuge (Remi). The supernatant was discarded and the cell pellet was suspended gently without damaging the cells in 1ml of ice cold 0.1M CaCl₂ and the suspension was kept on ice for an additional 90 min and centrifuged at 2500 rpm at 4°C for 10 min. The supernatant was discarded carefully and the cell pellet was resuspended in 1ml of ice cold 0.1M CaCl₂. About 150 µl suspension of competent cells was aliquoted in 1.5 ml microfuge tubes, frozen and stored at -80°C.

Transformation

2 µl of the plasmid DNA was added into the thawed tube containing 200µl *E coli* HB101 competent cells. This was then mixed gently by tapping the tube and the transformation mixture thus obtained was incubated on ice for 20 min. During this incubation period, LB medium was pre-warmed to 42°C. Heat-shock was given to the transformation mixture by placing the tube in water bath set at 42°C for 45 sec followed immediately by incubating on ice for 2 min.

To this transformation reaction mixture, 250 µl of pre-warmed Luria Bertani medium was added. The competent cells were allowed to recover for 1.30 hours at 37°C with agitation on a horizontal shaker. 100 µl of the transformation mixture was then plated on LB-agar plates containing Vancomycin (30µg/ml) and incubated at 37°C for overnight.

Results and Discussion

In the present study a total of 56 bacteria were isolated, of which 32 from fish 12 from shrimp and 12 from clam Among the 105 isolates recovered from the samples collected, (90.47%) tested positive for Methicillin resistance by disc diffusion.

Minimum Inhibitory Concentration (MIC)

For maximum number of bacterial isolates MIC for ampicillin ranged from 2 to 64 µg with maximum at 64 µg shown by *Kluyvera intermedia* and minimum 2 µg shown by various strain like *Acinetobacter johnsonii*, *Edwarsiella spp* and *Pseudomonas alcaligenes*. The minimum value of MIC obtained for ampicillin was 0.016 µg for *Bacillus circulans*.

For methicillin, out of 16 isolates 10 were completely resistant. The strains which were resistant to methicillin were *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Acinetobacter johnsonii*, *Streptococcus spp*, *Edwarsiella spp*, *Pseudomonas alcaligenes* etc. For other strains MIC for methicillin ranged from 5 to 30 µg with maximum value 30 µg shown by *Flavobacterium breve* and minimum 5 µg shown by various isolates like *Bacillus circulans*, *Enterobacter aerogenes* and *Penaeibacillus lautus*. The minimum value of MIC for methicillin was 0.1 µg for *Enterococcus phoeniculicola*. For maximum number of strains MIC for Vancomycin

ranged from 2 to 128 µg with maximum ranged 128 µg for *Streptococcus equi* and minimum 2 µg for *Enterococcus phoeniculicola*. The minimum MIC value for vancomycin was 0.064 µg by *Enterobacter aerogenes* (Fig.1).

Similarly for other strains, the maximum MIC value reported for ciprofloxacin and streptomycin was 240 µg, while for carbenicillin the value was 128 µg and for polymyxin B and erythromycin the MIC value was 120 µg. The MIC results for various antibiotic used against different bacterial isolate are shown in the Table 1.

Mutation frequency

The mutation frequencies at which two vancomycin susceptible strains of *Klebsiella oxytoca* and *Enterobacter aerogenes* mutated to develop resistance when exposed to vancomycin, were determined at drug concentration of 5, 10, 15 and 20 times of their respective MIC's 0.256µg and 0.064 µg. Mutants' resistance to vancomycin isolated at 5, 10 and 15 times the MICs frequencies, is given in Table 2. However, at 20 times the MIC, the mutation frequency of *Klebsiella oxytoca* was not detectable (ND), which was the limit of detection of this experiment. Since the MIC concentration of *Enterobacter aerogenes* was less (0.064 µg) the mutation frequencies obtained was less compared to *K. oxytoca* and mutation was not recorded at 15 and 20 times of MIC for the isolate (Table 3.).

