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Full Length Research Paper

Plasmid profile of human *Escherichia coli* isolated from five major geopolitical zones of Nigeria

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Plasmid exchange between different strains of bacteria of the same species or different strains of different species is a recognized source for the rapid spread of antimicrobial resistance. In this study, 89 human isolates of *Escherichia coli* from five major geopolitical zones of Nigeria were isolated and tested against 14 antibiotics by the disk diffusion method. Resistance plasmids were extracted and separated by agarose gel electrophoresis for profiling. In all, 42 different antibiotics resistance pattern was observed, with all the isolates showing resistance to at least four or more drugs tested. Plasmid profiling revealed that the isolates contained various size of R-plasmids. A total of 146 plasmids were detected with molecular sizes ranging from 1 to 120 KB. Of all the plasmids detected, the 120 KB plasmid occurred most frequently in all the geopolitical zones. Although some strains exhibited different antibiotic resistance patterns, some of their plasmids had similar migration patterns on agarose gel electrophoresis. Multiple resistances were conferred by R-plasmids of different sizes. The high prevalence of antibiotic resistance conferring plasmids observed in this study may be due to the increasing widespread use of antibiotics.

Key words: Plasmid profile, *Escherichia coli,* antibiotic resistance, Nigeria.

INTRODUCTION

Escherichia coli belongs to the family Enterobacteriaeceae. It is one of the main causes of both nosocomial and community acquired infections in humans. The organism is therefore of clinical importance and can be isolated from various specimens (Karlowsky et al., 2004; Khachatryan et al., 2008; Johnson and Nolan, 2009; Johnson et al., 2009; Nsofor and Iroegbu, 2012; Nsofor and Iroegbu, 2013).

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment. This therefore demands the need for periodic screening of common bacterial pathogens for their antibiotic susceptibility profiles in different communities. According to Umolu et al. (2006), *E. coli* isolates in Nigeria are highly resistant to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazole. The widespread occurrence of drug resistant *E. coli* and other pathogens in our environment has necessitated the need for regular monitoring of antibiotics susceptibility trends to provide the basis for developing rational prescription programs, making policy decisions and assessing the effectiveness of both (Omigie et al., 2006).

Molecular tools have been used to correlate animal associated pathogens with similar pathogens affecting humans and to clearly demonstrate transferable resistant genes carried by plasmids common to both animals and humans (Pitout et al., 2009; Abbassi- Ghozzi et al., 2012; Ben Aissa and Al-Gallas, 2007). The possibility of antibiotic resistance genes circulating among humans

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constitutes a direct threat to public health. This threat prompts research into emerging resistance mechanisms, novel approaches to antimicrobial efficacy and stringent control measures in the prudent use of antimicrobials in human medicine.

Comparative sequence analyses of different types of antimicrobial resistance genes suggest that they originated and diversified in environmental communities, from which they were mobilized and propagated into taxonomically and ecologically distant bacterial populations. Plasmid exchange between *E. coli* strains is a recognized source for the rapid spread of antimicrobial resistance phenotypes (Gakuya et al., 2001; Smith et al., 2003; Fang et al., 2008). The potential significance of plasmids in disseminating antimicrobial resistance genes is further enhanced by the association of plasmids with mobile genetic elements, such as transposons, integrons, and insertion (IS) elements (Bennett, 2004; Toleman et al., 2006, Bennett 2008, Pitout et al., 2009). To better understand the evolution and dissemination of resistance phenotypes from clinical, agricultural, and environmental settings, it is therefore necessary to perform molecular analysis of resistant isolates at three different levels, comparing whole genomes, single plasmids, and individual resistance gene cassettes. In this study, plasmids isolated from antibiotics resistant *E .coli* obtained from the major tertiary hospitals located in the five geopolitical zones of Nigeria was analyzed by agarose gel electrophoresis.

MATERIALS AND METHODS

Specimen collection, cultivation and identification of *E. coli*

Sample collection, cultivation, identification of *E. coli* and antibiotics susceptibility testing was based on our previous published work (Nsofor and Iroegbu, 2013). Briefly, human fecal specimens were collected at the University of Port Harcourt Teaching Hospital, Port Harcourt, Rivers State (south-south), Abia State University Teaching Hospital, Aba, Abia State (south east), Lagos State University Teaching Hospital, Ikeja, Lagos state (south west), National Hospital, Abuja (north-central) and Military Reference Hospital, Kaduna State (north-north). All sampling procedures were in accordance with guidelines of the National Health Research Ethics Committee, Nigeria (www.nhrec.net). The specimens were streaked directly on Eosin Methylene Blue agar (EMB) (Oxoid, England). No antibiotic was included in the EMB agar plates used for the cultivation. The inoculated plates were incubated overnight at 37°C. A single colony on EMB with green metallic sheen taken to be *E. coli* was selected from an individual fecal sample for further characterization. *E. coli* was fully identified using conventional microbiological tests-Indole positive, Methyl red positive and Citrate negative (Cheesbrough, 2000).

