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PCR Detection of Extended Spectrum β-Lactamase from Some Gram Negative Bacteria of Clinical Source

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Abstract

Beta-lactamase-producing microorganisms present distinct challenges in health care systems around the world as a result of the extensive utilization of broad-spectrum antibiotics. The objective of this research endeavor was to ascertain the antibiotic resistance profile and presence of β-lactamase genes in Gram-negative bacteria that had been previously identified and collected from laboratory work benches at the Department of Microbiology and Parasitology, UCH, located in Ibadan, South-West Nigeria. The Kirby-Bauer disc diffusion method was used to assess antibiotic susceptibility on all the isolates, and the double disc synergy test was used to validate extended spectrum beta-lactamase (ESBL) production phenotypically. The PCR techniques were used to detect β-lactamase genes. The sample distribution of the forty clinical isolates were collected from the work benches and the distribution was: urine (17), blood (9), wound (6), sputum (4), amniotic fluid (3) and tracheal aspirate (1). Klebsiella pneumoniae was the most prevalent organism with 17 isolates (42.5%). Out of the 40 isolates that were obtained, 21 were ESBL producers and the distribution was as follows: Klebsiella pneumoniae (9), Escherichia coli (6), Enterobacter cloacae (2), Enterobacter aerogenes (1), Pseudomonas aeruginosa (1), Acinetobacter baumannii (1) and Hafnia alvei (1). The majority of the clinical isolates showed considerable resistance to antibiotic classes that were tested, with amoxicillin-clavulanate showing the highest resistance rate at 65%. Out of all the ESBL producers, 35.3% had bla_{TEM}, 29.4% had bla_{CTX-M}, 23.5% had bla_{SHV}, and 11.8% had bla_{CMY}.
AmpC β-lactamases, Antibiotic resistance, Disc diffusion, Gram-negative bacteria, Polymerase chain reaction.

Keywords: AmpC β-lactamases, Antibiotic resistance, Disc diffusion, Gram-negative bacteria, Polymerase chain reaction.

INTRODUCTION

The development of effective antibiotics has been a game-changer in the fight against bacterial illnesses. Nevertheless, numerous bacterial species have developed antibiotic resistance or

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multidrug resistance due to the extensive use of antibiotics. Unfortunately, drug-resistant diseases, such as multidrug-resistant gram-negative bacteria, are notoriously hard to detect with standard diagnostic procedures, and there is a severe shortage of novel antibiotics (Muteeb *et al.,* 2023). According to Hussain *et al.* (2021), the predominant mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria is by the synthesis of β-lactamases. The two most frequent types of β-lactamases are ESBLs and AmpC β-lactamases (Shaaban *et al.,* 2022).

AmpC β-lactamases have gained importance for over than forty years since their discovery as one of the enzymes responsible for antibiotic resistance in Gram-negative bacteria (Zhou *et al.,* 2022). AmpC β-lactamases are either plasmid or chromosomal mediated. The increased presence of plasmid mediated AmpC β-lactamases worldwide is becoming of great concern because most clinical laboratories and physicians remain unaware of their clinical importance, and therefore have been responsible for several nosocomial outbreaks (Xiong *et al.,* 2021). Without accurate laboratory detection and reporting of such resistant phenotypes and strains producing plasmid-mediated AmpC, treatment of Gram-negative infection may remain suboptimal (Flannery *et al.,* 2022).

ESBLs are most often a plasmid mediated heterogeneous group of β-lactamase enzymes that confer resistance to a wide range of commonly used β-lactam antibiotics (Bush and Bradford, 2020). TEM and SHV type ESBLs used to be the dominant ESBL genotypes (Dirar *et al.,* 2020). However, in the past decade, the CTX-M type ESBLs have become the most widely distributed and globally dominant genotypes (Castanheira *et al.,* 2021). Infections caused by ESBL-producing Gram negative bacteria are associated with increased morbidity and mortality. This scenario is linked to inappropriate, indiscriminate or delayed antimicrobial treatment (Husna *et al.,* 2023).

In Nigeria, extended-spectrum cephalosporins and fluoroquinolones are widely used as broad-spectrum antibiotics and remain the drugs of choice to treat infections caused by various Gram-negative pathogens (Nwafuluaku *et al.,* 2021). Therefore, this study is aimed to carry out phenotypic and genotypic characterization of β-lactamase genes and determine the antibiotic resistance pattern in Gram- negative bacteria obtained from University College Hospital, Ibadan, Oyo State, Nigeria.

Figure 1: Geological Map of Ibadan

MATERIALS AND METHODS

Study Area

The University of Ibadan (UI), situated in the vibrant city of Ibadan, the capital of Oyo State, Nigeria, serves as the primary research institution for this study. UI is located at approximately Latitude 7° 23' 28.19" N and Longitude 3° 54' 59.99" E.

Collection of samples

The isolates were obtained from different clinical samples such as sputum, amniotic fluid, urine, tracheal aspirate, wound and blood at the Department of Microbiology and Parasitology, University College Hospital (UCH), Ibadan, Oyo State. Ethical approval was sought before sample collection to adhere to ethical standards and institutional guidelines.

Sterilization of Media and Materials

Muller Hinton agar was prepared following the manufacturer's instructions and sterilized using autoclave at 121°C for 15 minutes. The media were purchased from Oxoid Limited. All glass wares were washed with detergent, rinsed, dried, wrapped in aluminum foil, and sterilized in a hot air oven at 180°C for 6 hours.

Identification of Isolates

The isolates were identified using the MicrobactTM Gram Negative System Identification Kit (Thermo Scientific-Oxoid, UK). The results were transcribed in to a code and organisms were identified using a computerized profile register.

Determination of Antibiotic Resistance

The experiment was conducted using the disc diffusion method (Krishnan *et al.,* 2019), following the instructions provided by CLSI (2018) guidelines on Muller-Hinton agar after normalization of broth to 0.5 McFarland standard to test all the isolates against the panel of eleven different antibiotics. The antibiotics utilized were ciprofloxacin (5 µg), aztreonam (30 µg), gentamicin (10 µg), cefpodoxime (10 µg), Cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), azithromycin (15 µg), amoxicillin-clavulanate (30 µg), cefepime (30 µg), cefuroxime (30 µg) (Oxoid, UK).

Phenotypic Detection of ESBL

The clinical isolates were examined for potential production of extended-spectrum beta-lactamase (ESBL) using the double disc synergy test (DDST) in accordance with the standards set by the Clinical Laboratory Standards Institute (CLSI, 2018), Figure 2.

