Objective: Infections of the urinary tract remains one of the most common bacterial infections with many implicated organisms being Gram-negative, which are increasingly resistant to antimicrobial agents. The aim of the study was to evaluate the resistance of ESBL producing Gram-negative enterobacteriaceae to commonly prescribed antibiotics and the prevalence of CTX-M genes from these isolates using polymerase chain reaction (PCR).

Methods: The isolates were collected from urine over a period of 4 mo and studied, and were identified using Microgen Identification Kit (GN-ID). Susceptibility testing was performed by the modified Kirby Bauer disc diffusion method, and results were interpreted according to Clinical and Laboratory Standard Institute (CLSI). Extended-Spectrum Beta-Lactamase (ESBL) production was detected by the double-disc synergy test (DDST). Molecular characterization was based on the isolates that were positive for the phenotypic detection of ESBL.

Results: Sixty one (61) isolates of Gram-negative uropathogens were identified. Of these, 19 (31.2%) were E. coli, 15 (24.6%) were Salmonella arizonae, Klebsiella pneumoniae were 7 (11.5%), Klebsiella oxytoca were 3 (4.9%), Enterobacter gergoviae were 6 (9.8%), 4 (6.6%) were Citrobacter freundii, 4 (6.6%) were Serratia marcescense, and 1 (1.6%) were Enterobacter aerogenes, Proteus mirabilis, and Edwardsiella tarda each. Analysis of the bacterial susceptibility to antibiotics revealed most of them to be generally resistant to cotrimoxazole (73.3%), nalidixic acid (66.7%), nitrofurantoin (30%). Of the 15 ESBL producers, 11 (73.3%) were harbouring CTX-M genes.

Conclusion: The study revealed a high susceptibility to nitrofurantoin, whereas susceptibility to cotrimoxazole was lowest. It further portrays a high prevalence of enterobacteriaceae isolates harbouring bla CTX-M genes in Sokoto metropolis.

Keywords: Resistance, Prevalence, Gram-negative, Urinary Tract Infections, CTX-M

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 "抗微生物 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 sulphhydryl 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
identified. The study was carried out in the Pharmaceutical Microbiology Laboratory of Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Usman Danfodiyo University, Sokoto.

**Chemicals and reagents**
The culture media and antibiotic discs used in this study were all sourced from Oxoid, UK and prepared as per the manufacturer’s instruction. Identification kit used is sourced from Microgen ID Kit, GN-ID, UK, while the plasmid extraction kit is from ZymoPURE™, and primers were sourced from Inqaba Biotech, South Africa.

**Inclusion criteria**
Included in the study were urine samples from outpatients with UTIs with age group ≥ 18 y. A UTI in this study was defined as a positive urine culture ≥ 10⁵ colony-forming unit/ml (cfu/ml) of pure bacterial growth.

**Collection of clinical isolates**
A total of three hundred and sixty-five (365) non-repetitive urine samples were collected over four (4) months from patients. Early morning mid-stream clean catch urine samples were collected by patients in sterile disposable containers. Before urine collection, patients were counseled on how to collect urine samples by observing all aseptic conditions to avoid contamination. Urine samples were inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar using a calibrated loop and incubated under aerobic conditions for 18-24 h at 37 °C. Pure cultures of the individual isolates were obtained by sub-culturing on nutrient agar (NA).

**Identification of bacterial isolates**
An 18-24 hour pure culture of the bacterial isolate to be identified was used. Oxidase test was carried out on the isolate before strip inoculation. Only oxidase negative isolates were considered. A loopful was emulsified from an 18-24 hour culture in 3 ml sterile 0.9 % saline for the GN A microwell strip (Microgen Identification Kit) and was mixed thoroughly. Using a sterile Pasteur pipette, 3-4 drops (approximately 100µl) of the bacterial suspension was added to each well of the strip(s). The GN A microwell strips were read after 18-24 h incubation for Enterobacteriaceae according to manufacturer’s instruction.

**Antibiotic susceptibility testing (AST)**
The susceptibility of the isolates to seven (7) commonly prescribed antibiotics was determined by the modified Kirby Bauer disc agar diffusion on Mueller Hinton agar (MHA). The antibiotic used were ciprofloxacin (CIP, 5 µg), norfloxacin (NOR, 10 µg), gentamicin (CN, 30 µg), nalidixic acid (NA, 30 µg), cefoxitin (CTX-M, 25 µg), nitrofurantoin (F, 300 µg), and amoxicillin/clavulanic acid (AMC, 30 µg). Results were interpreted according to CLSI guidelines [17].

**Screening for ESBL production**
Multidrug-resistant (MDR) isolates were further screened for ESBL production. ESBL screening was performed by disc diffusion using cefotaxime (CTX, 30µg) and ceftazidime (CAZ, 30µg) on MHA for the phenotypic confirmatory test. The test organisms were inoculated on the surface of MHA plates. The zones in mm shown below for respective antibiotics indicate the susceptibility of the isolates to ESBL production. A zone of ≥ 15 mm in diameter is considered as phenotypic positive for ESBL production. The sensitivity of the isolates to seven (7) commonly prescribed antibiotics was determined by the modified Kirby Bauer disc agar diffusion on Mueller Hinton agar (MHA). The antibiotic used were ciprofloxacin (CIP, 5 µg), norfloxacin (NOR, 10 µg), gentamicin (CN, 30 µg), nalidixic acid (NA, 30 µg), cefoxitin (CTX-M, 25 µg), nitrofurantoin (F, 300 µg), and amoxicillin/clavulanic acid (AMC, 30 µg). Results were interpreted according to CLSI guidelines [17].

**Statistical analysis**
Data were analyzed using Microsoft Excel.

**RESULTS**
Three hundred and sixty-five (365) urine samples were analyzed over a 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 Enterobacteriaceae with K. pneumoniae 11.5%, Enterobacter gergoviae 9.8%, 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

| Gene | Size (bp) | Nucleotide sequence (5’-3’) | Annealing T °C | Reference |
|------|-----------|-----------------------------|----------------|-----------|
| CTX-M | 909 | F: TCTTCAGAAATAAGGATCCG<br>R: CCGTTCCCGATTTACAAAC | 65 | [18] |

Table 2: Thermal cycling conditions for PCR

| Step             | Temperature °C | Time           | No. of cycles |
|------------------|----------------|----------------|---------------|
| Initial denaturation | 94             | 3 min          | 1             |
| Denaturation      | 94             | 15 seconds     | 30            |
| Annealing         | 65             | 30 seconds     | 1             |
| Extension         | 72             | 30 seconds     | 1             |
| Final extension   | 72             | 5 min          | 1             |
| Final hold        | 10             |                |               |

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

Fig. 2: Percentage susceptibility of gram-negative uropathogens to commonly prescribed antibiotics CIP = Ciprofloxacin, NOR = Norfloxacin, CN = Gentamicin, NA = Nalidixic acid, SXT = Co-trimoxazole, F = Nitrofurantoin, AMC = Amoxicillin/clavulanic acid
DISCUSSION

Although UTIs caused by ESBL producing Gram-negative Enterobacteriaceae are a cause of concern due to clinical failure of 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 gentamycin (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-M beta-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-M beta-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.

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AUTHORS CONTRIBUTIONS

Busayo Olalekan Olayinka, Nuhu Tanko and Rebecca Olaajumoke 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 Olaajumoke Bolaji and Eugene Ong Boon Beng.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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