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*Original Article*

# Phenotypic and PCR-Based Detection of ESBL Genes in Gram-Negative Bacteria Isolated from Suppurative Wound Infections at CHUD-BA, Benin

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## Abstract

Introduction: Wound infections represent a major therapeutic challenge, particularly in low- and middle-income countries where antimicrobial resistance is rapidly increasing. Gram-negative bacilli are of special concern due to their ability to produce extended-spectrum β-lactamases (ESBLs), which compromise β-lactam therapy. However, molecular data from northern Benin remain scarce. This study aimed to determine the antimicrobial resistance profiles and detect major ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$) among Gram-negative bacteria isolated from suppurative wound infections at CHUD-BA, Benin.

Methodology: A prospective study was conducted from June 2021 to February 2022. Gram-negative bacteria were isolated from infected wound samples. Identification was performed using standard biochemical tests, the API 20E system, and oxidase testing where appropriate. Antimicrobial susceptibility testing followed EUCAST 2021 guidelines. Biofilm production was assessed using the quantitative microtiter plate assay. ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$) were detected by PCR using appropriate controls.

Results: Out of 107 pus samples, 51/107 (47.7%) yielded Gram-negative bacilli. *Escherichia coli* accounted for 24/51 (47.1%) isolates, followed by *Klebsiella pneumoniae* (11.76%), *Klebsiella ornithinolytica* (9.80%), and *Klebsiella oxytoca* (7.84%). The isolates exhibited multidrug resistance, and 39.6% of the gram-negative bacilli were biofilm producers. ESBL genes were detected as follows: *bla*$_{CTX-M}$ (17/50; 34%), *bla*$_{TEM}$ (14/50; 28%), and *bla*$_{SHV}$ (4/50; 8%).

Conclusions: The high prevalence of multidrug resistance, ESBL genes, and biofilm-forming capacity among Gram-negative wound isolates underscores the urgent need for antimicrobial stewardship and infection control strategies in Northern Benin.

**Key words:** infection; suppuration; Gram-negative bacteria; bacterial resistance; ESBL.

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## Introduction

Wound care represents an essential component of healthcare practice and plays a critical role in reducing morbidity and improving patient quality of life. Wounds range from acute lesions, such as surgical incisions, burns, and traumatic injuries, to chronic wounds, including diabetic foot ulcers, venous leg ulcers, and pressure injuries. While most wounds are colonized by microorganisms from the skin microbiota, infection occurs when microbial proliferation overwhelms host defenses, leading to delayed healing and an increased risk of complications [1].

The burden of chronic and infected wounds is particularly pronounced in sub-Saharan Africa, where delayed presentation to healthcare facilities, limited access to diagnostic laboratories, and empirical antibiotic use contribute to poor clinical outcomes [2,3]. The estimated global prevalence of chronic wounds is approximately 2.21 per 1,000 individuals [4], and several African studies have documented a high incidence of wound infections caused by multidrug-resistant organisms in both community and hospital settings [5,6].

Among the microorganisms associated with wound infections, Gram-negative bacteria are of particular concern due to their capacity to acquire and disseminate antimicrobial resistance mechanisms. Studies conducted in West and Central Africa have documented a rising prevalence of multidrug-resistant Gram-negative bacilli in wound and surgical site infections,

particularly *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus spp.*, and *Pseudomonas aeruginosa* [6–8].

Extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacilli represent a major therapeutic challenge. ESBL enzymes, particularly those encoded by the *blaTEM, blaSHV,* and *blaCTX-M* genes, hydrolyze third-generation cephalosporins and other β-lactam antibiotics, thereby limiting treatment options [9]. The predominance of *blaCTX-M*-type ESBLs has been widely reported across African countries, including Nigeria [7], Ghana [8], Senegal [10], and Burkina Faso [11]. In addition to enzymatic resistance mechanisms, Gram-negative bacteria can form biofilms within wound environments, enhancing their persistence and tolerance to antimicrobial agents and host immune responses [12,13].

The development of wound infection is multifactorial and influenced by host-related factors such as immune status, diabetes, and malnutrition, as well as wound characteristics (acute versus chronic) and environmental conditions [14]. In many African healthcare systems, the limited availability of laboratory diagnostic services promotes reliance on empirical antibiotic therapy, which may inadvertently select for resistant strains and accelerate the spread of antimicrobial resistance [15].

