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Emergence of Fluoroquinolone Resistance in Pigs, Nigeria

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Abstract**—**A comparison of resistance to quinolones was carried out on isolates of Shiga toxin-producing *Escherichia coli*O157:H7 from cattle and *mecA* and *nuc* genes harbouring *Staphylococcus aureus* from pigs. The isolates were separately tested in the first and current decades of the 21st century. The objective was to demonstrate the dissemination of resistance to this frontline class of antibiotic by bacteria from food animals and bring to the limelight the spread of antibiotic resistance in Nigeria. A total of 10 isolates of the *E. coli* O157:H7 and 9 of *mecA* and *nuc* genes harbouring S. aureus were obtained following isolation, biochemical testing, and serological identification using the Remel Wellcolex *E. coli* O157:H7 test. Shiga toxin-production screening in the *E. coli* O157:H7 using the verotoxin *E. coli* reverse passive latex agglutination (VTEC-RPLA) test; and molecular identification of the *mecA* and *nuc* genes in *S. aureus*. Detection of the *mecA* and *nuc* genes were carried out using the protocol by the Danish Technical University (DTU) using the following primers *mecA*-1:5'-GGGATCATAGCGTCATTATTC-3', *mecA*-2: 5'-AACGATTGTGACACGATAGCC-3', *nuc*-1: 5'-TCAGCAAATGCATCACAAACAG-3', *nuc*-2: 5'-CGTAAATGCACTTGCTTCAGG-3' for the *mecA* and *nuc* genes, respectively. The *nuc* genes confirm the *S. aureus* isolates and the *mecA* genes as being methicillin-resistant and so pathogenic to man. The fluoroquinolones used in the antibiotic resistance testing were norfloxacin (10 µg) and ciprofloxacin (5 µg) in the *E. coli* O157:H7 isolates and ciprofloxacin (5 µg) in the *S. aureus* isolates. Susceptibility was tested using the disk diffusion method on Muller-Hinton agar. Fluoroquinolone resistance was not detected from isolates of *E. coli* O157:H7 from cattle. However, 44% (4/9) of the *S. aureus* were resistant to ciprofloxacin. Resistance of up to 44% in isolates of *mecA* and *nuc* genes harbouring *S. aureus* is a compelling evidence for the rapid spread of antibiotic resistance from bacteria in food animals from Nigeria. Ciprofloxacin is the drug of choice for the treatment of Typhoid fever, therefore widespread resistance to it in pathogenic bacteria is of great public health significance. The study concludes that antibiotic resistance in bacteria from food animals is on the increase in Nigeria. The National Food and Drug Administration and Control (NAFDAC) agency in Nigeria should implement the World Health Organization (WHO) global action plan on antimicrobial resistance. A good starting point can be coordinating the WHO, Office of International Epizootics (OIE), Food and Agricultural Organization (FAO) tripartite draft antimicrobial resistance monitoring and evaluation (M&E) framework in Nigeria.

Keywords**—**Fluoroquinolone, Nigeria, resistance, *Staphylococcus aureus*.

I. Introduction

THE WHO has described fluoroquinolones as critically important medicines for the treatment of human infections and advised prudent use in both human and veterinary medicine [1]. Quinolones including their fluorinated derivatives are widely used as the drug of choice in the treatment of urinary tract infection and typhoid fever. This extensive use along with non-therapeutic use in animal production has in part been responsible for the spread of resistance to fluoroquinolones. Several reports have described fluoroquinolones resistance in food animals [2]-[4] as well as commensal bacteria populations from humans, animals and environment [5]. On the other hand, the role of plasmid-mediated quinolone resistance (PMQR) gene, other resistant genes and mechanisms have produced widespread transfer of quinolone resistance between unrelated bacteria [5].

Pork is the most preferred source of animal protein in Makurdi, Nigeria, and virtually all residents consume it. However, pork has been reported in many parts of the world to be a vehicle for spread of pathogens that cause food borne illnesses due to its contamination during the slaughtering process [6]. For verification of slaughter hygiene conditions in the daily practice, the microbial status of carcasses is often determined by monitoring indicator organisms on carcasses at the end of slaughter [7]-[9], some of which include *Escherichia coli, Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus,* and *Campylobacter jejuni.* Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasing cause of health care-associated and livestock-associated infections worldwide [10]. The confirmation of the presence of the *mecA* gene has been the "golden standard" for detection of methicillin resistant *S. aureus* (MRSA) worldwide [11].

Antimicrobial resistance is a threat to humanity's way of life. Individual countries must recognize this threat and join in the global effort at developing strategies for antimicrobial resistance. This study has the objective of highlighting an important aspect of antibiotic resistance which is the resistance to a frontline antimicrobial agent, as well as increasing awareness of the menace in Nigeria.

II. Materials and Methods

A. Ethical Considerations

Ethical approval for the studies was granted by the Research Ethics Committee of the Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Certificate no. 99/051 and 015/101.