Plasmid extraction and transformation

Transformation was done with isolated strains containing the resistance gene encoded plasmid as the donor cells and the *E.coli* HB101 strains as the recipients (Chen and Dubnau, 2004). A total of 105 strains were segregated during the study period and among these, 15 were used for the plasmid extraction. Out of 15 selected multidrug

resistant isolates, 10 strains showed detectable plasmids, and these 10 strains were used for the plasmid transformation study. The names of the strains and their plasmid profile are given in Table 4 and in figure 2 respectively. The plasmid from *Escherichia coli* V.157 was used as marker to determine the size of the plasmid (Macrina *et al.*, 1982.)

Plasmid analysis revealed that there were detectable plasmids in 10 isolates out of 15 selected multidrug resistant isolates. Five of the isolates possessed no plasmid, two of the isolates viz., *Bacillus circulans* and *Streptococcus spp* possessed single plasmids of size 4.4 (lane 4) and 6.6 kb (lane 5), respectively.

Acinetobacter johnsonii was found to harbour 6 plasmids of size ranging from 2 to 23kb (lane 6). *Enterobacter aerogenes* (lane3) and *Enterococcus phoeniculicola* (lane 7) was found to carry four numbers of plasmid each. Four of the isolates *Klebsiella pneumonia* (lane1), *Klebsiella oxytoca* (lane 2), *Enterobacter aerogenes*, *Enterococcus phoeniculicola* harboured a similar sized common plasmid of size 4.4kb.

After the transformation process the isolates were exposed to varying concentrations of 18 different antibiotics. The transformed strains showed resistance to different antibiotics to which they were initially susceptible. The list of antibiotics to which it gained resistance after transformation is given below in Table 5.

Among all the different isolates from the study area, the most commonly and frequently encountered strain was *Klebsiella pneumonia* and *Klebsiella oxytoca*. Among them, *Klebsiella pneumoniae* was the predominant strain in all samples. Being clinical isolates, their presence in significant numbers distinctly point to their source as the hospital effluents.

Table.1 Showing MIC results for various antibiotics used against different bacterial isolate

Test	Hi comb MIC strips												
	Isolated strains												
Name	<i>Enterococcus phoeniculicola</i>	<i>Bacillus circulans</i>	<i>Acinetobacter johnsonii</i>	<i>Streptococcus equi.</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella oxytoca</i>	<i>Acinetobacter johnsonii</i>	<i>Flavobacterium breve</i>	<i>Kluyvera intermedia</i>	<i>Edwardsiella Spp</i>	<i>Serratia automophila</i>	<i>Pseudomona alcaligenes</i>	<i>Enterobacter aerogenes</i>
AMP	0.016	0.016	2	8	-	-	2	4	64	2	4	2	4
AK	0.01	0.01	0.1	0.1	256	4	0.1	0.01	0.1	0.1	-	0.1	-
AZM	0.10	0.01	0.1	0.01	-	-	.1	0.01	0.01	0.1	0.5	0.1	0.5
CB	1	4	-	-	-	16	-	-	32	128	4	-	4
C	0.01	0.1	0.1	0.1	-	10	0.1	10	0.01	0	1	0.1	1
CIP	0.01	0.01	0.1	0.01	240	10	0.1	0.01	0.004	0.01	0.1	0.1	0.1
COT	10	0.01	10	5	-	-	10	.1	5	0.1	-	-	-
CPM	0.1	-	0.1	1	-	7.5	0.1	1	-	30	5	0.1	5
E	0.01	0.1	120	5	-	-	120	5	5	-	0.1	120	0.1
GEN	0.01	0.01	0.1	0.1	-	2	0.1	1	10	5	-	0.1	-
K	0.1	0.1	1	5	-	60	1	1	1	10	5	1	10
MET	0.1	5	-	-	-	-	-	30	-	-	5	-	5
NA	0.1	0.1	0.1	0.1	-	-	0.1	0.1	0.1	5	0.1	0.1	5
NIT	0.1	10	10	5	-	-	10	-	10	-	5	10	5
PB	5	5	5	5	120	5	5	0.1	10	-	5	5	5
S	0.1	0.1	1	0.1	240	3	1	0.1	0.1	10	1	1	1
TE	0.01	0.01	0.1	0.01	-	0.25	0.1	0.01	0.01	0.1	0.1	0.1	0.01
VA	2	4	64	128	-	0.246	64	32	-	-	-	64	0.064

Table.2 Range of mutation frequencies for vancomycin sensitive strain of *Klebsiella oxytoca*

