Plasmid Profile and Multidrug Resistance Pattern of *Escherichia coli* Isolated from Swine in Abia State

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Abstract: Plasmid is known to play a very vital role in the emergence of multi-drug resistant bacteria in veterinary and human health. A total of 90 isolates of *Escherichia coli* out of 100 swab samples were recovered from cloacal swabs of swine (neonate, piglet and adults). All the isolates were morphologically and biochemically identified, while 20 of the representative isolates were confirmed using molecular-studies, and used for plasmid profile analysis. Twelve antibiotics were used for the study. All the isolates were resistant to amoxicillin (100%). Resistance to other antibiotics were as follows; Oxacillin (96%), Erythromycine (76%) and Streptomycin (68%). The demonstration of multi-drug phenotype cuts across the various age range of the swine. The bacteria isolates were sensitive to Gentamycin (100%), Ciprofloxamine (92%), Ceftazidine (92%) and Oxfloxacin (88%). There was multidrug resistance of *E. coli* (22.4%) with the predominant resistance patterns being CIP-OXF-CET-CEZ-GEN-CXM. There was Plasmid cure of all the bacteria isolates, an indication that resistance was chromosomally mediated. The study revealed that there were multi-drug resistant strains of *E. coli* in the studied Swine. Molecular detection of *E. coli* showed bands with amplicon size of 160 bp. The absence of visible bands for the plasmids despite the fact that the isolates were resistance to antibiotics, implies that chromosomal genes may be responsible for conferring resistance to antibiotics. From the study, swine may serve as a reservoir for *E. coli* strains carrying antimicrobial resistant genes.

• Keywords: Plasmid profile, Molecular detection, Multi-drug resistance, *Escherichia coli*, Swine.

Introduction

The emergence and distribution of multi-drug resistant (MDR) bacteria in environments pose a risk to animal and human health (Roest *et al*., 2007). Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic resistant microorganisms in both veterinary and human medicine (Neu, 1992; Witte, 1998). The acquired resistance occurs both in pathogenic non- pathogenic bacteria endogenous flora of exposed animals (Piddock, 1996; Van den Bogard, 1997; Van den Bogard and Stobberingh, 1999). These microorganisms may be shed in faeces leading to contamination of soil, food and aquatic environments. Though use of antimicrobial agents has been confirmed as a successful means of combating against bacterial contamination and infection, it's widespread use has produced a reservoir of antimicrobial agents and MDR microorganisms. The occurrence and persistent of antimicrobial resistant bacteria in animals is accompanied by co-contamination of the environment leading to a great health alarm (Grobbel *et al*., 2007; Martinez, 2009).

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment (Hassan. 1995). According to Albinu *et al*. (2004), *E. coli* is highly resistant to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazole.

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The high incidence of drug resistant *E. coli* and other pathogens in our environment has made it needful for regular antibiotics susceptibility monitoring with the objective of making available alternative rational prescription and therapy (Omigie *et al*., 2006). The mechanisms of resistance involves polymorphisms in antimicrobial targets that reduce vulnerability, gene encoding efflux system and proteins that fortify target sites or drug modifiers (Wright, 2007).

The property of multi-drug resistance could be transferred via conjugation from resistant strains of *E. coli* in a food animal to another by means of plasmid which occurs in cytoplasm of the donor bacterium which multiply independently of the chromosomal DNA (Cole *et al*., 2005; Kozak *et al*., 2009). Thus, a new bacterium with resistance factor emerges that is resistant to one or more antimicrobial agents (Buxton and Fraser, 1977). Factors responsible for resistance can be transferred via mobile genetic elements, such as plasmids, transoposons or prophages thereby making it possible for horizontal transfer within and between bacteria species (Davics and Davies, 2010), especially in environments such as the gut microbiome (Smillie *et al*., 2011). These have been collectively described as antimicrobial resistance (Marshall and Levy, 2011).

E. coli is a member of the family enterobactericeae, which includes many genera, such as *Salmonella*, *Shigella* and *Yersinia*. Though *E. coli* is a normal flora of healthy animals, it 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 species of farm animals and their environment (Hoyle *et al*., 2006).

