Vol. 13(25), pp. 400-406, July, 2019
DOI: 10.5897/AJMR2019.9137
Article Number: 9C06F2661540
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Extended spectrum beta-lactamase production and plasmid mediated quinolone resistance among lactose non-fermenting *Enterobacteriaceae* isolated from poultry sources in Calabar, Nigeria

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Received 8 May, 2019; Accepted 2 July, 2019

This study investigated the co-carriage of plasmid mediated quinolone resistance (PMQR) and extended spectrum beta-lactamase (ESBL) producing lactose non-fermenting (LNF) *Enterobacteriaceae* isolated from poultry birds. This was a descriptive cross-sectional study carried out between September, 2016 and March, 2017. The Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility patterns. ESBL screening disc kit was used to detect ESBL activities. Detection of ESBL and PMQR genes was carried out by means of polymerase chain reaction. In total, 207 LNF *Enterobacteriaeae* isolates were recovered from the cloacal swabs of poultry birds within the Calabar Metropolis. ESBL-producing isolates were 162 (78.3%) while fluroquinolone resistant isolates were 194 (93.7%). Among the ESBL-producing isolates, resistance to Ciprofloxacin, Norfloxacin, Levofloxacin, Ofloxacin and Nalidixic acid was 55 (34.2%), 26 (16.1%), 35 (21.7%), 50 (31.1%), and 162 (100%), respectively. About 65% of the quinolone resistant isolates were positive for at least one of the PMQR and ESBL genes in this study. Strict antimicrobial screening, surveillance of resistant isolates as well as the judicious practice of antimicrobial administration in the poultry setting with special emphasis on fluoroquinolones is advised given the high prevalence of co-existent ESBL and PMQR genes.

Key words: LNF enterobacteriaeae, Extended spectrum beta-lactamases, quinolone resistance.

INTRODUCTION

A serious concern is arising on the coexistence of Extended beta lactamase (ESBL) and Plasmid-mediated quinolone resistance (PMQR) producing non-lactose fermenting *Enterobactericeae* in animal husbandry which could be dangerous to humans especially in strains that may not be routinely screened for antibiotic resistance

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(Oghenevo et al., 2016; Yangkam and Bassey, 2015).

The past decade has witnessed a rise in the use antimicrobial agents in both the clinical and the veterinary setting with the concomitant increase in the development of antimicrobial resistance (Orji et al., 2005; Schwarz et al., 2001; Xiong et al., 2018). Resistance is common to the most frequently used antibiotics. The frequently recommended antimicrobial agents for common infections caused by *Enterobacteriaceae* are beta-lactams and quinolones/fluoroquinolones (in severe cases) (Bajaj et al. 2016).

Resistance to quinolone is chromosome-mediated via a mutation of the DNA gyrase encoding genes (*gyrA* and *gyrB*) and the topoisomerase IV encoding genes (*par*C and *parE*) (Strahilevitz et al., 2009). However, there seems to be a linkage between resistance to quinolone and beta lactam antibiotics. Production of ESBL is plasmid mediated. Resistant plasmids carrying genes encoding for ESBL usually carry genes encoding for quinolone resistance and this has given rise to PMQR in the *Enterobacteriaceae* family (Ni et al., 2016; Sun et al., 2014).

Three mechanisms for PMQR have been detected since 1988 (Tran and Jacoby 2002). They include, plasmid genes *qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S which codes for the quinolone resistance proteins (Qnr) of the pentapeptide repeat family that protects DNA gyrase and topoisomerase from quinolone inhibition, acetylation of quinolones with an appropriate amino nitrogen target by a variant of the common aminoglycoside acetyltransferase AAC(6')-Ib, thus reducing their activity and the plasmid mediated *oqx*AB and *qep*A genes used for efflux pump mechanisms (Tran and Jacoby 2002; Jacoby et al., 2014).

These plasmid-mediated mechanisms only provide low-level resistance that by itself does not exceed clinical breakpoint for susceptibility but nonetheless facilitates selection for higher-level resistance and makes infections by pathogens much more difficult to treat (Pourahmad Jaktaji and Mohiti, 2010). Currently, there is a dearth of data regarding the co-carriage of ESBL and PMQR genes in *Enterobacteriaceae* from poultry sources in Nigeria. Therefore, this study aimed at investigating the prevalence of PMQR and ESBL determinants in lactose non-fermenting *Enterobacteriaceae* from poultry sources in Calabar, Nigeria.

