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Original Article

ESβL and MβL Production in Gram-Negative Bacteria Isolated From HIV Seropositive Individuals

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Abstract

Background: Extended-spectrum β-lactamase (ESβL) or metallo-β-lactamase (MβL) production by gram-negative bacteria in immunocompromised patients poses a serious therapeutic challenge for infection control and is associated with infections with a higher morbidity/mortality, especially in developing countries. This study aimed to phenotypically evaluate the production of ESβL as well as MβL in 75 gram-negative bacterial isolates from clinical samples of the human immunodeficiency virus (HIV) positive individuals.

Methods: Bacterial identification was by chromogenic media, analytical profile index 20 E, and 20 NE kits, and ESβL production was tested by double-disc synergy test (DDST) and combination disc method, while MβL production was screened with imipenem ethylene diamine tetra-acetic acid (EDTA) combined disc and EDTA-disc potentiation with ceftazidime.

Results: Altogether, 57 isolates (76.0%) produced ESβL either with DDST (6), combination disc method (49), or both (2). DDST detected the ESβL enzyme in 10.7% of the tested isolates which were all *Pseudomonas aeruginosa*. None of the bacterial isolates revealed MβL production with the imipenem/imipenem-EDTA method, whereas 26.7% of tested isolates produced MβL with EDTA-disc potentiation using ceftazidime out of which 65.0% were *P. aeruginosa*. Moreover, ESβL/MβL co-production was evident in 22.7% of the tested bacterial isolates with *P. aeruginosa* constituting 64.7%.

Conclusion: ESβL and MβL co-production among the studied isolates indicates a heightened resistance to β-lactam antibiotics, suggesting grave health consequences, especially in immunocompromised individuals with already limiting therapeutic options in the region. The study revealed higher ESβL production compared to MβL production in isolates, with the predominating producing specie being *P. aeruginosa*, and higher ESβL and MβL detection by the combination disc method and EDTA-disc potentiation using ceftazidime, respectively.

Keywords: HIV, Extended-spectrum β-lactamase, Metallo-β-lactamase, Double-disc synergy test, Gram-negative bacteria

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Introduction

Gram-negative organisms, especially the family of Enterobacteriaceae, generally cause both community and nosocomial infections. They are also implicated in secondary infections associated with high mortality in individuals infected with human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (1). The global occurrence of extended-spectrum β-lactamase (ESβL) and metallo-β-lactamase (MβL) production in Enterobacteriaceae is increasing steadily, especially among HIV patients (2) as the over-dependence on β-lactam antibiotics in the hospital setting has led to increased resistance to them in bacteria (3).

ESβLs are enzymes with hydrolytic capabilities to split open the beta-lactam unit of the penicillins, thereby deactivating them. ESβLs can hydrolyze many penicillins, cephalosporins (from first to the fourth generation), and monobactams (aztreonam), excluding the carbapenems or cephamycins (4,5). MβLs, on the other hand, are an Amber class B carbapenemase that hydrolyses all the above antimicrobials along with carbapenems (6) but is powerless against monobactams (7). Various gene clusters of MβLs such as bla_{IMP}, $bla_{NDM,}$ and bla_{VIM} are present on plasmids carried by a wide range of medically relevant bacteria (6).

ESβL and MβL production in bacteria is recurrently linked to co-resistance to non-β-lactam drugs, and thus, displays multidrug resistance to many other antibiotic

classes along with β-lactam antibiotics (3,8-10). This ability may significantly impair the therapy of serious and life-threatening infections and also restrict the management options for infections, especially in immunocompromised individuals.

The prevalence of ESβL and MβL-producing bacteria is on the global rise as the etiology of AIDS, antibiotic resistance patterns, and antibiotic resistance mechanisms in gram-negative bacteria are constantly changing (11). There is evidence that the occurrence of carbapenemase-producing bacteria in Africa is common, and it has also been reported in Nigeria (12,13). Olaitan et al (12) reported the incidence of a multidrug-resistant, OXA-23-producing strain of *Acinetobacter baumannii* among clinical isolates in Ibadan, Southwest Nigeria. The spread of ESβL and MβL-producing bacteria poses a significant threat to health practitioners and the public. HIV infection predisposes infected individuals to opportunistic infections due to immunosuppression, and this is usually a principal cause of morbidity and even mortality (14,15).

With challenges for prevention and curative therapy, HIV-induced immune compromise significantly intensifies the menace of bacterial infections, with possibilities for re-occurrence. This study, therefore, aimed to investigate the production of ESβL and MβL in 75 gram-negative rods recovered from skin and rectal swabs of HIV seropositive patients.

