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Characterization of Microbial Resistome in Bacteria Isolated from Human, Environmental and Animal sources using DNA Microarray Technology and Genome Sequencing

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Keywords

Antimicrobial resistance, Antibiotic resistance, Beta-lactam resistance, Carbapenemase, Carbapenem-resistance, Extended spectrum β-lactamase, Jimma Medical Center, KPC, Methicillin resistant *S. aureus*, Multidrug resistance, NDM, OXA, and Resistome.

Abstract

Background: Antimicrobial resistance (AMR) is a growing threat to public health globally. The impact is even worse in resource constrained countries. The occurrence of antimicrobial resistant bacteria in animals, the environment, and apparently healthy humans exacerbates the problem and serves as a reservoir for further dissemination. In the study area, Jimma, Ethiopia, there is no comprehensive data about the prevalence, diversity, and distribution of AMR in various sectors. Therefore, the current study aimed to address the existing scarce data related to AMR and provide comprehensive information on the matter.

Methods: A cross-sectional study design was employed to understand the prevalence, diversity, and distribution of AMR in bacteria isolated from various sources. All the bacterial isolates were re-identified with matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS). Antibiotic susceptibility testing (AST) was determined using the Kirby-Bauer disk diffusion method and Etest strips. The phenotype ESBL screening was done by double disc synergetic test (DDST) and Mast disks. Molecular characterization of Gram-negative bacteria (GNB) was performed by multiplex polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) microarray techniques.

Result: A wide range of bacterial species were identified in samples obtained from patients, animals, the environment, and apparently healthy humans. *E. coli* (22.9%) was the most predominant isolate followed by *Klebsiella* species (21.1%), *Enterobacter* species (10.7%), and *Acinetobacter* species (12.5%). In GNB, a high rate of resistance against ampicillin (90%), cefuroxime (82%), amoxicillin-clavulanic acid (76%), piperacillin (75%), and cefotaxime (74%) was observed. Extended spectrum beta-lactamase (ESBL) producers were isolated in all sample categories. However, the prevalence and diversity were variable. The highest proportion was exhibited in clinical samples (76.6%) followed by environmental (49.2%) and animal samples (28.2%). The molecular analysis of GNB showed that *bla*CTX-M and *bla*NDM were the predominant acquired ESBLs and carbapenemase encoding genes, respectively. The co-existence of multiple resistance genes was observed in a lot of isolates.

Conclusions: The findings revealed a high rate of resistant bacterial species in clinical, environmental, animal, and human samples from apparently healthy subjects. Various genes encoding for beta-lactam resistance were identified in all sample categories, predominantly was *bla*CTX-M and *bla*NDM.

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List of abbreviations and acronyms

AIDS: Acquired immunodeficiency syndrome

ALF: AmpC-type β-lactamase discovered in Alcaligenes faecalis

AMR: Antimicrobial resistance

AST: Antibiotic susceptibility testing

CTX-M: Cefotaxime-Munich

DNA: Deoxyribonucleic acid

erm: Encode ribosomal methyltransferases

ESBL: Extended spectrum β-lactamase

GNB Gram-negative bacteria

GPB Gram-positive bacteria

HIV: Human immunodeficiency virus

IMP: Imipenemase

IRB: Institutional Review Board

JMC: Jimma Medical Center

KPC: *Klebsiella pneumoniae* carbapenemase

MALDI-TOF MS: Matrix-assisted laser desorption ionization-time of flight mass spectrometry

MDR: Multi-drug resistance

MIC: Minimum inhibitory concentration

MLST: Multilocus sequence typing

NDM: New Delhi metallo-β-lactamase

NICU: Neonatal intensive care units

OXA: Oxacillinases

PCR: Polymerase chain reaction

SHV: Sulfhydryl reagent variable Enzyme

TEM: Temoniera strain of *Escherichia coli*

UK: United Kingdom

USD: United States Dollar

VIM: Verona integron-encoded metallo-beta-lactamase imipenemase

WGS: Whole genome sequencing

WHO: World Health Organization

XDR: Extensively drug resistance

List of publications

Paper I: Gashaw M, Gudina EK, Ali S, Gabriele L, Seeholzer T, Alemu B, Froeschl G, Kroidl A, Wieser A. Molecular characterization of carbapenem-resistance in Gram-negative isolates obtained from clinical samples at Jimma Medical Center, Ethiopia. Frontiers in Microbiology 2024.;15:1336387.
https://doi.org/10.3389/fmicb.2024.1336387

Paper II: Gashaw, M.; Gudina, E.K.; Tadesse, W.; Froeschl, G.; Ali, S.; Seeholzer, T.; Kroidl, A.; Wieser, A. Hospital Wastes as Potential Sources for Multi-Drug-Resistant ESBL-Producing Bacteria at a Tertiary Hospital in Ethiopia. Antibiotics 2024, 13, 374. https://doi.org/10.3390/antibiotics13040374

Paper III: Gashaw M, Ali S, Berhane M, Tesfaw G, Eshetu B, Workneh N, Seeholzer T, Froeschl G, Kroidl A, Wieser A, Gudina EK. Neonatal sepsis due to multi-drug-resistant bacteria at a tertiary teaching hospital in Ethiopia. *Pediatr Infect Dis J* 2024;43:687–693. https://doi.org/10.1097/INF.0000000000004364

1. My contribution to the publications

1.1 Contribution to paper I

My contribution to the paper " Gashaw M, Gudina EK, Ali S, Gabriele L, Seeholzer T, Alemu B, Froeschl G, Kroidl A, Wieser A. Molecular characterization of carbapenem-resistance in Gram-negative isolates obtained from clinical samples at Jimma Medical Center, Ethiopia. Frontiers in Microbiology 2024.;15:1336387." involves various stages of the research process. I played a leading role in formulating the study concept, designing the study, and data curation both at Jimma and Munich. The data curation included specimen inoculation, inspection, bacteria identification using classical methods, AST using Kirby-Bauer disk diffusion technique and saving the isolates at JMC Microbiology laboratory. Additionally, I contributed to the inoculation, re-identification of isolates using MALDI TOF MS, AST using Kirby-Bauer disk diffusion technique, and automated reading using ADAGIO (BioRad) at Max von Pettenkofer Institute (MvPI), Medical Microbiology Laboratory in Munich, Germany. I also conducted ESBL screening using mast disc and determined the MIC of carbapenem antibiotics using Etest strips. Furthermore, I performed DNA extraction and participated in the characterization of carbapenem-resistant bacterial isolates using multiplex PCR. Finally, I took the leading role in manuscript writing, starting from preparing the initial draft and finalizing it for publication. I also took full responsibility for preparing the response letter to the editor's and reviewers' comments, suggestions, and feedback. Throughout all these activities, I received guidance and support from my supervisors and other co-authors.

1.2 Contribution to paper II

I have contributed significantly to the paper "Gashaw, M.; Gudina, E.K.; Tadesse, W.; Froeschl, G.; Ali, S.; Seeholzer, T.; Kroidl, A.; Wieser, A. Hospital Wastes as Potential Sources for Multi-Drug-Resistant ESBL-Producing Bacteria at a Tertiary Hospital in Ethiopia. Antibiotics 2024, 13, 374". My involvement was active at various stages of the research process. I took a leading role in the development of the study concept, study design, and data curation, both at Jimma and Munich. This included tasks such as sample collection, specimen processing, isolation, identification, and conducting AST using the Kirby-Bauer disk diffusion technique at JMC microbiology laboratory. Additionally, I participated in inoculation, re-identification of the isolates using MALDI TOF MS, AST

using the Kirby-Bauer disk diffusion method, and automated reading using ADAGIO (Bio-Rad) at MvPI Medical Microbiology Laboratory in Munich, Germany. I conducted ESBL screening using the DDST and determined the MIC of carbapenem antibiotics using Etest strips. Furthermore, I was involved in DNA extraction and the characterization of *E. coli* strains using DNA microarray techniques. Moreover, I took the lead in the manuscript development, from producing the initial draft to finalizing it for publication. I also took full responsibility for preparing the response letter, addressing the comments, suggestions, and feedback from the editor and reviewers. Throughout these tasks, I received guidance and support from my supervisors and other co-authors similar to other articles.

1.3 Contribution to paper III

I have made substantial contributions to the publication of the paper " Gashaw, M.; Ali, S.; Berhane, M.; Tesfaw, G.; Eshetu, B.; Workneh, N.; Seeholzer, T.; Froeschl, G.; Kroidl, A.; Wieser, A.; Gudina, E.K. Neonatal Sepsis Due to Multidrug-resistant Bacteria at a Tertiary Teaching Hospital in Ethiopia. The Pediatric Infectious Disease Journal 43(7): p 687-693, July 2024. I actively participated in the development of study concepts, study design, and data curation, both at Jimma and Munich. For instance, I participated in specimen processing, bacterial isolation, identification, and AST. I was also responsible for saving the isolates using storage media at JMC Microbiology Laboratory. In Munich, at MvPI, Medical Microbiology Laboratory, I participated in inoculation and re-identification of the isolates using MALDI TOF MS and conducted AST using Kirby-Bauer disk diffusion technique and automated reading using ADAGIO (Bio-Rad). I also performed ESBL screening using mast discs and DDST, as well as determining the MIC of carbapenem antibiotics using Etest strips. Moreover, I played a significant role in the manuscript write-up, from preparing the initial draft to finalizing it for publication. I took full responsibility for preparing the response letter, addressing the comments, suggestions, and feedback provided by the editor and reviewers. Throughout these tasks, I received valuable guidance and support from my supervisors and other co-authors.

2. Introduction

2.1 Background

AMR is the ability of microorganisms to develop resistance against antimicrobial drugs that were once effective in treating infections caused by them. This phenomena could be observed in bacteria, viruses, parasites, and fungi [1]. It can arise through diverse mechanisms [2]. These mechanisms include mutations, enzymatic inactivation, efflux pumps, reduced permeability, target modification, or acquisition of resistance genes [3-7]. Mutations can alter the genetic material of bacteria and make changes in their cellular components that could be the target site for the drugs [8]. Enzymatic inactivation on the other hand involves the production of enzymes by bacteria that can chemically modify or inactivate antimicrobial drugs [4]. Efflux pumps are cellular machinery that actively pumps out antimicrobial drugs from bacterial cells and subsequently reduce its concentration within the cell [9]. Reduced permeability refers to altering the structure of bacterial cell membranes that limit the entry of antimicrobial drugs [6]. Target modification occurs when the bacteria undergo changes on specific antimicrobial targets on the bacterial cell [10, 11]. Lastly, bacteria can acquire resistance genes from other bacteria through horizontal gene transfer via different mechanisms such as conjugation, transformation, or transduction, which provides them with genetic instructions to resist the effects of antimicrobial drugs [12-14]. Through these various mechanisms, bacteria have developed the ability to survive and multiply even when exposed to antimicrobial drugs.

There are numerous encoding genes that are commonly found in both GPB and GNB, contributing to their antibiotic resistance [15, 16]. In GPB, there is for example the mecA gene, which confers methicillin resistance in *S. aureus.* There are also vanA and vanB genes, which are associated with vancomycin resistance in *Enterococcus* species [17, 18]. Additionally, erm genes such as ermB and ermC are responsible for resistance to macrolide antibiotics in GPB [19]. Similarly, in GNB, there are several resistance encoding genes that make them resistant to various antibiotics [20]. For instance, *bla*TEM, *bla*CTX-M, and *bla*SHV genes are frequently associated with the production of ESBLs, resulting in resistance to a broad range of β-lactam antibiotics including penicillins and cephalosporins [21, 22]. Furthermore, carbapenemase-encoding genes like *bla*KPC, *bla*NDM, VIM, IMP, ALF, and *bla*OXA are responsible for conferring resistance to carbapenem antibiotics as well as other beta-lactams [22-24].

These resistant mechanisms can function either independently or in combination with each other, making the bacteria resistant to multiple antibiotics [11]. Therefore, understanding the mechanisms of AMR is of utmost importance for developing effective strategies to combat AMR [11, 25]. By targeting specific mechanisms, it is possible to decrease AMR development and spread. This could help to preserve the efficacy of currently available antimicrobial drugs that are used in the treatment of bacterial infections routinely [26]. The presence of these resistance encoding genes in bacteria has become a global concern in clinical settings, as they could easily transfer from one bacteria to another and limit the efficacy of antibiotics and pose challenges to the treatment of bacterial infections [27, 28].

The emergence and spread of antibiotic resistance are a complex and dynamic process influenced by various factors [29]. These factors include inappropriate utilization of antimicrobial drugs both in humans and animals, poor infection prevention and control measures, limited access to clean water and sanitation, international travel and globalization, trade, lack of regular AMR surveillance and monitoring, decline in the development of new antimicrobial drugs compared to the rising resistance rates, use of chemicals and fertilizers in agriculture, wind, and many others [30, 31]. Therefore, addressing these multifaceted risk factors require a comprehensive approach involving collaborations among healthcare professionals, policymakers, researchers, and the public [31].

Figure 1: One-Health schematic representation of AMR dissemination

Accurate and timely diagnosis of AMR and its determinants is crucial for guiding appropriate treatment decisions, optimizing patient outcomes, and most importantly preventing the spread of resistant microorganisms [32, 33]. Nowadays, there are several methods

available for diagnosing AMR [34]. Phenotypic AST, which includes the Kirby-Bauer disk diffusion method (qualitative) and the MIC method (quantitative) are commonly used. Genotypic testing, such as PCR and DNA sequencing, allows for the detection of specific genetic markers or encoding genes associated with resistance [35-38]. Molecular methods such as multiplex PCR and DNA microarrays are also utilized for AMR diagnosis [36, 39, 40]. Additionally, rapid diagnostic tests are becoming increasingly popular in recent years [41]. However, it is important to note that the availability and utilization of different diagnostic methods may vary depending on the healthcare setting and resources available [42, 43]. Moreover, the interpretation of these test results require expertise and an understanding of local resistance patterns and their clinical correlations [42].

2.2 Statement of the problem

The increasing prevalence of AMR is a growing global concern that has a substantial impact on public health, healthcare systems, and the global economy [44, 45]. According to a systematic analysis, bacterial AMR was responsible for an estimated total of 4.95 million deaths globally in 2019, with 1.2 million deaths being directly attributed to it [46]. If effective measures are not implemented, this number could escalate to 10 million deaths by 2050, surpassing the mortality caused by cancer [47, 48]. Furthermore, the economic impact of AMR is becoming substantial [49, 50]. Treating infections caused by resistant pathogens often requires more expensive and prolonged treatment regimens, including the use of second-line or last-resort antibiotics [49]. As a result, healthcare costs escalate significantly for individuals and healthcare systems [51]. According to recent studies, it is estimated that by 2050, the global economic impact of AMR could reach 300 billion to more than 1 trillion USD if appropriate actions are not taken [51-53].

Low-income countries show a particularly heavy burden of AMR due to several contributing factors [54, 55]. These factors include high disease burdens, limited resources, and inadequate access to quality healthcare [55]. In these countries, infectious diseases such as tuberculosis, malaria, HIV/AIDS, and diarrheal diseases are highly prevalent [56]. Treating these diseases often requires antimicrobial therapy, and the emergence of resistant strains complicates their management [57]. As a result, treatment failures, increased rates of illness and death, and higher healthcare costs are observed in alarming rates in these settings [58]. The limited access to healthcare in these countries also contributes to inappropriate use and suboptimal dosing of antimicrobial drugs, further raising the risk of AMR development [55]. Limited resources could also contribute to inadequate infection prevention and control measures and a limited or a lack of data regarding the prevalence and trends of AMR within healthcare settings [59]. These conditions promote the transmission of resistant pathogens [60]. In addition, poverty in these countries further exacerbates the problem, as there are high numbers of vulnerable populations, including children, the elderly, and immunocompromised individuals [61]. This vulnerability, exacerbated by malnutrition, intensifies the rates of illness and death caused by AMR [62].

AMR poses a substantial healthcare burden in sub-Saharan countries, leading to detrimental consequences for public health [63]. In 2019, there were approximately 1.05 million deaths associated with AMR bacterial infections, with 250,000 deaths directly attributed to AMR [64]. The high prevalence of AMR bacterial pathogens in these countries

has made the treatment of common bacterial infections, such as pneumonia, urinary tract infections, and bloodstream infections, increasingly challenging due to the limited effective antimicrobials [65]. Moreover, the rise of AMR has adverse effects on the economy, impacting sectors such as agriculture, livestock production, and trade [66]. In sub-Saharan countries that heavily rely on agriculture, the loss of effective antimicrobials for treating animal diseases poses a significant threat to food security and livelihoods [66-68].