MATERIALS AND METHOD

This study was carried out within the Calabar metropolis in Cross River State, Nigeria. Calabar is the capital city of Cross River State in the South-South geopolitical zone of Nigeria. Cross River State shares boundaries with Benue State to the North, Ebonyi and Abia States to the west and to the east by the Republic of Cameroun. The city is administratively divided into Calabar municipal and Calabar South Local Government Areas (LGAs). Calabar covers a surface area of about 406 km^2 (157 $mile^2$) and a population of 371,022 at the 2006 census.

Study design

This was a descriptive cross-sectional study designed to investigate the prevalence of PMQR and ESBL determinants in lactose non-fermenting *Enterobacteriaceae* from poultry sources. This study was carried out in the Department of Microbiology, University of Calabar teaching Hospital, Calabar, Nigeria from September 2016 to March 2017.

Ethical consideration

The Ethical Committees of the selected hospital approved the protocol for this study. Approval was also obtained from the Cross River State Ministry of Health, conveyed via CRS/MH/ CGSE-H/018/Vol/123 and the Health Research Ethical Committee of University of Calabar.

Isolation and identification of species

The samples included cloacal swabs obtained from healthy birds in major poultry farms and markets within the Calabar metropolis. Sample collection method was by random sampling. Sterile cotton gauze moisturized with 70% alcohol was used to clean the surrounding of the cloaca of the birds, and a sterile cotton swab was inserted about two inches into the cloaca and whirled for about two seconds. The used swab sticks were stored in 10% buffered peptone water prior to transportation to the laboratory within four hours.

The samples inoculated on MacConkey agar and Xylose lysine deoxycholate agar. Lactose non-fermenting *Enterobacteriaceae* were identified using standard culture methods and conventional biochemical tests. The DNA of each lactose non-fermenting isolate was extracted and used for the amplification of the 16s rRNA region of the DNA of the isolates using a specific primer set (27F: 5'-AGAGTTTGATCMTGGCTCAG-3', 1492R: 5'-CGGTTACCTTGTTACGACTT-3'), by means of the ABI 9700 Applied Biosystem thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba biotec, South Africa (Taq polymerase, DNTPs, $MgCl_2$), the primer sets at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation at 95ºC for 5 min, denaturation at 95ºC for 30 s, annealing at 52ºC for 30 s, extension at 72ºC for 30 s for 35 cycles and final extension at 72ºC for 5 min. The product was resolved on a 1% agarose gel at 120V for 20 min and viewed by means of a UV trans-illuminator (Inqaba biotec, Pretoria - South Africa).

DNA sequence analysis was performed using direct sequencing of both strands by means of the BigDye Terminator kit on a 3510 ABI sequencer (Inqaba biotecl, Pretoria - South Africa). The obtained DNA sequences were edited using TraceEdit. Highly similar sequences were downloaded from GenBank in the National Center for Biotechnology Information using BLASTn (http://www.ncbi.nlm.nih.gov/blast). The Lactose non-fermenting isolates were confirmed by a 100% match with the 16s rDNA of the downloaded sequences. One hundred and seventy-two (172), lactose, non-fermenting *Enterobacteriaceae* were identified following 16S rRNA sequence analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out using commercially available antimicrobial discs types (LiofilChem Diagnostic ID USA) which included; Ciprofloxacin (CIP-5 µg),

Norfloxacin (NOR-10 µg), Levofloxacin (LEV-5 µg), Ofloxacin (O-5 µg), Nalidixic acid (NA-30 µg), Clotrimoxazole (CLO-50 µg), Amikacin (AK-30 µg), Imipenem (IMI-10 µg), Chloramphenicol (C-30 µg), Cefpodoxime (PX-10 µg), Ceftazidime (CAZ-30 µg), Cefotaxime (CTX-30 µg), Ceftriaxone (CRO-30 µg), Cefepime (FEP-30 µg), Aztreonam (ATM-30 µg). The quality control strain used was *E. coli* ATTC 25922. The susceptibility of the isolates to the antimicrobial agents was determined by means of the Kirby-Bauer disk diffusion method as described by the Clinical and Laboratory Standard Institute (Ferreira et al., 2011)

