Plasmid Profile of Multidrug Resistant Isolates from Water and Fish

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ABSTRACT

Plasmids are extrachromosomal genetic material that can replicate independently and usually harbour antibiotic resistance genes. This study evaluated the presence and sizes of plasmid from multiple antibiotic resistant (MAR) isolates from water and fish using acridine orange as a plasmid curing agent. The minimum inhibitory concentration (MIC) of acridine orange to the 22 MAR ranged between 0.625 – 5.0 mg/mL. Majority (86.4 %) of the MAR isolates lost their resistant and became susceptible to more antibiotics indicating that their resistance was plasmid encoded. As much as 68.4 % of the isolates had one or more plasmid bands with sizes varying from 1.0 -3.0 kbp. The presence of plasmids - carrying MAR isolates in fish and river may heighten the dissemination of antibiotic resistance genes to the environment which will pose a serious threat to the human health.

Keywords: Multiple Antibiotic Resistant, Plasmid, Acridine orange, Plasmid bands.

INTRODUCTION:

Antimicrobial resistance (AMR) is a major threat to the effective treatment and prevention of a wild range of infections which are caused by bacteria, fungi and viruses. Clinical outcomes of patients are mostly worsened by AMR and there is a high risk of death caused by AMR. Acceleration in the emergence and spread of drug- resistant pathogens is on the increase.[1] and the aquatic environment is not an exception to this.[2] The use of antibiotics in human medicine and also in intensive animal husbandry for disease management and animal welfare directives may be a reason for the selective pressure exerted on bacteria which leads to a rapid division rate to retain only resistant organisms in their population,[3,4] and under the conditions of continued antimicrobial pressure, resistant strains propagate and spread.[5] Aquaculture settings have been hypothesized to serve as sources of antibiotic resistance genes which promotes the likelihood of growing resistance to antibiotics in human and animal pathogens.[4]

Plasmids are small circular, double-stranded DNA molecules which ranges in different sizes and can exist independently of the chromosome. Some plasmids can accommodate about 400 or more genes and resistance plasmids carries one or more antibiotic resistance genes.[6] Many resistance plasmids are transferred by conjugation, encoding functions which promotes cell- to – cell DNA transfer of the bacterial resistance genes.[7] Many resistance plasmids which are conjugative are usually about 30 kbp in size or larger, others are mobilizable when they are helped by a conjugative plasmid co-resident in the cell and they are usually less than 10 kbp in size.[8]

Plasmids harbour AMR genes (ARGs), they are self-replicating elements of DNA and often transmissible between bacteria. [9] They carry a considerable variety of genes including those that confer antibiotic

resistance and resistance to a number of toxic heavy metals, those that provides enzymes that expand the nutritional ability of the cells, virulence determinants that permit invasion of and survival in animal systems, and functions that enhance the capacity to repair DNA damage [8,10]. Resistance plasmids are plasmids that carry one or more antibiotic resistance genes and plasmid-encoded antibiotic resistance encompasses most of classes of antibiotics currently in clinical use and includes resistance to many that are at the forefront of antibiotic therapy.

AMR organisms could either be chromosomally or plasmid mediated. The plasmid mediated resistances are lost when the organism is treated with acridine orange. Plasmid replication is inhibited by various agents that can intercalate between the bases of DNA, particularly acridine orange, without inhibiting the chromosomal DNA replication. Such inhibition can lead to loss of the plasmid.[11] The study verified the availability of resistance plasmid in the multidrug drug resistant bacterial from fish and river water as reported by Aliyu and his team. [12]

