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Full Length Research Paper

Extended-Spectrum β-Lactamases-Producing *Klebsiella pneumoniae* from Orthopaedic Wounds

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ABSTRACT

This hospital-based prospective study characterized and determined the frequency of extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* from orthopaedic wounds of 200 patients at an Orthopaedic hospital in south-eastern Nigerian between July 2017 and February 2018. Wound samples were collected aseptically using sterile swab sticks, and processed using standard microbiological techniques. Antibiotic susceptibility test was evaluated using Kirby-Bauer disc diffusion method while ESBL-producing *K. pneumoniae* isolates were confirmed using double disc synergy test (DDST). The twenty-five of the *K. pneumoniae* suspected ESBL producers were further tested using double disc synergy test (DDST). Molecular characterization using Polymerase Chain reaction (PCR) method was used to detect the genes encoded in the *K. pneumoniae* isolate. *Klebsiella pneumoniae* was isolated from 80 (40%) the 200 collected wound swab samples. Prevalence of infection with *K. pneumoniae* isolates was higher in elderly patients, with males more frequently infected than females. Also, orthopaedic leg wounds had the highest infection rate. *Klebsiella pneumoniae* isolates were highly resistant to the tested beta lactam antibiotics. Imipenem, a carbapenem, was the most active antibiotic against *K. pneumoniae* isolates. Of the twenty-five samples subjected to DDST, Ten were confirmed positive, giving a prevalence of 40.0%. Molecular characterization with PCR method also detected three genes encoded in *K. pneumoniae* isolate namely, Temoniera (TEM), Sulfhydryl variant (SHV) and Cefotaximase (CTX-M) genes. It is therefore imperative to curtail the continued increasing frequency of ESBL-producing *K. pneumoniae* in circulation through strict infection control measures and judicious use of antibiotics.

Keywords: Orthopaedic wounds, ESBL, *K. pneumoniae*, Imipenem, Nigeria

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative rod in the Enterobacteriacea family. Beta lactamases are bacterial enzymes that inactivate beta-lactam rings of beta-lactam antibiotic such as the penicillin and its derivatives, first, second, third, fourth generation cephalosporins, carbapenems and the monobactams. Extended-spectrum beta-lactamases (ESBL) have emerged as an important mechanism of resistance in Gram-negative bacteria.

ESBL pose a major hindrance in the therapeutic outcome of patients resulting in a significant clinical challenge if remained undetected (Hijazi *et al*., 2016) and makes it obligatory to identify the prevalence and epidemiology of *K. pneumoniae* in hospitals, and thereby control the spread of these strains, and determine suitable preventive measures and treatment policies (Jadhav *et al*., 2012). Wound is a breach on the skin and the exposure of subcutaneous tissue

following loss of skin integrity. In the event of infection, a wound fails to heal, the patient suffers increased trauma, treatment cost rise, and general wound management practices become more resource demanding (Feglo *et al*., 2016). Infection continues to be a major complication of wounds that are resistant to many clinically useful antibiotics and infections by these bacteria results in increased morbidity, mortality, and longer hospital stays (Feglo *et al*., 2016) This study will serve as a guide to the empirical choice of antibiotics during treatment of infections caused by *K. pneumoniae*. This study is therefore designed to isolate, characterize the enteric bacteria *K. pneumoniae* in orthopaedic wounds; determine its prevalence and frequency of ESBL-production, and genotypically screen for the presence of beta lactamase associated genes in ESBL namely $bla_{TEM,}$ $bla_{SHV,}$ and bla_{CTX-M}in the isolates.

MATERIALS AND METHODS

Study Area and Population

The study was carried out at an Orthopaedic Hospital South East, Nigeria. Analysis of the collected wound swab samples was done at the diagnostic Microbiology laboratory department of the orthopaedic hospital used for the work. 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. Oral-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.

Collection of samples

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 orthopedic hospital used, and processed within 1hour of sample collection.

Isolation and identification of bacteria

Wound swab specimen were aseptically streaked onto differential media (MacConkey agar) and Blood agar plates in duplicate using wire loops and incubated at 37^0C, both aerobically and in micro-aerophilic conditions for 24h and 48h respectively. The bacteria colonies isolated on media plates were identified phenotypically using morphological, physiological, Gram staining and biochemical test as described by Cheesbrough (2014). The molecular detection of *K. pneumoniae* gene was by PCR method.