Drug	Mutation frequencies at			
	5 × MIC	10 × MIC	15 × MIC	20 × MIC
Vancomycin (R ₁)	3.5-3.84×10 ⁻³	2.15-2.33×10 ⁻³	0.24-1.1×10 ⁻³	ND
(R ₂)	3.2-3.6×10 ⁻³	2.0-2.1×10 ⁻³	0.16-0.9×10 ⁻³	ND

Table.3 Range of mutation frequencies for vancomycin sensitive strain of *Enterobacter aerogenes*

Drug	Mutation frequencies at			
	5 × MIC	10 × MIC	15 × MIC	20 × MIC
Vancomycin (R ₁)	1.53-1.86×10 ⁻³	0.15-0.55×10 ⁻³	ND	ND
(R ₂)	1.23-1.43×10 ⁻³	0.11-0.21×10 ⁻³	ND	ND

Fig.1 Showing methicillin resistant *Klebsiella pneumoniae*

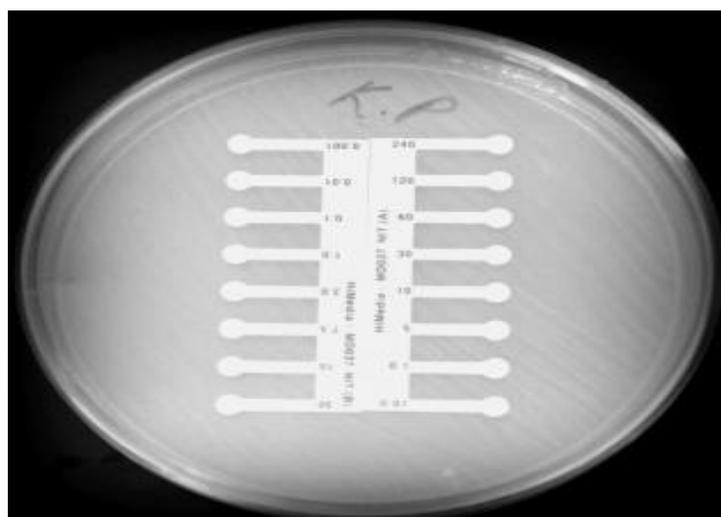


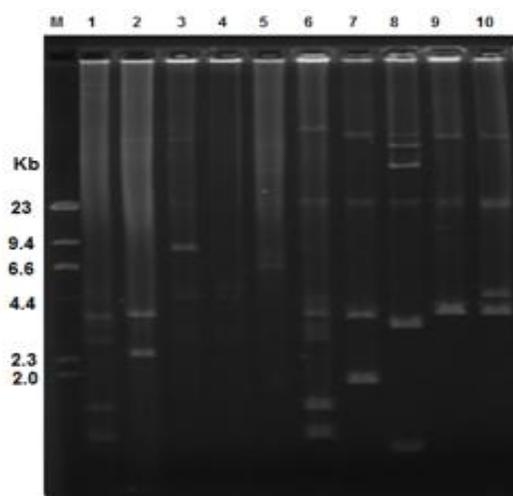
Table.4 Table of Plasmid profilation

S.No	Name of the strains
1	<i>Klebsiella pneumoniae</i>
2	<i>Klebsiella oxytoca</i>
3	<i>Enterobacter aerogenes</i>
4	<i>Bacillus cirulans</i>
5	<i>Streptococcus equi</i>
6	<i>Acinetobacter johnsonii</i>
7	<i>Enterococcus phoeniculicola</i>
8	<i>Pseudomonaalcaligenes</i>
9	<i>Serratia antomophila</i>
10	<i>Flavobacterium breve</i>

Table.5 Result of transformation of resistant gene

Antibiotic	Name of the isolate	
	<i>Enterobacter aerogenes</i>	<i>Enterococcus phoeniculicola</i>
AMP	+	+
AK	+	-
AZM	-	-
CB	-	+
C	+	-
CIP	-	+
COT	+	-
CPM	+	-
E	+	+
GEN	+	+
K	-	-
MET	-	-
NA	-	+
NIT	-	-
PB	+	-
S	-	-
TE	-	+
VA	-	-

Figure.2 Showing plasmid profilation



Minimum Inhibitory Concentration (MIC)