In Ethiopia, a country burdened by poverty, war, and conflicts, AMR poses a serious threat to public health, just as it does in many other low-income countries worldwide [69, 70]. The presence of AMR complicates the treatment of infectious diseases, making it increasingly challenging to effectively manage and control them [69]. This, in turn, raises the risk of mortality and morbidity among the population [71]. One of the primary challenges faced in Ethiopia is the limited availability of appropriate diagnostic tools and well-equipped laboratory facilities [72, 73]. As a result, healthcare providers may face difficulties in accurately diagnosing infections and determining the most appropriate course of treatment [74]. This could lead to a tendency for over-prescription and improper use of antibiotics [64, 75]. Moreover, this malicious practice could contribute to the development and spread of AMR [76]. Therefore, conducting AMR-related research in Ethiopia is of paramount importance to gain insights into the prevalence, patterns, and drivers of resistance within the country [77]. Such research can inform policy makers, guide the development of effective interventions, and contribute to global efforts in combating AMR [78].

In this respect, this study aims to provide base line data to understand the magnitude of AMR and deliver valuable insights into the specific challenges and factors influencing AMR in Jimma, Ethiopia. The study was done at JMC and its vicinities, a large tertiary, referral, and teaching hospital responsible for serving to a catchment area of over 20 million population in Southwest Ethiopia. Its significant size and comprehensive services make it a vital healthcare hub in the region. As a tertiary and referral hospital, it offers specialized medical care and handles complex cases referred from the surrounding region, ensuring access to advanced treatments and specialized expertise. Therefore, conducting research in this setting, it becomes possible to uncover the underlying issues associated with AMR, comprehending its true magnitude, the contributing factors to its development and spread. Consequently, this approach allows for the development of targeted and context-specific strategies to mitigate the impact of AMR on public health, benefiting the large population within the region and Ethiopia at large.

3. Objectives

3.1 General objective

- To identify and characterize the microbial resistome in bacteria isolated from human, environmental, and animal sources using phenotypic testing, DNA microarray, and genome sequencing.

3.2 Specific objectives

- To assess the resistance profiles of bacteria isolated from neonates suspected of having infections at JMC.
- To assess the prevalence and carbapenemase expression among GNB isolated from patients at JMC.
- To assess the prevalence and distribution of specific antibiotic resistance encoding genes among the bacteria isolated from different sources.
- To assess the impact of environmental factors, such as sewage, surface swabs, and houseflies in the spread of AMR.
- To evaluate the potential transmission dynamics of antibiotic-resistant *E. coli* strains obtained from different sources and their implications for public health and environmental management.

4. Methods and materials

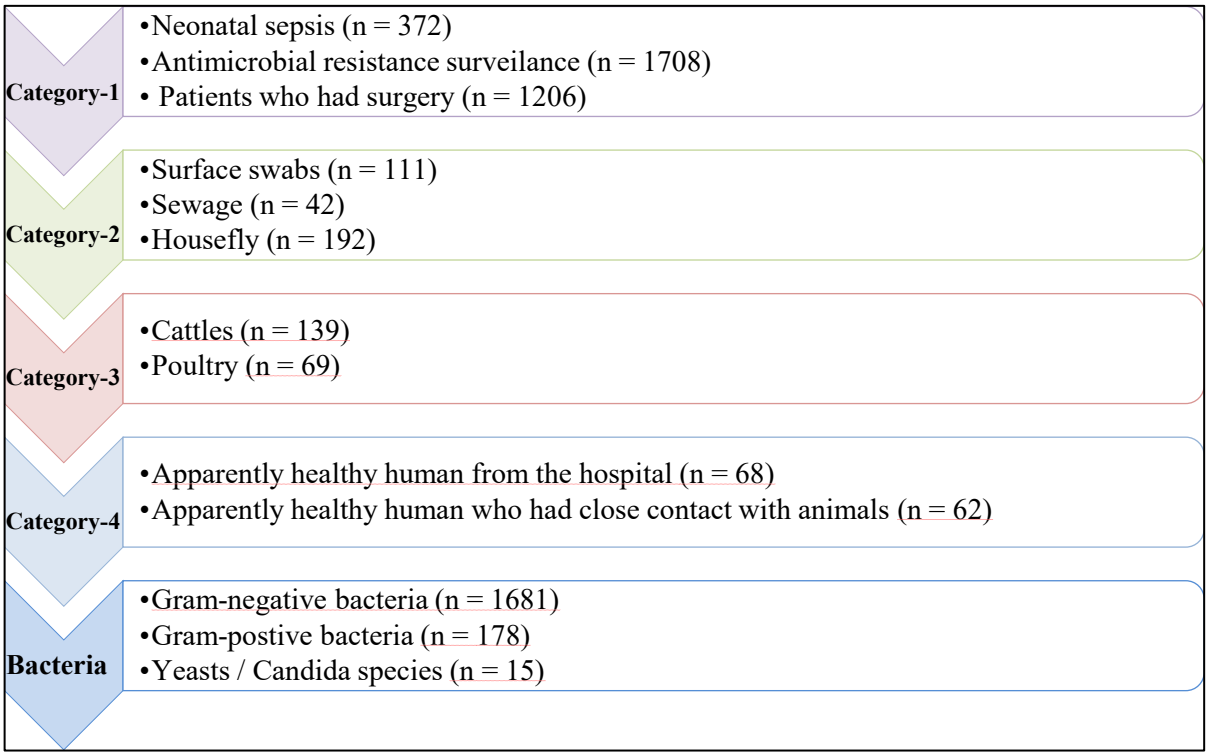
4.1 Data collection and description of bacterial sources

In this study, we have conducted a comprehensive collection of information from various sources. Firstly, we gathered socio-demographic and clinical data, along with associated risk factors, from the patients who participated in the study. Furthermore, clinical samples were obtained from patients suspected of having infections and were subjected to culture and AST following standardized operating procedures (SOPs). The entire process of specimen collection, incubation, and microbiological analysis was meticulously described in the respective articles. Secondly, environmental samples, including surface swabs, houseflies, and sewage samples, were collected and processed in accordance with the JMC microbiology laboratory's SOPs. Thirdly, we collected feces and droppings from cattle and poultry, respectively. Lastly, fecal samples were collected from apparently healthy individuals who were closely working and living with animals or involved in patient care at JMC. By gathering and analyzing this diverse range of samples, we were able to gain a comprehensive understanding of the burden of AMR, as well as the types and mechanisms of resistance involved in bacteria obtained from these samples.

Our research project had a specific focus on analyzing bacteria obtained from a wide range of samples collected during multiple studies conducted at JMC, a large tertiary, referral, and teaching hospital that serves a population of over 20 million in its catchment area, as well as its surrounding areas. The analysis encompassed various sample categories, including: (1) clinical samples such as blood, urine, stool, cerebrospinal fluid (CSF), wound swabs, ascitic fluid, pleural fluid, abscess, peritoneal fluid, and synovial fluid; (2) environmental samples, consisting of surface swabs, houseflies, water, and sewage samples; (3) bacteria obtained from animal samples, specifically feces and droppings; and (4) fecal samples collected from apparently healthy humans who have close contact with animals or who are involved in patient care within the hospital as described in detail in Figure 2.

Up on now, we have successfully published three articles. These articles cover diverse topics, including neonatal sepsis caused by MDR bacteria, the molecular characterization of carbapenem-resistant GNB obtained from clinical samples, and an investigation into the prevalence of MDR-ESBL producing GNB in hospital wastes. Moreover, we are currently in the process of preparing a fourth article that aims to evaluate the extent of AMR and the associated transmission risk. This evaluation involves the sequencing of *E. coli* strains isolated from clinical, animal, environmental, and apparently healthy human samples.

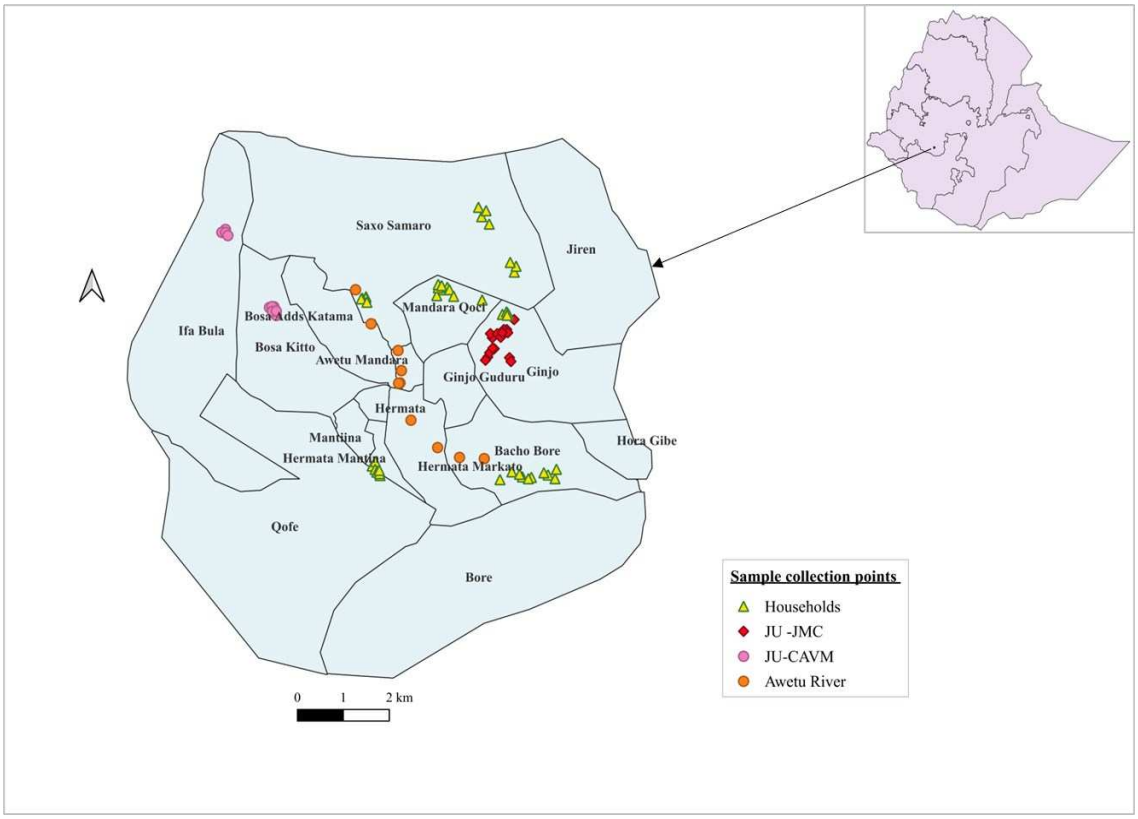
Figure 2: Describing the study projects and respective participants from which all bacterial isolates were obtained.



4.2 Study sites

The study was conducted in Jimma town, Ethiopia, specifically at JMC and its surrounding areas. The study recruited patients who showed signs of infection and had requested culture tests. Caregivers of these patients were also included in the study. Moreover, environmental samples, including surface swabs, houseflies, and sewage samples from both the hospital and its vicinity, were collected, and subjected to analysis. In various sections of Jimma town, households that owned cattle were selected as participants. Subsequently, samples of human feces, animal feces, and droppings were collected. Additionally, sewage and water samples were obtained from different locations within the town. Furthermore, samples from cattle and poultry were collected from Jimma University College of Agriculture and Veterinary Medicine (JU-CAVM), as well as Jimma University Farm Demonstration (Figure 3).

Figure 3: Map of Jimma town at which animal, environmental, and human samples were collected.



4.3 Methods used in Paper I

A cross-sectional study was conducted to determine the resistance profile and the molecular epidemiology of carbapenem resistance among GNB isolated from clinical specimens collected from patients suspected of infection at JMC. A total of 1,794 clinical samples, including blood, urine, wound swabs, cerebrospinal fluid (CSF), ascitic fluid, pleural fluid, abscess, peritoneal fluid, and synovial fluid were collected from patients suspected of having bacterial infections. In total, 846 Gram-negative bacterial isolates were obtained by culturing these clinical samples on Columbia 5% Sheep Blood-, Chocolate-, and MacConkey-agar plates. The laboratory analyses were performed both at JMC microbiology laboratory and MvPI Medical Microbiology Laboratory in Munich. The tests included identification of bacteria, phenotypic antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method and Etest strips, phenotype screening for ESBL with Mast disks, and identifying the presence of carbapenemase genes, such as *bla*KPC, *bla*NDM, *bla*OXA, *or bla*VIM using multiplex PCR. Statistical analysis was performed using Microsoft Office 2016 excel sheets and GraphPad Prism version 8.4.3. to assess the

frequency and proportion of the antibiotic resistance phenotypes and carbapenem resistance genes exhibited by the bacterial isolates.

4.4 Methods used in Paper II

A cross-sectional study design was conducted at JMC and its surrounding areas to investigate the role of hospital waste as potential sources for MDR-ESBL producing bacteria. Hospital waste samples were collected from various sources including surface swabs, sewage, and housefly samples using appropriate sampling techniques. The waste samples were processed and cultured on Columbia 5% Sheep-Blood and MacConkey agars at JMC microbiology laboratory. The isolated bacteria were re-identified using MALDI TOF MS at MvPI Medical Microbiology Laboratory in Munich and then AST was done using Kirby-Bauer disc diffusion technique. The ESBL screening was performed by DDST. The MDR profiles of the isolates were determined by the classical definition of their resistance to at least one antibiotic in three various antibiotic classes. DNA microarray technique was employed to detect and identify ESBL, AMPC, TEM, SHV, and carbapenemase encoding genes among E. coli strains isolated from surface swab, sewage and housefly samples. Data analysis involved calculating the prevalence of multidrug-resistant-ESBL-producing bacteria in different types of hospital waste was done. These methods provided valuable insights into the potential role of hospital wastes as reservoirs for multidrug-resistant ESBL-producing bacteria at JMC, Ethiopia.

4.5 Methods used in Paper III

An observational longitudinal study was carried out on 372 neonates admitted to the NICU of JMC with a clinical diagnosis of sepsis. The participants were recruited after obtaining consent from their parents or care givers and were followed until discharge or death. During their follow up, patient related data, including demographic information, clinical characteristics, and laboratory results, were collected using case report forms (CRF). Additional data on risk factors, such as gestational age, birth weight, and exposure to antibiotics, were collected from the medical records and analyzed to identify potential associations with MDR.

Blood and/or CSF cultures were performed to identify potential etiologies at JMC microbiology laboratory. This yielded a total of 152 potential pathogens. Re-identification of

the isolates were performed with MALDI TOF MS at MvPI Medical Microbiology Laboratory in Munich, and then AST was performed using the Kirby-Bauer disk diffusion method to determine the resistance patterns of the bacterial isolates to twenty-five antibiotics. The prevalence of multi-drug resistant-ESBL bacteria was determined considering resistance to at least one antibiotic in three different classes of antibiotics and considering the results from both the double disc synergy test (DDST) and the ESBL phenotype analysis with mast discs.

4.6 Statistical analysis

The data was double entered using EpiData software version 4.6. The Data analysis was performed using Microsoft Office 2016 excel sheets and GraphPad Prism version 8.4.3. Descriptive statistics were employed to summarize the characteristics of microbiological data, such as growth rates, proportion of resistance to various antibiotics, and mechanisms of resistance or proportion of resistance encoding genes.

4.7 Ethical considerations

Following the acquisition of ethical approval from the Institutional Review Board of Jimma University Institute of Health (protocol numbers: IHRPGO/495/2018 & IHRPGO/1087/21) and the Medical Faculty of Ludwig-Maximilians-Universität of Munich, Germany (Opinion No: 21–0157), the study was conducted in adherence to the approved protocols. The study participants (where applicable) were provided with a clear and comprehensive explanation of the study's purpose, as well as the associated risks and benefits. The researchers ensured that all participants had a thorough understanding of how their data would be utilized in the study before proceeding, and their questions and concerns were addressed until they were fully satisfied. Then after, written informed consent was obtained from individual patients, care givers, or guardians before the study was carried out. For participants who could not read the information sheet and the consent form had been read and interpreted till they properly understand. After ensuring their proper understanding of the study’s’ risks and benefits, they were asked to provide consent to participate in the study. The information was kept confidential and anonymized throughout the data analysis, interpretation, and manuscript write-up phases.

Additionally, for animal and environmental samples, proper communication with owners and/or respective stakeholders was employed to get permission prior to data collection.

The findings of the study were provided to the respective stake holders, and other concerned bodies to avail the information and use it for further intervention and studies.

5. Results

5.1 Frequency of bacteria

Through microbiological analysis, a wide range of bacterial species were identified in samples obtained from patients, animals, the environment, and apparently healthy humans. Among the clinical samples, the most frequently isolated bacteria were *E. coli* (22.9%), followed by *Klebsiella* species (21.1%), *S. aureus* (12.7%), *Acinetobacter* species (12.5%), *Enterobacter* species (10.7%), and others. Similarly, in environmental samples, *E. coli* (29.3%) was the predominant bacteria, along with *Klebsiella* species (11.9%), *Providencia* species (11.9%), *Proteus* species (11.4%), *Enterobacter* species (6.2%), *Acinetobacter* species (8.9%) and many more. Animal samples also predominantly contain *E. coli* (57.6%), *Enterobacter* species (15.6%), *Klebsiella* (14.9%), *Acinetobacter* species (3.6%) and others. However, in apparently healthy human fecal samples, a less diverse range of bacteria was identified, with *E. coli* accounting for 86.8% and *Klebsiella* species for 11%. Although certain bacterial strains were consistently present across all sample sources, their prevalence and abundance varied (Table 1).