Antimicrobial Susceptibility Test (AST)

Antimicrobial susceptibility test was performed on each *K. pneumoniae* isolate using Kirby Bauer disc diffusion method as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2020, 2017; CLSI, 2013a; Gharavi *et al*., 2021). *Klebsiella pneumoniae* from the samples were sub-cultured onto nutrient agar and incubated at 37^0C for 24h for purification. The isolates were standardized to the turbidity equivalent of 0.5 McFarland's standard. *Klebsiella pneumoniae* isolates were tested on Mueller-Hinton agar against the following antibiotic discs: Amoxicillin + clavulanic acid (30µg), imipenem (10µg), Cefotetan (30µg), cefotaxime (30µg), ceftazidime (30µg), Cefpirome (30µg), Cefoxitin (30µg), Aztreonam (30µg) (Oxoid UK). Results or the zones of inhibitions were measured with a meter rule and recorded as susceptible or resistant according to approved guidelines (Gharavi *et al*., 2021; CLSI, 2020, 2017; CLSI, 2013b).

Confirmation of ESBL production by double disk synergy test (DDST)

This was done by the DDST according to CLSI recommendations CLSI (2017). In this test, third generation separate cephalosporins (ceftazidime 30µg and cefotaxime 30µg discs alone) and in combination with amoxicillin-clavulanic acid (30/10µg) as the center disc were placed 15 mm apart side by side on the inoculated Mueller-Hinton agar (MHA) plates and incubated at 37^0C for 24h. After incubation, inhibition zone diameter (IZD) was measured, and a 5mm or more increases in IZD for ceftazidime and cefotaxime tested in combination with amoxicillin-clavulanic acid versus its zone when ceftazidime and cefotaxime tested alone confirms ESBL production (Gharavi *et al*., 2021; CLSI, 2020, 2017; CLSI 2013b; EUCAST 2013).

Molecular characterization of *Klebsiella pneumoniae* using PCR method

Bacteria isolates that took the characteristics of *K. pneumoniae* morphologically and *biochemically* were subjected to molecular characterization as confirmatory test. A conventional single or linear polymerase chain reaction (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 (^{0}C): Melting temperature; F: Forward; R: Reverse. Source: Parajuli *et al.* (2016)

Bacteria plasmid DNA template extraction was done by boiling method. The PCR was done in a mixture containing: 19µl double distilled water, 4µl PCR master mix, 0.5µl (F) primer, 0.5µl (R) primer and 1µl 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 from 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 litres (10µl) 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 *K. pneumoniae* were subsequently presented.

RESULTS AND DISCUSSION

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

Klebsiella pneumoniae was isolated from 80 (40%) of the 200 collected wound swab samples. Twenty (25 %) of the *K. pneumoniae* isolates were confirmed to be ESBL producers. *Klebsiella pneumoniae* isolated was highest 38 (47.5%) among the age group 31-60 years, but was of equal occurrence 21 (26.25%) among the age groups 1-30 years and 61 years and above (Table 3). The *K. pneumoniae* isolates were mostly prevalent among elderly patients while its frequency was slightly higher among males than females but there was no significant gender differences between patients infected with *K. Pneumoniae* (P=0.243) (Table3). It was noticed that wounds of accident victims had the highest bacterial prevalence of 18 (22%) while the lowest was observed in samples from ulcer patients 4 (5%).

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

Characteristics of bacteria isolates from wounds	
Morphology:	
Form	Irregular Circular rods
Surface	Glistening Moist
Colour	Creamy, Pinkish
Margin	Swarming, Entire
Biochemical:	
Gram	Negative -
Oxidase	Negative -
Citrate	Positive +
Lactose	Positive +
Hydrogen Sulphide (H_2S)	Negative -
Methyl Red	Negative -
VogesProskauer (VP)	Positive +
Nitrate	Positive +
Indole	Negative -
Urea	Positive +
Microscopic:	
Motility	Negative -
Organism	*K. pneumoniae*

