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ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF EXTENDED SPECTRUM BETALACTAMASE PRODUCERS IN GRAM-NEGATIVE UROGENITAL ISOLATES IN KANO, NIGERIA

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ABSTRACT
The emergence of resistant strains of urogenital extended spectrum beta-lactamase producing isolates has presented a serious set back in the treatment option for urogenital tract infection. Emergence and spread of these strains resulted in treatment failure and disease complications. This study was aimed to determine the prevalence of ESBL producers in Gram-negative urogenital isolates and their susceptibility to 10 selected antimicrobial agents [aztreonam, cefoxitin cefepime, levofloxacin, ciprofloxacin, kanamycin, gentamicin, streptomycin, clarithromycin and erythromycin.]. A total of 500 isolates of Escherichia coli, Proteus mirabilis, Proteus vulgaris, Klebsiella pneumoniae, Klebsiella aerogenes, and Pseudomonas aeruginosa were collected from Microbiology Department of Aminu Kano Teaching Hospital (AKTH), Kano. The isolates were tested against third generation cephalosporins using Clinical Laboratory Standard Institute (CLSI) recommended, WHO modified Kirby-Bauer disc diffusion method. Isolates with reduced susceptibility to cefpodoxime, cefpotaxime and ceftriaxone were considered to be a possible ESBL producers and were confirmed using double disc synergy method. The number of ESBL producers in 500 urogenital gram negative isolates was found to be 77/500 (15.4%). Out of the 77 ESBL producing urogenital isolates 47 (61%) isolates which include E. coli, (33, 70%), K. aerogene (3, 6%), K. pneumoniae (6, 13%), P. mirabilis (9%) and P. aeruginosa (2%) were subjected to antimicrobial susceptibility test using CLSI recommended, WHO modified Kirby Bauer disc diffusion method. Zone diameters were interpreted using European Committee on Antimicrobial Susceptibility Testing breakpoint tables for interpretation of zone diameters. The overall result demonstrates high resistance rate (≥60%) to the selected antibiotics. The isolates were found to be more susceptible to Cefoxitin (40%), and Cefepime (38%); followed by Kanamycin (32%), Levofloxacin (28%), and Ciprofloxacin (28%). With Gentamicin (16%) Clarithromycin (11%), Streptomycin (9%), Aztreonam (4%) and Erythromycin (2%), showing low antimicrobial activity against the isolates. The result of this study shows that multidrug resistant ESBLs producing strains are present among urogenital bacterial pathogens in Kano. It is recommended that urogenital ESBLs isolates treatment option be based on antimicrobial susceptibility results.
Keywords: Extended Spectrum Beta-lactamases, Prevalence, Gram-negative urogenital isolates, Antimicrobial susceptibility.

INTRODUCION

Urinary tract infections (UTIs) have been reported to affect up to 150 million individuals annually worldwide, while antibiotic resistance in Urogenital pathogens is increasing worldwide in both outpatients as well as hospitalized patients (Stamm, 1999). It varies according to geographic locations and is directly proportional to the use and misuse of antibiotics (Stamm, 1999). And the plasmids responsible for ESBL production also confer resistance to other several antimicrobial agents, this cause limitation in the design of treatment alternatives (Jacoby and Medeiros, 1991). Resistant Urogenital tract infections if not properly treated can lead to serious complications such as Pelvic Inflammatory Diseases (PID), ectopic pregnancy, abscess formation, Fallopian tube obstruction, Epididymochitis, Orchitis, and the involvement of kidneys causing nephritis (Health Professor, 2008).

Multidrug resistance among many organisms has become a big challenge to infectious disease management. It is increasingly being reported in bacteria and is often mediated by genetic mobile elements such as plasmids, transposons, and integrons (Dessen *et al*., 2001). Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site specific recombination, and they have an intergrase gene (int), a nearby recombination site (attI), and a promoter, (pant) (Hall, 1997). Integrons seem to have a major role in the spread of multidrug resistance in gram-negative bacteria but integrons in gram-positive bacteria have also been described (Dessen *et al*., 2001). The knowledge of susceptibility pattern of resistant urogenital tract infection is necessary not only for optimal patient management but also for institution of appropriate infection control measures to prevent the spread of these organisms.

MATERIALS AND METHODS

A total of 500 isolates of *Escherichia coli, Proteus mirabilis, Proteus vulgaris, Klebsiella pneumoniae, Klebsiella aerogenes,* and *Pseudomonas aeruginosa* were collected from Microbiology Department of Aminu Kano Teaching Hospital (AKTH), Kano.

Confirmatory Tests

The following tests were carriedout to confirm the identity of the isolates.

- Indole test
- Urease test
- Citrate utilization test
- Kligler iron agar test

Detection of ESBLs Producing Isolates

The isolates were tested against third generation cephalosporins (cefpodoxime, cefpotaxime and ceftriaxone) using Clinical Laboratory Standard Institute (CLSI) recommended, WHO modified Kirby Bauer disc diffusion method (Lalitha, 2001). Zone diameters were interpreted using the revised National Committee on Clinical Laboratory Standard document (NCCLS, 1998). Isolates with reduced susceptibility to cefpodoxime (≤ 17mm) cefpotaxime (≤ 27mm) and ceftriaxone (≤ 25mm) were considered to be possible ESBL producers.

Phenotypic Confirmatory Test

Phenotypic confirmatory Test was carried-out using Double Disc Synergy Test. Disks containing the standard 10µg of cefpodoxime and 30µg of cefpotaxime/ceftriaxone, are placed 15mm apart (edge to edge); with amoxicillin-clavulanic acid disk containing 10µg of the later compound mounted exactly at their centre. After 16-20 hours of incubation at 35^{o}C, any enhancement of the zone of inhibition between a beta-lactam disk and that containing the beta-lactamase inhibitor is indicative of the presence of an ESBL (Coudron *et al.,* 1997).

Antimicrobial Susceptibility Test

Out of the 77 ESBL producing urogenital isolates 47 (61%) isolates which include *E. coli* (33, 70%), *K. aerogene* (3, 6%), *K. pneumoniae* (6, 13%), *P. Mirabilis* (4, 9%) and *P. aeruginosa* (1, 2%) were subjected to antimicrobial susceptibility test using CLSI recommended, WHO modified Kirby Bauer disc diffusion method (Lalitha, 2001). Commercially prepared antimicrobial susceptibility discs from Oxoid company were used and they include: Cefepime, Cefoxitin, Aztreonam, Gentamicin, Streptomycin, Kanamycin, Erythromycin, Clarithromycin, Ciprofloxacin and Levofloxacin.

Procedure

Using a sterile wire loop 3 to 5 well isolated colonies of similar appearance were picked (by touching the top of each colony with the wire loop) from a freshly prepared overnight culture. The colonies were transferred and emulsified in a tube containing 3 to 5ml of nutrient broth. The preparations were incubated at 35^{o}C until the growth exceeds the turbidity of 0.5 Mc Farland standard (usually within 2 to 6 hours). The turbidity of the actively growing broth culture was adjusted with sterile nutrient broth to obtain a turbidity optically comparable to that of 0.5 Mc Farland standard. This was achieved visually in adequate light by comparing the turbidity of the inoculum tube and the 0.5 Mc Farland standard mixed immediately against a card with a white background and contrasting black lines. Optimally, within 15 minutes after adjusting the turbidity of the inoculum, sterile swab was dipped in to the suspension. The excess fluid was removed from the swab by pressing and rotating it against the side of the tube above the level of the suspension. The dried surface of the MHA plate was inoculated by evenly streaking over the surface of the plate in three directions, rotating the plate approximately 60^{0} to ensure even distribution of the inoculum. With the Petri dish lid in place the medium was allowed to soak for 3 to 5 minutes, to allow any excess surface moisture to be absorbed. Using a sterile needle mounted in holder, the 10 antimicrobial discs under investigation were evenly distributed on the inoculated plates with the aid of a template. Two plates were used for each isolate; the first plate carries 6 antimicrobial discs while the second carries 4 making 10 antimicrobial discs per isolate. The discs were placed 15mm from the edge of the plate and 25mm from disc to disc. The discs were lightly pressed down to ensure contact with the medium. The plates were allowed for sometime (not more than 30 minutes) for the antibiotic to be diffused in to the medium. The plates were then inverted and incubated aerobically at 35^{0}C for 16 to 18 hours (overnight). The plates were examined after 16 to 18 hours of incubation. For satisfactory confluent lawn growth. Plates with too heavy and or too light growth were rejected and the test repeated. The diameter of the zones of complete inhibition (as judged by an unaided eye) were measured to the nearest whole millimetre, using a ruler held on the back of an inverted petri dish. Zone diameters were interpreted using European Committee on Antimicrobial Susceptibility Testing breakpoint tables for interpretation of zone diameters (EUCAST, 2011).