MATERIALS AND METHODS

Plasmid Curing with Acridine

Twenty-two AMR organisms as reported by Aliyu and his team [12] were collected and plasmid curing was carried out. The method described by Udeze and co-workers [13] was employed to cure the resistant isolates. In this determination, the sub-Minimum Inhibitory Concentration (Sub-MIC) of acridine orange for each of the twenty-two multiple antibiotic resistant isolates was used. A 1.5g of acridine orange was weighed and dissolved in 150 mL of sterile distilled water in a standard flask to have a concentration of 10 mg/mL. Colonies from 24 hour cultures were emulsified in sterile normal saline to form a standard inoculum. Five millilitre, 5 mL of double strength (DS) sterile nutrient broth was measured in the first test tube while the remaining nine test tubes had 5 mL each of sterile single strength nutrient broth. Five millilitre, 5 mL of the stock acridine orange was measured and transferred into the first tube containing DS nutrient broth to have a concentration of 5000 µg/mL. From the first tube, 5mL was transferred to the second tube and serially diluted up to the tenth tube which had concentration of 9.8 µg/mL. Using a sterile micropipette, 0.1 mL of standard inoculum was added to all the test tubes. Another set of ten tubes serially diluted as above but without any organism inoculated was also prepared. All the tubes were incubated at 37 °C for 24 hours. The inoculated tubes were then compared with the set that was not inoculated to determine the sub-MIC tube. The isolate from the sub-MIC tube was sub-cultured on Nutrient Agar and incubated for 24 hours at 37 °C and the susceptibility of the twenty-two resistant isolates after acridine treatment was re-determined using agar diffusion method. The diameter of zones of inhibition was recorded and compared with the values before plasmid curing with acridine.

Plasmid Profile of Multiple Antibiotic-Resistant Isolates

Bacterial Cell Preparation

Fresh culture of nineteen (19) bacterial isolates which became susceptible to more antibiotics after acridine treatment were used. Their original colonies (pre-treatment) were picked from a 24-hour culture and inoculated in 5 mL of sterile Luria-Bertani (LB) broth and incubated at 37 °C for 24 hours. The bacterial cells were then harvested by centrifugation at 10,000 rpm for 5 minutes in an Eppendorff tube. The supernatant was discarded and cells harvested. The step was repeated for higher yield.[14]

Plasmid DNA Extraction

The harvested cell pellets were vortexed in a Stuart vortex mixer for few seconds and

re-suspended in 100 µL of Buffer 1 (50mM Tris-HCl, 10mM EDTA, 100µg/mL RNas A, pH 8.0) then properly mixed to form homogenous suspension. To each of the Eppendorff tube, 200 µL of Buffer 2 (1% Sodium Dodecyl Sulphate, SDS, 0.2M NaOH) was added and the caps were closed. The solution was mixed and stored in freezer for 5 minutes. A 150 µL of ice-cold Buffer 3 (3.0 M Potassium acetate, pH 5.2) was added to each Eppendorff tube, the caps were closed and then inverted for few times to mix the solution. The tubes were again kept on ice for 5 minutes after which they were placed in the centrifuge and spun at 10,000 rpm for 2 minutes. The supernatant was then transferred into clean 1.5 mL Eppendorff tube and precipitate discarded. To each tube of supernatant an equal volume (about 400 µL) of isopropanol was added. The caps were closed and the content mixed vigorously. The tubes were left to stand at room temperature for 2 minutes, and then centrifuged at 10,000 rpm for 5 minutes. The supernatant was carefully removed and discarded.

The DNA pellet was then washed with 200 µL of 95 % Ethanol and mixed by inversion several times. The tubes were again centrifuged at 10,000 rpm for 2-3 minutes. The supernatant was then carefully removed and discarded. The tubes were left with the caps open under vaccum for 15-20 minutes to dry off the last traces of alcohol.

Gel Electrophoresis

A 1.0 g of agarose was weighed and dissolved in 100 mL of TBA (Tris-Boric Acid) buffer and heated until agarose gel was fully dissolved. It was then cooled to about 45 °C. Thereafter, the gel solution was poured into assembled gel holder with 20 place comb and allowed to solidify. The comb was wiggled a few times and lifted straight up. The gel holder lifted carefully and submerged in the electrophoresis tank containing TBA buffer with the wells closer to the negative electrode. Five microliter, 5.0 µL each of loading dye was added to the plasmid preparations from the different bacterial isolates and loaded into different wells using micropipette. A standard 100 bp molecular ladder was loaded into the first well. The gel was then run for 2 hours at 75 Volts.

