ORIGINAL PAPER

Nig. J. Pharm. Res. 2015, 1, pp. 59-65
ISSN 0189-8434

Prevalence And Antimicrobial Susceptibility Of Extended Spectrum Beta-Lactamase (Esbl) Producing Gram-Negative Uropathogens In Sokoto, Nigeria

*T. Nuhu[1B-D], R. O. Bolaji[2E] and B. O. Olayinka[2A,F]

[1]Department of Pharmaceutics and Pharmaceutical Microbiology, UsmanuDanfodiyo University, Sokoto.
[2]Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria.

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Extended Spectrum Beta-Lactamases (ESBLs) producing Gram-negative uropathogens is now a major source of concern worldwide.
Objectives: The study was conducted to determine the prevalence and susceptibility to antimicrobial of ESBL-producing Gram-negative uropathogens in Sokoto, Nigeria.
Materials and Methods: A total number of Three Hundred and Sixty Five (365) urine samples were analyzed between November, 2014 and February, 2015. Antimicrobial susceptibility testing was determined using the modified Kirby Bauer method. Confirmation of ESBL phenotype was performed by Double-Disc Synergy Test (DDST) method.
Results: Gram-negative uropathogens constitute 60.9% out of the 105 positive cultures. Male patients were 54.7%, while females were 45.3%. Age group 19-40 constitutes 54.7%, while age group 41-60 was 32.8%, and 61 years and above accounted for 12.5%. The isolates were highly resistant to Cotrimoxazole (71.9%), but highly susceptible to Nitrofurantoin (70.3%). Out of the 64 Gram-negative uropathogens *E.coli* constitute 29.7%, followed by *Salmonella arizonae* (23.4%), *Klebsiella oxytoca* (10.9%), *Enterobacter gergoviae* (9.3%), *Serratia marscense* (6.3%), and *Citrobacter freundii* (6.3%). *Klebsiella pneumoniae* (4.7%). Others account for 1.6% each (*Enterobacter aerogenes, Proteus mirabilis, Pseudomonas aeroginosa, Edwardsiella tarda, Burkholderia pseudomallei* and *Acinetobacter iwoffii*). Fifteen (15) (83.3%) were phenotypically confirmed using the Double-Disc Synergy Test (DDST) as ESBL producers of which *E.coli* account for 26.7%, *Enterobacter gergoviae* (20%), *Enterobacter aerogenes* (6.7%) *Klebsiella oxytoca* (6.7%), *Klebsiella pneumoniae* (6.7%), *Citrobacter freundii* (13.3%), while *Serratia marscense, Edwardsiella tarda, Acinetobacter iwoffii* accounted for (6.7%) each.
Conclusions: Our findings document the presence of ESBL-producing Gram-negative uropathogens in Sokoto. *E.coli* and *Enterobacter gergoviae* were the predominant ESBL producers. Nitrofurantoin remains active in the majority of the isolates.

Keywords: ESBLs, Gram-negative uropathogens, Antimicrobial susceptibility.

INTRODUCTION

Urinary tract infections (UTIs) is one of the most common infections encountered in the human population, with about 150 million estimated per year worldwide (Gupta, 2001). Lamido *et al*., (2010) stated that UTIs accounts for a significant part of the work load in clinical microbiology laboratories and enteric bacteria (in particular, *Escherichia coli*) remained the most frequent cause of UTI. Getenet and Wondewosen, 2011, stated that UTI that occur in a normal genitourinary tract with no prior instrumentation are considered uncomplicated, whereas complicated infections have structural and functional abnormalities, including instrumentation such as indwelling catheters. Hooton, 2012 classify episodes of acute cystitis and pyelonephritis occurring in healthy premenopausal, non-pregnant women with no history suggestive of an abnormal urinary tract as uncomplicated, whereas all others are classified as complicated. Although the distribution of pathogens that cause UTI is changing.