Table 1: Distribution of bacterial isolates obtained from clinical, animal, environmental, and apparently healthy human samples.

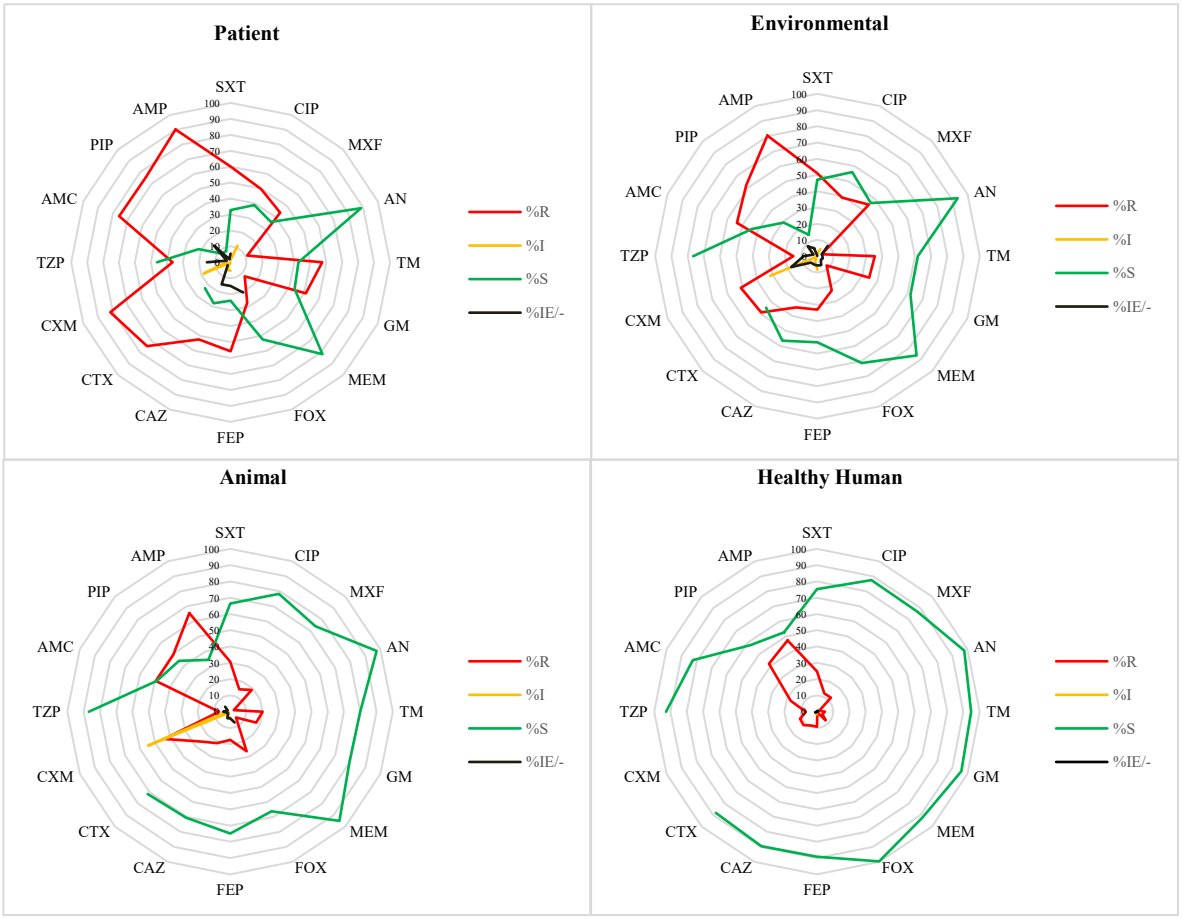
Bacterial	**Clinical**		**Environmental**		**Animal**		**Healthy Human**	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
E. coli	231	22.9	108	29.3	159	57.6	118	86.8
Klebsiella species	213	21.1	44	11.9	41	14.9	15	11.0
Acinetobacter species	126	12.5	19	5.1	10	3.6	-	-
Enterobacter species	108	10.7	23	6.2	43	15.6	-	-
Proteus species	76	7.5	42	11.4	4	1.4	-	-
Pseudomonas species	47	4.7	1	0.3	7	2.5	-	-
S. marcescens	15	1.5	-	-	3	1.1	-	-
C. freundii	7	0.7	5	1.4	5	1.8	-	-
L. adecarboxylata	5	0.5	2	0.5	-	-	1	0.7
M. morganii	5	0.5	14	3.8	-	-	-	-
R. ornithinolytica	3	0.3	9	2.4	-	-	-	-
C. sakazakii	2	0.2	1	0.3	-	-	1	0.7
M. odoratimimus	2	0.2	-	-	1	0.4	-	-
S. maltophilia	2	0.2	1	0.3	-	-	-	-
C. koseri	1	0.1	-	-	-	-	-	-
E. hermannii	1	0.1	2	0.5	1	0.4	-	-
Providencia species	2	0.2	44	11.9	1	0.4	-	-
Aeromonas species	-	-	10	2.7	-	-	1	0.7
Salmonella species	-	-	1	0.3	1	0.4	-	-
Others GNB	-	-	29	7.9	-	-	-	-
S. aureus	128	12.7	14	3.8	-	-	-	-
S. haemolyticus	15	1.5	-	-	-	-	-	-
Other GPB	21	2.1	-	-	-	-	-	-
Total	**1010**	**100**	**369**	**100**	**276**	**100**	**136**	**100**

Other GNB - *Kluyvera* species (9), *Wohlfahrtiimonas chitiniclastica* (9), *Pantoea* species (3), *Pruralibacter gergoviae* (3), *Escherichia fergusonii* (1), *Hafnia alvei* (1), *Igantzschineria indica* (1), *Moellerella wisconsensis* (1), *Pectobacterium carotovorum* (1), **Other GPB** - *Staphylococcus epidermidis* (6), *Staphylococcus sciuri* (3), *Staphylococcus xylosus* (3), *Staphylococcus cohnii* (3), *Staphylococcus hominis* (3), *Staphylococcus lugdunensis* (1), *Staphylococcus pasteuri* (1), *Staphylococcus warneri* (1).

5.2 Antibiotic susceptibility test results

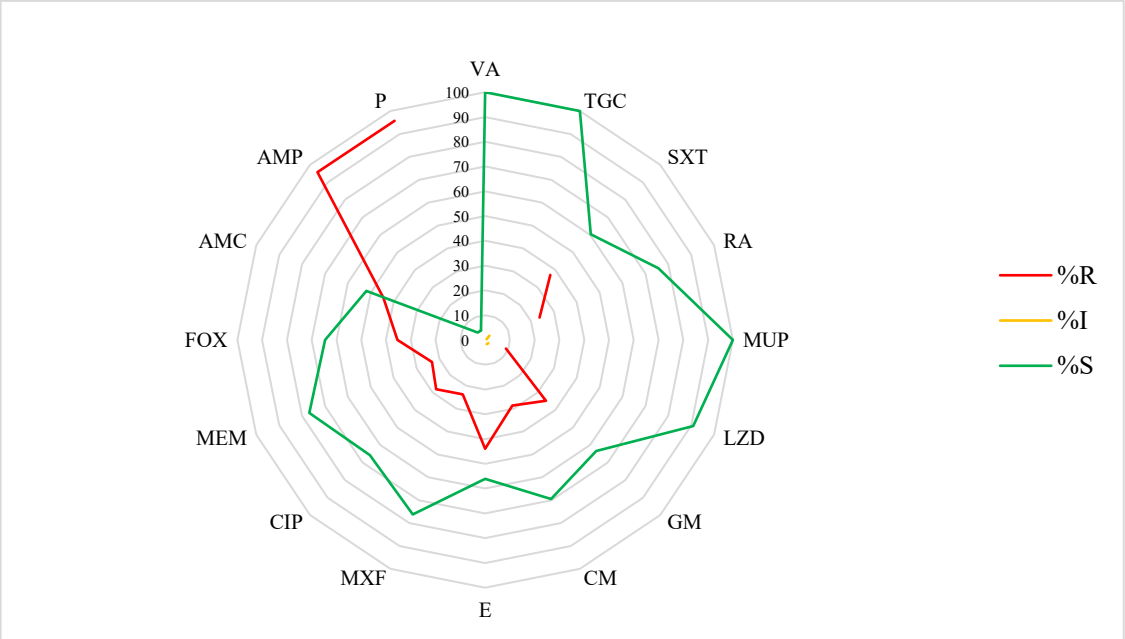
The results of the AST conducted on bacteria isolated from various sample categories revealed a concerning level of resistance. In particular, GNB isolated from clinical samples exhibited a high rate of resistance against ampicillin (90%), cefuroxime (82%), amoxicillin-clavulanic acid (76%), piperacillin (75%), and cefotaxime (74%). Similarly, GNB isolated from environmental samples also displayed a substantial rate of resistance to these antibiotics. On the other hand, GNB obtained from animals and apparently healthy human samples demonstrated a lower rate of resistance. Despite the variation in the prevalence and distribution of resistant bacterial isolates among the different sample categories, the study revealed a substantial presence of resistance to both first- and second-line antibiotics (Figure 4). Additionally, in clinical samples, the AST result of GPB revealed that most of *S. aureus* strains were penicillinase producers with a high level of resistance to penicillin /ampicillin (95.7% each), and amoxicillin-clavulanic acid (44.5%). Overall, 35.4% of GPB were methicillin-resistant based on the phenotypic result of cefoxitin. Among the Gram-positive isolates 43.9% and 28.7% of them were resistant to erythromycin and clindamycin, respectively (Figure 5).

Figure 4: The proportion of antibiotic resistant (including intrinsic resistant) Gram-negative bacterial isolates obtained from clinical, environmental, animal, and apparently healthy human samples.



Key: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; MXF, moxifloxacin; CIP, ciprofloxacin; GM, gentamicin; TM, tobramycin; AN, amikacin; and SXT, sulfamethoxazole-trimethoprim. All intermediate or increased exposure and susceptible results were excluded from the analysis.

Figure 5: The proportion of antibiotic resistant Gram-positive bacterial isolates obtained from clinical samples.

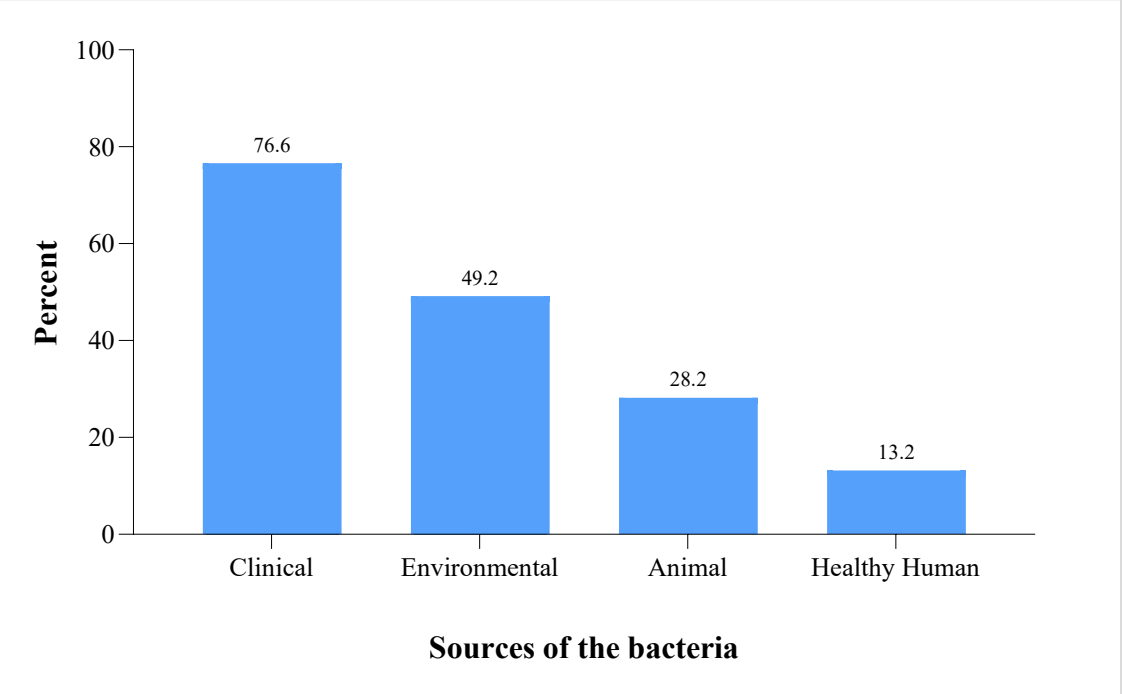


Key: P, penicillin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; MEM, meropenem; CIP, ciprofloxacin; MXF, moxifloxacin; E, erythromycin; CM, clindamycin; GM, gentamicin; LZD, linezolid; MUP, mupirocin; RA, rifampicin; SXT, sulfamethoxazole-trimethoprim; TGC, tigecycline; and VA, vancomycin.

5.3 The proportion of Extended Spectrum Beta-Lactamases (ESBL)

The presence of ESBL producing GNB were identified in all categories of samples, including clinical, environmental, animal, and apparently healthy human samples. It was determined using DDST. The analysis revealed a high prevalence of ESBL-producing GNB in clinical samples, with these strains constituting 76.6% of the isolates. Similarly, environmental samples exhibited a prevalence rate of 49.2%, indicating a significant presence of ESBL-producing GNB in the environment. Animal samples and apparently healthy human samples showed lower rates of 28.2% and 13.2%, respectively, but still demonstrated the presence of ESBL-producing strains. This consistent presence of ESBL-producing strains across all sample types underscores their widespread distribution and highlights their significance in terms of (Figure 6).

Figure 6: Distribution of ESBL-producing Gram-negative bacterial isolates obtained from clinical, animal, environmental, and apparently healthy human samples.



5.4 Molecular characterization of *E. coli* isolated from various sources.

The characterization of the resistome in bacteria isolated from human, environmental, and animal sources using DNA microarray techniques and multi-locus sequence typing revealed several key findings; In the Kirby-Bauer disk diffusion test, it was found that 41.6% (254/611) of *E. coli* isolates displayed resistance to at least one of the tested β-lactam antibiotics. Among these resistant isolates, 96.1% (244/254) showed genotypic matches with their corresponding phenotypic resistance. Notably, the remaining 3.9% (10) of the strains did not have any identified genes that could explain their resistance. Therefore, their resistance could involve untested genes or alternative mechanisms such as penetration issues or export pumps. Regarding the distribution of those isolates with genotypically confirmed beta-lactamase resistant genes, a high number of them originated from patient specimens 66.8% (151/226) followed by environmental samples which accounted for 54.6% (59/108) of the cases (Table 2).

Overall, we detected the presence of encoding genes for four different subtypes of *bla*CTX-M enzymes: *bla*CTX-M-9, *bla*CTX-M-15, *bla*CTX-M-32, and a separate group 1 *bla*CTX-M (non-identified or none defined). The predominant subtype, *bla*CTX-M-15, was present in 74.2% (181/244) of the *E. coli* strains. Of these, 69.1% (n = 125) of the

strains were from patient samples and the remaining 18.2% (n = 33) were from the environmental. The next most prevalent subtype was the *bla*TEM-(WT) expressing gene, accounting for 59.4% (145/244) of the strains. Among these, 61.4% (89) originated from patient sources, while 24.8% (36) were from environmental samples (Table 2).

Table 2: Distribution of carbapenemase and extended spectrum beta-lactamase encoding genes of *Escherichia coli* isolated from clinical, environmental, animal, and apparently healthy human samples in Jimma, Ethiopia.

Types of antimicrobial resistance gene	**Source of *E. coli* strains**				**Total**
	Patients % (n = 226)	**Healthy humans % (n = 118)**	**Animals % (n = 159)**	**Environmental % (n = 108)**	**% (n = 611)**
Carbapenemase encoding genes	**3.5 (8)**	**0**	**0.6 (1)**	**5.6 (6)**	**2.5 (15)**
NDM	2.2 (5)	0	0	5.6 (6)	**1.8 (11)**
OXA-48	0.9 (2)	0	0.6 (1)	0	**0.5 (3)**
OXA-48 + NDM	0.4 (1)	0	0	0	**0.2 (1)**
ESBL encoding genes	**59.7 (135)**	**11.0 (13)**	**9.4 (15)**	**37.9 (41)**	**33.4 (204)**
CTX-M group 1 type-15	55.3 (125)	9.3 (11)	7.5 (12)	30.6 (33)	**29.6 (181)**
CTX-M group 1 type-9	1.3 (3)	0.9 (1)	1.3 (2)	2.7 (3)	**1.5 (9)**
CTX-M group 1, ND	1.8 (4)	0	0.6 (1)	2.8 (3)	**1.3 (8)**
CTX-M group 1 type-32	1.3 (3)	0	0	0	**0.5 (3)**
CTX-M group 1 type-15 + 9	0	0.9 (1)	0	1.8 (2)	**0.5 (3)**
AMPC encoding genes	**7.1 (16)**	**0**	**3.1(5)**	**5.6 (6)**	**4.4 (27)**
CMY II (n = 11)	4.0 (9)	0	0.6 (1)	0.9 (1)	**1.8 (11)**
ACT/MIR (n = 10)	2.2 (5)	0	1.3 (2)	2.8 (3)	**1.6 (10)**
DHA (n = 5)	0.9 (2)	0	0.6 (1)	1.9 (2)	**0.8 (5)**
ACT/MIR + DHA (n = 1)	0	0	0.6 (1)	0	**0.2 (1)**
TEM/SHV encoding genes	**41.2 (93)**	**5.1 (6)**	**8.2 (13)**	**34.3 (37)**	**24.4 (149)**
blaTEM- (WT) (n = 144)	39.4 (89)	5.1 (6)	8.2 (13)	33.4 (36)	**23.6 (144)**
blaSHV-(WT) (n = 4)	1.8 (4)	0	0	0	**0.6 (4)**
blaTEM-104K + 164C (n = 1)	0	0	0	0.9 (1)	**0.2 (1)**
Total	**66.8 (151)**	**11.9 (14)**	**12.6 (20)**	**54.6 (59)**	**39.9 (244)**

5.5 Co-occurrence of antibiotic resistance encoding genes in *E. coli*

The most common coexisting β-lactam encoding genes were *bla*CTX-M-15 and *bla*TEM (WT), which were found in 34.8% (85/244) of *E. coli* strains. Moreover, two of the isolated *E. coli* strains were co-harboring four different encoding genes (such as NDM + CMY II + CTX-M-15 + TEM and OXA-48 + NDM + CTX-M-15 + TEM) while 8.2% (20/244) of the strains carried three and 44.3% (108/244) carried two encoding genes

(Table 3). As expected, *E. coli* strains in the study exhibited a high prevalence of phenotypic resistance to penicillins and cephalosporins.

Table 3: The co-existence of resistance genes in *E. coli* isolated from patients and other sources.

Types of antimicrobial resistance gene	**Sources of the resistance strains**		**Total**
	Patients (n = 151)	**Others (n = 93)**	**% (n = 244)**
Co-existed with carbapenemase producing strains	**5.3 (8)**	**7.5 (7)**	**6.2 (15)**
NDM + CTX-M group 1 type-15 + *bla*TEM	1.3 (2)	2.2 (2)	**1.6 (4)**
NDM + CTX-M group 1 type-15	0.66 (1)	3.2 (3)	**1.6 (4)**
OXA-48 + CTX-M group 1 type-15 + *bla*TEM	1.3 (2)	1.1 (1)	**1.2 (3)**
NDM + CMY II + CTX-M group 1 type-15 + *bla*TEM	0.66 (1)	0	**0.4 (1)**
OXA-48 + NDM + CTX-M group 1 type-15 + *bla*TEM	0.66 (1)	0	**0.4 (1)**
NDM + CTX-M group 1, ND + *bla*TEM	0	1.1 (1)	**0.4 (1)**
NDM	0.66 (1)	0	**0.4 (1)**
ESBL producing strains	**85.4 (129)**	**65.6 (61)**	**77.9 (190)**
CTX-M group 1 type-15 + *bla*TEM	39.1(59)	28.0 (26)	**34.8 (85)**
CTX-M group 1 type-15	31.1(47)	20.4 (19)	**27.1 (66)**
CTX-M group 1 type-9	2.0 (3)	5.4 (5)	**3.3 (8)**
CTX-M group 1 type-15 + CMY II + *bla*TEM	2.6 (4)	0	**1.6 (4)**
CTX-M group 1 type-15 + CMY II	1.3 (2)	2.2 (2)	**1.6 (4)**
CTX-M group 1, ND + *bla*TEM	2.0 (3)	0	**1.2 (3)**
CTX-M group 1 type-15 + *bla*TEM + *bla*SHV	2.0 (3)	0	**1.2 (3)**
CTX-M group 1 type-32 + *bla*TEM	2.0 (3)	0	**1.2 (3)**
CTX-M group 1, ND	0.66 (1)	2.2 (2)	**1.2 (3)**
CTX-M group 1 type-15 + 9 + *blaTEM*	0	2.2 (2)	**0.8 (2)**
CTX-M group 1 type-15 + ACT/MIR	1.3 (2)	0	**0.8 (2)**
CTX-M group 1 type-15 + *bla*SHV	0.66 (1)	0	**0.4 (1)**
CTX-M group 1 type-15 + DHA	0.66 (1)	0	**0.4 (1)**
CTX-M group 1, ND + ACT/MIR + DHA	0	1.1 (1)	**0.4 (1)**
CTX-M group 1 type-15 + ACT/MIR + *bla*TEM	0	1.1 (1)	**0.4 (1)**
CTX-M group 1 type-9 + ACT/MIR + *blaTEM*	0	1.1 (1)	**0.4 (1)**
CTX-M group 1 type-15 + 9	0	1.1 (1)	**0.4 (1)**
CTX-M group 1 type-15 + DHA + *blaTEM-104K* + 164C	0	1.1 (1)	**0.4 (1)**
AMPC encoding genes	**4.0 (6)**	**5.4 (5)**	**4.5 (11)**
ACT/MIR	2.0 (3)	3.2 (3)	**2.4 (6)**
CMY II + *bla*TEM	1.3 (2)	0	**0.8 (2)**
DHA	0	2.2 (2)	**0.8 (2)**
DHA + *bla*TEM	0.66 (1)	0	**0.4 (1)**
TEM encoding genes	**5.3 (8)**	**21.5 (20)**	**11.5 (28)**
*bla*TEM- (WT)	5.3 (8)	21.5 (20)	**11.5 (28)**

5.6 Molecular characterization of carbapenem-resistant other Gram-negative isolates obtained from clinical samples

A total of 155 isolates were included in the analysis, and the presence of genes associated with carbapenem resistance was determined using multiplex PCR. The molecular analysis revealed a high prevalence of carbapenemase resistance encoding genes among GNB obtained from clinical samples. Out of the total isolates, 69.0% (n = 107) were found to exhibit at least one carbapenemase resistance encoding gene. Among the identified carbapenemase genes, the most frequently detected gene was *bla*NDM, which accounted for 21% (37/179) of the total detected genes. This was followed by *bla*VIM and *bla*KPC42, accounting for 15% (26/179) and 8% (14/179) of the detected genes, respectively. Coexistence of two or more carbapenemase encoding genes was observed in 46.7% (50/107) of the isolates, as shown in Table 4. The molecular analysis provides valuable insights into the prevalence and diversity of carbapenem resistance mechanisms among the analyzed GNB isolates.

Table 4: Distribution of carbapenemase-coding genes among Gram-negative bacteria (n = 107)

Bacteria	**Carbapenem resistance genes**	**Resistance strains**	
		n	**%**
Acinetobacter baumannii complex	OXA-51	41	38.3
	VIM + OXA-51	13	12.2
	NDM + OXA-51 + OXA-23	8	7.5
	NDM + OXA-51 + OXA-58	5	4.7
	NDM + OXA-51 + VIM	5	4.7
	NDM + OXA-51	3	2.8
	NDM + OXA-51 + OXA-58 + VIM	1	0.9
	NDM + OXA-23	1	0.9
	OXA-51 + OXA-23	1	0.9
	VIM + OXA-51 + OXA-58	2	1.9
	NDM + KPCu	1	0.9
Acinetobacter haemolyticus	NDM + OXA-23	1	0.9
Enterobacter cloacae	KPC42	2	1.9
E. coli	NDM	5	4.7
	OXA-48	2	1.9
	NDM + OXA-48	1	0.9
Klebsiella pneumoniae	KPC42	5	4.7
	KPC42 + NDM	3	2.8
	KPC42 + VIM	2	1.9
Klebsiella variicola	KPC42 + VIM	2	1.9
	NDM + VIM	1	0.9
Pseudomonas aeruginosa	NDM	1	0.9
Pseudomonas mendocina	NDM	1	0.9

6. Discussion

These findings highlight the widespread presence of genotypic and phenotypic resistance to β-lactam and carbapenem antibiotics in GNB isolated from patient, apparently healthy human, environmental, and animal samples. However, the prevalence and the distribution of these MDR bacteria varied among different categories of samples. For example, the prevalence of resistant bacteria in clinical samples was higher than in bacteria from environmental and animal sources. Human isolates may exhibit resistance due to selective pressures from clinical settings, imprudent antibiotic usage, poor infection prevention and control practices, and/or lack of regular surveillance programs [79, 80]. This underscores the potential impact on public health, as it can lead to treatment failure and serve as a potential source for further dissemination of resistant bacterial strains within healthcare settings and/or the wider community [81].

Furthermore, the presence of resistant bacteria in environmental and animal samples emphasizes the potential transmission or spread of antibiotic resistant bacteria between different reservoirs, posing challenges for both human and veterinary medicine [82, 83]. The environment and animals may harbor resistance by contamination from clinical settings, agricultural practices, or pollution [84]. The presence of these bacteria in animal and environmental samples raises concerns about the potential zoonotic transmission and environmental contamination [85]. These findings emphasize the urgent need for comprehensive strategies aimed at the prevention and control of antibiotic resistance across multiple sectors. Understanding these source-specific resistance profiles can guide targeted interventions and preventive measures in each context.

We also identified a diverse range of antibiotic-resistant bacterial isolates, including *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Acinetobacter* spp., and many others, in all categories of samples. From characterization of these bacteria, we have identified a wide range of resistance genes among them. This indicates the pervasive presence of antibiotic resistance within microbial populations of all sources. The discovery of shared or similar resistance genes in bacterial strains across different sources holds significant importance. It suggests the existence of transmission pathways and highlights the interconnected nature of antibiotic resistance [86]. Horizontal gene transfer within the same or different species may play a crucial role in the dissemination of resistance genes, potentially lead-

ing to heightened AMR in human pathogens [84, 86]. This finding emphasizes the necessity of adopting a One Health approach to combat antibiotic resistance, taking into account the interrelationships between humans, animals, and the environment.

For example, the molecular characterization of *E. coli* strains from these three sample categories revealed the presence of acquired carbapenemase and ESBL encoding genes, such as *bla*NDM, and CTX-M group 1 type-15, CTX-M group 1, ND, *bla*TEM, and AMPC encoding genes like ACT/MIR and DHA. It is concerning to note that despite the presence of carbapenem resistant and ESBL-producing bacteria in various sample sources, there is an absence or lack of proper strategies to combat the spread of AMR in healthcare facilities and the wider community in the study area. These findings underscore the importance of implementing effective antibiotic stewardship and infection prevention measures to mitigate the spread of MDR bacteria and minimize the risk of infections in healthcare settings [87, 88]. Additionally, the multiplex PCR analysis of other Gram-negative bacteria isolated from clinical samples exhibited a high rate of both inherent and acquired carbapenemase genes. Among them, the most frequently identified genes were *bla*NDM, *bla*VIM, and *bla*KPC42. On the other hand, due to its intrinsic presence in *A. baumannii*, the *bla*OXA-51-like gene was the predominant inherent encoding gene. Co-harboring of various acquired genes was observed in many Gram-negative bacterial isolates such as *Acinetobacter* spp, *E. coli*, *Klebsiella* spp, and others. The most common acquired coexisting genes were *bla*NDM + *bla*OXA-23. As a result of all of this, bacteria become resistant to first-line and second-line antibiotics that are routinely prescribed in the study setting. The finding highlights the urgent need for alternative treatment options and the implementation of stringent infection prevention and control measures [88].

The genetic variability of resistance genes, as revealed through DNA microarray technique and multiplex PCR, provides a deeper understanding of the mechanisms driving the spread of antibiotic resistance. The identification of mutations, mobile genetic elements, and plasmids associated with resistance genes helps elucidate the routes and mechanisms of horizontal gene transfer [89]. This knowledge is crucial for designing effective interventions to prevent the dissemination of resistance genes and combat the development of antibiotic resistance [88]. Moreover, the co-occurrence of multiple resistance genes within individual isolates further highlights the complexity of antibiotic resistance mechanisms [90]. Identifying potential reservoirs of resistance encoding genes is crucial in clinical, environmental, and animal sources. This identification it could help to explain

the potential reservoirs for human infections, which can lead to treatment challenges and increased morbidity and mortality. For instance, in the current finding, the resistance spectrum of identified bacterial strains across all sectors from the same area and same time frame may explain the transmission of MDR bacteria from one sector to another [91]. Therefore, strategies aimed at monitoring and mitigating the transmission of resistance genes from these reservoirs to human pathogens are crucial for preventing the further spread of AMR [44, 91].

The findings of this study regarding the presence, diversity, and transmission dynamics of antibiotic-resistant bacterial genes hold significant implications for public health. The identification of shared resistance genes across different sources, including humans, animals, and the environment, indicates the existence of transmission routes and underscores the interconnectedness of antibiotic resistance. This highlights the importance of implementing robust surveillance systems, infection prevention and control measures, and the separation of human and animal dwellings. Additionally, it emphasizes the need for effective sewage and waste management practices to avoid environmental contamination. Furthermore, the development of new antimicrobials is crucial in mitigating the impact of antimicrobial-resistant bacterial infections and safeguarding public health. Overall, these findings reinforce the urgent need for comprehensive strategies to address antimicrobial resistance and protect public well-being.

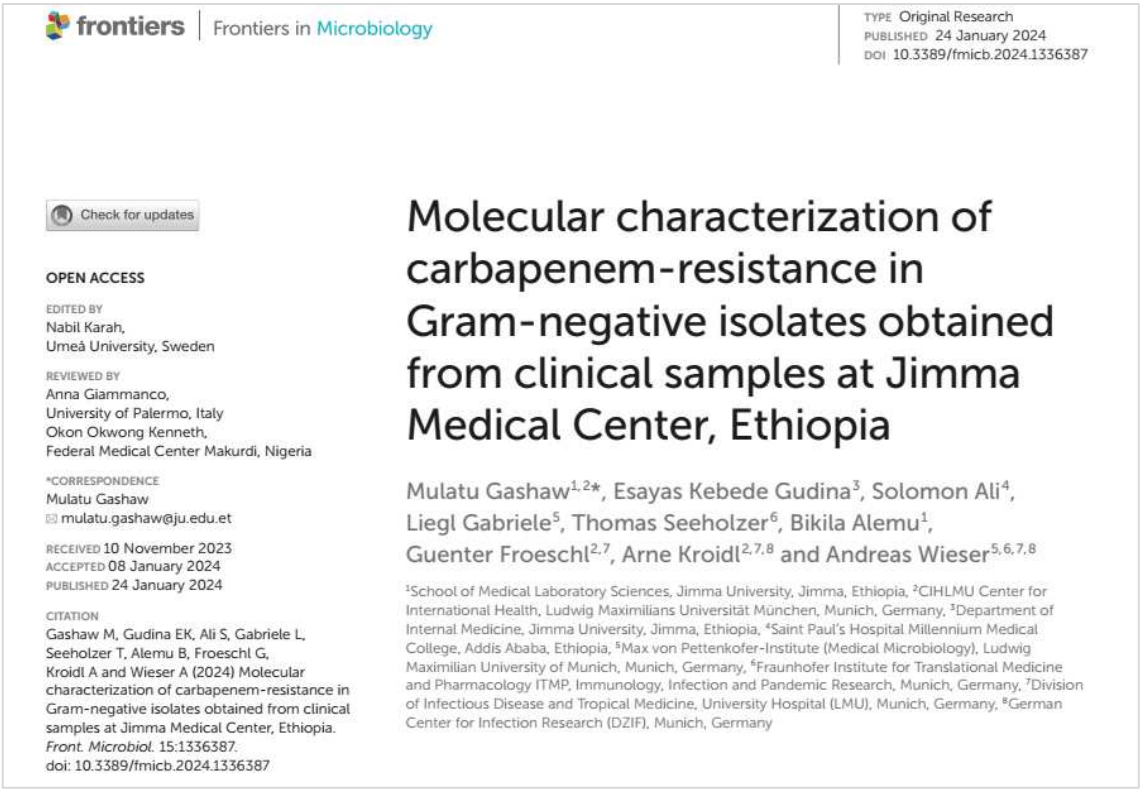
The study has limitations that should be considered while interpreting its finding. Firstly, it did not investigate the specific factors that contribute to the presence of MDR bacteria in environmental and animal samples. This could help to understand the associated risk factors and develop appropriate strategies to mitigate AMR. Secondly, the study did not thoroughly examine the extent of transmission risks posed by these samples, both in terms of spreading drug-resistant bacteria to patients within the hospital and the potential dissemination to the wider community. However, we plan to perform phylogenetic analysis on *E. coli* strains isolated from all categories of samples and compare their clonality. Thirdly, we did molecular analysis to detect the resistance encoding genes on some the Gram-negative bacterial isolates. As a result, the findings may not reflect the actual distribution of all resistance encoding genes.