The electrophoresis tank was disconnected and the gel containing separated plasmid was visualized under a Trans-illuminator UV light of wavelength 302 nm. This was then photographed with a Polaroid camera and documented using BioRad gel electrophoresis documentation system. After photographing, the distance of migration of each plasmid was determined relative to the standard DNA ladder loaded in the first well.[15] The value of the DNA marker used was matched with the distance of migration of the bands in each isolates and the plasmid sizes of the isolates were determined using the Bio-rad image lab software.

RESULTS

The susceptibility profile after the treatment of the multiple antibiotic resistance organisms with acridine was carried out and the result revealed that the minimum inhibitory concentration (MIC) of acridine is 625 µg/mL to 40.9 % of the twenty-two organisms tested. This is followed by 1250 µg/mL (36.4 %) and the highest MIC was 5000 µg/mL for 18.2 % of the organisms. The susceptibility of the multi-drug resistant organism is presented in Tables 2 and 3. As shown in these tables, the sensitivity of most of the isolates to some of the test antibiotics changed. A number of the isolates became sensitive to at least two (2) more drugs. Increased sensitivity was exhibited against Amoxicillin- clavulanate, Cefuroxime, Gentamicin, Nitrofurantoin,Chloramphenicol and Co-trimoxazole. About 13.6 % of the organisms (*Klebsiella pneumoniae* and *Escherichia coli*) did not show any change in number nor pattern of antibiotic resistance. After plasmid curing

there were decreases in the number of isolates that were still resistant however these decreases were only 4.5 % (each of Tetracyline and Erythromycin), 9.1 % (Ampicillin) and 23.1 % in Co-trimoxazole. Fourteen and ten more isolates became more sensitive to Cefuroxime and Amoxicillin respectively (Table 4). In plasmid analysis, nineteen isolates belonging to seven bacterial strains that became susceptible to at least one or more antibiotics after curing were selected for DNA plasmid analysis. A total of 13 (68.4 %) isolates showed one or two plasmid bands and their molecular weights were in the range of 1.6 – 3.2 kbp (Table 5 and plate1) while 31.6 % (6 isolates) showed no visible bands and 26.3 % of the isolates had two plasmid bands while 42.1 % showed only one detectable band.

Table 1: MIC and Sub-MIC Values of Resistance Isolates from Water and Fish After Curing.

Isolate No.	Organism	MIC (µg/mL)	Sub-MIC (µg/mL)
2	*Serratia marcescens*	625	312.5
3	*Escherichia coli*	2500	1250
7	*Klebsiella pneumoniae*	625	312.5
20	*Klebsiella pneumoniae*	625	312.5
21	*Enterobacter gergoviae*	1250	625
24	*Klebsiella pneumoniae*	5000	2500
25	*Klebsiella pneumoniae*	1250	625
43	*Salmonella spp.*	625	312.5
45	*Escherichia coli*	5000	2500
47	*Escherichia coli*	5000	2500
48	*Escherichia coli*	625	312.5
61	*Serratia rubidaea*	625	312.5
62	*Escherichia coli*	5000	2500
66	*Klebsiella pneumoniae*	1250	625
68	*Klebsiella pneumoniae*	625	312.5
69	*Klebsiella pneumoniae*	1250	625
70	*Klebsiella pneumoniae*	1250	625
71	*Klebsiella pneumoniae*	625	312.5
73	*Klebsiella pneumoniae*	1250	625
75	*Salmonella spp.*	625	312.5
76	*Shigella sonnei*	1250	625
79	*Escherichia coli*	1250	625

Table 2: Resistance Pattern of Selected Bacteria Species Isolated from Water and Fish Before and After Curing

