INTRODUCTION

Urinary Tract Infections (UTIs) are a serious public health issue, particularly in the developing world where there is a high level of poverty, ignorance and poor hygienic practices [1]. UTIs are the most prevalent bacterial infections in humans with Gram-negative pathogens most especially E. coli, which is regarded as the most important cause of nosocomial infections [2], both in adults as well as pediatric groups [3].

Wagenlehner and Naber [4] (2006) further noted that there are two important aims in the antimicrobial treatment of UTIs; an effective rapid response to therapy and prevention of recurrence of the patient treated and prevention of emergence of resistance to antimicrobial chemotherapy.

Since antibiotics have been introduced into clinical medicine, antibiotic-resistant bacteria have evolved. In 2016, the World Health Organization officially stated that “antimicrobial resistance is a global and societal challenge and threat”. The constant increase of simultaneous resistance to various classes of antibiotics significantly reduces the possibility of treatment of infections caused by ESBL producers [5-7] have also reported that the management of UTIs has become increasingly challenging due to the production of ESBLs. They tend to be a worrying global public health issue due to their associated higher morbidity and mortality. Hence, they represent a clear and present higher danger to public health [8].

The production of ESBLs is one of the most prevalent resistance mechanisms in Gram-negative bacilli. ESBLs are enzymes whose rates of hydrolysis of the extended-spectrum beta-lactam antibiotics such as ceftazidime, cefotaxime, or aztreonam are >10% than that for benzylpenicillin [9]. ESBLs are predominantly described in K. pneumoniae and E. coli, but recently the enzymes were found in other genera of Enterobacteriaceae family [6, 10].

Initially, the Temoneira (TEM) and sulphydryl variants (SHV) were recognized as the main ESBLs, but in recent times, the cefotaxime-Munich (CTX-M) become more prominent and considered the most prevalent beta-lactamases found in clinical isolates of E. coli globally [11]. Currently, the three major ESBL types are TEM, SHV, and CTX-M [9].

There are reported cases of CTX-M producing uropathogens isolates [12], and in orthopedic patients [13] from Nigeria. The CTX-M enzymes are known as an increasingly serious public health concern worldwide and have been noted to be the cause of outbreaks throughout the world [14]. These CTX-M genes are usually present in large plasmids that also carry additional resistance genes, but have been found on plasmids ranging in size from 7 to 430 kb [9].

The ongoing global spread and increased prevalence of CTX-M-type ESBL in Enterobacteriaceae is of great concern [15]. Due to the explosive dissemination of CTX-M around the world and increasing description worldwide, Canton et al. [16] have referred to it as the “CTX-M pandemic”.

The study evaluates the resistance of the ESBL producing Gram-negative Enterobacteriaceae to commonly prescribed antibiotics and investigates the prevalence of CTX-M genes from these isolates using PCR.

MATERIALS AND METHODS

Approval to carry out this study was obtained from the ethics committee of the Specialist Hospital Sokoto (SHS) with approval number ESHS/239839. The sample collection was based on
Identification of bacterial isolates
Using a sterile Pasteur pipette, 3-microwell strip (Microgen Identification Kit) and was mixed thoroughly.
sub-culturing on nutrient agar (NA).

Screening for ESBL production
Antibiotic susceptibility testing (AST)
microwell strips were read after 18 -24 h incubation for bacterial suspension was added to each well of the strip(s). The GN A
a calibrated wire loop and incubated under aerobic conditions for 18-
inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar using all aseptic c onditions to avoid contamination. Urine samples were
patients were counseled on how to collect urine samples by observing patients in sterile disposable containers. Before urine collection,
morning mid -stream clean catch urine samples were collected by
Enterobacteriaceae samples were collected over four (4) months from patients. Early
µg). Results were interpreted according to CLSI guidelines [17].