Materials and Methods

Isolate Selection

The bacterial isolates for this study comprised 75 randomly selected and non-repeated gram-negative bacilli isolated from samples obtained from HIV seropositive individuals in an earlier study (16,17). Each strain was unique in terms of species identification or resistance pattern even when more than one isolate was recovered from the same patient.

Previously, the selected bacterial isolates were identified by conventional microbiology techniques, including Gram staining, growth patterns on MacConkey agar, ChromoBio TBX, and Hi Chrome agar, and rapid biochemical tests were conducted with the Analytical Profile Index API 20E and 20NE (bioMérieux, France). The preserved isolates stored at -20°C in Tryptone soy broth containing 15% glycerol were revived in nutrient broth and incubated at 35 ± 2°C overnight. Fresh cultures were then streaked onto sterile MacConkey agar, incubated at 35 ± 2°C overnight, and used for subsequent analyses.

Human Immunodeficiency Virus Viral Load and CD4 T Cell Count

HIV viral load and CD4 T cell count were determined as previously described (17). HIV viral load was evaluated by the Amplicor HIV-1 monitor which is an *in vitro* nucleic acid amplification test (Roche version 1.5, Switzerland), while CD4 T cell count was measured by flow cytometry using the CyFlow Counter SL-3 (Partec, Germany). Data gathered for age, weight, height, CD4, and viral load is presented using descriptive statistics.

Screening of Isolates for the Production of Extended-spectrum β-lactamase

The Double-Disc Synergy Test

This was done as a universal disc diffusion technique using Mueller-Hinton agar (MHA). The latter was inoculated with the test organism. The test isolate was suspended in Ringer solution and standardized to 0.5 McFarland turbidity standard, and inoculation was done using a sterile swab stick to create a lawn of the isolate. Antibiotic discs (Mast Diagnostics) of aztreonam, cefotaxime, ceftriaxone, and piperacillin (30µg each) were arranged at a distance of about 30mm from each other (center-to-center) on the surface of the agar around a ceftazidime/clavulanic-acid (20 µg/10 µg) and subsequently incubated overnight at 37°C (18).

A probable ESβL production was indicated by the expansion of the clear zone of inhibition towards the ceftazidime/clavulanic-acid disc, signifying a synergy between clavulanic acid and any one of the antibiotics employed in screening (18). A negative reaction was recorded when the inhibition zone did not expand around the ceftazidime/clavulanic-acid disc. For the control organisms, American Type Culture Collection isolates *Klebsiella pneumoniae* ATCC 700603 (positive) and *Escherichia coli* ATCC 25922 (negative) strains were used.

The Combination Disc Method

This was done with pairs of discs containing piperacillin with/without tazobactam as well as cefotaxime with/without clavulanic acid (Mast Diagnostics). The preparation of each test organism and inoculation of MHA was performed as described above. The two discs were then placed on opposite sides of the plate inoculated with the test organism. The discs were arranged in such a way that the piperacillin was placed next to piperacillin/tazobactam, and the cefotaxime was placed next to cefotaxime/clavulanate, leaving a space of about 25mm between them. The plates were incubated at 37°C overnight, and the diameters of inhibition zones were then measured (19). ESβL production was implied if the clear zone of inhibition around each combination disc was not less than 5mm larger than that of the cephalosporin alone, or if the clearing around the combination disc was approximately 50% larger than that of the single disc due to the presence of the tazobactam or clavulanic acid (19).

Screening of Isolates for the Production of Metallo-β-Lactamase

Imipenem-Ethylene Diamine Tetra-acetic Acid Combination Disc Method

The isolates were screened phenotypically for the production of MβL using imipenem supplemented with ethylene diamine tetra-acetic acid (EDTA) (20,21). The antibiotics used were imipenem (10 µg) and imipenem-

EDTA, (10 µg/750 µg). Antibiotic/EDTA combined discs were formulated locally by dispensing 20 µL of the 0.1M EDTA (Sigma Chemicals) solution onto 10 µg imipenem discs to attain the required concentration of 750 µg in each disc. These discs were then dried slowly in an oven at 70°C and stored in airtight containers devoid of desiccants at -20°C until required. The standardized broth culture of the test strain prepared as previously described was inoculated on a sterile MHA plate using sterile cotton-tipped applicators and was allowed to dry. One imipenem (10 µg) disc was then dispensed on the surface of the already inoculated MHA agar plate, with another EDTA/imipenem combination disc set at least 25 mm away from it. Incubation was done at 37°C for 18 to 24 hours. MβL-producing strains were confirmed by visually observing and measuring the clear zones of inhibition, which was indicated by an expansion of ≥7 mm around the combination disc as against the single imipenem disc (21).