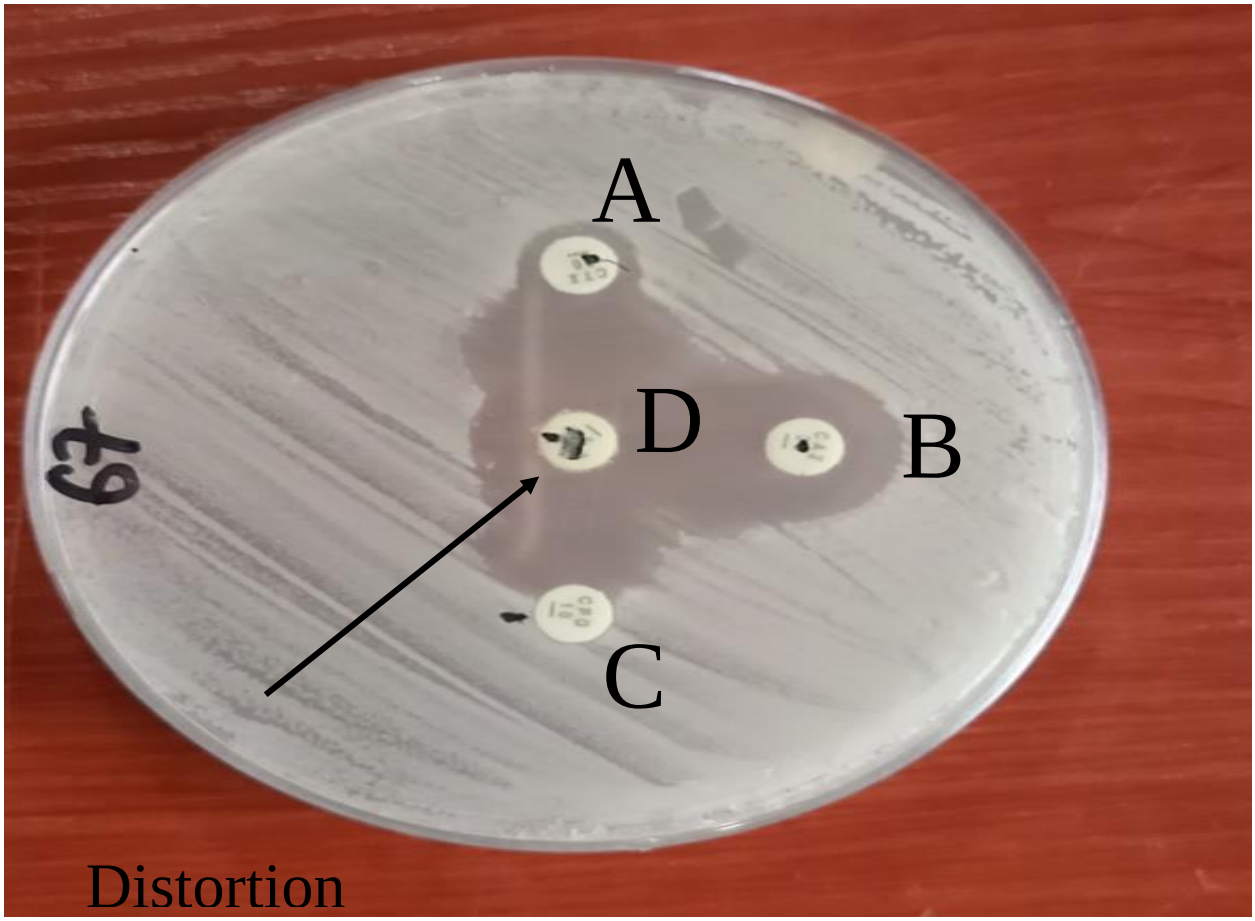

Figure 2:Double Disc Synergy Test (DDST) for detection of ESBL production
Note: A larger area of inhibition or distortion surrounding ceftazidime, cefpodoxime, and cefotaxime when compared to amoxicillin clavulanate showed a positive result for extended-spectrum beta-lactamase (ESBL) synthesis.
Key: A = Cefotaxime (CTX); B = Ceftazidine (CAZ); C= Cefpodxime (CPD); D = Amoxicillin Clavulanate (AMC)

Identification of ESBL and AmpC genes in isolates that produce ESBLs

The genomic DNA of the bacterial isolates that exhibited a positive phenotypic ESBL test was extracted using a Zymo Research Bacterial DNA MiniPrepTM Kit (Zymo Research Corporation, USA) according to the instructions provided by the manufacturer. The detection of genes was performed using the conventional PCR technique with specified primers. The nucleotide sequences of the target genes are displayed in Table 1.

Table 1: Sequences of primers in detecting extended spectrum andAmpCβ-lactamase genes

Target genes	Sequences (5′ – 3′)	Reference
TEM Primers	F-GCATCTTACGGATGGCATGA R-GTCCTCCGATCGTTGTCAGAA	Roschanski *et al.,* 2014
SHV Primers	F-TCCCATGATGAGCACCTTTAAA R-TCCTGCTGGCGATAGTGGAT	Roschanski *et al.,* 2014
#CTX-A Primers	F-CGGGCRATGGCGCARAC R-GCRCCGGTSGTATTGCC	Roschanski *et al.,* 2014
#CTX-B Primers	F-ACCGAGCCSACGCTCAA R-CCGCTGCCGGTTTTATC	Roschanski *et al.,* 2014
CMY Primers	F-GGCAAACAGTGGCAGGGTAT R-AATGCGGCTTTATCCCTAACG	Roschanski *et al.,* 2014

Note: #CTX-A was designed for the detection $bla_{\text{CTX-M-1 and}}$ $bla_{\text{CTX-M-9}}$;
#CTX-B was designed for the detection of $bla_{\text{CTX-M-2 and}}$ $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-25}}$

RESULTS

Among the 40 clinical isolates, 17 were identified as *Klebsiella pneumoniae*, 11 were *Escherichia coli*, 6 were *Pseudomonas aeruginosa*, 4 were *Enterobacter* spp., and there was 1 each of *Hafnia alvei* and *Acinetobacter baumannii*. The analysis of organisms obtained from different clinical samples in this study revealed that urine had the highest prevalence of clinical Gram-negative bacteria, accounting for (17/42.5%) The sequence of prevalence was followed in that order by blood (9/22.5%), wound (6/15%), sputum (4/10%), amniotic fluid (3/7.5%), and tracheal aspirate specimen (1/2.5%) had the lowest prevalence.

Figure 3 displays the resistance rates of Gram-negative bacteria derived from clinical sources against specific drugs. Among the 40 isolates, the highest resistance rates were observed for amoxicillin clavulanate, with 26 isolates (65%) being resistant. This was followed by cefepime, with 25 isolates (62.5%) showing resistance. Additionally, 22 isolates (55%) were resistant to gentamicin, while 21 isolates (52.5%) exhibited resistance to aztreonam, cefoxitin, ceftazidime, cefotaxime, and ampicillin sulbactam. Out of the clinical Gram-negative bacteria, only 20 (50%) exhibited resistance to ciprofloxacin, cefpodoxime, and azithromycin, respectively.

Among the 40 isolates that were examined for ESBL production, 21 were found to be positive, resulting in an overall prevalence rate of 52.5%. The prevalence of ESBLs was highest in *Acinetobacter baumanni* (100%) and *Hafnia alvei* (100%) respectively, followed by *Enterobacter* spp. (75%), *Escherichia coli* (54.5%), *Klebsiella pneumoniae* (52.9%), and the lowest frequency of ESBL producing bacteria was observed in *Pseudomonas* spp. (16.7%).

The PCR technique was used for the molecular characterization of the isolates' resistance genes. After screening for ESBL-associated genes (bla_{SHV}, bla_{TEM}, and bla_{CTX-M}), only 18 of 21 phenotypic ESBL-positive isolates (85.7%) tested positive, suggesting that the phenotypic tests were quite sensitive. Among ESBL positive isolates, the bla_{TEM} gene was the most common at 35.3%, followed by bla_{CTX-M} at 29.4%, bla_{SHV} at 23.5%, and bla_{CMY} at 11.8%. Both *Klebsiella pneumoniae* 50 and *Escherichia coli* 192 showed a strong presence of all three gene groups. Only four (22.2%) of the isolates had the bla_{TEM} gene alone; the rest, with the exception of one *Klebsiella* spp. isolate, had the bla_{CTX-M} gene in conjunction with the bla_{TEM} and/or SHV/CMY genes. Table 2 provides a summary of the β-lactamase gene distribution across ESBL producers.

Table 2: ESBL and AmpC β-lactamase gene profile of bacteria isolated from clinical samples

Isolate identity	Source	Beta-lactamase genes detected
K. pneumoniae 134	Urine	bla_{CTX-M},bla_{TEM}
K. pneumoniae 131	Urine	bla_{TEM}
K. pneumoniae 67	Urine	bla_{TEM}
K. pneumoniae 129	Urine	bla_{CTX-M}, bla_{SHV}
K. pneumoniae 24	Sputum	bla_{CTX-M}
K. pneumoniae 193	Sputum	bla_{TEM}, bla_{SHV}
K. pneumoniae 0001	Sputum	bla_{TEM}, bla_{SHV}
K. pneumoniae 50	Blood	bla_{CTX-M}, bla_{TEM}, bla_{SHV}
K. pneumoniae 53	Blood	bla_{SHV}
E. coli 12	Urine	bla_{TEM}, bla_{CMY}
E. coli 191	Urine	bla_{TEM}
E. coli 17	Blood	bla_{CTX-M}, bla_{SHV}
E. coli 192	Blood	bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{CMY}
E. coli 13	Tracheal aspirate	bla_{CTX-M}, bla_{TEM}, bla_{SHV}
E. coli 190	Amniotic fluid	None
E. cloacae 002	Wound	bla_{CTX-M}, bla_{CMY}
E. cloacae 195	Wound	bla_{CTX-M}, bla_{CMY}
*E. aerogenes*196	Wound	bla_{CTX-M},bla_{TEM}
Pseudomonas spp. 94	Urine	bla_{TEM}
A. baumannii 56	Urine	None
H. alvei	Wound	None