Isolate No.	Organism	No of Antibiotics	
		Before curing*	After curing
2	*Serratia marcescens*	6	3
3	*Escherichia coli*	6	3
7	*Klebsiella pneumoniae*	6	3
20	*Klebsiella pneumoniae*	7	4
21	*Enterobacter gergoviae*	6	4
24	*Klebsiella pneumoniae*	6	5
25	*Klebsiella pneumoniae*	5	4
43	*Salmonella spp.*	6	3
45	*Escherichia coli*	7	7
47	*Escherichia coli*	6	3
48	*Escherichia coli*	5	4
61	*Serratia rubidaea*	6	4
62	*Escherichia coli*	6	4
66	*Klebsiella pneumoniae*	7	4
68	*Klebsiella pneumoniae*	6	6
69	*Klebsiella pneumoniae*	5	4
70	*Klebsiella pneumoniae*	7	4
71	*Klebsiella pneumoniae*	7	7
73	*Klebsiella pneumoniae*	5	3
75	*Salmonella spp.*	6	3
76	*Shigella sonnei*	6	4
79	*Escherichia coli*	5	4

*Source: Aliyu and his team [12]

Table 3: Resistance Profile of Selected Bacteria Species Isolated from Water and Fish.

Isolate No.	Isolates	Resistance Profile	
		Before curing	After curing
2	*Serratia marcescens*	AMP-AMC-CN-TE-E-CXM	AMP-TE-E
3	*Escherichia coli*	AMP-AMC-F-CN-TE-E	AMP-TE-E
7	*Klebsiella pneumoniae*	AMP-AMC-CN-TE-E-CXM	AMP-TE-E
20	*Klebsiella pneumoniae*	AMP-AMC-CN-TE-E-CXM-C	AMP-AMC-E-CXM
21	*Enterobacter gergoviae*	AMP-AMC-CN-TE-E-CXM	AMP-AMC-TE-E
24	*Klebsiella pneumoniae*	AMP-AMC-CN-TE-E-CXM	AMP-AMC-TE-E-CXM
25	*Klebsiella pneumoniae*	AMP-CN-TE-E-CXM	AMP-CN-TE-E
43	*Salmonella spp.*	AMP-AMC-TE-E-SXT-CXM	AMP-TE-E
45	*Escherichia coli*	AMP-AMC-TE-E-SXT-CXM-C	AMP-AMC-TE-E-SXT-CXM-C
47	*Escherichia coli*	AMP-AMC-CN-TE-E-CXM	CN-TE-E
48	*Escherichia coli*	AMP-TE-E-SXT-CXM	AMP-TE-E-SXT
61	*Serratia rubidaea*	AMP-AMC-TE-E-SXT-CXM	AMP-TE-E-SXT
62	*Escherichia coli*	AMP-F-TE-E-CXM-C	AMP-TE-E-C
66	*Klebsiella pneumoniae*	AMP-AMC-CIP-TE-E-SXT-CXM	AMP-TE-E-SXT
68	*Klebsiella pneumoniae*	AMP-CIP-TE-E-SXT-CXM	AMP-CIP-TE-E-SXT-CXM
69	*Klebsiella pneumoniae*	AMP-TE-E-SXT-CXM	AMP-TE-E-SXT
70	*Klebsiella pneumoniae*	AMP-AMC-CIP-TE-E-SXT-CXM	AMP-TE-E-SXT
71	*Klebsiella pneumoniae*	AMP-AMC-CIP-TE-E-SXT-CXM	AMP-AMC-CIP-TE-E-SXT-CXM
73	*Klebsiella pneumoniae*	AMP-TE-E-SXT-CXM	AMP-TE-E
75	*Salmonella spp.*	AMP-AMC-TE-E-SXT-CXM-C	TE-E-SXT
76	*Shigella sonnei*	AMP-AMC-TE-E-SXT-C	AMP-AMC-TE-C
79	*Escherichia coli*	AMP-TE-E-SXT-C	AMP-TE-E-SXT

Table 4: Percentage Differences in The Antibiotic Susceptibility Pattern of Isolates Before and After Curing

Antibiotics	No. of Isolates Resistant out of 22		% Decrease
	Before Plasmid curing	After Plasmid curing	
Ampicillin	22/22	20/22	9.1
Amox. / Clav.	16/22	06/22	62.5
Nitrofurantoin	3/22	0/22	100
Gentamicin	7/22	02/22	71.4
Ciprofloxacin	4/22	02/22	50
Tetracycline	22/22	21/22	4.5
Erythromycine	22/22	21/22	4.5
SMZ/TMP	13/22	10/22	23.1
Cefuroxime	19/22	05/22	73.7
Chloramphenicol	6/22	03/22	50

Amox. / Clav. : Amoxicillin / Clavulanic acid combination.
SMZ/TMP: Sulphamethoxazole trimethoprim combination.

