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Molecular epidemiology of extended-spectrum beta-lactamase producing Gram-negative bacteria among surgical site infection patients in Ethiopia: a multicenter prospective study

Seble Worku^{1,2,3*} , Tamrat Abebe¹, Alemseged Abdissa³, Ayenew Berhan², Ashenafi Alemu³, Getachew Tesfaye Beyene³, Berhanu Seyoum³, Adane Mihret^{1,3} and Göte Swedberg⁴

Abstract

Extended-spectrum beta-lactamases (ESBLs) producing Gram-negative bacteria are public health threats. This study aims to characterize ESBL-producing Gram-negative bacteria (GNB) isolated from surgical site infection patients. A multicenter cross-sectional study was conducted at four hospitals located in central (Addis Ababa), southern (Hawassa), northern (Debre Tabor), and Southwestern (Jimma) parts of Ethiopia. A wound culture was performed among 752 surgical site infection (SSI) patients where 286 GNB were confirmed using MALDI-TOF; of which 135 were subjected to whole genome sequencing using the Illumina (HiSeq 2500) system. The overall genotypic frequencies of ESBL-producing GNB were 57.8%. The detection of ESBL-producing GNB at Hawassa, Debre Tabor, Jimma, and Addis Ababa was 21.5%, 19.3%, 29.6%, and 29.6%, respectively. The detection frequency of *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV} genes was 51.8%, 40%, and 8.1%, respectively. The most frequently detected ESBL gene was *bla*_{CTX-M-15} (56.4%). Both *bla*_{TEM-1B} (41%) and *bla*_{SHV-187} (5.1%) were the most frequently detected variants of *bla*_{TEM} and *bla*_{SHV} respectively. The molecular epidemiology of ESBL-producing GNB among surgical site infection patients in Ethiopia showed many variants of ESBL genes. Good antimicrobial stewardship and standard bacteriological laboratory services are necessary for the effective treatment of ESBL-producing GNB.

Keywords Multicenter study, Molecular epidemiology, ESBL, Gram-negative bacteria, SSI, Ethiopia

*Correspondence:

Seble Worku
workuseble@gmail.com

¹Department of Microbiology, Immunology and Parasitology, College of Health Sciences, School of Medicine, Addis Ababa University, Addis Ababa, Ethiopia

²Department of Medical Laboratory Science, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia

³Bacterial and Viral Diseases Research Directorate, Armauer Hansen Research Institute, Addis Ababa, Ethiopia

⁴Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden



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Introduction

Gram-negative bacteria (GNB) are one of the major world's public health problems due to high antibiotic resistance to expanded-spectrum β -lactam antibiotics (ESBL) [1, 2], and surgical site infection (SSI) is health-care-associated infection [1, 2]. The emergence of antimicrobial resistance, especially from GNB that produce extended-spectrum β -lactamases, is a major worldwide health concern because it commonly results in the failure of empirical antibiotic therapy, which raises morbidity and mortality rates of SSI patients [1, 3].

The challenges to controlling the global epidemiology of SSI are the lack of standardized criteria for diagnosis of infection and the emergence of antimicrobial resistance (AMR) among bacterial pathogens [3, 4]. Prolonged antibiotic prophylaxis and overuse of antibiotics are significantly associated with increased risk of drug resistance pathogens [5]. Many studies particularly in GNB showed that multidrug-resistant (MDR): acquired non-susceptibility to at least one agent in three or more antimicrobial categories, extended-spectrum β -lactamases (ESBL), and carbapenemase-producing causes a serious health concern in hospitals, which in turn worsens the problem by the lack of new antimicrobial drugs having an effect against them [6–9]. These ESBLs are usually plasmid-encoded and located on different transferable genetic elements and can hydrolyze many antibiotics which complicate the treatment of many hospitalized patients [3–5]. The most important β -lactamases are cephalosporinases like (AmpC β -lactamases), ESBLs and the carbapenemases like metallo- β -lactamases (MBLs) [10]. Generally, all beta-lactamase variants are classified into four classes, A (serine penicillinases), B (metallo-beta-lactamases), C (cephalosporinases (acinetobacter-derived cephalosporinase or ADC) and D (oxacillinases), which give resistance to penicillins, most β -lactams, cephalosporins and cloxacillin, respectively [11]. The most common ESBL genes include TEM (named Temoniera in Greece), SHV (sulphydral variable), and CTX-M (reference to its preferential hydrolytic activity against cefotaxime, CTX as its acronym, M for Munich) [4]. The most common GNB particularly ESBL and carbapenemase-producing associated with surgical wound infection are *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, which showed increasing drug-resistance mechanism [12, 13]. Antibiotic resistance, including MDR, ESBL, and carbapenemase-producing are major problem of bacterial pathogens isolated from SSI in hospitals and increasing every year [14, 15]. The high prevalence of SSI and the increasing incidence of MDR of isolates to antimicrobials was alarmingly high and reduced the therapeutic options, increased the treatment costs, increased hospitalization time, and mortality rates (18, 21, 22), so the emergence

of MDR is becoming a challenge for health personnel to treat hospital-acquired infections [12]. SSIs are currently a worldwide problem due to the rapid spread of resistant microbes affecting the effectiveness of antimicrobials [13]. In hospitals, 30–50% of antibiotics are prescribed for surgical prophylaxis, with 30–90% of these antibiotics being misused [16, 17]. In prophylaxis, antimicrobial agents, particularly β -lactams, are the most commonly used types (23, 24). Even though it is a natural phenomenon, this misuse and overuse increase selection pressure, favouring the emergence of drug-resistant bacteria especially ESBL and carbapenems-producing bacterial, making the choice of empirical therapy more difficult and expensive, and poses a serious threat to public health, thus increasing the global risk of SSI [3, 4, 18]. SSI is the most commonly reported case of nosocomial infections in low and middle-income countries [4]. The overall SSI rate in Ethiopia has been reported to be between 14.8% and 20% at various teaching hospitals [5, 16, 17, 19] and in general surgical wards 38% of surgical patients [6]. In developing countries, including Ethiopia, published reports on bacterial pathogens and their antibiotics resistance patterns of frequently causing SSIs and the associated factors among all age groups of SSI at four hospitals in Ethiopia are scarce (22). Moreover, virtually all earlier reports depend on phenotypic laboratory methods to characterize pathogenic bacteria and studies done at single sites, with limited sample sizes (9, 23, 24). A systematic reviews and meta-analyses study published by Birhanu Y et al. (25) focused on the pooled prevalence of SSI and its aetiology in Ethiopia.

Thus, the goal of this research was to fill in the gaps that were found. Mainly the study aimed to show the magnitude of ESBL-producing GNB and associated risk factors among surgical site infection patients in four hospitals, in Ethiopia.

Materials and methods study design, study area, study population, and sample size

A hospital based cross-sectional multicenter study was conducted between July 2020 and August 2021 and briefly described in our previous published work by Worku S et al. [19]., a multicentre cross-sectional study was conducted between July 2020 and August 2021 at four selected Hospitals in Northern, Central, Southern, and Southwest Ethiopia. The study was conducted in purposively selected Hospitals in Ethiopia, namely, Debre Tabor Comprehensive Specialized Hospital (DTCSH), Hawassa University Comprehensive Specialized Hospital (HUCSH), Jimma University Specialized Hospital (JUSH), and Tikur Anbessa Specialized Hospital (TASH) (Fig. 1).

