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ARTICLE

Escherichia coli as Possible Agents of Spread of Multidrug Resistance in Port Harcourt, Rivers State.

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Abstract

Multidrug resistance (MDR) continues to be a growing global issue. The problem of MDR is fuelled in part by the spread of the genes encoding resistance horizontally which is linked particularly to conjugation involving plasmids. Studies have demonstrated the presence of plasmids in drug resistant isolates, few have shown a link between these plasmids and drug resistance via plasmid curing especially in our locale. This study set out to explore this link in *Escherichia coli* isolates from Port Harcourt, Nigeria. Plasmid curing was done on a selection of clinical and non-clinical bacteria using acridine orange and antibiotic susceptibility testing carried out on both cured and uncured variants. Data generated was analysed to ascertain the multiple antibiotic resistance (MAR) index and MDR of each isolate. Data was then compared to ascertain effects of plasmid curing on antibiotic resistance of the isolates. Results revealed a decrease in resistance to 7 of 8 antibiotics following plasmid curing. The highest change was noted in ceftazidime (40%), followed by ofloxacin (26.7%). Plasmid curing caused a shift in MAR index values of isolates from higher to lower indices. At MAR index values of ≤0.25 occurrence increased from 5% to 36.7% while at MAR index values ≥0.75, occurrence reduced from 29.9% to 10.0%. A reduction in the degree of MDR was noted (from 55% to 36.7%). Strikingly, the reduction in MDR level of non-clinical isolates was 30% as opposed to 3.4% in the clinical isolates. This study shows a link between plasmids and antibiotic resistance. For the non-clinical isolates, the high-level link between MDR and plasmid carriage could indicate a higher use of antimicrobials in non-clinical rather than clinical settings. Additionally, it could be an indicator for a higher risk of the transfer of MDR determinants from non-clinical sources to human populations in our locale.

Keywords: Plasmid curing, Nigeria, MDR, spread.

1.0 Introduction

The issue of drug resistance is currently a worldwide one. In recent years, this problem has increased in scope and severity, with the evolution of multidrug resistant (MDR) pathogens (Roca et al., 2015). This problem is fuelled in part by the spread of the genes encoding resistance via horizontal gene transfer. In a bid to tackle this problem, studies have focused on controlling or reducing the development of drug resistant pathogens which occurs via the acquisition of resistant genes. The greatest influence on the spread of these drug resistance genes is thought to be exerted by conjugation, which involves plasmids.

Studies demonstrating the presence of plasmids in drug resistant pathogens, often point at a possible role of these plasmids in the drug resistance. Plasmid curing experiments whereby plasmid curing is accompanied by a loss of antibiotic resistance, more clearly points at a link between the plasmids and drug resistance. As early as 1963, plasmids were shown to play a role in antibiotic resistance (Watanabe 1963). Over the years, the presence of plasmids has been linked with resistance to several drug classes, with plasmids thought to carry the genes for most clinically significant resistance (Foster 1983, Carattoli 2009, Jacoby et al., 2014).

Some research contributing to the current pool of information linking antibiotic resistant genes and plasmids have been carried out in Nigeria. These studies employed direct molecular detection of some of these plasmids via PCR (Ogbolu et al., 2013, Ogbolu et al., 2016), plasmid isolation and DNA-DNA hybridization (Soge et al., 2006), transformation experiments (Ojo et al., 2003, Ojo et al., 2006) and complete DNA sequencing of isolated plasmid (Sumrall et al., 2014, Fortini et al., 2015). Majority of Nigerian studies however, have simply demonstrated the presence of various plasmids in drug resistant bacteria. Only a few studies assayed for possible links between these plasmids and drug resistance using plasmid curing experiments, particularly in clinical isolates. Fewer even, have looked at isolates from our particular geographical location (Awopeju et al., 2015, Horsfall et al., 2017).

Both studies carried out in Rivers State, noted in literature to have carried out plasmid curing in drug resistant clinical isolates in Port Harcourt, Nigeria, focused on organisms exhibiting extended spectrum beta lactamase (ESBL) resistance, specifically in *Escherichia coli* and *Klebsiella pneumoniae*. This present study therefore aimed at assessing the relationship between drug resistance and plasmid carriage in a cross section of *E. coli* isolates. Establishing a link between drug resistance and plasmids from isolates in our locale, would contribute to the limited epidemiological information from this region. Additionally, it would provide baseline data for subsequent studies and information on possible risk of future development of drug resistance.