B. Sample Collection

Between January, 2000 and November, 2006 *E. coli* O157:H7 isolates were obtained from cattle on farms in North-western Nigeria (n=1,800). This was done using a cross-sectional design. Later, from January to November, 2015,

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swab samples were obtained from pigs at slaughter in Makurdi, North-central, Nigeria (n=241).

C. Isolation of E. coli O157:H7 and Staphylococcus aureus

The samples from cattle were cultured [12]. Colourless colonies on CR-SMAC agar were selected and biochemically identified using standard procedures. Presumptive identification of *E. coli* O157:H7 was carried out using the Remel Wellcolex *E. coli* kit. (Remel Europe Ltd., UK). The performance of the test and control latex reagents was confirmed using fresh overnight cultures of a control strain EHEC EDL 933.The production of Shiga toxins 1 and 2 (Stx 1 and Stx 2) by isolates that were serologically detected using the Vero Toxin *E. coli* Reverse Passive Latex Agglutination (VTEC-RPLA) test kit (Oxoid Ltd., England). While the sample from pigs were enriched and plated on baird parker agar supplemented with egg yolk tellurite (Oxoid Ltd., England). Typical colonies were subcultured and tested for beta-haemolysis based on [13]. Beta-haemolytic isolates were subjected to DNase [14] and microgen Staph- ID identification [15].

D. Identification of mecA and nuc Genes in S. aureus

2.4.1 DNA extraction was carried out using the ZR Fungal/Bacterial DNA mini prepkit (ZymoBIOMICS™ Epigenetics®, USA), based on the manufacturer's recommendation.

1) Detection of mecA and nuc Genes

A monoplex PCR assay for detection of *mecA* and *nuc* genes (species specific) were carried out using the protocol described by [16]. Briefly, amplification of the target genes was performed in a total reaction volume of 25 µl containing 2.5 µl of 10 X PCR buffer, 1 µl MgCl2 (25 mM), 0.25 µl of each dNTP (25 mM each), and 0.25 µl of 2U Taq DNA polymerase, 0.25 µl of each primers (130 µg/ml), 15.75 µl of PCR water and 5 µl template DNA. The PCR amplification was carried out with an initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min and a final extension at 72°C for 10 min. Of the amplified products, 10 µl were electrophoresed in 1% agarose, stained with ethidium bromide using a 100 bp ladder (Thermoscientific®) and visualized with a UV transilluminator.

E. Fluoroquinolone Resistance Testing of E. coli O157:H7 and Methicillin-Resistant S. aureus

In vitro susceptibility tests were performed by the standardized single disc diffusion method [17] in Muller-Hinton Agar (MHA) (Oxoid Ltd., England) with antimicrobial disc using a recommended *E. coli* O157:H7 reference strain (EDL 933) as the control organism. The antimicrobial agents tested and their disc concentrations in micrograms were as follows: ciprofloxacin (CIP5) and norfloxacin (NOR10) with the *E. coli* O157:H7. The isolates of methicillin-resistant *S. aureus* (MRSA) were tested using ciprofloxacin (CIP5).

From a pure culture of the organisms on SMAC agar, five colonies of the organisms to be tested were transferred with a wire loop to a test tube containing 4 ml of tryptone soya broth. All the five colonies were colourless, similar in morphology and were picked successively. The tubes were incubated at 37°C for 2 hours. The suspension was standardized by diluting with 0.85% saline to a density visually equivalent to the McFarland standard 0.5 (a turbidity standard prepared by add 0.5ml of 1% barium chloride solution to 99.5 of 1% sulphuric acid). This equates to approximately 108 enterobacteriaceae organisms per ml. MHA plates were prepared and dried in an incubator, cotton swabs were immersed into the culture and the entire surface of MHA was spread evenly with the suspension. The inoculum was allowed to dry with the plates covered for 10 minutes. Using aseptic techniques, each disc was placed and pressed gently to ensure full contact with the agar surface. The inverted plates were incubated immediately at 37°C for 24 hours.

The diameter of the zone of inhibition of growth including the diameter of the disc was measured to the nearest millimetre. The zone produced by each isolate was compared with that produced by the control strain (EDL 933). A strain was considered sensitive if the diameter of its zone of inhibition of growth by the antimicrobial agent was greater or equal to 4 mm less than that of the control culture. The average diameter of the inhibition zone produced by the control strain was 16 mm, so a zone was moderately sensitive if its zone of inhibition was 11-12 mm and resistant if it showed no zone if inhibition of growth or if the diameter of its zone was less or equal to 10 mm [18].

III. Results

A. Identification of nuc and mecA Genes in S. aureus

A total of 40 isolates from all the locations combined out of the 54 isolates positive for *Staphylococcus aureus* based on biochemical test were selected for polymerase chain reaction (PCR). A total of 30 (75%) isolates were positive for *Staphylococcus aureus* showing amplification of *nuc* gene at 255 bp (Fig. 1) and nine (22.5%) isolates were positive for the mecA gene (Fig. 2).