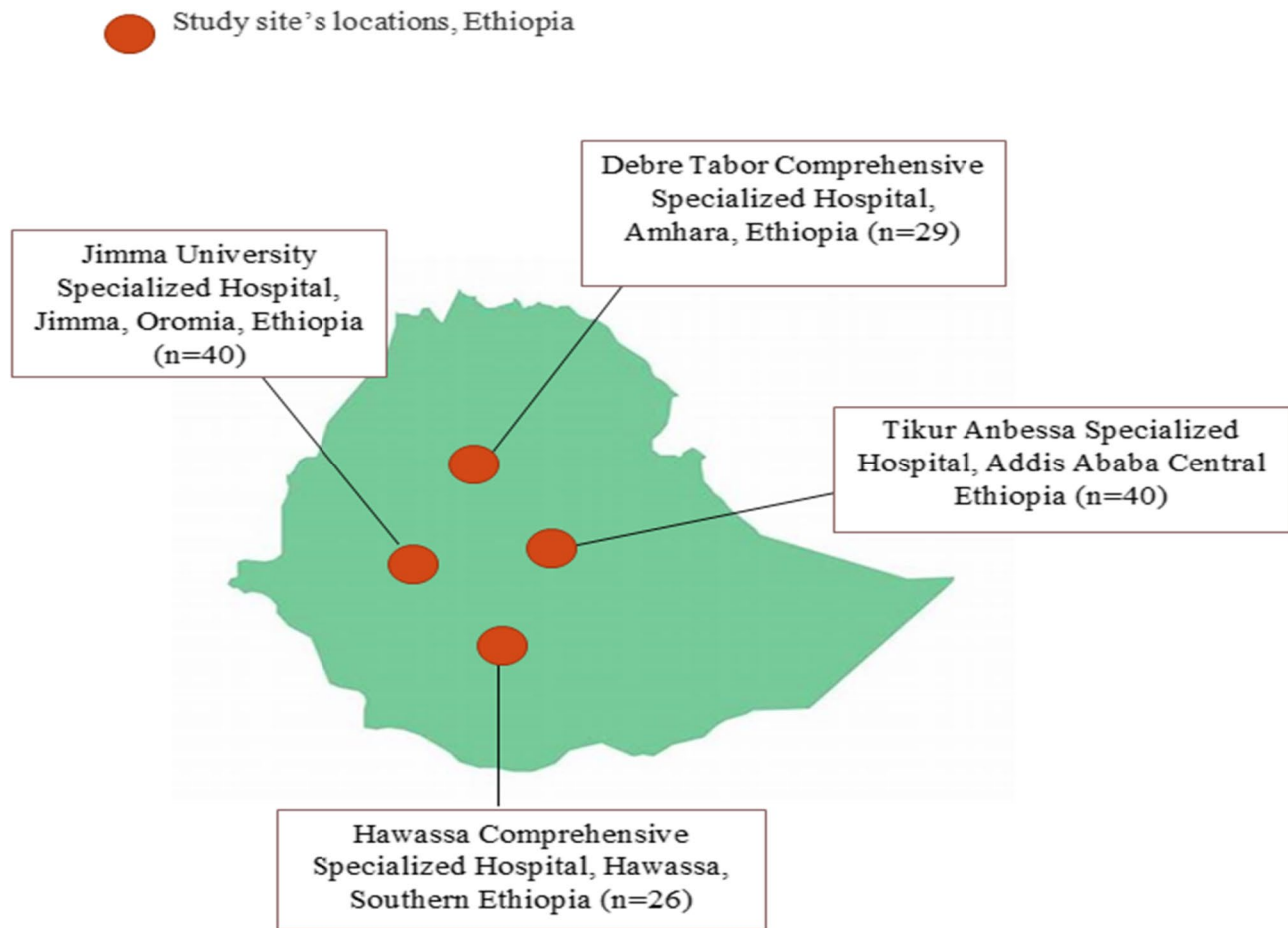


Fig. 1 Map of the geographic locations of the four referral hospitals selected

Wound culture and Gram-negative bacteria isolation and identification

The attending physician's decision was used to identify the eligible SSI patients, and their socio-demographic and possible risk factor data was gathered using questionnaire as describes in our previous published work by Worku S et al. [19]. All surgical patients, irrespective of age, operated during the study period and who developed sign and symptoms of surgical site infection within 30 days or within 1 year if there is implant and who gave consent and/or assent to participate in the study included. All patients, who develop surgical site infections later than 30 days after the operation without implant, infected burn wounds, and patients with infection of an episiotomy, excluded additionally patients who received therapeutic antimicrobials during hospitalization prior to surgery for comorbid conditions, patients with a history of previous admission for a similar surgical procedure and patients with incomplete medical information excluded.

A total of 286 Gram-negative bacteria isolated from clinically diagnosed cases of surgical site infection from different wards in all hospitals. Specimens were collected

aseptically on the first day that patients presented with clinical evidence of infection, such as purulent drainage from an incision or drain, prior to cleaning the wound with antiseptic. Trained personnel collected pus, pus aspirates, and wound swabs using sterile syringes with needles or sterile cotton-tipped swabs. Before collection, the area around the infected site was cleaned with 10% povidone iodine to minimize contamination from skin commensals. Following this, the wound was rinsed with normal saline. All wound swabs were then placed into modified Stuart's Transport Medium and immediately transported to the bacteriology laboratory for culture and drug susceptibility testing, ensuring delivery within one hour.

Bacterial identification was performed using a standardized laboratory protocol. At each study site, the isolates were characterized by their colony characteristics, Gram staining, and conventional biochemical tests. Members of the family *Enterobacteriaceae* and other Gram negative bacteria were identified using the following biochemical tests: Indole Production, Hydrogen Sulfide (H₂S) Production, Citrate Utilization, Motility Test, Urease Test, Oxidase Test, Carbohydrate Utilization

Tests, Triple Sugar Iron (TSI) Test, Lysine Decarboxylase (LDC) Test, Malonate Test. Specifically, *Klebsiella ozaenae* was identified from *Klebsiella pneumoniae* using malonate biochemical test. *P. aeruginosa* was differentiated from other *Pseudomonas species* using *P. aeruginosa* Screen 80 tablet (Rosco, DK- 2630) cetrimid selective media [6].

All bacterial strains were stored at $-70\text{ }^{\circ}\text{C}$ and transported to the Armauer Hansen Research Institute (AHRI) and then all the bacterial isolates were re-identified and confirmed by using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and latter brought to, Sweden, and the Karolinska Institute, Stockholm, Sweden. Each laboratory test was performed in accordance with the well-known protocols and carefully recorded. Each MALDI-TOF MS path contained strains for quality control using *E. coli* ATCC 25,922. Of 286 Gram-negative bacteria isolated from all study sites 135 Gram-negative bacteria were selected for whole Genome Sequencing (WGS) based on their antibiotics resistance pattern due to shortage of budget.

All positive cultures were identified based on their colony characteristics on the respective media, Gram-staining reactions, and confirmed through a series of biochemical tests following standard methods as outlined by Cheesbrough (2006).

