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Full Length Research Paper

Extended-Spectrum β-Lactamases-Producing *Escherichia coli* from Orthopaedic Wounds

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ABSTRACT

The study, aimed at determining the prevalence and antibiotic resistance pattern of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* from wound infections was done between July 2017 and February 2018 at an Orthopaedic Hospital in South-Eastern Nigeria. Two hundred wound swabs collected using sterile swab sticks were aseptically inoculated primarily on MacConkey and blood agar plates, respectively and uniformly streaked at interval with flaming sterile wire loops. Incubation was at 37°C for 24 hours under aerobic and in microaerophilic conditions for bacterial growth. Antibiotic susceptibility test was evaluated by Kirby-Bauer disc diffusion method. ESBL-producing forty-nine (24.5%) *E. coli* isolates were obtained from the 200 swab samples. Forty-five (22.5%) of *E. coli* isolates subjected to Double disc synergy testing (DDST), confirmed the 18 isolates to be ESBL-producers. The Polymerase chain reaction (PCR) detected three genes Temoniera, Sulfhydryl variant, and Cefotaximase, mostly carried in *E. coli* plasmid, thus confirming the presence of *E. coli*. Elderly patients and males had highest prevalence of wound infections, especially on legs. Imipenem 5 (10.2%) was most active on *E. coli* isolates but there was a high frequency of multidrug-resistant pathogenic *E. coli*. Antibiotic susceptibility test showed that *E. coli* was highly resistant to Cefpirone 41 (83.7%), Cefoxitin37 (79.6%), Ceftazidime 35 (72.4%), Amoxicillin/Clavulanic acid 45(91.8%), Cefotetan 44 (89.8%), Cefotaxime 43 (88.8%), and Aztreonam 32 (66.3%). Indiscriminate use of third generation cephalosporins and monobactams must therefore be avoided. Currently, Imipenem has proved to be a better option for the treatment of orthopaedic wounds infections with *E. coli*.

Keywords: Orthopaedic wounds, *Escherichia coli*, Imipenem, antibiotic resistance, Nigeria

INTRODUCTION

Infections by Extended-spectrum beta-lactamases (ESBL)-producing organisms are causing significant diagnostic and therapeutic problems in affected patients both in the hospital and community at large. *Escherichia coli*, a member of the family Enterobacteriaceae is a Gram-negative, facultative anaerobic bacterium (Clement. *et al*., 2013). The enzymes Extended spectrum beta lactamases (ESBL) inactivate beta lactam antibiotics such as Penicillins and its derivatives, first, second, third and fourth generation Cephalosporins, Carbapenems, and monobactams. The enzymes are easily and quickly inducible from one organism to another within the members of the Enterobacteriaceae family, making them emerging pathogens (Lee *et al*., 2010; Rodriguez-Villalobos *et al*., 2010). Antimicrobial resistance can increase complications and costs associated with procedures and treatment.

Antimicrobial resistance among pathogens of wound infections is on the increase (Tumane and Wasnik, 2013). The consequences of infection due to ESBL-producing Enterobacteriaceae (ESBL-E) are well known. Wound infections due to ESBL-E have led to increased length of hospital stay, treatment failures, increased hospital costs, improper antibiotic use, and most notably, increased mortality and morbidity and health care costs (Alo *et al*., 2012). The control of wound infections has become more challenging due to widespread bacterial resistance to antibiotics and to a greater incidence of infections caused by Enterobacteriaceae family. In recent times, susceptible bacterial pathogens such as members of the Enterobacteriaceae family are spontaneously developing resistance to these first-line choice drugs used for treatment of severe infections. This is a public health concern (CLSI, 2017, 2020). This cross-sectional prospective study was designed to investigate the prevalence of ESBL-producing *E. coli* in wound infections in an orthopaedic hospital in South-Eastern Nigeria. Next was to determine the antimicrobial pattern, and screen for ESBL genes such as bla_{TEM}, bla_{SHV}, $bla_{CTX-M,}$ using PCR method.

MATERIALS AND METHODS

Study Area and Population

The study was carried out at an Orthopaedic Hospital in South East, Nigeria. Analysis of the collected wound swab samples was done at the diagnostic Microbiology laboratory department of the Institution. The study was an institution based cross sectional study conducted for a period of eight months from July 2017 to February 2018. The study was approved and granted by the joint committee on human research publications and ethics of the hospital. The study populations were all the patients that had wound infections and attending clinics at the in and out patients departments of the hospital during the study period. Verbal informed consent was obtained from the patients admitted in different wards and the out patients department, or their legal relatives after due explanation of the study protocols and purpose.