Table 3: Prevalence of *K. pneumoniae* isolated in relation to age and gender

Characteristics	Number of samples (%)	*K. Pneumonia* (%)
Age (years):		
1 – 30	50 (25.0)	21 (26.25)
31 – 60	93 (46.5)	38 (47.50)
61 and above	57 (28.5)	21 (25.25)
Total	200 (100)	80 (100.0)
Gender:		
Male	100 (50.0)	42 (52.50)
Female	98 (49.0)	38 (49.50)
Missing value	2 (1.0)	0 (0.00)
Total	**200** (100)	**80 (100.0)**

All the *K. pneumoniae* isolates tested were highly resistant to amoxicillin/clavulanic acid 49 (61.25%), aztreonam 46 (57.5%), cefoxitin 47 (58.75%), cefotaxime 65 (81.25%), ceftazidime 55 (68.75%), cefpirome 54 (67.5%) and imipenem 14 (17.5%). *Klebsiella pneumoniae* isolates were highly resistant to the tested

beta-lactam antibiotics used while imipenem, a carbapenem, was the most active antibiotic against the *K. pneumoniae* isolates (Table 4).

Table 4: The antibiotic susceptibility profiles of *K. Pneumonia* isolates and break point recommendations for detecting ESBLs

Antimicrobial agents			Resistant %	Resistant (mm)	Sensitive (mm)
Amoxicillin/Clavunate	AMC	20/10 µg	49 (61.25)	≤13	≥18
Aztreonam	ATM	30 µg	46 (57.5)	≤17	≥21
Cefoxitin	FOX	30 µg	47 (58.75)	≤14	≥18
Cefotaxime	CTX	30 µg	65 (81.25)	≤22	≥26
Ceftazidime	CAZ	30 µg	55 (68.75)	≤17	≥21
Cefotetan	CTT	30 µg	48 (60.0)	≤12	≥16
Cefpirome	CPO	30 µg	54 (67.5)	≤14	≥18
Imipenem	IMP	10 µg	14 (17.5)	≤13	≥16

Twenty five (25) samples were tested using Double disc synergy test. Ten (10) samples were confirmed positive, with prevalence of 40.0% (Table 5).

Table 5: Prevalence of confirmed beta-lactamase producing *K. pneumoniae* (DDST)

Gender	*K. pneumoniae*	
	No Tested	No Positive (%)
Male	17	6 (35.2)
Female	8	4 (50.0
Total	**25**	**10 (40.0)**

Inhibition zone diameter (IZD) for ceftazidime and cefotaxime tested in combination with amoxicillin-clavulanic acid versus its zone when ceftazidime and cefotaxime tested alone confirms ESBL production (Plates 1 and 2).

Detection of *K. pneumoniae* genes by PCR with primers Cefotaximase (CTX-M), Sulfhydryl variant (SHV) and Temoniera (TEM) are recorded in Table 6.

The resultant DNA bands were visualized under ultra violet illumination using image analysis system, and photographed with digital camera to demonstrate TEM and SHV bands of *K. pneumoniae* isolates (Plate 3).

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

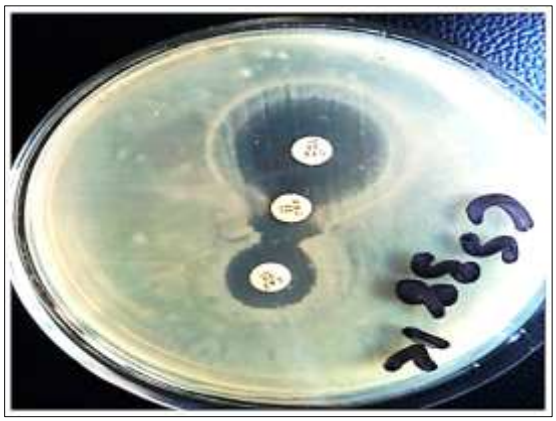

Plate 2: The IZD for ceftazidime and cefotaxime tested alone

Table 6: The PCR detection of *K. pneumoniae* with CTX-M, SHV and TEM genes

Bacteria isolates	*Cefotaximase* CTX-M		*Sulfhydryl variant* SHV		*Temoniera* TEM	
	No. +ve	%	No. +ve	%	No. +ve	%
K. pneumoniae(n = 10)	0	0.0	5	50.0	9	90.0