7. Conclusion

The characterization of bacteria isolated from human, environmental, and animal sources using DNA microarray technique, multiplex PCR, and multilocus sequence typing provides valuable insights into the presence, diversity, and transmission dynamics of antibiotic resistance. The findings highlight the widespread occurrence of resistance genes, interconnectedness of AMR across different sources, and the complexity of resistance mechanisms. Moreover, diverse acquired carbapenemase and beta-lactamase encoding genes were observed in GNB, with the predominant presence of *bla*NDM and *bla*CTX-M group 1, respectively. This research is fundamental for understanding the complexity of AMR and developing targeted interventions to preserve the effectiveness of antimicrobial therapies. The findings underscore the importance of a One Health approach and highlight the need for comprehensive strategies to combat AMR in diverse settings and populations. The research findings serve as a foundation for evidence-based strategies and interventions to preserve the effectiveness of antibiotics, protect human and animal health, and safeguard the environment. Therefore, implementation of rigorous waste management practices, strengthening surveillance systems, promoting One-health approaches, enhancing antimicrobial stewardship, improving infection prevention- and control measures, educating and raising awareness among healthcare providers and the community, and fostering international collaboration to support research and development on AMR are crucial and highly recommended to mitigate AMR and its public health threats. Continued research and concerted efforts are necessary to combat antibiotic resistance and ensure the availability of effective treatments for future generations. By implementing these recommendations, we can collectively address the growing threat of antibiotic resistance.

8. Publications

8.1 Paper I



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Molecular characterization of carbapenem-resistance in Gram-negative isolates obtained from clinical samples at Jimma Medical Center, Ethiopia

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Molecular characterization of carbapenem-resistance in Gram-negative isolates obtained from clinical samples at Jimma Medical Center, Ethiopia

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Background: In resource-constrained settings, limited antibiotic options make treating carbapenem-resistant bacterial infections difficult for healthcare providers. This study aimed to assess carbapenemase expression in Gram-negative bacteria isolated from clinical samples in Jimma, Ethiopia.

Methods: A cross-sectional study was conducted to assess carbapenemase expression in Gram-negative bacteria isolated from patients attending Jimma Medical Center. Totally, 846 Gram-negative bacteria were isolated and identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Phenotypic antibiotic resistance patterns were determined using the Kirby-Bauer disk diffusion method and Etest strips. Extended-spectrum β-lactamase phenotype was determined using MAST disks, and carbapenemases were characterized using multiplex polymerase chain reactions (PCR).

Results: Among the isolates, 19% (157/846) showed phenotypic resistance to carbapenem antibiotics. PCR analysis revealed that at least one carbapenemase gene was detected in 69% (107/155) of these strains. The most frequently detected acquired genes were *bla*NDM in 35% (37/107), *bla*VIM in 24% (26/107), and *bla*KPC42 in 13% (14/107) of the isolates. Coexistence of two or more acquired genes was observed in 31% (33/107) of the isolates. The most common coexisting acquired genes were *bla*NDM + *bla*OXA-23, detected in 24% (8/33) of these isolates. No carbapenemase-encoding genes could be detected in 31% (48/155) of carbapenem-resistant isolates, with *P. aeruginosa* accounting for 85% (41/48) thereof.

Conclusion: This study revealed high and incremental rates of carbapenem-resistant bacteria in clinical samples with various carbapenemase-encoding genes. This imposes a severe challenge to effective patient care in the context of already limited treatment options against Gram-negative bacterial infections in resource-constrained settings.

KEYWORDS

carbapenem-resistant, carbapenemases, *bla*OXA, *bla*NDM, ESBL, Jimma

Introduction

Gram-negative bacteria (GNB), such as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, are common culprits in healthcare-associated infections (Sikora and Zahra, 2020). Carbapenem resistance is increasing at alarming rates in these organisms (Beshah et al., 2023). The resistance can arise from various mechanisms, including the production of carbapenemase enzymes, decreased permeability of the bacterial cell wall, increased efflux pump activity, alterations in outer membrane porins, and target site mutations that reduce affinity to carbapenems (Aurilio et al., 2022). These mechanisms can act individually or in combination, leading to the development of multidrug-resistant strains that pose significant challenges in treating infections caused by these bacteria (Das, 2023). GNB have the ability to acquire and express a variety of carbapenemase genes (Dwomoh et al., 2022; Tenover et al., 2022; Tilahun et al., 2022). These genes can spread within or between different bacterial species through horizontal transfer of plasmids, conjugative transposons, or integrons (Hammoudi Halat and Ayoub Moubareck, 2020). As a result, carbapenem resistance in GNB is a major public-health concern worldwide. The most common carbapenemases identified in GNB include oxacillinases (OXA), *Klebsiella pneumoniae* carbapenemase (KPCs), and metallo-beta-lactamases (MBLs), including New Delhi metallo-β-lactamase (NDM) and Verona integron-encoded metallo-beta-lactamase imipenemase (VIM) (Rabaan et al., 2022). These enzymes can break down carbapenem antibiotics, and develop resistance not only to carbapenems, but also to many other beta-lactam antibiotics, such as penicillins, cephalosporins, and monobactams (Jean et al., 2022).

Infections with these pathogens are associated with high rates of mortality and morbidity since treatment options are limited to a few last-resort antibiotics that often come with many side effects (Caston et al., 2022). Furthermore, infections with carbapenem-resistant GNBs increase healthcare cost and the length of hospital stays (Van Duin, 2017). Such infections are major concerns for critically ill patients, immunocompromised individuals, and those with comorbidities (Aleidan et al., 2021; Di Carlo et al., 2021). In resource-constrained countries, including Ethiopia, the public health impact is even worse due to the lack of reserve treatment options (Alemayehu et al., 2023; Beshah et al., 2023).

Rapid and reliable detection of carbapenem-resistant GNB is critical for appropriate laboratory-guided patient management, for surveillance, and for applying effective evidence-based infection prevention and control practices (Nordmann and Poirel, 2019; Shanmugakani et al., 2020). A combination of phenotypic detection and genotypic confirmation of carbapenemase-expressing genes by polymerase chain reaction (PCR) is recommended (Rabaan et al., 2022).

However, due to lack of technical expertise, specialized equipment, and reagents, detecting and tracking the molecular epidemiology of carbapenem-resistant bacterial isolates is difficult in low-income countries (Nordmann and Poirel, 2019; Shanmugakani et al., 2020). As a result, data on the burden of infections with carbapenem-resistant bacterial species and associated outcomes is scarce in Sub-Saharan African countries, including Ethiopia (Stewardson et al., 2019). Therefore, this study aimed to determine the extent of carbapenemases among GNBs obtained from clinical samples using both phenotypic and genotypic techniques.

Materials and methods

Study setting, design, and time

A cross-sectional study was conducted to detect the carbapenemase genes in carbapenem-resistant GNB obtained from patients treated at Jimma Medical Center (JMC). JMC is an 800-bed teaching hospital in southwest Ethiopia with a catchment population of over 20 million. All patients from whom samples were sent for culture and antibiotic susceptibility test as part of routine clinical care were recruited prospectively for the study.

Clinical sample collection

Clinical samples (blood, cerebrospinal fluid [CSF], wound swabs, ascitic fluid, pleural fluid, abscess, peritoneal fluid, and synovial fluid) were collected aseptically by the clinicians, nurses or laboratory professionals. Other clinical samples such as urine, stool, and sputum were collected by the patients themselves after proper instruction was provided. Samples were then transported within 1 h after collection to the JMC microbiology laboratory for analysis.

Bacterial isolation and identification

All clinical specimens, except for blood, were inoculated on 5% Colombia Sheep Blood, Chocolate, and MacConkey agars and incubated aerobically at 35–37°C for 18–22 h. Blood samples were collected and added to BD BACTEC bottles (Becton Dickinson, Sparks, MD, USA) and then incubated for 5 days at 35–37°C in the BD BACTEC™ FX40 (Becton Dickinson, Sparks, MD, USA) automated culture machine. If growth was observed, it was sub-cultured on 5% Colombia Sheep Blood, Chocolate, and MacConkey agars in similar environmental conditions for further analysis. Subsequently, all positive pure cultures were tested for antimicrobial susceptibility. Isolates were picked off the plates and kept at −80°C in storage media containing skimmed milk, tryptone soya, glucose, glycerol, and distilled water until they were transported to Max von Pettenkofer Institute, Hospital Hygiene, and Medical Microbiology Laboratory in Munich, Germany. There, the isolates were re-identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker, Germany).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out according to the Kirby-Bauer disk diffusion technique using 16 antibiotics (Bio-Rad, France) (Supplementary Table S1). Reading of the results was done using the ADAGIO 93400 automated system (Bio-Rad, France) and interpreted as resistant (R), intermediate (I), and susceptible (S) based on the respective breakpoints for specific organisms in the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021).

Phenotypic detection of ESBLs

ESBL phenotype identification was carried out using MAST disks (Mast Group, UK) on all isolates ($n = 648$) that were non-susceptible to beta-lactam antibiotics such as cefotaxime, cefoxitin, cefepime, piperacillin/tazobactam, or meropenem in the Kirby-Bauer disk diffusion technique. The results were interpreted using the Mast Disks Combi D68C ESBL/AmpC calculator spreadsheet (Mast Group, UK) and reported as negative, positive, or inconclusive for ESBL or/and AmpC. Isolates with reports of "Further work required" or "Equivocal" or that grew toward all disks with below 9 mm of inhibition zone were grouped together as "inconclusive."

Detection of carbapenem resistance using Etest strips

All bacterial isolates that were intermediate or resistant to meropenem in the Kirby-Bauer disk diffusion method were tested with ertapenem Etest strips for *Enterobacterales* and meropenem Etest strips (both BioMérieux Deutschland GmbH) for non-lactose fermenting Gram-negative rods. According to EUCAST's breakpoints for meropenem, an isolate was considered intermediate if the MIC value was between 2 and 8 mg/L and resistant when the MIC was greater than 8 mg/L. Bacterial isolates with MIC values greater than 0.5 mg/L were interpreted as resistant to ertapenem. Otherwise, all the remaining strains were considered susceptible to meropenem or ertapenem, respectively (EUCAST, 2021).

Detection of carbapenemase encoding genes using PCR

The DNA was extracted from 3 to 5 fresh pure colonies of the respective bacterial isolate and extracted using High Pure PCR template preparation kit (Roche, Germany) following the manufacturer's instruction. The quantity, purity, and concentration of the extracted DNA were measured by Nano-Drop ND-100 (Thermo Fisher Scientific, Wilmington, USA). Excluding the intrinsic carbapenem-resistant *S. maltophilia*, all the remaining isolates ($n = 155$) that were resistant to carbapenem antibiotics and/or showed inconclusive results in ESBL phenotypes by Mast disks (Mast Group, UK) were characterized by multiplex PCR to detect the carbapenemase encoding genes using specific primers and probes (Supplementary Table S2) used in previous studies (Kruttgen et al., 2011; Huang et al., 2012) and kindly provided by the molecular diagnostics of the Max von Pettenkofer Institute by Schubert S. and Gross B. Reference strains carrying *bla*OXA-48 (*K. pneumoniae* ATCC-BAA-2524), *bla*KPC (*E. coli* ATCC-1101362), and *bla*NDM (*K. pneumoniae* ATCC-BAA-2146) were used as positive controls.

Statistical analysis

The data was entered and analyzed using Microsoft Office 2016 excel sheets and GraphPad Prism version 8.4.3. Tables and graphs were used to display the frequency of phenotypic antibiotic resistance patterns and the distribution of carbapenemase encoding genes among phenotypically carbapenem-resistant bacterial pathogens.

Ethical considerations

The study was carried out with the approval of both Jimma University Institute of Health Institutional Review Board, Ethiopia (protocol numbers: IHRPGO/495/2018 & IHRPGO/1087/21) and the Ethics Committee of the Medical Faculty of Ludwig-Maximilians-Universität of Munich, Germany (Opinion No: 21–0157). Written informed consent was obtained from study participants and parents or guardians in case of neonates, infants, and children before enrollment in the study. All the information was kept confidential and recorded anonymously. The culture results were sent back timely to the treating physicians to provide the recommended medical attention to the respective patients.

Results

Frequency of Gram-negative bacterial isolates

A total of 1,794 clinical specimens were processed during the study period. Of these, 953 specimens collected from 894 patients were positive resulting in the isolation of 1,010 bacterial strains. The majority of isolates (846/1,010) were GNB, which were the only one included in the current study. A single bacterial pathogen was identified in 896 specimens, while two and three isolates were detected in the remaining 55 and 2 clinical samples, respectively. Overall, more than 30 different species of GNB were identified. The most commonly identified bacterial pathogen was *E. coli* accounting for 27% (231/846) of the GNB isolates, followed by *K. pneumoniae* 19% (163/846), *A. baumannii* complex 15% (126/846), and *E. cloacae* complex 13% (108/846) (Supplementary Table S3). More than 75% (643/846) of the GNB were isolated from admitted patients. Of these, 32% (206/643) were from the neonatal intensive care unit (NICU), 27% (184/643) from surgical, 27% (173/643) from pediatric, and 12% (80/643) from medical wards.

Antimicrobial resistant pattern of Gram-negative bacteria

In Kirby-Bauer disk diffusion technique, a remarkable prevalence of non-susceptibility was observed against cefuroxime, ampicillin, and piperacillin, with rates reaching 100% (846/846), 92% (763/827), and

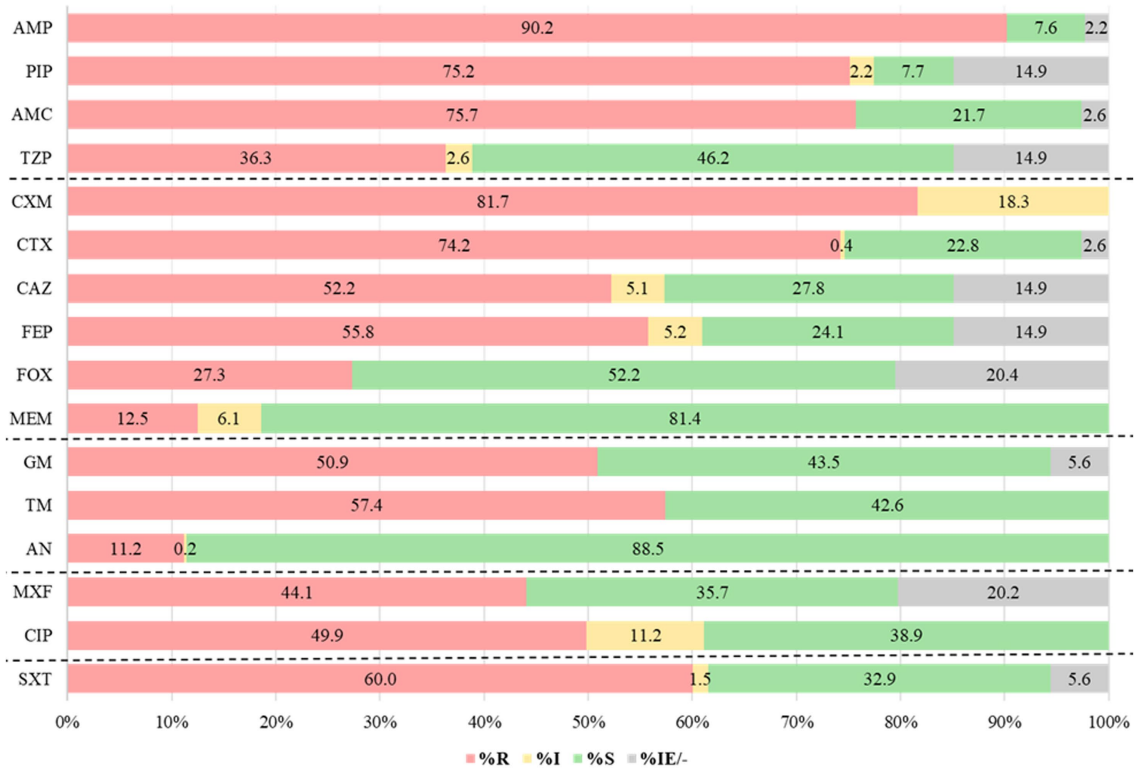

FIGURE 1
Antibiotic resistance patterns for Gram-negative bacteria ($n = 846$). AMP, ampicillin; PIP, piperacillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; GM, gentamicin; TM, tobramycin; AN, amikacin; MXF, moxifloxacin; CIP, ciprofloxacin; SXT, Trimethoprim- sulfamethoxazole; R, resistant; I, intermediate; S, susceptible, IE: insufficient evidence; and "–" No breakpoints.

91% (655/720) respectively. Among the tested antibiotics, meropenem and amikacin showed the least resistance, 18% (149/846) and 12% (97/846), respectively. The isolates also exhibited a high rate of resistance to trimethoprim-sulfamethoxazole (60%), aminoglycosides (11–57.4%), and fluoroquinolones (55.3–61.1%) (Figure 1).