Phenotypic confirmatory test for ESBL production
A suspension of each test organism was prepared in freshly prepared 0.9% normal saline to give an inoculum equivalent to a 0.5 McFarland. The test organisms were inoculated on the surface of MHA plates. Confirmation of the ESBL phenotype was performed by DDST using antibiotic discs containing two cephalosporins and amoxicillin/clavulanate. The discs were ceftazidime, CAZ (30 µg), AMC (30 µg) and cefotaxime, CTX (30 µg). A sterile needle was used to place the CTX and CAZ on the agar at a distance of 20 mm center to center from a combination disc of AMC. The plates were incubated at 37 °C and were examined for an extension of the edge of the zone of inhibition of antibiotic discs towards the disc containing AMC. It is interpreted as synergy and considered positive or the presence of ESBL.

Bacteria cell preparation
Luria and Bertani (LB) broth was used, which is a rich medium that is commonly used to culture members of the Enterobacteriaceae or as a general-purpose bacterial culture medium for a variety of facultative organisms. Single colonies were picked from freshly streaked isolates on NA and inoculated into 5 ml LB broth medium and incubated overnight at 37 °C. Bacterial cells were then harvested by centrifugation at 4 °C, 16,000 rpm in a refrigerated micro-centrifuge for 30 seconds at 37 °C in an Eppendorf’s tube. The supernatant was then decanted and cells harvested.

Plasmid extraction
Plasmid extraction was carried out using ZymoPURE™ Miniprep Kit according to the manufacturer’s instructions. To ascertain that plasmids were extracted, the extracted plasmids were subjected to agarose gel electrophoresis.

Amplication of CTX-M genes
Amplification of plasmid DNA fragments was carried out usingDream Taq™DNA polymerase (enhanced Taq DNA Polymerase optimized for high throughput PCR applications). Dream Taq™PCR master mix (2X) was vortexed and centrifuged for 30 seconds at 8,000 rpm. The thin-walled PCR tube was then placed on an ice pack and the following components were added for each isolate for the single reaction of 10 µl: 0.2 µl of Dream Taq™ PCR master mix was added in the PCR tube. Then dNTP Mix 2 mmol each 0.5 µl were added, forward primers; CTX-M 0.5 µl of the forward primers were calculated and added. Then 0.5 µl CTX-M of the reverse primers were calculated and added. A 3.0 µl of template DNA (plasmid DNA), 10X PCR Taq buffer 1 µl were added. The nuclease-free water was added in the PCR tube to make up a total volume of 10 µl. The samples were vortexed gently and spin down. For 15 isolates, the forward and reverse primers, Taq buffer, dNTP, Taq polymerase, water, and template were multiplied by 15, making a total sum of 150 µl Dream Taq™ PCR master mix (2X) i.e. for forwarding 7.5 µl CTX-M, and reverse primer: 7.5 µl CTX-M.

Statistical analysis
Data were analyzed using Microsoft Excel.

RESULTS
Three hundred and sixty-five (365) urine samples were analyzed over 4 mo period. After the investigations, the results showed that the percentage of Gram-negative isolates was 16.7%. The prevalence of isolates in females was 42 (68.8%) and in males 19 (31.2%). The Gram-negative isolates consist mainly of E. coli (31.1%), Salmonella arizonae 24.6%, Klebsiella oxytoca 4.9%, Klebsiella pneumoniae 11.5%, Enterobacter gergoviae 98%, Citrobacter freundii 6.6%, Serratia marcescens 6.6%, and 1.6% were Enterobacter aerogenes, Proteus mirabilis, and Edwardsiella tarda each. The percentage distribution of the Gram-negative isolates is shown in fig. 1.
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Annealing °C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M</td>
<td>909</td>
<td>F: TCTTCAGAATAGGAATGCC</td>
<td>65</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCGTTTCCGCATACAAAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Thermal cycling conditions for PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>94</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>15 seconds</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Final</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Antimicrobial susceptibility testing

The Gram-negative uropathogens were generally resistant to cotrimoxazole (73.3%), nalidixic acid (66.7%), norfloxacin (53.5%), ciprofloxacin (50.5%), and gentamicin (48.6%). The percentage susceptibility of the Gram-negative uropathogens is shown in fig. 2.

