Plasmid-mediated multiple antibiotic resistance among *Klebsiella pneumoniae* and *Escherichia coli* in a tertiary hospital in Nigeria

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Abstract

Introduction: *About 34 and 31% respectively of Klebsiella pneumoniae and Escherichia coli from clinical samples were isolated and identified on the basis of morphology, growth and biochemical characteristics. Urinary samples had the highest occurrence of K. pneumoniae and E. coli with 50 and 64.2% incidence rate respectively.*
Results: *Although there was no significant difference in their susceptibility to all the antibiotics (p>0.05), the results of their susceptibility profile showed that K. pneumoniae was more susceptible to augmentin (73.5%), followed by ofloxacin (64.7%) and Cefixime (61.8%). Moreover, E. coli showed highest susceptibility to nitrofuratoin (75%), followed by Cefixime (74.2%) and ceftriaxone (71). The results of their antibiotic resistance pattern revealed that both K. pneumoniae and E. coli showed multi-drug resistant (MDR) phenotype against the tested antibiotics. Both K. pneumoniae and E. coli showed highest resistance against cefuroxime (76.5 and 83.9% respectively) followed by gentamycin (67.7 and 77.4% respectively). However, both K. pneumoniae and E. coli exhibit similar resistant pattern to all the class of antibiotics under investigation (p>0.05). The MAR indexes for the antibiotics used against both K. pneumoniae and E. coli showed that nitrofurantoin has the lowest MAR index (0.018), while the highest was found for cefuroxime (0.08). Curing of resistant markers in K. pneumoniae was variable; but the highest resistant marker cured was that of cefuroxime (76.9%). In E. coli, cloxacillin resistant markers were not cured, but resistant markers to other antibiotics were cured variably. The result further revealed that the frequency of resistant markers cured in K. pneumoniae and E. coli were not significantly different (p>0.05). A total of 18 plasmids with molecular weight ranging from 0.56 to >23.13kbp were observed in the selected multi-drug resistant (MDR) isolates of K. pneumoniae and E. coli.*
Conclusion: *Four different plasmid profile groups were detected for selected MDR isolates of K. pneumoniae with 23.13kbp as the most abundant plasmid. Only one (1) plasmid profile group for MDR E. coli was detected.*

Keywords: Plasmid, MDR, *K. pneumoniae, E. coli.*

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No conflicts of interest have been declared by the authors

Introduction

An increase in the emergence of multidrug-resistant (MDR) bacteria in recent years is worrisome to the world population. The presence of antibiotic resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria[1, 2]. Plasmid-associated resistance genes have been discovered for majority of known antimicrobials [3, 4], and it is not uncommon for a single plasmid to simultaneously mediate resistance to five or six antimicrobials.

Escherichia coli is a common enteric commensal of mammals and a common cause of human infection. As such, *E. coli* strains are routinely exposed to a wide range of antimicrobial agents. The *E. coli*, worldwide, have developed resistance to antimicrobial agents and the phenomenon is increasing both in outpatients and hospitalized patients [5, 6], with a propensity for plasmid carriage[7].

Klebsiella is the oldest genus known among the *Enterobacteriaceae* family; the normal habitat of this bacteria is the intestinal tract of human and animal, but may be transferred to another site causing a wide range of infections, as in burns, wounds, respiratory tract and urinary tract infections; these infections become difficult to treat because of the increased ability of *Klebsiella* to resist different types of antibiotics [8]. The vast majority of *Klebsiella* infections, however, are associated with hospitalization. As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus[9]. The present study was carried out with an objective to examine the incidence of multidrug resistant strains of *E.coli* and *K. pneumoniae,* which were recovered from patients attending a tertiary care hospital in Nigeria.

Materials and Methods

Thirty four (34) and thirty one (31) multi drug resistant strains of *Klebsiella pneumoniae* and *Escherichia coli* respectively isolated by standard procedures[10] from 100 clinical specimens sent to Medical Microbiology Laboratory of University of Benin Teaching Hospital (UBTH) were evaluated in this study.

