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Plasmid Conjugation in *E. coli* and Drug Resistance

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Abstract

The emergence of multidrug resistance in clinical *Escherichia coli* has been associated significantly with plasmid mediated genes in carriers; which is an important cause of morbidity and mortality in developing countries. This study aimed at determining the antibiotics susceptibility pattern of *E. coli* isolates claimed to be multidrug resistance using disc diffusion method. It also determined the presence of transferable resistance plasmids through conjugation and evaluated the medical significance of plasmid encoding *E. coli* and drug resistance. The result showed that majority of the multidrug resistance in clinical microorganism was as a result of the acquisition of plasmid-carrying antibiotic-resistance genes, acquired through conjugal transfer of plasmids which has greatly contributed to the rapid spread of antibiotic resistance among *E. coli* isolates which could lead to complication in therapy and limit treatment options, increase in mortality rate, high economic burden and longer hospital stays. To cub this, it is imperative to checkmate the rate at which over the counter drugs are sold and antibiotic misused in animal feeds. This will play a key role in decreasing the emergence of resistant bacteria strains within our environment.

Key words: *E. coli*, conjugation, transconjugation, plasmid, multidrug resistance

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Introduction

Many documentary evidences have showcased the medical implications and impact of horizontal gene transfer in bacteria. The morphology and characteristics of *E. coli* and related bacteria has also been explicitly evaluated to play a key role in the study of gene transfer through an existing population (Kroll, *et al.,* 2010; Willey, *et al.,* 2008; APEC, 2011; Hudault, *et al.,* 2001, CDC, 2007). Bacterial plasmids serve as a helper plasmid, based on the naturally occurring pRK plasmid containing the genes necessary for encoding conjugation machinery; because of their ability to possess transfer origin (oriT) regions from different plasmid groups, transposable genetic elements, repeat elements, cryptic prophages, and bacteriophage remnants (Hemantha and Sayontan, 2012; Lukjancenko, *et al.,* 2010), which enable new strains of *E. coli* to evolve through the natural biological processes of mutation, gene duplication and horizontal gene transfer (Todar, 2012). Plasmids are circular, autonomously self replicating extra-chromosomal DNA found in bacteria (Dubey, 2009). They are considered as transferable genetic elements, or "replicons", capable of autonomous replication within a suitable host. According to Jennifer, *et al.,* (2011), bacteria plasmids are key vectors of horizontal gene transfer, mediating the mobilization of gene from one bacterial to another and enable a competent bacterial to take up naked DNA and to spread within and between bacteria species by conjugation, which facilitates the rapid dissemination of potentially beneficial genes (like resistance to antibiotic; R-plasmid) through a bacterial population. The more the copy number of resistance plasmid present in a bacterial cell, the higher the resistant ability of the bacteria (Pray, 2008). It should be noted that plasmids may contain properties belonging to more than one functional class (Kandavelou and Chandrasegaran, 2008). Most bacteria R-plasmid confer resistance to antibiotics by way of one or more of the following mechanisms which they code for: (1) Producing an enzyme capable of destroying or inactivating the antibiotic. (2) Altering the target site

receptor for the antibiotics to reduce or block its binding. (3) Preventing the entry of the antibiotic into the bacterium and/or actively transporting the antibiotic out of the bacterium. (4) Modulating gene expression to produce more of the bacteria enzyme altered by the antibiotic (Arias and Murray, 2009; Willey *et al.*, 2008). Bacterial infections are usually treated with antibiotics (Willey, *et al.,* 2008). However, the antibiotic sensitivity of different strains of *E. coli* varies widely because of the possession of R-plasmid. As with most Gram-negative organisms, *E. coli* is resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which may be used to treat *E. coli* infection include amoxicillin, as well as other semi synthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides (Reid, *et al.,* 2001). Antibiotic resistance is a growing global problem (Jennifer, *et al.,* 2011). Some of this is due to overuse of antibiotics in humans, but some of it is probably due to the use of antibiotics as growth promoters in animal feeds. Also prescription of antibiotics for non-bacterial infection (colds, influenza, most upper respiratory infections etc) or prescribing the 'newest' antibiotics in the market when older "brands" may still be effective. These simply increase the rate at which the natural selection for resistance occurs (Soulsy, 2005). This research aimed at evaluating the correlation between plasmid encoded in *E. coli*, its transferability through conjugation and the health implications.