Prevalence of ESBL phenotypes

All 648 bacterial isolates that were non-susceptible (tested intermediate or resistant) to one of the β-lactam antibiotics were further analyzed for ESBL phenotypes using Mast disks (MAST group UK). The analysis revealed that 66% (425/648) of the isolates produced extended-spectrum beta-lactamases (ESBL), 7% (47/648) had both ESBL and AmpC phenotypes, and 3% (19/648) showed only an AmpC phenotype (Figure 2). The remaining 24% (157/648) of the isolates showed inconclusive results when read with Mast disks combi D68C ESBL/AmpC calculator spreadsheets (Mast group, UK).

More than 75% (491/648) of the isolates that showed resistance to β-lactam antibiotics in the disk diffusion technique were confirmed as ESBL and/or AmpC phenotypes by Mast disks (Mast group, UK). As shown in Table 1, all *Citrobacter* species, *K. oxytoca*, *Proteus* species, *S. marcescens*, *M. morganii*, *C. sakazakii*, *L. adecarboxylata*, *M. odoratimimus*, and *P. stuartii* were confirmed as ESBL producers. Furthermore, the prevalence of ESBL production was observed in 93% (127/137) of *K. pneumoniae*, 94% (134/142) of *E. coli*, and 97% (98/101) of *Enterobacter* isolates. The remaining 24% (157/648) of the isolates showed inconclusive results, primarily *A. baumannii* complex, and *P. aeruginosa* which accounted for 71% (87/122) and 98% (42/43) of the respective isolates as shown in Table 1.

Carbapenem minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of carbapenem antibiotics, specifically ertapenem for *Enterobacterales* and meropenem for non-lactose fermenting GNB, was determined using Etest strips. This was done for all isolates ($n = 155$) that were tested carbapenem-resistant in the Kirby-Bauer disk diffusion method and/or showed inconclusive results in the Mast disk analysis. Accordingly, 79% (105/133) of non-lactose fermenting isolates and 100% (24/24) of the lactose fermenting isolates showed intermediate or resistant phenotypes against meropenem or ertapenem Etest strip, respectively (Figure 3).

Molecular epidemiology of carbapenemase-expression in Gram-negative bacteria

The PCR analysis revealed that 69% (107/155) of the carbapenem non-susceptible isolates carried at least one carbapenemase-encoding

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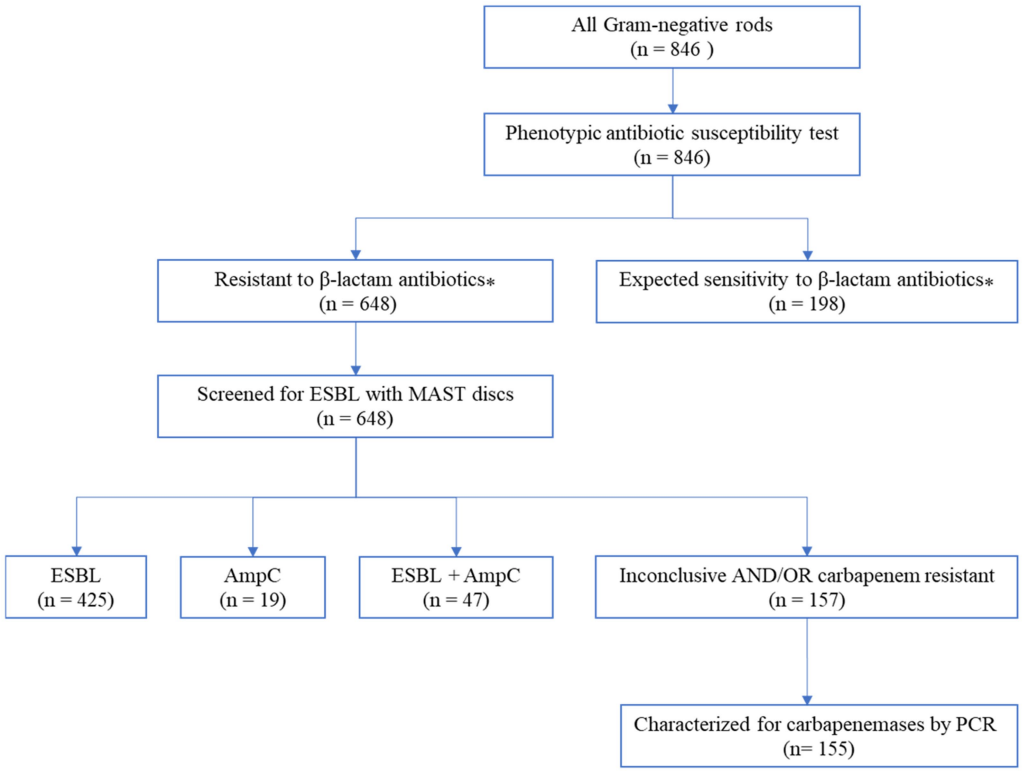

FIGURE 2
Flow diagram of the laboratory analysis to detect carbapenem-resistant Gram-negative bacteria. *The antibiotic susceptibility test result to selected beta-lactam antibiotics such as cefotaxime, cefoxitin, cefepime, piperacillin/tazobactam, or meropenem; intrinsic resistances according to EUCAST are considered as expected; values with insufficient evidence according to EUCAST were not taken into account (EUCAST, 2021).

TABLE 1 Proportion of ESBL phenotypes in Gram-negative bacteria (*n* = 648).

Bacteria	ESBL		AMPC		ESBL and AMPC		Inconclusive	
	n	%	*n*	%	*n*	%	*n*	%
A. baumannii complex (*n* = 122)	14	11.5	1	0.8	20	16.4	87	71.3
Citrobacter species (*n* = 8)	5	NA	3	NA	0	0.0	0	0.0
Enterobacter species (*n* = 101)	85	84.2	6	5.9	7	6.9	3	3.0
E. coli (*n* = 142)	119	83.8	6	4.2	9	6.3	8	5.6
K. oxytoca (*n* = 9)	9	NA	0	0.0	0	0.0	0	0.0
K. pneumoniae (*n* = 137)	120	87.6	2	1.5	5	3.6	10	7.3
K. variicola (*n* = 21)	13	61.9	0	0.0	5	23.8	3	14.3
Proteus species (*n* = 34)	34	100.0	0	0.0	0	0.0	0	0.0
P. aeruginosa (*n* = 43)	1	2.3	0	0.0	0	0.0	42	97.7
Pseudomonas species (*n* = 4)	2	NA	0	0.0	0	0.0	2	NA
S. marcescens (*n* = 14)	14	NA	0	0.0	0	0.0	0	0.0
M. morganii (*n* = 5)	5	NA	0	0.0	0	0.0	0	0.0
Other GNRs (*n* = 8)	4	NA	1	NA	1	NA	2*	NA
Total (*n* = 648)	425	65.6	19	2.9	47	7.3	157	24.2

Other Gram-negative rods (GNRs): *Cronobacter sakazakii* (1), *Leclercia adecarboxylata* (2), *Myroides odoratimimus* (2), *Providencia stuartii* (1), and **Stenotrophomonas maltophilia* (2); NA, not applicable. Percentage is not calculated if the denominator is less than 20.

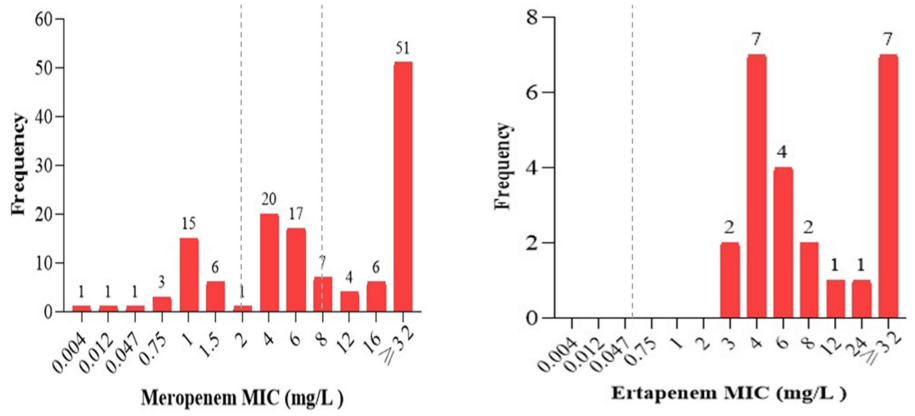

FIGURE 3
The frequency of carbapenem minimum inhibitory concentrations of all strains tested resistant in Kirby-Bauer disk diffusion or having inconclusive results in the Mast Disk assay. The MICs of meropenem ranging from (0–2 mg/L), (2–8 mg/L), and > 8 mg/L were interpreted as sensitive, intermediate, and resistant; ertapenem MIC values ≤0.5 mg/L and > 0.5 mg/L were interpreted as sensitive and resistant, respectively, as indicated in the broken lines according to EUCAST breakpoints (EUCAST, 2021).

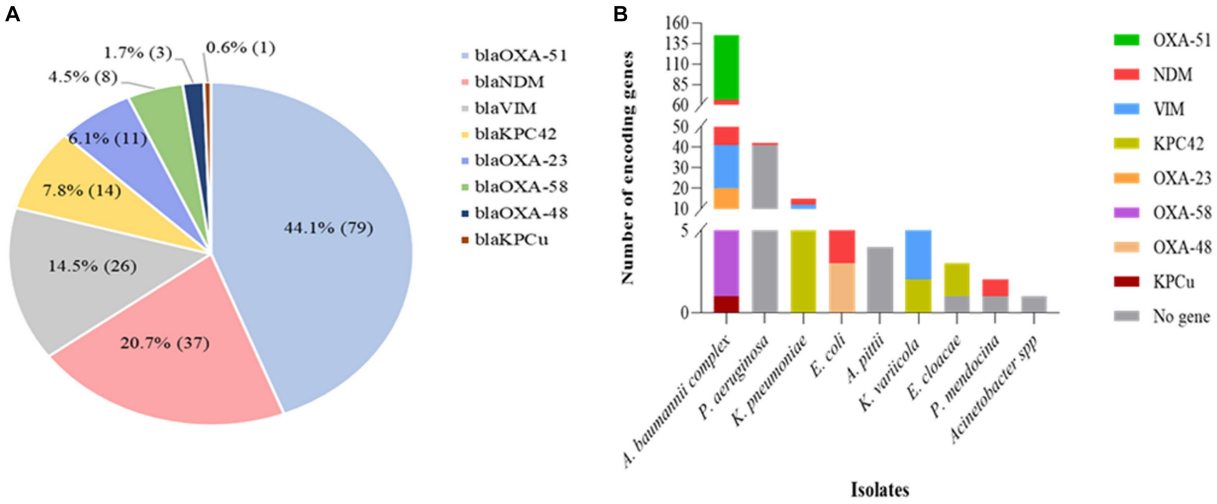

FIGURE 4
Distribution of carbapenemase encoding genes in various Gram-negative bacterial isolates with phenotypic resistance against carbapenems, as determined by PCR analysis. **(A)** The relative proportion of carbapenemase encoding genes ($n = 179$) as indicated in the pie chart. **(B)** The distribution of carbapenemase determinants in carbapenem-resistant isolates ($n = 155$). The PCR analysis revealed the presence of several types of carbapenemase determinants in many of the bacterial species. As a result, more than one carbapenemase determinant or mechanism of resistance was identified in 49 of the isolates.

gene, including both inherent and acquired genes. Among the acquired carbapenemase genes, the most frequently identified gene was *bla*NDM, constituting 21% (37/179) of the total detected genes. This was followed by *bla*VIM and *bla*KPC42, accounting for 15% (26/179), and 8% (14/179) respectively (Figure 4A). Regarding the distribution of carbapenemase-encoding genes, *bla*NDM was detected in various strains including *A. baumannii* (24), *E. coli* (6), *K. pneumoniae* (3), *K. variicola* (1), *P. aeruginosa* (1), *P. mendocina* (1) and *A. haemolyticus* (1). On the other hand, due to its intrinsic presence in *A. baumannii*, the *bla*OXA-51-like gene was exclusively found in *A. baumannii* strains (79) (Figure 4B). Conversely, no carbapenemase-encoding genes could be detected in 31% (48/155) of carbapenem-resistant isolates. *P. aeruginosa* was the most common, accounting for 85% (41/48) of them (Figure 4B).

Co-harboring of two or more acquired genes was observed in 31% (33/107) of the isolates, with *A. baumannii* being the predominant strain, accounting for 70% (23/33) of those isolates. Multiple gene coexistence was also detected in *A. haemolyticus* (1), *E. coli* (1), *K. pneumoniae* (5), and *K. variicola* (3) strains. The most common acquired coexisting genes were *bla*NDM + *bla*OXA-23, observed in 24% (8/33) of the isolates (Table 2).

Discussion

Our study revealed high proportions of ESBL and carbapenemase producing Gram-negative pathogens, primarily *E. coli*, *K. pneumoniae*, *E. cloacae* complex, *A. baumannii* complex, and *P. aeruginosa* in

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TABLE 2 Frequency and distribution of carbapenemase-coding genes among Gram-negative bacteria ($n = 107$).

Bacteria	AST using Etest strips		Carbapenem resistance genes (n)	Resistance strains % (n)
	Antibiotic	MIC (mg/L)		
Acinetobacter baumannii complex ($n = 81$)	MP	≤2 (22)	OXA-51	38.3 (41)
		2–8 (19)		
		≤2 (4)	VIM + OXA-51	12.2 (13)
		2–8 (6)		
		>8 (3)		
		>8 (8)	NDM + OXA-51 + OXA-23	7.5 (8)
		2–8 (1)	NDM + OXA-51 + OXA-58	4.7 (5)
		>8 (4)		
		>8 (5)	NDM + OXA-51 + VIM	4.7 (5)
		2–8 (1)	NDM + OXA-51	2.8 (3)
		>8 (2)		
		>8 (1)	NDM + OXA-51 + OXA-58 + VIM	0.9 (1)
		>8 (1)	NDM + OXA-23	0.9 (1)
		>8 (1)	OXA-51 + OXA-23	0.9 (1)
		2–8 (1)	VIM + OXA-51 + OXA-58	1.9 (2)
		>8 (1)		
		>8 (1)	NDM + KPCu	0.9 (1)
Acinetobacter haemolyticus ($n = 1$)	MP	>8 (1)	NDM + OXA-23	0.9 (1)
Enterobacter cloacae ($n = 2$)	ETP	>0.5 (2)	KPC42	1.9 (2)
E. coli ($n = 8$)	ETP	>0.5 (5)	NDM	4.7 (5)
		>0.5 (2)	OXA-48	1.9 (2)
		>0.5 (1)	NDM + OXA-48	0.9 (1)
Klebsiella pneumoniae ($n = 10$)	ETP	>0.5(2)	KPC42	4.7 (5)
		>0.5(3)		
		>0.5(3)	KPC42 + NDM	2.8 (3)
		>0.5(2)	KPC42 + VIM	1.9 (2)
Klebsiella variicola ($n = 3$)	ETP	>0.5(2)	KPC42 + VIM	1.9 (2)
		>0.5 (1)	NDM + VIM	0.9 (1)
Pseudomonas aeruginosa ($n = 1$)	MP	>8 (1)	NDM	0.9 (1)
Pseudomonas mendocina ($n = 1$)	MP	>8 (1)	NDM	0.9 (1)

Interpretation: Meropenem, MIC value ≤ 2 mg/L → S, 2-8 mg/L → I, > 8 mg/L → R; and Ertapenem, MIC value ≤ 0.5 mg/L → S, >0.5 mg/L → R, and screening cut-off for both antibiotics MIC > 0.12 mg/L.

comparison to previous studies conducted worldwide (Chen et al., 2021; Jean et al., 2022). In most low-income countries, carbapenems are considered the last-resort antibiotics, as other antibiotics like colistin and polymyxin B are not available. Carbapenem-resistant infections are increasing at alarming rates worldwide (Hammoudi Halat and Ayoub Moubareck, 2020), and this trend is even worse in low-income countries (Stewardson et al., 2019) including Ethiopia (Sewunet et al., 2022; Tilahun et al., 2022). Inadequate infection prevention and control measures, lack of proper hand hygiene, insufficient isolation precautions, and limited regular AMR surveillance (Ali et al., 2018; Eshetu et al., 2019) contribute to this problem.

More than three-fourths (76.6%, 648) of the isolates were tested resistant to one or more beta-lactam antibiotics such as cefotaxime, cefoxitin, cefepime, piperacillin/tazobactam, or meropenem. Among all isolates, 59% (499/846) showed ESBL phenotypes, and 19% (157/846) were carbapenem-resistant phenotypically. Our findings indicate an increase in ESBL phenotypes in Jimma compared to previous reports of 50–51% in 2016 (Gashaw et al., 2018; Zeynudin et al., 2018). The observed high prevalence of ESBL-producing isolates could be explained by the high rate of nosocomial infections among hospitalized patients (Ali et al., 2018). The lack of proper infection prevention and control practices (Sastry et al., 2017; Maki and Zervos, 2021), along with horizontal gene transfer (Da Silva and Domingues, 2016) and the spread of resistant genes within local microbial populations may contribute to the high rate of beta-lactam resistance. Additionally, the high rates of *Acinetobacter* and *Pseudomonas* species which are

intrinsically resistant to many beta-lactam antibiotics could explain this increase.