Despite advances in molecular diagnostics, conventional culture-based techniques remain the cornerstone of routine clinical microbiology laboratories. These methods allow for bacterial isolation, identification, and antimicrobial susceptibility testing, thereby guiding targeted therapeutic decisions. While culture-based approaches primarily detect cultivable pathogens, molecular techniques provide additional insight into specific resistance determinants and the genetic basis of antimicrobial resistance. In the present study, molecular analysis was deliberately focused on detecting clinically relevant ESBL resistance genes rather than on comprehensive characterization of the wound microbiota.

Given the well-established association between Gram-negative bacteria, ESBL production, and multidrug resistance in African hospital settings, generating context-specific epidemiological data is essential. Local surveillance data are critical to inform antimicrobial stewardship strategies and strengthen infection prevention and control policies in developing countries.

Accordingly, this study aimed to characterize the antimicrobial resistance profiles and detect the major ESBL-associated resistance genes (*blaTEM*, *blaSHV*, and *blaCTX-M*) among Gram-negative bacteria isolated from suppurative wound samples at the Departmental University Hospital of Borgou-Alibori (CHUD-BA), Benin. By integrating phenotypic antimicrobial susceptibility testing with targeted molecular detection of resistance genes, this work contributes to reinforcing regional antimicrobial resistance surveillance efforts and supporting evidence-based clinical management.

## Methodology

*Study Design, Setting, and Period*

A prospective cross-sectional study was conducted over nine months, from June 2021 to February 2022, at the Departmental University Hospital of Borgou–Alibori (CHUD-BA), a major public referral hospital in northern Benin.

The study focused exclusively on Gram-negative bacterial isolates recovered from clinically infected suppurative wounds. These wounds comprised both acute forms, including postoperative infections, traumatic wounds, and burns, as well as chronic forms such as diabetic foot ulcers, pressure ulcers, and venous ulcers. A wound was considered infected based on clinical criteria, including purulent discharge, local signs of inflammation (pain, redness, and swelling), and/or delayed healing, as assessed by the attending clinicians.

*Sample Size and Eligibility Criteria*

The sample size corresponded to all consecutive suppurative wound samples received in the microbiology laboratory during the study period that met the inclusion criteria (n = 107). As this was a hospital-based surveillance study, no prior statistical power calculation was performed; all eligible cases during the study timeframe were included.

Eligible participants were patients presenting with clinically infected suppurative wounds, provided that sufficient sample material was available for bacteriological analysis and that culture yielded Gram-negative bacterial growth. Non-suppurative wounds were excluded, as were duplicate isolates obtained from the same patient. Samples considered contaminated or those yielding exclusively Gram-positive bacteria were also excluded from analysis.

*Ethical Considerations*

This study was conducted on bacterial isolates obtained during routine diagnostic procedures. No additional specimens were collected for research purposes, and no identifiable patient data were recorded. The study protocol was reviewed and

approved by the Doctoral School of Life and Earth Sciences of the University of Abomey-Calavi (UAC). The study complied with national ethical standards.

*Bacteriological Examination and Identification*

For primary culture, samples were inoculated onto Eosin Methylene Blue (EMB) agar and blood agar [16-17]. EMB agar, a selective and differential medium, was used to isolate and differentiate Gram-negative enteric bacteria based on lactose fermentation, whereas blood agar allowed broader recovery of wound pathogens, including non-fastidious Gram-negative organisms [16-17]. All inoculated plates were incubated aerobically at 37°C for 18–24 hours [16-17]. Mueller–Hinton agar was subsequently used for antimicrobial susceptibility testing.

Following incubation, colonies were examined microscopically using the Gram staining method to confirm Gram reaction and cellular morphology [16-17]. Presumptive *Enterobacteriaceae* isolates were identified using the API 20E identification system [18] in accordance with the manufacturer's instructions. Isolates not consistent with *Enterobacteriaceae*, including suspected *Pseudomonas* species, were further characterized through oxidase and catalase testing, assessment of growth characteristics on selective media, and standard biochemical reactions [17,19]. Identification of *Pseudomonas* species was based on oxidase positivity, characteristic colony morphology, and a biochemical profile consistent with non-fermenting Gram-negative bacilli [17,19].

