Molecular Characterization and Antibiotic Resistance profile of *Escherichia coli* from Food Producing Animals from Southwest Nigeria.

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Abstract: The global menace of superbugs is a major challenge to human and animal health with food animals as major vehicles in the spread of resistance genes via the food chain. Chicken and cattle were sampled in Ibadan, Nigeria, to determine the prevalence and diversity of antibiotic resistant *Escherichia coli*. Raw milk samples were from 70 cattle, faecal samples from another 70 cattle and 50 cloaca swabs from free-range chickens were randomly collected for this study. *E. coli* isolates were confirmed by PCR method following DNA extraction using β-d-glucuronidase-encoding gene *uid*A primer.A 352 base pair (bp) specific product was amplified from all of the *E. coli* tested (n= 26 from milk; n= 60 from fecal, and n= 10 from cloaca) and these were tested for susceptibility to nine antimicrobial agents. Forty-one (42.7%) were sensitive to all the nine antimicrobial agents while the remaining 55 (57.3%) were resistant to one or more antibiotics. Twenty-five unique resistant patterns were detected. This study indicated prevalence of resistant *E. coli* in food animals with or without antibiotic treatment history. Acquisition and spread of resistance factors could be due to misuse of antibiotic agents or through horizontal gene transfer (HGT). There is need for national surveillance, monitoring and control of distribution and diversity of foodborne resistance bacteria.

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1 Introduction

Infection due to micro-organisms impose serious constrains on animal production all over the world due to mortality and reduced productivity. *Escherichia coli* is an unbiquitous organism found in the gastrointestinal tract of human and animals resulting from faecal contamination during food animal slaughter, it is often found in the soil water and for a number of *E. coli* strains are recognized as important pathogens of livestock such as cattle and poultry and some of them can cause severe human diseases such as hemorrhagic colitis and uremic syndrome (Ferens and Hovde 2011). Food borne are usually linked with the consumption of unhygienic processed or uncooked food of animal origin, including beef, chicken, unpasteurized and contaminated post pasteurization dairy products (Paton and Paton 1998; Hussein 2007). Commensal *E. coli* is often used as indicator bacteria for monitoring and assessing the level of antimicrobial resistance in the community (Kijima-Tanaka et al 2003). The ease of transferability of resistance trait among bacteria through mobile genetic elements and the preponderance of *E. coli* in the faeces and environment may facilitate the development and spread of antimicrobial resistance (Kang et al 2005; Lee et al 2006).

Overdependence on antimicrobial usage in animal production, as well as unhygienic practices leading to food contamination are important factors responsible for the emergence of antimicrobial resistance in bacteria of animal origin and their eventual transmission to humans (WHO 2000). Treatment of bacterial infections often requires antimicrobial therapy depending on susceptibility of the pathogens and pharmacokinetics of the drug for achieving desired therapeutic concentration at the site of infection and thus clinically efficacy (Mc kellar et al 2004). However, veterinary practitioners have a limited choice of antimicrobial for use in food animal production due to antimicrobial resistance, food safety and human health concerns (Van den Bogaard and Stobberingh 2000). The world health organization (WHO) warned that we are almost approaching post antibiotic era, Thus, this called for monitoring of the use antimicrobial agent in the food producing animals and the determination of potential factors that influence the level of resistance in pathogens (Aarestrup 2005). Considering the level of misuse of antimicrobial agents in animal production in Nigeria,

the objective of this study was to determine the prevalence molecular characterization and antibiotic susceptibility of *Escherichia coli* isolates from livestock products in Nigeria.

2. Material and Methods

Collection and identification of *E.coli.*

Cloaca swabs (n=50) were collected from free-range chickens, milk samples (n=70), and cattle fecal samples (n=70) were collected between June 2010 and April 2011 from metropolitan abattoirs and in the vicinity of Molete, Akinyele and Bodija areas of Ibadan Oyo State, southwestern Nigeria). Milk samples (5 ml) were collected directly into sterile containers from the udder after an initial stream of fore-milk was discarded. Fecal samples were collected directly from the rectum into sterile containers. Samples were stored in a cooler with ice packs for delivery to the Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria, isolation and biochemical characterization.

Isolation and identification

The samples were inoculated into sterile Tryptone soya broth (TSB) without antibiotics and cultures were subsequently incubated (37°C, 18 h) after which a loopful was incubated on 7% sheep blood agar and MacConkey agar (MAC). All colonies that were Lactose fermenters on MacConkey agar were confirmed as *E. coli* [Gram-negative rod, motile, catalase negative, oxidase negative, Indole positive; Barrow and Felthman, (2005)] and were further subcultured on EMB agar (Merick, Germany). Green metallic sheen isolates were considered to be *E. coli* and were stored on agar slants at -4°C until they were transported to the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, for molecular characterization and antibiotic susceptibility testing.