In previous studies conducted in Ethiopia, the rate of carbapenem resistance among Gram-negative rods was low ranging 1.7–15.1% (Misha et al., 2021; Tekele et al., 2021; Seman et al., 2022; Tilahun et al., 2022; Alemayehu et al., 2023). However, our findings showed an increase in resistance to carbapenems (18.6%). Our current study revealed high rates of phenotypic carbapenem resistance among *Acinetobacter* (71.3%) and *Pseudomonas* species (97.7%, 42/43), compared to a previous study conducted in the same area in 2016, where resistance rates were 56.4 and 7.3% for *Acinetobacter* and *Pseudomonas* isolates, respectively (Sewunet et al., 2022). This increase in resistance may be attributed to the increasing use of carbapenems at the hospital and poor infection control measures. Infections caused by such resistant isolates greatly limit the treatment options. Therefore, addressing the rising threat of carbapenemase-producing *Acinetobacter* and *Pseudomonas* species requires a multifaceted approach including the implementation of effective infection prevention and control measures, promotion of antimicrobial stewardship programs to ensure appropriate antibiotics use, and development of new antibiotics effective against these resistant strains (Mulani et al., 2019; Jean et al., 2022).

Additionally, it is important to identify the determinants of carbapenem resistance in bacterial pathogens. While many isolates express a carbapenemase, others may develop resistance due to other mechanisms such as porin loss (Atrissi et al., 2021). In our study, we investigated both the phenotypic resistance and the presence of carbapenemase genes. In *A. baumannii*, we found the presence of intrinsically encoded *bla*OXA-51-like genes, as well as the acquired *bla*NDM and *bla*KPC encoding genes. We did not investigate any regulatory phenotypes involved in increased expression of *bla*OXA-51-like enzymes, so we can only speculate on their role in the phenotypically resistant isolates, possibly in combination with permeability issues or efflux pumps. Nevertheless, in the case of *P. aeruginosa,* the observed carbapenem resistance could not be linked to the carbapenemases tested in the study. Instead, it is more likely that the resistance is due to porin loss as suggested by a previous study (Atrissi et al., 2021).

Similar to previous studies conducted in Egypt (Abouelfetouh et al., 2019) and South Africa (Anane et al., 2020), PCR analysis revealed that all *A. baumannii* isolates carried the *bla*OXA-51-like genes. In 13.6% (11/82) and 9.9% (8/82) of *Acinetobacter* strains, *bla*OXA-23-like and *bla*OXA-58-like genes were detected, respectively. The prevalence of *bla*OXA-51-like gene in our study was higher than reported in a previous study in Jimma (63.1%) (Sewunet et al., 2022). This can be explained by the higher proportion of *A. baumannii* strains that currently dominate nosocomial infections as compared to previous studies. All 79 *A. baumannii* isolates carried the intrinsic *bla*OXA-51-like gene, but 22 of them were phenotypically susceptible to meropenem according to the MIC values. This can be explained by the intrinsic low efficiency of *bla*OXA-51, which is not easily detected by phenotypic methods, as reported in previous studies (Hu et al., 2007; Nigro and Hall, 2018).

The New Delhi metallo-beta-lactamase (NDM), classified as group B in the Ambler classification, is an enzyme that can break down a wide range of beta-lactam antibiotics, including carbapenems. It was first reported in Ethiopia in 2017 in *A. baumannii* strains (Pritsch et al., 2017). Back then, it could only be detected in some isolates of *Acinetobacter baumannii*, with no evidence of its presence in other isolates. However, NDM is no longer limited to *Acinetobacter* species and has been found in various GNB, such as *K. pneumoniae*, *K. variicola*, *E. coli*, *P. aeruginosa*, and *P. mendocina* (Legese et al., 2022; Seman et al., 2022; Sewunet et al., 2022; Tufa et al., 2022). This enzyme is particularly concerning because it can rapidly spread between different bacterial species through horizontal gene transfer, leading to the emergence of extensively drug-resistant infections (Da Silva and Domingues, 2016). It is also frequently associated with other antibiotic resistance determinants and may be transferred alongside them. Our study detected the *bla*NDM gene in 34.6% of carbapenemase positive isolates, which is comparable to a study conducted in Kenya where 30% of the isolates carried the NDM gene (Villinger et al., 2022). The other commonly acquired carbapenemase gene identified in our study was *bla*KPC42, which was found in all carbapenem-resistant *K. pneumoniae* (10) and two of the three carbapenem resistant *K. variicola* strains. It has not been previously reported in Ethiopia but has been frequently reported in other parts of the world (Miranda et al., 2018).

Most of the *A. baumannii* isolates in our study harbored two (19) or three (21) carbapenemase genes. Moreover, five *K. pneumoniae* and three *K. variicola* isolates carried two carbapenemase genes. In total, 50 of the isolates carried multiple carbapenemase genes (*bla*OXA-51, *bla*NDM, *bla*VIM, *bla*OXA-23, *bla*OXA-58, *bla*KPC42, *bla*OXA-48, and *bla*KPCu), which is consistent with other studies conducted in Ethiopia where multiple carbapenemase determinants have been reported (Legese et al., 2022; Sewunet et al., 2022). In general, the prevalence of NDM in *Acinetobacter* and other GNB has been increasing globally in recent years (Sands et al., 2021; Awoke et al., 2022; Seman et al., 2022).

There are certain limitations to our study that should be considered when interpreting the results. First, the study was conducted in a single tertiary level facility, which may not fully represent the diversity of antimicrobial resistance patterns in the broader community or other healthcare settings in the region. Second, the PCR analysis was performed on isolates that were phenotypically resistant to carbapenems in the disk diffusion method and/or showed inconclusive results in the Mast disk analysis. This approach may have excluded some isolates with reduced carbapenem susceptibility that were not detected by the phenotypic resistance, potentially underestimating the true burden of carbapenem resistance in the study area. Third, we did not investigate if the resistance against carbapenems observed in some *A. baumannii* strains was due to overexpression of OXA-51 or other metabolic or regulatory changes such as loss of permeability or increased efflux.

Conclusion

Our study demonstrated a high rate of carbapenem resistance among GNB, primarily in *Acinetobacter* species. The majority of this resistance was attributed to carbapenemases, probably along with other factors. Consequently, treating infections caused by these pathogens in this region may prove challenging due to limited treatment options. To address this issue, it is essential to revise treatment strategies in order to effectively manage infections caused by resistant strains. Moreover, it is imperative to uphold diligent surveillance, apply optimal infection prevention and control strategies,

and promote antimicrobial stewardship practices to effectively manage and combat the dissemination of carbapenem-resistant bacteria.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was approved by the Institutional Review Board (IRB) of Jimma University Institute of Health, Ethiopia and The Ethics Committee at the Medical Faculty of LMU Munich, Germany. Written informed consent was also obtained from patients, parents, or guardians prior to recruitment in the study.

Author contributions

MG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. EG: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing. SA: Conceptualization, Supervision, Writing – review & editing. LG: Data curation, Writing – review & editing. TS: Writing – review & editing. BA: Data curation, Writing – review & editing. GF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. AK: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. AW: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1336387/full#supplementary-material

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8.2 Paper II

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MDPI

Article

Hospital Wastes as Potential Sources for Multi-Drug-Resistant ESBL-Producing Bacteria at a Tertiary Hospital in Ethiopia

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Abstract: The hospital environment is increasingly becoming an important reservoir for multi-drug-resistant (MDR) Gram-negative bacteria, posing serious challenges to efforts to combat antimicrobial resistance (AMR). This study aimed to investigate the role of hospital waste as a potential source of MDR ESBL-producing bacteria. Samples were collected from multiple sources within a hospital and its vicinity, including surface swabs, houseflies, and sewage samples. The samples were subsequently processed in a microbiology laboratory to identify potential pathogenic bacteria and confirmed using MALDI-TOF MS. Bacteria were isolated from 87% of samples, with the predominant isolates being *E. coli* (30.5%), *Klebsiella* spp. (12.4%), *Providencia* spp. (12.4%), and *Proteus* spp. (11.9%). According to the double disc synergy test (DDST) analysis, nearly half (49.2%) of the bacteria were identified as ESBL producers. However, despite exhibiting complete resistance to beta-lactam antibiotics, 11.8% of them did not test positive for ESBL production. The characterization of *E. coli* revealed that 30.6% and 5.6% of them carried *bla*CTX-M group 1 type-15 and *bla*NDM genes, respectively. This finding emphasizes the importance of proper hospital sanitation and waste management practices to mitigate the spread of AMR within the healthcare setting and safeguard the health of both patients and the wider community.

Keywords: hospital waste; MDR; ESBL; NDM; CTX-M; Gram-negative bacteria

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Abstract: The hospital environment is increasingly becoming an important reservoir for multi-drug-resistant (MDR) Gram-negative bacteria, posing serious challenges to efforts to combat antimicrobial resistance (AMR). This study aimed to investigate the role of hospital waste as a potential source of MDR ESBL-producing bacteria. Samples were collected from multiple sources within a hospital and its vicinity, including surface swabs, houseflies, and sewage samples. The samples were subsequently processed in a microbiology laboratory to identify potential pathogenic bacteria and confirmed using MALDI-TOF MS. Bacteria were isolated from 87% of samples, with the predominant isolates being *E. coli* (30.5%), *Klebsiella* spp. (12.4%), *Providencia* spp. (12.4%), and *Proteus* spp. (11.9%). According to the double disc synergy test (DDST) analysis, nearly half (49.2%) of the bacteria were identified as ESBL producers. However, despite exhibiting complete resistance to beta-lactam antibiotics, 11.8% of them did not test positive for ESBL production. The characterization of *E. coli* revealed that 30.6% and 5.6% of them carried *bla*CTX-M group 1 type-15 and *bla*NDM genes, respectively. This finding emphasizes the importance of proper hospital sanitation and waste management practices to mitigate the spread of AMR within the healthcare setting and safeguard the health of both patients and the wider community.

Keywords: hospital waste; MDR; ESBL; NDM; CTX-M; Gram-negative bacteria

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1. Introduction

Multi-drug-resistant (MDR) bacteria, which produce both extended spectrum beta-lactamase (ESBL) and carbapenemase, pose a significant and persistent global health threat [1]. This phenomenon has resulted in increased rates of morbidity, mortality, and escalated healthcare expenditures [1,2]. The presence of these bacteria in healthcare facilities and their surroundings further exacerbates the problem [3]. Contaminated surfaces, hospital sewage, and other environmental factors within the hospital have been identified as potential reservoirs and sources of MDR bacteria due to their close proximity to patients and healthcare workers. Furthermore, houseflies have the potential to mechanically transmit MDR bacteria to both patients and the wider community [3–5]. In resource-limited settings like Ethiopia, where healthcare infrastructure and waste management systems are

suboptimal, the risk posed by hospital waste as a reservoir for MDR bacteria is becoming a pressing concern [1,6].

Hospital sewage serves as a conduit for the disposal of various waste materials, including fecal matter, biological wastes, biopsy specimens, clinical sample leftovers, and discarded medical supplies, potentially carrying a myriad of pathogenic bacteria [7,8]. Such sewage can contain MDR bacteria originating from infected or colonized patients, making them a reservoir for the spread of drug-resistant strains within the hospital and to the community [8]. The complex microbial niche in sewage provides opportunities for gene transfer and genetic recombination, facilitating the acquisition and spread of resistance determinants among bacteria [9,10]. Hospital environments, particularly those with inadequate sanitation and waste management practices, can attract houseflies, increasing the risk of MDR bacteria being disseminated by these insects [6,11]. They can carry bacteria on their body surfaces and within their digestive systems, facilitating their dissemination from contaminated sources to other locations [11,12].

Previous studies conducted in the same study area have reported a high prevalence of ESBL-producing bacteria and carbapenem-resistant strains among Gram-negative bacteria isolated from clinical samples [13–17]. The prevalence rates for ESBL producers range from 50 to 80%, while carbapenem-resistant strains range from 10 to 20% [13–17]. Notably, ESBL production is commonly observed in bacteria such as *E. coli*, *K. pneumoniae*, *K. variicola*, *E. cloacae*, and many others [14,15,17]. Similarly, the emergence of carbapenem resistance is frequently detected in Gram-negative bacteria such as *A. baumannii*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* [16,17]. These resistant strains have been associated with healthcare-associated infections, posing a serious threat to effective antimicrobial therapy [18]. As a result, they contribute to increased morbidity, mortality, and healthcare costs [18,19].

Jimma Medical Center, located in Ethiopia, is a tertiary hospital that serves as a referral center for the southwest region of the country and plays a crucial role in providing essential healthcare services to a substantial population [20]. However, the potential contribution of hospital waste to the spread of MDR bacteria in this setting remains poorly understood. Therefore, understanding the dynamics and sources of MDR bacterial isolates from hospital sewage, houseflies, and environmental samples provides valuable insights into the prevalence, genetic characteristics, and potential transmission routes of drug-resistant bacteria within healthcare settings and, more importantly, to the community. Thus, based on the evidence, appropriate infection control measures can be implemented to prevent their spread and reduce the burden of MDR infections. Therefore, this study aimed to provide insights on potential reservoirs for MDR and ESBL-positive pathogenic Gram-negative bacteria within the environment of Jimma Medical Center.

2. Results

2.1. Proportion of Bacterial Growth

The microbiological analysis revealed the presence of potential pathogenic bacteria in samples obtained from houseflies, hospital rooms and medical device surface swabs, and sewage samples. A total of 345 samples, including 111 surface swabs and 42 sewage samples collected in 2019 and 192 housefly samples collected in 2021, were examined. The overall isolation rate was 80.9% (95% CI: 77.2% to 84.6%), with a 100% isolation rate from housefly and sewage samples. However, potentially pathogenic Gram-negative bacteria were isolated from 40.5% (n = 45) of hospital rooms and medical device surface swab samples (Figure 1).

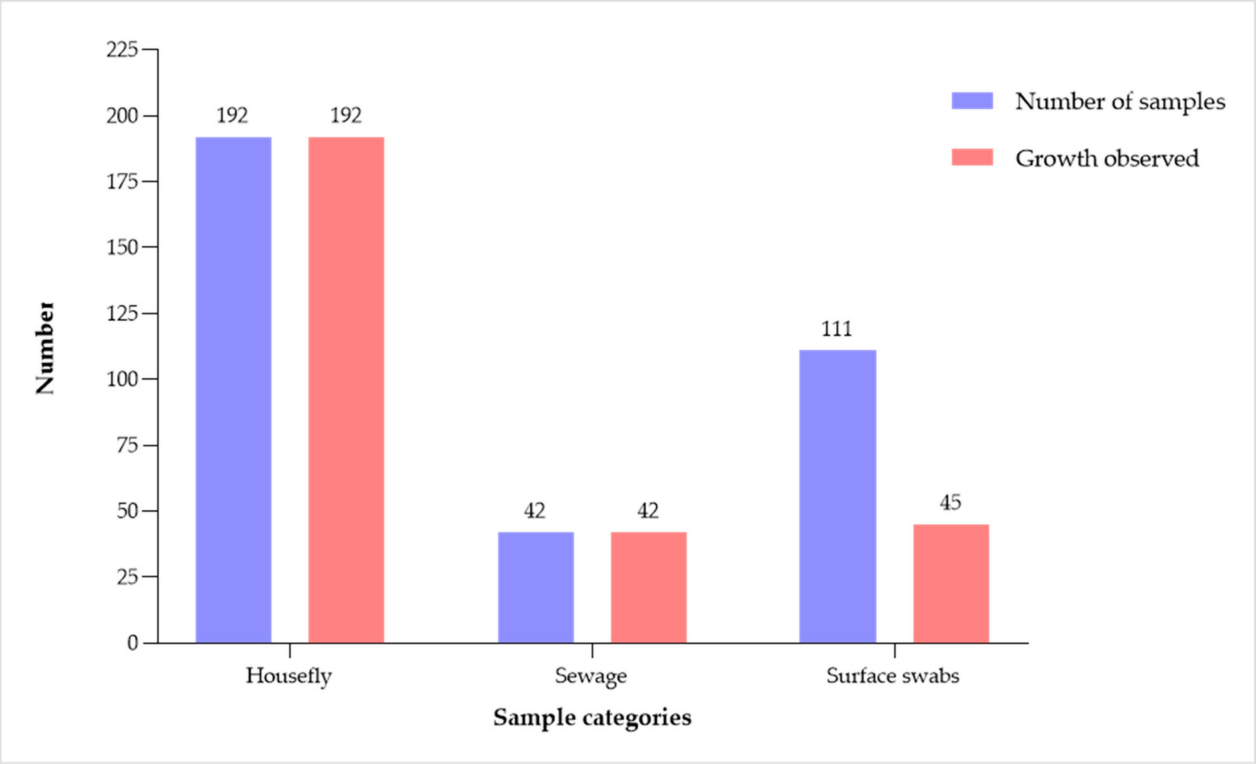

Figure 1. The proportion of aerobic bacterial growth obtained from housefly, sewage, and surface swab samples.