2.0 Materials and Method

2.1 Collection and Identification of samples and clinical Isolates

Environmental and food samples analysed in this study were obtained from a variety of sources (Table 1) while clinical isolates were obtained from the University of Port Harcourt Teaching Hospital, Alakahia, Rivers State, Nigeria. Samples and clinical isolates were analysed by plating on Eosin Methylene Blue agar. Organisms showing the characteristic green metallic sheen were purified and identified using standard biochemical tests (Cheesbrough 2000, Cowan and Steel 1985).

Table 1: Sources of Non-clinical Isolates

Environmental	Water Puddle
	Soil Sample
	Poultry Faecal matter
	Poultry beddings
	Rabbit beddings
Food	Local African Salad

All samples. were obtained from the Choba environ of the University of Port Harcourt.

2.2 Plasmid Curing

This was undertaken on all isolates with the aim of eliminating any plasmids present in the cell (Ojo et al., 2014). In brief, this process involved growing each organism in Mueller Hinton broth containing 0.1 mg/ml of acridine orange, for 24 hr at 37˚C.

2.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing of both cured and uncured variants of each isolate was done using the standard disc diffusion testing method (Bauer et al., 1966). In brief, an inoculum corresponding to 0.5 McFarland standard was seeded onto a Mueller Hinton Agar plate, and relevant antibiotic multidisc applied. Zones of inhibition were determined for each test antibiotic after 24 hr incubation at 37˚C and the susceptibility profile determined for each isolate based on the NCCLS standard (NCCLS 2000). The standard discs used was the Abtek Gram negative test disc containing Ceftazidime (30 µg), Cefuroxime (30 µg), Gentamicin (10 µg), Cefixime (5 µg), Ofloxacin (5 µg), Augmentin (30 µg), Nitrofurantoin (300 µg) and Ciprofloxacin (5 µg).

Multiple antibiotic resistance (MAR) index was determined using a/b where a is number of antibiotics for which resistance was observed and b is total number of antibiotics tested. This ranges from 0 to 1 and provides an indication of level of resistance exhibited by each isolate. Additionally, isolates were defined as multidrug resistant if resistant to three or more drug classes (Cookey and Otokunefor 2016).

3.0 Results

3.1 Bacterial Identification

Following bacterial isolation and identification, sixty isolates were determined to be *Escherichia coli*, thirty from clinical sources and thirty from non-clinical sources.

3.2 Antibiotic Susceptibility Profile

Susceptibility testing of both sets of isolates prior to plasmid curing revealed 25 unique susceptibility profiles in total (Table 2). Of these 25 antibiograms, 9 were unique to the non-clinical isolates and 12 to clinical isolates, with 5 antibiograms common to both sets of isolates. No isolate was fully sensitive and only 3.3% (2/60) of total isolates were resistant to all of the 8 antibiotics. Information from the antibiograms showed that both sets of isolates had a similar level of diversity, though clinical isolates had a slightly higher level of diversity.

Table 2: Antibiotic Susceptibility Profile of Bacterial Isolates

S/No	Antibiotic Resistance Patterns	Non-Clinical Isolates	Clinical Isolates
1.	AUG	-	2
2.	NIT	-	1
3.	AUG-CFM	-	1
4.	AUG-CRX	-	3
5.	AUG-OFL	-	1
6.	AUG-CAZ-CRX	7	-
7.	AUG-CPR-CRX	-	1
8.	CAZ-CRX-GEN	-	1
9.	CAZ-CFM-CRX	1	-
10.	AUG-CAZ-CPR-CRX	1	-
11.	AUG-CAZ-CFM-CRX	3	6
12.	AUG-CPR-CRX-OFL	-	1
13.	AUG-CAZ-CFM-CPR-CRX	3	1
14.	AUG-CAZ-CFM-CRX-GEN	1	1
15.	AUG-CAZ-CFM-CRX-OFL	1	-
16.	AUG-CAZ-CFM-CRX-NIT	1	1
17.	AUG-CAZ-CPR-CRX-OFL	1	-
18.	AUG-CFM-CPR-CRX-NIT	1	-
19.	AUG-CFM-CPR-CRX-OFL	-	2
20.	AUG-CPR-CRX-GEN-OFL	-	3
21.	CAZ-CFM-CPR-CRX-OFL	-	1
22.	AUG-CFM-CPR-CRX-NIT-OFL	1	-
23.	AUG-CAZ-CFM-CPR-CRX-OFL	2	2
24.	AUG-CAZ-CFM-CPR-CRX-GEN-OFL	5	2
25.	AUG-CAZ-CFM-CPR-CRX-GEN-NIT-OFL	2	-
	DIVERSITY	0.91	0.94