Antibiotics susceptibility testing

The antibiotics susceptibility pattern of the isolates was determined using the disk diffusion method (Cheesbrough, 2000), on Mueller-Hinton agar (Oxoid, England). Inhibition zone diameter values were interpreted using standard recommendations of the Clinical Laboratory Standard Institute (CLSI, 2006). Susceptibility was tested against ampicillin (10 µg), amoxycillin/ clavulanic acid (20/10 µg), Cefoxitin (30 µg), cefpodoxime (10 µg), cefpirome (30 µg), ceftriaxone (30 µg), cefotaxine (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), nitrofurantoin (30 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (10 µg) and tetracycline (30 µg), (Oxoid, England). *Escherichia coli* ATCC 25922 was included as a reference strain.

Plasmid profile

Plasmid DNA was extracted using the alkaline SDS method (Kado and Liu, 1981). The isolates were grown on Luria-Bertani (LB agar) overnight at 37°C. About 10-50 isolated colonies were suspended in 75 µl of lysis buffer (3% SDS, 50 Mm Tris-Base pH 12.6) and incubated in a 55°C water bath for 1 h for cell lysis. After the lysis, 50 µl of phenol: chloroform (1:1, v/v) was added to the bacterial suspension. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation (14,000 rpm for 15 min). Avoiding the precipitate at the interface, the upper aqueous phase (40nµl) was transferred to another set of 1.5 ml microcentrifuge tubes and was mixed with 7 µl of 6x loading dye (Invitrogen) for electrophoresis. Agarose gel electrophoresis was performed with 0.8% agarose in 1x TAE buffer (121 Tris-Base, 22.55 glacial acetic acid and 50 ml of 0.5 M EDTA pH 8.0) on a horizontal gel apparatus at 100 V/cm^2 for 3 h. The BAC-Tracker Super coiled DNA (Invitrogen) and 1 KB plus DNA ladders (Invitrogen) were included for the estimation of plasmid sizes. The gel was stained with 5 µl of 10 mg/ml of ethidium bromide, visualized by UV transilluminator (Fisher Scientific) and photographs were taken with gel imager (Alpha Innotech Corporation,San Leandro, CA, USA).

RESULTS

The antibiotic susceptibility testing results show 42 different antibiotics resistance profiles, with all the isolates showing resistance to at least four or more of the drugs tested. Among the 89 isolates tested, were 94.4% that showed resistance to ampicillin; 85.5% to cotrimaoxazole, 92.1% to cephalothin; 78.7% to streptomycin, 70.8% to nitrofurantoin; 79.8 to tetracycline; 67.4% to chloramphenicol; 74.2% to amoxicillin clavulanic acid; 61.8% to cefpirome; 52.8% to cefpodoxime; 46.1 % to cefotaxine; 46.1% to ceftriaxone; 31.5% to cefoxitin; 38.2% to nalidixic acid and 24.7% to gentamycin.

Taking the susceptibility pattern source after source, the isolates from the south east zone of Nigeria showed a unique pattern of resistance; they were resistant to ampicillin and cotrimaoxazole (91.3% each); gentamycin and nalidixic acid (17.4% each); tetracycline and amoxicillin clavulanic acid (87% each); chloramphenicol and ceftriaxone (47.8% each); streptomycin, cefpodoxime and cefotaxine (65.2% each) (Table 1). A similar pattern was observed in the isolates from the south west zone; 93.1% of the isolates were resistance to cotrimaoxazole, tetracycline and cephalothin respectively. Also, common resistance pattern of 66.7% to cefpodoxime and nitro-furantoin; 33.3% to ceftriaxone and gentamycin; and 53.3% to cefoxitin and nalidixic acid respectively was

Table 1. Percentage Antibiotics Resistance Pattern of *E. coli* isolated from human specimens in different regions of Nigeria**.**