Hawkey, (2008) stated that since the introduction of antibiotics in the 1950s, there has been a steady and significant increase in the number of resistant strains of

bacteria. Antibiotic resistance is a specific type of drug resistance in which a microorganism has the ability of withstanding the killing or inhibitory effects of antibiotics (Al-Jebouri and Mdish, 2013).

Antimicrobial resistance among pathogenic bacteria is increasing worldwide especially against beta-lactam drugs, due to the production of beta-lactamase enzymes (NNIS, 2003), which destroy the beta-lactam ring of these antibiotics, thus preventing the actions of Penicillin Binding Proteins (PBPs). Beta-lactams are a group of antibiotics acting on the cell wall of a bacterial cell. These include the penicillins, cephalosporins, carbapenems and monobactams. The beta-lactam antibiotics like penicillin have a beta-lactam ring which can be hydrolyzed by beta-lactamases resulting in ineffective compound. These beta-lactamases are now capable of hydrolyzing not only penicillins but also cephalosporins, monobactams and carbapenems, hence these are called extended spectrum beta-lactamases (ESBLs) (Bush, 2001).

A commonly used working definition is that, ESBLs are beta-lactamases capable of conferring bacterial resistance to the penicillins; first-, second- and third-generation cephalosporins; and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by beta-lactamase (β-lactamase) inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). They have been detected in other Gram-negative Bacilli such as *Proteus* spp., *Salmonella* spp., *Pseudomonas aeruginosa* and other *Enterobacteriaceae* (Vendana and Honnavar, 2009).

In addition, ESBL-producing organisms exhibit co-resistance to many other classes of antibiotics, resulting in limitation of therapeutic option (Alipourfard and Nilufar, 2010). Alipourfard and Nilufar, (2010) also stated that patients suffering from infections caused by ESBL-producing organism are at risk of treatment failure if extended spectrum cephalosporins are prescribed.

ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Paterson, 2000).

There are several groups of ESBLs with similar behavior but different evolutionary histories. The largest groups are the mutants of TEM and SHV beta-lactamases, with over 150 members. The second largest group of ESBLs is the CTX-M enzymes.

This study was carried to prospectively determine the prevalence of ESBL in Gram-negative producing uropathogen at Specialist Hospital Sokoto. It was further aimed to know about the type of Gram-negative uropathogens responsible for UTIs and their antibiotic susceptibility patterns.

METHODS

Study Population

Three hundred and sixty five (365) early morning mid-stream urine were collected from patient aged ≥ 18 years presenting to the hospital with dysuria, frequency or urgency of less than seven (7) days urination, with or without fever or flank pain who volunteered to be included in the study were considered after getting approval from Specialist Hospital Sokoto Research Ethic Committee. The sampling was carried out between November, 2014 and February, 2015.

For this study, patient's demographic data were recorded. Patients on admission and with urinary tract instrumentation were excluded. Isolate for which it was impossible to discriminate between contamination and infection were excluded from the analysis.

All urine samples yielding growth of Gram-negative *Enterobacteriaceae*were selected. Only one positive culture per patient was included in the study.

Media Preparation

The media used in this work include Cysteine Lactose Electrolyte Deficient (CLED) Agar, Nutrient Agar (NA), and Mueller Hinton Agar (MHA), all sourced from Oxoid, UK. The media were prepared based on manufacturer's instruction and sterilized by autoclaving for 15 minutes at 121℃.

Sample Collection

Early morning mid-stream clean catch urine samples were collected using sterile disposable containers with screw caps. Prior to urine collection, patient were counseled on how to collect urine sample by observing all aseptic conditions to avoid contamination.

Culturing and Isolation of Bacteria from Urine Samples

Sterile petri-dishes containing 20ml prepared CLED Agar was allowed to set and their surfaces dried in an incubator at 40℃ for 5 minutes. Urine samples were cultured on CLED Agar using streak plate method and allowed to stay for 30 minutes and incubated in aerobic condition for 18-24 hours at 37℃. Cultures without any colony at the end of 18-24 hours incubation were discarded.

