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SOME RESISTANT GENES ASSOCIATED WITH DIARRHOEA IN RIVERS STATE

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ABSTRACT

Extended-spectrum beta-lactamse (ESBL)-producing organisms are now increasing among clinical isolates worldwide and isolation and detection of ESBL- producing strains is crucial for the selection of most effective antibiotic for treatment of infections. Due to the increasing rise of antibiotic resistance to some commonly used antibiotics for diarrhoea treatment and the limited information about the epidemiology of ESBL producing bacteria in Rivers State, *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* isolated from 120 diarrhoeic stool samples were investigated for the presence of resistance genes. In this study Plasmid DNA was extracted from bacteria and PCR amplification of the genes SHV, CTX-M and OXA was done using their specific primers. The result showed the presence of 100%, SHV, 10% CTX-M and 10% OXA genes amongst the enteric bacteria isolated from the diarrhoeic stool samples with SHV gene highest in prevalence. Therefore, proper management of prescription of antibiotics and also identification of ESBL producing bacteria in a community is important for effective prevention and treatment of infectious diseases.

KEYWORDS: Extended-spectrum beta-lactamse, Antibiotic resistance, diarrhoea, bacteria.

INTRODUCTION

In Nigeria, cephalosporin is usually the drug of choice in treating infections by gram negative bacteria in the family of the *Enterobacteriaceae*[1], however resistance to these drugs is of great concern worldwide because new resistance mechanisms are emerging and spreading globally increasing the cost of health care and threatening the ability to threat common infectious diseases which can result in prolonged illness and death. Some bacteria mainly gain resistance by producing some enzymes known as beta-lactamases.[2] Extended spectrum beta lactamases (ESBL's) are enzymes capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from common beta-lactamases such as TEM and SHV-type enzymes that have under gone one or more amino acid substitutions near the active site of the enzyme, thus increasing their affinity for and hydrolytic activity against third generation cephalosporins and monobactams.[3, 4] ESBLs are usually described as enzymes that are mediated by genes located on plasmids though some are also found in transposons or integrons, which enables their movement from one bacterium to another. Multidrug-resistant (MDR) bacterial strains harbouring ESBLs have been reported worldwide but there is insufficient information about these MDR bacteria in Nigeria. Microorganisms that produce ESBL play a very important role in infection control and are responsible for spreading resistance to other gram negative bacteria which are the major cause of outbreaks throughout the world. Extensive use of new antibiotics such as cephalosporins leads to the development of new ESBL's. ESBLs that are mostly found in Enterobacteriaceae are TEM, SHV and CTX-M although the majority of ESBL producing strains are *Escherichia coli* and *Klebsiella pneumonia.*[5-8] *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumonia* have been found to be associated with diarrhoea in Rivers State but there is inadequate information about the epidemiology of ESBL in *Enterobacteriaceae* in the State. This study is carried out to investigate for the presence of ESBL in gram negative bacteria isolated from diarrhoeic stool samples in Rivers State.

MATERIALS AND METHODS

Ethical Approval

Following Ethical approval and written informed consent from the Ethics Committee of the University of Port Harcourt Teaching Hospital, Rivers state Hospitals Management Board Port Harcourt and each participant, 250 stool samples were obtained from diarrhoeic patients in two major tertiary health facilities in Rivers State from November 2014 to October 2016.

Bacteria isolation and antimicrobial susceptibility testing

The diarrhoeic stool samples were inoculated onto MacConkey agar, *Salmonella Shigella* agar, Deoxycholate Citrate agar, Nutrient agar, Xylose Lysine Deoxycholate and Thioglycholate Citrate Bile Salt agar. The agar plates were incubated aerobically at 37ºC for 24hours for the growth of pure single colonies and identification of suspicious colonies was done using standard microbiological procedures.[9]

Antimicrobial susceptibility testing was done using the Kirby –Bauer disk diffusion procedure using the following antimicrobial agents: Ampicillin 30µg, Septrin 30 µg, Nalidixic acid µg, Ceporex 10 µg, Streptomycin 30 µg, Gentamycin 10 µg, Augumentin 30 µg, Ciproflox 10 µg, Perflacine 10 µg, Tarivid 10 µg, Norfloxacin 10 µg, Amoxil 20 µg, Rifampicin 20 µg, Erythromycin 30 µg, Chloramphenicol 30 µg, Ampiclox 20 µg and Levofloxacin 20 µg. The results were recorded according to criteria of Clinical Laboratory Standards Committee[10] and the multidrug resistant strains were investigated for the presence of ESBL genes.