Transferring 4 to 5 confirmed *Salmonella* colonies in a tube containing 2.5 ml sterile normal saline by means of a sterile inoculating loop to prepare a bacterial lawn. The suspension was vortexed and its turbidity compared with barium chloride (0.5 McFarland Turbidity Standard; 1.0×10^8 CFU/µL). The optical density of the standard was regularly monitored with a spectrophotometer at λ=625nm and 1cm light path (ODλ=0.08 - 0.1) (Cheesbrough, n.d.). One hundred micro liters (100 µL) of the inoculum was spread on Iso-sensitest agar plates. The excess inoculum was siphoned with sterile Pasteur pipettes. Plates were allowed to dry at room temperature in a laminar flow hood. The discs containing predetermined amounts of the antimicrobial agents were then dispensed onto the bacterial lawn using a pair of sterile forceps and gentle pressure applied to ensure complete contact with the agar. The disks were placed 15 mm away from the edge of the plate and 25 mm apart from each other. The plates were inverted within 15 min after the discs were dispensed, and incubated at 37°C for 16 to 18 h. After incubation, they were examined by reading the diameters of the inhibition zones and interpreted in accordance with the description of the United States Clinical and Laboratory Standard Institute (Pallecchi et al. 2012).

Phenotypic ESBL detection

The screening test for the detection of ESBL activity was carried out by means of the ESBL screening disc kit (Cefotaxime, Cefotaxime + Clavilanic acid, Cefotaxime + Clxacillin and Cefotaxime+Clavilanic acid + Cloxacillin) by LiofilChem Diagnostic, ID. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 was used as positive and negative control strains respectively for ESBLs production. (Mathai et al., 2002).

Molecular detection of ESBLs

All the isolates that were phenotypically resistant to the beta-lactam antimicrobial agents used in this study were screened for some relevant ESBL encoding genes (bla_{SHV}, bla_{OXA} and bla_{CTX-M}) by means of polymerase chain reaction (Abrar et al., 2019).

The bla_{CTX-M}, bla_{SHV}, bla_{OXA} genes were amplified using the following primer sets respectively: CTX-M/F: 5'-CGCTTTGCGATGTGCAG-3' and CTX-M/R: 5'-ACCGCGATATCGTTGGT-3', SHV/F: 5'-CGCCTGTGTATTATCTCCCT-3' and SHV: 5'-CGAGTAGTCCACCAGATCCT-3', OXA/F: 5'-AGCCGTTAAAATTAAGCCC-3' and OXA/R: 5'-CTTGATTGAAGGGTTGGGCG-3' on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 µL for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba biotec, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 min; denaturation, 95ºC for 30 s; annealing, 52ºC for 30 s; extension, 72ºC for 30 s for 35 cycles and final extension, 72ºC for 5 min. The PCR product was resolved on a 1% agarose gel at 120 V for 20 min and visualized on a UV trans-illuminator (Inqaba biotech, South Africa).

Molecular detection of PMQR genes

All the isolates that were phenotypically resistant to the fluoroquinolone antimicrobial agents used in this study were screened by means of polymerase chain reaction for the detection of *qnr*A, *qnr*B, *qnr*S and *qep*A (Chen et al. 2012; Kao et al. 2016).

Statistical analysis

The relationships between the lactose non-fermenting *Enterobacteriaceae* isolates and fluoroquinolone resistance and PMQR determinants were evaluated using the Chi-square or Fisher's test where necessary. The data generated in this study was analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 22.0.). P-values of less than 0.05 (< 0.05), was considered statistically significant.

RESULTS

Antibiotics resistance patterns are shown in Table 1. A total of 207 Lactose Non-Fermenting (LNF) *Enterobacteriaeae* isolates were recovered from the cloacal swabs of poultry birds within the Calabar Metropolis. *Proteus mirabilis* was the most common bacterial isolate 118 (57.0%), followed by *Salmonella enterica* 39 (18.8%) while *Providencia rettgeri* 8 (3.9%) was the least common species.

All the isolates showed varied resistance to the 11 antibiotics and were most resistant to Nalidixic acid (93.7%), ceftazidime 179 (86.5%), cefotaxime 182 (87.9%) and cefpodoxime 179 (86.5%). Among the fluoroquinolones used in this study, resistance to nalidixic acid was the most prominent while resistance to Norfloxacin was the least. Among the beta lactam antibiotics, resistance to cefotaxime was the highest.

ESBL was phenotypically detected in 162(78.3%) of the 207 isolates. In Table 2 are shown the frequency of ESBL genes among the *Enterobacteriaceae* isolates under study. The genes bla_{CTX-M} and bla_{SHV} were the highest occurring 119(57.5%), while bla_{OXA} was the least ESBL gene detected 27(13%).

