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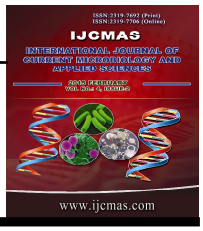

Original Research Article

Plasmid Profile and Antimicrobial Resistance Ratings of *Escherichia coli* Isolates from Pigs and Poultry Birds in Abia State, Nigeria

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Keywords

Plasmid profile, Antimicrobial ratings, *Escherichia coli*, Food animals, Multi drug resistance, Resistance genes

A B S T R A C T

Multiple antimicrobial resistances may be acquired through mobile genetic elements such as plasmids which play an essential role in facilitating the transfer of the resistance genes contributing to the creation of multi drug-resistant phenotype. In the present study, 310 *E. coli* isolates from pigs and 270 isolates from poultry birds were tested for antimicrobial resistance and plasmid profiles. The results showed that more than 45% of the isolates exhibited multi-dry resistance. Thirty percent (30%) were found to possess plasmids. Some isolates possess single sized plasmid others had multiple plasmids which ranged from 600bp-1517bp. The isolates were resistant to Augmentin (80%), septrin (100%), ceporex (100%) and ampicillin (100%). It also shows that the isolates were sensitive to ceproflox (100%) and gentamycin (70%). This studies show that multiple drug resistant strain harboured plasmids of varying sizes. Antimicrobial susceptibility studies revealed that food animals from Nigeria contain multiple antimicrobial resistant strains of *E.coli* which may serve as a reservoir for antimicrobial resistance genes in food animals.

Introduction

The importance of farm animals in the spread of resistance genes to human populations is increased by worldwide reports of mobile genetic elements in animals raised for human consumption (Roest *et al*., 2007). *Escherichia coli* of animal origin with resistance to antimicrobials and multiple antimicrobials have been widely documented (Mora *et al*., 2005).

When exposed to antimicrobial compounds, bacteria evolve resistance mechanisms. These include polymorphisms in antimicrobial targets that reduce vulnerability, as well as genes encoding

efflux systems, drug modifiers or proteins that fortify target sites (Wright, 2007; Davies and Davies, 2010). Resistance determinants can be transferred via mobile genetic elements, such as plasmids, prophages or transposons, allowing horizontal transfer within and between bacterial species (Davies and Davies, 2010), particularly in environments such as the gut micro biome (Smillie *et al*., 2011), and have collectively been dubbed the antimicrobial resistome (Wright, 2007; Marshall and Levy 2011). The transfer of resistance genes into the gut can come from diverse environments, for example, from soil bacteria (Forsberg *et al*., 2012).

The flora of healthy animals has also been implicated as a reservoir of antibiotic resistance genes (de Jong *et al*., 2012) and resistance transfer has been shown to occur between different animal species on farm premises (Hoyle *et al*., 2006). *E.coli* has been indicated as a possible reservoir for antimicrobial resistance genes and might play a role in the spreading of such determinants to other bacteria (Ahmed *et al*., 2010).

Previous studies have explored the pig gut resistome (Looft *et al*., 2012), but population-scale studies are still lacking. Since antimicrobials are widely used in medicine (Goodssens *et al*., 2005) and food production (Barton, 2000; Davies and Davies, 2010; Marshall and Levy 20011; Aarestrup, 2012); there is need to understand the variation of the resistome using the molecular epidemiology of resistant plasmid.

Materials and Methods

Sample Collection and Bacteriology

Thirty-three commercial farms keeping broiler or laying hens and twenty-four commercial farms keeping pigs from various zones of Abia-State were randomly selected for the study. Fresh faecal material was collected via cloacae swab and rectal swab from poultry birds and pigs respectively. They were kept in an icepack while being transported to the laboratory. The samples were processes on the same day of collection.

After overnight incubation, growths on MacConkey agar (MCA) plates suggestive of *E.coli* were further streaked onto Eosin Methylene Blue (EMB) agar and subjected to IMV_iC tests as described by Quinn *et al* (1994).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed by the standard disk diffusion (Bauer *et al*., 1966). At least five morphologically similar colonies were gently touched with a sterile wire loop and were transferred into 5ml of sterile nutrient broth in universal tubes. The broth was incubated with shaking at 37^oC, until the visible turbidity was equaled to 0.5 McFarland standards (Andrews, 2006). The density of the organism suspension was adjusted to 0.5 McFarland by adding sterile distilled water. The adjusted suspension was used within 15 minutes to inoculated Mueller-Hinton agar plates by dipping a sterile cotton-wool swab into the suspension. Excess liquid was removed by turning the swab against the side of the container. The inoculums were spread evenly over the entire surface of the plate by swabbing in three directions (Andrews, 2006). The plates were allowed to dry before applying the antimicrobial discs (Optudisc-Nig). Following incubation, clear zones surrounding antimicrobial disks were measured and compared to reported zones established by manufacturer of the antimicrobial disks. The following

antimicrobials were tested Ofloxacin (OFL, 10mcg), Peflacin (Pef, 10mcg), Ciproflox (Cip, 10mcg), Augmentin (Aug, 30mcg), gentamycin (Gen, 10mcg), Streptomycin (Str, 30mcg), Ceporex (Cep, 10mcg), Nalidixic acid (Nac, 10mcg), Septrin (Sep, 30mcg) and Ampicillin (Amp, 30mcg). Isolates were determined to be sensitive, intermediate resistance or resistance based on their respective zones sizes as described by CLSI (2010). All the strains used in this study did not harbour any virulence genes commonly associated with pathogenic *E.coli*, when tested by polymerase chain reaction (PCR).

Plasmid analysis

Plasmid DNA extraction was done according to the protocol of Birnboim and Doly (Birnboim and Doly, 1979). Purified plasmids from multi drug resistant isolates were used to transform chemically competent *E.coli*. K-12 by heat shock (Sambrook *et al*., 2000). Transformed and cured isolates were analyzed on selective media containing four different antimicrobials (10mcg OFL, 30mcg Str, 30mcg Amp and 10mcg Cep) after incubation at 37^{o}C for 24 hours. After 5 hours of sub-cultured, transformed and cured colonies, their antimicrobial resistance ratings and plasmid pattern was analyzed.

Plasmid DNA isolation was done by mini ultra-prep plasmid kit (AB gene, Epsom, Surrey KT 19 GAP, UK). 0.8% agarose gel was used to run the plasmid DNA. *E.coli* K-12 control plasmid DNA was loaded at each run. Plasmid DNA was visualized after ethidium bromide staining in UV Tran illuminator (bioradfel documentation system, USA). The molecular masses of the unknown plasmid DNA were assessed by comparison of their mobilities with those of a super coiled DNA ladder with known molecular masses. The photo capt. Mw program was used to determine the molecular weights of the plasmid band and to analyze the plasmid profile.

Result and Discussion

Table 1 shows the sensitivity ratings of *E.coli* before subjecting the organisms to curing and transformation process. This was done to prove if antimicrobial resistance of this organism was mainly as a result of the presence of R-plasmid or selective pressure on the organism as a result of antimicrobial use in animal production or both. This shows that the organisms were resistant to Augmentin (80%), Septrin (100%), Ceporex (100%) and Ampicillin (100%). It also shows that the organisms were sensitive to ceproflox (100%) and gentamycin (70%).

Table 2 shows the resistance ratings of *E.coli* post-curing process. After the removal of the resident DNA from the isolates, sensitivity test was done on the organism to check if there are variations in the sensitivity ratings pre-, and post-curing processes. When Table was compared with Table 1 it showed that some isolates that was sensitive to certain antimicrobial agents during pre-curing were resistant after though not significant ($P>0.05$). This signified that resistance of the isolates to certain antimicrobial agents was not as a result of the presence of R-plasmids in the DNA.

Table 3 shows resistance ratings of *E.coli* post-transformation. There was significant difference among *E.coli* isolates between pre-transformation and post-transformation. This showed that resistance of the *E.coli* isolates was partially due to the presence of R-plasmids, but also due to bacteria mutation as a result of misuse of some of these agents in animal production. The

control organism *E.coli* K-12 was used in the transformation process.

Plate 1 show result of the plasmid profile of ten (10) representatives of *E.coli* isolates analyzed with 0.8% agarose gel. Isolates Es 7, Es5 and Es4 have plasmid with size 600bp. L is a 100-1517 bp DNA ladder while others are isolates that do not have plasmid. Analysis of plasmid DNA revealed that only three (3) of the ten (10) representatives of *E.coli* harbours plasmids. Also small plasmids, which appeared as bright bands mostly below the band of chromosomal DNA on the gel, were used in typing analysis because large plasmids might be lost during cell storage and culturing or plasmid extraction.

Exposure to antimicrobial agents is a major factor with regard to development of antimicrobial resistance. Thus animals and animal products could be significant sources of resistant bacteria for the human population (Feinman, 1998). However, the ease with which bacteria acquire new resistance genes by self-transmissible and mobilizable plasmids and conjugative transposons may represent a more significant contribution to the increasing incidence of resistant strains (Nikolich *et al*., 1994).

The results from this study showed high resistance frequencies in non-pathogenic *E.coli* from poultry birds and pigs in Abia-State, Nigeria. All *E.coli* isolates were shown to be 100% resistant to Sep, Cep, and Amp. Earlier studies by Miles *et al* (2006), showed that 100% resistances were reported in *E.coli* isolates of chicken and guile origin. Administration of antimicrobial agents to poultry birds and pig, have provided a selective pressure which explains the detection of resistant bacteria and as a result, many bacteria associated with poultry and piggery products are commonly resistant to antimicrobial agents (Turtura *et al*; 1990). Previous studies have revealed that there is a link between the use of antimicrobial agents in poultry and other food producing animals and the emergency of human pathogens with decreased susceptibilities or complete resistance to antimicrobials used for treatment of human infections (Bager *et al*., 1997). *E.coli* strains are routinely exposed to a wide range of antimicrobial agents and have a very wide natural distribution and a propensity for plasma carriage (Sherley *et al*., 2003).

We isolated a collection of multi resistance plasmids from non-pathogenic isolates of *E.coli*. Plasmids harbouring multiple antimicrobial resistance determinants, transferred resistance against 6 of tested antimicrobials (except pet, gen, cep and Nac) when transferred from non-pathogenic *E.coli* to susceptible *E.coli* K-12 strain. Comparing different antimicrobials, we have shown that resistance gene abundance and penetration on average are higher for drugs used in animals, even when compensating for differences in how many resistance genes are known. This is consistent with expectations from previous research into a,"farm-to-fork" connection (Marshall and Levy, 2011).

Comparison of plasmid profiles is a useful method for assessment of the possible relatedness of individual clinical isolates of a particular bacteria species for epidemiological studies (Dutta *et al*., 2002). In this study, about 40% of the representative isolates harbour plasmids. Most profiles were characterized by the presence of small plasmids of 2 to 3kbp, 4-5kbp, and 6 to 7kbp. They were detected in about 97% of all the representative isolates. The resistance observed during the post curing process (sensitivity) showed that