Primers and PCR assay

E.coli were sub cultured overnight in Luria Bertani broth (Merck,Germany) and geromic DNA was extracted using boiling method. PCR reactions were performed in a total volume of 25 µl, including 1.5mM MgCl2, 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µm of each dNTP (Fermentas), 1 µm primers, 1 IU of Taq DNA polymerase (Fermentas), and 5 µl (40–260 ng/µl) of DNA. Amplification reactions were carried out using a DNA thermo-cycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) as follows: Three min at 95 °C, 35 cycles each consisting of 1 min at 94 °C, 90 s at ~55 °C (show in Table 1) and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. Amplified samples were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100 bp DNA ladder, Fermentas) was used as a size standard. Strains of *E. coli* O157:K88ac:H19, CAPM 5933 and *E. coli* O159:H20, CAPM 6006 were used as positive controls.

The primer sequences are**:**

uidA Forward**:** GTTGAACTGCGTGATGCG and

uidA Reverse**:** TGCGAGGTACGGTAGGAG

Antibiotic susceptibility testing

The antibiotic resistance profile of the isolates was determined using breakpoint assays on LB Agar plates. The agar was autoclaved, cooled to 55 °C and the specific breakpoint concentrations of antibiotics as shown in table I. were added before agar was poured into petri dishes (150 × 15 mm). The bacterial isolates were retrieved from freezer stocks and transferred from 96-well plates into another 96-well plate with sterile LB broth using a pin replicator and incubated overnight at 37 °C. Cultured isolates were then re-transferred onto LB plates containing the antibiotics breakpoints and incubated over night at 37 °C. Isolates were scored as "1" for growth (resistant) or "0" for no growth (susceptible) for each antibiotic plate. LB plate without antibiotic was used to confirm successful transfer of culture to agar plates using the 96-well replicator, while sensitive strain of *E. coli* K12was included as a negative control. Isolates of bacteria that were resistant to >3 classes of antibiotics were considered MDR.

Table I: Breakpoint Concentrations of Antibiotics used for susceptibility test

Antibiotic	Breakpoint Concentration
Ampicillin	32 ug/mL
Amoxicillin/Clavulanic Acid	32/16 ug/mL
Cefoxitin	32ug/mL
Chloramphenicol	32 ug/mL
Florfenicol	16ug/mL
Nalidixic Acid	32ug/mL
Kanamycin	64ug/mL
Streptomycin	16ug/mL
Sulfamethoxazole/Trimethoprim	12/8 ug/mL

3. Results

A 352 base pair region of *E. coli* chromosome was amplified from all 96 (50.5%) *E. coli* isolates tested in this study. Figure 1 showed results of PCR on some of the samples. Ten isolates of the bacterium were isolated and confirmed from free ranged chicken cloaca swabs, 26 milk borne isolates and 60 from faecal samples of cattle (Table II). Majority of the isolates exhibited multiple antibiotic resistance, the

percentage resistance of the isolates to antibiotics includes streptomycin (39.6%), sulfamethoxazole/trimethoprim (28.5%), ampicillin (28.1%), amoxillin clavulanic acid (17.7%), kanamycin (13.5%), chloramphenicol (12.5%), cefoxitin 9.4%), florfenicol (6.3%) and nalidixic acid (3.13%) as shown in Table III. However, 41 (42.7%) were sensitive to all the antimicrobial agents. Twenty-five resistant patterns were obtained in all as shown in Tables IVa and IVb below.