Ethylene Diamine Tetra-acetic Acid-disc Potentiation Using Ceftazidime

For the EDTA-disc potentiation with ceftazidime, a modification of the methods by Behera et al was used (22,23). Lawns of the test isolates were created on MHA plates, and a 6mm blank disc was placed on the agar surface along with ceftazidime (30 µg) no less than 25 mm from the blank disc. A 10 µL aliquot of EDTA solution (0.5M) was carefully dropped onto the blank disc, and the plates were covered and then incubated at 35°C overnight. Augmentation of the zone of inhibition in the space between the EDTA disc and the ceftazidime disc compared with the size of the clear zone on the other side of the ceftazidime disc was inferred to indicate MβL production.

Results

Gram-Negative Bacterial Distribution in the Human Immunodeficiency Virus Seropositive Patients

The selected isolates, as described in a previous study (16,17), were obtained from 46 HIV-positive patients, comprising 36 females and 10 males (78.3% and 21.7%, respectively), and their ages ranged from 7 to 60 years with a mean of 37.8 years. A total of 26 (56.5%) had commenced antiretroviral therapy, while 43.5% of them were antiretroviral therapy-naive. Moreover, the CD4 T cell count varied widely between 7 cells/µL as the lowest value and 1108 cells/µL as the highest value, with a mean of 483.4 cells/µL. Furthermore, the viral load values ranged between 54 and 4042 HIV-1 RNA copies/mL with a mean value of 553.9 copies/mL (Tables 1 and 2).

A total of 75 non-repetitive gram-negative bacterial isolates belonging to 8 different genera comprising of 10 bacterial species, namely, *Pseudomonas aeruginosa* (49), *Salmonella typhi* (15), *Enterobacter aerogenes, Klebsiella pneumoniae,* and *Serratia liquefaciens* (2 isolates each), as well as *Chryseomonas luteola*, *Citrobacter freundii*, *Enterobacter cloacae, Serratia marcescens, and Proteus mirabilis* (1 isolate each) were assessed phenotypically to detect ESβL and MβL production. All the isolates selected for ESβL and MβL screening were recovered from skin swabs (80%) except *S. typhi* isolates which were cultured from rectal swabs (20%).

Extended-spectrum β-lactamase Production in the Gram-Negative Isolates

Altogether, 57 (76.0%) out of 75 isolates were positive for ESβL production either with the double-disc synergy test (DDST), combination method, or both. Forty-nine of these isolates were positive with the combination method only and six organisms with both methods, while

Table 1. Distribution of HIV Seropositive Participants

Gender	Age Group (y)	CD_4 (cells/µL), n=46				VL (copies/mL), n=46		
		≤200	201–350	351–500	>500	≤400	>400	ND
Male	≤19	0	1	0	1	1	0	1
	20-39	0	1	0	1	0	1	1
	40-59	3	2	0	1	4	0	2
	≥60	0	0	0	0	0	0	0
Female	≤19	0	0	0	0	0	0	0
	20-39	4	5	2	12	12	3	8
	40-59	1	2	3	6	6	3	3
	≥60	1	0	0	0	0	0	1

Note. HIV: Human immunodeficiency virus; VL: Viral load; ND: Not determined.

Table 2. Profile of CD_4 T-Cell Counts and HIV-1 Plasma VL of the Participants

Variables	Male		Female	
	On ART	Not on ART	On ART	Not on ART
Mean CD_4 T-cell count (cells/µL)	333.67 (n=3)	476.71 (n=7)	545.52 (n=23)	411.54 (n=13)
Mean VL values (HIV-1 RNA copies/mL)	131.5 (n=2)	205.50 (n=4)	767.42 (n=19)	190.2 (n=5)

Note. HIV: Human immunodeficiency virus; VL: Viral load; ART: Anti-retroviral therapy.

only 2 organisms were positive with the DDST method alone. The DDST for ESβL production detected the enzyme in 10.7% (8 out of 75) of the isolates (Figure 1). Six (75.0%) out of these were obtained with the synergy of ceftazidime/clavulanic acid with only one other disc, 4 with only piperacillin (50.0%), and 2 with only ceftriaxone (25.0%), as depicted in Table 3. The remaining 2 ESβL-positive isolates were obtained with synergy with 3 other discs, namely, piperacillin/ceftriaxone/cefotaxime and aztreonam/ceftriaxone/cefotaxime, respectively. It is noteworthy that all the ESβL-positive isolates detected with the DDST screening method were *P. aeruginosa.* Overall, 55 (73.3%) isolates tested positive for ESβL production using the combination disc method. ESβL production was detected in 47 (85.5%) of the isolates with piperacillin and piperacillin/tazobactam combination only, including *P. aeruginosa* (59.6%), *S. typhi* (23.4%), *Enterobacter aerogenes* and *Serratia liquefaciens* (4.3%), and each of *Chryseomonas luteola, Citrobacter freundii, Klebsiella pneumonia,* and *Proteus mirabilis* at 2.1%. ESβL production was detected in only 3 *P. aeruginosa* (5.4%) strains using cefotaxime/clavulanate (Table 3). However, 5 (9.1%) out of the ESβL producing strains were identified with the use of the two combinations employed in this study, including *P. aeruginosa, Enterobacter cloacae, S. typhi,* and *S. marcescens*.

Metallo-β-Lactamase Production in the Gram-Negative Isolates

MβL production was detected in none of the isolates when screened with imipenem/imipenem-EDTA, whereas, 20 (26.7%) of the isolates showed a positive result when screened with EDTA-disc potentiation using ceftazidime (Figure 1), 13 (65.0%) of which were *P. aeruginosa*, 6 (30.0%) were *S. typhi*, and the last isolate was *S. marcescens* (5.0%).

Extended-spectrum β-Lactamase and Metallo-β-Lactamase Co-Production

ESβL and MβL co-production was observed in 22.7% (17/75) of the screened isolates, and 11 (14.7%) of these were *P. aeruginosa.* Other species were *S. typhi* (6.7%) and *S. marcescens* (1.3%).

Discussion

This study was designed to detect the production of ESβLs and MβLs in 75 gram-negative bacteria that were isolated from various samples obtained from HIV-positive individuals using phenotypic screening methods. The advent and spread of ESβL- and carbapenemase-producing gram-negative bacteria is regarded as a major public health issue, most especially in immunocompromised individuals as found in HIV seropositive patients.

The current study revealed an elevated number of ESβL producers amongst the screened isolates, with *P. aeruginosa* predominating. In total, 76.0% of the screened isolates produced ESβL, whereas only 26.7% of isolates produced MβL with EDTA-disc potentiation using ceftazidime. Of these ESβL producers, 65.0% were *P. aeruginosa* as ESβL production was detected in 67.3% (33/49) of tested *P. aeruginosa* strains and constituted 60.0% of all the isolates which tested positive with the combination disc method (33/55), while MβL production was evident