MATERIALS AND METHODS

Identification and Biochemical Characterization of *E. coli* and *Proteus mirabilis:* Clinical isolates of *E. coli* confirmed to be multidrug resistant and *Proteus mirabilis* from asymptomatic patience were collected from Medical Microbiology Department of Ahmadu Bello University Teaching Hospital, Shika. The isolates were inoculated onto nutrient agar and incubated at 37^{o}C for 24hrs. Further identification and biochemical characterization was carried out using standard microbiology methods (De silva *et al*., 2001; Ellis and Goodacre, 2006; Chakraborty and Nishith, 2008).

Antibiotics Susceptibility Test and MIC: Antibiotics susceptibility test and MIC of the multiple drugs resistant *E. coli* to amoxicillin-clavulanic acid and ceftriaxone were also carried out (Hadley, 2002; Cowan and Steel, 1993; Barrow and Feltham, 1993; Cheesbrough, 2000).

Conjugation Study: The conjugation study was carried out by transferring the resistant strains of *E. coli* isolates that were resistant to amoxicillin-clavulanic acid and ceftriaxone to *Proteus mirabilis* using the methods described by Onaolapo *et al*., (1997) with some modifications: The minimum inhibitory concentration (MIC) of the test antibiotics against the sensitive *Proteus mirabilis* was determined. The resistant isolates of *E. coli* were grown in sterile 5ml nutrient broth each at 37°C for 18 hours. Amoxicillin-clavulanic acid and ceftriaxone sensitive *Proteus mirabilis* was sub-cultured into the sterile nutrient broth and incubated at 37°C for 18 hours. The overnight cultures of the potential donor (R+) *E. coli* resistant isolates and the recipients (R-), *Proteus mirabilis* was mixed in a ratio 1:10 respectively in 5ml volume of sterile nutrient broth and incubated in a static incubator at 37°C for 18 hours to increase plasmid copy number through conjugation. One loop full of the transconjugants, organisms {resistant *E. coli* (R+) and *Proteus mirabilis* (R-)} was sub-cultured on separate MacConkey agar plates incorporated with MIC of amoxicillin-clavulanic acid and ceftriaxone against recipient *P. mirabilis* separately and incubated at 37°C for 24 hours. The plates were examined for the presence or absence of growth and characteristics of *Proteus mirabilis*. Original culture of *Proteus miralibis* and *E. coli* separately were standardized and streaked on MacConkey agar containing MIC of amoxicillin-clavulanic acid and ceftriaxone as positive control. The colonies of the transconjugants (*Proteus mirabilis*) observed were aseptically picked and transferred into nutrient agar slant and subcultured into sterile nutrient broth after which the MICs of amoxicillin-clavulanic acid and ceftriaxone against the transconjugant were determine as described by Lennette *et al.*, (1990).

Curing of Transconjugants: The curing of transconjugants *Proteus mirabilis* carrying the plasmid was carried out by treating the *Proteus mirabilis* transconjugant with an acridine orange dye as described by Onaolapo (1986): Each of the transconjugants was grown overnight in sterile nutrient broth and incubated at 37°C for 18hours in a static incubator. The overnight culture of *Proteus mirabilis* transconjugants was standardized (106 cfu/ml). A stock solution of acridine orange in sterile distilled water (10,000µg/ml) was prepared and vortexed properly. Five mililiter (5ml) of the solution was

dispensed into sterile test tubes. Ten microliter (10µl) of standardized transconjugated *Proteus mirabilis* (10^6cfu/ml) were inoculated into the solution of acridine orange and incubated at 37°C for 18 hours. The overnight culture of transconjugated *Proteus mirabilis* in acridine dye was sub-cultured onto MacConkey agar and the colonies obtained were assessed for their antibiotic sensitivity against the antibiotics used initially. The MIC method was also used to determine whether the resistant pattern had changed or not.

Result

Conjugation Studies

Result showed that the *Proteus mirabilis* used for this study is only resistant to tetracycline but susceptible to all other antibiotics used for this study (Table 1). Table showed that all the resistant isolates are multidrug resistant i.e resistant to at least 9 and above antibiotics used for this study.