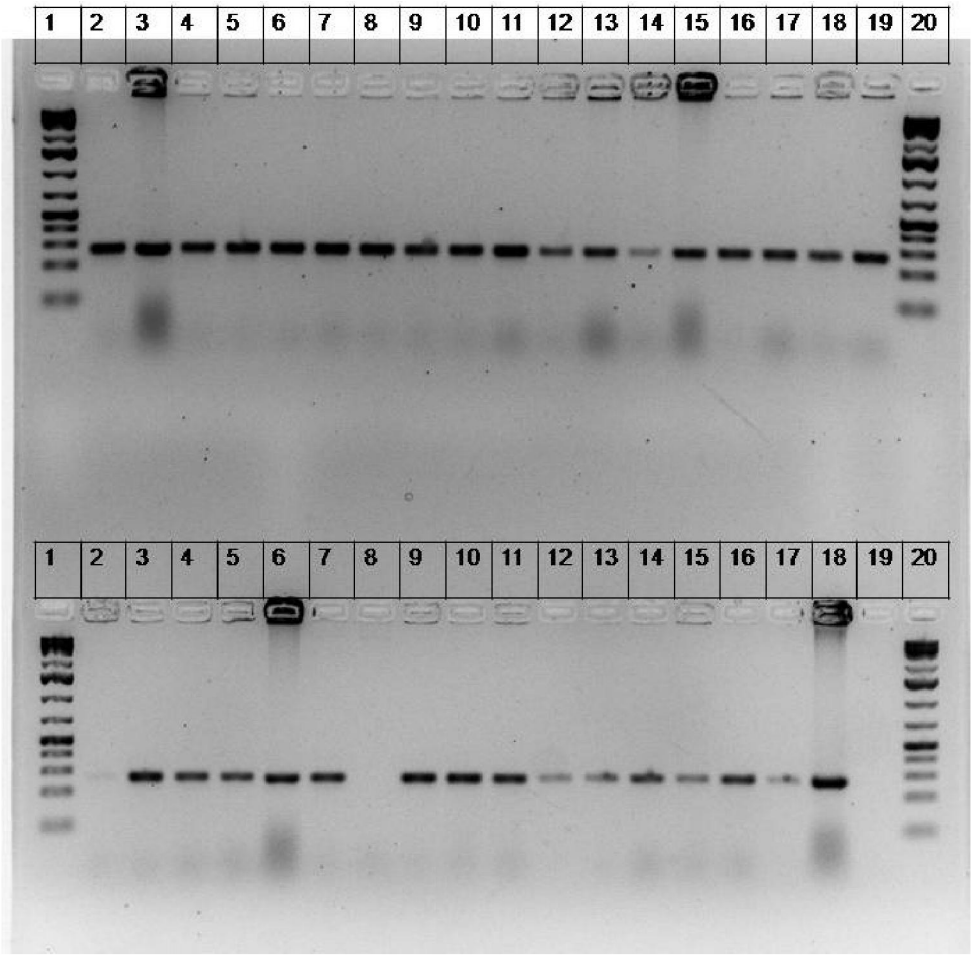

Figure 1: PCR amplification from *Escherichia coli*. An 352 bp product was amplified from the *E. coli* plasmid or genome using the β-d-glucuronidase-encoding gene *uid*A primer. Products were electrophoresed on 1% agarose gel in 1_TBE buffer. Lanes 1 and 20 were 100 bp DNA ladder. Lanes 2–19 show the PCR products amplified *E. coli.*

Table II: *Escherichia coli* isolated from raw milk, cattle faeces and chicken cloaca

Type of sample collected	No of *E. coli* isolated
Raw milk from cattle	26 (37.14%)
Faecal sample of cattle from abattoir	60 (85.71%)
Cloaca swab from local chicken	10 (20%)

Table III: Antimicrobial resistance profile of *Escherichia coli* isolated from bovine raw milk and cattle faeces.

Antimicrobial agents	Resistant *E. coli*
Streptomycin	28 (39.58%)
Sulfamethoxazole/Trimethoprim	37 (38.54%)
Ampicillin	27 (28.13%)
Amoxicillin/Clavulanic acid	17(17.71%)
Kanamycin	13 (13.54%)
Chloramphenicol	12 (12.5%)
Cefoxitin	9 (9.38%)
Florfenicol	6 (6.25%)
Nalidix acid	3 (3.13%)

Table IVa: Antimicrobial resistance patterns of *E.coli* isolates from the cow milk, faeces of cattle and cloaca swab of chicken in Ibadan, Oyo State, Nigeria

Resistance Group	Resistance Pattern	Source of isolate		
		Cow milk	Faece of cattle	Chicken cloaca swab
R1	Sulfa/Tri, Kana, Amp, Amoxi/Clavu, Strep Cefo Choram	0	1	0
R2	Sulfa/Tri, Flor Amp Amoxi/Clavu Strept Cefo	0	1	0
R3	Sulfa/tri, Amp Amoxi/Clavu Strept Cefo Chloram	0	1	0
R4	Flor Amp Amoxi/Clavu Strept Cefo	1	0	0
R5	Sulfa/Tri Flor Amp Amoxi/Clavu Cefo	0	1	0
R6	Sulfa/Tri Nali Kana Amp Strept	1	0	0
R7	Sulfa/Tri Amp Amoxi/Clavu Strept Cefo	1	0	0
R8	Sulfa/Tri Kana Amp Strept Chloram	3	6	0
R9	Sulfa/Tri Kana Strep Chloram	0	1	0
R10	Sulfa/Tri Amp Amoxi/Clavu Strept	1	2	0
R11	Sulfa/Tri Flor Amp Amoxi/Clavu	1	0	0
R12	Sulfa/Tri Nali Amp Strept	1	1	0
R13	Sulfa/Tri Amoxi/Clavu Cefo	1	0	0
R14	Sulfa/Tri Amp Strept	1	1	0
R15	Amoxi/Clavu Strept Cefo	0	1	0
R16	Sulfa/Tri Amp Amoxi/Clavu	0	2	0
R17	Kana Strept	0	1	0
R18	Amoxi/Clavu Cefo	1	0	0
R19	Sulfa/Tri Amoxi/Clavu	0	1	0
R20	Amp Strept	0	1	0
R21	Sulfa/Tri Strept	0	2	0
R22	Amoxi/Clavu	1	0	0
R23	Flor	0	2	0
R24	Sulfa/Tri	5	2	0
R25	Strept	2	8	0
R26	Susceptible to all	15	16	10