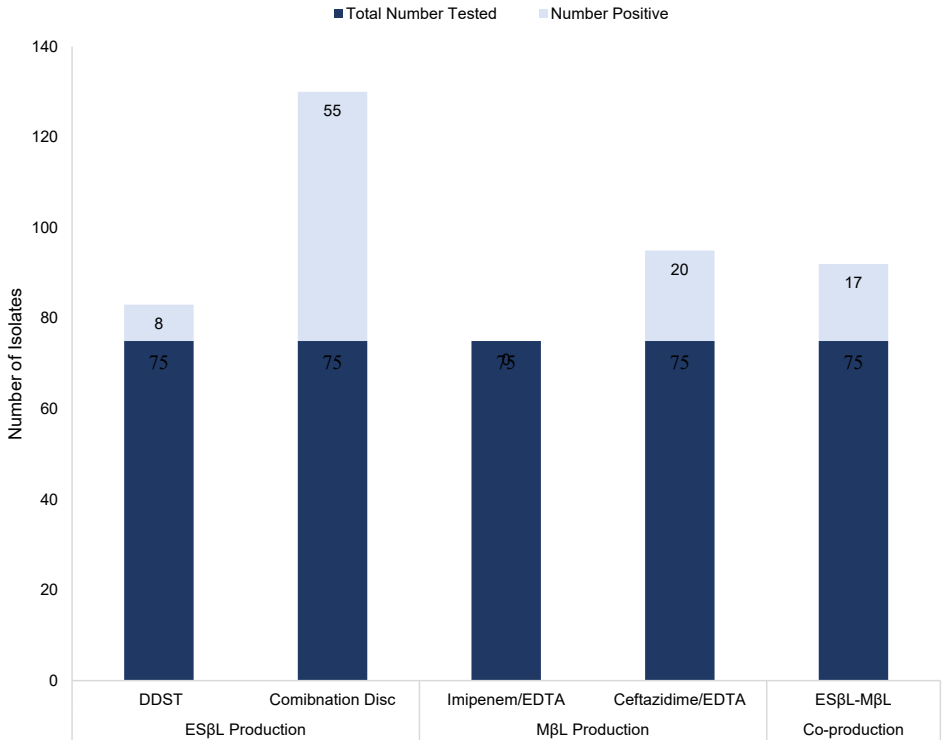

Figure 1. Frequency of Occurrence of the ESβL and MβL Producers. *Note.* ESβL: Extended spectrum β-lactamase; MβL: Metallo-β-lactamase; DDST: Double-disc synergy test; EDTA: Ethylene diamine tetra acetic acid

Table 3. Frequency of Occurrence of the ESβL and MβL Producing Gram-Negative Bacterial Isolates

Bacterial Specie	Total Number Tested	ESβL									MβL	
		DDST					Combination Disc				Combination Disc	EDTA-Disc Potentiation
		1	2	3	4	Number Positive	PRL/ PTZ	CTX/ CTCV	Both	Number Positive	Imipenem/ EDTA	Ceftazidime/ EDTA
Chryseomonas luteola	1	0	0	0	0	0	1	0	0	1	0	0
Citrobacter freundii	1	0	0	0	0	0	1	0	0	1	0	0
Enterobacter aerogenes	2	0	0	0	0	0	2	0	0	2	0	0
Enterobacter cloacae	1	0	0	0	0	0	0	0	1	1	0	0
Klebsiella pneumoniae	2	0	0	0	0	0	1	0	0	1	0	0
Proteus mirabilis	1	0	0	0	0	0	1	0	0	1	0	0
Pseudomonas aeruginosa	49	6	0	2	0	8	28	3	2	33	0	13
Salmonella typhi	15	0	0	0	0	0	11	0	1	12	0	6
Serratia liquefaciens	2	0	0	0	0	0	2	0	0	2	0	0
Serratia marcescens	1	0	0	0	0	0	0	0	1	1	0	1
Total	75	6	0	2	0	8	47	3	5	55	0	20

Note. ESβL: Extended spectrum β-lactamase; MβL: Metallo-β-lactamase; DDST: Double-disc synergy test; PRL: Piperacillin; PTZ: Piperacillin with tazobactam; CTX: Cefotaxime; CTCV: Cefotaxime with clavulanic acid; EDTA: Ethylene diamine tetra acetic acid; "1-4": Detection with 1, 2, 3, or 4 different discs.

in 26.5% (13/49) of screened *P. aeruginosa* strains and made up 65.0% of all MβL positive organisms using the EDTA-ceftazidime disc potentiation test. Different values have also been recorded by various authors from several studies. This corresponds with a study by Begum et al (24) in which a prevalence value of 37.8% was reported for ESβL-producing gram-negative bacteria, with 90.2% being *P. aeruginosa,* highlighting the high rate of ESβL production in this species. The rates reported in our study are, however, higher than those in Basak et al (25) at 40% and 11.2% for ESβL and MβL production, respectively.

Various reports of MβL production prevalence rates have been recorded ranging from 69.5% in imipenem-resistant *P. aeruginosa* isolates (26), 54.0% (27), and 43.6% in clinical isolates (28), and all values were much higher than those obtained in our study. However, these values were lower than those reported for MβL production from *Pseudomonas* species by Franco et al (29) who reported MβL production of 77.0%. More recently, phenotypic testing of gram-negative bacterial isolates suggested the presence of an ESβL and a carbapenemase in 14.0% of isolates (30), while Rani et al (31) detected ESβL production in 37.3% of the isolates in their study. At variance with these rates is the report of 0.2% MβL production by *Pseudomonas* species (32), while Thapa et al (7) reported MβL production in 5.8%, prevalently from *Acinetobacter calcoaceticus baumannii* complex followed by *P. aeruginosa.* More recently, another study (33) reported ESβL and carbapenemase prevalence rates of 61.0% and 28.0% from Ghana, respectively, a rate more in agreement with that recorded in the present study.