Identification and screening for ESBL production in gram-negative uropathogens

The result of the identification and screening for ESBL production in Gram-negative uropathogens found that out of the 61 Gram-negative isolates, (18) 29.5% were potential ESBL producers, while 70.5% were not ESBL producers.

Phenotypic confirmation of ESBL production in gram-negative uropathogens using DDST

Upon analyzing the 18 isolates for phenotypic ESBL confirmation using DDST, 15 (83.3%) were confirmed ESBL producers.

Amplification of CTX-M on gel electrophoresis

The plasmid DNA PCR of the CTX-M gene on ESBL isolates revealed the position of the amplification products, which were estimated with the position of the molecular weight marker as shown in fig. 3. Eleven (11) of the 15 isolates were found in the region of the expected amplicon of 909 bp.

![Fig. 1: Percentage distribution of gram-negative isolates from urine](image1)

![Fig. 2: Percentage susceptibility of gram-negative uropathogens to commonly prescribed antibiotics](image2)
The study revealed a high susceptibility to nitrofurantoin by the urinary tract pathogens in the Sokoto metropolis. Hence the need for empirical treatment, the occurrence of ESBL positive strains expressing MDR to antibiotics has remained the dominant problem in the therapy of infections caused by Gram-negative bacilli [6, 19]. The overall frequency of ESBL producing Enterobacteriaceae among urinary tract pathogens in this study was 83.3%. However, in comparison to our study, a combination of low and high isolation rate was recorded in many studies: 44% in Saudi Arabia [20], 66.7% in India [21], 67.9% in Portugal [22], and 84% in Turkey [23].

Our study showed isolates were resistant to the commonly used antibiotics such as cotrimoxazole (73.3%), nalidixic acid (66.7%), norfloxacin (53.3%), ciprofloxacin (50.5%), and a lesser rate in gentamicin (48.6%) and amoxicillin/clavulanate (45.7%) with least in nitrofurantoin (31.4%). A similar data is a comparison with a study in India, in which isolates showed high resistance to ampicillin, cephalosporins, quinolones, and cotrimoxazole, but comparatively less resistance to gentamicin, levofloxacin, nitrofurantoin, netilmicin and imipenem [24, 28].

CTX-Mbeta-lactamases are often observed in clinical isolates of E. coli and K. pneumoniae [9]. This is in line with our study, as out of the 15 isolates, eleven harbor CTX-Mbeta-lactamases, with E. coli (33.3%), Enterobacter aerogenes (13.3%), while Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, and Citrobacter freundii with 6.7% each. Rezaei et al., [25] from Iran observed low prevalence of CTX-M (28%) and [26] reported a 28.8% prevalence of CTX-M in another study, CTX-M (5.1%) gene was responsible for ESBL production [14]. The data are however lower to our study (73.3%). A similar prevalence to our study was recorded by [27], in which CTX-M (74%) enzymes were the most common ESBL types.

Our study demonstrates an increasing trend in the emergence of ESBL in community-acquired UTI. Therefore, it is of great concern that the Gram-negative uropathogens carrying CTX-M are widespread in the Sokoto metropolis. Hence the need for antimicrobial stewardship and guidance for the management of these complex MDR infections can never be overemphasized.

CONCLUSION

The study revealed a high susceptibility to nitrofurantoin by the Gram-negative uropathogens, whereas susceptibility to cotrimoxazole to these isolates was lowest. It further portrays a high prevalence of Enterobacteriaceae isolates harboring CTX-M genes; thus a demonstration of the emergence of ESBL in community-acquired UTI in the study area.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

Busayo Olalekan Olayinka, Nuhu Tanko and Rebecca Olajumoke Bolaji conceptualized the study, while the methodology was carried out by Busayo Olalekan Olayinka and Nuhu Tanko. Formal analysis and investigation were done by Nuhu Tanko and Rebecca Olajumoke Bolaji. Writing of the original draft was prepared by Nuhu Tanko and Busayo Olalekan Olayinka. Review and editing were done by Eugene Ong Boon Beng. The study was supervised by Busayo Olalekan Olayinka, Rebecca Olajumoke Bolaji and Eugene Ong Boon Beng.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES