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The genetic relatedness of drug resistant *E.coli* isolates of human and animal origin in Nigeria

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Molecular epidemiology of human and animal ecovariants of *Escherichia coli* from different regions of Nigeria were studied using their antibiotic susceptibility patterns, plasmid profile and pulsed-field gel electrophoresis (PFGE). *E. coli* was isolated using eosin methylene blue agar (EMB) and identified by conventional microbiological technique. The isolates were tested against 14 antibiotics using the disc diffusion method. PFGE was performed using XbaI as restriction enzyme according to pulse net protocol. Overall, 42 different antibiotics resistance clusters were observed, with each isolate showing resistance to at least four or more drugs tested. Fingerprinting of 140 isolates by PFGE technique and subsequent cluster analysis revealed a diverse *E. coli* population belonging to 47 distinct subtypes. Cluster analysis of the 120 KB plasmid bearing isolates indicated that these isolates belonged to one unique clonal group with ≥80% genetic similarity to each other, their animal or human origin, geographical distribution and clinical or non-clinical source notwithstanding. The sharing of drug resistant strains between human and animal population has shown that identical clones are circulating among human and animal population in the study area.

Key words: *Escherichia coli*, epidemiology, animal ecovariants, cluster analysis.

INTRODUCTION

Antimicrobial drugs have played an indispensable role in decreasing illness and death associated with infectious diseases in animals and humans (Daniel et al., 2012). However, selective pressure exerted by antimicrobial drug use also has been the major driving force behind the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria (Aarestrup et al., 2008). In addition, resistance has developed after advent of every major class of antimicrobial drugs, varying in time from as short as 1 year (penicillin) to >10 years (vancomycin) (Levy and Marshall, 2004).

Escherichia coli is usually a commensal bacterium of humans and animals. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis and septicemia (von Baum and Marre, 2005; Sodha et al., 2006). Therapeutic options vary depending on the type of infection. For example, for urinary tract infections, trimethoprim/sulfamethoxazole and fluoroquinolones are treatments of choice (Taur and Smith, 2007), whereas for Shiga toxin-producing *E. coli* infections, antimicrobial drug therapy is not recommended (Igarashi et al., 1999). *E. coli* is sometimes used as a sentinel for monitoring antimicrobial drug resistance in fecal bacteria because it is found more frequently in a wide range of hosts, acquires resistance easily (Erb et al., 2007), and is a reliable indicator of resistance in salmonellae (Womack et al., 2010).

Molecular tools have been used to correlate animal associated pathogens with similar pathogens affecting

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humans and to clearly demonstrate transferable resistant genes carried by plasmids common to both animals and humans (Pitout et al., 2009). The possibility of antibiotic resistance genes circulating among humans, animals and the environment 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 and animal 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 human and animal *E. coli* strains is a recognized source for the rapid spread of antimicrobial resistance phenotypes (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 (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 epidemiological analysis of resistant isolates at three different levels when comparing whole genomes, single plasmids and PFGE.

Although, few studies have evaluated antimicrobial resistant *E. coli* in Nigeria (Okeke et al., 1999, Aibinu et al., 2004, and Umolu et al., 2006), most available data are specific to strains that are pathogenic either to human or animals. Little or no data exist on the molecular epidemiology of human or animal drug resistant *E. coli* isolates in Nigeria. This study, is therefore, aimed at determining the genetic relatedness of drug resistant *E.coli* isolates of human and animal origin in Nigeria.