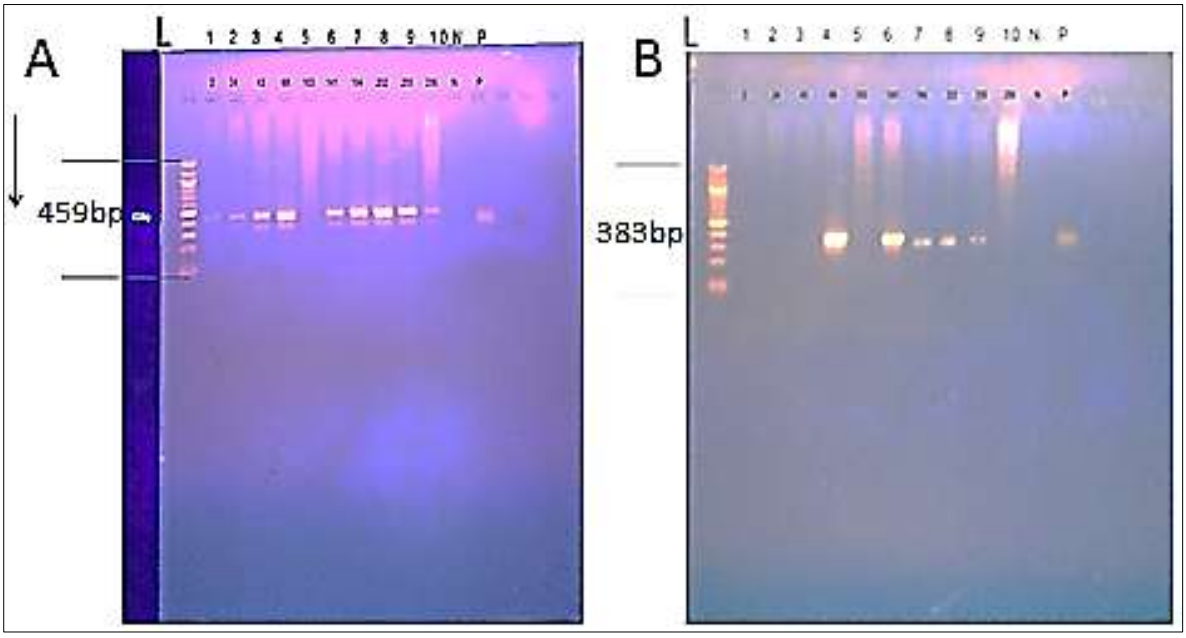

Plate 3: The TEM and SHV bands of *K. pneumoniae* isolates

A: Duplex PCR profiles for bla_{TEM} of *K. pneumoniae* isolates. Lane marked P = positive control and lane marked N= negative control. Lane 5 is negative while other lanes (bands) are positive for bla_{TEM}.

B: Duplex PCR profile for $bla_{SHV\ of}$ *K. pneumoniae* isolates Lane P = positive control, Lane N= negative control. Lanes 4, 6, 7, 8, 9 are positive for bla_{SHV} while lanes 1, 2, 3, 5, 10 are negative

This study showed that a total of 80 (40%) *K. pneumoniae* was isolated from the 200 wound swab samples collected. The result of this work agrees with that of Iroha *et al*. (2017) and correlated positively with the findings of Muhammad *et al*. (2008) who reported that *K. pneumoniae* is the leading causative agent of orthopaedic wound infections at the institution used. This work on the other hand disagrees with the findings of Chukwura *et al*. (2010) as *Pseudomonas aeruginosa*, followed by *Escherichia coli*, and *K. pneumoniae* was the most prevalent bacteria found in wounds at institution used for the research. This was supported also by Japoni *et al*. (2010) who reported that *Pseudomonas aeruginosa* was the most frequent bacteria isolated from wounds.

In striking contrast, Hisham *et al*. (2013) declared in their study that the most predominant of bacteria isolated from burn wound patients were *Staphylococcus aurous*, followed by *Pseudomonas aeruginosa*, and *K. pneumoniae*. Girma *et al*. (2013) reported *Proteus* species as the most prevalent for wound infections which disagrees with this work and that of Chukwura *et al*. (2010).

