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PLASMID PROFILE AND ANTIMICROBIAL RESISTANCE RATINGS OF *ENTEROCOCCI* ISOLATES FROM PIGS AND POULTRY BIRDS IN ABIA STATE, NIGERIA

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ABSTRACT
Our aim was to isolate and investigate the resistance ratings of enterococci poultry and pig isolates to various antimicrobial agents as well as to determine their plasmid profiles. Antimicrobial resistance ratings and the plasmid profiles of Enterococci isolated from poultry birds and pigs were analyzed. Three hundred and thirty enterococci isolates from poultry birds and pigs were obtained from the three zones in Abia State. Antimicrobial resistance ratings, transformation, curing and plasmid extraction for enterococci were done. The result showed that in both animal species multi-resistance to antimicrobials occurred in more than 40% of enterococci isolates. The enterococci isolates were resistant to floxapen (90%), ceprofloxacin (70%) and norfloxacin (80%). It also showed that the organisms were sensitive to lincocin (100%), chloramphenicol (85%) and gentamicin (75%). There were significant differences (P<0.05) in some reactions of some *Enterococcus* isolates to certain antimicrobial agents especially to chloramphenicol, rifampicin and gentamicin. Some isolates that was sensitive to gentamicin, rifampicin and chloramphenicol during pre-curing were resistant after curing though not significant (P>0.05). There was significant difference (P<0.05) among the isolates during pre-transformation and post-transformation process. Plasmid profile analysis of *Enterococcus* spp. revealed plasmid DNA bands ranging in size from 800 to 2000bp which appeared as bright bands. Large plasmid were lost during cell storage, some were plasmid less. No correlation could be made between plasmid patterns and antimicrobial resistance. The studies show good prospects for further research in the same area to explore and assign definite cause for antimicrobial resistance and multi-drug resistance.

Keywords: *Enterococcus* spp., plasmid, poultry birds, pigs, antimicrobial resistance.

PROFIL PLASMIDIQUE ET LES EVALUATIONS DE LA RESISTANCE AUX ANTIMICROBIENS ISOLATS ENTEROCOCCI DES PORCS ET VOLAILLES A L'ETAT D'ABIA, NIGERIA.

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RESUME:
Notre but était d'isoler et d'examiner les évaluations des isolats entérocoque des volailles et des porcs aux plusieurs agents antimicrobiens aussi bien que déterminer leurs profils plasmidique. Les évaluations de la résistance aux microbiens et les profils plasmidiques d'Entérocoque isolé des volailles et des porcs ont été analysés. Trois cent trente entérocoque isolats des volailles et des porcs ont été obtenus de trois zones différentes a l'État d'Abia. Les évaluations de la résistance aux antimicrobiens, la transformation durcissement et plasmide extraction pour entérocoques ont été faits. Le résultat a montré que dans les deux espèces animales, multirésistance aux antimicrobiens se produit dans plus de 40% des isolats d'entérocoques. Les isolats d'entérocoques étaient résistants a floxapen(90%), également Ceprofloxacin (70%), et norfloxacin(80%). Il a aussi montre que les organismes étaient sensibles a Lincocin(100%), Chloramphenicol(85%) et la gentamicine(75%). Il y avait des différences significatives (P<0,05) dans certaines réactions des isolats d'enterolocoques aux certains agents antimicrobiens en particulier a chloramphénicol, a rifampicine et gentamicine. Certains isolats qui étaient sensibles a la gentamicine, a rifampicine et chloramphénicol pendant pré- durcissement étaient résistants après le durcissement bien qu'insignifiant (P>0,05). Il y avait une différence significative (P<0,05) parmi les isolats lors de la pré- transformation et post-transformation. L'analyse du profil plasmidique d'*Enterococcus spp.* a révélé les bandes d'ADN plasmidique allant de la taille de 800 a 2. 000 bp qui semblait bandes brillantes. Les grands ont été perdus lors de stockage de cellules,, certains étaient moins plasmide. Pas de corrélation entre schémas de plasmides et la résistance aux antimicrobiens. Les recherches montrent de bonnes perspectives pour des

recherches plus approfondies dans la même domaine d'explorer et d'attribuer la cause précise pour la résistance. Aux antimicrobiens et la multirésistance.