RESULTS

The overall prevalence of ESBL producing urogenital gram negative isolates in the study was found to be 15.4% (77/500) as shown in Table 1. The highest prevalence was found among *Escherichia coli* isolates (23.3%; 51/219), followed by *Klebsiella aerogenes* (16.0%; 4/25), *Klebsiella pneumoniae* (12.1%; 12/99), *Proteus mirabilis* (8.1%; 9/111), and *Pseudomonas aeruginosa* (2.5%; 1/40). ESBLs were not detected in *Proteus vulgaris* (0%, 0/6). Among urogenital samples, highest prevalence was recorded in catheter tips (26.7%), followed by Endocervical swab (21.1%), urine (14.6%) urethral swabs (10%), HVS (8.3%). No ESBL producers were detected in semen sample (Table 2).

The prevalence of ESBL producers in hospitalised patients (20.1%; 43/214) was higher compared to non-hospitalized patients (11.9%; 34/286) as shown in Tables 3 and 4 respectively.

Table 1 : Prevalence of ESBL producers among Gram-negative urogenital isolates

Bacteria isolates	ESBL Production		Number of isolate screened	% prevalence
	positive	negative		
Escherichia coli	51	168	219	23.3
Klebsiella aerogenes	4	21	25	16.0
Klebsiella pneumoniae	12	87	99	12.1
Proteus mirabilis	9	102	111	8.1
Proteus vulgaris	0	6	6	0.0
Pseudomonas aeruginosa	1	39	40	2.5
Total	**77**	**423**	**500**	**15.4**

Table 2: Prevalence of ESBL producers among the urogenital samples

Bacteria isolates	ESBL Production		Number of isolate screened	(%) prevalence
	positive	negative		
Urine	59	346	405	14.6
High vaginal swab	1	11	12	8.3
Endocervical swab	4	15	19	21.1
Urethral swab	1	9	10	10
Semen	0	9	9	0.0
Catheter tip	12	33	45	26.7
Total	**77**	**423**	**500**	**15.4**

Table 3: Prevalence of ESBLs among the urogenital Isolates (U. I.)in non-hospitalized patients

Bacteria isolates	ESBL Production		Number of isolate screened	% prevalence
	positive	negative		
Escherichia coli	21	104	125	16.8
Klebsiella aerogenes	1	9	10	10
Klebsiella pneumoniae	6	53	59	10.2
Proteus mirabilis	6	62	68	8.8
Proteus vulgaris	0	4	4	0.0
Pseudomonas aeruginosa	0	20	20	0.0
Total	**34**	**252**	**286**	**11.9**

Table 4: Prevalence of ESBLs among the urogenital isolates (U. I.) in hospitalized patients

Bacteria isolates	ESBL Production		Number of isolate screened	% prevalence
	positive	negative		
Eschrechia coli	30	64	94	32
Klebsiella aerogenes	3	12	15	20
Klebsiella pneumoniae	6	34	40	15
Proteus mirabilis	3	40	43	7
Proteus vulgaris	0	2	2	0.0
Pseudomonas aeruginosa	1	19	20	5
Total	**43**	**171**	**214**	**20.1**

The overall susceptibility result demonstrate high resistance rate to the selected antibiotics. The isolates were most susceptible to Cefoxitin (40%), followed by cefepime (38%); Kanamycin (32%), Levofloxacin (28%), and ciprofloxacin (28%). The isolates demonstrated low susceptibility to Gentamicin (16%) Clarithromycin (11%), streptomycin (9%), Aztreonam (4%) and Erythromycin (2%) as shown in Table 5.