MATERIALS AND METHODS

Study population

The study population included humans (who were either ill or presumptively healthy) and variety of apparently healthy domestic livestock viz: cattle, goats, swine and chicken obtained from five geopolitical zones of Nigeria viz: South-East, South-West, South-South, North-Central and North-North. In the South-South and South-East, clinical specimens were collected at the University of Port Harcourt Teaching Hospital, Port Harcourt, Rivers State and the Abia State University Teaching Hospital, Aba, Abia State, respectively. The Lagos State University Teaching Hospital, Ikeja, Lagos was the site of specimen collection for the South-West, while the National Hospital, Abuja and Military Reference Hospital, Kaduna State were the sources of specimens from the North-Central and North-North respectively. All samples from these hospitals were clinical specimens from patients who were having gastroenteritis or similar illness. Healthy undergraduate students of Madonna University, Elele were included in the study for the isolation of human commensal *E. coli.* These individuals reported no exposure to antibiotics for six months prior to sampling and each person received an explanation of the study objectives and consent form for inclusion in the study. All sampling procedures were in accordance with guidelines of the National Health Research Ethics Committee, Nigeria (www.nhrec.net). All the animals included in this study were (at the time of specimen collection) not showing any sign of ill-health. The cattle and goat specimens came from the Obinze livestock market Owerri, Imo State while the Madonna University Poultry, Anambra State was the source of poultry specimens. The specimens from swine came from a farm located at the Ogborhil area of Abia state. There was no documented evidence of antibiotics use in the farms from which the specimens were collected, although the management of the poultry farm indicated occasional antibiotics use at this facility.

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

Fecal droppings were randomly collected from goats, cattle, pigs and chicken; and care was taken to avoid collecting more than one fecal sample per individual animal. Feces were packed in a sterile plastic container and were transported to the laboratory in ice-box. One gram of each animal's feces was homogenized in 9 ml of sterile saline solution, then the volume of the homogenate was made up to 10 ml to get a 10% suspension. The contents were mixed thoroughly and 10-fold serially diluted and 0.2 ml inoculums from each dilution plated out on Eosin Methylene Blue agar (EMB) (Oxoid, England). Human fecal specimens were streaked directly on EMB agar. 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), tetracycline (30 µg), gentamicin (10 µg), cefpodoxime (10 µg), cefoxitin (30 µg), cefpirome (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), sulfamethoxazole-trimethoprim (10 µg) (cotrimaoxazole), cephalothin (30 µg), nitrofurantoin, ceftriaxone (30 µg) and cefotaxine (30 µg) (Oxoid, England). *E. coli* ATCC 25922 was included as a reference strain.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using XbaI (New England Biolabs) according to pulse net protocol (www.cdc.gov/pulsenet/protocols.htm). Briefly, DNA fragments were resolved by electrophoresis in 1% SeaKem Gold agarose gels with a CHEF DRII machine (Bio-Rad), using 0.5× Tris-borate-EDTA as the buffer. Gels were run for 18 h at 14°C, using a linearly ramped switching time from 2.2 to 63.8 s and a voltage of 6.0 V/cm^2. After electrophoresis, the gels were stained in 400 ml of deionized water containing 40 µl of 10 mg/ml of ethidium bromide for 20 min on a rocker and distained three times for 20 min each with distilled water. Bands were visualized by UV transilluminator (Fisher Scientific) and photographed using Alpha imager (Alpha Innotech Corporation,San Leandro, CA, USA). Digitalized gel images were saved and subjected to analysis with