*Antibiotic susceptibility testing*

Antimicrobial susceptibility testing was performed by the solid medium diffusion method [20]. Bacterial inocula were adjusted to 0.5 McFarland standard (~1–2 × $10^8$ CFU/mL). Plates were incubated at 37°C for 18–24 hours. Inhibition zone diameters were measured in millimeters and interpreted according to EUCAST 2021 breakpoints [20]. The antibiotic panels were selected based on EUCAST recommendations and antibiotics commonly prescribed in Benin.

A total of 22 antibiotics (Bio-Rad Laboratories, California, USA) were tested on 47 *Enterobacteriaceae* strains, namely: amoxicillin + clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), cefepime (FEP, 30 µg), cefixime (CFM, 5 µg), aztreonam (ATM, 30 µg), cefoxitin (Fox, 30 µg), ceftazidime (CAZ, 10 µg), ceftriaxone (CTR, 30 µg), cefuroxime (CXM 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), ertapenem (ETP, 15 µg), gentamycin (CN, 10 µg), imipenem (IPM, 10 µg), levofloxacin (LEV, 5 µg), netilmicin (NET, 10 µg), nitrofurantoin (F, 100 µg), norfloxacin (NOR, 10 µg), pefloxacin (PEF, 5 µg), piperacillin (PIP, 30 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), ticarcillin (TIC, 75µg), tobramycin (TOB, 10 µg).

Furthermore, fifteen antibiotics were tested against two *Pseudomonas* spp. strains.: These included amoxicillin–clavulanic acid (AMC, 30 µg), aztreonam (ATM, 30 µg), cefepime (FEP, 30 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 10 µg), ceftriaxone (CRO, 30 µg), cefuroxime (CXM, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), ertapenem (ETP, 15 µg), gentamicin (CN, 10 µg), imipenem (IPM, 10 µg), levofloxacin (LEV, 5 µg), meropenem (MEM, 10 µg), ticarcillin–clavulanic acid (TCC, 75/10 µg), and piperacillin–tazobactam (TZP, 110 µg).

*Biofilm Formation*

Biofilm formation was evaluated using the quantitative microtiter plate assay, based on the method originally described by Christensen *et al.* [21] and subsequently modified and standardized by Stepanović *et al.* [22]. This assay is widely applied for the assessment of biofilm production in both Gram-positive and Gram-negative bacteria.

Briefly, 10 µL of an 18-hour bacterial culture was inoculated into 150 µL of Brain Heart Infusion (BHI) broth in sterile 96-well polystyrene microplates and incubated at 37 °C for 24 hours under static conditions to allow biofilm development. Following incubation, planktonic cells were gently removed, and each well was washed three times with approximately 200 µL of sterile physiological saline (0.9% NaCl) to eliminate non-adherent bacteria.

The adherent biofilm was fixed and stained with 0.1% (w/v) crystal violet for 10 minutes. Excess stain was removed by washing, and the plates were air-dried at room temperature. Biofilm formation was determined by the presence of crystal violet–stained adherent biomass on the well surface. When applicable, the bound dye was solubilized and quantified spectrophotometrically according to the criteria described by Stepanović *et al.* [22], allowing classification of isolates as non-, weak, moderate, or strong biofilm producers.

*Molecular detection of resistance and virulence genes*

Extraction of bacterial DNA

Genomic DNA was isolated using a protocol adapted from Rasmussen and Morrissey [23] and subsequently applied by Sina *et al.* [24]. Briefly, preserved bacterial strains were cultured on Mueller–

Hinton (MH) agar and incubated at 37 °C for 24 hours. From these cultures, DNA was extracted as follows: a 24-hour preculture was prepared in 1 mL of liquid MH medium within an Eppendorf tube. After incubation, the tubes were centrifuged at 12,000 rpm for 5 minutes, and the supernatant was discarded. The resulting pellet was resuspended in 500 µL of 1X BER buffer, homogenized by repeated pipetting with a wide-tip cone (1000 µL), and heated in a dry bath at 100 °C for 30 minutes. The suspension was centrifuged again at 12,000 rpm for 5 minutes, and the supernatant was transferred to a fresh tube. To this, 500 µL of chilled 96% ethanol (4 °C) was added, followed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was carefully removed, and the pellet was air-dried. Finally, DNA was rehydrated in 50 µL of sterile distilled water and stored at 4 °C for immediate use or at −20 °C for long-term preservation.