Table 1: Antibiotic Susceptibility Profile of Clinical Isolate of *Proteus mirabilis*

Organism	CAZ	CRO	F	OFX	CXM	NA	TE	AML	CIP	SAM	C	CL	AMC	CN
P. mirabilis	S	S	S	S	S	S	R	S	S	S	S	S	S	S

S = Sensitive R = Resistant Ampicillin-sulbactam (SAM), Amoxicillin (AML), Amoxicillin-Clavulanic acid (AMC), Cefalexin (CL), Ceftriaxone (CRO), Ceftazidine (CAZ), Cefuroxime (CXM), Ciprofloxacin (CIP), Nalidixic Acid (NA), Ofloxacin (OFX), Gentamicin (CN), Tetracycline (TE), Chloramphenicol (C), Nitrofurantoin (F).

Table 2: Antibiotic Susceptibility Profile of Resistance Gene Donor

Lab Code	Resistance Pattern	MARI	No. Resistance	Resistant category
S45	CAZ, CL, AMC, CRO, OFX, CXM, NA, TE, AML, CIP, SAM	0.8	11	MDR
U64	CAZ, CRO, F, CXM, NA, TE, AML ,CIP, SAM, C, CL, AMC,CN	0.9	13	MDR
U58	CAZ, OFX, CXM, NA, TE, AML, CIP, SAM, AMC	0.6	9	MDR
S57	CAZ, F, OFX, CXM, NA, TE, AML,CIP,SAM,CL,AMC	0.8	11	MDR
U60	F, OFX, CXM, NA, TE, AML, C, CL, AMC, CIP, SAM	0.8	11	MDR
URO2	CAZ, CRO, OFX, CXM, TE, AML, SAM, CL,AMC	0.6	9	MDR
W15	CAZ,CRO,OFX, CXM, NA, TE, AML, SAM, C, CL, AMC,CN	0.9	12	MDR
eaB2	CAZ, CRO, OFX, CXM, NA, TE, AML, CIP, SAM, CL, AMC	0.8	11	MDR
B7	CAZ, F, CRO, CXM, TE, AML, SAM, AMC, C	0.6	9	MDR
B3	CAZ, CRO, F, OFX, NA, CXM, AML, SAM, AMC	0.6	9	MDR

Keys: Ampicillin-sulbactam (SAM), Amoxicillin (AML), Amoxicillin-Clavulanic acid (AMC), Cefalexin (CL), Ceftriaxone (CRO), Ceftazidine (CAZ), Cefuroxime (CXM), Ciprofloxacin (CIP), Nalidixic Acid (NA), Ofloxacin (OFX), Gentamicin (CN), Tetracycline (TE), Chloramphenicol (C), Nitrofurantoin (F).

Table 3: Minimum Inhibitory Concentration of the 10 Multidrug Resistant Isolates of *E. coli* to Ceftriaxone and Amoxicillin-Clavulanic Acid Isolated in ABUTH Shika, Zaria.

Isolates	Ceftriaxone MIC in µg/ml	Amoxicillin-Clavulanicacid
URO2	500	500
B2	500	500
B7	250	500
B3	500	500
S45	125	500
S57	125	500
U58	125	250
U60	125	500
U64	500	500
W15	500	500

The minimum inhibitory concentration (µg/ml) of the 10 most multidrug resistant *E. coli* isolated from different sources in A.B.U Teaching Hospital Shika. The 10 isolates were picked for MIC based on multidrug resistance from different sources. The serial dilutions for the MIC in µg/ml were as follow: 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, and 0.98. The MIC in µg/ml of the selected isolates rage from 125 to 500.

Table 4: MIC of ceftriaxone and amoxicillin-clavulanic acid in µg/ml against *Proteus mirabilis* before and after (transconjugation)

P. mirabilis Isolates	Ceftriaxone Conc.		Amoxicillin-Clavulanic acid Conc.	
	Before	After	Before	After
URO_{2p}	0.97	500	15.63	500
B_{2p}	0.97	500	15.63	500
B_{7p}	0.97	31.25	15.63	500
B_{3p}	0.97	500	15.63	500
$S4_{5p}$	0.97	31.25	15.63	500
$S5_{7p}$	0.97	500	15.63	500
U_{58p}	0.97	250	15.63	250
U_{60p}	0.97	31.25	15.63	500
U_{64p}	0.97	500	15.63	500
W_{15p}	0.97	500	15.63	500

These results showed that there was a significant transfer of resistant gene, encoded by plasmid from the donor (clinical isolates of *E. coli*) to the recipient *P. mirabilis*.

Plasmid Curing: The curing of the transconjugants with acridine orange showed significant changes in the antibiotics sensitivity pattern of all the tested transconjugants as showed in figure 1 below while the MIC values of ceftriaxone and amoxicillin-clavulanic acid to the tested transconjugants also decreased significantly when compared with those obtained in the untreated transconjugants as shown in table 5 below.