Plasmid extraction and quantification

Plasmid DNA was extracted from 10 isolates which were multi drug resistant during the antimicrobial susceptibility testing using Zyppy Plasmid Miniprep kit (Zymo Biotech, SA) according to the manufacturer's instructions to check for plasmid mediated transfer of antimicrobial resistance genes. The extracted plasmids were quantified using the Nano-drop 1000 spectrophotometer. Two microliter of the extracted products was placed on lower pedestal and the concentration was read from the Nano-drop software on a computer system.

PCR amplification

The ESBL genes of the plasmid were amplified using specific primers for SHV, OXA and CTX-M respectively as shown in table 1. It was done on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 20 microlitres for 35 cycles. The PCR mix included: the X1 Dream taq Master mix supplied by Inqaba, South Africa containing dNTPs, $MgCl_2$, buffer and Taq polymerase. The primers at a concentration of 0.2M, the extracted ESBL gene served as template and 7.68 microlitres of Dnase free waster was used to make it up to a final volume of twenty microliters. The PCR conditions were as follows: Initial denaturation, 95ºC for 3 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes after which the machine keeps the amplicons cool at 4^{o}C. PCR products were detected by agarose gel electrophoresis using 2% agarose gel stained with ethidium bromide and DNA of known molecular weight.

Table 1: Primers for ESBL gene amplification.

NAME	SEQUENCE	BARCODE	LENGTH
CTX-M/F	CGCTTTGCGATGTGCAG	S3D8E	17 BASES
CTX-M/R	ACCGCGATATCGTTGGT	S3D8F	17 BASES
SHV/F	CGCCTGTGTATTATCTCCCT	S3D8A	20BASES
SHV/R	CGAGTAGTCCACCAGATCCT	S3D8B	20 BASES
OXA- 1/F	AGCCGTTAAAATTAAGCCC	S3D90	19 BASES
OXA- 1/R	CTTGATTGAAGGGTTGGGCG	S3D91	20 BASES

RESULTS

Of the 250 samples inoculated, 50% *Escherichia coli*, 13.33% *Pseudomonas aeruginosa*, 20% *Staphylococcus aureus* and 10% *Klebsiella pneumoniae* were isolated and confirmed from only 120 of the diarrhoeic stool samples using standard microbiological procedures and 8 samples had no significant growth.

Fifty-two (43.33%) were found to be sensitive to all gram negative commonly used antibiotics used and 20(16.66%) were found to be sensitive to gram positive antibiotics. Eight (6.67%) were found to be sensitive to one of each of the gram positive and gram negative antibiotic used and 40(33.33%) isolates were found to be multi drug resistant to the most frequently used antibiotics to treat diarrhoea in Rivers State.

Ten out of the 40 multidrug resistant genes investigated for the presence of ESBL genes revealed all 10 (100%) multi drug resistant strains harbouring SHV resistant gene with size 293bp (figure 1). One (10%) harboured CTX-M resistance gene with size 550bp (figure 2) and 1 (10%) harboured OXA resistant gene with size 908bp (figure 3).

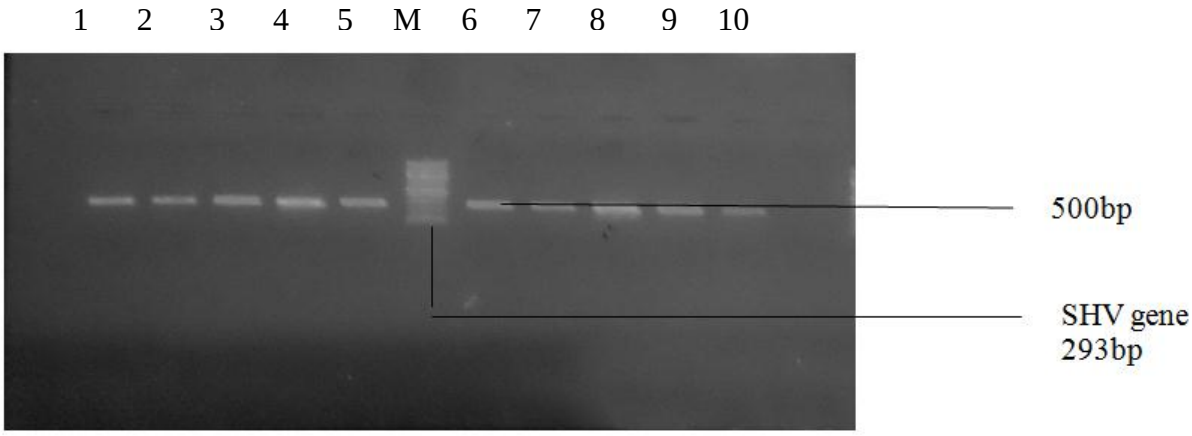

Figure 1: Gel electrophoresis showing the Extended β-lactamase of the *SHV* gene from diarrhoeic stool. Lanes 1-10 represents the positive bands while lane M represents the 100bp Quick-Load DNA molecular ladder.