Among the isolates tested, *Pseudomonas aeruginosa* was 100% resistant to all the antibiotics used in the study, followed by *P. Mirabilis* [Cefoxitin (75%), cefepime (75%), and Kanamycin (75%)], *K. pneumoniae* [Cefoxitin (67%), cefepime (67%). and Kanamycin (66%)] and *E. coli* [Cefoxitin (58%), cefepime (61%), and Kanamycin (49%)] as shown in Table 5.

Table 5: Susceptibility Pattern of ESBL Producing Isolates to Some elected Antibiotics (%)

	Antibiotic sensitivity (%)																											
Isolates	**Cefepime**		**Aztreonam**			**Cefoxitin**		**Kanamycin**			**Streptomycin**			**Gentamicin**			**Levofloxacin**			**Ciprofloxacin**			**Clarithromycin**			**Erythromycin**		
	R	**S**	**R**	**I**	**S**	**R**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
E. coli	61	39	94	3	3	58	42	48	16	36	76	15	9	62	23	15	64	6	30	67	6	27	70	18	12	91	9	0
K. aerogenes	33	67	100	0	0	33	67	33	0	67	67	0	33	50	50	0	67	0	33	33	33	33	33	33	33	67	0	33
K. pneumoniae	67	33	83	0	17	67	33	66	17	17	100	0	0	33	33	33	50	50	0	50	17	33	100	0	0	100	0	0
P. aeruginosa	100	0	100	0	0	100	0	100	0	0	100	0	0	-	-	-	100	0	0	100	0	0	100	0	0	100	0	0
P. mirabilis	75	25	100	0	0	75	25	75	25	0	100	0	0	-	-	-	25	25	50	75	0	25	100	0	0	100	0	0
Total	**62**	**38**	**94**	**2**	**4**	**60**	**40**	**3**	**15**	**32**	**80**	**11**	**9**	**58**	**26**	**16**	**59**	**13**	**28**	**61**	**11**	**28**	**74**	**15**	**11**	**92**	**6**	**2**

Key :- R : resistant isolate, **I :** intermediate sensitive isolates, **S :** susceptible isolates

DISCUSSION

Low prevalence of ESBLs was recorded in this study when compare with the prevalence in some studies. In India Babypadmini and Appalaraju (2004), studied a total of 411 urinary isolates. ESBL production was 41% in *E.coli* and 40% in *K. pneumoniae*. In Nigeria, Olanitola *et al.* (2007) analysed 50 urinary isolates for ESBL production. A prevalence of 30%, (15/50) was observed. In Kano, Nigeria, Yushau *et.al,* (2007), reported 9.5% prevalence of ESBL producers in a study on 747 enterobacteriaceae isolates using double disc synergy test. The prevalence was lower than the figure obtained in this study, though the study was carried-out on enterobacteriaceae clinical isolates the findings of this study thus indicate an increase in prevalence of ESBLs in Kano.

The result of this study show that ESBL producers are present among urogenital bacterial pathogens in Kano and they occur at an alarming rate in some species. And the incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past few years resulting in limitation of therapeutic options; with outbreaks involving ESBL strains been reported all over the world, making them emerging pathogens (Ananthkrishnan, *et al.,* 2000). Resistant urogenital tract infections, if not properly treated, can lead to complications that may results in permanent and or temporary infertility, Pelvic Inflammatory Diseases (PID), ectopic pregnancy, abscess formation, Fallopian tube obstruction, epididymitis, orchitis, and the involvement of kidneys causing nephritis (Health Professor, 2008).

The overall prevalence of ESBL producers in hospitalized patients (20.1) was found to be double the prevalence in nonhospitalized patients (11.9). This is in line with findings around the word. Lautenbach *et al.,* (2001) in their study on risk factors for infection and impact of resistance outcomes reported that Infection and colonization with ESBL producing organisms are usually hospital-acquired especially in intensive care units. Other hospital units that are at increased risk include surgical wards, paediatrics, neonatology, rehabilitation units and oncology wards (Bermudes *et al*., 1997). Urinary tract infection in out patients is more common among individuals with recent hospitalization and or catheterization. And also among Individuals with no hospital contact due to inappropriate treatment (Health Protection Agency 2008).