Table 5: Minimum inhibitory concentrations of ceftriaxone and amoxicillin-clavulanic acid in µg/ml before and after curing the transconjugants (*Proteus mirabilis*).

Isolates	Ceftriaxone Conc.		Amoxicillin/Clavulanic acid Conc.	
	Before	After	Before	After
URO_{2p}	500	1.95	500	62.5
B_{2p}	500	0.97	500	0.97
B_{7p}	31.25	0.97	500	0.97
B_{3p}	500	0.97	500	31.25
$S4_{5p}$	31.25	0.97	500	62.5
$S5_{7p}$	500	0.97	500	31.25
U_{58p}	250	0.97	250	0.97
U_{60p}	31.25	0.97	500	31.25
U_{64p}	500	0.97	500	31.25
W_{15p}	500	3.91	500	125

This result showed that there were significant changes in the cured transconjugants after treatment with acridine dye.

Determination of the Level of Significance of Transconjugants Before and After Plasmid Curing: The results of the level of significant between ceftriaxone and amoxicillin-clavulanic acid in µg/ml before and after curing the transconjugants is as shown in table 6 and 7 below.

Table 6: Ceftriaxone t-Test: Pairing Two Sample for Means

	Before curing	After curing
Mean	334.375	1.362
Variance	49707.03125	0.896373333
Observations	10	10
Pearson Correlation	0.34175594	
Hypothesized Mean Difference	0	
Df	9	
t Stat	4.730200477	
P(T<=t) one-tail	0.000536798	
t Critical one-tail	1.833112923	
P(T<=t) two-tail	0.001073595	
t Critical two-tail	2.262157158	

Table 7: Amoxicillin-Clavulanic acid t-Test: Paired Two Sample for Means

	Before curing	After curing
Mean	475	37.791
Variance	6250	1451.664
Observations	10	10
Pearson Correlation	0.339563	
Hypothesized Mean Difference	0	
Df	9	
t Stat	18.38368	
P(T<=t) one-tail	9.53E-09	
t Critical one-tail	1.833113	
P(T<=t) two-tail	1.91E-08	
t Critical two-tail	2.262157	

In both results, the t calculated are in both tables greater than the t critical at p value < 0.05, hence, there is a significant difference in the MIC of ceftriaxone and amoxicillin-clavulanic acid in µg/ml before and after curing the transconjugants.

Antibiotic Sensitivity Profile of *P. mirabilis* Transconjugants before Curing

The antibiotic susceptibility of the transconjugants before curing is as shown in figure 1 below. *Percentage Sensitivity Pattern of Transconjugant P. mirabilis after Curing:* The antibiotic susceptibility pattern of the transconjugants after curing is as shown in figure 2 below. The result shows that the transconjugants after curing were still 100% resistant to SAM and AMC, 90% to CAZ, 80% to AML,CXM, TE and CL, 60% to AMP, 50% to NA while none of the transconjugants show any resistant to CIP, F, OFX, CN, C and CRO after curing with acridine dye.