Members of the family *Enterobacteriaceae* and other gram negative bacteria were identified using the following biochemical tests: Indole Production, Hydrogen Sulfide (H_2S) Production, Citrate Utilization, Motility Test, Urease Test, Oxidase Test, Carbohydrate Utilization Tests, Triple Sugar Iron (TSI) Test, Lysine Decarboxylase (LDC) Test, Malonate Test. Specifically, *Klebsiella ozaenae* was identified from *Klebsiella pneumoniae* using malonate biochemical test. *P. aeruginosa* was differentiated from other *Pseudomonas species* using *P. aeruginosa* Screen 80 tablet (Rosco, DK- 2630) cetrimid selective media [6].

Antimicrobial susceptibility testing

The antibiotics susceptibility test performed onto Muller-Hinton agar (Oxoid) by using the Kirby-Bauer disk diffusion technique. Using a sterile wire loop, 3–5 pure colonies were transferred to a tube containing 5 mL of sterile normal saline (0.85% NaCl) and gently mixed until a uniform suspension formed. Standard inoculum density was adjusted to 0.5 McFarland units. The excess broth suspension was removed by taping against the tube wall. The bacterial suspension was swabbed on MHA surface by using sterile swab then a set of antibiotic discs placed with sterile forceps at least 24 mm apart from one another [6]. All antibiotics discs were OXOID products (Oxoid Ltd, UK), and susceptibility of Gram-negative isolates was tested against: ampicillin (10 μg), gentamicin

(10 μg), amikacin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), cefepime (30 μg), tetracycline (30 μg), amoxicillin + Clavulanate (20/10 μg), Trimethoprim-sulfamethoxazole (1.25/23.75 μg), ampicillin-sulbactam (10/10 μg), aztreonam (30 μg), meropenem (10 μg), Imipenem (10 μg), ertapenem (30 μg). Gram-positive isolates were tested against penicillin (10units), ampicillin (10 μg), vancomycin (30 μg), erythromycin (15 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), clindamycin (30 μg), erythromycin (15 μg), doxycycline (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), and oxacillin (5 μg), tetracycline (30 μg) as described in our published work by Worku S et al. [19]. Following that, the plates were incubated at $37\text{ }^{\circ}\text{C}$ for 18–24 h. Each zone of inhibition was measured to the nearest millimeter, and classified as sensitive, intermediate, or resistant using the standard technique [6].

DNA extraction, whole genome sequencing and the identification of resistance genes

DNA was extracted manually using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), based on the manufacturer's instructions. In short, two to six pure colonies growing on cystine lactose electrolyte deficient agar were used to extract DNA. After that, the DNA content was determined using QubitTM3.0 (Thermo Scientific, Waltham, MA, USA), and samples were stored at $-20\text{ }^{\circ}\text{C}$ until WGS was completed. All the isolates were subjected to WGS at the Science for Life Laboratory, Solna, Sweden. A 96-well WGS plate was filled with 20 μL of each DNA sample. Sequencing libraries were generated using Nextera XT (Illumina kits) and short-read sequencing was run on Illumina (HiSeq 2500) systems with a 150 bp insert size paired end sequencing protocol at the Science for Life Laboratory. CLC Genomic workbench version 23 (QIAGEN, Hilden, Germany) was used for the genome assembly. With the assembled genomes, the acquired antimicrobial resistance genes were identified using the ResFinder 4.1 web tool at the Center for Genomic Epidemiology <http://www.genomicepidemiology.org/> (accessed on August 2023) using a threshold of 90% and 60% coverage. Each WGS run included quality control. Reports are available at file:///Users/goteswed/Downloads/846125_QC_4.html. The raw sequence data are deposited at NCBI as BioProject: PRJNA1117753.

Quality control

The validity of the study was guaranteed by the quality control procedures carried out during the entire laboratory work process. Every specimen was gathered in compliance with the standard operating procedure (SOP). To guarantee the accuracy of the data, double data entry methods were used. Control strains were inoculated to

assess the performance of all prepared media and the effectiveness of antibiotics, *E. coli* (ATCC®25922), for each new batch of agar plates. In addition, the sterility of culture media was checked by incubating 5% of the prepared media at 37 °C for 24–48 h. Gram-stain and biochemical tests reagent were checked against control strains of *E. coli*. The 0.5 McFarland standard densitometer was used. *Klebsiella pneumoniae* ATCC® 700,603 screening and confirmatory tests for ESBLs (positive), and *E. coli* ATCC 25,922 (ESBL negative) were used as quality control (QC) in all tests. Additionally, each MALDI-TOF MS run contained strains for quality control using *E. coli* (ATCC® 25922). Prior to multiplexing, each primer pair underwent a monoplex PCR validation.

Data analysis

The data were checked for completeness, missing values, and coding of questionnaires entered into Research Electronic Data Capture (RED-Cap) and exported to STATA version 25.0. The frequencies of the, ESBL producers, resistance genes and the co-occurrence of multiple ESBL genes and other variables were calculated. Bivariate and multivariate logistic regressions were assessed to see the association of sociodemo-graphic and possible risk factors of ESBL producing GNB. Statistical significance was considered at *p*-values

Results

Socio-demographic characteristics

In the present study, a total of 752 patients investigated for surgical site infection and 286 were Gram-negative

Table 1 Socio-demographic data of the patients investigated for surgical site infection at four different hospitals in Ethiopia

| Variables | Characteristics | Total |
|-----------------------------|-----------------|-----------|
| | | N (%) |
| Sex | Male | 74 (54.8) |
| | Female | 61 (45.2) |
| Age (in years) | | 34 (25.2) |
| | 19–40 | 62 (45.9) |
| | 41–60 | 29 (21.5) |
| | ≥ 61 | 10 (7.4) |
| Hospital | HUCSH | 26 (19.3) |
| | DTCSH | 29 (21.5) |
| | JUSH | 40 (29.6) |
| | TASH | 40 (29.6) |
| Preoperative hospital stay | ≤ 7 | 57 (42.2) |
| | > 7 | 78 (57.8) |
| Previous use of antibiotics | Yes | 78 (57.8) |
| | No | 57 (42.2) |
| Nature of surgery | Elective | 39 (28.8) |
| | Emergency | 96 (71.1) |
| Duration of operation | ≤ 1 h | 57 (42.2) |
| | > 1 h | 78 (57.8) |

bacteria of these based on the antibiotics resistance pattern 135 isolates were enrolled WGS from four different hospitals. The male participants were 54.8% while the females were 45.2%. The patients’ ages ranged from five days to 70 years with a mean age of 30.1 years (Table 1). The number of patients from Hawassa Comprehensive Specialized Hospital (HUCSH) was 26, and the numbers from Debre Tabor Comprehensive Specialized Hospital (DTCSH), Jimma University Specialized Hospital (JUSH), and Tikur Anbessa Specialized Hospital (TASH) were 29, 40 and 40, respectively (Table 1).

Frequencies and distributions of GNB at different hospitals

From the 752 patients, wound cultures were performed at each study site, and a total of 286 GNB were isolated from all the study sites and among them 135 were subjected for whole genome sequencing (WGS) based on the antibiotics resistance pattern (Table 2).

