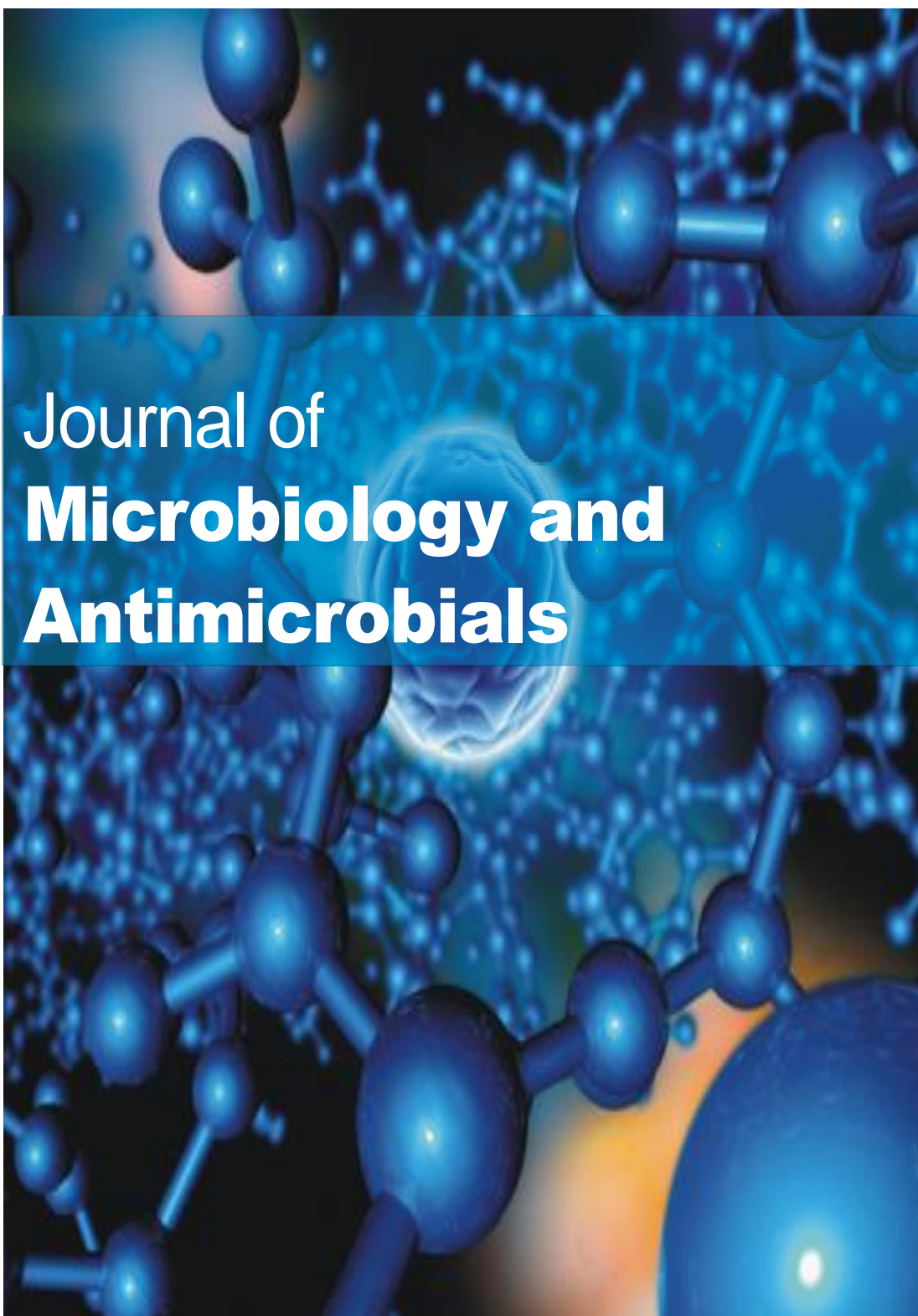


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Full Length Research Paper

Plasmid curing of antibiotic resistant *Escherichia coli* isolates from urine and stool samples

Oriomah Churchill* and Akpe Azuka Romanus

Department of Biology, School of Basic Studies, Nigeria Maritime University, Okerenkoko, Delta State, Nigeria.