Sample source	Antibiotic														
	AMP	SXT	GN	NA	TE	S	C	KF	F	AMC	CRO	CPO	CPD	CTX	FOX
SE	91.3	91.3	17.4	17.4	87.0	65.2	47.8	95.7	70.0	87.0	47.8	74.0	65.2	65.2	39.0
SW	86.7	93.3	33.3	53.3	93.3	100	73.3	93.3	66.7	60.0	33.3	46.7	66.7	40.0	53.3
SS	100	76.5	29.4	23.5	58.8	88.2	76.5	88.2	58.8	82.4	41.2	76.5	47.1	41.2	41.2
NC	100	100	20.0	100	80.0	80.0	80.0	100	100	80.0	40.0	60.0	60.0	40.0	20.0
NN	96.6	79.3	27.6	44.8	75.8	72.4	70.0	90.1	72.4	65.5	52.2	55.2	41.4	41.4	10.3

AMP, Ampicillin; SXT, Cotrimaoxazole; GN, Gentamycin; NA, Nalidixic acid; TE, Tetracycline; S, Streptomycin; C, Chloramphenicol; KF, Cephalothin; F, Nitrofurantoin; AMC, Amoxycillin; CRO, Ceftriaxone; CPO, Cefpirome; CPD, Cefpodoxime; CTX, Cefotaxine; FOX, Cefoxitin SE, South-East; SW, South-West; SS, South-South; NC, North-Central; NN, North-North.

Table 2. The plasmid profiles of the isolates according to geographical locations.

Plasmid profile (KB)	Frequency of distribution according to location/zone				
	South - East N=48 (%)	South -South N=29 (%)	South -West N=27 (%)	North Central N=06 (%)	North -North N=36 (%)
1.0	19 (39.6)	01 (3.4)	04 (14.8)	01 (16.7)	01 (2.8)
1.4	04 (8.3)	00	03 (11.1)	00	02 (5.6)
1.5	00	08 (27.6)	01 (3.7)	01 (16.7)	03 (8.3)
1.8	00	02 (6.9)	02 (7.4)	00	00
2.0	00	03 (10.3)	01 (3.7)	00	06 (16.7)
2.5	01 (2.0)	03 (10.3)	01 (3.7)	01 (16.7)	04 (11.1)
3.0	02 (4.2)	03 (10.3)	02 (7.4)	01 (16.7)	01 (2.8)
4.0	03 (6.3)	00	02 (7.4)	00	00
4.5	00	01 (3.4)	00	00	01 (2.8)
5.0	00	00	00	00	06 (16.7)
7.0	00	01 (3.4)	00	01 (16.7)	02 (5.6)
10.0	00	01 (3.4)	01 (3.7)	01 (16.7)	03 (8.3)
20.0	00	02 (6.9)	00	00	00
95.0	03 (6.3)	00	02 (7.4)	00	00
120	16 (33.3)	04 (13.8)	07 (26.0)	00	06 (16.7)

N= number of plasmids.

observed among these isolates (Table 1). The isolates from the south-south zone showed a unique resistance to cephalosporins; 41.2% to cefoxitin, ceftriaxone and cefotaxine respectively and a very high resistance of 76.5% to cefpirome-a third generation cephalosporin (Table 1).

Plasmid profiling

A total of 146 plasmids were detected with molecular sizes ranging from 1 to 120 KB. Of all the plasmids detected, the 120 KB plasmid was most frequent, with 23% occurrence rate. All the isolates bearing this plasmid also harbored one or more smaller plasmids, and they were resistant to six or more antibiotics including cefpirome, a third generation cephalosporin. In general, 15 different plasmid profiles were observed with plasmids 2.5, 3 and 120 KB occurring in all the geographical zones (Table 2).

Similar to what was observed in antibiotics resistance, the plasmid distribution in isolates from the south-east zone showed a unique pattern. Of the 48 plasmids detected from the isolates in this zone, a total of seven plasmid profiles was observed, with the 1 and 120 KB plasmids being most frequent (39.6 and 33.3%) respectively; the remaining plasmids harbored by these isolates occurred at a considerably low rate; 2.0-8.3% (Table 2). The isolates from other regions were more diverse in terms of plasmid distribution, though, 11 plasmid profiles were observed in the isolates from south-south, south-west and north-north respectively and there was an even distribution across the different plasmid sizes observed in this study. In the south-south, the 1.5 KB plasmid occurred highest (27.6%), while the 120 KB plasmid was most frequent in the isolates from the south-