some isolates harboured resistance genes in their chromosomal DNA. The resistance ratings of *Escherichia coli* during pre-curing process when compared with the resistance ratings during post-curing indicate variations. The resistance observed during post-transformation showed that some antimicrobial resistance isolates were plasmid mediated. A reported resistance testing in both post-curing and post-transformation showed that resistant isolates possess resistant genes in both plasmid and chromosal DNA. When compared with the resistance ratings at pre-curing and pre-transformation it showed that multi-resistance of *E.coli* to antimicrobial agents is mainly through selective pressure. There was a significant difference ($P<0.05$) between the resistance ratings during pre-curing and pre-transformation process. This is an agreement with Gossens *et al* (2005), who stated that the wide spread use of antimicrobials plays a significant role in the emergence of resistant bacteria. The various isolates that harboured plasmids have sizes between 600kp and 0.6kbp to 2.0kbp. Forty percent of *E.coli* isolates harbored plasmid with size between 0.8kbp and 1.6kbp. Antimicrobial agent – resistant *E. coli* of animal origin has been proposed to constitute an important potential source of R plasmids for indigenous *E. coli* in the human gut and, subsequently, for human pathogens (Linton, 1986). In part, apparently related plasmids have been found in epidemiological unrelated *E. coli* isolates from humans and pigs (Jorgensen, 1983). This study showed that some isolates of *E. coli* that possess antimicrobial resistance were seen to habouring plasmids. The presence of plasmids in the isolates did not have any correlation with the antimicrobial pattern. We conclude that the high prevalence of multi-drug resistance detected in our study is a matter of concern, since, there is a large reservoir of antimicrobial resistance genes within the zones, and that the resistance genes within the zones, and that the resistance genes were easily transferable to other strains.

Table.1 Resistance ratings of Escherichia coli pre-curing and pre-transformation processes

Isolates/drugs	Peflacine (10mg)	Gentamycin (10mg)	Augmentin (30mg)	Ceproflox (10mg)	Septrin (30mg)	Ceporex (10mg)	Streptomycin (30mg)	Ampicillin (30mg)	Ofloxacin (10mg)	Nalidixic acid (30mg)
E.coli 1	S	SS	SS	S	R	R	R	R	R	S
E.coli 2	S	S	R	S	R	R	R	R	R	S
E.coli 3	S	S	R	S	R	R	R	R	R	SS
E.coli 4	S	S	R	S	R	R	R	R	R	SS
E.coli 5	S	S	R	S	R	R	R	R	R	R
E.coli 6	S	R	R	S	R	R	SS	R	SS	R
E.coli 7	R	S	R	S	R	R	SS	R	SS	R
E.coli 8	SS	S	R	S	R	R	R	R	R	R
E.coli 9	S	S	R	S	R	R	R	R	SS	SS
E.coli 10	R	SS	SS	S	R	R	SS	R	R	SS