Table IVb: Summary of resistance pattern for bacterial isolates

Resistance	**No. of existence of the resistance**
Mono resistance	4
Double resistance	5
Triple resistance	4
Quadruple resistance	4
Quintuple resistance	5
Sextuple resistance	2
Septuple resistance	1
Total	25

4 Discussions

In this investigation, out of 190 specimens collected, 96(50.5%) *Escherichia coli* isolates were identified. None of the *E. coli* strain isolates was identified as *E. coli* 015:H7. The isolates comprised of ten of from free ranged local chicken, 26 from bovine milk samples and 60 from faecal samples of cattle.

The use of antibiotics in animal production and the attendant selection of resistant bacteria has been well studied (Aarestrup 2000; Angulo et al 2000; O'Brien 2002; Butaye et al 2003; Asai et al 2005; Anonymous 2007; Castanon 2007; Diarra et al 2007; Diarrassouba et al 2007). However, besides the simple principle that provided exposure to an antimicrobial agent can select for a resistant bacterium, the selection and dissemination of antimicrobial resistance is a complex phenomenon, which should be examined with ecological and population perspectives. Many antibiotic resistance genes in these bacteria have been identified on mobile genetic elements such as plasmids, transposons and bacteriophages, allowing their dissemination among bacteria in the gut or in extra-intestinal environments.

Antibiotic resistance spread from food animals to humans could be as a result of antibiotic resistance bacteria from a carrier animal that entered into meat production chain to infect humans through ingestion and also ingestion of unpasteurized dairy product. *Escherichia coli* resistance bacteria may be due to drug inactivating enzymes which are usually R-plasmid mediated (Robicsek et al 2006; Poehlsgaard and Douthwaite 2005), or due to genetic mutation

causing a change in a particular protein of the 30, ribosome submit (Poehlsgaard and Douthwaite 2005). Occasionally, mammary glands may be infected by *Escherichia coli* due their contact with faeces of the cows. The consumption of such infected milk by humans can cause outbreak watery diarrhoea (Lynch et al 2009). Resistance due to diminished uptake of antibiotic by the bacteria cell have been reported to be plasmid mediated (CDC 2013). However, colonization of the intestinal tract with resistant *E. coli* from chickens has been shown in human volunteers *(*Linton et al 1977) and resistance to the same drugs has been described previously in programs undertaken in different countries that monitor bacterial resistance in veterinary medicine (Heuer and Hammerun 2005; Asai et al 2006).

E. coli isolates are frequent contaminants of food of animal origin and in this study, this microorganism was recovered from 50 cloacae swabs sample of free ranged local chicken, 70 bovine milk samples and 70 faecal sample of cattle at abattoir, in addition, most of the isolates showed a multi-resistant phenotype indicating that *E. coli* isolates originating from food animals could be a reservoir of antimicrobial resistance. However, all the ten *Escherichia coli* isolated from the free ranged local chicken were all sensitive to all the nine antibiotic used in this study This showed that these free ranged chicken were not been exposed to antibiotics during their lifetime. In a similar study carried out in Thailand, all isolated *E. coli* from Thai broilers were found to be resistant to tetracycline, ampicillin and erythromycin in agar disk diffusion assays (Mooljuntee et al 2010). Increasing in age of animals has been associated with a progressive decline in antimicrobial resistance in *E. coli* isolated from cattle (Watson et al 2012). The presence of resistance groups unique to milk can be explained in terms of likely contamination from the environment and humans during handling and milking (Boor and Murphy 2002).

From this present study, it was observed that the *Escherichia coli* isolates showed high rate of resistance to streptomycin, sulfamethoxazole/trimethoprim and ampicillin while greater number were susceptible to cefoxitin, florfenicol and nalidixic acid. It was observed that all the *E. coli* strain isolated from chicken were susceptible to all the nine antibiotic tested in this study, this might be because these birds are local free range and they have not been subjected to any antibiotic before the samples collection. According to the literature, there is evidence that selective pressure exerted on bacteria to develop resistance has created concern that antimicrobial use may contribute to the persistence and spread of virulence in microorganisms. In *E. coli,* drug-resistance genes seem to be linked to virulence factors, creating the potential for antimicrobials to co-select for virulence genes [Rosengren et al 2009]. In this regard, as demonstrated in this study, the monitoring of antibiotic resistance and virulence in bacteria from environmental sources, such as milk and feacal samples from cows, would help to identify relevant factors that contribute to the spread of resistant bacteria and would support the prudent use of antibiotics.

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