OCCURRENCE OF EXTENDED SPECTRUM BETA-LACTAMASES AMONG CLINICAL BACTERIA ISOLATED FROM DAURA GENERAL HOSPITAL, KATSINA STATE, NIGERIA

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Abstract
Background: Bacterial resistance to antibiotics is a major global challenge in the diagnosis and treatment of infectious diseases. ESBLs are enzymes that confer resistance to third and fourth generation cephalosporins that are produced to counter resistance to normal betalactamase enzymes.
Aim: This work was aimed at detecting the occurrence of ESBLs among clinical bacterial isolates at the study site.
Methods: Three hundred and ninety nine (399) Gram negative bacterial isolates were collected from the study site and identified using standard biochemical tests. The isolates were screened for ESBLs using Clinical Laboratory Standards Institute (CLSI) breakpoint and confirmed using Double Disc Synergy Test (DDST). The standard antibiotic discs used were augmentin (AMC 30µg), cefotaxime (CTX 30µg) and ceftazidime (CAZ 30µg) [Oxoid, England].
Results: The results of CLSI breakpoint test showed that 206 (51.62%) were positive for ESBLs which include; *Proteus* spp 88(22.05%), *E. coli* 40(10.02%), *Klebsiella* spp 48(12.03%), *Citrobacter* spp 18(4.51%), *Providencia* spp 4(1.01%), *Shigella* spp 6(1.50%), *Salmonella* spp 2(0.50%). ESBLs confirmation using DDST revealed that 119 (57.76%) were positive for ESBL production viz; *Proteus* spp 66(32.04%), *E. coli* 8(3.88%), *Klebsiella* spp 28(13.59%), *Citrobacter* spp 12(5.82%), *Providencia* spp 2(0.97%), *Shigella* spp (31.46%), *Salmonella* spp *0* (0.00%) giving an overall ESBLs occurrence of 29.82%.
Conclusion: The high occurrence of ESBLs observed among the clinical isolates implies that the enzymes occur at an alarming rate which may lead to high patient mortality due to treatment failure.
Keywords: Detection, ESBLs, CLSI, DDST, Bacterial isolates, Hospital

Introduction

Betalactam antibiotics are those antibiotics that share a common betalactam ring in their structure.

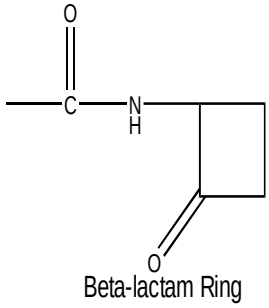

Figure1:

Examples of such antibiotics are penicillins and cephalosporins. Cephalosporins are also β-lactam antibiotics in which the β-lactam ring is fused with dihydrothiazine ring.

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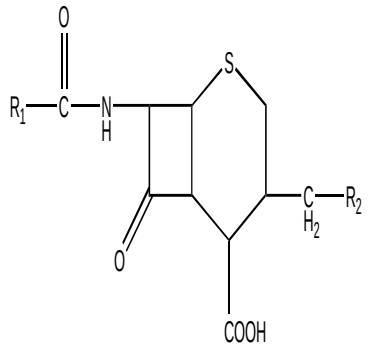

Figure 2: Basic Cephalosporin Ring Structure (7-Aminocephalosporan ic acid)

Third generation cephalosporin had been developed in response to increased prevalence of β-lactamases in certain organisms, but resistance to these extended β-lactamases emerged quickly. The first of these enzymes, capable of hydrolyzing the newer β-lactams sulfhydryl variable 2 (SHV 2) was found in a single strain of *Klebsiella ozaenae* in Germany (Paterson and Bonomo, 2005).

Extended spectrum β-lactamases (ESBLs) are betalactamases capable of conferring bacterial resistance to Penicillins, first, second and third generations cephalosporin and aztreonam (but not cephamycins) or (carbapenems) by hydrolysis of these antibiotics, and are inhibited by β-lactamase inhibitors such as clavulanic acid. First described in Germany (1983) and France (1985) among *Klebsiella* spp., ESBLs exist in every region of the world and in most genera of enterobacteria. Currently, ESBLs are becoming major threats for patients in the hospitals, long term care facilities and community (Taneja and Sharma, 2008).

In practical terms, detection of ESBL in a clinical significant isolate, whether mediated by Teimoneira (TEM), SHV, or Cefotaxime (CTX-M) genes should mean therapeutic resistance to an extended spectrum cephalosporin (indeed, to all cephalosporins, aztreonam, and penicillins). The treatment of choice of such circumstances should be the use of carbapenems or cephamycins depending on the sensitivity result (MacKenzie *et al.,* 2002).