Specimen collection, culturing, and identification of bacterial isolates

A total of 200 wound swabs were collected from patients at the outpatient and in-patient departments of the hospital with sterile cotton swab sticks. The collected wound swab samples were immediately transported to the Microbiology laboratory unit of the Institution and processed within 1 hour of sample collection. Wound swab specimens were aseptically streaked on MacConkey and Blood agar plates, incubated at 37°C, both aerobically and in micro-aerophilic conditions for 24 hr. and 48 hr. respectively. The suspected *Escherichia coli* colonies were phenotypically identified using morphological, biochemical and molecular characteristics (Clement *et al.*, 2013).The following biochemical tests namely oxidase, Indole, Motility, H_2S Production, VogesProskauer (VP), methyl red, sugar fermentation test, nitrate, citrate utilization, and urea test were conducted to confirm the results obtained from macroscopy and microscopic examination of the isolates.

Antimicrobial Susceptibility Test (AST)

Antimicrobial susceptibility test was performed on each identified *E. coli* isolates using Kirby Bauer disc diffusion method as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2017. The *E. coli* from the samples were sub-cultured onto nutrient agar and incubated at 37°C for 24 hr. The isolates were standardized to the turbidity equivalent of 0.5 McFarland's standard. The *E. coli* isolates were tested on Mueller-Hinton agar against the following antibiotic sensitivity discs: Amoxicillin + clavulanic acid (30µg), imipenem (10 µg), cefotetan (30µg),

cefotaxime (30µg), ceftazidime (30 µg), cefpirome (30µg), Cefoxitin (30µg), Aztreonam (30µg) (Oxoid UK). Results, or rather the zone of bacteria inhibition diameter were measured with a meter rule and recorded as susceptible or resistant according to the CLSI guidelines (Gharavi *et al*., 2021; CLSI 2017, CLSI 2013).

Confirmation of ESBL production by Double Disk Synergy Test (DDST)

This was done by the DDST according to CLSI recommendations (CLSI 2017; CLSI 2014; CLSI 2013). Briefly *E. coli* isolates suspected of producing ESBL, after being screened with the beta lactam antibiotics, were swabbed on Mueller- Hinton (MH) agar plates. A disc containing amoxicillin/clavulanic acid (30µg) was placed on the center of the MH agar plates; ceftazidime (30 µg), ceftriazone (30µg) and cefotaxime (30µg) were placed adjacent to the central disc at a distance of 15mm. After an overnight incubation at $37^{\circ C}$, a ≥5 mm increase in the inhibition zone diameter(IZD) for either of the cephalosporins tested in combination with the central disc versus its zone diameter when tested alone confirms ESBL production phenotypically by the DDST method (Gharavi *et al.*, 2021; CLSI 2017, 2020 CLSI 2013; EUCAST 2013).

Molecular characterization of *E. coli* using PCR method

Bacteria isolates that took the characteristics of *E. coli* morphologically and *biochemically* were subjected to molecular characterization as confirmatory test. A conventional single or linear PCR technique was used to detect the Temoniera (TEM) genes Sulfhydryl variant (SHV) and Cefotaximase (CTX-M) genes that encode ESBL production in the test bacterial isolates, using specific reverse and forward primers (Table 1) as described by Parajuli *et al*. (2016) with little modification. The primers were supplied by integrated DNA technologies, 1710 commercial park, Coralville, Iowa 52241, USA.

Table 1: Primers for the, bla-TEM, bla-SHV genes and bla-CTX-M

Gene	Primers (5'-3')	Amplicon Size (bp)	TM (˚c)
TEM	F:5'-GAGACAATAACCCTGGTAAAT-3' R:5'-AGAAGTAAGTTGGCAGCAGTG-3'	459	45
SHV	F:5'-GTCAGCGAAAAACACCTTGCC-3' R:5'-AGAAGTAAGTTGGCAGCAGTG-3'	383	45
CTX-M	F:5'-GAAGGTCATCAAGAAGGTGCG-3' R:5'-GCATTGCCACGCTTTTCATAG-3'	560	45

Key: TM (˚c): Melting temperature; F: Forward; R: Reverse. Source: Parajuli *et al.* (2016)