Characterization of Isolates

Isolates were sub-cultured unto Nutrient Agar plates and incubated at 37℃ for 18-24 hours. Isolates from pure culture were characterized by:

a. Gram stain: according to Cheesbrough (2006), a thin smear of each culture was made, air dried and heat fixed. Each slide was flooded with crystal violet and allowed to stay for 60 seconds before washing with water. Gram's iodine was added to each slide and was allowed to remain for 60 seconds and washed with water. Each slide was slightly tilted and ethyl alcohol was gently used to cover the smear on the slide so that it runs off the edge of the slide and was rinsed with water. The slides were counter-stained with safranin for 30

seconds and then rinsed with water. The slides were air-dried and viewed using a microscope.

b. Identification of bacterial isolates: bacterial isolates were identified using a commercial identification kit of Microgen GN-ID systems (Microgen Bioproducts Limited, UK). Suspension of the organisms were made in a sterile 0.9 % ($^{w}/_{v}$) sodium chloride and mixed thoroughly until the turbidity was equivalent to 0.5 McFarland according to the manual that accompanied the kit. The results were measured using the Microgen Identification System Software (MID-60).

Antibiotic Sensitivity Testing

Antibiotic Sensitivity Testing of the isolates was determined using the modified Kirby Baeur method. The discs (Oxoid, UK) are Ciprofloxacin (CIP, 5 µg), Norfloxacin (NOR, 10 µg), Gentamicin (CN, 30 µg), Nalidixic acid (NA, 30 µg), Cotrimoxazole (SXT, 25 µg), Nitrofurantoin (F, 300 µg), and Amoxicillin/Clavulanic acid (AMC, 30 µg).

The diameter of the zones of inhibition produced by each antibiotic on the discs were measured using a ruler, and the result recorded in milliliters and interpreted as resistant, intermediate or sensitive to the antibiotic agent used. Intermediate and resistant strains were categorized together as resistant. Results were interpreted according to the CLSI (2012) interpretative chart.

ESBL producing isolates by phenotypic method

Gram-negative bacilliisolates were sreened for ESBL production, and then phenotypic confirmatory test using CLSI guidelines 2012. ESBL screening was performed by disc diffusion using Cefpodoxime (CPD, 10 µg), Cefotaxime (CTX, 30 µg) and Ceftazidime (CAZ, 30 µg). The tests were interpreted according to CLSI guidelines (2012).

The zones shown below for respective antibiotic indicate potential ESBL producer.

Cefopodoxime (CPD) ≤ 17

Cefotaxime (CTX) ≤ 27

Ceftazidime (CAZ) ≤ 22

If any isolate is suspected as ESBL producer, phenotypic confirmatory test will be carried out.

Confirmation of ESBL phenotype was performed by Double Disc Synergy Test (DDST) method using antibiotic discs containing two cephalosporins and Amoxicillin/Clavulanic acid. The discs (Oxoid, UK) were CAZ 30 µg, AMC 30 µg and CTX 30 µg. Using a sterile needle, Cefotaxime (CTX, 30 µg) and Ceftazidime (CAZ, 30 µg) were placed on the agar at a distance of 20 mm center to center from a combination disc of Amoxicillin/Clavulanic acid (AMC, 20:10 µg). The plates were incubated at 37℃ and were examined for an extension of the edge of zone of inhibition of antibiotic discs toward the disc containing AMC 30 µg. This is interpreted as synergy and considered positive or presence of an ESBL.

Statistical Analysis

Data were analyzed using Microsoft Excel 2010 version. Discrete values were expressed as percentages. Descriptive statistics were used to summarize patient characteristics and the prevalence of antimicrobial resistance.

RESULTS

A total number of 365 urine samples were collected for this study. There are 105 positive cultures. Gram-negative uropathogens account for 64/105 (60.9 %), while 41/105 (39.1 %) for Gram-positive.