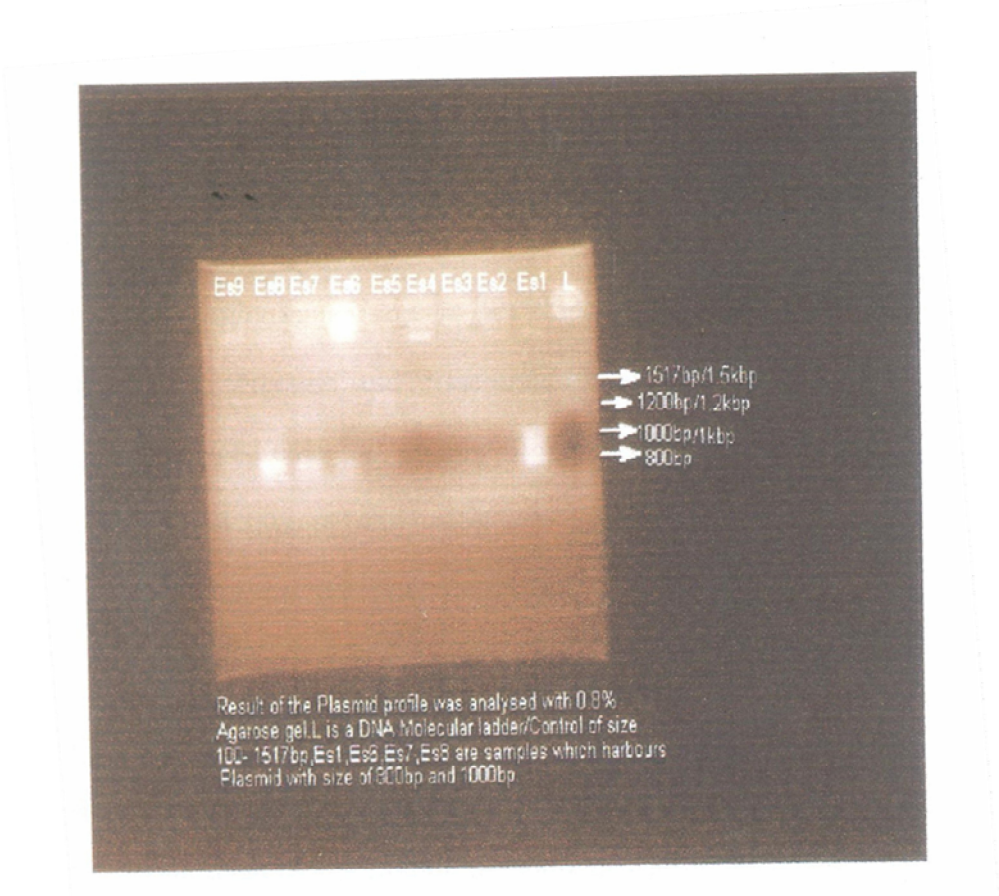
Table.2 Resistance ratings of Escherichia coli post-curing process

Isolates/drugs	Peflacine (10mg)	Gentamycin (10mg)	Augmentin (30mg)	Ceproflox (10mg)	Septrin (30mg)	Ceporex (10mg)	Streptomycin (30mg)	Ampicillin (30mg)	Ofloxacin (10mg)	Nalidixic acid (30mg)
E.coli 1	SS	R	R	S	R	S	SS	R	S	S
E.coli 2	SS	S	R	S	R	S	S	R	S	S
E.coli 3	S	R	R	S	SS	S	S	R	S	SS
E.coli 4	SS	SS	R	S	R	S	SS	R	S	R
E.coli 5	S	R	R	SS	R	S	SS	R	S	R
E.coli 6	S	SS	R	S	R	S	S	R	S	R
E.coli 7	SS	S	R	S	R	R	S	R	S	SS
E.coli 8	S	S	R	S	R	S	S	R	S	S
E.coli 9	S	S	R	S	R	S	S	R	S	S
E.coli 10	SS	R	R	S	R	S	S	R	SS	SS
E.coli- 12 (control)	S	S	S	S	S	S	S	S	S	S

Table.3 Resistance ratings of Escherichia coli post-transformation

Isolates/drugs	Peflacine (10mg)	Gentamycin (10mg)	Augmentin (30mg)	Ceproflox (10mg)	Septrin (30mg)	Ceporex (10mg)	Streptomycin (30mg)	Ampicillin (30mg)	Ofloxacin (10mg)	Nalidixic acid (30mg)
E.coli 1	S	SS	S	S	R	S	S	R	S	S
E.coli 2	S	S	S	S	S	S	S	S	S	S
E.coli 3	S	S	R	S	S	S	S	S	S	S
E.coli 4	S	S	S	S	S	S	S	S	S	S
E.coli 5	S	S	S	S	R	S	S	S	S	S
E.coli 6	S	S	S	S	R	S	S	R	S	S
E.coli 7	S	S	S	S	R	R	S	R	S	S
E.coli 8	S	S	S	S	S	S	S	R	S	S
E.coli 9	S	S	R	S	S	S	S	S	S	S
E.coli 10	S	S	S	S	S	S	S	S	S	S
E.coli K-12 (control)	S	S	S	S	S	S	S	S	S	S

Plate.1 Plasmid profile of E.coli isolates



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