Highest prevalence was found among *Escherichia coli* (23.3%; 51/219) and followed by *Klebsiella aerogenes* (16.0%; 4/25), *Klebsiella pneumoniae* (12.1%; 12/99), *Proteus mirabilis (*8.1%; 9/111) and *Pseudomonas aeruginosa* (2.5%; 1/40). This is due to the fact that ESBL producing isolates are most commonly *Klebsiella spp*, and *Escherichia coli* (CDC 2010). Other isolates of *Enterobacteriaceae*, such as *Salmonella sp* and *Proteus mirabilis*, and isolates of *Pseudomonas aeruginosa* produce ESBLs but not as common as in *Klebsiella* species and *E. coli* (CDC 2010).

The prevalence of ESBL producers among the urogenital samples was found to be highest in catheter tip (26.7%), followed by Endocervical swab (21.1), urine (14.6), urethral swab (10), and high vaginal swab (8.3). This because of the associated risk factors for infection / colonization with ESBL producing organisms which include presence of vascular or urinary catheters, undergoing hemodialysis, prior exposure to antibiotics (*e.g.,* quinolones, trimethoprim-sulfamethoxazole, aminoglycoside and metronidazole), prior ceftazidime or azteronam administration and prior residence in a long term care facility [Bradford (2001), Patterson (2001), Paterson *et al* (2005) and Lautenbach *et al.,* (2001)].

The urogenital ESBL producing isolates were most susceptible to cefoxitin 40%, and cefepime 38% due to the fact that cefepime exhibits more stability to hydrolysis by ESBLs than the 3rd generation cephalosporins; and cefoxitin is not hydrolyzed by the ESBLs. Resistant ESBLs mutant were also reported which are resistant to cephamycins (Pangon, *et al.,* 1989). When compared to other studies Gunserene et al., (1999) reported similar result of 38% susceptibility to cefepime, Sorlozano et al., (2007) found higher susceptibility of 91% and 80% to cefoxitin and cefepime respectably, and Casellas et al., (2003) found lower susceptibility of 24% to cefepime.

Levofloxacin and ciprofloxacin were also found to be relatively effective (28%, and 28% respectively) as it were reported by Iroha et al., (2008) (ciprofloxacin 45%) Sorlozano et al., (2007) (ciprofloxacin 28%, Levofloxacin 27%); and; Gunseren et al., (1999) [Amikacin 36%, and ciprofloxacin 47%]. Considering the susceptibility pattern of the fluoroquinolones (ciprofloxacin, levofloxacin) above newer fluoroquinolones are unlikely to confer added benefits due to the increase fluoroquinolones resistance among ESBLs producing isolates.

Kanamycin was most susceptible (32%) among aminoglycosides, followed by Gentamicin (16%), and streptomycin (9%). Kanamycin is not readily available in this locality and mostly preserved as second line injectable drug (treatment of Tuberculosis) that is why resistance was not as high as in Ggentamicin and streptomysin which are the most common aminoglycosides used in the treatment of bacterial infection.

The genes encoding the ESBLs production especially CTX-M type E. coli are often found to be associated with genetic structures that are genetically linked to class 1 integrons which integrates antibiotic resistance gene cassettes responsible for resistance to b-lactams, aminoglycosides, chloramphenicol, sulphonamides, tetracycline, and ciprofloxacin. (Bonnet, 2004 and Pitout *et al.,* 2004). This may explain the reason of multidrug resistance pattern of the urogenital ESBL producing isolates investigated in this study.

CONCLUSION

- The results of this study show that ESBL producers are present among urogenital bacterial pathogens in Kano and they occur at an alarming rate in some species.
- ESBL producers are more prevalent in hospitalized patients than non-hospitalized patients.
- Catheter tip, Endocervical swab, and urine samples have a higher prevalence of ESBL producers among urogenital samples.
- The overall susceptibility result demonstrate high rate of resistance among the isolates to the selected antibiotics.
- The result of this study shows that Multidrug resistant ESBLs producing strains are present among urogenital bacterial pathogens in Kano.

Recommendation

In order to reduce the risk of treatment failure in urinary infection, it is recommended that urogenital isolates with reduced susceptibily to third generation cephalosporins should be screened for ESBL production and treatment option be based on antimicrobial susceptibility results.

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