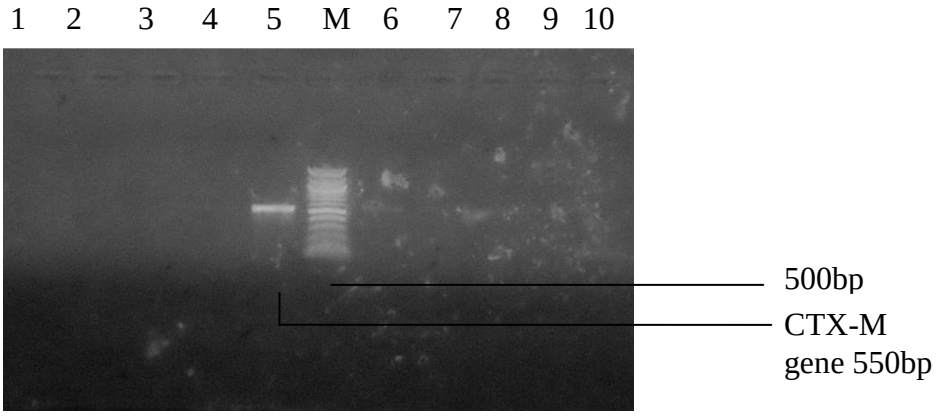

Figure 2: Gel electrophoresis showing the Extended β-lactamase of the *CTX-M* gene from diarrhoeic stool. Lanes 5 represents the positive band. Lane M is the 1000bp Quick-Molecular DNA ladder.

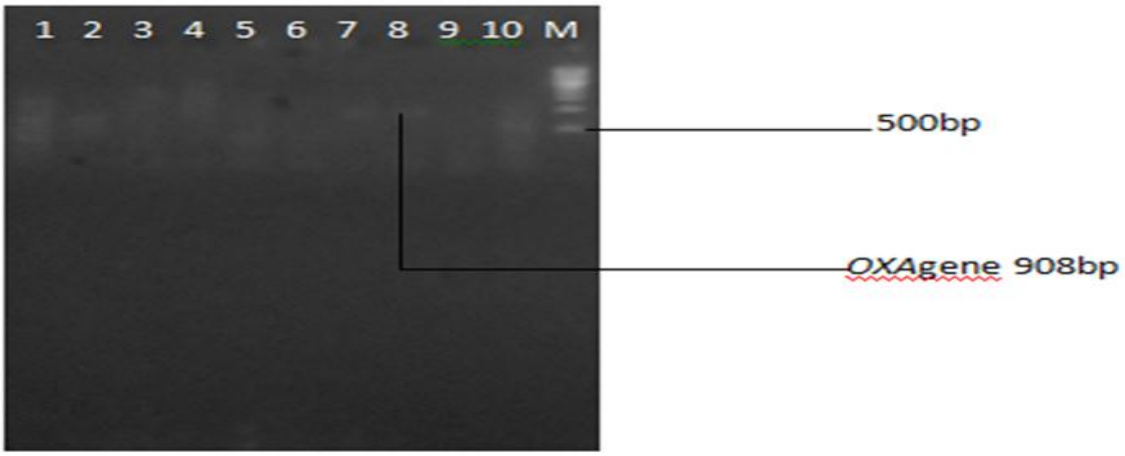

Figure 3: Gel electrophoresis showing the Extended β-lactamase of the *OXA* gene from diarrhoeic stool. 8 represent the positive band. Lane M is the 500bp Quick-Molecular DNA ladder.