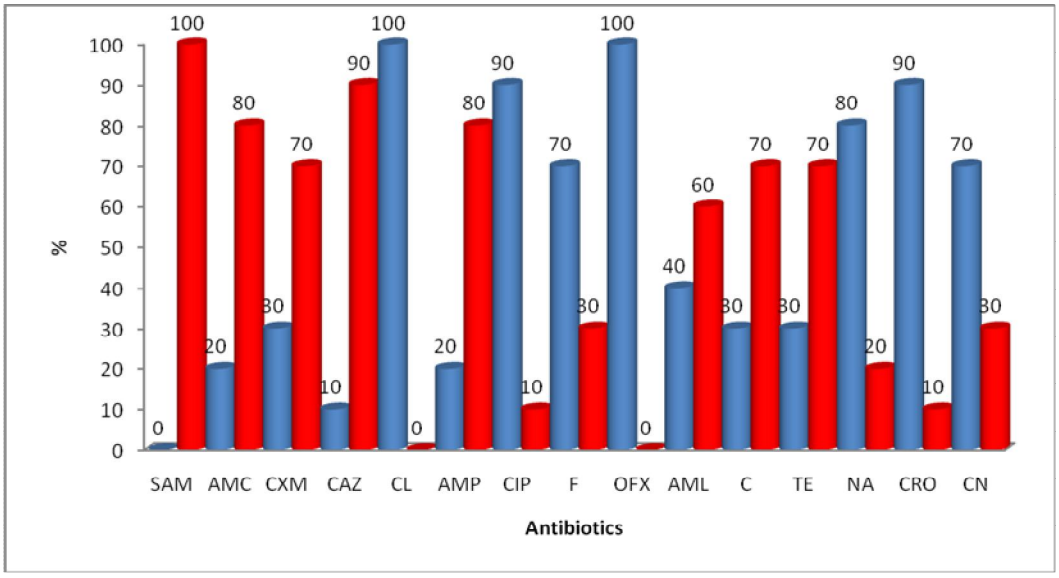


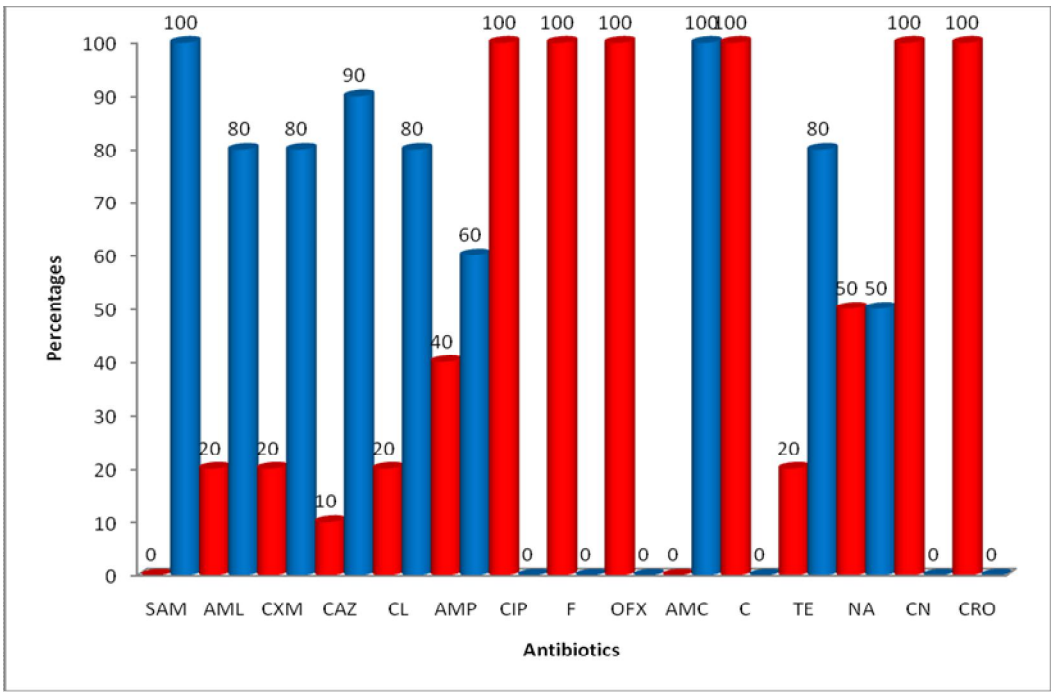
Figure 1: Sensitivity profile of transconjugant *P. mirabilis* before curing.

Keys: Red colour = Resistance, Blue colour = Sensitive, Ampicillin-sulbactam (SAM), Amoxicillin (AML), Amoxicillin-Clavulanic acid (AMC), Cefalexin (CL), Ceftriaxone (CRO), Ceftazidine (CAZ), Cefuroxime (CXM), Ciprofloxacin (CIP), Nalidixic Acid (NA), Ofloxacin (OFX), Gentamicin (CN), Tetracycline (TE), Chloramphenicol (C), Nitrofurantoin (F).

Discussion

The minimum inhibitory concentration (µg/ml) of amoxicillin –clavulanic acid against the 10 multidrug resistant *E. coli* isolates from different sources in A.B.U Teaching Hospital Shika, showed that 5 of the isolates were resistant to ceftriaxone and 9 were resistant to amoxicillin-clavulanic acid at MIC of 500µg/ml. Two (2) of the isolates were found to be resistant to ceftriaxone and one to amoxicillin-clavulanic acid at MIC of 250 µg/ml respectively. These suggest that the isolates have high resistance to amoxicillin-clavulanic acid than ceftriaxone at the same MIC level. It should be noted that organisms can show high MIC value of >2000 µg/ml to even gentamicin and fluoroquinolones which is the beacon of hope in the treatment of *E. coli* infections (Maria, *et al.,* 2006). This showed that amoxicillin-clavulanic acid commonly prescribed for *E. coli* infections is losing its therapeutic confidence leading to the use of "reserved" antibiotics for complication. Soon or later most antibiotics prescribed might also loose there's leading to complication in therapy and limit treatment options.