The present study was conducted to isolate, molecularly detect and evaluate the antimicrobial resistance pattern of *E. coli* and also to determine the most effective drug against the tested *E. coli*..

Materials and Methods

Collection of samples

Cloacae swab samples were collected from different piggery farms located in Umungasi, Abayi, Osisioma, Ogbor Hill, Umuojima and Ama-Ogbonna all within Aba North, Aba South, Obi-Ngwa, and Osissioma Local Government Area of Abia State, using sterile swab stick. A cross sectional survey study was employed. Random sampling method was adopted in choosing the swine from each pen. A total of 100 samples were used for the study. Samples collected were transported to the veterinary laboratory of Michael Okpara University of Agriculture for processing.

Isolation and identification of *E. coli*

The samples were cultured as described by Quinn, *et al.* (1994), on blood agar, MacConkey agar and Eosin Methylene Blue agar within 4hrs of sample collection and incubated at 37°C for 24hrs. The isolates obtained were sub-cultured on Eosin Methylene Blue agar and incubated for 24hrs to obtain a pure culture of *E. coli*. Isolate that is ≥120 colony forming units (CFU) of each faecal swab after incubation at 37°C for 24hr were considered significant. A combination of colonial morphology, motility tests and Gram staining was conducted to determine the cultural characteristics of *E. coli*. The biochemical test conducted was as described by Cheesbrough, (2004) and include; catalase test, oxidase test, glucose fermentation tests and hydrolysis of arginine.

Antiobiotic Susceptibilty Test

The study made use of commercially available antibiotic discs (Oxoid, Basingstoke, United Kingdom) to evaluate the susceptibility pattern of the isolates. Twelve different antibiotics disc with various concentrations were used and they include Augmentin AUG (30 µg), Oxfloxacin OXF (5 µg), Ceftriazone CET (30 µg), Ciprofloxacin CIP (5µg), Tetracycline TET (30µg), Streptomycin STR (10µg), Eythromycine ERY (10 µg), Gentamycin GEN (30 µg), Amoxicillin AM (25 µg), Oxacillin OXA (1 µg), Ceftazidium CEF (30 µg) and Cefixime CXM (5 µg). The agar disc diffusion technique (Bauer *et al.* 1966). After incubation at 37°C for 24hrs, zones of inhibition were measured using a transparent meter rule. The susceptibility of isolates to antibiotics was interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004). The zones of inhibition were compared with that of *E. coli* strain type ATCC 25922 used as the control.

Plasmid DNA Isolation and Profiling

Escherichia coli strains isolated were inoculated on nutrient agar and incubated at 37°C for 24hrs. Alkaline lysis method (Zymogen UK) was employed in resistant plasmid DNA extraction. Three colonies of *E. coli* were picked and centrifuged and the supernatant was discarded. Three different buffers (B_1, B_2, B_3) of 200 µl and 400 µl were added to the pelleted cells at various times and thoroughly mixed by vortex before centrifugation at 16,000 x g for 2-3 minutes. The supernatant was loaded inside the zymo-spin column and centrifuged for 30 seconds and the flow through was discarded while W_1 buffer containing 200 µl was introduced into the column via a collection tube and centrifuged for 30 seconds. Thereafter, plasmid W_2 buffer containing 400 µl was added and centrifuged for 60 sec. The procedure as described by Ranjbar, (2007) was employed. The spin column was introduced into a new micro centrifuge tube and 50 µl of DNA E_1 buffer was added and centrifuged for 30-40 seconds. The extracted DNA was electrophoresed on a 0.8% agarose gel stained with ethidium bromide. The molecular studies were conducted in Lahore Medical Research Centre and Diagnostic Laboratory, Benin.

Preparation of agarose gel

The procedure described by Bikandi *et al.* (2004) was employed.Agrose (0.8 g) was dissolved in 100ml. Tris EDTA buffer in order to prepare a 0.8% agarose gel. The mixture was heated for 3-5min in a microwave to completely dissolve and allowed to cool to 56 °C before 8µl of ethidium bromide was added. Pour the agarose gel into the electrophoresis chambers and allow to it solidify.