The results obtained from the distribution of bacteria with respect to age groups across the wound samples collected revealed that the age group 31-60 years harboured more bacteria isolates, followed by age group 61years and above. This study agrees with the work of Nwankwo *et al*. (2015) in Kano. In their study, they reported high prevalence of bacteria isolation among elderly patients, which they attributed to low immunity, and risk factor for wound infections. This did not agree with the work of Iroha *et al*. (2017) as younger patients harboured more bacteria in wounds.

Further analysis showed that males had the higher prevalence of *K. pneumoniae* from orthopaedic wounds (52.5%) while females had a slightly lower prevalence of (47.5%). Occupational involvement might be the cause of the slight increase in population of male over the female.

This work reported results with higher prevalence of wound infections among out-patients (62.5%) as compared to the in-patients (37.5%). In this case of patients' status, where higher bacterial growth was recorded among the wounds of out-patients than the in-patients, this trend of results could be as a result of patients waiting for a long time before seeking medical attention, and as such, leading to heavy growth of bacteria and heavy infections. In this work, there were more out-patients studied than the in-patients and disagreed with the findings from similar work (Chukwura *et al*., 2010; Iroha *et al*., 2017).

That Orthopaedic wounds on the legs had the higher prevalence of bacteria than the hands and chest/neck agrees with the work of Iroha *et al*. (2017). The prevalence of bacterial isolates from type of wounds indicated that accident victims had the highest value, while the least was that of ulcer patients. This work agrees with the work of Iroha *et al*. (2017), in terms of accident cases, but disagrees with the least prevalence which they claim were patients with burn wounds.

This study reported very high resistant frequency of *K. pneumoniae* to panel of second and third generation cephalosporins. This agrees with similar works done in Ghana, West Africa (Iroha *et al*., 2017; Saana *et al*., 2014; Obeng-Nkrumah *et al*., 2013). The present work disagrees with the publication of Muhammad *et al*. (2008) that all infections studied at the same orthopedic hospital were treated with the prophylactic antibiotics of first generation cephalosporins such as cefazoline. In our study, Imipenem had a very good activity against *K. pneumoniae*. This result is similar to that found in India (Prakash and Saxena, 2014), in Karachi (Abdullah *et al*., 2013), that *K. pneumoniae* susceptibility to imipenem was 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. However, the suggestion (Oteo *et al*., 2010) that beta-lactam/lactamases inhibitors combination may be used in treating ESBL producing infections contradicts the current study, because of high resistance in amoxicillin/clavunate with a high probability of therapeutic failure.

In our DDST, 40% were confirmed ESBL-producers but in an earlier work done in Eastern Nigeria, Afiukwa *et al*. (2010) reported 4.4% prevalence of ESBL-producers which fell short of the values obtained in our study. Our work confirmed there is a high prevalence of ESBL producing bacteria in circulation at the orthopaedic hospital where the study was done, and that the ESBL genes most prevalence in the hospital is bla_{TEM} while bla_{SHV} was moderate but bla_{CTX-M} was insignificant. Finding from our molecular identifications agrees with that from a teaching hospital in Ghana (Obeng-Nkrumah *et al.*, 2013) while it disagrees with findings from Lebanon and India where bla_{CTX-M} genes were mostly positive (Chakraborty, *et al*., 2020).

Conclusion

The outcome of this work, gave the most frequently encountered enteric bacteria in orthopaedic wounds as *K. pneumoniae.* A high level of resistance to multiple classes of antibiotics especially the beta-lactams was noted among the *K. pneumoniae* ESBL producers. The ESBL-producing *K. pneumoniae* in orthopaedic wounds was highly resistant to ceftazidime, cefotaxime, cefpirome, cefotetan, cefoxitin, aztreonam, and amoxicillin/ clavulanic acid. Also, a high level of confirmed ESBL producers

existed among the *K. pneumoniae isolates*. Imipenem is still very active against *K. pneumoniae* and could be a better option for the treatment of infected orthopaedic wounds. Since all three ESBL genes $bla_{TEM,}$ bla_{SHV}, and bla_{CTX-M} in *K. pneumoniae* tested were demonstrated in this study, strict infection control measures, and judicious use of antimicrobials are highly recommended to lower the frequency of ESBL-producing *K. pneumoniae*.

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