Detection of *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV}

Genes Among all Gram-negative bacteria, *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} were detected in 50.8%, 40.1% and 8% of the samples at least once (Fig. 2). Among the GNB isolated at each hospital, the detection of *bla*_{CTX-M} at DTCSH, HUCSH, JUSH, and TASH was 21.1%, 19.7, 39.4%, and 19.7% respectively. The *bla*_{TEM} was (30.9%) and (25.5%) at JUSH and DTCSH detected. At TASH *bla*_{SHV} (54.5%) detection was higher while not detected at HUCSH (Fig. 2).

E. coli was the most frequent isolate and harbored high frequencies of *bla*_{CTX-M}, (52.2%), *bla*_{TEM} (40.3%) and *bla*_{SHV} (8.2%) genes (Fig. 3). Similarly, most GNB were found to have *bla*_{CTX-M}, and *bla*_{TEM} gene families with different detection rates between the strains. The detection of *bla*_{SHV} in *E. cloacae* (42.9%), *K. pneumoniae* (20.8%) and *S. dysenteriae* (16.7%) was high, while there was low detection of *bla*_{SHV} among *E. coli* (5.1%). No *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes were detected in the rare isolates of *Alcalignes faecalis*, *Acinetobacter soli*, *Pseudomonas plecoglossicida*, *Morganella morganii*, *Enterobacter asburiae* and *Enterobacter bugandensis* (Fig. 3).

Molecular epidemiology of ESBL producing Gram-negative bacteria

Of 135 GNB subjected to WGS, 57.8% encoded at least one ESBL gene. At least one ESBL gene was detected among 83.3% of *Pseudomonas aeruginosa*, 83.3% of *E. cloacae*, and 66.7% *Klebsiella pneumoniae*, 57.9% *E. coli* and 42.1% of *Acinetobacter baumannii* (Fig. 4).

The frequencies of ESBL, and non ESBL producing GNB detected at DTCSH, HUCSH, JUSH and TASH were 21.5%, 19.3%, 29.6% and 29.6%, respectively. Among the patients who showed wound culture positivity for

Table 2 Frequency and distribution of GNB isolated from the patients investigated for SSI and subjected for whole genome sequence in four Ethiopian hospitals

| Gram- negative bacteria | DTCSH n (%) | HUCSH n (%) | JUSH n (%) | TASH n (%) |
|--|-------------|-------------|------------|------------|
| <i>Escherichia coli</i> (n = 39) | 11 (28.2) | 9 (23.1) | 10 (25.6) | 9 (23.1) |
| <i>Acinetobacter baumannii</i> (n = 38) | 4 (10.5) | 12 (31.6) | 9 (23.7) | 13 (34.2) |
| <i>Klebsiella pneumonia</i> (n = 24) | 7 (29.2) | 2 (8.3) | 6 (25) | 9 (37.5) |
| <i>Enterobacter cloacae</i> (n = 6) | 1 (16.7) | 1 (16.7) | 2 (33.3) | 2 (33.3) |
| <i>Shigella dysenteriae</i> (n = 6) | 2 (33.3) | - | 1 (16.7) | 3 (50) |
| <i>Pseudomonas aeruginosa</i> (n = 6) | 1 (16.7) | 1 (16.7) | 2 (33.3) | 2 (33.3) |
| <i>Enterobacter hormaechei</i> (n = 4) | - | 1 (25) | 3 (75) | - |
| <i>Acinetobacter pittii</i> (n = 2) | - | - | 1 (50) | 1 (50) |
| <i>Acinetobacter soli</i> (n = 2) | - | - | 2 (100) | - |
| <i>Citrobacter 1sedlakii</i> (n = 1) | - | 1 (100) | - | - |
| <i>Acinetobacter lactucae</i> (n = 1) | - | - | 1 (100) | - |
| <i>Pantoea ecurina</i> (n = 1) | - | - | 1 (100) | - |
| <i>Alcalignes faecalis</i> (n = 1) | - | 1 (100) | - | - |
| <i>Pseudomonas plecoglossicida</i> (n = 1) | - | 1 (100) | - | - |
| <i>Morganella morganii</i> (n = 1) | - | - | - | 1 (100) |
| <i>Enterobacter asburiae</i> (n = 1) | - | - | 1 (100) | - |
| <i>Enterobacter bugandensis</i> (n = 1) | - | - | - | 1 (100) |
| Total (n = 135) | 26 (19.3) | 29 (21.5) | 39 (28.9) | 41 (30.4) |

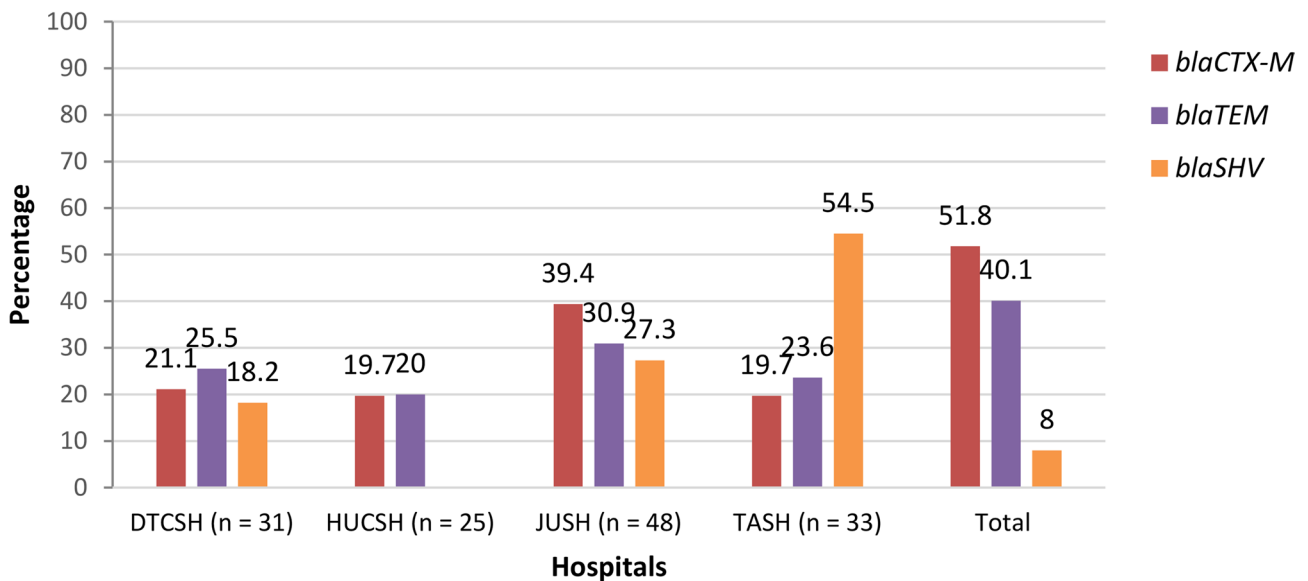


Fig. 2 Frequency of *bla_{CTX-M}*, *bla_{TEM}* and *bla_{SHV}* families detected at least once from GNB subjected to WGS per study site. DTCSH: Debre Tabor Comprehensive Specialized Hospital, HUCSH: Hawassa University Comprehensive Specialized Hospital, JUSH: Jimma Specialized Hospital, and TASH: Tikur Anbessa Specialized Hospital

GNB, the possible risk factors for the increased ESBL producing GNB were assessed, and the multivariable analysis showed statistically significant association only between the previous use of antibiotics (p-value = 0.003) independent variables and frequencies of ESBL producing GNB (Table 3).

ESBL genes

Several variants of *bla_{CTX-M}* that are ESBL were detected among the GNB sequenced from all the study sites

(Table 4). The most frequent gene was *bla_{CTX-M-15}* with an overall detection rate of 44.4%. The frequency of *bla_{CTX-M-15}* at HUCSH, DTCSH, TASH, and JUSH was 15%, 23.3%, 20% and 41.7%, respectively. On the other hand in addition to *bla_{CTX-M-15}*, three other *bla_{CTX-M}* variants were found at HUCSH while *bla_{CTX-M14b}* found in all hospital sites (Table 4). *E. cloacae* (66.7%) were the most common *bla_{CTX-M-15}* producer followed by *K. pneumoniae* (62.5%). On the other hand *E. cloacae*, *K. pneumoniae*, *A. baumannii*, and *E. coli* harbored

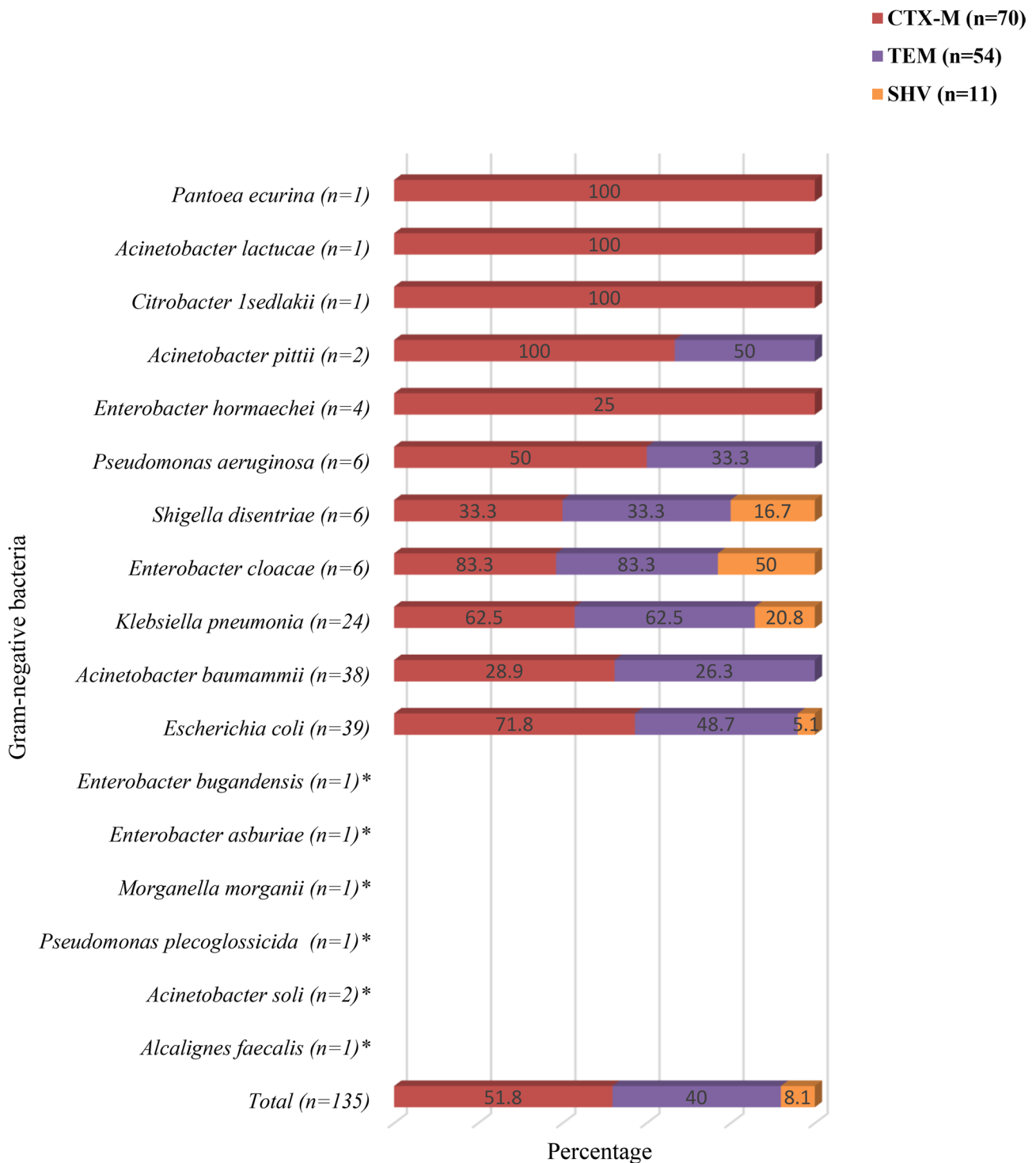


Fig. 3 Frequency and distribution of Gram-negative bacteria that encoded *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes detected using the whole genome sequence. * No *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} detected

*bla*_{TEM-1B} with the frequencies of 66.7%, 50%, 23.7% and 16% (Fig. 5).

The detection of *bla*_{CTX-M-15} from, *E. coli*, *S. dysenteriae*, and *A. baumannii* was 56.4%, 33.3%, and 21%, respectively. The detection of *bla*_{SHV-187} from *E. cloacae*

was 50% followed by 20.8% *K. pneumoniae* (Fig. 5). The *bla*_{SHV-55,114,142, 64,184,186,194and210} was another ESBL gene detected at 4.2% frequency from a single *K. pneumoniae* at TASH only. The *bla*_{ACT-15} and *bla*_{ACT-16}, *bla*_{GES-11},