The average age was 39.8 years old and patient's age ranged from 19 to 73 years old. Among the patients with Gram-negative uropathogens, male patients were predominant 35/64 (54.7%) while isolates from females were 29/64 (45.3 %). Female to male ratio is 1:1.21. patients' age distribution due to isolated Gram-negative pathogens showed that age group 19-40 had 35/64 (54.7 %), while age group 41-60 had 21/64 (32.8 %) and age 61 years and above accounted for 8/64 (12.5 %) of the isolates as presented in Table 1.

High rate of resistance was recorded against Cotrimoxazole (71.9%), followed by Nalidixic acid (67.2%), then Ciprofloxacin (54.7%), Norfloxacin (53.1%), Gentamicin (50%), Amoxicillin/Clavulanic acid (48.4%), and Nitrofurantoin (29.7). High sensitivity was recorded against Nitrofurantoin (70.3%), followed by Amoxicillin/Clavulanic acid (51.6%). For Gentamicin, 50% of the isolates were sensitive, 46.9% to Norfloxacin, 45.3% to Ciprofloxacin and 32.8% to Nalidixic acid. Lowest sensitivity was shown towards Cotrimoxazole by only 28.1% of the isolates (Table 2).

Rate of resistance to the majority of tested antibiotics varied from 29.7% to 71.9%, whereas rate of sensitivity varied from 28.1% to 70.3% (Table 2).Analysis of the result according to percentage of UTI isolate among the Gram-negative uropathogen, revealed that *E. coli* was the highest with 19/64 (29.7%), followed by *Salmonella arizonae* with 15/64 (23.4%), then *Klebsiella* spp (*Klebsiella oxytoca* (10.9%) and *Klebsiella pneumonia* (4.7%)) with 10/64 (15.6%). *Enterobacter* spp (*Enterobacter gergoviae* (9.3%) and *Enterobacter aerogenes* (1.6%)) account for 7/64 (10.9%), whereas *Serratia marscense* and *Citrobacter freundii*account for 4/64 (6.3%) each. Others accounted for 1/64 (1.6%) each and include *Proteus mirabilis, Pseudomonas aeroginosa, Edwardsiella tarda, Burkholderia pseudomallei* and *Acinetobacter iwoffii* as shown in Table 3.

From the results of phenotypic screening for ESBL, all Gram-negative isolates were screened, and 18/64 (28.1%) were potential ESBL producers as depicted in Table 3. The 18 isolates displayed Multi-Drug Resistance

(MDR), which is resistance to three or more classes of antibiotics. Upon analyzing the 18 isolates for phenotypic ESBL confirmation using the Double-Disc Synergy Test (DDST), 15/18 was confirmed ESBL producers. Frequency of ESBL production among Gram-negative organisms revealed that E. coli accounted for 4/15 (26.7%), Enterobacter spp 4/15 (26.7%), Klebsiella spp 2/15 (13.3%), Citrobacter freundii 2/15 (13.3%), while Serratia marscense, Edwardsiella tarda, and Acinetobacter iwoffi accounted for 1/15 (6.7%) each as presented in Table 4.

Table 1: Patients' age distribution due to Gram-negative uropathogens

Age Group	**E.C**	**S.A**	**K.S**	**E.S**	**C.F**	**S.M**	**E.T**	**P.M**	**Ps.A**	**A.I**	**B.P**
19-40	12.5	12.5	14.1	3.1	4.7	3.1	1.6	0	0	1.6	1.6
41-60	14.1	6.4	0	4.7	1.6	3.1	0	1.6	1.6	0	0
≥ 61	3.1	4.7	1.6	3.1	0	0	0	0	0	0	0
	29.7	**23.5**	**15.7**	**10.9**	**6.3**	**6.2**	**1.6**	**1.6**	**1.6**	**1.6**	**1.6**

E.C – *E. coli*, **S.A** – *Salmonella arizonae*, **K.S** – *Klebsiella* spp, **E.S** – *Enterobacter* spp, **C.F** – *Citrobacter freundii*, **S.M** – *Serratia marscense*, **E.T** – *Edwardsiella tarda*, **P.M** – *Proteus mirabilis*, **Ps.A** – *Pseudomonas aeruginosa*, **A.I** – *Acinetobacter iwoffii*, **B.P**– *Burkholderia pseudomallei*