Ceftazidime (CAZ), Cefuroxime (CRX), Gentamicin (GEN), Cefixime (CFM), Ofloxacin (OFL), Augmentin (AUG), Nitrofurantoin (NIT) and Ciprofloxacin (CPR).

3.3 Plasmid curing based variation in degree of antibiotic resistance

Antimicrobial susceptibility testing of the different isolates revealed a range of susceptibilities to the various antibiotics (Fig. 1). Prior to plasmid curing, the highest level of resistance was noted against Augmentin and the lowest against Nitrofurantoin. Bacterial resistance to majority of the antibiotics (87.5%, 7/8) was affected by the plasmid curing procedure. In all cases, a >10% reduction was observed. The highest reduction in bacterial resistance was 40%, noted in the resistance against Ceftazidime. This was followed by a difference of 26.7% noted in ofloxacin.

3.4 Plasmid curing and MAR Index

MAR Index represents a general picture of the degree of resistance of individual isolates. Plasmid curing of test isolates caused a shift in MAR Index from the high indices to the lower indices with a higher representation of MAR Index values of 0 to 0.25 in cured isolates in comparison to the uncured variants (Figure 2). The occurrence of isolates at MAR index values of ≤0.25 increased from 5% before curing to 36.7% post curing while at higher MAX index values (≥0.75), occurrence reduced from 29.9% to 10.0%.

3.5 Plasmid curing and Multidrug Resistance (MDR)

Similarly, a change in the degree of MDR due to plasmid curing was noted. In total, 55% of isolates were found to be MDR prior to plasmid curing, but after plasmid curing only 36.7% of isolates were observed to be MDR. A higher variation in levels of MDR was noted with the non-clinical isolates, with a greater than 30% reduction following curing (Figure 3).