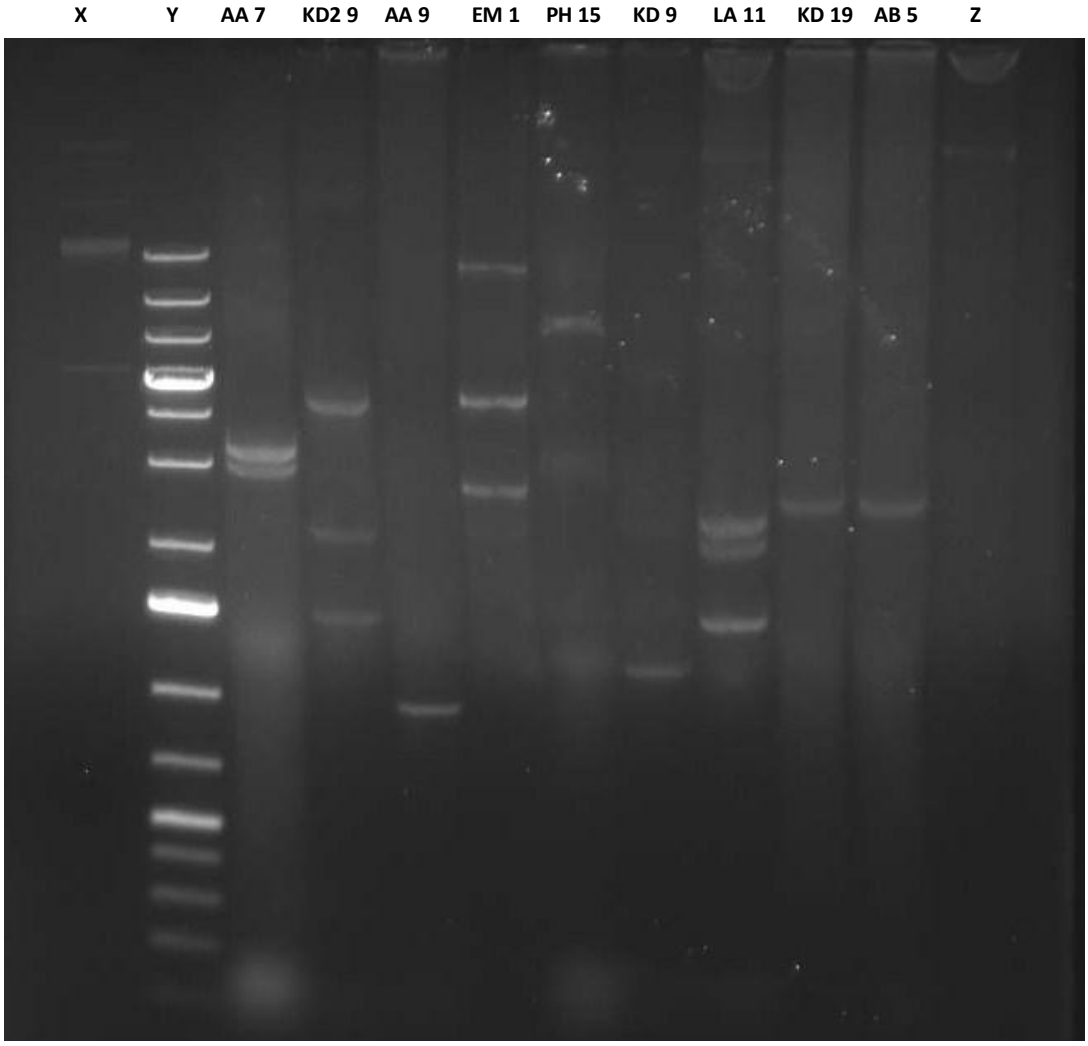

Figure 1. The plasmid profile of first group of *E. coli* Isolates from human specimens. X= Bac-tracker super coiled DNA Ladder, Y=1KB plus DNA Ladder, Z=120KB Plasmid positive control. In this figure, all isolates harbor plasmids with strains KD29 (North-North), and EM1 (South-South) harboring three plasmids each while strain LA11 (South-West) harborfour plasmids including the 120 KB plasmid.

west. Two isolates from the south-south harbored the 20 KB plasmid which was not present in any other isolate; also the 5 KB plasmid was only detected in the isolates from the north-north zone. The isolates from this zone harbored the 2, 5 and 120 KB plasmids at the same rate of 16.7% respectively. Six plasmids were detected from the isolates from the north-central zone, with each isolate harboring one plasmid at a time (Table 2). Figures 1 to 6 show the detailed gel images of some of the plasmid profiles.