Among the ESBL-producing isolates, the rate of resistance to ciprofloxacin, norfloxacin, levofloxacin, ofloxacin and nalidixic acid was respectively 55 (34.2%), 26 (16.1%), 35 (21.7%), 50 (31.1%) and 162 (100%). The resistance to these fluroquinolone anitbiotics was significantly higher when compared to the non-ESBL-producing isolates ($P<0.05$). However, among the ESBL-producing isolates, there was no significant difference in the rate of fluoroquinolone resistance when compared with beta-lactam resistance ($P>0.05$).

Of the 207 isolates, 194 were resistant to quinolone antibiotics. About 65.5% (127) of the quinolone resistant

Table 1. Antimicrobial resistance pattern of LNF *Enterobacteriaceae* species isolated from poultry sources.

Antibiotics	Percentage (%) resistance					Total (n=207)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=118)	*Proteus vulgaris* (n=22)	*Providencia stuartii* (n=20)	*Providencia rettgeri* (n=8)	
Ciprofloxacin	48.7	22.9	36.4	5.0	0.0	26.6
Norfloxacin	20.5	13.6	13.6	0.0	0.0	13.0
Levofloxacin	51.3	6.8	36.4	0.0	12.5	17.9
Ofloxacin	84.6	7.6	31.8	0.0	12.5	24.2
Nalidixic acid	100	94.1	90.9	80.0	100	93.7
Ceftazidime	97.4	84.7	77.3	80.0	100	86.5
Ceftriaxone	87.2	62.7	54.5	100	0.0	58.9
Cefotaxime	87.2	87.3	77.3	100	100	87.9
Cefpodoxime	87.2	84.7	77.3	100	100	86.5
Cefepime	87.2	90.7	36.4	0.0	0.0	72.0
Aztreonam	94.9	82.2	86.4	35.0	0.0	77.3
Imipenem	38.5	16.9	4.5	0.0	0.0	17.4
Amikacin	41.0	44.9	9.1	0.0	0.0	34.3
Clotrimoxazol	89.7	70.3	68.2	30.0	50.0	69.1
Chloramphenicol	41.0	52.5	0.0	20.0	12.5	40.1

Table 2. Prevalence of ESBL-producing genes in LNF Enterobacteriaceae isolated from poultry sources.

Gene	No.(%) detection					Total (n=207)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=118)	*Proteus vulgaris* (n=22)	*Providencia stuartii* (n=20)	*Providencia rettgeri* (n=8)	
*bla*OXA	13 (33.3)	14 (11.9)	0	0	0	27 (13.0)
*bla*SHV	24 (61.5)	82 (69.5)	12 (54.5)	1 (5.0)	0	119 (57.5)
*bla*CTX-$_M$	33 (84.6)	70 (59.3)	16 (72.7)	0	0	119 (57.5)

Table 3. Prevalence of PMQR genes in LNF Enterobacteriaceae isolated from poultry sources.

Gene	No.(%) detection					Total (n=194)
	Salmonella enterica (n=39)	*Proteus mirabilis* (n=111)	*Proteus vulgaris* (n=20)	*Providencia stuartii* (n=16)	*Providencia rettgeri* (n=8)	
*qnr*A	3(7.7)	0	0	0	0	3(1.5)
*qnr*B	36(92.3)	61(55.0)	20(100)	1(6.3)	3(37.5)	121(62.4)
*qnr*S	20(51.3)	7(6.3)	0	0	0	27(13.9)
*qep*A	14(35.9)	9(8.1)	1(4.5)	2(10.0)	8(100)	34(17.5)

isolates were positive for at least one of the PMQR genes and ESBL genes used in this study. In Table 3 are shown the distribution of PMQR genes in the 194 quinolone resistant isolates. The *qnr*B gene was the most common PMQR gene detected 121 (62.4%), followed by *qep*A 34 (17.5%), *qnr*S 27 (13.9%) and *qnr*A 3 (1.5%).

Using the *S. enterica* isolates as an example, in table 4 is illustrated the co-existence of the ESBL and PMQR genes in LNF Enterobacteriaceae. All 39 *S. enterica* isolates demonstrated resistance to fluoroquinolones while 34 isolates were found to produce ESBL. Among the 34 ESBL-producing isolates, 41% (14) carried at least one PMQR gene, that is, one PMQR gene was co-carried with ESBL gene in the plasmid.