Table 2: Summary of results of resistance/sensitivity profile to antimicrobial agents among Gram-negative bacilli

Antimicrobial agents	Percentage of Isolates	
	Susceptibility (%)	Resistant (%)
Ciprofloxacin	54.7	45.3
Norfloxacin	53.1	46.9
Gentamicin	50	50
Nalidixic acid	32.8	67.2
Cotrimoxazole	28.1	71.9
Nitrofurantoin	70.3	29.7
Amoxicillin/Clavulanic acid	51.6	48.4

Table 3: Common Gram-negative uropathogens and screening for ESBL production in Gram-negative bacilli

Organism isolated	Number (%)	Potential ESBL producers (%)	ESBL negative (%)
E. coli	19 (29.7)	6 (33.3)	13 (28.3)
Salmonella arizonae	15 (23.4)	0 (0.0)	15 (32.6)
Klebsiella spp	10 (15.6)	2 (11.1)	8 (17.4)
Enterobacter spp	7 (10.9)	4 (22.2)	3 (6.5)
Serratia marscense	4 (6.3)	1 (5.6)	3 (6.5)
Citrobacter freundii	4 (6.3)	3 (16.7)	1 (2.2)
Proteus mirabilis	1 (1.6)	0 (0.0)	1 (2.2)
Pseudomonas aeroginosa	1 (1.6)	0 (0.0)	1 (2.2)
Edwardsiella tarda	1 (1.6)	1 (6.7)	0 (0.0)
Burkholderia pseudomallei	1 (1.6)	0 (0.0)	1 (2.2)
Acinetobacter iwoffii	1 (1.6)	1 (6.7)	0 (0.0)
Total	**64**	**18**	**46**

Table 4: Phenotypic confirmation for ESBL production in Gram-negative bacilli using DDST

Isolate	Confirmed ESBL positive	Confirmed ESBL negative	Total
E. coli	4 (26.7%)	2	6
Enterobacter spp	4 (26.7%)	0	4
Klebsiella spp	2 (13.3%)	0	2
Citrobacter freundii	2 (13.3%)	1	3
Serratia marscense	1 (6.7%)	0	1
Edwardsiella tarda	1 (6.7%)	0	1
Acinetobacter iwoffi	1 (6.7%)	0	1

Antimicrobial drug resistance is a threat to the general population. This is due to very limited treatment options available for these pathogens. The widespread and inappropriate use of antibiotics is recognized as a significant contributing factor to the spread of bacterial resistance and the development of resistance to antimicrobial agents (Mincey and Parkulo, 2001). Problems associated with ESBL-producing isolates include multidrug resistance, difficulty in detection and treatment, and increased mortality of patients （Alipourfard and Nilufar, 2010）. This also made treatment of ESBL producing pathogens difficult and costlier.

Our study was based on laboratory investigation and includes only patients attending the general out-patient department (GOPD) of Specialist Hospital Sokoto. In this present study, out of 105 positive isolates, 60.9% (64/105) were Gram-negative bacilli. Our study revealed that males were susceptible to UTI than females. This is contrary to most studies where statistically females are more prone to UTIs than males. Reasons for the higher prevalence of UTIs in male than females may be due to influence of culture of the people of Sokoto where opposite sex even husband and wife hardly discuss issues that have to do with sexual organs in public (Nuhu, 2015). With this, some females prefer to seek medical attention from traditional herbalist, while some females prefer to seek medical

attention from drug vendor and sometimes in community Pharmacies. In most cases, females present to hospitals with complicated UTIs beyond the traditional herbalist or drug vendor or the community Pharmacists. However, patients with complicated UTIs are excluded in this study. Also, from the onset of sample collection, the numbers of male volunteer were more than that of the female.