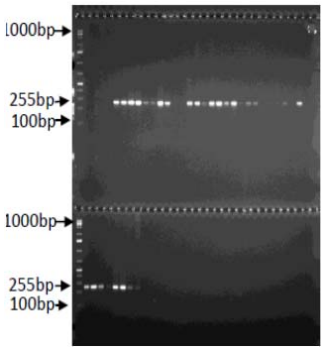

Fig. 1 Gel picture showing PCR for detection of *nuc* gene to confirm *Staphylococcus aureus* species from isolates. Lane M: 100 bp molecular ladder (ThermoScientific). Lanes 1-40 are presumptive *Staphylococcus* isolates. Thirty lanes showed amplification of the *nuc* gene at 255 bp (DTU) specific for *Staphylococcus aureus*

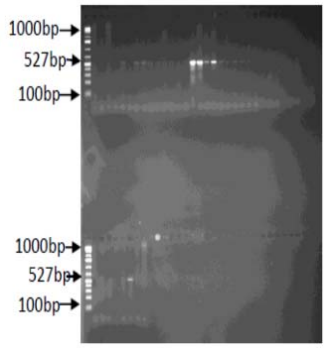

Fig. 2 Gel picture showing PCR for detection of *mecA* gene to confirm methicillin-resistant *Staphylococcus aureus* strains from isolates. Lane M: 100 bp molecular ladder (ThermoScientific). Lanes 1-40 are representatives of isolates. Nine lanes showed amplification of the *mecA* gene at 527bp (DTU) specific for methicillin-resistant *Staphylococcus aureus*

B. Detection of Shiga Toxin-Producing E. coli O157:H7 and MRSA and Fluoroquinolone Resistance of the Isolates

Up to 0.6% (10/1800) isolates of *E. coli* O157:H7 were confirmed as Shiga toxin-producing based on the result of the VTEC- RPLA test (Table I) and 3.7% (9/241) of MRSA were obtained based on detection of *mecA* gene (Fig. 2, Table I). Fluoroquinolone resistance was not detected in the 10 isolates of *E. coli* O157:H7, as all the phenotypes tested were susceptible to both norfloxacin and ciprofloxacin. However, 44% (4/9) of the MRSA tested were resistant to ciprofloxacin (Table I).

TABLE I
EMERGENCE OF FLUOROQUINOLONE RESISTANCE IN PIGS, NIGERIA

Time frame	No. of isolates tested		No. positive	% positive
	E. coli O157:H7	MRSA		
1st decade (2000-2010)	10	-	0	0
2nd decade (2011-date)	-	9	4	44

MRSA – Methicillin-resistant Staphylococcus aureus

IV. DISCUSSION

The study identified the two categories of *E. coli* Shiga toxins. The Shiga toxin 1 (Stx1) was in 10% (1/10) and Shiga toxin 2 (Stx2) in 90% (9/10) of the isolates confirming pathogenicity and membership of STEC strains [19]. A similar lop-sided ratio of production of Stx1: Stx2 has been reported [20]. Resistance to fluoroquinolones, norfloxacin and ciprofloxacin was not detected in a population of *E. coli* O157:H7 in the dawn of the 21st century in the cattle study. This could have been the case since fluoroquinolone preparations were not yet available for non-therapeutic use in animals in the country. The availability and indiscriminate use of the antibiotic explains the emergence of phenotypes of *S. aureus* that are resistance to fluoroquinolones in pigs. Extensive use and emergence of fluoroquinolone resistance in food animals has been reported by [5].

Methicillin resistance is the mechanism of *S. aureus* to overcome anti-staphylococcal agents. Methicillin resistant *S. aureus* (MRSA) is an increasing cause of health and livestock associated infection worldwide [10]. The altered penicillin-binding protein (PBP2a) is associated with methicillin resistance and this protein has reduced affinity for beta-lactam antibiotics which is encoded by the *mecA* has been the 'golden standard' for detection of methicillin resistant *S. aureus* (MRSA) worldwide [11]. This study provided evidence of MRSA by the detection of *mecA* gene in 3.7% (9/241) of the isolates tested; similar findings have also demonstrated and reported in studies performed by [21]-[24]. The *nuc* gene encodes the thermonuclease enzyme of *S. aureus* its amplification is used for speciation of *S. aureus* [25], [26]. This gene was amplified in all the isolates that harboured the *mecA* gene in this study.

The study has demonstrated that resistance to fluoroquinolones is very high in isolates of MRSA from pigs in Nigeria. Similar high or extremely high resistance to fluoroquinolones in food animals have been reported only in *E. coli* populations [27], making this finding of public health significance.

V. CONCLUSION AND RECOMMENDATIONS

A. Conclusion

The study concludes that extensive and indiscriminate use of fluoroquinolones in humans and in food animals has led to the emergence of fluoroquinolones resistance in Nigeria.

B. Recommendation

It is important that Nigeria should be a part of the WHO, OIE, FAO initiative for addressing antimicrobial resistance. A first step could be implementation of M&E of antimicrobial resistance protocols that are designed specifically for the country.

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