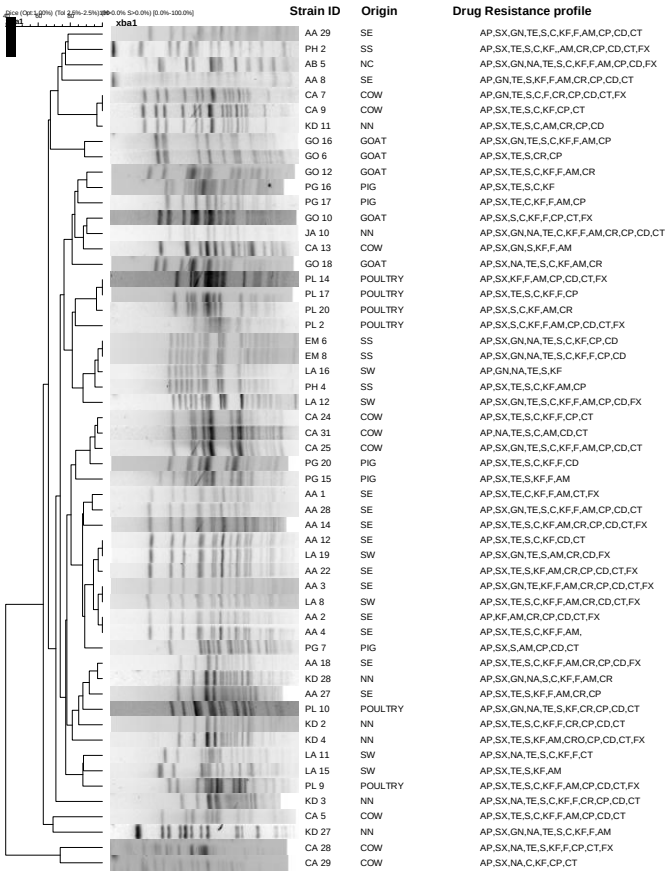

Figure 1. The genetic relatedness of *E. coli* Isolates from humans and animals in Nigeria. AP = Ampicillin, SX = Cotrimaoxazole; GN = Gentamycin, NA = Nalidixic acid, TE = Tetracycline. S = Streptomycin, C = Chloramphenicol, KF = Cephalothin, F = Nitrofurantoin, CR = Ceftriaxone, AM = Amoxycillin clavulanic acid, CP = Cefpirome, CD = Cefpodoxime, CT = Cefotaxine, FX = Cefoxitin, SE = South-East, SW = South-West, SS = South-South, NN = North-Central, NC = North-Central.

BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by using the unweighted pair group for arithmetic means average (UPGMA) based on Dice coefficients to quantify the similarities.

RESULTS AND DISCUSSION

The *E. coli* isolates were studied for genetic relatedness using PFGE technique. PFGE fingerprints and cluster analysis showed that, the 140 isolates fingerprinted formed a relatively diverse population belonging to 47 distinct PFGE subtypes. These isolates belonged to one unique clonal group with >80% similarity with each other despite diverse hosts (human vs. animals) or sample sources (geographical regions or clinical vs. non-clinical) (Figure 1).

Considering the genetic relatedness of the isolates from various sample sources, those from south-east showed a unique trend. The 19 isolates fingerprinted belonged to 8 distinct PFGE subtypes with 57.9% showing ≥80% genetic relatedness. This indicates a dissemination of single clonal group in the south east. The isolates from the south-west showed a slightly similar trend to the south east. Of the 14 isolates that were type-able by PFGE, 7 distinct PFGE subtypes were observed with 50% showing ≥80% genetic relatedness. In the south-south zone, 4 isolates (28.6%), were 90% genetically related, the highest genetic diversity observed in human isolates was recorded in the isolates from north-north zone. Of the 23 isolates fingerprinted, 13 distinct PFGE subtypes were observed.

Generally, the isolates from animal specimens were more genetically diverse when compared to those from human specimens. Of the 24 isolates from cattle specimens fingerprinted, 11 distinct PFGE subtypes was recorded with 33.3% of the isolates showing ≥80% genetic relatedness. In goat specimens, 5 out of 16 isolates (31.3%); in pig, 4 out of 14 (28.6%); and in poultry specimens, 2 out of 9 isolates (22.2%) were ≥80% genetically related, respectively

One of the strengths of the current research is that we restricted our analysis to only one *E. coli* isolate per fecal sample thereby maximizing biological independence between isolates. Furthermore, sampling was geographically distributed. The independence between isolates was consistent with generally high diversity of PFGE profiles observed in this study. There were some groupings for human isolates, but these included isolates from multiple geographic locations suggesting a potential common source for human isolates.

However, there was a correlation between antibiotic resistance and PFGE profile; cluster analysis indicated that, these isolates belonged to one unique clonal group with >80% similarity, each of the isolates being resistant to six or more antibiotics (Figure 1). This indicates that there are shared *E. coli* clones circulating among human and animal population. Most studies of antibiotic resistance in animal agriculture have been directed toward pathogenic bacteria (Bottner et al., 1995; DebRoy and Maddox, 2001). The findings of this PFGE typing provide a unique perspective on the role of commensal *E. coli* as a potential reservoir of resistance genes for multiple antibiotics. Monitoring resistance in commensal bacteria, such as *E. coli*, is important, as they can gain access to the food chain. Zhao et al. (2001) reported the presence of extended-spectrum cephalosporin-resistant *E. coli* and *Salmonella* spp. in retail ground meat, signifying the public health importance of this issue. Nonpathogenic multidrug-resistant strains of *E. coli* in the intestinal microflora serve as an important reservoir of mobile resistance genes which can be transferred in the intestines to other bacterial species, including pathogens such as *Salmonella* spp. (Hoyle et al., 2004). This can be an important mechanism for acquiring antibiotic resistance in pathogenic bacteria that pose a challenge