Detection of genes coding for the ESBL phenotype in *Enterobacteriaceae*

PCR was performed in a total volume of 20 µL, containing 2 µL of 10 × PCR buffer, 2 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 1 µL of each primer (10 pmol), 0.5 µL of Taq DNA polymerase (5 U/µL), and 5 µL of template DNA. Amplification was carried out with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 52 °C for 30 seconds, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. The different primers used are listed in Table 1 [25]. The genes *blaTEM*, *blaSHV*, and *blaCTX-M* were selected because they represent the most prevalent ESBL determinants globally and are commonly reported in African *Enterobacteriaceae*.

Positive control strains harboring ESBL genes were included in each PCR run to verify assay performance and amplification accuracy. Negative controls consisting of the complete reaction mixture without template DNA were also incorporated to detect potential contamination. To ensure reliability, PCR assays yielding negative results were repeated for confirmation. Amplified products were analyzed by agarose gel electrophoresis and visualized under ultraviolet illumination.

**Table 2.** Distribution of samples by sex and age.

| Variable | Percentage (%) |
|---|---|
| **Sex** | |
| F | 42.1 |
| M | 57.9 |
| **Age (years)** | |
| [0-10] | 7.5 |
| [11-20] | 14.9 |
| [21-30] | 25.2 |
| [31-40] | 22.4 |
| [41-50] | 7.5 |
| [51-60] | 10.3 |
| [61-70] | 9.4 |
| [71 and more] | 2.8 |

*Data analysis*

Data were coded and entered using Microsoft Excel 2013. Statistical analyses were performed with GraphPad Prism 8. Categorical variables were expressed as frequencies and percentages. Associations were evaluated using appropriate statistical tests, and $p < 0.05$ were considered statistically significant.

## Results

*Sociodemographic characteristics of patients*

A total of 107 patients with clinically infected wounds were included in the study. The age of patients ranged from 1 to 92 years, with a mean age of 24 years. The largest age group was 21–30 years, representing 27 patients (25.23%). Male patients predominated, accounting for 62 cases (57.94%), while 45 patients (42.06%) were female (Table 2).

*Gram-negative bacteria diversity in suppurative wounds*

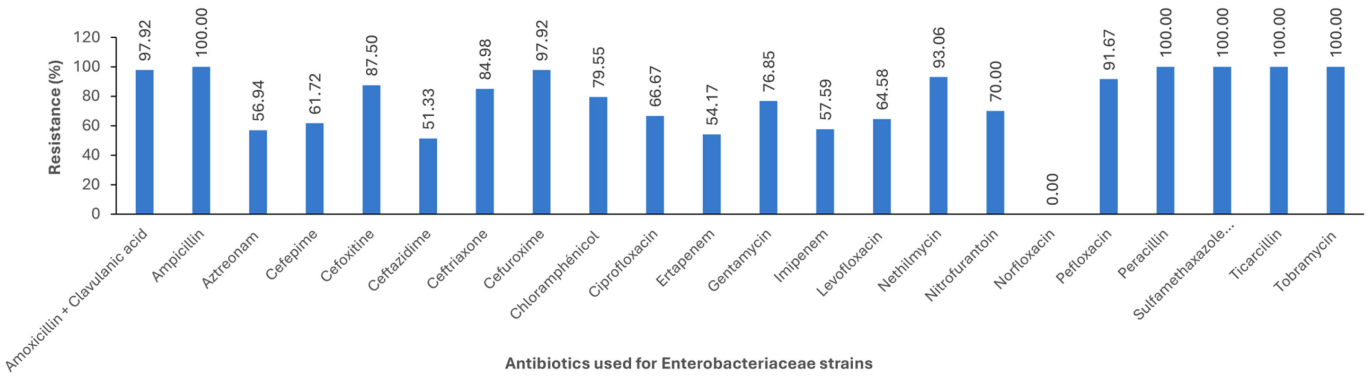
Of the 107 suppurative wound samples analyzed, 51 isolates (47.7%) were identified as Gram-negative bacteria and were included in subsequent analyses. Among these isolates, *Escherichia coli* was the most frequently identified species, accounting for 24 isolates (47.05%). Klebsiella pneumoniae represented 6 isolates (11.76%), followed by *Klebsiella ornithinolytica* with 5 isolates (9.80%) and *Klebsiella oxytoca* with 4 isolates (7.84%). The remaining 12 isolates (23.53%) comprised other species, including *Enterobacter spp*., *Citrobacter spp.*, *Proteus spp*., *Yersinia spp*., and *Pseudomonas spp*.

All isolates were recovered on EMB and blood agar

**Table 1.** Primers used for the chain polymerization reaction.

| Genes | Primers | Sequences | Reference |
|---|---|---|---|
| $bla_{TEM}$ | OT-F | 5'-ATTGGGTGCACGAGTGGGTTAC-3' | [25] |
| | OT-R | 5'-ATAATTGTTGCCGGGAAGCTAG-3' | |
| $bla_{SHV}$ | SHV-F | 5'-CGCCGGGTTATTCTTATTTGTCGC-3' | |
| | SHV-R | 5'-TCTTTCCGATGCCGCCGCCAGTCA-3' | |
| $bla_{CTX-M}$ | CTX-F | 5'-CGCTTTGCGATGTGCAG-3' | |
| | CTX-R | 5'-ACCGCGATATCGTTGGTAAT-3' | |

**Figure 1.** Resistance of *Enterobacteriaceae* strains to antibiotics.




media. Identification of *Yersinia* species was based on colony morphology, Gram staining, and biochemical profiling using the API 20E system in combination with standard biochemical reactions. The detailed distribution of bacterial species is presented in Table 3.

**Table 3.** Frequency of bacteria isolated according to species.

| Bacteria isolated | Percentage (%) |
|---|---|
| *Escherichia coli* | 47.05 |
| *Klebsiella pneumoniae* | 11.76 |
| *Klebsiella ornithinolytica* | 9.80 |
| *Klebsiella oxytoca* | 7.84 |
| *Enterobacter aerogenes* | 3.92 |
| *Enterobacter cloacae* | 3.92 |
| *Citrobacter freundii* | 3.92 |
| *Pantoea* spp. | 1.96 |
| *Proteus mirabilis* | 1.96 |
| *Proteus vulgaris* | 1.96 |
| *Pseudomonas luteola* | 1.96 |
| *Pseudomonas oryzihabitans* | 1.96 |
| *Yersinia* spp | 1.96 |

*Antibiotic susceptibility profiles*

Resistance Profile of *Enterobacteriaceae* (n = 49)

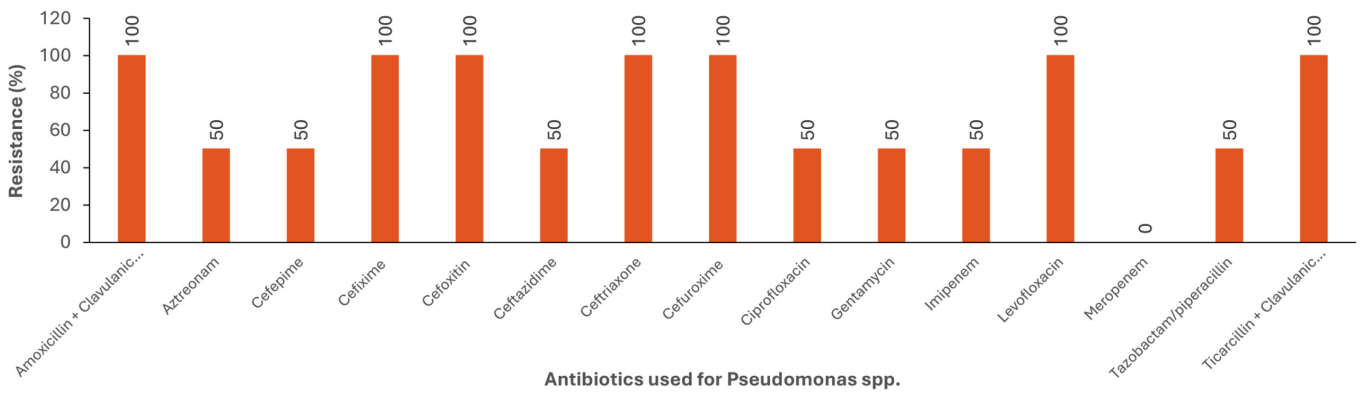
Of the 51 Gram-negative isolates recovered, 49 belonged to the order *Enterobacteriaceae*, and resistance rates were calculated based on these 49 isolates. Very high resistance rates were observed to several commonly used antibiotics. All isolates (49/49; 100%) were resistant to ampicillin, piperacillin, ticarcillin, sulfamethoxazole-trimethoprim, and tobramycin. Resistance to amoxicillin–clavulanic acid and cefuroxime was also notably high, each observed in 48 of 49 isolates (97.9%). In addition, 45 of 49 isolates (91.7%) were resistant to pefloxacin. Lower resistance rates were recorded for norfloxacin, as detailed in Figure 1.