It is generally thought that patients having infection caused by ESBLs producing organisms are at increased risk of treatment failure with extended spectrum β-lactam antibiotics (NCCLS, 2005).

Current therapy for strains of enterobacteriaceae that express ESBLs is limited to imipenem, however, there have already been reports of therapeutic failure of this drug with strains that produce multiple β-lactamase (Ahmed *et al.*, 1999). There are limited therapeutic options left which result in high mortality and facilitate further nosocomial spread of ESBLs producing organisms as such strains expressing ESBLs present a host of challenges as we head into 21st century.

The extended spectrum of β-lactamases production is increasing rapidly and globally, due to the frequent prescription of β-lactam antibiotics (Paterson and Bonomo, 2005). This calls for the need to detect the occurrence or otherwise of ESBLs producing organism.

Extended spectrum β-lactamases are an increasing cause of multi drug resistant infection throughout the world, bacteria carrying such enzymes have been recognized as a cause of health care associated infection and the incidence of such organism also appears to be increasing in the community, which can pose a major threat to life, are often difficult and expensive to treat and can delay discharge from hospital.

The site for isolate collection was General Hospital Daura, Katsina State. The reason for choosing this hospital is that patients from twelve Local Government areas in Daura Senatorial zone patronize the center as well as patients from neighboring Niger Republic coupled with the high number of drug hawkers in area.

This study was aimed at detecting the presence or otherwise of ESBL producing enterobacteriaceae at Daura General Hospital.

The objectives of the study are to identify members of enterobacteriaceae from clinical isolates available at General Hospital, Daura, Katsina State and also to screen the identified isolates for the production of extended spectrum β-lactamases (ESBLs) using clinical laboratory standard institute (CLSI) criteria and confirm it by Double Disc Synergy Test (DDST).

Materials And Methods

Sampling Site

The site for isolate collection was General Hospital Daura, Katsina State. Daura Town is located between latitude 13° - 14° North between longitude 8° - 9° East. It is situated close to Nigerian border with Niger Republic. The hospital received both in-patients and out-patients from the neighbouring local governments and Niger republic (Atanda et al., 2014).

Ethical clearance

The ethical approval letter was received following approval by hospital management in order to enable the research to be conducted in the Microbiology Unit of the Pathology Department of the same hospital.

Sample size

The minimum sample size was three hundred and ninety nine (399) Gram negative enterobacteria isolates based on calculation using the formula for determining minimum sample size for prevalence study i.e.

$$n = \frac{Z^2(pxq)}{d^2}$$ (McDonald, 2008)

Where

n= Sample Size

Z= Confidence level 95% (1.96)

p= Proportion of resistant species in actual population

d= Minimum sampling error = 5% (0.05)

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q= 1 - p

Based on the work of Bell *et al.* (2002), prevalence of 15% was recorded making P= 0.15, q=1-0.15 = 0.859 and the substituting into the above formula

$n = (1.96)^2 (0.15X0.85)/(0.05)^2$

n = 3.8416(0.128)/0.0025

n = 0.4898/0.0025 = 195.92 = 196

A total of 399 Gram negative bacteria isolated from various clinical samples were obtained from the Microbiology Laboratory of General Hospital Daura, Katsina State, which include *Proteus* spp n=157, *Escherichia coli* n=95, *Klebsiella* spp n=86, *Citrobacter* spp n=30, *Salmonella* spp n=14, *Shigella* spp n=11, *Providencia* spp n=6, *Enterobacter* spp n=3 and *Morganella* spp n=2.

Biochemical Tests for Identification of the Enterobacterial Isolates

The biochemical tests were carried out to confirm the identity of the Gram negative organisms obtained based on standard protocols.

Indole Test

The test organism was inoculated into a Bijou bottle containing about 3ml of sterile peptone water. The inoculated peptone water was then incubated for 24hrs at 37°C.

Test for indole was achieved by the addition of 0.5ml of Kovacs regent, shaken gently and then examined for positive result within 10 minutes. Positive result was observed by the appearance of red colour in the surface layer while absence of red color in the surface layer indicated negative result (CLSI 2012).

Urease Test

The test organism was inoculated into 3ml of urea agar medium in a bijou bottles. The inoculated medium was then incubated for 24hrs at 37°C

Pink color was then observed for positive result while absence of pink color indicates egative result (CLSI, 2012).