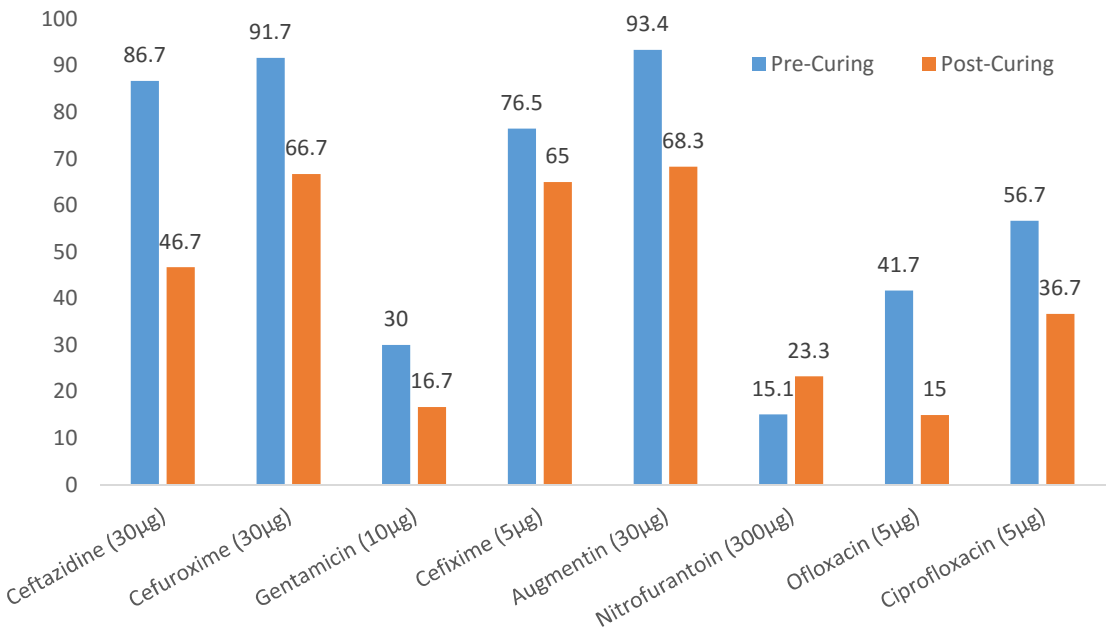

Figure 1: Effect of plasmid curing on antibiotic resistance

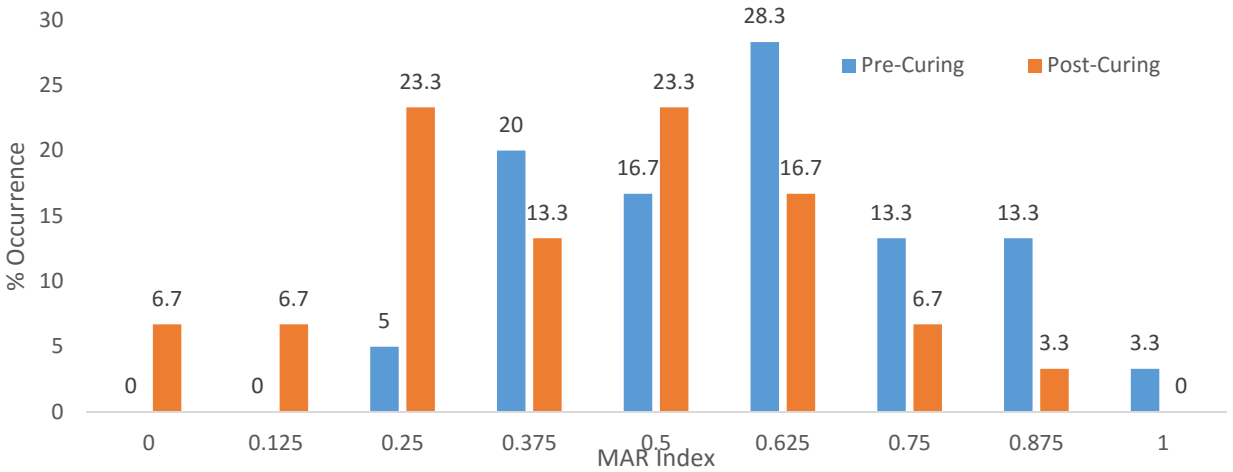

Figure 2: Effect of plasmid curing on MAR Index

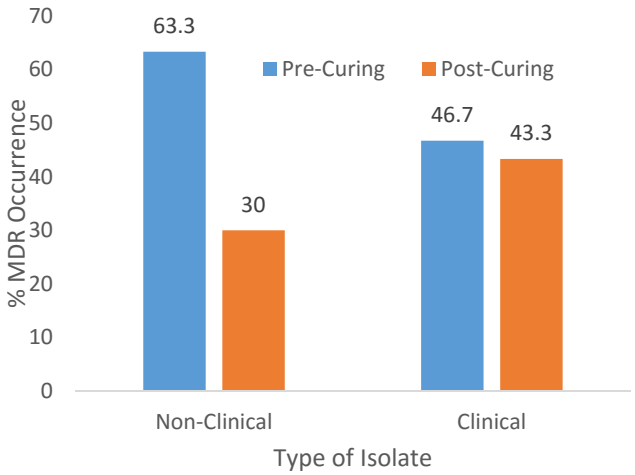

Figure 3: Effect of plasmid curing on Multidrug Resistance

4.0 DISCUSSION

Plasmids are extrachromosomal genetic elements capable of replicating independently of the chromosome. They could be conjugative, capable of transfer from one host to another. These mobile genetic elements have contributed immensely to the spread of resistance genes. While some resistance genes have been found encoded on chromosomes, in some cases they have been described in association with plasmids. Examples of which include resistant genes against beta-lactams, vancomycin, quinolones, sulphonamides and polymyxins (Carattoli 2009, Sultan et al., 2018). These plasmids are of different types, some of which may be associated with more than one antibiotic resistant gene and in some cases, resistance to a single antibiotic may be associated with more than one plasmid (Buckner et al., 2018).