In conjugation studies, the preliminary antibiotics susceptibility evaluation of the recipient *Proteus mirabilis* showed that *P. mirabilis* was resistant only to tetracycline, out of the 14 antibiotics commonly prescribed for *E. coli* associated infections in A.B.U Teaching Hospital Shika, Zaria. The MIC of ceftriaxone and amoxicillin-clavulanic acid to the clinical isolate of *Proteus mirabilis* was 0.97µg/ml and 15.63µg/ml respectively. Phenotypic evaluation of transconjugant *Proteus mirabilis* on MacConkey agar impregnated with 31.25µg/ml of ceftriaxone and amoxicillin-clavulanic acid showed that the recipient isolate received resistant characteristic features of the donor (e.g *E. coli*). This behaviour is characterized by the presence of a conjugative plasmid (Somkiat, *et al.,* 2007), which might reflect the multifactorial nature of virulence, and provide a selective advantage to virulent *E. coli* in antibiotics environment (Johnson, *et al.,* 2002).



Keys: Red colour = sensitive, Blue colour = resistance

Figure 2: Sensitivity parttern of transconjugant *P. miriabilis* after curing

The sensitivity profile of the transconjugant *P. mirabilis* showed that the transconjugants were 100% resistant to ampicillin-sulbactam, 90% resistant to ceftazidine, 80% to amoxicillin-clavulanic acid and ampicillin, 70% to cefuroxime, chloramphenicol, tetracycline, 60% amoxicilline, 30% to gentamicin and nitrofurantoin, 20% to nalidixic acid, 10% to ceftriaxone and ciprofloxacin and 0% to ofloxacin. This showed that there was a transferable genetic property located on the plasmid via conjugation which will influence an increase in mortality rate; high economic burden and longer hospital stays as most antibiotics might not be effective when required leading to therapeutic failure due to high rate of resistance.

After curing with acridine dye, the result of the MIC clearly showed that the resistant properties of the transconjugant *P. mirabilis* was plasmid encoded to ceftriaxone and amoxicillin-clavulanic acid because there was a significant reduction in the activity of the test antibiotics against the cured transconjugant *P. mirabilis*. However, high MIC value of amoxicillin-clavulanic acid was observed after curing. This may probably be due to mutation in the chromosome of the transconjugant isolates. Similar observation has been reported by other workers (Mohammad and Nishat, 2011).

The sensitivity profile of the transconjugants after curing was 100% to ampicillin-sulbactan and amoxicillin-clavulanic acid, 90% to ceftaxidine, 80% to amoxicillin, cefuroxime, tetracycline and cefalexin, 60% to ampicillin and 50% to nalidixic acid. However, none of the transconjugants showed any resistance to ciprofloxacin, nitrofurantoin, ofloxacin, gentamicin, chloramphenicol and ceftriaxone. This observation probably suggests that there might be a frame shift mutation on the chromosome that influenced the resistant properties of the cured transconjugants to express the resistant trait displayed in this study.

Conclusion and Recommendations

Having observed the rate of transfer of genetic material in *E. coli* and related microorganism like *Proteus mirabilis* through plasmid conjugation which lead to a higher drug resistant in the transconjugants (receptor), which may probably increase through an existing population when antibiotics are misused, immediate regime “checkup” on antibiotics to be prescribed for *E. coli* associated infections will be of greater impact, in reducing the rate of resistance within ABUTH, Shika. It is therefore important

to curb these problems of antibiotic overuse, through rational use of antibacterial agents and constant antimicrobial sensitivity surveillance should be encouraged to help clinicians provide safety and effective empiric therapies. This will serve as an important means of reducing the selective pressure that will help reduce emergence and re-emergence of resistant organisms and reduce the transfer of acquired genetic materials and also quick recovery of patients in the hospitals. Also, sound knowledge of the local distribution of pathogens, their susceptibility patterns and prompt initiation of effective antimicrobial treatment are essential in patients suffering from multidrug resistance infections caused by *E. coli* through alertness, awareness campaign and improvement in health care services, sanitation and hygienic character in all sectors of life.

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