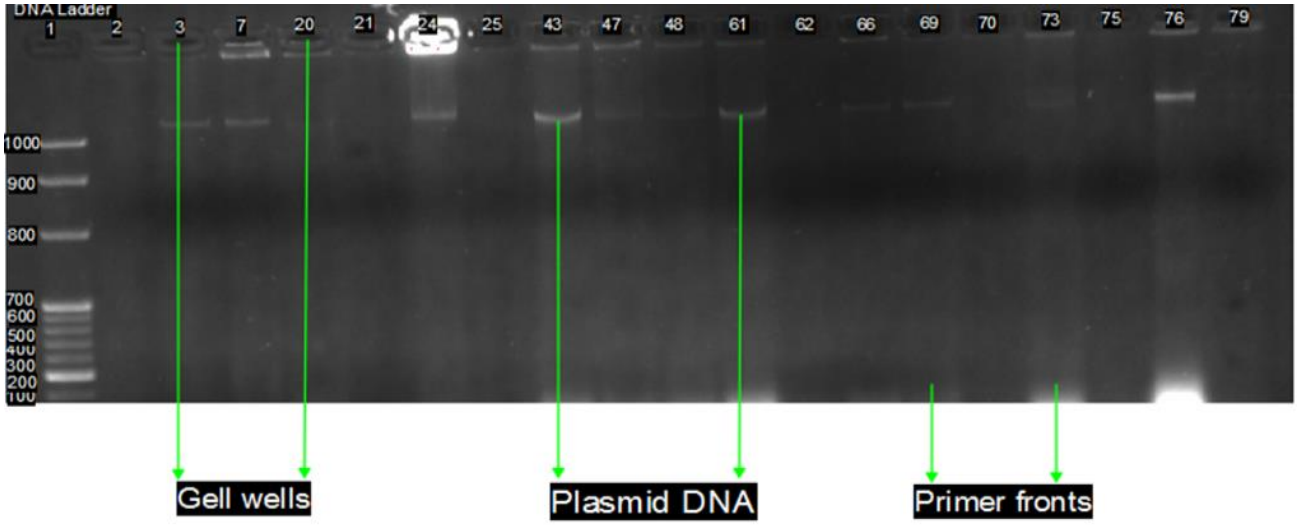

Plate 1: 1.0 % Agarose Gel Electrophoresis of 100 Base Pair and Plasmid DNA Isolated from Multidrug Resistant Isolates from Water and Fish.

Key:

Lane 1: Marker standard
Lane 2: Isolate2. *Serratia marcescens*
Lane 3: Isolate 3. *Escherichia coli*
Lane 4: Isolate 7. *Klebsiella pneumoniae*
Lane 5: Isolate 20. *Klebsiella pneumoniae*
Lane 6: Isolate 21. *Enterobacter gergoviae*
Lane 7: Isolate 24. *Klebsiella pneumonia*
Lane 8: Isolate 25. *Klebsiella pneumonia*
Lane 9: Isolate 43. *Salmonella* spp
Lane 10: Isolate 47. *Escherichia coli*
Lane 11: Isolate 48. *Escherichia coli*
Lane 12: Isolate 61. *Serratia rubidaea*
Lane 13: Isolate 62. *Escherichia coli*
Lane 14: Isolate 66. *Klebsiella pneumoniae*
Lane 15: Isolate 69. *Klebsiella pneumoniae*
Lane 16: Isolate 70. *Klebsiella pneumoniae*
Lane 17: Isolate 73. *Klebsiella pneumoniae*
Lane 18: Isolate 75. *Salmonella* spp
Lane 19: Isolate 76. *Shigella sonnei*
Lane 20: Isolate 79. *Escherichia coli*

Table 5: Comparison of Plasmid Number and Sizes of Some Antibiotic Resistant Bacteria Isolated from Water and Fish.