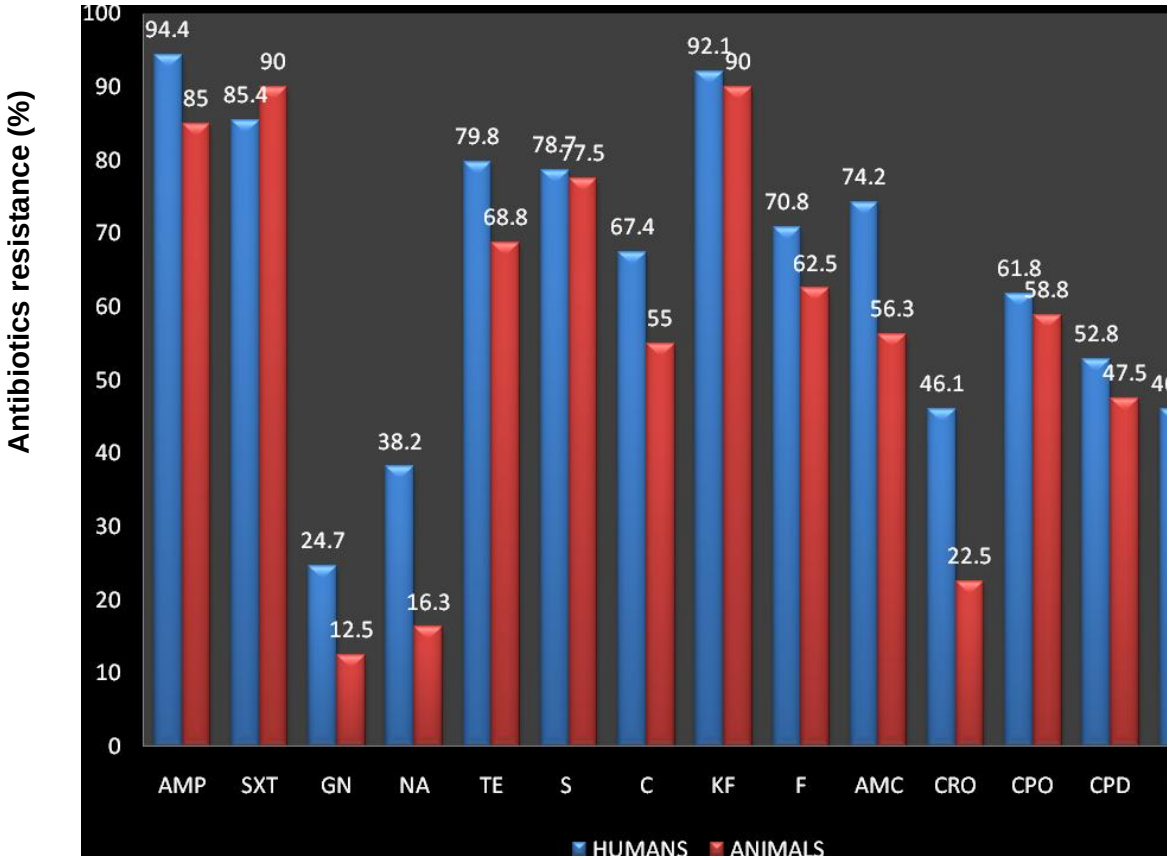

Figure 2. Percentage antibiotic resistance of *E. coli* isolates from humans and animals in Nigeria. AMP = Ampicillin, SXT = Cotrimaoxazole, GN = Gentamycin, NA = Nalidixic acid, TE = Tetracycline, S-Streptomycin, C = Chloramphenicol, KF = Cephalothin, F = Nitrofurantoin, AMC = Amoxycillin clavulanic acid, CRO = Ceftriaxone, CPO = Cefpirome, CPD = Cefpodoxime, CTX = Cefotaxine, FOX = Cefoxitin.

for effective antibiotic therapy.

In conclusion, the sharing of drug resistant strains between human and animal population as shown by PFGE in this study proved that identical clones are circulating among human and animal population in the study area (Figure 2). This suggests that commensal *E. coli* from animals can perhaps play a dynamic role in the ecology of multidrug resistance in the Nigerian human population.

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