Plasmid DNA detection

This was conducted by loading the first well with 10 µl of the molecular markers while the second well was loaded with 2 µl of loading dyes mixed with 8 µl of the plasmid DNA extract. Both were subjected to electrophoresis in a horizontal tank at 90 V for 60 min. After electrophoresis, plasmid DNA bands were visualized by fluorescence ultraviolet light trans-illuminator and analysed using a photo documentation system. The molecular weights were estimated using molecular weight standard of the maker.

Plasmid DNA curing

The plasmid cure was carried out according to the procedure described by Vivyan *et al.* (1972), in which 9 ml of freshly prepared nutrient broth was inoculated with 1 ml of pure *E. coli* culture that was grown in Luria Bertanii broth containing antibiotics and incubated at 37°C for 24 hrs. The medium was left for 2-3 hrs to allow the organism to grow and then 1 ml of 10% sodium dodeocyl sulphate (SDS) curing agent was added and incubated at 37°C for 24 hr. post impregnated with antibiotic on Muller Hinton agar plate impregnated with antibiotic disc and incubated at 37°C

for 24hrs. The zone of inhibition was read and recorded and compared with the pre-antibiogram.

Results

Table 1: Isolation rate of *E. coli* from neonates, piglets and adult pigs in Abia State

Age range	Isolation rate	% Isolation
Neonate (2-7 days)	28 (30)	96.7
Piglet (3-6months)	33(35)	91.4
Adult Above 12months	30(35)	85.7
Total	90(100)	91.3

Table 2: Antibiotic Susceptibility profile of *Escherichia coli* isolates obtained from test swine

Antibiotic types	No resistance (%) (n=50)	No sensitive (%) (n=50)
Ciprofloxacin (5µg)	4 (8)	46 (92)
Oxfloxacin (55µg)	6 (12)	44 (88)
Cefixime (5µg)	12 (24)	38 (76)
Ceftriaxone (30µg)	5 (10)	45 (90)
Ceftazidime (30µg)	4 (8)	46 (92)
Amoxicillin (25µg)	50 (100)	0 (0)
Augmentin (30µg)	30 (60)	20 (40)
Oxacillin (1µg)	48 (96)	2 (4)
Gentamycin (30µg)	0 (0)	50 (100)
Erythromycin (10µg)	38 (76)	12 (24)
Tetracycline (30µg)	32 (64)	18 (24)
Streptomycin (10µg)	34 (68)	16 (32)

Key: No=number, %=percentage

Table 3: Multi drug resistant patterns of *Escherichia coli* isolated from swine

Isolates	Multi drug resistant pattern
N5	AUG-TET-CXM-CIP-OXF
N7	CET-CEZ-CXM-OXA-OXF
N16	CXM-CIP-OXF-TET-OXA
N18	CXM-CIP-OXF-STR-ERY-AUG
N19	GEN-OXF-TET-ERY-AMX
N20	CXM-TET-STR-CEZ-AMX
N21	OXA-GEN-CEZ-STR-ERY-OXF
N22	CET-AUG-ERY-AMX-OXA-OXF
N23	CXM-AUG-TET-STR—OXF-GEN
N24	AUG-CIP-TET-AMX-CXM-GEN
N25	CET-AUG-OXF-STR-AMX-TET-OXA
N26	CXM-CEZ-ERY-TET-AUG-STR-OXA
N27	CIP-TET-STR-AMX-GEN-AUG-OXF
N28	CEZ-STR-CIP-OXF-OXA-CET-OXF
N29	CXM-ERY-AMX-TET-STR-OXF-CIP

N30	AMX-AUG-OXA-ERY-TET-STR-CIP
N32	CIP-OXF-CET-CEZ-CEZ-GEN-CXM-
N33	AMX-TET-STR-OXF-OXA-CEZ-OXA-CXM
N36	AMX-TET-OXF-AUG-OXA-CEZ-GEN-CXM

Table 4: Plasmid curing profile of Mutidrug resistant E.coli Isolates .

Key: AUG= Augmentin, TET = Tetracycline, CXM = Cefurixime, CIP = Ciprofloxacin, CET = Ceftriaxone, CEZ = Ceftazidim, OXF = Oxfloxacin, OXA = Oxacillin, Ery = Erythromycin, STR= Streptomycin, GEN = gentamycin,

Variables	No with plasmid (n=10)	No cured (%)
Neonates (2-7 days)	10	10(100)
Weaned (3-6 months)	10	10(100)
Adult (above 12 months)	10	10(100)

Key: No=number, %=percentage
AMX = Amoxicillin.

Table 4: Plasmid profile of multidrug resistant *Esherichia coli* isolates

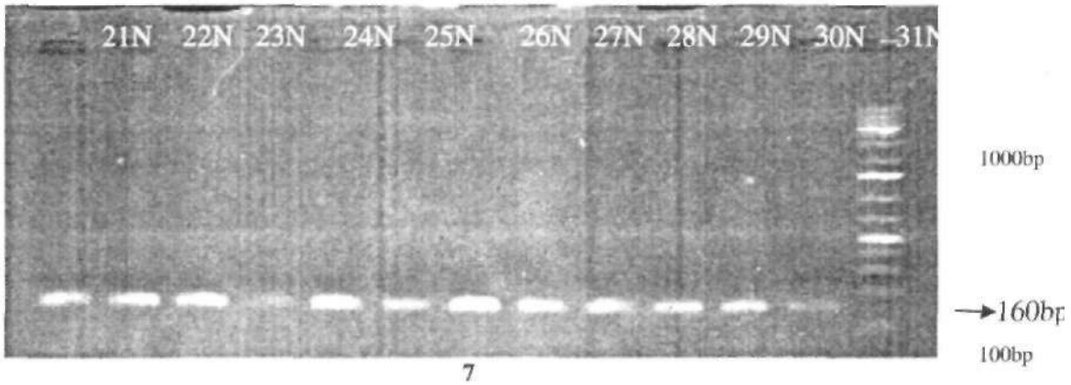

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Plate 1:Polymerase chain reaction results of bacterial isolates analysed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L is 100-1000bp DNA ladder (molecular marker). Samples N21-N32 positive for *E. coli* with bands at 160bp

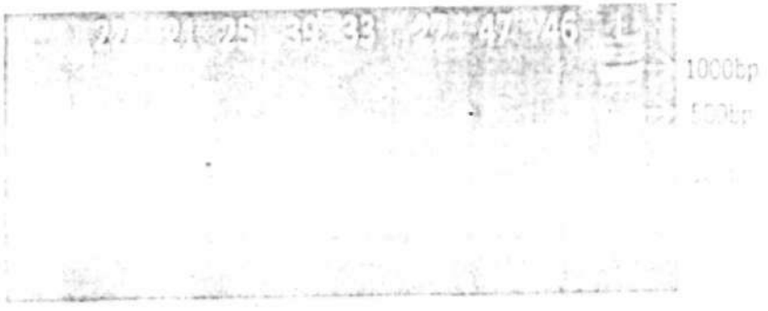

Plate 2: 0.8% agar gel electrophoresis of analysed plasmids extracted from multidrug resistant *E.coli* isolates. Ladder at 100-1000bp.

A total of 100 swab samples were collected and plated on Eosin methylene blue, colonies of isolated bacteria that have gave greenish metallic sheen characteristics of *E.coli* was use were collected to confirm the organisms. Table 1 shows the distribution patterns and isolation rate of *E.coli* from pigs of various age range. The highest isolation rate of *E. coli* was recorded in neonates with isolation rate of 96.7%, while adult pigs (above 12 months) had the lowest isolation rate of 85.7%. The prevalence isolation rate range of the swine is from 85.7% to 96.7% with an overall prevalence of 91.3%. This may suggest that the immunity of the neonates could be lower, while the adult pig is more resistance to *E. coli* organism. The spectrum of antimicrobial resistance in descending order for 12 antibiotic agent were Amoxicillin, Oxacillin, Erythromycin, Streptomycin,. Tetracycline, Augmentin, Cefixime, Oxfloxacin, Ceftriaxone, Ceftazidime, Ciprofloxacin and Gentamycin. All the isolates were found resistant to Amoxicillin while 96%, 76% and 68% were resistant to Oxacillin, erythromycin and streptomycin, respectively.