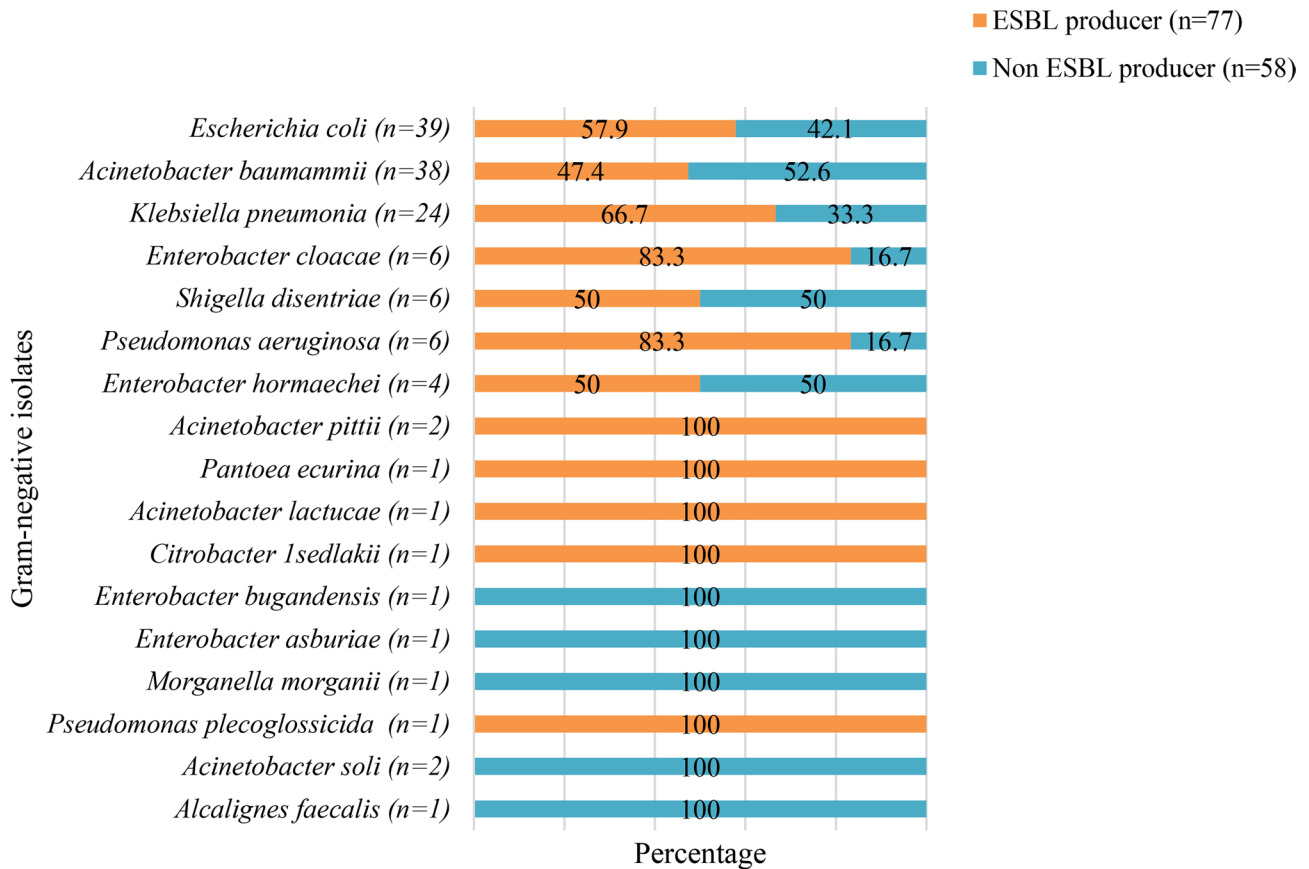


Fig. 4 Frequency and distribution of Gram-negative bacteria that are ESBL and carbapenemase producers and non-producers

Table 3 Frequency of Gram-negative bacteria that harboured at least one ESBL gene in relation to patient characteristics

| Variables | Characteristics | Total N (%) | ESBL gene | | CRO (95% CL) | P-value | AOR (95% CL) | P-value |
|-----------------------------|-----------------|----------------|----------------|----------------|-------------------|---------|------------------|---------|
| | | | Positive n (%) | Negative n (%) | | | | |
| Sex | Male | 74 (54.8) | 35 (47.3) | 39 (52.7) | 0.629 (0.31-1.26) | 0.2 | 0.62 (0.29-1.31) | 0.207 |
| | Female | 61 (45.2) | 39 (63.9) | 22 (36.1) | | | | |
| Age (in years) | | 34 (25.2) | 17 (72.7) | 17(27.3) | 1 | | | |
| | 19-40 | 62 (45.9) | 26 (78.9) | 36 (21.1) | | | | |
| | 41-60 | 29 (21.5) | 10 (88.9) | 19 (11.1) | | | | |
| | ≥ 61 | 10 (7.4) | 6 (60) | 4 (40) | | | | |
| Hospital | DTC SH | 29 (21.5) | 14 (48.3) | 15 (51.7) | | | | |
| | HUC SH | 26 (19.3) | 16 (61.5) | 10 (38.5) | | | | |
| | JUSH | 40 (29.6) | 31 (77.5) | 9 (22.5) | | | | |
| | TASH | 40 (29.6) | 17 (42.5) | 23 (57.5) | | | | |
| Preoperative hospital stay | ≤ 7 | 57 (42.2) | 43 (75.4) | 14 (24.5) | 1 | | | |
| | > 7 | 78 (57.8) | 69 (88.5) | 9 (11.5) | | | | |
| Previous use of antibiotics | Yes | 78 (57.8) | 74 (74.9) | 4 (5.1) | 8.54 (2.70-26.98) | 0.00 | 27 (3.0-250) | 0.003 |
| | No | 57 (42.2) | 39 (68.4) | 18 (31.8) | | | | |
| Nature of surgery | Elective | 39 (28.8) | 25 (64.1) | 14 (35.9) | 0.96 (0.32-1.49) | 0.3 | | |
| | Emergency | 96 (71.1) | 53 (55.2) | 43 (44.8) | | | | |
| Duration of operation | ≤ 1 h | 57 (42.2) | 27 (47.4) | 30 (52.6) | 1.1 (0.55-2.2) | 0.8 | | |
| | > 1 h | 78 (57.8) | 45 (57.7) | 33 (42.3) | | | | |

Commonly detected beta-lactamase

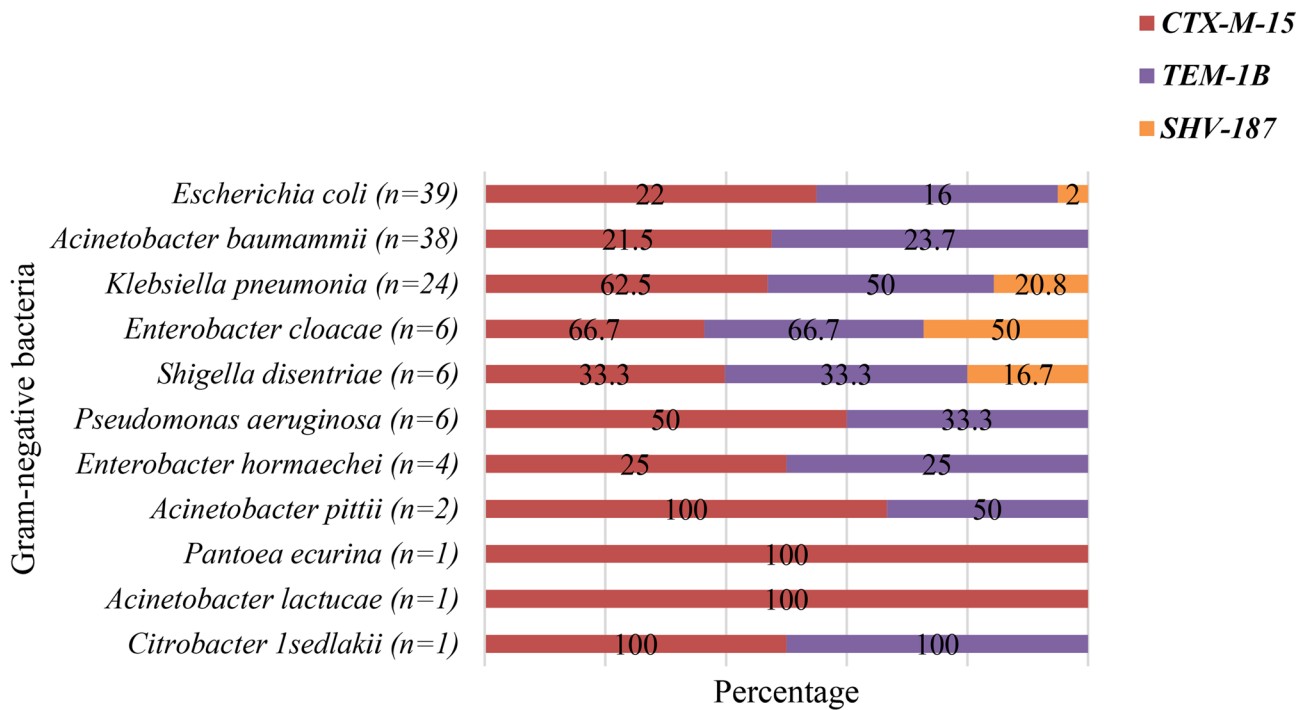


Fig. 5 Frequency and distribution of *bla*_{SHV-187}, *bla*_{CTX-M-15} and *bla*_{TEM-1B} that were detected frequently among Gram-negative bacteria