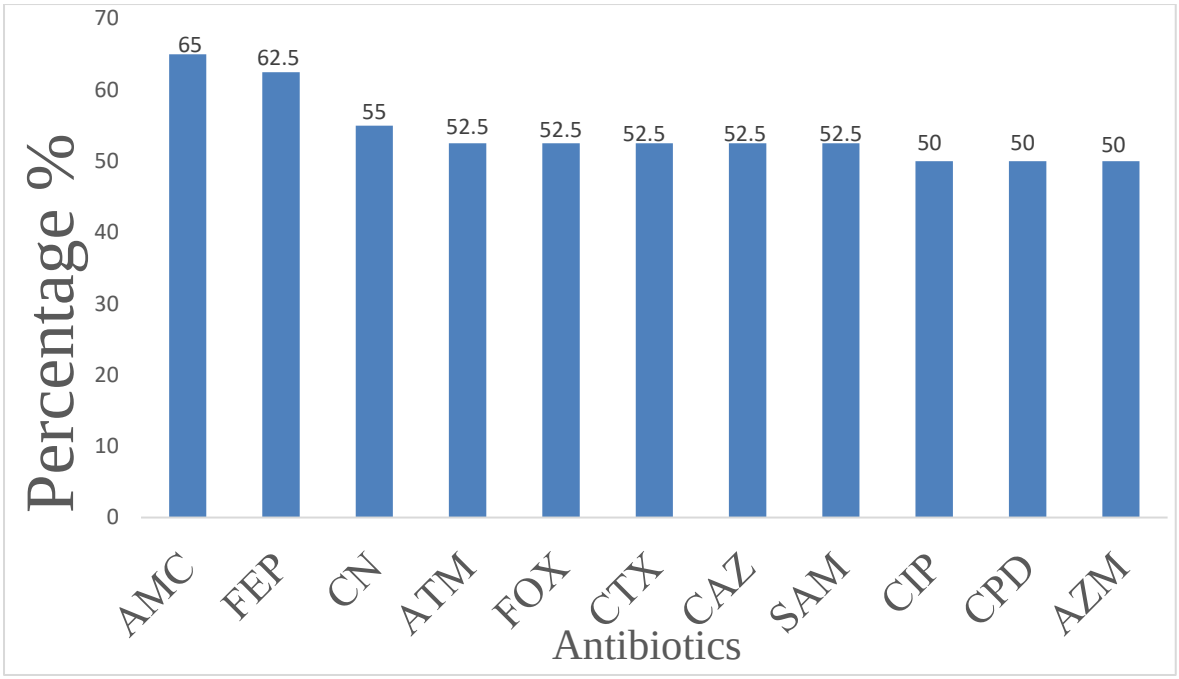

Figure 3: Resistance of Clinical Gram-negative bacteria to selected classes of antibiotics
Key: AMC = Amoxicillin-clavulanate; FEP = Cefepime; CN = Gentamicin; ATM = Aztreonam; FOX = Cefoxitin; CTX = Cefotaxime; CAZ = Ceftazidime; SAM = Ampicillin sulbactam; CIP = Ciprofloxacin; CPD = Cefpodoxime; AZM = Azithromycin

DISCUSSION

The rate of antibiotic resistance is increasing at a faster pace than the development of new treatments in clinical practice, which is causing a worldwide health burden (Salam *et al.,* 2023). Extended Spectrum Beta-Lactamase (ESBL) synthesis by bacteria is the predominant method that has rapidly disseminated globally and has been recently observed in clinical settings (Husna *et al.,* 2023).

The study revealed that *K. pneumoniae* accounted for 42.5% of the clinical isolates, making it the most frequently observed, followed by E. coli at 27.5%. This finding aligns with the study conducted by Wyres and Holt (2018), identified *K. pneumoniae* as the prevailing organism in clinical environments.

Clinical Gram-negative bacteria were most common in urine (42.5%) and blood samples (22.5%) among the clinical specimens. This finding is in agreement with that of Elbadawi *et al.* (2020), who also discovered that clinically-obtained Gram-negative bacteria were most common in blood (16.4% prevalence) and urine (61.2%) specimens, though there were differences in the percentages or values obtained due to variables such as the amount of samples analyzed and the duration of the research. Isolates of *Klebsiella pneumoniae* were the most common in the urine, which is in line with the findings of Hasan *et al*. (2021), who found that Klebsiella pneumoniae was the leading cause of UTIs in humans.

The prevalence of antibiotic resistance among the clinical isolates in this study was comparable to that reported by Ebrahim-Saraie *et al*. (2019). Out of all the isolates, most of them exhibited considerable resistance to certain classes of antibiotics. Additionally, a significant number of isolates had reduced sensitivity to potentially effective medicines such amoxicillin clavulanate, cefepime, gentamicin, cefotaxime, and cefpodoxime.