Citrate Utilization Test

Using a sterile straight wire loop suspension of the organism in normal saline was streaked first at the slope surface of the Simmons citrate and the butt of the medium was stabbed.

The inoculated media was then incubated for 24hrs at 37°C. Bright blue colouraton indicates positive result while absence of colour change indicated negative result (CLSI, 2012).

Triple Sugar Ion (TSI) Agar Test

Using a straight sterile wire loop, the butt of the TSI agar in a test tube was stabbed and then the slope was streaked. The test tube was then incubated for 24hrs at 37^0C.

The test-tube was then observed and recorded (Cheesbrough, 2008).

Standard Antibiotics

The standard antibiotics used were augmentin (AMC 30µg, 12/2018), cefotaxime (CTX 30µg, 12/2018) and ceftazidime (CAZ 30µg, 12/2018) [Oxoid, England].

Clinical Laboratory Standard Institute Test for ESBL Screening

Emulsified inocula from the BHIA (Brain Heart Infusion agar) plates in test tube of sterile normal saline that matched the turbidity of 0.5 Mc Farland standards were swabbed on the surface of prepared Mueller-Hinton Agar (Oxoid, England). This was followed by placing the disks of amoxicillin/clavunate AMC 20 µg/10 µg respectively i.e. augmentin (Oxoid, England), independent of one another. The plates were incubated at 37° for 24hrs before observation for the measurement of zone of inhibition using vernier caliper. Zone diameters ≤ 25mm for cefotaxime and ≤ 22mm for ceftazidime were interpreted as positive for the presence of ESBLs (CLSI, 2012).

Double Disc Synergy Test for ESBL Confirmation

All isolates suspected for ESBL production were further confirmed using double disc synergy test as described by Jarleier *et al.* (1998). One loopful of inoculum was emulsified from the BHIA plates in test tubes of sterile normal saline to match the prepared 0.5 Mc Farland standard. The emulsions were swabbed on to the surface of prepared Mueller-Hinton Agar (Oxoid, England). This was followed by placing a disc of amoxicillin/clavunalate AMC (20 µg/ 10µg respectively) i.e. augmentin (Oxoid, England) at the centre of the inoculated agar plate and a disc of cefotaxime (CTX) (30 µg) [Oxoid, England] 20mm (center to center). Another antibiotic disc of ceftazidime (CAZ) [30 µg, Oxoid, England], was also placed on the plate 20mm (center to center) from the augmentin disk. The plates were incubated at 37°C for 18-24hrs before observation for clear extension of the edge of either or both cefotaxime and ceftazidime inhibition zone toward the augmentin disk, which was interpreted as synergy indicating the presence of an ESBL (CLSI, 2012).

Statistical Analysis

The results obtained were subjected to chi-square test. The contingency level of the chi-square test was 7 by 2, i.e. number of rows r = 7, and number of column c = 2, (c by r), to determine whether there is significant difference or not between the result of screening and confirmation tests.

Results

The result of biochemical tests was compared with standard table to identify the isolated bacteria (Table 1). A total of three hundred and ninety nine (399) Gram negative bacteria were identified as members of the family enterobacteriaceae (Table 2).

Two hundred and six (206) of the identified enterobacteriaceae representing 51.62% were positive for ESBLs. These include *Proteus* spp 88 (22.05%), *E. coli* 40 (10.02%), *Klebsiella* spp 48 (12.03%), *Citrobacter* spp 18 (4.51%), *Providencia* spp 4 (1.01%), *Shigella* spp 6 (1.50%), *Salmonella* spp 2 (0.50%), as indicated in Table 3.

Confirmatory test using Double Disk Synergy Test (DDST) showed that one hundred and nineteen (119) representing (57.76%) were positive for ESBL production.

These include *Proteus* spp 66 (32.04%), *E. coli* 8 (3.88%), *Klebsiella* spp 28 (13.59%), *Citrobacter* spp 12 (5.82%), *Providencia* spp 2 (0.97%), *Shigella* spp 3 (31.46%), *Salmonella* spp *0* (0.00%) as indicated in Table 4.

There exists a significant difference in ESBL production among the bacterial species when the results were subjected to chi square statistical analysis, because the calculated value (23.29) was greater than the table value (12.59) at 5% confidence level and 6 degree of freedom. Out of the 399 isolates screened for ESBL production, 119 were confirmed to produce ESBL giving an overall prevalence of 29.82% (Table 5).