Received 4 October, 2018; Accepted 21 June, 2019.

The origin of bacteria resistance to antibiotics can either be chromosomal or extra-chromosomal (plasmid mediated) and one way of determining the origin of bacterial drug resistance is by plasmid elimination. In this study, the antibiotic susceptibility of seven *Escherichia coli* isolates (numbered 1-7) from urine and stool samples, were assessed using the disk diffusion method. The 10 antibiotics used were: nitrofurantoin (100 µg), ciprofloxacin (5 µg), tetracycline (50 µg), norfloxacin (10 µg), amoxicillin (20 µg), ofloxacin (5 µg), chloramphenicol (10 µg), cefuroxime (30 µg), ampicillin (10 µg) and gentamicin (10 µg). All isolates (100%) were observed to have shown resistance to ampicillin. Isolate No 6 was resistant to 70% of the antibiotics while isolate No 7 was observed to be resistant to 30% of the antibiotics. In order to determine if the resistance is plasmid mediated or chromosomal-borne, two of the isolates (29%) that showed resistance to more than one antibiotic were subjected to acridine orange mediated plasmid elimination. Isolate No 6 lost its resistance to 5 out of the 7 antibiotics (71%) while isolate number 7 lost its resistance to 2 out of the 3 antibiotics (67%) after the curing. Loss of resistance after the plasmid curing was an indication that the resistance was plasmid-mediated while the resistance mechanism for those that retained their resistance after plasmid curing was chromosomal-borne. It was suggested that further studies be done for the characterization of resistance plasmids on *E. coli* and policies be set that will minimize the indiscriminate use of antibiotics.

Key words: Antibiotics, chromosomal, *Escherichia coli*, plasmid, resistance, sensitivity, susceptibility.

INTRODUCTION

Escherichia coli is a rod-shaped, Gram-negative facultative aerobic, coliform bacterium of the genus *Escherichia* which is classified as member of Enterobacteriaceae within the Gamma Proteobacteria class, found in the lower intestine of warm blooded organisms (Tenailon et al., 2010). Despite most strains being commensal inhabitants of the intestine, there are

also some extraintestinal pathogenic *E. coli* (ExPEC) bacteria which have the ability to cause diverse and serious diseases, such as urinary tract infections (UTIs) and bacteremia (Wiles et al., 2008; Ron, 2010; Kanayama et al., 2015). Treatment of these infections with the use of antibiotics has become a global concern due to the emergence of resistant *E. coli* strains which is

*Corresponding author. E-mail: oriomahchurchill@gmail.com, Tel: +2348138298636.

rapidly on the increase (Peralta et al., 2007; Ngwai et al., 2010; Wang et al., 2017).

Currently, two general mechanisms of resistance can be found in *E. coli*; those associated with mutations in the chromosome and those related to plasmids. Mutation in the chromosome may alter the target site and reduce the intracellular accumulation of the antibiotics. It can also lead to the production of enzymes that degrade antibacterial drugs (Miller et al., 2014). The plasmids (R plasmids) have been reported to be the most frequent causes of antibiotic resistance in most bacteria including the *E. coli* which have exhibited resistance to beta lactam antibiotics, aminoglycosides and fluoroquinolone (Carattoli, 2013; Miller et al., 2014; Zhang et al., 2014). The plasmids which are extra-chromosomal materials also allow the movement of genetic materials, including antimicrobial resistant genes between bacterial species and genera through gene exchange processes thereby causing a rapid increase in antibiotic resistance (Carattoli, 2013).

Since antibiotic resistance in bacteria can either be chromosomal or plasmid mediated, subjecting the bacterial cells to processes that lead to plasmid elimination is one way of determining the general mechanisms of bacterial drug resistance. Thus, this is a preliminary study that was designed to determine if the antibiotic resistance in the *E. coli* isolates was plasmid mediated or chromosomal-borne by treatment of the resistant isolates with acridine orange and carrying out susceptibility tests on the antibiotics tested.

MATERIALS AND METHODS

Sample collection

Seven clinical isolates of *E. coli* from urine and stool samples were collected from the diagnostic laboratory centre of the Department of Medical Laboratory Sciences, Ambrose Alli University, Ekpoma and coded as isolate no. 1-7. Isolates were inoculated on nutrient agar plate and incubated at 37°C for 24 h before they were confirmed using Gram's reaction, indole, motility, methyl red, nitratase and oxidase tests.