E. coli was found to be the most prevalent Gram-negative bacteria. This is consistent with reports from other studies (Kolawole *et al*., 2009; Oladeinde *et al*., 2011; Getenet and Wondewosen, 2011). In this study, the second most prevalent Gram-negative bacteria was *Salmonella arizonae*, this is contrary to most reports. *Salmonella arizonae* is an uncommon human pathogen with serious infections reported in hosts with impaired immune function or systems caused by conditions such as collagen vascular diseases requiring immune suppressive therapy, malignancy, organ transplantation, HIV infection and a young age of less than 7 years (Stefano *et al*., 2011). In 2005, Jeffrey *et al*., stated that serious infection has not been documented in a healthy human adult, isolation of should *Salmonella arizonae* prompt evaluation of the immune status in an apparently healthy individuals. The Center for Disease Control and Prevention (CDC) and National Center for HIV, Sexual Transmitted Diseases (STDs) and Tuberculosis prevention in 1987, classified *Salmonella* infection as an AIDS indicator. To the best of our knowledge, our findings represent the first documented infection in which *Salmonella arizonae* was isolated from urine in Sokoto metropolis. However, detection of ESBLs among members of the genius Salmonella is rare (Morosini *et al*., 1995). This is in line with our study, as none of the *Salmonella arizonae* was found to produce ESBL.

In this study, the most infected age group with Gram-negative uropathogen was 19-40 years. This may be attributed to increase sexual activity among these age groups. Factors for increasing incidence of UTI in young age are associated with high unprotected sexual activity and history of recurrent UTIs. This is in line with the authors' earlier view, as most patients in the age group are married, sexually active and therefore the probability of high incidence of UTIs.

Our findings revealed that the Gram-negative isolates displayed various level of resistance to the mostly prescribed antibiotics used in empirical treatment of UTIs. All gram-negative isolates portray a high susceptibility to Nitrofurantoin, whereas susceptibility to Cotrimoxazole was low. Wariso *et al*., 2010 reported in another study South-South Nigeria, in which susceptibility to Cotrimoxazole by all uropathogens was 7.1%. This could be due to it misuse, abuse and free access. Low levels of resistance observed in Nitrofurantoin may be due to low level of prescription by physicians. In addition, Hejer, 2012 reported that AFSSAPS has recommended restricting the use of Nitrofurantoin to girls 16 years of age or older and women who have documented cystitis due to susceptible organisms and when no other antibiotic with a better risk-benefit ratio can be used orally. This according to AFSSAPS is owing to the occurrence of severe hepatic and pulmonary side effect reported with Nitrofurantoin. Despite the reasons given, our study has shown that Nitrofurantoin remains effective against the majority of the isolates.

There is great variation in prevalence of ESBL producing organisms from one place to another and even over time for a given place (Faisal *et al*., 2011). The present study showed that out of 64 tested isolates, 15 (23.4%) were ESBL producers. Our results was higher in comparison to study carried in Kano (North-West Nigeria), where ESBL accounted for 15.4% (Tijjani *et al*., 2012), and lower in comparison to other study carried out in Nigeria (30%. Olanitola *et al*., 2007).

Our findings, showed highest frequency for ESBL production in *E. coli* and *Enterobacter* spp (26.7%) each. Tijjani *et al*., reported an almost similar prevalence of *E.coli* with 23.3%. Faisal *et al*., reported a higher frequency of ESBL production in *Klebsiella* spp (84.6%), followed by *E. coli* (68.5%), *Enterobacter* spp (36.8%) and *Proteus mirabilis* (28.5%). Venkatadri *et al*., reported ESBL production in *Klebsiella pneumoniae* (76%) and *E. coli* (50.9%). This is far higher than our findings in Sokoto.

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Address for correspondence:

T. Nuhu
Department of Pharmaceutics and Pharmaceutical Microbiology, UsmanuDanfodiyo University, Sokoto.
E-mail: nuhu.tanko@yahoo.com

Conflict of Interest: None declared
Received: 28 July, 2015
Accepted: 12 December, 2015