Preparation of inoculums

The overnight cultures of the test organisms were inoculated onto peptone water and vortex thoroughly. The turbidity of the bacterial suspensions were then adjusted and compared with 0.5 McFarland standard. The 0.5 McFarland standards was prepared by adding 0.5 ml of 1.2% (wt/vol) barium chloride dihydrate (Bacl2.2H2O) solution to 99.5 ml of 1% sulphuric acid. The turbidity standard was then aliquot into test tubes identical to those used to prepare the inoculums suspension.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on Mueller—Hinton agar (HiMedia, India) by using commercial antibiotic disks. Commercially available antimicrobial discs used included: ciprofloxacin (30µg), ceftriaxone (10µg), ofloxacin (25µg), amoxicillin-clavulanate (25µg), cefuroxime (30µg), gentamycin (10µg), ceftazidime (30µg), cloxacillin (10µg) and cefixime (10µg). The results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS)[11]

Multiple antibiotic resistance indexes

The Multiple Antibiotic Resistance index (MARI) for an antibiotic was calculated thus: [Number of antibiotics resistance to the isolates / (Number of antibiotics x Number of isolates)].

Curing

Curing of the drug resistant isolates was done using 10% sodium dodecyl sulphate (SDS) as described by Akortha *et al*[12]. The resistant isolates were grown for 24hours at 34^{o}C in nutrient broth containing 10% SDS. After 24hrs, the broth cultures were agitated to

homogenize the content and were sub-cultured unto Mueller-Hinton agar (MHA) plates. The plates were incubated at 37°C for 24hrs after which colonies were screened for antibiotic resistance by the disk diffusion method. Cured markers were determined by comparison between the pre- and post-curing antibiogram of isolates.

Plasmid DNA isolation

Selected strains of *Klebsiella pneumoniae* and *Escherichia coli* that were cured of their resistance markers were subjected to plasmid DNA isolation according to the protocol of Birboim and Doly[13]. Five millilitres of overnight culture was transferred to eppendorf tubes and centrifuged for 5 minutes at 8,000g to pellet the cells. The supernatant was discarded, the cell pellet re-suspended in 200ìl of solution A (50mM glucose, 10mM EDTA, 25mM Tris-hydrochloride pH 8.0), and incubated at 370C for 10 minutes. After thorough vortexing, 400ìL of freshly prepared solution B (0.2M NaOH, 4% sodium dodecyl sulphate) was added, the sample mixed by inverting the tubes rapidly and left for another 10 minutes at room temperature. To this suspension, 300ìl ice cold solution C (3M Sodium acetate pH 4.8) was added to stop the cell lysis. The suspension was incubating on ice for 10 minutes, and then centrifuged for 5 minutes at 3, 000xg. Supernatant was transferred to new eppendorf tube and 700ìl (1ml) chilled chloroform was added, mixed by vortexing and left for 10 minutes. It was subsequently centrifuged for 10 minutes at 3,000xg to precipitate the DNA. The supernatant was poured off, 1ml of cold 70% ethyl alcohol added, kept in ice bath for 10 minutes and supernatant again discarded with ethanol. The tubes were inverted on a paper towel to drain the remaining traces of liquid and the pellets were resuspended in 100ìl TE buffer (10mM Tris, 1mM EDTA, pH 8.0) and kept in freezer.

Agarose gel electrophoresis of plasmid DNA extracts was carried out on 0.8% (w/v) agarose gel in a 0.5x concentration of Tris-borate EDTA (TBE) buffer, stained with ethidium bromide. 20ìl of each plasmid extract was then loaded into an agarose well after mixing with 2ìl of bromophenol blue. A DNA Hind III DNA molecular weight marker was loaded into one of the wells as standard. The gel was thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for 35 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound Ethidium bromide under a short wave UV light transilluminator and the photograph was taken. The DNA bands were matched with those for lambda DNA Hind III digest molecular weight markers. The approximate molecular weight of each plasmid was consequently obtained by extrapolation on graphical plots of molecular weight of marker against the distance travelled by the respective band [10].

Statistical Analysis

Mann-Whitney test and Duncan Multiple Range Test (DMRT) were used to test for significant differences in all the data obtained. All statistical analyses were carried out using the SPSS 17.0 window based program. Significance difference and non- significance difference was defined when pd 0.05 and pe 0.05 respectively.

Results

The results in Table 1 revealed that the incidence of *Klebsiella pneumoniae* (34%) is higher than *Escherichia coli* (31%) in the clinical

Table 1: Frequency of *E. coli* and *K. pneumoniae*

Clinical samples	*K. pneumoniae*	*E. coli*
	(%)	(%)
Wound swab	2(5.9)	7(22.6)
Blood	6(17.7)	2(6.5)
Urine	17(50)	20(64.5)
High vagina swab	-	2(6.5)
Ear swab	2(5.9)	-
Catheter tip	6(17.7)	-
Endocervical swab	1(2.9)	-
Total	34(34)	31(31)

samples. The result further showed that urinary samples had the highest occurrence of *K. pneumoniae* and *E. coli* with 50% and 64.2% incidence rate respectively. *K. pneumoniae* was not isolated in high vagina swab (HVS), while *E. coli* was not isolated in ear swab, catheter tips and Endocervical swab (ECS).

Table 2: Resistance profile of *K. pneumoniae* and *E. coli* and MARI for the antibiotics

Antibiotic	*K. pneumoniae*		*E. coli*		
	No. Resistant (%)	No. Sensitive (%)	No. Resistant (%)	No. Sensitive (%)	MARI for antibiotics
Augmentin	9(26.5)	25(73.5)	23(74.2)	8(25.8)	0.049
Cefixime	13(38.2)	21(61.8)	8(25.8)	23(74.2)	0.032
Nitrofuratoin	7(41.2)	10(58.8)	5(25)	15(75)	0.018
Ciprofloxacin	17(50)	17(50)	13(41.9)	18(58.1)	0.046
Ceftriaxone	19(55.9)	15(44.1)	9(29)	22(71)	0.043
Ofloxacin	12(35.3)	22(64.7)	17(54.8)	14(45.2)	0.044
Cefuroxime	26(76.5)	8(23.5)	26(83.9)	5(16.1)	0.08
Gentamycin	23(67.7)	11(32.3)	24(77.4)	7(22.6)	0.072
Ceftazidime	23(67.7)	11(32.3)	22(71)	9(29)	0.069
Cloxacillin	20(58.8)	14(41.2)	13(41.9)	18(58.1)	0.050

Key: *MARI = Multiple antibiotic resistant index*

The results of their antibiotic susceptibility pattern revealed that *K. pneumoniae* was more susceptible to augmentin (73.5%), followed by ofloxacin (64.7%) and Cefixime (61.8%) while *E. coli* showed highest susceptibility to nitrofuratoin (75%), followed by cefixime (74.2%) and ceftriaxone (71). However, there was no significant difference in their susceptibility to all the antibiotics ($p>0.05$). The results of their antibiotic resistance pattern revealed that both *K. pneumoniae* and *E. coli* showed multi-drug resistant (MDR) phenotype against the tested antibiotic.

K. pneumoniae and *E. coli* showed highest resistance against cefuroxime (76.5 and 83.9% respectively) followed by gentamycin (67.7 and 77.4% respectively) (Table 2). The result of

Table 3: Plasmid curing analysis of resistant *K. pneumoniae* and *E. coli*

Antibiotics	*K. pneumoniae*			*E. coli*		
	No. resistant (Pre-curing)	No. resistant (Post-curing)	No(%) cured	No. resistant (Pre-curing)	No. resistant (Post-curing)	No(%) cured
Augmentin	9	4	5(55.6)	23	8	15(65.2)
Cefixime	13	7	6(46.2)	8	7	1(12.5)
Nitrofuratoin	7	2	5(71.4)	5	1	4(80)
Ciprofloxacin	17	9	8(47.1)	13	8	5(38.5)
Ceftriaxone	19	10	9(47.4)	9	2	7(77.8)
Ofloxacin	12	4	8(66.7)	17	12	5(29.4)
Cefuroxime	26	6	20(76.9)	26	11	15(57.7)
Gentamycin	23	13	10(43.5)	24	18	6(25)
Ceftazidime	23	8	15(65.2)	22	10	12(54.5)
Cloxacillin	20	9	11(55)	13	13	0(0)

statistical analysis revealed that both *K. pneumoniae* and *E. coli* exhibit similar resistant pattern to all the class of antibiotics under investigation (p>0.05).

The multiple antibiotic resistances (MAR) index for each antibiotic used against *K. pneumoniae* and *E. coli* was calculated. The result showed that cefuroxime (0.08) has the highest MAR index closely followed by gentamycin (0.072) and ceftazidime (0.069), while the lowest MAR index was found for nitrofurantoin (0.018).

The plasmid curing analysis of multi-drug resistant *K. pneumoniae* and *E. coli* are shown in Table 3. Curing of resistant markers in *K. pneumoniae* was variable; but the highest resistant marker cured was that of cefuroxime (76.9%), followed by that of nitrofuratoin (71.4%) and ofloxacin (66.7%). In *E. coli*, *pneumoniae* and *E. coli* were not significantly different (p>0.05).

A total of 18 plasmids with molecular weight ranging from 0.56 to >23.13kbp were observed in the selected multi-drug resistant (MDR) strains of *K. pneumoniae* and *E. coli*. Four different plasmid profile groups were detected for selected MDR strains of *K. pneumoniae* with 23.13kbp as the most abundant plasmid. Only one (1) plasmid profile group for MDR *E. coli* was detected. The number of organisms per plasmid profile group vary from 1-9 for MDR *K. pneumoniae*.

Discussion

E. coli and *Klebsiella* spp. are members of the Enterobacteriaceae family that exist in the human intestine and are involve in nosocomial infections[14]. The findings that *K. pneumoniae* was the most isolated Gram negative organisms from clinical samples as shown in this study were in conformity with previous studies[15, 16]. Contrary to the findings in this study however,

Table 4: Plasmid profile analysis of selected strain

Isolates	Lab no.	No. of plasmid	Size of plasmid (kbp)
K. pneumoniae	3	1	23.13
	5	1	23.13
	8	1	23.13
	23	1	23.13
	24	2	0.56, 23.13
	25	1	9.42
	28	1	23.13
	30	1	23.13
	36	2	23.13, >23.13
	37	1	23.13
	41	1	23.13
	44	1	23.13
E. coli	2	1	23.13
	4	1	23.13
	6	1	23.13
	32	1	23.13
	33	1	23.13
	46	1	23.13

cloxacillin resistant markers were not cured, but resistant markers to other antibiotics were cured variably. The result further revealed that the frequency of resistant markers cured in *K.*

many other reports showed that *E. coli* was the most abundant Gram negative organisms isolated from clinical samples [17, 18 and 19]. Urinary samples had the highest isolation rate of *K. pneumoniae* and *E. coli* as shown in this study with the highest incidence on *K. pneumoniae*. This finding is contrary to the report of Razaghi *et al*. [20] who in their study revealed that respiratory samples had the highest isolation of *K. pneumoniae* and *E. coli*. In the same vein, other studies showed that *E. coli* was the most predominant pathogen from urinary samples, closely followed by *K. pneumoniae* [19, 21 and 22].

In this study, 75% *E. coli* and 58.8% *K. pneumoniae* were susceptible to nitrofurantoin. This finding is comparably higher than those reported by Ramesh *et al*. [23] and Mohammed *et al*. [17]. *K. pneumoniae* and *E. coli* in this study had their highest occurrence from urinary sample. Coincidentally, nitrofurantoin is a urinary antiseptic. Therefore, nitrofurantoin can be employed as a reliable oral alternative agent for urinary tract infections.

Gonzalez and Spencer [24] reported that aminoglycosides have a good activity against clinically important Gram negative bacilli. In this study however, only 32.3% *K. pneumoniae* and 22.6% *E. coli* are susceptible to gentamycin. This finding is in agreement with previous report from India and Israel [25, 26]. The high resistance to gentamycin by *K. pneumoniae* (67.7%) and *E. coli* (77.4%) in this study might not be unconnected with the increased use of the drug. Miller *et al*. (27) reported that the pattern of resistance to aminoglycosides is affected by selective pressure in different regions. Resistance to aminoglycosides is frequently due to the acquisition of modifying enzymes such as acetyltransferases, phosphorylases and adenylyltransferases, among others [28].

Resistance to fluoroquinolones in Gram-negative bacteria has increased in recent years, probably caused by excessive and inappropriate use of these drugs [29], particularly, due to under-dosing and mono-therapy against moderately susceptible pathogens. The observed resistance to ciprofloxacin and ofloxacin (fluoroquinolones) in this study was 50% and 35.3% respectively for *K. pneumoniae* while 41.9% and 54.8% respectively for *E. coli*. This is similar to the findings reported in Canada [30], USA [31], Palestine [32] and Turkey [33].

In this study, it was observed that an average of 59.6% and 52.4% of *K. pneumoniae* and *E. coli* showed resistance to at least one of the four cephalosporins (Cefixime, ceftriaxone, cefuroxime and ceftazidime) used in this study, and their resistance was found to co-exist with the resistance to other antibiotics. This is closely similar to the findings of Shiju *et al*. [34]. Resistance to â-lactams is said to be mediated by â-lactamase enzymes which are capable of hydrolyzing and inactivating a wide variety of â-lactams including penicillins [35]. The genes encoding â-lactamases can be located on the bacterial chromosomes, plasmids, or on transposons [36]. In *Klebsiella* spp. and *E. coli* isolates, resistance to â-lactam may also occur due to modified outer membrane permeability [37].

The result of the present study indicate that *K. pneumoniae* and *E. coli* exhibit resistance traits against penicillins (augmentin and cloxacillin), with *K. pneumoniae* exhibiting even higher resistance than *E. coli.* This finding is closely similar to previous report [38].

The MAR indexes result revealed that cefuroxime (0.08) has the highest MAR index closely followed by gentamycin (0.072) and ceftazidime (0.069). This indicates that these antibiotics were highly resistant among *K. pneumoniae* and *E. coli*. However, the lowest MAR index was found for nitrofurantoin (0.018) indicating highest susceptibility among the tested organisms. The MAR index in this study is an indication that the organisms isolated in this study are grossly exposed to antibiotic. This implies that patients from whom these organisms are isolated comes from an environment where antibiotics are severally used.

This MDR phenotype by both *K. pneumoniae* and *E. coli* may be due to plasmids harboring several resistance genes which are transferred from one bacterium to another [39]. Mathai *et al*. [40] have linked such resistance pattern to the presence of integrons.The result of curing analysis in this study showed that loss of resistant markers among MDR *K. pneumoniae* and *E. coli* were variable. This implies that most resistant markers on these isolates could have been acquired either from other bacterial species in their environment or inherent as the case may be. However, all *E. coli* isolates were not cured of cloxacillin resistant markers, which imply that cloxacillin resistant markers on these species may be inherent.

The most common plasmids encountered in this study were 23.13kbp. This is contrary to findings from other researchers [41, 42], but similar to reports from some previous studies[43, 44].The findings of this study raises assumption that this plasmid is one of the most stable plasmids among these isolates. Plasmids bearing one or more resistance genes have the ability to code for enzymes that destroy or modify drug[45]. The high resistance shown by *K. pneumoniae* and *E. coli* in this study may be due to the presence of R-plasmids as shown by loss of resistance markers after curing. The emergence of R-plasmid in this study could be due to wide spread use of antibiotics which is caused by misuse and abuse of antibiotics due to easy accessibility to drug outlets, self-medication and poor patient's compliance to antibiotic therapy. The predominant resistance plasmids involved in this study may be very prone to transfer, as suggested by its occurrence in several strains of *K. pneumoniae and E. coli*. This plasmid may have entered these strains that have a particular ability to persist in the guts of patients [46], on the hands of health care workers, and or in the health care environment [47].

Conclusion

This study highlights widespread antimicrobial resistance patterns and diverse plasmid profile of *K. pneumoniae and E. coli* from Nigeria. It is hoped that this information will be a useful baseline for further epidemiological investigations. The high MDR in this region is a cause for concern. Further molecular studies may have to be conducted to establish the basis of this MDR. Strict antibiotic policy should be adopted in hospitals to estimate the impact of high resistance in bacteria and to take steps at reducing it. There are several possible methods for overcoming resistance and includs reduced use of antibiotics, use of synergistic combinations, addition of an anti-resistance factor, attacking the underlying disease, improving the hygienic measures and regular surveillance studies [48, 49].

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