Overall, these findings highlight a markedly high prevalence of multidrug resistance among *Enterobacteriaceae* isolates. Ampicillin, Fosfomycin/trometamol, piperacillin, sulfamethoxazole-trimethoprim, ticarcillin, and tobramycin resistance was observed in 51/51 (100%) isolates. The strains exhibited 97.9% resistance to amoxicillin + clavulanic acid, 97.9% to cefuroxime, and 91.7% to pefloxacin.

Resistance Profile of *Pseudomonas* spp. (n = 2)

Two isolates (3.9%) were identified as *Pseudomonas* species. Both isolates (2/2; 100%) exhibited resistance to amoxicillin–clavulanic acid, ceftriaxone, cefuroxime, levofloxacin, and ticarcillin–

**Figure 2.** Antibiotic resistance of *Pseudomonas* spp.



clavulanic acid. However, both remained susceptible to meropenem.

The earlier statement indicating that not all *Pseudomonas* strains were resistant to antibiotics was inaccurate (Figure 2). The present results instead demonstrate a multidrug-resistant phenotype with preserved susceptibility to carbapenems. Given the very small number of *Pseudomonas* isolates (n = 2), these findings should be interpreted with caution.

*Biofilm formation among gram-negative bacilli*

Among the 51 Gram-negative isolates evaluated, 20 (39.2%) demonstrated visible biofilm formation using the qualitative microtiter plate assay (Figure 3). Biofilm production varied according to species. All *Yersinia* species isolates (2/2; 100%) formed biofilms. Biofilm was also detected in 3 of 5 *Citrobacter* species isolates (60%) and in 4 of 7 *Enterobacter* species isolates (57.1%). Among *E. coli* isolates, 8 of 24 (33.3%) were biofilm producers. In contrast, no biofilm formation was observed in *Proteus mirabilis* (0/3), *P. vulgaris* (0/2), or *Pseudomonas luteola* (0/1). Analysis of variance showed no significant difference between biofilm-forming and non-biofilm-forming bacilli ($p > 0.9999$).

*Detection of genes coding for ESBL production*

Among the 49 *Enterobacteriaceae* isolates screened for extended-spectrum β-lactamase (ESBL) genes, *blaCTX*-M was the most frequently detected, present in 17 isolates (34.7%), followed by *blaTEM* in 14 isolates (28.6%) and *blaSHV* in 4 isolates (8.2%). Co-occurrence of multiple ESBL genes was observed in a subset of isolates, with four isolates (8.2%) harboring both *blaTEM* and *blaCTX*-M, and one isolate (2.0%) carrying *blaTEM*, *blaSHV*, and *blaCTX*-M simultaneously. The distribution of ESBL genes varied by species (Figure 4). Among five *Enterobacter* species isolates, three (60%) carried *blaCTX-M*. In *Escherichia coli*, ten of 24 isolates (41.7%) harbored *blaTEM*, while three isolates (12.5%) carried *blaSHV*.