Table 5 Frequency and distribution of other *bla*_{TEM} and *bla*_{SHV} variants detected at four Ethiopian hospitals

| | | Total n (%) | DTCSH n (%) | HUCSH n (%) | JUSH n (%) | TASH n (%) |
|-----------------------|-------------------------------|-------------|-------------|-------------|------------|------------|
| TEM variants detected | <i>bla</i> _{TEM-1B} | 47 (34.8) | 11(23.4) | 9 (19.1) | 17 (36.2) | 10 (21.3) |
| | <i>bla</i> _{TEM-33} | 3 (2.1) | 1 (33.3) | 1 (33.3) | | 1 (33.3) |
| | <i>bla</i> _{TEM-219} | 1 (0.7) | 1 (100) | | | |
| | <i>bla</i> _{TEM-1 C} | 1 (0.7) | 1 (100) | | | |
| SHV variants detected | <i>bla</i> _{SHV-187} | 11 (8.1) | 2 (18.1) | - | 3 (27.3) | 6 (54.5) |
| | <i>bla</i> _{SHV-82} | 1(0.7) | | | | 1(100) |
| | <i>bla</i> _{SHV-139} | 1(0.7) | | | | 1(100) |
| | <i>bla</i> _{SHV-163} | 1(0.7) | | | | 1(100) |
| | <i>bla</i> _{SHV-182} | 1(0.7) | | | | 1(100) |
| | <i>bla</i> _{SHV-187} | 1(0.7) | | | | 1(100) |
| | <i>bla</i> _{SHV-188} | 1(0.7) | | | | 1(100) |

*bla*_{PAO} and *bla*_{CMY-72} were the other rare ESBL genes detected (Table 4).

Non-ESBL β-Lactamase variants of *bla*_{TEM} and *bla*_{SHV}

Although some variations of *bla*_{TEM} and *bla*_{SHV} are thought to be common ESBL determinants, the majority of variants found in this investigation were distinct broad-spectrum beta-lactamase genes that weren't ESBL (Table 5). The most frequently detected *bla*_{TEM} variant was *bla*_{TEM-1B} (34.8%) and the frequencies were 19.1%, 21.3%, 23.4% and 36.2% at HUCSH, TASH, DTCSH and JUSH, respectively. The other three *bla*_{TEM} variants were detected at DTCSH. While no other *bla*_{TEM}

variants were detected at JUSH (Table 5). Among the *bla*_{SHV} variants that are not ESBL, the most commonly found one was *bla*_{SHV-187} (8.1%), which was first identified at TASH (54.5%) and then at JUSH (27.3%). Table 5 shows this information. At HUCSH, it was not detected, but at DTCSH, 18.1% of it was. Only at TASH were the other *bla*_{SHV} variants detected. The common producers of *bla*_{SHV-187} were *E. cloacae* (50%) followed by *K. pneumoniae* (20.8%), while *bla*_{SHV-187} was rare among *E. coli* (5.1%) (Fig. 4).

Table 6 Co-occurrence of multiple ESBL genes and ESBL genes with other common non-ESBL variants of *bla_{TEM}* and *bla_{SHV}*

| Combinations of genes | Total <i>n</i> | DTCSH <i>n</i> | HUSH <i>n</i> | JUSH <i>n</i> | TASH <i>n</i> |
|---|-------------------|-------------------|------------------|------------------|------------------|
| CTX-M-15*TEM-1B | 21 | 5 | 4 | 8 | 4 |
| CTX-M-15*ACT15 | 1 | | | 1 | |
| CTX-M-2* CTX-M-27 | 1 | | 1 | | |
| CTX-M-15*PAO | 1 | | | 1 | |
| CTX-M-14b*TEM1B | 2 | 1 | 2 | | 1 |
| CTX-M-15*TEM1C | 1 | 1 | | | |
| <i>bla_{SHV-187}</i> * CTX-M-15 | 2 | | | 1 | 1 |
| TEM1B*VAN | 1 | 1 | | | |
| TEM-1B *CTX-M-15*ACT-15 | 2 | 1 | | 1 | |
| TEM-1B *CTX-M-15*ACT-16 | 2 | | | 2 | |
| TEM-33 *TEM-169*CTX-M-15 | 2 | | 1 | | 1 |
| SHV-187*SHV-106*CTX-M-15 | 1 | 1 | | | |
| SHV-187 *TEM-219*CTX-M-15 | 1 | 1 | | | |
| <i>bla_{SHV-187}</i> * TEM-1B*CTX-M-15 | 6 | 2 | | 2 | 2 |
| TEM-1B *CTX-M-15*CTX-M-14b | 4 | 1 | | 2 | |
| TEM-1B *CTX-M-15*ACT-16*CMY6 | 1 | 1 | | | |
| SHV-55*SHV-82*SHV-114*SHV- 139*SHV-142*SHV-184*SHV- 186*SHV-194*SHV-210 | 1 | | | | 1 |

Co-occurrence of multiple ESBL genes

Multiple ESBL genes co-occurring or one ESBL gene co-occurring with additional *bla_{TEM}* and *bla_{SHV}* variations were found in various Gram-negatives (Table 6). It was discovered that GNB carrying the *bla_{CTX-M-15}* gene also harboured non-ESBL variations of *bla_{TEM}* and *bla_{SHV}* as well as multiple more ESBL genes. The co-occurrence of *bla_{CTX-M-15}* and *bla_{TEM-1B}* (*n*=21) was the most frequent gene combination followed by *bla_{CTX-M-14B}* and *bla_{TEM-1B}*. While the 3 combinations of *bla_{SHV-187}* * *bla_{TEM-1B}* * *bla_{CTX-M-15}* were detected among 6 GNB, the 4 *CTX-M-15* * *TEM-1B* * *ACT-16* * *CMY6* gene combination was detected from 1 GNB (*E.coli*) (Table 6).

Discussion

Infections caused by ESBL-producing Gram-negative bacteria are increasing at an alarming rate and have become a serious public health threat worldwide. The current study is showing the molecular epidemiology of ESBL producing Gram-negative bacteria among patients investigated for surgical site infection at four referral hospitals located in the Amhara region, Addis Ababa, southern region and Oromia region of Ethiopia. In the present study, a significant association between ESBL producing GNB infection and previous history of used antibiotics. Unlike our study, a report showed that Chronic illnesses and insertion of medical instruments into the body increase the likelihood of producing ESBL [7]. Of all the GNB subjected to WGS, 57.8% encoded at least one ESBL gene. Similar with our study, the previous reports

from Nigeria 58% [8] and Gaza also showed 59% occurrence of ESBL [9]; On the other hand, this study, higher than the previous systematic reports of pooled prevalence of ESBL-producing GNB in Ethiopia was 50% [18] and a lower detection rate was reported when it compared with study conducted in Jimma 63.4% [20]. These results, variation, revealed that the prevalence of ESBL gene types can vary between locations or geographical regions, over use of antibiotics use and time.