DISCUSSION

Worldwide, there is an increase in ESBL producing microorganisms in clinical isolates. Different publications have reported that faecal carriage of broad-spectrum β lactamase-producing commensal *Enterobacteriaceae* from humans is rising.[11-14] The most predominant family of ESBLs reported among commensal *E. coli* is the CTX-M family, with the CTX-M-9 cluster being the most common cluster worldwide.[15]

This study shows the presence of 40(33.33%) isolates from diarrhoeic stool samples to be multi drug resistant to the most frequently used antibiotics to treat diarrhoea in Rivers State. Ten out of the 40 multidrug resistant genes investigated for the presence of ESBL genes revealed all 10 (100%) multi drug resistant strains harbouring SHV, 1 (10%) harboured CTX-M and 1 (10%) harboured OXA resistant genes respectively. The high level of multidrug resistance found among the isolates might be as a result of the presence of resistance gene found amongst the strains especially in *Klebsiella pneumoniae* that was transferred to the other bacteria strains. Studies by Nester and his colleagues in 2004 reveals that *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* can acquire resistance plasmid in a mixed culture.[16] A study in 2008 also states that resistance conferred by ESBLs is often associated with resistance to other classes of antibiotics.[17]

The prevalence of ESBL producing microorganisms in clinical isolates is different in different geographic locations**.** Studies in Portugal by Machado and his colleagues over a period of 2 years confirmed ESBL in 39% of *Enteobacteriaceae* isolates.[18] About 38-39% of *E. coli* isolates from United Arab Emirate were also identified as having ESBL.[19] In Nigeria ESBL has also been reported in clinical isolates of *Enterobacteriaceae*.[20-22] High prevalence of ESBL has been reported from Latin America, Turkey and India though low rates have also been reported in Korea, Japan, Malaysia, Netherland and Singapore.[4, 23 - 26]

Our finding showed SHV gene are wide spread in Rivers State because of the high prevalence 10 (100%) of the gene detected despite the low level of *Klebsiella pneumoniae* isolated from the diarrhoeic stool samples. This is in agreement with a study in 2017 by Obasi and colleagues which identified *Klebsiella pneumoniae* isolates with resistance to β- lactams from pharmaceutical waste water in south Western Nigeria.[27] In Nigeria, SHV, TEM and CTX-genes have also been reported in *Enterobacter* spp., *Klebsiella* spp. and *Escherichia coli*.[28-30]

Livermore in 1995 reported that SHV gene is said to be derived from *klebsiella species* and has been found to confer resistance to broad-spectrum penicillins such as ampicillin, tigecycline and piperacillin but not to the oxyimino substituted cephalosporins.[5] The high prevalence of the SHV gene might be as a result of the gene evolving as a chromosomal gene in *Klebsiella spp* incorporating into a plasmid and spreading to the other *enterobacteria species* isolated.

A low prevalence 10% of CTX-M resistance gene was also detected in this study contrary to the high prevalence detected in developed part of the world. Studies in 2007 by Mendonca and his colleagues revealed 68% while that of Lavigne and his colleagues revealed 66% prevalence of ESBL positive E.coli in France and Portugal respectively.[31-32] A study by Gazouli and his colleagues in 1998 reveals that CTX-M gene can be acquired by the horizontal gene transfer from other bacteria using genetic apparatuses such as conjugative plasmid or transposon and that they preferentially hydrolyze cefotaxime and has been found in isolates of *Salmonella enterica* serovar, *Typhimurium*, *E. coli* mainly and some other species of *Enterobacteriaceae*.[33] Shaikh and his colleagues revealed in their study that *E. coli* is more endemic, and *K. pneumoniae* is more epidemic and their mobile genetic elements, usually plasmids.[34] *E.coli* shows great level of resistance to penicillin derivative drugs like ampicillin and amoxicillin[35] and can also acquire resistance from the environment. High levels of *E.coli* resistance is reported in Africa especially Nigeria.[36]

This study also reveals 10% isolates harbouring OXA resistant gene. According to Evans and Amyes in 2014, OXA-type β-lactamases are relatively rare and are always plasmid mediated but in some cases has migrated into *enterobacteriaceae* becoming a major cause of carbapenem resistance.[37] They have the ability of hydrolyzing oxacillin and cloxacillin and predominantly occur in *Pseudomonas aeruginosa* but have been detected in many other Gram-negative bacteria such as *E.coli* isolates.[38 - 39]

High rates of resistance to broad spectrum antibiotics such as third generation class of cephalosporins was found amongst the strains due to presence of SHV, CTX-M and OXA genes. The implication of this now is that treating infections by these strains of bacteria will be a great challenge because treatment options will be limited to expensive and sometimes toxic drugs.

CONCLUSION

It can be deduced from the results that SHV, CTX-M and OXA resistance genes are responsible for multi drug resistance in diarrhoea treatment in Rivers state with SHV gene having the highest percentage of resistance. Therefore, proper management of prescription of antibiotics and also identification of ESBL producing bacteria in a community is important for effective prevention and treatment of infectious diseases.

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