Klebsiella pneumoniae and *Klebsiella oxytoca* showed resistance to almost all the antibiotics tested for MIC and for a few to

which they were sensitive, the concentration of antibiotic was very high. For *Klebsiella pneumoniae* MIC range for amikacin, ciprofloxacin, polymyxin B and streptomycin are 256 µg, 240 µg, 120 µg and

240 µg, respectively, whereas for *K. oxytoca* the value for carbenecillin, chloramphenicol and kanamycin are 16µg, 10 µg and 60 µg, respectively. Kumar *et al.*, (2006) and Siddiqui *et al.* (1992) reported MIC >256µg/ml and >500µg/ml for ampicillin in *Enterobacteriaceae* isolates.

Nadia (2008) showed that the MIC range of tetracycline, ciprofloxacin and azithromycin for *Staphylococcus aureus* were 0.12 mg/ml to 32 mg/ml. In the present study the MIC concentration for *Klebsiella pneumoniae* for amikacin, ciprofloxacin, polymyxin B and Streptomycin was found to be in the range < 240 µg which is much higher than the value recommended by Clinical and Laboratory Standards Institute (CLSI) standard (CLSI, 2009). Other strains like *Klebsiella oxytoca*, *Acinetobacter johnsonii*, *Streptococcus spp*, *Serratia antomophila* and *Pseudomonas alcaligenes* also showed resistance upto 4, 0.0, 8, 9, 4 and 2 µg respectively. However, these isolates were not as resistant to different antibiotics as *K. pneumonia* as the concentration of the antibiotics at which these isolates were sensitive was lesser than that for *K. pneumoniae* but still higher than the recommended value.

To our knowledge this is the first report of incidence of multi drug resistant *Klebsiella spp* isolated from the aquaculture farms. The strain which showed resistance is highly pathogenic to human health. Thus the MIC results suggest that the untreated sewage released with the hospital discharge may be the source for the bacteria to acquire and disseminate the resistance, and therefore pose a serious potential threat to human health.

Mutation frequency

Mutation frequency gives an idea of the rate at which resistance to a particular antibacterial is likely to develop during

therapy. *K. oxytoca* and *Enterobacter aerogenes* were exposed to vancomycin at 5, 10, 15 and 20 times their respective MICs. The isolates developed resistance at significant lower frequencies. For *Klebsiella oxytoca* colonies appeared upto 15 times of MIC whereas for *Enterobacter aerogenes* they appeared only upto 10 times of MIC. Similar kind of results was observed by Barnes *et al* (1992) when they exposed *Aeromonas salmonicida* to 15 to 20 times of the MIC. A previous study conducted by Stamm (1989) did not find any difference in mutation frequency between sarafloxacin and oxolinic acid. Mutation frequency results obtained in the present study can be explained by the 7day incubation period employed, compared to only 48 hr incubation in the study by Stamm (1989), as mutants to vancomycin were rarely seen at 48 hours of observation even in the present study, indicating that with frequent or longer duration of exposure to antibiotics is a sufficient cause for bacteria to mutate and acquire resistance.

Plasmid extraction and transformation

Plasmids are important vehicles for carrying antibiotic resistance genes (Bennett *et al.*, 2008). High organic load and diverse bacterial communities present in the environment present a unique opportunity for the evolution and transfer of antibiotic resistance genes. Plasmids can harbour genes that confer resistance to most clinically significant antibiotic classes such as macrolides, tetracyclines, cephalosporins, fluoroquinolones, aminoglycosides and β-lactam (Bennett *et al.*, 2008; Martinez, 2009).

In the present study 10 isolates were used for the extraction of plasmid, out of which seven strains found to harbour one to six plasmids ranging from 4.4 to 23 kbp. The isolates which were taken for plasmid extraction are the ones which either pose potential threat to

human health or are emerging bacterial fish pathogens like *K. pneumoniae*, *Acinetobacter johnsonii* etc. The strain *Acinetobacter johnsonii* isolated from the lakeshore fish sample was reported to carry highest number of six plasmids while *Enterobacter aerogenes* and *Enterococcus phoeniculicola* was found to carry four numbers of plasmids each. Similar results were reported by Li *et al.* (1999), Molina and Galetti (2002), Shafiani and Malik (2003) and Wang *et al.* (2006). This suggests that antibiotic resistance is encoded on high molecular weight multiple plasmids, and can easily spread in the community. However, in the present study it was noticed that the strains like *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes* and *Enterococcus phoeniculicola* which were harbouring more numbers of plasmids showed increased resistance to antibiotics. It has been reported that the isolate harbouring the single high molecular weight plasmid are responsible for multidrug resistance in different strains of *Aeromonas* strain (Roy *et al.*, 2003; Sorum *et al.*, 2003).