DISCUSSION

Plasmids are the major mechanism for the spread of antibiotic resistant genes in bacterial populations (Smith et al., 2003; Johnson and Nolan, 2009; Bennett, 2008; Shames et al., 2009). Multiple resistance genes are harbored on resistance plasmids (R-plasmids), some of which are conjugative (Lloyd et al., 2007). The improper and unnecessary use of antimicrobial drugs in human and veterinary medicine also promotes development of resistant strains with R-plasmids. Rasko et al. (2008), reported that both pathogenic and non-pathogenic strains resistant to drugs may be transported from animals to humans via food. Such strains act as an important source for *in vivo* transmission of R-plasmids to drug sensitive strains in the animal bowel mainly through conjugation (Pitout et al., 2009). Other workers reported that transmitssion of resistance plasmids of *E. coli* from poultry to human commonly occurs (Thumbikat et al., 2009). Understanding the molecular epidemiology of resistance plasmids has been a major issue since investigators/scientists became aware of its role in the spread of antimicrobial drug

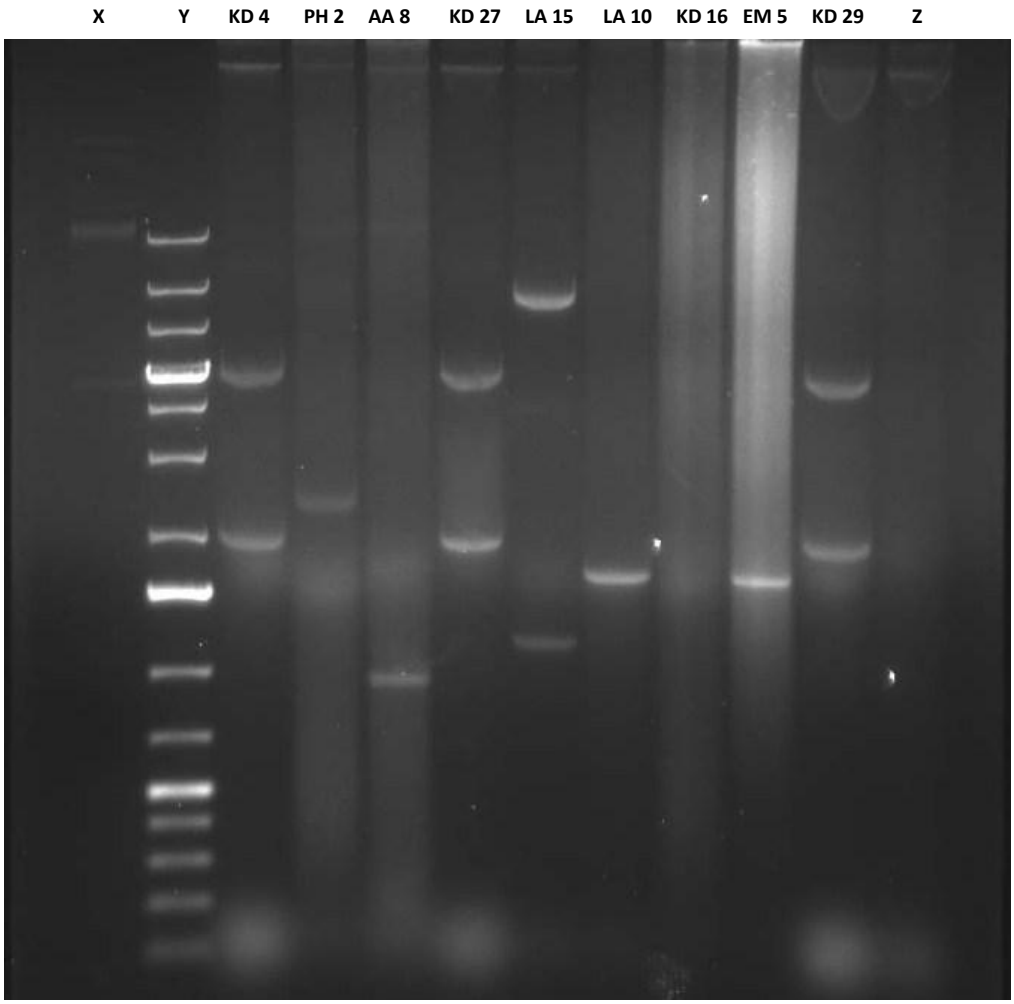

Figure 2. The plasmid profile of second group of *E. coli* Isolates from human specimens. X= Bactracker super coiled DNA Ladder, Y=1KB plus DNA Ladder, Z=120KB Plasmid positive control. This gel image shows that all isolates apart from LA 10, KD 16 and EM 5 harbor the 120 KB plasmid. Isolate KD 16 (North-North) had no plasmid.

resistance. However, understanding this epidemiology has been complex because of the diversity and dynamic nature of these elements. The plasmid replication system, which dictates the plasmid's behavior (host range, copy number) is the major plasmid landmark from a biologic standpoint; it is used for plasmid classification and identification (Oswald et al., 1994). However, their number (plasmid copies) also plays a critical role in imparting various characteristics to the pathogen, such as resistance towards different antibiotics.