About 50% of the antibiotics used demonstrated sensitivity to *E.coli* which ranges from76%-96%. The isolates from neonates were most sensitive to Amoxicillin, Oxacillin, Ciprofloxacin, Ceftazidine and erythromycin ($p<0.05$). Sensitivity to Gentamycin, Ceftazidime, Ciprofloxacin and Ceftriaxone was significantly high in isolates obtained from adult pigs above 12 months of age ($p<0.05$).

Table 3 shows MDR pattern of *E. coli*. There were nineteen resistant patterns in this study with CIP-OXF-CET-CEZ-GEN-CXM (N33) as the most predominant pattern occurring in 22.4%, this was followed by CXM-CEZ-ERY-TET-AUG-STR-OXA (N22) as the second most predominant resistant pattern in 11.2% and the least occurring pattern was AMX-TET-SYR-OXF-OXF-OXA-CEZ-CXM (N34) at 0.8%.Table 4 shows Plasmid curing of multidrug resistant *E. coli* to detect whether the antibiotic resistance was as a result of plasmid or chromosomal effect. The isolates were all cured 100%.Plate 1 shows the molecular confirmation of *E. coli*. Ten *E. coli* isolates were amplified and showed marked bands with amplicon size of 160bp while two isolates N24 and N32 were poorly amplified. The absence of any band as shown in plate 2 indicates that resistance were chromosomally mediated.

Discussion

Though *E. coli* commonly occurs as normal flora of most acceptable thresh-hold, it may becomes pathogenic to the animal host. The high isolation rate of *E. coli* in this study may be due to poor hygienic practices. Resistance commonly observed among the antibiotic class of penicillin and macrolides as observed in this study and the sensitivity to antibiotic class of aminoglycoside, cephalosporins and quinolones is in agreement with Akingbade *et al.* (2014). This may be most likely due to the presence of cephalosporinase and penicillinase enzymes which destroy the Beta-lactam ring structure of the antibiotics and this is in agreement with the findings of Fontana *et al.*. (2000) and Livermore (1995). The antimicrobial susceptibility profiles of 50 *Escherichia coli* isolates obtain from swine is shown in Table 2. High resistance of *E. coli* to antibiotics like Augmentine, Amoxicillin and erythromycin is in disagreement with the finding of Igbal *et al.* (2002). *E. coli* resistance to the quinolones in this study is 10% and is in agreement with the findings of Farooqi *et al.*. (2000). This may be due to *E. coli* organisms using different mechanisms to develop resistance such as ability to modify the antibiotics target site, presence of inhibitory enzymes, possession of efflux pumps, and acquisition of resistant plasmids and mutation of the drug receptor site. The high antimicrobial resistance shown in this study may be due to factors such as inappropriate usage of antibiotics and this is in agreement with Eduardo *et al.* (2008) and Yah *et al.* (2006).

The nineteen resistant patterns observed in this study with these isolates displaying resistance to 3 or more classes of antibiotics is suggestive of multidrug resistance of *E. coli* to these antibiotics. This observed difference in resistance pattern as shown in swines may likely be due to abuse of antibiotics. Gentamycin followed by Ciprofloxacine and Ceftaziditu are the best drug of choice for the treatment of *E. coli* and this finding is consistent with that of Akingbade *et al.* (2014). *E.coli* resistance to Ciprofloxacin and Ofloxacine at 8% and 12% is in agreement with the report of Oteo *et al.*. (2005) in Netherland and Alex *et al.*. (2001) in Germany.

The high resistance of *E. coli* to Amoxicillin and Tetracycline at (>64%) is closely related to that reported by Umolu *et al.*. (2006). *E. coli* that were mult- drug resistant at pre-antibiogram stage were later found to be susceptible at the post antibiogram stage. This suggest that plasmid borne multidrug resistant gene may have been denatured by the sodium dodecycle sulphate used as the curing agent. Molecular confirmation evidence by amplification of bands with amplicon size of 160bp is closely related to the findings by Nathalie *et al.* (2001), but at variance with Sadjia *et al.* (2003), who confirmed amplicon size of 200bp. This difference in amplicon sizes may be due to different primers that were used. The isolates showed resistance to antibiotics but did not possess any plasmid, suggesting that chromosomal DNA may be responsible for carrying genes that confer resistance to antibiotics. It is very important that treatment should be based on recent laboratory test result of isolate.

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