The results of transformation experiment of bacterial plasmid showed that antibiotic resistance in bacterial species is transferable to other bacterial genera (*E. coli*) through plasmids. Similar previous reports on transformation experiments were reported in plasmid of *Vibrio* isolates and from *Penaeid* shrimp (Liu *et al.*, 1999; Molina and Galetti, 2002). The results of the transformation experiment in the study using selected isolates as the donor and the *E. coli* HB 101 as the recipient, indicates that the majority of the plasmid associated resistance markers were transferred to the *E. coli* strain which was confirmed by antibiotic sensitivity test. In the study it was observed that *E. coli* which was initially sensitive to all the 18 different antibiotics showed resistance to more than 10 antibiotics after transformation. This revealed that the plasmid carry the resistant gene which can be transferred from one to other spp.

In conclusion, the present study indicates how the resistance get acquire and transform from the terrestrial to the aquatic organism. Stillthere is a long-standing debate over the exact role that antimicrobials play in the current antibiotic resistance crisis.

It is in everyone's best interest to slow down the development of antimicrobial resistance, but not a s single group can do it all alone. Although the precise combination of actions required to arrest the current global increase in resistance is unknown, essential elements must include as follows:

- 1) elimination of unnecessary use, overuse, and abuse of antimicrobial agents in all sectors;
- 2) universal adherence to principles of judicious use;
- 3) collection and analysis of data on antimicrobial use;
- 4) surveillance of antimicrobial resistance in all potential reservoirs;
- 5) mechanisms for identification of and rapid response to dangerous resistance trends;
- 6) application of infection control strategies, including hygiene and immunization, in human and animal settings; and
- 7) promoting aggressive research and development of new antimicrobial agents.

Acknowledgement

The authors would like to highly acknowledge the support rendered by Centre of Aquatic Animal Health Management, School of Aquaculture and Biotechnology, Kerala University of Fisheries and Ocean Studies for smooth running of the research work.

References

Barnes, D. E., A. E. Tomkinson and A.R. Lehmann (1992). "Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular

- hypersensitivity to DNA-damaging agents." *Cell* 69(3): 495-503.
- Bauer, A.W., Kirby, W.M.M., Serris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology* 45: 493-496.
- Bennett, J. W., C. K. Murray, RL Homes and James H. Jorgensen. (2008). "Diminished vancomycin and daptomycin susceptibility during prolonged bacteremia with methicillin-resistant *Staphylococcus aureus*." *Diagnostic microbiology and infectious disease* 60(4): 437-440.
- Elkin, C. J., P. M. Richardson, H. M. Fourcade, N. M. Hammon, M. J. Pollard, P. F. Predki, T. Glavina, and T. L. Hawkins. 2001. High-throughput plasmid purification for capillary sequencing. *Genome research* 11:1269-1274.
- Farrar WE Jr, Eidson M, Guerry P, Falkow S, Drusin LM, Roberts RB. Interbacterial transfer of R factor in the human intestine: in-vivo acquisition of R-factor-mediated kanamycin resistance by a multiresistant strain of *Shigella sonnei*. *J Infect Dis*. 1972; 126:27-33.
- Ines Chen and David Dubnau (2004), "Dna uptake during bacterial transformation". *Nature Reviews Microbiology* 2, 241-249.
- Kumar, M S., V. Lakshmi and R. Rajagopalan (2006). "Occurrence of extended spectrum beta-lactamases among Enterobacteriaceae spp. isolated at a tertiary care institute." *Indian Journal of medical microbiology* 24(3): 208.
- Levy SB. The challenge of antibiotic resistance. *Sci Am*. Available at: www.sciam.com/1998/0398issue/0398levy.html#link6. Accessed April 16, 2001
- Li, L., H. Yuan, C. D. weaver, J. Mao, G.H. Farr III, D.J. Sussman, J.Jonkers, D. kimelman and D. Wu. (1999). "Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt- mediated regulation of LEF- 1." *The EMBO Journal* 18(15): 4233-4240.
- M Lee, D. Heseck, B. Blazque, E. Lastochkin, b Boggesss, Jed F. Fisher and Shahriar Mobashery. (2014). "Catalytic spectrum of the penicillin-binding protein 4 of *Pseudomonas aeruginosa*, a nexus for the induction of β lactam antibiotic resistance" *Journal of the American Chemical Society* 137 (1): 190-200.
- Macrina, F. L., J. A. Tobian, KR Jones, RP Evans (1982). "A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*." *Gene* 19(3): 345-353.
- Martinez, J. L., A. Fajardo, L Garmendia, A Hernandez, J F Linares, L Martinez-Solana and M.B. Sanchez. (2009). "A global view of antibiotic resistance." *FEMS Microbiology Reviews* 33(1): 44-65.
- Meyer M, Kolpin DW, Bumgarner JE, Varns JL, Daughtridge JV. Occurrence of antibiotics in surface and groundwater near confined animal feeding operations and wastewater treatment plants using radioimmunoassay and liquid chromatography/electrospray mass spectrometry. Paper presented at 219th meeting of the American Chemical Society; March 26-30, 2000; San Francisco, CA (abstr 1:106)
- Molina, W. F. and P. M. Galetti-Jr (2002). "Robertsonian rearrangements in the reef fish *Chromis* (Perciformes, Pomacentridae) involving chromosomes bearing 5S rRNA genes." *Genetics and Molecular Biology* 25(4): 373-377.
- Nadia Liassine, Patricia Zulueta-Rodriguez, Celine Corbel, Christine Lascols,