Isolate No.	Isolates	No. of Plasmid Bands	Plasmid sizes (bp)
2	*Serratia marcescens*	1	2,613
3	*Escherichia coli*	2	1,637; 2,633
7	*Klebsiella pneumoniae*	2	1,671; 2,709
20	*Klebsiella pneumoniae*	1	2,746
21	*Enterobacter gergoviae*	1	2,784
24	*Klebsiella pneumoniae*	1	1,778
25	*Klebsiella pneumoniae*	0	Nil
43	*Salmonella spp.*	2	1,754; 2,922
47	*Escherichia coli*	1	2,983
48	*Escherichia coli*	1	3,025
61	*Serratia rubidaea*	2	1,828; 3,067
62	*Escherichia coli*	0	Nil
66	*Klebsiella pneumoniae*	2	1,932; 3,175
69	*Klebsiella pneumoniae*	1	1,986
70	*Klebsiella pneumoniae*	0	Nil
73	*Klebsiella pneumoniae*	0	Nil
75	*Salmonella spp.*	0	Nil
76	*Shigella sonnei*	1	2,142
79	*Escherichia coli*	0	Nil

DISCUSSION

Contaminated drinking water and food are major sources of enteric pathogens, causing several waterborne disease outbreak and the consumption of water with presence of antibiotic- resistant bacteria is a major public health concern as antibiotic-resistant bacteria could be transferred to humans, contributing to the spread and persistence of antibiotic-resistant bacteria in environments.[16,17] Result from this study shows the curing of plasmids from some antibiotic resistance isolates with a reduction in the number of antibiotics which they showed resistance to. This implies that the resistance of these cured isolates were plasmid encoded while those other isolates which showed no curing effect might have had chromosomal resistance or there could have been changes in the genetic make-up of the organisms.

Plasmid curing using acridine orange increased susceptibility to antibiotic from 62 % to 100 % depending on the antibiotic. The presence of multiple antibiotic resistance among enteric bacteria isolates from aquatic environment has also been reported by Mervat and co-researchers,[18] who investigated antimicrobial resistance profiles of enterobateriaceae isolated from Rosetta Branch of River Nile, Egypt. The plasmid DNAs obtained from the multidrug resistance isolates on gel electrophoresis in this study showed thirteen isolates harbouring eighteen plasmid bands ranging between 1-3 kbp (Table 5 and Plate 1) while some isolates do not have plasmid as similarly reported by Olagoke's team, [19] who observed presence of plasmid in some organisms while others were plasmid less. This implied that the antibiotic resistance markers associated were plasmid encoded and might be transmitted easily amongst bacteria.[20] The bands observed did not occur randomly which shows that the plasmids carry information for characteristics which are essential for the bacteria to survive in their environment within the host.[21] This is also in line with the work of Abdellatif research group [22] who reported 82.0, 50.0 and 87.0 % of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively to harbour plasmid with different sizes and number. All the thirteen isolates which showed plasmid bands were observed to be resistant to Ampicillin, Tetracycline and Erythromycin. Other resistance isolates with plasmid bands showed varying levels of resistance to amoxicillin- clavulanic acid, gentimicin, cefuroxime, ciprofloxacin, chloramphenicol and sulphamethoxazole/ trimethoprim. There is an ever growing resistance of the family enterobacteriaceae to many commonly prescribed antibiotics especially in the developing countries and much of this problems are shown to be due to the presence of transferrable plasmids encoding MDR and their dissemination among different enterobacterial species.[23]

CONCLUSION

The presence of plasmid carrying multiple antibiotic resistance isolates in fish and water could serve as means of resistance gene transfer which will pose a serious threat to public health.

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