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Distribution of β-Lactam resistant Gram-negative bacteria isolated from clinical and environmental sources in two tertiary hospitals in Makurdi, Benue State, Nigeria

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For appropriate control of infections, it is necessary to possess updated awareness about occurrence of the causative agents. Gram-negative bacteria are considered important microorganisms that cause hospital infections. Clinical multidrug resistant Gram-negative bacteria were obtained from clinical samples including urine, high vaginal swab (HVS), wound swab (WS), stools, ear swab (ES), endocervical swab (ECS), sputum and blood, from Federal Medical Centre (FMC) and Benue State University Teaching Hospital (BSUTH) located at Makurdi. Sewer wastewater and sediment samples were also collected from both hospitals using standard sampling techniques and bacteria were isolated using pour plate technique. Identification was done using API 20E kit. Out of the 403 clinical bacteria obtained, 271 were from FMC and 132 from BSUTH; of these, 299 were confirmed Gram-negative (218 from FMC and 81 BSUTH, respectively). Thirty-nine Gram-negative bacteria were also isolated from the sewer samples, that is, from the environmental samples. Pooled frequencies of Gram negative bacteria isolated from clinical samples in both hospitals were: Urine (56.9%), HVS (11.7%), WS (11.4%), stools (7.7%), ES (6.0%), ECS (3.3%), sputum (2.3%) and blood (0.7%). The identified bacteria from the clinical samples from FMC and BSUTH were *Escherichia coli* (92; 55), *Pseudomonas* sp. (104; 17), *Klebsiella* sp. (19; 5) and *Proteus* sp. (3; 4) respectively.

Key words: Antibiotic resistance, Gram-negative bacteria, β-lactams.

INTRODUCTION

The use of antibiotics for the treatment of bacterial infections is very important; however, increase in the rate at which bacteria develop resistance to these agents all over the world is a public health challenge because the antimicrobial agents become less effective (Neu, 1992; Witte, 1998; Alhaj et al., 2007). Antibiotic resistance in

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pathogenic bacteria has been an increasing medical problem for decades (Mazel and Davies, 1999; Kastner et al., 2005). The development of antibiotic resistance by bacterial pathogens may be due to selection or acquiring resistance determinants even when the organisms were not directly exposed to the antibiotics. In addition, potential and obligate pathogens acquire resistance determinants which are spread among species and genera (Kastner et al., 2005). The intestine of humans and animals are especially favored in settings that allow association of densely packed microorganisms (Salyers et al., 2004; Kastner et al., 2005).

The beta–lactamases target the peptidases of bacterial cell-wall in biosynthetic processes (Ittoo et al., 2010). The beta-lactam antibiotics are the largest and most commonly used group of antimicrobial agents all over the world which are distinguished by a chemical structure known as the beta-lactam ring. Based on this, they can be divided into five groups, depending on the ring structure fused to the beta-lactam ring (Penicillins, cephalosporins, carbapenems, monobactam and beta-lactamase inhibitors) (Walsh, 2003). The beta-lactam antibiotics work by blocking the peptidoglycan of the cell wall component, through transpeptidation inhibition of penicillin binding proteins (Walsh, 2003; Andes and Craig, 2005; Chambers, 2005). Toxicity to the beta-lactams is very low in animals since penicillin binding proteins are not found in their cells, but allergy against penicillins and other beta-lactams can be very serious (Weiss and Adkinson, 2005). The spectrum of action can be narrow or broad and targets both Gram-positive and Gram-negative bacteria. Resistance against beta-lactams is primarily mediated by a structural change of the penicillin binding proteins (leading to lower affinity of the drug) or by bacterial production of enzymes cleaving to the beta-lactam ring. Other mechanisms include decreased permeability or active transportation via efflux pumps (Chambers, 2005).

The β-lactamases is the collective name of enzymes that open the β-lactam ring by adding a water molecule to the common β-lactam bond, and this inactivates the β-lactam antibiotic from penicillin to carbapenems. This hydrolyzation was first observed in 1940 by Abraham and Chain as penicillinase in a strain of *Escherichia coli* (Abraham and Chain, 1940). Although, the clinical effect of such hydrolyzation was not noted until the beginning of the 1950s, when the first β -lactam-resistant *Staphylococcus aureus* isolates appeared in hospitals (Kirby, 1944).

Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the gram-staining method of bacterial differentiation (Baron et al., 1996). They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall pack in between an inner cytoplasmic cell membrane and a bacterial outer membrane. The gram-negative bacteria include *E. coli*, as well as many pathogenic bacteria, such

as *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis,* and *Yersinia pestis.*

Based upon a number of different observations including that the gram-positive bacteria are the major reactors to antibiotics and that gram-negative bacteria are, in general, resistant to them, it has been proposed that the outer cell membrane in gram-negative bacteria (diderms) evolved as a protective mechanism against antibiotic selection pressure (Gupta, 2011). The diderm bacteria can also be further differentiated between simple diderms lacking lipopolysaccharide (LPS); the archetypical diderm bacteria, in which the outer cell membrane contains lipopolysaccharide; and the diderm bacteria, in which the outer cell membrane is made up of mycolic acid (for example, Mycobacterium) (Desvaux et al., 2009).

The proteobacteria are a major phylum of gram-negative bacteria, including *E. coli, Salmonella, Shigella,* and other *Enterobacteriaceae, Pseudomonas, Moraxella, Helicobacter, Stenotrophomonas, Bdellovibrio*, acetic acid bacteria, and *Legionella*. Other notable groups of gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria. Medically relevant gram-negative bacilli include a multitude of species. Some of them cause primarily respiratory problems (*Klebsiella pneumoniae, Legionella pneumophila, P. aeruginosa*), primarily urinary problems (*E. coli, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens*), and primarily gastrointestinal problems (*Helicobacter pylori, Salmonella enteritidis, Salmonella* Typhi). Gram-negative bacteria associated with hospital-acquired infections include *Acinetobacter baumannii*, which cause bacteremia, secondary meningitis, and ventilator-associated pneumonia in hospital intensive-care units. The aim of this work is to illustrate the distribution of β-Lactam resistant Gram-negative bacteria from clinical and environmental sources in two healthcare facilities.

MATERIALS AND METHODS

Sample site/collection

Approval was obtained from the two hospitals before the commencement of the study. Ethical approval was obtained from the government of Benue State of Nigeria Ministry of Health and Human Services with reference number MOH/STA/204/VOL.1/31. Clinical isolates (Stock culture) were obtained from the laboratory benches of the Medical Microbiology Department by 10th March to 3rd June, 2016. Also, samples of wastewater and wastewater sediments were collected in the month of September, 2016 from sewers (gutters) from the two tertiary hospitals. Sites selected for the study were drains from various wards which includes; The Theatre, Female Surgical Ward, Pediatric Ward, Male and Female Medical Wards, Amenity Ward (ward block), Resident doctors hostel and cafeteria, Laboratory (Chemical Pathology, Microbiology, Hematology and Histopathology), Administrative Block, and Accident and Emergency Ward (A and E) BSUTH, whereas FMC samples sites include Laboratory, A Ward (Male Ward 18 years and above), Gynecology Ward, Theatre and Female Ward. Samples were routinely subcultured onto slants prepared from nutrient agar.

Wastewater and sediment samples

Wastewater and sediment samples were collected in the month of September, 2016. The water samples were collected into sterile bottles from the various units aseptically by using disposable micro pipette at each collection unit. Samples were safely transported by road to the laboratory, and immediately analysed.

Sediments were collected by wearing gloves and using clean hand trowel from different wastewater sampling sites to scoop sediments from the bottom of the sewers and introduced into sterile Bijou bottles. The trowel was properly cleaned using alcohol (70% ethanol) before using it in another site to avoid contamination.

Clinical bacterial isolates

Clinical bacterial isolates (Gram-negative multidrug resistant stock culture) were collected from stocks from the laboratory benches of the Department of Medical Microbiology of the hospitals listed above. Collection of clinical isolates was done between 10 March and 3 June, 2016. Isolates were confirmed using different laboratory synthetic media and biochemical tests were done using API 20E. The clinical samples were collected from samples of body fluids (urine and blood samples), swab (high vaginal, endocervical, wound, ear and sputum samples) and stool samples.

Isolation of β-lactam resistant Gram-negative bacteria from environmental sources

Beta lactam resistant Gram-negative bacteria were isolated from wastewater and wastewater sediments. This was done by supplementing peptone water with ampicillin antimicrobial susceptibility test disc 10 μ/g (Oxoid). Stock solution of peptone water was prepared according to manufacturer instructions. 5 ml each was dispensed into an incubating bottle sterilized at 121°C for 15 min and allowed to cool. The sterile ampicillin discs 10 μ/g were aseptically introduced into the sterile peptone water at 50°C to a final concentration of 60 μg/ml.

Water: For the wastewater samples, 1 ml each was introduced into the sterile incubating bottles containing sterile peptone water supplemented with ampicillin discs (60 μ/ml) and incubated for 18-24 h at 37°C.

Sediments: Serial dilutions were carried out with the sediment samples and 1 ml of 10^{-1} diluent was introduced into the sterile peptone water supplemented with ampicillin discs (60 μ/ml) and also incubated for 18-24 h at 37°C.

The 18-24 h incubated water and sediments samples above were subsequently streaked on MacConkey agar with the aid of sterile wire loop and incubated at 37°C for 18-24 h. This was done for all the wastewater and sediments samples.

Statistical analysis

Data obtained were subjected to frequencies and Chi-square analysis, using IBM Statistical Package and Service Solution (SPSS) version 20. The level of significance was defined as P ≤ 0.05.

RESULTS

A total of 403 clinical isolates reportedly, multidrug Gram-negative bacteria were collected from two (2) tertiary hospitals; Federal Medical Centre (FMC), and Benue State Teaching Hospital (BSUTH). Of these, two hundred and seventy-one (271) were from FMC, and 132 from BSUTH. The 403 clinical isolates were Gram stained; out of which two hundred and ninety-nine (299) bacteria were actually confirmed Gram-negative bacteria while 104 of the total of 403 isolates collected were eventually proven to be Gram-positive. Of these two hundred and ninety-nine (299) confirmed Gram-negative bacteria, 218 were from FMC while 81 were from BSUTH.

Of a total of 218 Gram-negative bacteria from FMC, urine had 127(58.26%), high vagina 33(15.14%), wound 26(11.93%), ear 11(5.05%), endocervical 10(4.59%), stool 8(3.67%) and sputum 3(1.38%). Out of a total 81 isolates from BSUTH, urine had 43(53.09%), stool 15(18.52%), wound 8(9.88%), ear 7(8.64%), sputum 4(4.94%), high vaginal swab and blood had 2(2.47%) each (Table 1).

The number/percentage distribution of probable identity of the isolates are shown in Table 2, *Pseudomonas* sp. had 102(46.79%) and 17(20.99%)*; E. coli* 94(43.12%) and 55(67.90%); *Klebsiella* sp. 19(8.72%), and 5(6.17%), while *Proteus* sp. had 3(1.38%) and 4(4.94%) from FMC and BSUTH, respectively. From FMC, *Pseudomonas* sp. was more abundant while from BSUTH, *E. coli* was more abundant in the clinical sample collected. *Proteus* sp. is the least dominant from the two hospitals.

The identified Gram-negative bacteria in the clinical samples from both hospitals were *E. coli*, *Pseudomonas* sp., *Klebsiella* sp., and *Proteus* sp. with a prevalence of 147(49.16%), 121(40.47%), 24(8.03%) and 7(2.34), respectively (Table 3).

Klebsiella sp. has higher percentage distribution in FMC compared to that in BSUTH with a distribution of 95(79.20%) and 25(20.80%) respectively while *Proteus* sp. is higher in BSUTH than FMC with a distribution of 20(57.10%) and 15(42.90%), respectively. The distribution is statistically significant (Table 4).

In the distribution of environmental bacteria isolated from wastewater and sediments from FMC, the highest bacterial distribution is *Proteus stuartii,* with a distribution of 15(100%), while the least is *Proteus vulgaris* with a distribution of 10(20%). From BSUTH, the highest bacterial distribution is *Citrobacter freudii* with a distribution of 100% while the least is *Proteus mirabilis* with a 25% distribution. The bacteria distribution from the two hospitals was statistically significant (Table 5).

Eleven (11) different bacterial species belonging to Six (6) genera were isolated from environmental sources (water and sediment) of the two hospitals which include; *E. coli*, *Shigella sonnei*, *Citrobacter diversus*, *C. freudii, Citrobacter koseri*, *Erwinia chrysanthemi*, *K. pneumonia*, *P. mirabilis*, *P. vulgaris*, *Providencia stuartii* and *Serratia liquifaciens*. Among the 11 bacterial isolated, *S. sonnei* has the highest frequency of isolates from water while the least bacterial isolate is *C. freundii* also from water; *C. koseri* is isolated from both water and sediment, *E.*

Table 1. Percentage distribution of specimen types and their sources in clinical Gram-negative bacteria from FMC and BSUTH.

S/N	Specimen type	BSUTH {No. (%)}	FMC {No. (%)}	Total {No. (%)}
1	Urine	43 (53.09)	127 (58.26)	170 (56.86)
2	High vagina swab	2 (2.47)	33 (15.14)	35 (11.71)
3	Wound swab	8 (9.90)	26 (11.93)	34 (11.37)
4	Stool	15 (18.52)	8 (3.67)	23 (7.69)
5	Ear swab	7 (8.64)	11 (5.05)	18 (6.02)
6	Endocervical swab	0 (0.00)	10 (4.59)	10 (3.34)
7	Sputum	4 (4.94)	3 (1.38)	7 (2.34)
8	Blood	2 (2.47)	0 (0.00)	2 (0.67)
	Total	81 (100.00)	218 (100.00)	299 (100.00)

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 2. Distribution of Gram- negative bacteria from FMC and BSUTH.

S/N	Gram-negative bacteria	BSUTH {No. (%)}	FMC {No. (%)}	Total {No. (%)}
1	*E. coli.*	55 (67.90)	94 (43.13)	147 (49.16)
2	*Pseudomonas* sp.	17 (20.99)	102 (46.79)	121 (40.47)
3	*Klebsiella* sp.	5 (6.17)	19 (8.72)	24 (8.03)
4	*Proteus* sp.	4 (4.94)	3 (1.38)	7 (2.34)
	Total	81 (27.09)	218 (72.91)	299 (100.00)

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 3. Distribution of Gram-negative bacterial in clinical samples from both FMC and BSUTH.

Sample	*Pseudomonas* sp. {No. (%)}	*E. coli* {No. (%)}	*Klebsiella* sp. {No. (%)}	*Proteus* sp. {No. (%)}	Total {No. (%)}
Urine	63 (21.07)	86 (28.76)	17 (5.69)	4 (1.34)	170 (56.86)
HVS	16 (5.35)	19 (6.35)	0 (0.00)	0 (0.00)	35 (11.71)
Stool	3 (1.00)	19 (6.35)	0 (0.00)	1 (0.33)	23 (7.69)
W/S	18 (6.20)	11 (3.68)	3 (1.00)	2 (0.67)	34 (11.37)
E/S	13 (4.35)	5 (1.67)	0 (0.00)	0 (0.00)	18 (6.20)
ECS	7 (2.34)	3 (1.00)	0 (0.00)	0 (0.00)	10 (3.34)
Sputum	1 (0.33)	2 (0.67)	4 (1.34)	0 (0.00)	7 (2.34)
Blood	0 (0.00)	2 (0.67)	0 (0.00)	0 (0.00)	2 (0.67)
Total	121 (40.47)	147 (49.16)	24 (8.03)	7 (2.34)	299 (100.00)

HVS- High Vagina Swab; W/S- Wound Swab; E/S- Ear Swab; ECS- Endocervical Swab.

Table 4. Percentage distribution of *Klebsiella*, *Proteus* and *Pseudomonas species* in clinical from BSUTH and FMC.

Bacterial species	Hospital		Total {No. (%)}
	BSUTH {No. (%)}	FMC {No. (%)}	
Klebsiella sp.	25 (20.8)	95 (79.20)	120 (100.00)
Proteus sp.	20 (57.10)	15 (42.90)	35 (100.00)
Pseudomonas sp.	85 (100.00)	0 (0.00)	85 (100.00)
Total	130 (54.2)	110 (45.80)	240 (100.00)

X^2 = 125.754, df = 2, p = 0.00; BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 5. Distribution of environmental Gram-negative bacterial from BSUTH and FMC.