Antibiotic sensitivity assay of bacterial isolates

Isolates were subjected to antibiotic screening by disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) (2017). Inocula were prepared by diluting overnight cultures in sterile NaCl (0.9%) suspension and then marched with McFarland turbidity index. Then, 0.1 mL of the bacterial suspension was then plated on to Mueller Hinton Agar and the commercially available antibiotic disks were placed on the lawn of the culture and the plates incubated overnight at 37°C. The ten antibiotics used were nitrofurantoin (100 µg), ciprofloxacin (5 µg), tetracycline (50 µg), norfloxacin (10 µg), amoxycilline (20 µg), ofloxacin (5 µg), chloramphenicol (10 µg), cefuroxime (30 µg), ampicillin (10 µg) and gentamicin (10 µg). The sensitivity, intermediate and resistance were determined by the diameter of the zone of complete growth inhibition around each disk according to the CLSI (2017) reference standards.

Plasmid curing analysis

Two of the isolates (29%); isolate no. 6 and 7 showing resistance to more than one antibiotic were selected for the plasmid curing experiment in order to determine if the resistance is plasmid-borne or chromosomal. The curing of the resistant *E. coli* was done using sub-inhibitory concentration of 0.1 mg/mL of acridine orange as described by Rasool et al. (2003) and Yah et al. (2007) with slight modification. Isolates were grown for 24 h at 37°C in Mueller-Hinton broth containing 0.1 mg/mL acridine orange. The broth was agitated to homogenize the content and loopful of the broth were inoculated on Mueller-Hinton Agar plate and antibiotic sensitivity testing was carried out as previously described. Presence or increase of zone of inhibition on the Mueller Hinton agar around the antibiotics where zone of inhibition were not initially observed or on antibiotics with lower zone of inhibition respectively when compared to the CLSI (2017) reference standard was an indication of a plasmid mediated resistance which has been cured, while the absence of zone of inhibition on Mueller Hinton agar was an indication of chromosomal resistance or plasmid not cured.

RESULTS

Antibiotic sensitivity assay of bacterial isolates

The results obtained from the antibiotics sensitivity of the *E. coli* isolates indicated that all isolates (100%) were resistant to ampicillin. However, all the isolates (100%) were susceptible to ciprofloxacin and gentamicin. Resistance to nitrofurantoin, tetracycline, norfloxacin, amoxycillin, ofloxacin, chloramphenicol and ampicillin (70% of the antibiotics) was observed for isolate no. 6 while isolate no. 7 was observed to be resistant to nitrofurantoin, tetracycline and ampicillin (30% of the antibiotics) as presented in Table 1.

Plasmid curing analysis and antibiotic sensitivity assay of bacterial isolates after curing

The two isolates (29%); isolate no. 6 and 7 with resistance to more than one antibiotic were subjected to plasmid curing. Isolate no. 6 lost its resistance to 5 (nitrofurantoin, norfloxacin, amoxycilline, ofloxacin and chloramphenicol) out of the 7 antibiotic (71%) as resistance to tetracycline and ampicillin (29%) was observed while isolate no. 7 lost its resistance to 2 (nitrofurantoin and ampicillin) out of the 3 antibiotics (67%) as resistance to tetracycline (33%) was observe after the curing. Although isolate no. 6 was still resistant to ampicillin, an increase in the diameter of zone of inhibition was observed as against what was recorded before curing as indicated in Table 2.

DISCUSSION

The indiscriminate use of antibiotics has increased the occurrence of antibiotic resistance in bacteria which has caused formidable global health challenges to man and

Table 1. Results of the antibiotics susceptibility test of the *Escherichia coli* isolates before curing.