In this study, plasmid profile analysis of the *E. coli* isolates by agarose gel electrophoresis showed a total of 146 different plasmid bands occurring in various combinations. The size of these bands ranged from 1-120 KB and most of the plasmids were shared among the isolates. Of all the plasmids detected the 120 KB plasmid (which can represent a diverse group of different plasmids) was most frequent, with 23% occurrence. All the isolates bearing this plasmid also harbored one or more smaller plasmids, and they were resistant to six or more antibiotics including Cefpirome, a third generation cephalosporin. This suggests that, there is correlation between size and number of plasmids to that of antibiogram of the isolates. This was later confirmed by DNA microarray, where, a remarkable number of *bla-CMY-2* gene was detected (data not shown). The *bla-CMY-2* gene, harbored in large plasmids is a AmpC beta-lactamase gene that confers resistance to third-generation cephalosporins (Daniel et al., 2009), thus, explaining the resistance to cephalosporins observed in this study.

The high resistance to Cefpodoxime and Cefpirome, observed in this study is of public health concern. This is probably the first documented evidence of such high resistance figure against any third or fourth generation cephalosporins in *E. coli* isolates from Nigeria. Okoli et al. (2005) reported the highest resistance rate of 31.5%

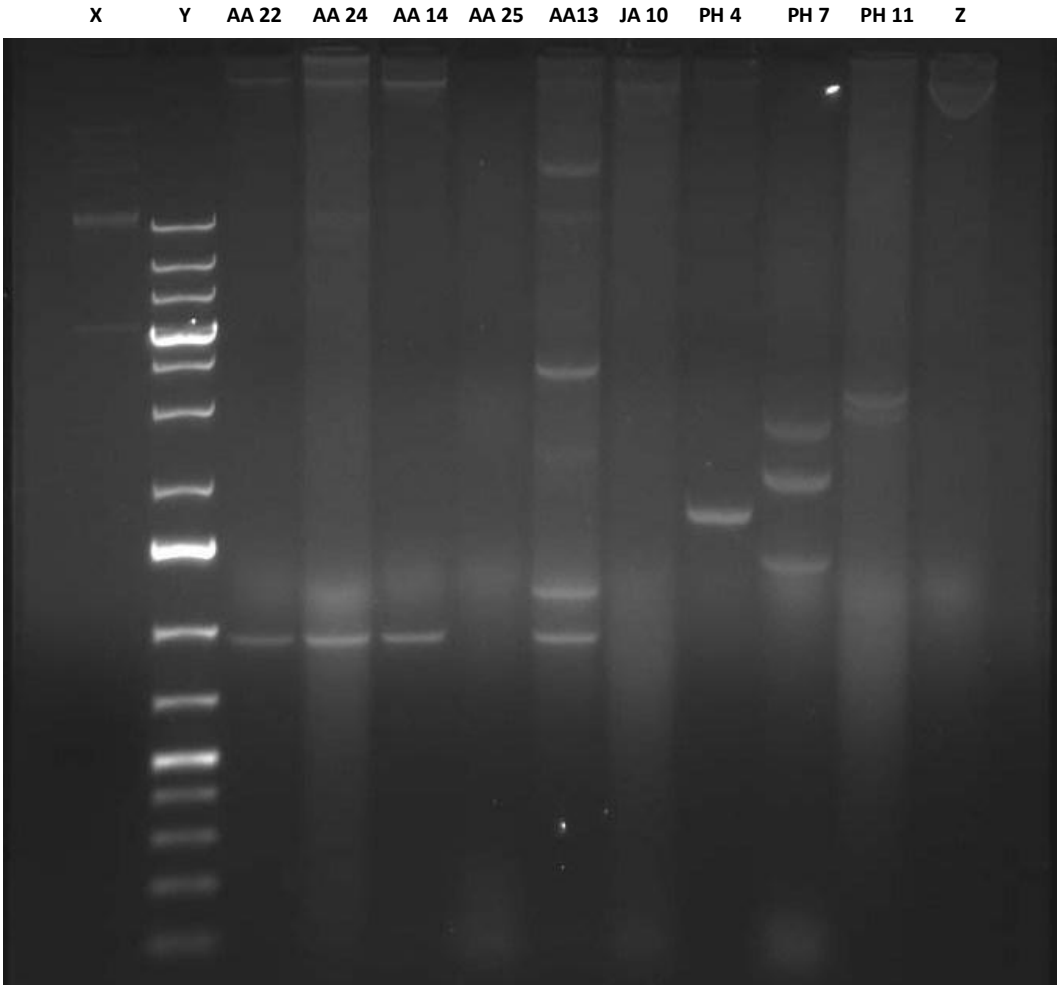

Figure 3. The Plasmid Profile of third group of *E. coli* Isolates from Human Specimens. X= Bac-tracker super coiled DNA Ladder, Y=1KB plus DNA Ladder, Z=120KB Plasmid positive control. Here, no plasmid was detected in isolate AA 25 (South-East) while the remaining isolates had one or more plasmids. The 120 KB plasmid was also prevalent in all the isolates apart from isolates PH 7 and PH 11 (South-South).