Mots- clés: *Enterococcus spp.*, Plasmide, Volailles, Porcs, la résistance aux antimicrobiens.

INTRODUCTION

Enterococcus species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals. Their intrinsic ruggedness allows them to persist and spread in the environment. Once viewed as a genus of minimal clinical impact, enterococci, have surfaced as organisms of importance due to the emergence of multi-drug-resistant strains that are associated with significant morbidity and mortality of human and animals (6). Currently, it is responsible for approximately 12% of all nosocomial infections in the United States (7, 8). Furthermore, their ability to acquire antimicrobial resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge for therapeutic measures (9). Multiple antimicrobial resistances in bacteria are most commonly associated with the presence of plasmids which contain one or more resistance genes.

These bacteria are of particular concern in human and animal medicine because some strains have constitutive antimicrobial resistance tracts (10). Another concern is that these organisms can transfer resistance genes to other bacterial species including pathogens (11). Transmission of resistance genes from normally more virulent pathogenic species to non-pathogenic organisms is very common with the animal and human intestinal tract micro-flora (12).

Although opinions differ in defining the source of resistant antimicrobial *Enterococcus* spp. increasing development of antimicrobial resistance and transferable resistance genes are points of concern (13). Furthermore, the use of antimicrobials perpetuated antimicrobial resistant plasmids in countries like Nigeria, where there is an unrestricted use of antimicrobials.

Understanding the molecular epidemiology of resistance plasmids has been a major issue since investigators/scientists became aware of its (plasmids) role in the spread of antimicrobial drug resistance. Molecular characterization of plasmids and other genetic elements are also epidemiologically useful. The plasmid replication system that dictates the plasmid's behavior (host range, cope number) is the major plasmid classification and identification (Novick, 1987). However, their number (plasmid copies) also plays a critical role in imparting various characteristics to the pathogen, such as resistance towards different antimicrobials. Therefore, the present study considered the use of *Enterococcus* species to evaluate antimicrobial resistant patterns in pigs and poultry. It was also designed to genetically characterize the isolates by using molecular techniques, such as plasmid profile analysis.

MATERIALS ANDMETHODS

Sample collection and bacterial isolation: samples were collected once each week from the three senatorial zones in Abia State. Pigs and poultry birds were randomly selected without bias in each farm visited. The faecal material was collected with a swab (EVE PON) from the rectum (pig), cloaca (poultry birds), and transported to the laboratory and were inoculated into enterococcosal broth and incubated at 35^{o}C for 24 hours. Presumptive identification of the *Enterococcus* species were performed by using the following characteristics; Gram stained reaction, colony morphology, growth and blackening of bile-aesculin agar, growth in the presence of 6.5% Nacl and growth at 10^{o}C and 45^{o}C, the presence or absence of catalase and acidification of glucose with the production of gas (1).

Antimicrobial susceptibility testing

All isolates identified as enterococci were tested by disk-diffusion method using Muller Hinton agar (DIFCO) as recommended by CLSI (2010). The selected antimicrobials included ciprofloxacin (10mcg), norfloxacin (10mcg), gentamycin (10mcg), lincocin (20mcg), streptomycin (30mcg), Rifampicin (20mcg), Erythromycin (30mcg), Ampiclox (20mcg), floxapen (10mcg) and chloramphenicol (30mcg) (Oxoid, UK). The sensitivity test was standardized using *E.faecalis* (ATCC 29212). Inhibition zone size was interpreted using standard recommendation of CLSI (4) as sensitive, intermediate resistance and resistance.

Plasmid isolation

The plasmid DNA of *Enterococcus* spp. were screened by the alkaline lysis method of Birnbom and Doly (2), modified by Maniatis (5). The products were then electrophoresed for 1 hour at 150V on a 0.8% agarose gel. After staining the gel with ethidium bromide ($0.5\mu g/ml$), the photograph was taken. Molecular mass of the plasmid was determined by approximate comparison with plasmid of known molecular weight, *E.coli* K-12 DHL that harboured 8 plasmid of 1.4 to 35.8 MDa (3).

Plasmid curing

Multiple resistant isolates were selected and submitted to plasmid curing according to Molina-Aja *et al* (7) with modifications. We use luria-Bertani broth (LB), supplemented with 0.85% Nacl and acridine orange at50$\mu g/ml$.Strains grown under constant shaking in LB medium for 24 hours at 30^{o}C were once again subjected to antimicrobial susceptibility testing against antimicrobials to which they were resistant. Resistance were classified as plasmid dependent when affected by plasmid curing

RESULTS

Table 1 shows the sensitivity ratings of *Enterococcus* before curing and transformation. This showed that the organisms were resistant to floxapen (90%), ciprofloxacin (70%) and norfloxacin (80%). It also showed that the organisms were sensitive to Lincocin (100%), chloramphenicol (85%) and gentamicin (75%).

In Table 2 there were significant differences (P<0.05) in some reactions of some *Enterococcus* isolates to certain antimicrobial agents especially to chloramphenicol, rifampicin and gentamicin. This indicates that some strains of *Enterococcus* contain multi-resistance plasmids. The *E.coli* k-12 that served as control was tested alongside with the *Enterococcus* isolates as shown in Table2. When Table 2 was compared with Table 1 it showed that some isolates that was sensitive to certain antimicrobial agents (gentamycin, rifampicin and chloramphenicol) during pre-curing were resistant after curing though not significant (P>0.05).

TABLE 1: RESISTANCE RATINGS OF *ENTEROCOCCUS* PRE-CURING AND PRE-TRANSFORMATION PROCESSES

Isolates/drugs	**Ciprofloxacin**	**Norfloxacin**	**Gentamycin**	**Lincocin**	**Streptomycin**	**Rifampicin**	**Erythromicin**	**Chloramphenicol**	**Ampicillin**	**Floxapen**
***Enterococcus* 1**	**R**	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**R**	**R**
***Enterococcus* 2**	**SS**	**R**	**S**	**S**	**R**	**S**	**SS**	**S**	**SS**	**R**
***Enterococcus* 3**	**R**	**R**	**S**	**S**	**SS**	**S**	**S**	**S**	**SS**	**R**
***Enterococcus* 4**	**R**	**R**	**SS**	**S**	**R**	**SS**	**S**	**S**	**SS**	**R**
***Enterococcus* 5**	**R**	**R**	**R**	**S**	**R**	**R**	**S**	**S**	**R**	**R**
***Enterococcus* 6**	**R**	**R**	**S**	**S**	**SS**	**S**	**S**	**S**	**R**	**R**
***Enterococcus* 7**	**R**	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**R**
***Enterococcus* 8**	**SS**	**SS**	**S**	**S**	**R**	**R**	**R**	**S**	**SS**	**R**
***Enterococcus* 9**	**SS**	**SS**	**S**	**S**	**SS**	**SS**	**R**	**SS**	**R**	**SS**
***Enterococcus* 10**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**R**

Key: 1-14mm = Resistant (R), 15-19mm = slightly sensitive (SS); 20-35mm = Sensitive (S) (CLSL, 2005).

Table 3 showed resistance ratings of *Enterococcus* species post-transformation. The control organism *E.coli* k-12 was used in the transformation process. There was significant difference (P<0.05) among the isolates during pre-transformation and post transformation process. Plate 1 show the result of the plasmid profile of ten (10) representative *Enterococcus* isolates analyzed with 0.8% agarose gel. L is a DNA molecular ladder/control of size 100-1517 bp: E_t1, E_t6, and of E_t8 are samples which harbor plasmid with size of 800bp and 2000bp. In this study only small plasmids, which appeared as bright bands mostly below the band of chromosomal DNA on the gel, were used in the typing analysis because large plasmids tend to be lost during cell storage and sub culturing or plasmid extraction.

TABLE 2: RESISTANCE RATINGS OF ENTEROCOCCUS POST-CURING PROCESS

Isolates/drugs	Ciprofloxacin (10μm)	Norfloxacin (10μm)	Gentamycin (10μm)	Lincocin (20μm)	Streptomycin (30μm)	Rifampicin (20μm)	Erythromicin (30μm)	Chloramphenicol (30μm)	Ampicillin (20μm)	Floxapen (20μm)
Enterococcus 1	R	R	R	SS	R	R	S	R	R	R
Enterococcus 2	S	R	R	S	R	R	S	R	S	R
Enterococcus 3	S	R	R	S	R	R	S	R	SS	R
Enterococcus 4	S	R	R	SS	R	R	S	R	S	R
Enterococcus 5	R	R	R	S	R	R	S	R	R	SS
Enterococcus 6	R	R	R	S	R	R	S	R	R	R
Enterococcus 7	R	R	R	SS	R	R	S	R	R	R
Enterococcus 8	S	S	R	S	S	R	SS	R	S	S
Enterococcus 9	S	S	SS	S	S	R	SS	R	S	S
Enterococcus 10	R	R	R	S	S	S	R	R	R	R
E.coli K-12 (control)	S	S	S	S	S	S	S	S	S	S

Key: 1-14mm = Resistant (R), 15-19mm = slightly sensitive (SS); 20-35mm = Sensitive (S) (CLSL, 2005).

DISCUSSION

The study of the prevalence of antimicrobial resistance in indicator micro flora can be very useful in monitoring and understanding the process of antimicrobial-mediated selection in individual host as well as in the general population (14). Multiple antimicrobial resistances in bacteria pathogens which are common phenomenon in developing countries, including Nigeria is most likely related to the frequency use over-the-counter drugs without proper or no medical supervision.

Multiple antimicrobial resistances may be acquired through mobile genetic elements such as plasmids, transposons and class 1 integrons (17, 16). Integrons play an essential role in facilitating the transfer of the resistance genes contributing to the creation of multi drug-resistant phenotype (15).

In this study, all *Enterococcus* isolates tested were resistant to at least one antimicrobial class. The percentages of resistant isolates were generally significant and higher than or occasionally comparable to those previously reported for enterococci recovered from pig farms and slaughter houses (k18). Although a large proportion of isolates were susceptible to gentamicin, lincocin, chloramphenicol and erythromycin, high level resistance to amino-glycosides was detected in a significant percentage of isolates, in accordance with previous reports (19). The prevalence of high level resistance to floxapen and streptomycin was much higher than high level resistance to norfloxacin. Because aminoglycosides are antimicrobial of choice for treating enterococci infections, in combination with cell-wall inhibiting antimicrobials (20), the possibility of disseminating through the food chain of genes conferring a high level of resistance to aminoglycosides in enterococci is of much concern.

Another finding with mentioning is the frequency of isolates (40%) with plasmid-mediated resistance. These plasmid resistance expressions (phenotypic detection) were derived from poultry birds and pigs. For McBride *et al*; (21), the presence of this type of mobile genetic element is common in enterococci, as they make up a substantial fraction of their genome, and responsible for much of the horizontal gene

transfer. It is noted that the isolates from the same sample site showed different ratings before and after curing.

TABLE 3: RESISTANCE RATINGS OF *ENTEROCOCCUS* SPECIES POST-TRANSFORMATION

Isolates/drugs	**Ciprofloxacin (10μm)**	**Norfloxacin (10μm)**	**Gentamycin (10μm)**	**Lincocin (20μm)**	**Streptomycin (30μm)**	**Rifampicin (20μm)**	**Erythromicin (30μm)**	**Chloramphenicol (30μm)**	**Ampicillin (20μm)**	**Floxapen (20μm)**
***Enterococcus* 1**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**S**
***Enterococcus* 2**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
***Enterococcus* 3**	**S**	**S**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**S**
***Enterococcus* 4**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
***Enterococcus* 5**	**S**	**S**	**S**	**S**	**S**	**SS**	**S**	**S**	**S**	**S**
***Enterococcus* 6**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
***Enterococcus* 7**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**
***Enterococcus* 8**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**S**
***Enterococcus* 9**	**S**	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**S**
***Enterococcus* 10**	**S**	**S**	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**S**
***E.coli* K-12 (control)**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**

Key: 1-14mm = Resistant (R), 15-19mm = slightly sensitive (SS); 20-35mm = Sensitive (S) (CLSL, 2005).