DISCUSSION

PMQR genes have been reported to be carried on mobile gene elements and can be easily transferred among

Table 4. Coexistence of ESBL and PMQR genes in *Salmonella enterica* (n=39) isolated from poultry sources

PMQR gene	ESBL genes	No. of isolates
*qnr*A	CTX-$_M$ + SHV	2
	CTX-$_M$ + OXA	1
*qnr*B	CTX-$_M$	7
	SHV	3
	CTX-$_M$ + SHV	10
	CTX-$_M$ + OXA	2
	CTX-$_M$ + SHV + OXA	11
*qnr*S	CTX-$_M$	4
	SHV	1
	CTX-$_M$ + SHV	8
	CTX-$_M$ + SHV + OXA	7
*qep*A	CTX-$_M$	1
	SHV	1
	CTX-$_M$ + SHV	4
	CTX-$_M$ + SHV + OXA	7

different bacterial strains and species (Osińska et al., 2016; Redgrave et al., 2014). This potential exacerbates the development of multi drug resistance because PMQR reportedly reduces microbial susceptibility to antibiotics and supports the occurrence of resistance-associated mutations on bacterial chromosomes, thus making *Enterebacteriaceae* infections much more difficult to treat. In this study, we look into the co-carriage of PMQR and ESBL genes in LNF *Enterebacteriaceae* isolates in Calabar, Nigeria.

All 207 LNF *Enterobacteriaceae* demonstrated varied degrees of resistance to the 11 antibiotics used in this study. However, the isolates were most resistant to Nalixidic acid and were least resistant to Imipenem, Levofloxacin and Norfloxacin. This partially agrees with a similar study carried out in Azerbaijan and Iran on ESBL-PMQR co-carriage where resistance to Nalixidic acid was highest (68.5%) closely followed by resistance to Levofloxacin (55%) and Norfloxacin (65%) (Azargun et al., 2018). The injudicious and common use of Nalixidic acid in comparison to other fluroquinolones, Levofloxacin and Norfloxacin could be the reason for such high resistance to Nalixidic acid in this region. Hence we recommend strict selection and rotation of antimicrobial agents coupled with the continuous monitoring of susceptibility profiles of antimicrobial agents to determine best treatment options

Our results further revealed that 78% (162) of the isolated produced ESBL and the most prominent genes of the 3 ESBL-producing genes tested were the *bla*$_{SHV}$ and the *bla*$_{CTX-M}$. This correlates with several studies that have reported both genes as the most prevalent ESBL-producers (Giske et al. 2008) There was also high prevalence of PMQR genes (65%) among the 194 isolates that were resistant to fluroquinolones with *qnr*B being the most prevalent. This agrees with several studies that have suggested of the added advantage given by PMQR genes to fluoroquinolone resistance.

Upon further analysis, we discovered that resistance to fluoroquinolones was significantly higher among ESBL-producing isolates than non-ESBL-producing isolates. This implies that co-carriage of ESBL and PMQR genes is associated with ESBL-producing isolates. This result agrees with several studies in Asia and Africa but disagrees with a few other studies, as specific effects of PMQR co-carriage on treatment outcomes has been difficult to document (Jacoby et al., 2014; Jiang et al., 2012; Shakya et al., 2013) Nonetheless, such high prevalence of multidrug resistance among ESBL-producing isolates poses a serious challenge to antimicrobial therapy.

About 65.5% (127) of the quinolone resistant isolates were positive for at least one of the PMQR genes and ESBL genes used in this study. To further buttress on the issue of co-carriage, our study using *S. enterica* isolates demonstrated that among the 34 ESBL-producing isolates, 41% (14) carried at least one PMQR gene, that is, at least one PMQR gene was co-carried with ESBL gene in its plasmid. This was in consonance with a previous study in which a high proportion of PMQR genes was observed among isolates possessing the ESBL genes (17).

The association of ESBL genes and PMQR genes are of importance for public health concerns. However, this

study did not include all the known ESBL genes and PMQR genes and molecular epidemiology was not performed, which could have further buttressed the evidences of the study. This study demonstrates high prevalence of LNF Enterobacteriaceae in a poultry setting. This agrees with several other studies done in USA and China (Projahn et al., 2018; Schwaiger et al., 2012). Due to the high prevalence of co-resistance to beta-lactam and fluoroquinolone antibiotics; we recommend the judicious practice of antimicrobial administration in the poultry setting with special emphasis on fluoroquinolones. We also recommend the continuous surveillance and monitoring of multidrug resistant isolates which should aid in proper antimicrobial administration in both humans and animals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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