Isolate no.	Source of isolate	Sex of patient	Age of patient (years)	Resistant pattern	Diameter of inhibition zone (mm) in the nearest whole number.									
					Antibiotics concentration present in the disc (µg).									
					N (100)	CIP (5)	TE (50)	NB (10)	AX (20)	OF (5)	C (10)	CF (30)	AM (10)	GN (10)
1.	Stool	Male	3	AM	19	32	16	24	20	24	24	32	10	20
2.	Stool	Male	4	AM	18	35	17	20	24	22	18	35	10	20
3.	Stool	female	9	AM	18	34	14	17	25	23	21	32	9	23
4.	Stool	Male	4	AM	18	32	17	24	25	24	21	33	10	19
5.	Stool	female	13	AM	17	33	13	16	23	24	22	31	10	20
6.	Urine	female	18	N, TE, NB, AX, OF, C, AM	0	30	0	10	0	10	12	32	0	18
7.	Stool	female	5	N, TE, AM	0	35	9	22	17	23	20	31	10	20

N=nitrofurantoin, CIP=ciprofloxacin, TE=tetracycline, NB= norfloxacin, AX= amoxycillin, OF=ofloxacin, C=chloramphenicol, CF=cefuroxime, AM=ampicillin GN=gentamicin.

Table 2. Results of the antibiotics susceptibility test of the multi-drug resistant *Escherichia coli* isolates after curing.

Isolate no.	Resistant pattern		Diameter of inhibition zone (mm) in the nearest whole number.									
			Antibiotics concentration present in the disc (µg).									
	Pre curing	Post curing	N (100)	CIP (5)	TE (50)	NB (10)	AX (20)	OF (5)	C (10)	CF (30)	AM (10)	GN (10)
6.	N, TE, NB, AX, OF, C, AM	TE, AM	18	33	0	21	13	26	16	31	13	18
7.	N, TE, AM	TE	17	33	10	22	16	25	26	33	19	20

N=nitrofurantoin, CIP=ciprofloxacin, TE=tetracycline, NB= norfloxacin, AX= amoxycillin, OF=ofloxacin, C=chloramphenicol, CF=cefuroxime, AM=ampicillin GN=gentamicin.

animals (Tzouveleakis et al., 2014). Previous studies have also shown the elevated trend in antibiotic resistance in *E. coli* (Johnson et al., 2007; Arathy et al., 2011; Nsofor et al., 2013; Yassin et al., 2017). The results obtained from the sensitivity test on the *E. coli* illustrated that all isolates were resistant to ampicillin. High level of ampicillin resistance by *E. coli* isolates had been reported (Nahid et al., 2008; Ngwai et al., 2010; Sheng et al., 2013). However, the multi-drug resistance that was observed in isolates no. 6 and 7 to nitrofurantoin, tetracycline, norfloxacin, amoxycillin, ofloxacin, chloramphenicol and ampicillin and nitrofurantoin, tetracycline and ampicillin respectively could be as a result of one or more drug resistance mechanisms that are being mediated by the bacterial plasmid or chromosome (Carattoli, 2013; Miller et al., 2014; Zhang et al., 2014). The plasmid curing assay on the two *E. coli* isolates revealed that 71 and 67% of the antibiotic resistance observed in isolate no. 6 and 7 respectively were plasmid-mediated, which is line with previous studies (Zaman et al., 2010; Vinay et al., 2013; Ojo et al., 2014). However, the 29 and 33% resistance for isolate no. 6 and 7 respectively that was still retained after the curing indicated chromosomal-borne resistant gene (Nguyen et al., 2014). Although resistance to

ampicillin was still retained by isolate no. 6, zone of inhibition of diameter 13 mm was observed as against none that was observed before the curing which may be an indication that the ampicillin resistant was being mediated by both the plasmid and the bacterial chromosome (Jesús et al., 2014).

Conclusion

This is a preliminary study showing the susceptibility and resistance of *E. coli* to the various tested antibiotics, where resistance to the most used antibiotic like ampicillin, nitrofurantoin, amoxycillin and tetracycline were observed. There was an indication from the curing experiment that most of the resistance shown to the antibiotics was plasmid-mediated, and this can easily be transferred from one strain to another or from one organism to another within the same environment. This study intends to carryout molecular study on the plasmid-borne and chromosomal-borne gene associated with antibiotic resistance using wide range of sample numbers. However, since overuse and misuse of antibiotics aid the development of resistance to antibiotics and most antibiotic resistance are plasmid

mediated which are capable of easily being transferred to non resistant microbial species, it is advisable to set up policies that will minimize the indiscriminate use of antibiotic especially the ones with lesser reports of the emergence of resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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