The *bla_{CTX-M-15}* detected from *E. cloacae* (66.7%), *K. pneumoniae* (62.5%), *E. coli* (56.4%), *P. aeruginosa* (50%), and other ESBL producing Gram-negative bacteria. This discovery suggests that other GNB have acquired the CTX-M genes. Similar findings have been reported in Jimma, Ethiopia [20]. Due to the rising incidence of MDR SSI and the scarcity of available antibiotics for treatment, the rise in bacteria generating ESBL enzymes in SSI patients is cause for worrying. More importantly, the high level of ESBL producing strains among SSI causing GNB in low-income countries is a major public health problem, due to the limited laboratory services and therapeutic options available. The emergence of ESBLs has been made possible by the presence of ESBL-encoding genes on plasmids and within transposons and insertion sequences [3].

In this study, *bla_{CTX-M}* (51.8%) was the most frequently detected ESBL family across the four referral hospitals. This finding was comparable with study conducted in Nigeria reported 48% [8]. This finding was lower than study performed in Jimma that reported 95.8% of *bla_{CTX-M}* [20]; however, a very low detection rate were reported in the Nepal [10] and Saudi Arabia 33% [12]. The *bla_{CTX-M-15}* (44.4%) was the most abundant ESBL gene detected among *bla_{CTX-M}*. These findings were comparable with different studies across the globe [3, 9, 12, 20].

The *bla_{CTX-M-15}* was very abundant at JUSH (41.7%), DTCSH (23.3%), and TASH (20%) while it was detected at a comparatively lower rate at HUCSH (15%). The detection of high levels of *bla_{CTX-M}* at JUSH may possibly be explained because the hospital is the main destination of patients referred from all Oromia region which is the largest region in Ethiopia.

Although the dissemination of *bla_{CTX-M-15}* was followed by *CTX-M-14B* in all hospitals, *bla_{CTX-M-2}*, *bla_{CTX-M-27}* and *bla_{CTX-M-169}* were detected only at DTCSH (northern). Today the *CTX-M-15* variant dominating worldwide, followed by *CTX-M-14*, and *CTX-M-27* is emerging in certain parts of the world [3].

In this study, *bla_{TEM}* (40%) was also detected in abundance. This result is comparable with study performed in Nigeria was 30.9% [8]. However the finding was lower than other studies [12]. Of several variants of *bla_{TEM}* detected, *bla_{TEM-1B}* (34.8%) was the most frequent. Broad-spectrum or inhibitor-resistant beta-lactamases

were the other *bla*_{TEM} variants. It is concerning that the majority of *bla*_{TEM} variants were co-detected with ESBL genes, such as *bla*_{CTX-M} variants and other ESBL genes, even though the majority of *bla*_{TEM} beta-lactamases detected were not ESBL.

Similarly, the detection of *bla*_{SHV} (8.1%) showed similarities with a study conducted in Burkina Faso were reported 5.9% of the *bla*_{SHV} genes and Saudi Arabia 2.1% [12] while this finding is lower than study conducted in Asella, Ethiopia 27.3% [13]. The ESBL variants of *bla*_{SHV} detected in this study were *bla*_{SHV-106}. The entire *bla*_{SHV} variant was detected at DTCSH. A similar result *bla*_{SHV-106} detection rate was reported in Portugal [14]. Whereas *bla*_{SHV-55}, *bla*_{SHV-114}, *bla*_{SHV-164}, *bla*_{SHV-184}, *bla*_{SHV-186}, *bla*_{SHV-186} and *bla*_{SHV-210} was another ESBL gene detected at 4.2% frequency from a single *K. pneumoniae* at TASH only. A similar SHV variant *bla*_{SHV-55} was detected among *K. pneumoniae* in Portugal [15].

The majority of bacteria that encoded ESBL also carried other beta-lactamase genes, and multiple ESBL genes were found in multiple cases. The *bla*_{CTX-M-15} gene co-occurred with several other ESBL genes and the non-ESBL variants of *bla*_{TEM} and *bla*_{SHV}. The co-occurrence of *bla*_{CTX-M-15} and *bla*_{TEM-1B} was the most frequently detected gene combination, followed by the *bla*_{SHV-187}**bla*_{TEM-1B}**bla*_{CTX-M-15} combination and *bla*_{TEM-1B}**bla*_{CTX-M-15}**bla*_{CTX-M-14b}. The findings of this co-occurrence of multiple ESBL genes were in agreement with the other studies. Thus, coexpression of different ESBL types is more common among GNB, which harbors CTX-M-type enzymes [21]. Alarmingly, these results demonstrated the spread of GNB harbouring numerous ESBL genes in the study areas.

Conclusions

A high genotypic frequency of ESBL producing GNB among SSI patients was detected. Different ESBL gene variants were found, with *bla*_{CTX-M-15} being the most frequently detected. In addition to the ESBL genes, diverse variants of *bla*_{TEM} and *bla*_{SHV} were detected, where *bla*_{TEM-1B} and *bla*_{SHV-187} being the most frequently detected variants in their respective families. Multiple combinations of ESBL genes were detected.

Limitation of the study

Due to financial limitations, only 135 isolates underwent WGS tested.

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Authors' contributions

S.W. was the primary researcher who conceived this study and was involved in data collection, laboratory investigation, data analysis, interpretation of the findings, drafting the manuscript, and write-up. A.B. and A.A. (Ashenafi Alemu) substantially participated in laboratory investigation. T.A., A.A. (Alemseged Abdissa), G.T.B., B.S., A.M., and G.S. substantially participated in the design of this study, reviewed the manuscript, and provided critical intellectual content. G.S. participated in the analysis and interpretation of the results. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data sets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical clearance and approval were obtained from Addis Ababa University's College of Health Sciences and AAREC, AAUMF03-008/2020. The Department of Medical Microbiology, Immunology, and Parasitology (DMIP) and the AHRI/ALERT Research Ethics Committee (AAREC) reviewed and approved the study, and institutional review board (IRB) approval was obtained from Addis Ababa University's College of Health Sciences and AAREC, AAUMF03-008/2020. The study was also approved by AHRI/ALERT Ethics Review Committee (protocol number: P0/2919) of the Armauer Hansen Research Institute and National Ethical Review committee (Ref No. MoE/17/246/767/23). A written permission letter was obtained from each study site before starting the data collection. The purpose and procedures of the study were explained to the study participants, participants' parents, or guardians before the commencement of the actual specimen collection. Those study participants who gave written informed consent and those children whose parents or guardians gave informed consent were selected and enrolled in this study. Results obtained from all patients were communicated to attending physicians and treated according to the protocol of the hospital. All patients' information was kept confidential by using an identifier/code to protect patient information from unauthorized person. In addition, the study was conducted in accordance with the declaration Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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