Table 1: Biochemical identification of bacterial isolates obtained from the study site

Isolates	**Indole**	**MR**	**VP**	**Cit**	**Urea**	**TSI**			
						Slant	**butt**	**H_2S**	**Gas**
Escherichia coli	+	+	-	-	-	Y	Y	-	+
Shigella spp	d	+	-	-	-	R	Y	-	-
Salmonella spp	-	+	-	-	-	R	Y	+	-
Proteus mirabilis	-	d	d	+	+	R	Y	+	d
Proteus vulgaris	+	+	-	d	+	Y	Y	+	+
Citrobacter spp	-	+	-	+	d	Y	Y	+	d
Enterobacter spp	-	-	+	+	-	Y	Y	-	+
Providencia spp	+	d	-	+	d	R	Y	-	d
Klebsiella spp	-	-	+	+	+/S	Y	Y	-	+
Pseudomonas spp	-	+	-	+	d	R	R	-	-
Morganella spp	+	+	-	-	+	R	Y	-	d

KEY
+ = Positive reaction
- = negative reaction
d = different strain give different result
Y = Yellow
R = Red-pink
+S = slow positive

Table 2: Occurrence of Gram negative organisms obtained from clinical dacterial isolates using biochemical tests

S/N	Isolates	Number Observed	% Occurrence
1	*Proteus* spp	152	38.00
2	*E. coli*	95	23.75
3	*Klebsiella* spp	86	21.50
4	*Citrobacter* spp	30	7.50
5	*Salmonella* spp	14	3.50
6	*Shigella* spp	11	2.75
7	*Providencia* spp	06	1.50
8	*Enterobacter* spp	03	0.75
9	*Morganella* spp	02	0.50
	TOTAL	**399**	**100**

Table 3: Prevalence of ESBLs among Enterobacteria isolates using CLSI tests

Isolate	Number screened	Number +ve	% occurrence
Proteus spp	152	88	57.89
E .coli	95	40	42.10
Klebsiella spp	86	48	55.81
Citrobacter spp	30	18	60.00
Providencia spp	6	4	66.66
Shigella spp	11	6	54.55
Salmonella spp	14	2	14.28
Enterobacter spp	3	0	00.00
Morganella spp	2	0	00.00
Total	**399**	**206**	**51.62**

Table 4: Prevalence of ESBLs among Enterobacteria isolates using Double Disc Synergy Test (DDST)

Isolate	Number screened	Number +ve	% occurrence
Proteus spp	88	66	75.00
E .coli	40	8	20.00
Klebsiella spp	48	28	58.33
Citrobacter spp	18	12	66.66
Providencia spp	4	2	50.00
Shigella spp	6	3	50.00
Salmonella spp	2	0	00.00
Total	**206**	**119**	**57.76**

Table 5: Prevalence of ESBL Producers among isolates collected from Daura General Hospital

Isolates	No. Screened	No of Confirmed ESBL	% confirmed ESBL
Proteus spp	152	66	43.42
E. coli	95	8	8.42
Klebsiella spp	86	28	32.55
Citrobacter spp	30	12	40.00
Providencia spp	6	2	33.33
Shigella spp	11	3	27.27
Salmonella spp	14	0	0.00
Enterobacter spp	3	0	0.00
Morganella spp	2	0	0.00
Total	**399**	**119**	**29.82**

Discussion

Out of the three hundred and ninety nine (399) Gram negative isolates obtained and subjected to biochemical tests, three hundred and ninety nine 399 (99.75%) were identified as members of the family enterobacteriaceae. High occurrence of Gram negative isolates was an indication of possible outbreak of infections since the organisms are pathogenic once found outside their natural habitat (Paterson and Bonomo, 2005), the high occurrence of enterobacteria may be due to poor hygienic practices, which may lead to the rapid spread of ESBLs and might result in possible epidemic that may not be curtailed by the normal drugs available.

Of the three hundred and ninety nine (399) enterobacterial isolates subjected to screening using Clinical Laboratory Standard Institute Criteria (CLSI) test. A total of two hundred and six (206) representing 51.62% were positive for ESBL using CLSI criteria (test). These include *Proteus* spp 88 (22.05%), *E. coli*, 40 (10.02%), *Klebsiella* spp, 48 (12.03%), *Citrobacter* spp, 18 (4.51%), *Providencia* spp, 4 (1.01%), *Shigella* spp, 6 (1.50%), *Salmonella* spp, 2 (0.50%) as indicated in Table 3. This may result from some of the enterobacteria acquiring genes responsible for ESBL production. Consequently, this may also lead to transfer of antibiotic resistant genes from one organism to another amongst members of the same specie or different species.