| Gram-negative bacterial species | Hospital | | Total No. (%) |
	BSUTH {No. (%)}	FMC {No. (%)}	
Citrobacter diversus	25 (55.60)	20 (44.40)	45 (100.00)
Citrobacter freundii	5 (100.00)	0 (0.00)	5 (100.00)
Citrobacter koseri	5 (50.00)	5 (50.00)	10 (100.00)
Erwinia chrysanthemi	0 (0.00)	5 (100.00)	5 (100.00)
E. coli	5 (33.30)	10 (66.70)	15 (100.00)
Klesiella pneumoniae	5 (33.30)	10 (66.70)	15 (100.00)
Proteus mirabilis	5 (25.00)	15 (75.00)	20 (100.00)
Proteus stuartii	0 (0.00)	15 (100.00)	15 (100.00)
Proteus vulgaris	40 (80.00)	10 (20.00)	50 (100.00)
Shigella liquefaciens	0 (0.00)	5 (100.00)	5 (100.00)
Shigella sonnei	5 (50.00)	5 (50.00)	10 (100.00)
Total	95 (48.70)	100 (51.3)	195 (100.00)

X^2 = 56.798, df = 10, p = 0.00; BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 6. Frequency of Gram-negative bacterial isolated from Environmental samples in BSUTH and FMC.

S/N	Gram-negative bacterial	Sources	BSUTH	FMC	Total
1	*Citrobacter diversus*	Water	2	2	4
		Sediment	3	2	5
2	*Citrobacter freundii*	Sediment	1	0	1
3	*Citrobacter koseri*	Water	1	0	1
		Sediment	0	1	1
4	*E. coli*	Water	0	1	1
		Sediment	1	1	2
5	*Erwinia chrysanthemi*	Water	0	1	1
6	*Klebsiella pneumonia*	Water	1	1	2
		Sediment	0	1	1
7	*Providencia stuartii*	Water	0	1	1
		Sediment	0	2	2
8	*Proteus mirabilis*	Water	1	1	2
		Sediment	0	2	2
9	*Proteus vulgaris*	Water	4	1	5
		Sediment	4	1	5
10	*Serratia liquefaciens*	Water	0	1	1
		Sediment	1	0	1
11	*Shigella sonnei*	Water	0	1	1
		Sediment	1	0	1
	Total		19	20	39

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

chrysanthemi from sediment and *S. liquefaciens* from water of equal distribution each (Table 6).

DISCUSSION

The most prevalent clinical bacterial isolate from the 299 confirmed Gram-negative bacteria from the two hospitals were *E. coli* 147(49.16%), followed by *Pseudomonas* sp. 121(40.47%), *Klebsiella* sp. 24(8.03%) and *Proteus* sp. 7(2.34%) as shown in Table 2. This is similar to a study conducted by Okesola and Ige (2012) who recorded *P. mirabilis* as the least dominant bacterial etiology of community acquired pneumonia. This study also correlates

with Rugira et al. (2016) who reported predominance of *E. coli* (51.2%) and *P. mirabilis* among the least occurrence of 2.3% in a tertiary hospital. El-Mahalawy et al. (2005) stated that it is important to recognize the importance of organisms like *E. coli, P. aeruginosa and Klebsiella* species as they cause higher mortality rates compared to Gram positive organisms.

These results indicate that the prevalence of urinary tract infection (UTI) is high in the two hospitals as urine samples (Tables 1 and 3) had the highest prevalence of bacterial isolates. A similar result was reported by Alauvdeen et al. (2017) who also isolated high prevalence of bacteria from urine. This may likely have been related to contamination of colonic bacteria (Ruston, 1997). The most prevalent bacterial isolate from the urine samples are *E. coli* from both hospitals. This result is similar with the report of Devanand and Ramchandra (2013) who reported high *E. coli* isolates from urine samples. Gram-negative bacteria, related to Enterobacteriaceae, in causing UTI have many factors which are responsible for their attachment to the uroepithelium. Their ability to colonize the urogenital mucosa with adhesins, pili, and fimbriae was established (Das et al., 2006).

Eleven (11) different bacterial species (Table 6) belonging to 6 genera were isolated which includes; *E. coli*, *S. sonnei*, *P. stuartii*, *Citrobacter diversus*, *K. pneumonia*, *C. freundii*, *C. koseri*, *E. chrysanthemi*, *P. mirabilis*, *P. vulgaris*, and *S. liquifaciens*. This is similar to the study of Mandal et al. (2011) who also isolated some of these organisms from the environment.

Conclusion

The study shows high level of distribution of *E. coli* and *pseudomonas* species in the clinical samples from the two hospitals while the wastewater and sediment of the two hospitals are contaminated with β-lactam resistant bacteria and can contribute to the spread of these bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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