Bacteria plasmid DNA template extraction was done by boiling method. The PCR amplification reaction was performed in a 25 µl mixture containing 18 µl of double distilled water (PCR grade), 4 µl PCR master mix, 0.5 (F) primer, 0.5 (R) primer, and 2 µl of DNA template. The PCR reaction mixtures were placed in a DNA thermal cycler for amplification, under programmed thermal and cycling conditions. For TEM genes, initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 45 seconds, followed by annealing at 55°C for 30 seconds initial elongation at 72°C for 2 minutes for SHV and CTX-M genes, the initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 45 seconds, followed by annealing at 55°C for 30 seconds, initial elongation at 72°C for 3 minutes and final elongation at 72°C for 2 minutes. For SHV and CTX-M

genes, the initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 45 seconds, followed by annealing at 60°C for 30 seconds, initial elongation at 72°C for 5 minutes. A 1.5% Agarose gel electrophoresis was run to determine the bands of the PCR products, using molecular markers, ethidine bromide and Bromphenol blue the indicator dye. Ten micro litre (10ul) of PCR amplified DNA negative and positive controls were added to agar walls. Electrophoresis was run in 1xTBE (1x Trace Borate EDTA) buffer at 90 Volts, for 45 minutes. The resulting DNA bands were visualized under ultra violet illumination using image analysis system, and photographed with digital camera and TEM and SHV bands of *E. Coli* were subsequently presented.

RESULTS AND DISCUSSION

Morphological, microscopic and biochemical characteristics of bacteria isolates from orthopaedic wounds confirmed that the organism is *E. coli* (Table 2).

Table 2: Morphological, biochemical, and microscopic characteristics of bacteria isolates from orthopaedic wounds

Characteristics of bacteria isolates from wounds	
Morphological:	
Form	Circular Rods
Surface	Smooth
Colour	Whitish, Pinkish
Margin	Entire
Biochemical:	
Gram	Negative -
Oxidase	Negative -
Citrate	Negative -
Lactose	Positive +
Hydrogen Sulphide (H_2S)	Negative -
Methyl Red	Positive +
VogesProskauer (VP)	Negative -
Nitrate	Positive +
Indole	Positive +
Urea	Negative -
Microscopic:	
Motility	Positive +
Organism	*E. coli*

Out of 200 samples of wound swabs collected from different patients and processed, 49 (24.5 %) *E. coli* were isolated. The distributions of bacteria with respect to age groups across the wound samples collected are shown in Table 3, which revealed that the age group 31 -60 years harboured more bacteria isolates, followed by 61 years and above. It can also be observed in Table 3 that males had a higher frequency of orthopaedic wounds (53.1%), while the female had a slightly lower frequency (46.9%).

Table 3: Frequency distributions of bacteria isolated from 200 orthopaedic wounds examined

Characteristics	Frequency	Frequency percentage
Age (years):		
1 – 30	12	24.5
31 – 60	23	46.9
61 and above	14	28.6
Gender:		
Male	26	53.1
Female	23	46.9
Site:		
Leg	23	46.9
Hand	17	34.7
Chest/neck	9	18.4
Status:		
Out- patient	26	53.1
In- patient	23	46.9
Total	**49**	**100.0**

The zones of bacteria inhibition diameters were also recorded as susceptible or resistant in Table 4. Double disc synergy test done on Twenty two (22) *E. coli* isolates that were most resistant to beta lactam antibiotics used, and nine (9) 40.9% were confirmed to be ESBL producing (Table 5). Inhibition zone diameter (IZD) for ceftazidime and cefotaxime tested in combination with amoxicillin-clavulanic acid versus its zone when ceftazidime and cefotaxine tested alone confirms ESBL production (Plates 1 and 2).

Table 4: Antibiotic susceptibility profiles of *E. coli* isolates and Break point recommendations for detecting ESBLs

Antimicrobial agents			Resistant %	Resistant (mm)	Sensitive (mm)
Amoxicillin/Clavunate	AMC	20/10 µg	45 (91.8)	≤13	≥18
Cefpirome	CPO	30 µg	41 (83.7)	≤14	≥18
Cefoxitin	FOX	30 µg	37 (79.6)	≤14	≥18
Cefotetan	CTT	30 µg	44 (89.8)	≤12	≥16
Cefotaxime	CTX	30 µg	43 (88.8)	≤22	≥26
Imipenem	IMP	10 µg	5 (10.2)	≤13	≥16
Aztreonam	ATM	30 µg	32 (66.3)	≤17	≥21
Ceftazidime	CAZ	30 µg	35 (72.4)	≤17	≥21