The detection of Gram-negative bacteria in urine, blood, amniotic fluid, tracheal aspirate, sputum, and wound specimens, which have the capability to create ESBL, is consistent with the findings of Gharavi *et al.* (2021), Ionescu *et al.* (2022) and Isogami *et al*. (2023) observed comparable rates of ESBL generation in bacteria in their respective studies, similar to the findings of our study. ESBL-producing Gram-negative bacteria were primarily detected in urine samples (33%) and to a lesser extent in blood samples (23.8%). This could be attributed to the increased quantity of urine and blood samples incorporated in this investigation.

The study found that 52.5% of the bacteria analyzed were producing ESBL. Notably, *Acinetobacter baumanni* and *Hafnia alvei* had the highest prevalence, with 100% of the isolates producing ESBL. This contradicts previous studies that identified *Escherichia coli* and *Klebsiella* species as the main ESBL producers (Naeem *et al*., 2021). It is worth noting that the number of isolates may have influenced these findings. The incidence of hospital-acquired infections caused by strains expressing bla_{TEM}, $bla_{\text{CTX-M}}$, and bla_{SHV} has significantly risen in the past decade (Mohamed *et al.,* 2020). Out of the ESBL phenotypic strains validated by PCR in this investigation, 21 strains showed positive genotypes for at least one of the ESBL genes investigated, accounting for 85.7% of the total. The *Klebsiella* isolates discovered in this investigation exhibited three forms of extended-spectrum beta-lactamases (ESBLs): SHV-type, CTX-M, and TEM.

The study identified $bla_{\text{TEM 12}}$ as the most prevalent gene (35.3%), followed by $bla_{\text{CTX-M 10}}$ (29.4%) and $bla_{\text{SHV 8}}$ (23.5%). This finding aligns with previous studies that have reported a high prevalence of ESBLs in hospital infections, with a significant proportion of these strains carrying the bla_{TEM} gene (Ibrahim *et al.,* 2021). Nevertheless, this contradicts the findings of Zeynudin *et al*. (2018) who identified the $bla_{\text{CTX-M}}$ gene as the most prevalent kind of extended-spectrum beta-lactamase (ESBL) in clinical settings worldwide. The discrepancy observed in this study, in relation to other studies on the prevalence rate of ESBLs, could be attributed to regional disparities. These disparities may arise from variations in the prevalence of TEM-type ESBLs across different geographic regions, differences in the level of healthcare facilities involved, variations in the type and quantity of antibiotics consumed, and disparities in the time period during which the isolates were collected (Mirkalantari *et al*., 2020).

Conclusion

ESBLs are regarded as one of the most significant mechanisms of antibiotic resistance. The high rates of resistance observed in this study are consistent with the prevalence of resistance genes, which greatly reduce the effectiveness of β-lactam antibiotics. *Klebsiella pneumoniae* was identified as the prevailing Gram-negative bacterium in this study. Most ESBL generating isolates exhibited resistance to drugs commonly employed for the treatment of various illnesses. This study demonstrated a significant occurrence of Extended-Spectrum Beta-Lactamase (ESBL) synthesis among Gram-negative bacteria found in clinical settings. This finding is supported by the identification of ESBL production in 21 out of 40 isolates, constituting 52.5% of the total clinical isolates. The prevalence of ESBL producers was highest in urine and blood samples. Beta-lactamase genes were absent in three of the ESBL-producing organisms, while AmpC genes were only found in isolates from urine and wound samples. This work presents additional evidence of the worldwide spread of bla_{TEM}, $bla_{\text{CTX-M}}$, and bla_{SHV} genes, highlighting the importance of implementing effective epidemiological surveillance.

The identification of ESBL production is crucial in both hospital and community isolates. Implementing effective infection-control measures and utilizing barriers are crucial considerations for limiting the transmission and occurrence of ESBL-producing bacteria. At the institutional level, it is necessary to adopt rapid diagnostic techniques for the effective monitoring and treatment of these strains in hospitals. Additionally, policies should be put in

place to restrict the indiscriminate use of broad-spectrum antibiotics, such as third- and fourth-generation cephalosporins and quinolones.

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