- Claude- James Soussy, Emmanuelle Cambau (2008). "First detection of plasmid quinolone resistance in the community setting and in hospitalized patients in Switzerland". *Journal of Antimicrobial chemotherapy*, 62 (5):1151-1152.
- National Research Council, Committee on Drug Use in Food Animals, Panel on Animal Health, Food Safety, and Public Health, Board on Agriculture, National Research Council, Food and Nutrition Board, Institute of Medicine. *The Use of Drugs in Food Animals: Benefits and Risks*. Washington, DC: National Academy Press; 1999
- Roy, I., S. Mitra, (2003). "Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery." *International Journal of Pharmaceutics*, 250(1): 25-33.
- Shafiani, S. and A. Malik (2003). "Tolerance of pesticides and antibiotic resistance in bacteria isolated from wastewater-irrigated soil." *World Journal of Microbiology and Biotechnology*, 19(9): 897-901.
- Shoemaker NB, Want GR, Salyers AA. Evidence for natural transfer of a tetracycline resistance gene between bacteria from the human colon and bacteria from the bovine rumen. *Appl Environ Microbiol*. 1992; 58: 1313–1320
- Siddiqui, M. N., I. Ahmed, Bader J. Farooqi and Mustaq Ahmed. (1992). "Myonecrosis due to *Aeromonas hydrophila* following insertion of an intravenous cannula: case report and review." *Clinical Infectious Diseases*, 14(2): 619-620.
- Sørum, H., M. Trine, (2003). "Integron-containing IncU R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*." *Antimicrobial agents and chemotherapy* 47(4): 1285-1290.
- Stamm, J. M. (1989). "In vitro resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620)." *Journal of Aquatic Animal Health* 1(2): 135-141.
- Swartz MN. Committee on Human Risk Assessment of Using Subtherapeutic Antibiotics in Animal
- Wang, J., S. M. Soisson, K. Young, W. Shoop and S. Kodali *et al.*, (2006). "Platensimycin is a selective FabF inhibitor with potent antibiotic properties." *Nature* 441(7091): 358-361.

How to cite this article:

Hemanta Pokhrel, Salini Gopi, Devika Pillai, C.G. Rakesh and Sneha, K.G. 2018. Determination of Minimum Inhibitory Concentration (MIC), Mutation Frequency and Plasmid extraction and Transformation of Antibiotic Resistant Bacterial Isolate from Aquatic Environment. *Int.J.Curr.Microbiol.App.Sci*. 7(07): 890-901.
doi: <https://doi.org/10.20546/ijcmas.2018.707.108>