Table 5: Prevalence of beta lactamase producing *Escherichia coli* (DDST)

	Escherichia coli	
Gender	Number tested	Number positive (%)
Male	13	5(38.46)
Female	9	4 (4.44)
Total	**22**	**9 (40.9)**

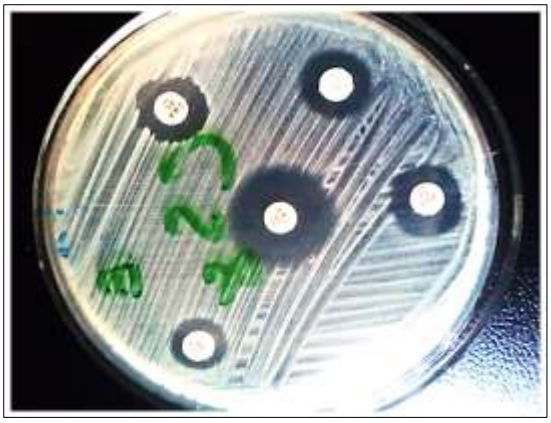

Plate 1: The IZD for ceftazidime and cefotaxime tested in combination with amoxicillin clavulanic acid

Plate 2: The IZD when ceftazidime and cefotaxine tested alone

Detections of *E. coli* isolates genes by PCR with primers Cefotaximase (CTX-M), Sulfhydryl variant (SHV) and Temoniera (TEM) are recorded in Table 6. The resultant DNA bands (Plate 3) were visualized under ultra violet illumination using image analysis system, and photographed with digital camera to demonstrate TEM and SHV bands of *E. coli* isolates as previously done with *Klebsiella pneumoniae* (Okwuonu and Chukwura, 2022).

Table 6: Molecular detection of *E. coli* genes by PCR using CTX-M, SHV and TEM primers

Bacteria isolates	*Cefotaximase* CTX-M		*Sulfhydryl variant* SHV		*Temoniera* TEM	
	No. +ve	%	No. +ve	%	No. +ve	%
Escherichia coli (n = 10)	1	10.0	2	20	10	100

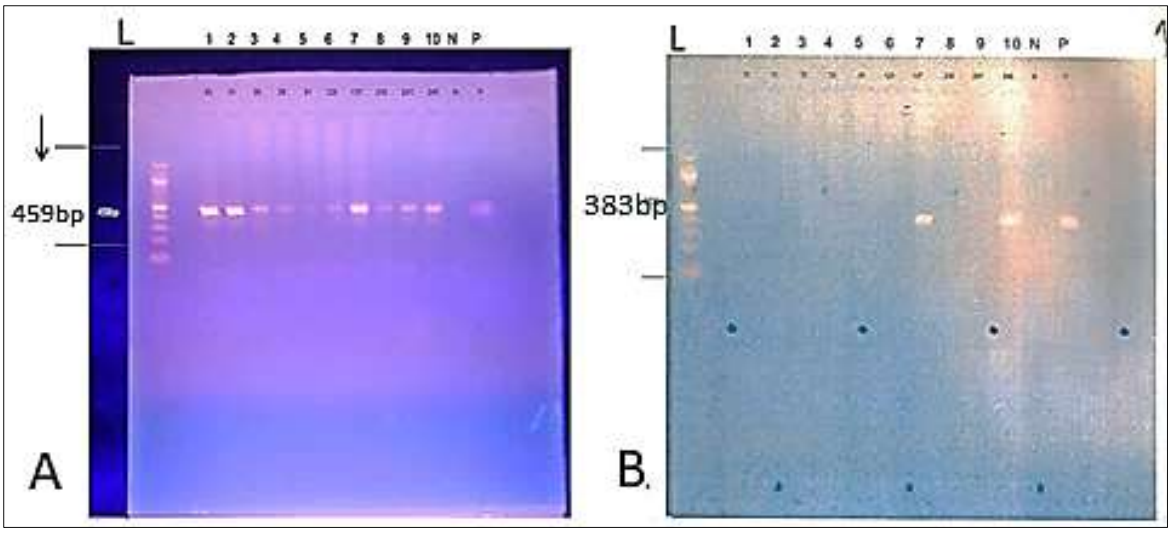

Plate 3: The TEM and SHV bands of *Escherichia coli* isolates.
[A] Duplex PCR profiles for bla_{TEM} of *E. coli* isolates. Lane marked P = positive control and lane marked N = negative control. All other lanes (bands) are positive for bla_{TEM}.
[B] Duplex PCR profile for bla_{SHV} of *E. coli* isolates. Lane 1, 2, 3, 4, 5, 6, 8, and 9 are negative for bla_{SHV} while lane 7 and 10 are positive.