to Cefuroxime (second generation cephalosporins). It is probable that even though these drugs belong to the third and fourth generation cephalosporins, they may have been introduced into clinical practice in the country. Current information indicates that, third and fourth generation cephalosporins resistance can also be plasmid mediated, involving the *blaCMY-2* plasmids, which is quite distinct from the known cephalosporins resistance genes. It is probable that this plasmid-mediated gene may have contributed to the wide distribution of high bacterial resistance to cephalosporins observed in this study. This assumption was later confirmed by DNA microarray, as a remarkable number of this gene was detected in this study (data not shown).

In *E.coli,* drug resistance increases as a function of time and their (microorganism's) exposure to many factors (antibiotics, chemicals, etc). Besides, the bacteria acquire resistance through different routes, such as plasmids or intrinsic resistance (inaccessibility of the target, multidrug efflux systems and drug inactivation), mutational resistance (drug target site modification, reduced permeability or uptake, metabolic by pass and repression of multidrug efflux), extrachromosomal or acquired resistance (drug target site modification, reduced permeability or uptake, metabolic by pass and repression of multidrug efflux). All these mechanisms of antibiotic resistance warrant a detailed investigation of multiple factors, with prioritization of the studies of molecular characterization.

In conclusion, 15 plasmid profiles (Table 2) were observed in the *E. coli* isolates, with the predominant 120 KB plasmid being distributed widely across both sample sources, indicating a striking diversity in these plasmids in Nigeria. Multi-drug resistance was a common feature in

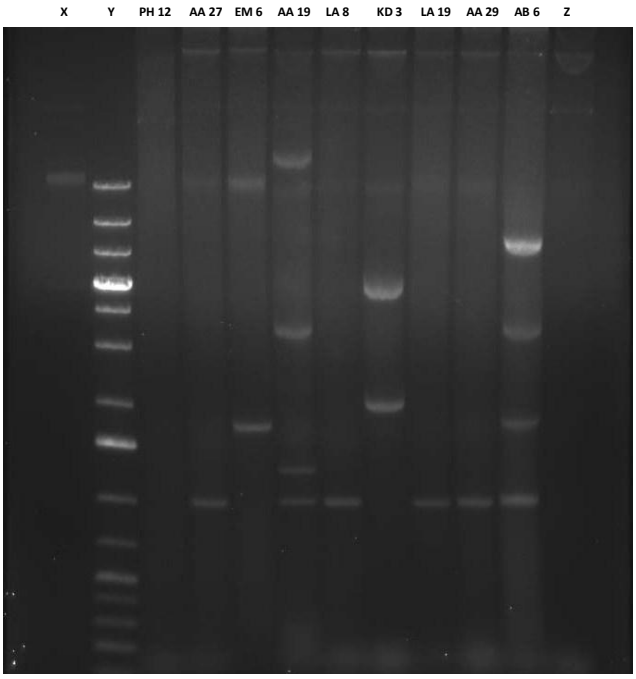

Figure 4. The Plasmid Profile of fourth group of *E. coli* Isolates from Human Specimens. X= Bac-tracker super coiled DNA Ladder, Y=1KB plus DNA Ladder, Z=120KB Plasmid positive control. In this gel image, isolate PH 12 (South-South) has no plasmid, while all the remaining isolates harbor multiple plasmids with the 120 KB plasmid present in all of them.