## Discussion

This study provides valuable insight into the epidemiology, antimicrobial resistance patterns, biofilm production, and ESBL genetic determinants of Gram-negative bacilli isolated from infected wounds in northern Benin. The findings reveal a concerning prevalence of multidrug-resistant (MDR) organisms, underlining the growing challenges in managing wound infections in this hospital setting.

The male predominance observed in this study (57.9%) is consistent with similar findings from Sudan and other African settings, where men are more frequently exposed to occupational trauma, road traffic injuries, and surgical procedures [26]. In contrast, studies from parts of Asia have reported higher infection rates among females, which may reflect differences in healthcare-seeking behaviors or underlying comorbidities [27]. The predominance of the 21–40 age group, which accounted for 47.7% of cases, aligns with observations from a Danish study that reported high infection rates among adults aged 16–60 years [28]. Adults in this age range are more likely to

**Figure 3.** Biofilm formation pattern of isolated Gram-negative bacilli.

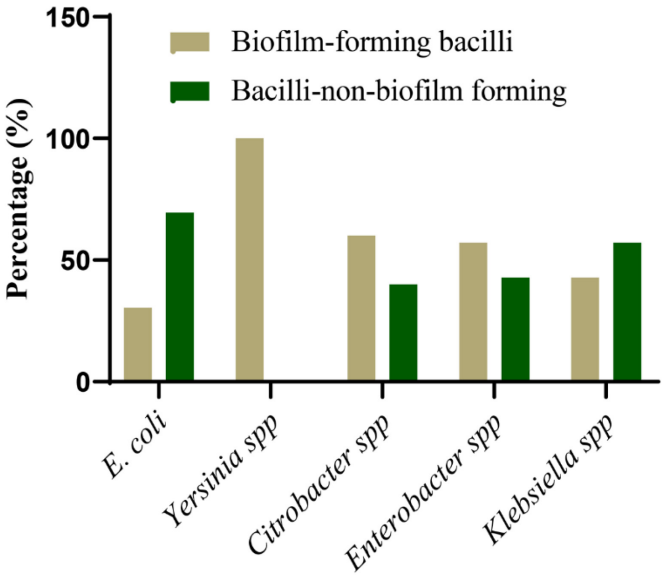
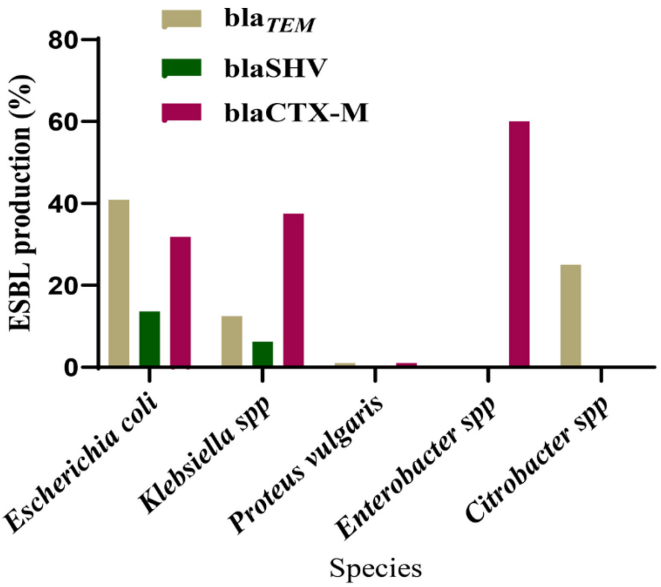



**Figure 4.** Percentage of identified *Enterobacteriaceae* species with *bla*TEM, *bla*SHV, and *bla*CTX-M genes.



experience trauma and undergo surgical procedures. In contrast, chronic wounds in older adults may be underrepresented due to referral patterns or limited access to tertiary care. These epidemiological patterns likely reflect regional sociocultural dynamics and healthcare access issues rather than intrinsic susceptibility.