Following curing in this study, the highest level of change in resistance was against ceftazidime. Resistance against ceftazidime has been shown to result from the activity of one of three beta-lactamases, some of which are plasmid mediated (Rains et al., 1995). Results of this research show that a high degree of ceftazidime resistance in the test isolates was plasmid mediated. Similar to this study, two other studies involving plasmid curing had reported that highest levels of change in resistance occurred in ceftazidime specifically or cephalosporins in general (Awopeju et al., 2015, Alkali et al., 2018, Orhue et al., 2017). Plasmid curing had less of an effect on levels of resistance (25% change) against cefuroxime which is a 2nd generation cephalosporin than ceftazidime. Similar to ceftazidime, resistance to cefuroxime is mediated by beta-lactamases, but one study reporting on cefuroxime resistance in *E. coli* noted that the dominant resistance mechanism was over production of a chromosomally encoded beta-lactamase (Schumacher et al., 1996). In contrast to ceftazidime resistance, resistance to cefixime in this study appeared to be more chromosomally related with only an 11.5% difference occurring following plasmid curing. Cefixime is also a 3rd generation cephalosporin, and was the first oral 3rd generation cephalosporin produced (Roche 1989).

For the fluoroquinolones, the 26.7% reduction in resistance noted against ofloxacin was similar to the 21% difference previously reported (Orhue et al., 2017) during a study involving plasmid curing experiments on Pseudomonas aeruginosa. Variations have however been noted with regards to ciprofloxacin. The 20% noted in this study was much higher than the 9% difference reported by Orhue and colleagues (Orhue et al., 2017) but much lower than the 60.6% reported by a study looking at plasmid curing in Staphylococci (Ojo et al., 2014), and another looking at plasmid curing in ESBL *E. coli* which reported a 45.2% change (Awopeju et al., 2015). Mutations in the chromosome have since been recognised as the predominant quinolone resistance mechanism (Yang et al., 2014). In more recent years, plasmid-mediated quinolone resistance genes have been described, particularly in Enterobacteriaceae (Martinez-Martinez et al., 1998). Variations therefore in plasmid curing levels of resistance could reflect varying levels of introduction of plasmid related resistance genes to the varying populations.

MAR index is generally an indication of the source of organisms, with a MAR index above 0.2 indicating that the organisms originated from an area with high antibiotic use (Davis and Brown 2016). The dramatic increase (31.7%) in occurrence of organisms at lower MAR index values noted in this study is a possible indication that plasmids played a key role in the resistance evolution in isolates assayed in this study.

One striking finding of this study is the association between plasmid curing and loss of MDR in non-clinical isolates. Unlike the clinical isolates where the level of multidrug resistant isolates reduced from 46.7% to 43.3%, for the non-clinical isolates a much higher reduction from 63.3% to 30% was noted pointing at a high-level association between the MDR genes and plasmids. Considering that plasmid maintenance is improved by presence of a selective pressure, this could indicate a higher use of antimicrobials in non-clinical rather than clinical settings. Non-clinical bacteria of medical importance have previously been noted to serve as possible reservoirs of MDR and the negative implications of the possible transfer of resistance genes between different bacterial types and from animal to human strains elucidated (Otokunefor et al., 2018, Seiffert et al., 2013, Card et al., 2017). Furthermore, such spread via plasmids has been demonstrated but in vivo and in vitro (Keelara and Thakur 2014, Martin et al., 2014, de Been et al., 2014, Card et al., 2017). This high-level link between MDR and plasmid carriage in this study could therefore point at a higher risk for the transfer of MDR determinants into human populations in our locale.

5.0 CONCLUSION

This study reports on an association between drug resistance and plasmids in *E. coli* isolated in Port Harcourt, Rivers State. Considering the mobile nature of plasmids and potential for transmission from one organism to another, these findings particularly in 3rd generation cephalosporins, could have major negative public health implications. An assessment of the relatedness of these isolates would be ideal to determine if there is a continuous introduction of plasmids to susceptible organisms or a spread of a single clone which had previously acquired the plasmid. The antibiogram typing has proven to be not very discriminatory in this case, probably as a result of the association between drug resistance and plasmids rather than chromosomes.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

Conception: KO
Design: KO
Execution: KO, VOO, CPN
Interpretation: KO, VOO, CPN
Writing the manuscript: KO

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