The prevalence of ESBLs is increasingly being reported worldwide, and it varies according to geographic location and is directly linked to the use and misuse of antibiotics. The *E. coli* isolated from wound samples at the orthopaedic hospital used for this study exhibited multidrug resistance traits. This could lead to difficulty in treatment, prolonged hospital stay, prolonged rehabilitation, morbidity, and mortality if not properly tackled (Lee *et al.*, 2010; Rodriguez-Villalobos *et al.*, 2010). The prevalence of *E. coli* in this study agrees with the results of the work of Iroha *et al*. (2017) and correlates positively with that of Muhammad *et al*. (2008) who reported that *E. coli* is one of the leading causative agents of orthopaedic wound infections at the institution investigated.

Socio-demographic results revealed that the age group of 31-60 years harboured more bacteria isolates, followed by 61 years and above. This study did agree with work of Nwankwo *et al*. (2015) who reported high prevalence of *E. coli* isolation among elderly patients which they attributed to low immunity, and risk factors for wound infection. This did not agree with the work of lroha *et al*. (2017), as younger patients harboured more *E. coli* in wounds. Results also revealed that males had a higher frequency of orthopaedic wounds (53.1%), while the females had a slightly lower prevalence (46.9%). Occupational involvement might be the cause of the slight increase in orthopaedic wound frequency of males over the females. This study showed results with higher prevalence of wound infections among out patients (55.1%, when compared to the in–patients (44.9%). This trend of results could be as a result of patients waiting for a longer time before seeking medical attention, and as such, leading to heavy growth of bacteria and heavy infections. This work disagreed with the findings of Iroha *et al*. (2017) and Chukwura *et al*. (2010), status wise.

This work reported very high resistance profile of *E. coli* to second and third generation cephalosporins. This agreed with the findings of similar works in Ghana (lroha *et al*., 2017; Saana *et al*., 2014; Obeng-Nkurumah *et al*., 2013) but partially disagreed with the study of Muhammad *et al*. (2008), who reported that all infections studied at the orthopaedic hospital were treated with the

prophylactic antibiotics of first generation cephalosporins such as cefazoline.

In our study, imipenem had a very good activity against *E. coli*. This result is similar to that found in lndia by Prakash and Saxena (2013), in Karachi by Abdullah *et al*. (2013) who reported susceptibility to imipenem between 97.7% and 100%. Oteo *et al*. (2010) recommended that carbapenems (imipenem and meropenems) could be used for the treatment of severe infections caused by ESBL-producing bacteria. On the other hand, the suggestions made by Oteo *et al*. (2010) that beta-lactam/lactamase inhibitors combination may be used in treating infections caused by ESBL-producing bacteria contradicts this current study, since there were high resistances in amoxicillin/clavulanic acid resulting in a high probability of therapeutic failure. Double disc synergy test (DDST) was done on Twenty two (22) *E. coli* isolates that were most resistant to the beta lactam drugs used and nine (9) 40.9% confirmed to be ESBL producing (see Table 5).

Polymerase chain reaction was used to detect the presence of three genes associated with ESBL production by *E.coli* namely, the Temoniera (bla_{TEM}), Sulfhydryl variant (SHV), and Cefotaximase (CTX-M). In a similar work done in India (Chakraborty *et al.*, 2020), a high rate of genotypically ESBL positive isolates (75%) was observed, indicating a high prevalence of ESBL producers, which agreed with this work. Chakraborty *et al.* (2020) reported CTX-M (60%) as the most predominant type of plasmid among the ESBL producers with 10% being TEM positive, and none harboured the SHV gene. This is in disagreement with our own findings.

Conclusion

The most frequently encountered Enterobacteriaceae in orthopaedic wounds studied was *Escherichia coli* and susceptibility testing using all classes of antibiotics was marked with gross resistance including the beta-lactam drugs. Double disc synergy test (DDST) was employed to confirm the suspected ESBL producers, of which nine of the Twenty-two tested were positive. The PCR test method used, analyzed three genes associated with ESBL production in *Escherichia coli* isolates and confirmed genotypically that TEM was the most predominant; SHV was moderately detected while CTX-M was very scanty. It is very important therefore to develop effective infection control and innovative strategies to reduce the spread of ESBL producing organism in both hospitals and communities.

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