Nearly half (47.7%) of the wound suppurations in our study were found to contain Gram-negative bacilli, which is lower than the proportions reported in some Asian hospital-based studies (over 80%) [29], but similar to findings in other African reports [26]. This supports the idea that Gram-negative organisms are becoming increasingly dominant in wound microbiota in various global settings. The predominance of *E. coli* and *Klebsiella* spp. in our study mirrors the recognized role of *Enterobacteriaceae* in wound infections [26], particularly in hospital environments. While *S. aureus* is a classical wound pathogen, its lower prevalence in this study may be due to the focus on Gram-negative isolates or because the study involved deeper suppurative infections rather than superficial colonization, where *S. aureus* is more commonly found. The diversity of species recovered, including *Enterobacter*, *Citrobacter*, *Proteus*, *Yersinia*, and *Pseudomonas*, highlights the polymicrobial and opportunistic nature of wound infections, particularly in resource-limited hospital settings.

The most alarming finding of this study is the extremely high resistance rates to commonly used beta-lactams and sulfamethoxazole-trimethoprim among *Enterobacterales* isolates. Resistance rates approaching or reaching 100% suggest long-standing antibiotic pressure and possible empirical overuse, a situation exacerbated by unrestricted antibiotic access, self-medication, and limited microbiological guidance in many low- and middle-income countries [30]. Similar resistance patterns have been reported in West Africa, indicating that this phenomenon is not isolated but part of a broader regional antimicrobial resistance crisis. Fluoroquinolone resistance was also notably high, exceeding rates observed in some high-income countries [31]. Given that fluoroquinolones are frequently prescribed for skin and soft tissue infections, this is particularly concerning. The two *Pseudomonas* spp. isolates, though multidrug-resistant, retained susceptibility to meropenem. This aligns with the intrinsic resistance mechanisms of non-fermenters like *Pseudomonas*, which employ efflux pumps and beta-lactamase production to resist many classes of antibiotics [32,33]. Although the small number of *Pseudomonas* isolates limits generalizability, these findings are consistent with known resistance patterns in non-fermenters.

One of the strengths of this study is the combined phenotypic and molecular assessment of resistance. Phenotypically, high resistance to third-generation cephalosporins was observed. Molecularly, the most prevalent ESBL gene was *blaCTX-M* (34.7%), followed by *blaTEM* (28.6%) and *blaSHV* (8.2%). The predominance of *blaCTX-M* is consistent with global trends, where *CTX-M*-type enzymes have largely replaced *TEM* and *SHV* as the dominant ESBL determinants [34]. The presence of *blaCTX-M* in 60% of *Enterobacter* spp. and *blaTEM* in 41.7% of *Escherichia coli* isolates supports the phenotypic observation of extended-spectrum cephalosporin resistance. The study's use of both phenotypic and molecular approaches is valuable, as phenotypic resistance confirms the clinical expression of resistance, while molecular detection identifies the underlying genetic mechanisms. This combined approach helps distinguish ESBL-mediated resistance from other mechanisms, such as *AmpC* overexpression or porin loss. However, a formal statistical correlation between phenotypic ESBL expression and gene presence was not conducted in this study, which would have strengthened the mechanistic interpretation of the results.

Biofilm formation was detected in 39.2% of isolates, a clinically significant finding, as biofilms are known to increase bacterial tolerance to antibiotics, protect bacteria from host immune responses, and promote horizontal gene transfer [35,36]. The high biofilm production rates in *Yersinia* (100%), *Citrobacter* (60%), and *Enterobacter* (57.1%) suggest that biofilm formation may contribute to the persistence and chronicity of wound infections in this setting. In contrast, the absence of biofilm formation among *Proteus* spp. (which are typically strong biofilm producers) is an interesting finding that may reflect strain-specific variation, the qualitative nature of the assay used, or suboptimal assay conditions. The qualitative crystal violet method provides a screening-level assessment of biofilm formation but lacks quantitative precision, meaning that biofilm prevalence may have been underestimated. These discrepancies highlight the need for more comprehensive biofilm analysis methods in future studies.

*Limitations*

This study was limited by the absence of sequencing confirmation of PCR products and the relatively small sample size. Future studies should

include larger cohorts and whole-genome sequencing approaches.

## Conclusions

The study demonstrates a high burden of multidrug-resistant Gram-negative bacilli in infected wounds, with blaCTX-M emerging as the dominant ESBL determinant. The combination of phenotypic resistance, molecular determinants, and biofilm formation underscores the complexity of wound infection management in this setting. Strengthened antimicrobial stewardship and expanded molecular surveillance are urgently needed.

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### Conflict of interest

No conflict of interest is declared.

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