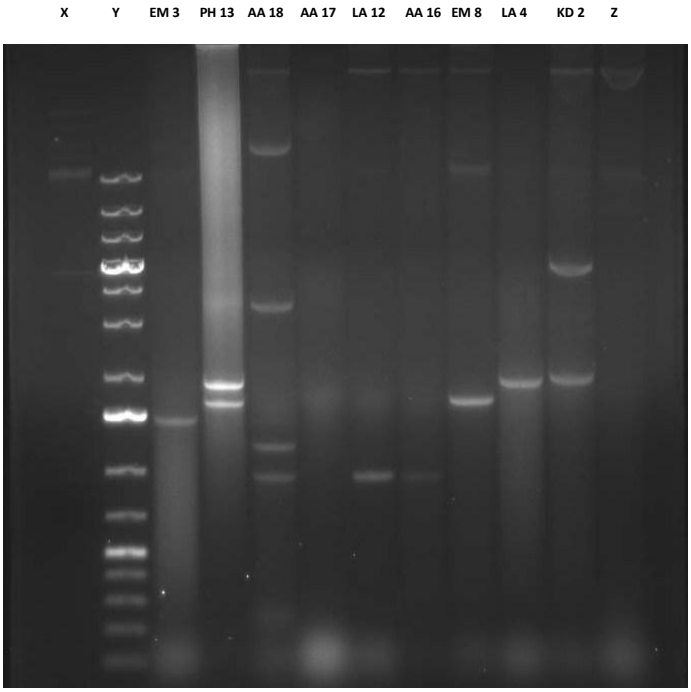

Figure 5. The plasmid profile of fifth group of *E. coli* Isolates from Human Specimens. X= Bac-tracker super coiled DNA Ladder, Y=1KB plus DNA Ladder, Z=120KB Plasmid positive control. This figure shows that, no plasmid was detected in isolate AA 17 (South-East). Isolate EM 3 has one plasmid, while the remaining isolates has two or more plasmids each.

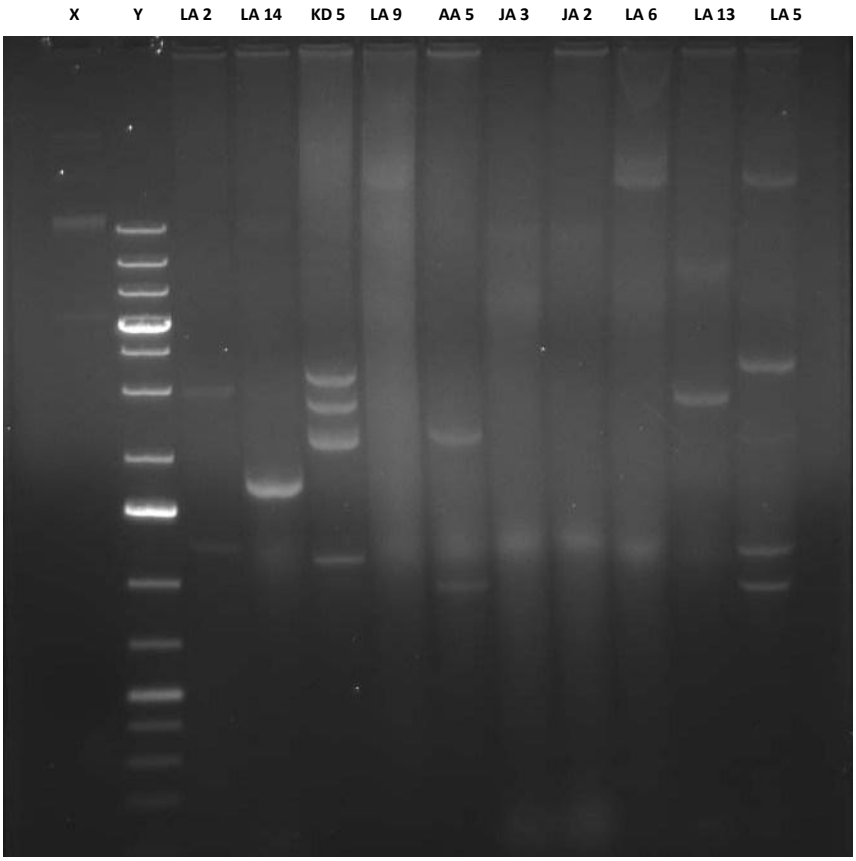

Figure 6. The Plasmid Profile of sixth group of *E. coli* Isolates from Human Specimens. X= Bac-tracker super coiled DNA Ladder, Y=1KB plus DNA Ladder. Here no plasmid was detected in isolate, JA 3 (North-North). Isolates LA 5 (South-West) and KD 5 (North-North) has four plasmids each, while the remaining isolates has one or two plasmids each.

these isolates, highlighting the fact that antibiotic resistance is prevalent in the study area. Thus, this study confirms the important role of plasmids in disseminating antibiotic resistance traits in *E. coli*.

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