There is quite a lot of data available on the prevalence of ESβL enzyme production among members of the family Enterobacteriaceae, however, there is a dearth of literature on the *P. aeruginosa* family. The phenotypic analyses endorsed by European Committee on Antimicrobial Susceptibility Testing (EUCAST) for detecting ESβL production in Enterobacteriaceae are not always appropriate for the detection of the same in *P. aeruginosa*. This could be a result of the variations in the families of ESβLs present in *P. aeruginosa* isolates and within the Enterobacteriaceae, possible co-production of MβLs, and the production and overexpression of natural AmpC cephalosporins. Further, carbapenem resistance in *P. aeruginosa* could be a result of the loss in permeability caused by the loss of the oprD porin, the improved regulation of a dynamic efflux system, or the production of MβLs (34). Carbapenem-hydrolysing MβLs have been widely studied and reported by various authors in numerous countries; hence, they are considered the most vital mechanism of resistance to carbapenems in *P. aeruginosa* (34,35).

No result was obtained for the detection of MβL production with the imipenem/EDTA combination even though this method has been reported to be more effective than the DDST or the EDTA-disc potentiation test (36). The reason for this is unknown but could be attributed to the existence of extra imipenem/EDTA combination resistance mechanisms in these isolates. If this is the case, this method may not detect MβL production as suggested previously (36).

A study from Tanzania reported that ESβL-producing bacterial strains were significantly more prevalent in HIV-infected children than in their HIV-negative counterparts as HIV-positive children were ten times more likely to be ESβL carriers than the control group of HIV-negative children (37). Few reports exist on the likelihood of HIV status being a risk factor for ESβL carriage; nevertheless, HIV-positive individuals are more susceptible to opportunistic infections, and are, therefore, expected to be more frequently on hospital admission, and tend to be administered more antimicrobials than HIV-negative persons.

The production of antibiotic-hydrolyzing enzymes

from bacterial isolates detected in this study is a strong indication of possible multidrug resistance, probably due to the constant use of multiple drugs associated with the category of patients from which the isolates were obtained. It is also likely that frequent visits to the hospital or hospitalization may impact colonization by microorganisms as a result of constant exposure. This agrees with reports of other authors who reported that patients during hospitalization acquired microorganisms at rates of 47.5% and 94.0%, respectively (38,39)

A salient fact is a probability that many of these microorganisms might have already acquired resistance to certain antibiotics, which is a trait easily transferable between organisms by horizontal gene transfer and common in the healthcare setting. This trend is worrisome because such bacterial strains are implicated in infections attributed to more severe illness and even death. Furthermore, since MβLs have hydrolytic capabilities against ß-lactams of all classes, and research is still ongoing in the search for nontoxic alternative remedies for infectious ailments, the sustained rise in their prevalence would portend serious public health challenges and could be a clinical catastrophe.

The presence and persistence of an ESβL- or MβL-producer is not only problematic for treatment but also creates a major challenge for the management and control of infections. Since their detection is challenging, their roles in spread within clinical settings and beyond as well as horizontal MβL gene transfer between pathogens go largely unnoticed, thereby posing substantial risks to colonizing hosts (3). The continued local surveillance of organisms capable of producing antibiotic-hydrolyzing enzymes in hospitals may play a valuable role in tracking emerging resistant traits in such strains and will help track outbreaks of infections by such strains. It will also aid therapeutic options in severely affected patients, ultimately reducing ineffective prescriptions and shortening hospital stays. Clinicians and microbiologists should also be regularly updated with local surveillance information.

Conclusion

The global emergence of ESβLs and MβL production among clinically important gram-negative bacteria is a growing problem, especially in Africa. Nevertheless, the magnitude of the problem is not fully conveyed because of the inadequate number of studies highlighting the need for detection and pinpointing resistance mechanisms, especially in Nigeria. Recommendations to combat this scourge in Nigeria include the routine screening, characterization, and reporting of the presence of gram-negative organisms with these traits in the healthcare setting, instigating antimicrobial stewardship programs, and prohibiting the sale of antimicrobial agents without a prescription. Other measures of control may include the improvement and continued development of essential infection control measures such as hand hygiene, equipping microbiology laboratories to detect the emergence of new strains, and the expansion of regional surveillance on gram-negative bacteria with ESβL and MβL producing traits both in the hospital and in the community.

Authors' Contribution

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Supervision: Folasade Muibat Adeyemi.
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Writing–review & editing: Folasade Muibat Adeyemi, Sunday Babatunde Akinde.

Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

The study was reviewed and approved by both the Ethical Review Board of the Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife, Osun State, Ile-Ife, as well as the Ondo State Specialist Hospital Management Board, Akure, Nigeria (Protocol Number ERC/2012/11/05).

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