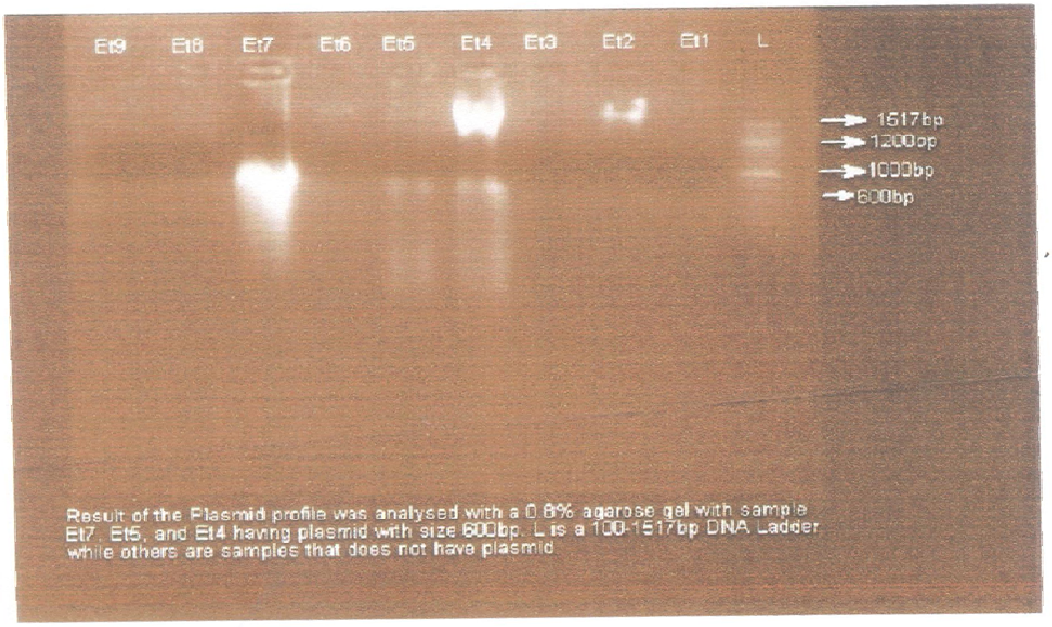

PLATE 1: PLASMID PROFILE OF *ENTEROCOCCUS* ISOLATES

Enterococci are prone to acquiring resistance to antimicrobials, either by mutation or by horizontal transfer of mobile genetic elements (Plasmids and transposons) (23). The reasons for the high number of plasmids in the resistance levels among several species of *Enterococcus* are still unknown (22).

Comparison of plasmid profiles could be a useful method for assessing relatedness of the clinical isolates with resistance to antimicrobial for epidemiological studies (16). In the present study, some of the plasmids were detected in most of the isolates, suggesting high rate of persistence of these plasmids in enterococci isolates. The maintenance of plasmids within the bacterial species is dictated by many environmental and genetic factors. Widespread usage of antimicrobials in Nigeria may cause significant variations in plasmids patterns amongst the enterococci isolates. Enterococci have intrinsic or acquired resistance to many commonly used antimicrobials. The resistances that cause the most severe therapeutic problems include high level resistance to floxapen, ceprofloxacine and norfloxacin. This, in turn, would limit the choice of antimicrobial (25). In the present study, the organisms were resistant to floxapen (90%), ciprofloxacin(70%) and norfloxcin (80%). On the other enterococci isolates were found to be sensitive to lincocin (100%), chloramphenicol (85%) and gentamycin (75%). The use of chloramphenicol in the treatment of Vancomycin Resistance Enterococci faecum (VREF) is limited due to side effects (24).

The resistance observed during post curing process showed that some isolates harboured resistance genes in their chromosomal DNA. The resistance ratings of *Enterococcus* during the 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 when this tests was repeated during post-curing and post transformation, it showed that resistant isolates possess resistant genes in both plasmid and chromosal DNA. Thirty percent of *Enterococcus* isolates harboured plasmids with size between 0.8kbp to 1.6kbp.This study showed that some isolates of *Enterococcus* that possess antimicrobial resistance were seen to be harbouring plasmids which did not have correlation with the antimicrobial resistance pattern. These observations imply that plasmid does not respect any boundaries, either between animals and human or bacterial species and genera, demonstrating the strong capacity of plasmids to be horizontally transmitted.

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