On subjecting the two hundred and six (206) isolates to confirmatory testing using Double Disk Synergy Test (DDST), one hundred and nineteen (119) representing 57.76% were positive for ESBL production. These include; *Proteus* species 66 (32.04%), *E. coli*, 8 (3.88%), *Klebsiella* spp, 28 (13.59%), *Citrobacter* spp 12 (5.82%), *Providencia* spp 2 (0.97%), *Shigella* spp, 3 (1.46%) and *Salmonella* spp, 0 (0.00%) as indicated in Table 4. This may be determined by the available number of isolates belonging to the same specie that is subjected to confirmatory test. Although, several reports showed that there is increasing prevalence of ESBLs worldwide, the extent of the problem is under recognized due to unawareness, poor laboratory detection and reporting as well as inadequate infections control measures, which may lead to transfer ESBLs among clinical isolates which vary from hospital to hospital within a single city (Bradford, 2001). There exists a significant difference in ESBLs production among the isolates when the results were subjected to chi-square statistical analysis because the calculated value (23.29) was greater than the table value (12.59) at 5% confidence level and 6 degree of freedom.

The highest prevalence of ESBL was found in *Proteus* spp 43.44% (66/152), *Citrobacter* spp 40.0% (12/30), followed by *Providencia* spp 33.33% (2/6), *Klebsiella* spp 32.55% (28/86), *Shigella* spp 27.27% (3/11) and then *E. coli* 8.42% (8/95) having the least prevalence, whereas *Salmonella* spp, *Enterobacter* spp and *Morganella* spp were not confirmed to be ESBLs producers.

The percentage prevalence of ESBL was higher in *Proteus* spp than other species of enterobacteriaceae involved in the study which confirms to the findings of Perilli *et al*. (2000) also agreed with the finding of Bradford (2001) which showed the occurrence and distribution of ESBL differs from country to country and from hospitals to hospitals.

On comparing the prevalence rate of ESBLs (29.82%) found in this study with the findings of similar studies, the prevalence of ESBL obtained in this study is lower when compared to a study by Borg *et al.* (2006) where they reported 70.0% from Egypt, whereas it is higher than the study by Yusuf *et al*. (2013) which reported prevalence of 15% in Kano State and the report of Aibinu *et al.* (2003) at Lagos indicated 20%. However, the prevalence was low when compared to that obtained in Benin (Edo State) which was 36.6% (Osazuwa and Osazuwa, 2011).

Comparison of the prevalence of ESBLs (29.82%) found in this study with the finding of similar studies around the world showed that the prevalence was lower than that obtained in United States of America (USA) which recorded 43% in 2003. In Europe ESBLs producing organisms have been spreading at an alarming rate, almost all the European countries has experienced outbreak with ESBL producing organism, as obtained in the General Hospital, Daura, Katsina State, Nigeria. A low prevalence was also recorded when compared to Asia 15% Bell *et al.* (2002). In Africa there is generally lack of comprehensive data with regard to ESBL producing organisms, notwithstanding the prevalence is low when compared to that obtained in Egypt with the majority of the isolates (70%) producing the enzyme.

The high rate of ESBL in African countries could be due to poorer social and economical situation, hospital overcrowding, lack of antimicrobial stewardship and excessive over the counter antibiotic usage and the under supported infections control measures (Villegas, 2008).

Conclusion

There is high occurrence of ESBLs among clinical bacteria isolated from Daura General Hospital with the rate being 29.82%. ESBLs producing bacteria occurs at an alarming rate particularly among *Proteus* spp *n*=66, *Klebsiella* spp n=28, and *Citrobacter* spp n=12.

Recommendations

Due to the spread of ESBL among bacterial pathogens, and the problem that may result from treatment failure due to infections with ESBL producing bacteria. It could be recommended that;

i). Similar study should be extended in the remaining other Hospitals of the two Senetorial zones of the Katsina State i.e. Katsina and Funtua General Hospitals respectively.

ii) The use of third generation cephalosporin especially at wide spread empiric level should be restricted either by formal restriction of availability or by education and increased availability for alternatives.

iii) Infection control strategies should be strictly adhered to, in hospital setting to prevent the spread of the ESBLs producers.

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