2.2. Profile of Isolated Gram-Negative Bacteria

Further analysis of the bacterial isolates revealed a diverse range of species in surface swabs, housefly, and sewage samples. A total of 37 different species of bacteria were identified in housefly samples, while 23 species were isolated in sewage samples and 11 species in surface swabs. Among the housefly samples, *Providencia* species (20.7%) were the most frequently isolated bacteria, followed by *Proteus* species (18.6%), *E. coli* (14.9%), and *Klebsiella* species (11.2%). *E. coli* (60%), *Aeromonas* species (15.6%), and *Acinetobacter* species (8.9%) were the predominant isolates in surface swabs. In sewage samples, *E. coli* (52.1%), *Klebsiella* (19.1%), and *Acinetobacter* species (9.6%) were frequently identified. However, it is noteworthy that MDR *E. coli*, *Klebsiella*, *Acinetobacter*, and *Enterobacter* species were consistently isolated from all sample types. Despite the consistent presence of these bacterial strains across all sample types, their prevalence and abundance varied (Table 1).

Table 1. The distribution of aerobic bacteria isolated from hospital rooms and medical device surface swabs, housefly, and sewage samples at a tertiary hospital in Ethiopia.

Bacteria	Housefly		Surface Swabs		Sewage		Total	
	n	%	*n*	%	*n*	%	*n*	%
E. coli	32	29.6	27	25.0	49	45.4	108	30.4
***Klebsiella* species**	24	54.5	2	4.5	18	40.9	44	12.4
***Providencia* species**	44	100	-	-	-	-	44	12.4
***Proteus* species**	40	95.2	2	4.8	-	-	42	11.8
***Enterobacter* species**	16	69.6	1	4.3	6	26.1	23	6.5
***Acinetobacter* species**	6	31.6	4	21.1	9	47.4	19	5.4
M. morganii	14	100	-	-	-	-	14	3.9
***Aeromonas* species**	1	NA	7	NA	2	NA	10	2.8
***Kluyvera* species**	7	NA	-	-	2	NA	9	2.5

Table 1. *Cont.*

Bacteria	Housefly		Surface Swabs		Sewage		Total	
	n	%	*n*	%	*n*	%	*n*	%
R. ornithinolytica	7	NA	-	-	2	NA	9	2.5
W. chitiniclastica	9	NA	-	-	-	-	9	2.5
C. freundii	5	NA	-	-	-	-	5	1.4
***Pantoea* species**	2	NA	-	-	1	NA	3	0.8
P. gergoviae	1	NA	-	-	2	NA	3	0.8
E. hermannii	1	NA	-	-	1	NA	2	0.6
L. adecarboxylata	1	NA	-	-	1	NA	2	0.6
C. sakazakii	-	-	-	-	1	NA	1	0.3
E. fergusonii	-	-	1	NA	-	-	1	0.3
Hafnia alvei	1	NA	-	-	-	-	1	0.3
I. indica	1	NA	-	-	-	-	1	0.3
M. wisconsensis	1	NA	-	-	-	-	1	0.3
P. carotovorum	1	NA	-	-	-	-	1	0.3
P. putida	1	NA	-	-	-	-	1	0.3
***Salmonella* species**	-	-	1	NA	-	-	1	0.3
S. maltophilia	1	NA	-	-	-	-	1	0.3
Total	216	60.8	45	12.7	94	26.5	355	100

Key: NA: not applicable, percentage was not calculated if the total number of bacterial isolates was less than 14.

2.3. Antibiotic Resistance Patterns

The results of the antibiotic susceptibility tests conducted on bacteria from all sample types combined revealed a significant level of resistance to several antibiotics. Specifically, a high rate of resistance was observed against cefuroxime, ampicillin, amoxicillin-clavulanic acid, piperacillin, and cefotaxime, with 100%, 61%, 44%, 42.2%, and 41.1%, respectively. Conversely, a low rate of resistance was observed against meropenem, amikacin, and piperacillin-tazobactam, representing 3.1%, 3.1%, and 8.6%, respectively. Furthermore, the double disc synergy test revealed that nearly half (49.2%) of the Gram-negative bacterial isolates were ESBL producers. A high proportion of ESBLs was observed in species such as *Acinetobacter*, *Proteus*, and *Providencia*, as indicated in Table 2. In general, an alarming level of resistance, ranging from 30% (in gentamicin) to 61% (in ampicillin), was observed to commonly used antibiotics, including beta-lactams, fluoroquinolones, and aminoglycosides, in Gram-negative bacteria isolated from various environmental samples of the medical center.

2.4. Molecular Epidemiology of ESBLs and Carbapenemase Expression in E. coli Strains

The findings of this study showed a high rate of ESBL- and carbapenemase-encoding genes among *E. coli* strains obtained from surface swabs, housefly, and sewage samples. A total of 66 *E. coli* strains were included in the analysis, and the presence of ESBL- and carbapenemase-encoding genes was determined using DNA microarray technology. The results revealed that 37.9% (n = 41) of the *E. coli* isolates exhibited at least one ESBL-encoding gene, with the predominant variant being CTX-M group 1 type-15. Additionally, 5.6% (n = 6) of the *E. coli* isolates carried carbapenemase genes, solely *bla*NDM. Among carbapenemase-encoding genes, five of them were found in housefly samples and the remaining one gene was detected from a surface swab. Similarly, a high rate of ESBL genes (43.9%) was detected in *E. coli* strains obtained from houseflies. However, 62.2% of the *bla*TEM genes were found in *E. coli* strains obtained from sewage samples (Table 3).

Table 2. The proportion of antibiotic-resistant Gram-negative bacteria obtained from surface swabs, housefly, and sewage samples at a tertiary hospital in Ethiopia.

Antibiotics	*E. coli*	*Klebsiella* spp.	*Providencia* spp.	*Proteus* spp.	*Enterobacter* spp.	*Acinetobacter* spp.	Ohers	Total
AMP	73.1	100	86.4	81.0	95.7	100	91.3	61.0
PIP	65.7	100	61.4	59.5	60.9	IE	59.6	42.2
AMC	38	43.2	100	21.4	91.3	100	64.3	44.0
TZP	19.4	31.8	9.1	0	17.4	IE	17.0	8.6
CXM	100	100	-	100	-	100	100	100
CTX	34.3	50.0	65.9	64.3	69.6	100	44.6	41.1
CAZ	31.5	47.7	59.1	26.2	56.5	-	40.4	27.9
FEP	34.3	40.9	40.9	64.3	65.2	-	42.1	30.3
FOX	13.9	13.6	15.9	0	100	-	46.4	18.5
MEM	24.1	2.3	0	0	4.3	31.6	4.3	3.1
MXF	35.2	38.6	61.4	76.2	56.5	-	52.2	34.7
CIP	30.6	40.9	47.7	66.7	30.4	100	37.5	33.9
TM	20.4	31.8	34.1	66.7	47.8	47.4	42.6	29.7
GM	16.7	31.8	36.4	66.7	65.2	52.6	32.6	30.1
AN	1.9	2.3	0	4.8	8.7	5.3	8.5	3.1
SXT	43.5	47.7	61.4	71.4	56.5	57.9	50.0	38.7
ESBL	38.9	43.2	61.4	64.3	52.2	68.4	37.5	49.2

Key: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; MXF, moxifloxacin; CIP, ciprofloxacin; GM, gentamicin; TM, tobramycin; AN, amikacin; SXT, sulfamethoxazole-trimethoprim; ESBL, extended spectrum beta-lactamase; spp., species; IE, insufficient evidence; and "-", no breakpoints. Only resistant isolates were included in the proportion analysis, while intermediate and susceptible results were excluded from the numerator. Additionally, rare bacterial isolates that do not have breakpoints in the EUCAST guidelines were excluded from the denominator in AST analysis. The resistance patterns of specific bacterial species are found in the Supplementary Table S1.

Table 3. Distribution of carbapenemase- and extended-spectrum-beta-lactamase-encoding genes of *Escherichia coli* isolated from surface swab, sewage, and housefly samples at Jimma.

Types of Antimicrobial Resistance Gene	Surface Swab (*n* = 10)	Housefly (*n* = 20)	Sewage (*n* = 36)	Total	
				(*n* = 66)	%
Carbapenemase encoding genes	**1**	**5**	**0**	**6**	**5.6**
NDM	1	5	0	6	5.6
ESBL encoding genes	**8**	**18**	**15**	**41**	**37.9**
CTX-M group 1 type-15	6	15	11	33	30.6
CTX-M group 1 type-9	2	0	2	3	2.7
CTX-M group 1, ND *	0	1	2	3	2.8
CTX-M group 1 type-15 + 9	0	2	0	2	1.8
AMPC encoding genes	**3**	**2**	**1**	**6**	**5.6**
CMY II (*n* = 11)	0	1	0	1	0.9
ACT/MIR (*n* = 10)	3	0	0	3	2.8
DHA (*n* = 5)	0	1	1	2	1.9
TEM/SHV encoding genes	**3**	**11**	**23**	**37**	**34.3**
***bla*TEM- (WT) (*n* = 144)**	3	11	22	36	33.4
***bla*TEM-104K + 164C (*n* = 1)**	0	0	1	1	0.9

Key: *—no specified CTX-M group-1, subtype enzymes.

3. Discussion

This study revealed that bacterial isolates were present in all sewage and housefly samples, as well as in 40.5% of surface swabs. Although the proportion of bacteria detected in surface swab samples was lower compared to housefly and sewage samples, it still indicates a substantial presence of bacteria that could serve as potential sources of infections within the healthcare facility. In our study, we identified a diverse range of antibiotic-resistant bacterial isolates, including *E. coli*, *Klebsiella* spp., *Providencia* spp., *Proteus* spp., *Enterobacter* spp., *Acinetobacter* spp., *Morganella morganii*, and many others, in all categories of samples. It is worth noting that a substantial proportion of these bacteria are known to be pathogenic, or at least facultative pathogens, and have been associated with healthcare-associated infections [21,22]. This emphasizes the potential role of the environment, as well

as houseflies, in perpetuating the spread of MDR pathogens, not only among patients, but to the wider community [6,23,24].

The microbiological analysis of surface swabs and sewage samples exhibited a wide array of bacterial strains, including MDR ESBL strains. Therefore, sewage was only streaked and analyzed with aerobic culture, so only the most prevalent aerobic Gram-negative bacteria would be detected. The molecular characterization of *E. coli* strains from these samples revealed the presence of acquired carbapenemase- and ESBL-encoding genes, such as (*bla*NDM) (1), CTX-M group1 type-15 (17) and CTX-M group 1, ND (2), and *bla*TEM, as well as AMPC-encoding genes, such as ACT/MIR and DHA (26). However, it is a common practice at the hospital to release sewage into the nearby stream without proper treatment. The high isolation rate of carbapenemase and ESBL bacterial strains in our study makes this practice highly hazardous. Additionally, in the hospital rooms, the floors are only mopped/cleaned twice daily with water and soap. In cases of suspected visible contamination, a 5% sodium hypochlorite solution diluted in water is used for cleaning. Such inadequate treatment and cleaning practices increase the risk of contamination for patients, healthcare providers, and caregivers in the healthcare facility, as well as water sources and the surrounding community [25–28]. In the community, transmission could occur through direct contact with contaminated surfaces and water or indirectly through animals that have direct contact with this contaminated water and environment [29]. The implications of this finding underscore the importance of implementing an effective sewage treatment system and proper cleaning practices of the hospital rooms and medical devices to mitigate the spread of MDR bacteria and minimize the risk of infections in healthcare settings.

In this study, it was found that houseflies harbor a diverse range of bacteria, including carbapenem-resistant strains and ESBL producers. Specifically, the analysis of *E. coli* strains using DNA microarray technology revealed the presence of acquired *bla*NDM genes and various ESBL-encoding genes in five and twenty *E. coli* strains, respectively. As a result, houseflies have been recognized as potential vectors for the transmission of MDR bacteria due to their attraction to waste areas such as open sewage systems, liquid and solid waste disposal sites, waste bins, and poorly cleaned toilets [30]. These insects can carry bacteria on their bodies and in their digestive systems, enabling them to spread pathogens from contaminated sources like sewage or decaying organic matter to other surfaces, including food, within a healthcare facility [30,31]. Moreover, houseflies can transport MDR bacteria from the environment into healthcare settings or vice versa [32,33]. Hence, the detection of MDR strains in the present study serves as a crucial warning, highlighting the necessity for implementing specific hygiene precautions.

The resistance spectrum of identified bacterial strains, as well as the detected resistance-encoding genes, was found to be similar to those observed in clinical samples from the same area [17]. This highlights the potential risk of transmission and the challenges in treating patients who acquire infections caused by these MDR bacteria transmitted through the hospital environment [17,34,35]. Of particular concern is the presence of the acquired *bla*NDM gene in this study, which encodes the New Delhi metallo-beta-lactamase and confers resistance to many beta-lactam antibiotics, including carbapenems, the last-resort antibiotics used to treat severe MDR bacterial infections [36]. It is worth noting that the acquired *bla*NDM gene can be horizontally transferred to other bacteria in the environment, further contributing to the dissemination of drug resistance [37–39]. Therefore, the high prevalence of drug-resistant bacteria in these samples underscores the urgent need for effective infection prevention and control strategies, including stringent hygiene practices and proper waste management to minimize bacterial contamination in areas prone to housefly infestation, such as toilets, sewage systems, waste bins, and the designated areas for liquid and solid waste disposal in healthcare facilities.

This study has limitations that should be considered when interpreting these findings. Firstly, it did not investigate the specific factors that contribute to the presence of MDR bacteria in hospital waste, such as the duration and storage conditions of the waste or

the impact of specific infection control practices. Understanding these factors could help in identifying associated risk factors and developing appropriate waste management strategies in the hospital. Secondly, this study did not thoroughly examine the extent of the transmission risks posed by these samples, including the spread of drug-resistant bacteria to patients within the hospital and the potential dissemination to the wider community. However, we plan to perform phylogenetic analysis on these bacterial strains and compare them to patient isolates [17]. Thirdly, we conducted molecular analysis to detect resistance-encoding genes on the most prevalent species, *E. coli* only. As a result, this part of the findings may not reflect the distribution of all resistance-encoding genes in other bacterial clades obtained from surface swabs, houseflies, and sewage. Furthermore, sewage was not analyzed using filtration and enrichment techniques. Thus, the real load of MDR bacteria in sewage will be higher than described in this study once the sensitivity of isolation is improved here. We made an intentional decision for this process to limit this study to the most prevalent and most problematic isolates. In depth analysis of the sewage is beyond the scope of this manuscript and is planned for future projects.

4. Materials and Methods

4.1. Description of the Hygiene Practice in Study Area

Hospital hygiene procedures at JMC include floor mopping/cleaning conducted twice daily as part of routine tasks by janitors in the wards, waiting areas, and corridors. The cleaning of windows and tiles is performed once a week. However, these cleaning activities lacked specific protocols and typically involved the use of detergent-based products, soap, or a diluted solution of 5% sodium hypochlorite (bleach) mixed with water at a ratio of 1:10. The diluted bleach solution was mainly used in areas with frequent contamination within the facility. The solid waste of the hospital is disposed of in open or closed waste bins without undergoing proper treatment, such as autoclaving. Then, the waste is transported to an incineration facility twice daily (morning and evening). It is stored there a day prior to incineration and left open, which can lead to the attraction of houseflies (Figure S1). Furthermore, the liquid waste and sewage system of the hospital are directly released into a nearby stream without undergoing any treatment, such as chemical inactivation, filtration, or UV irradiation, prior to discharge.

4.2. Study Design, Area, and Period

A cross-sectional study was conducted to assess the extent and distribution of MDR ESBL pathogenic gram-negative bacteria on surfaces, sewage, and houseflies at JMC during two specific periods: May to September 2019 and June to October 2021. To avoid bias, neither the janitors nor the healthcare providers were informed about the environmental sampling, which took place at random intervals during working days. Surface swab samples were collected from various wards within JMC, including the intensive care units (ICUs) and the operating theatres, as well as the recovery rooms. Additionally, the inpatient units, such as the surgical, medical, gynecological, maternity, pediatric, and ophthalmology wards, were sampled. Furthermore, sewage and housefly samples were collected from different points within the hospital, encompassing patient care areas, wards, laboratories, and waste disposal sites. It is important to note that these environmental sample collections were conducted during periods when no known outbreaks caused by Gram-negative bacteria were reported.

4.3. Sample Collection

The surfaces surrounding the patients' rooms and medical devices were sampled via swab. The following surfaces were chosen for sampling, if they were available for the individual patient: IV stands, inpatient floors, chairs, room sinks, walls, surgical tables, anesthesia tubes, forceps, chest tube sets, bedrails, bedside tables, toilet doorknobs, room doorknobs, electricity buttons, and cupboard knobs. Sterile cotton swabs pre-moistened in a sterile normal saline solution (0.9% NaCl) were used for sampling surfaces. At each site,

an area of approximately 4 cm^2 was swabbed in two directions at right angles to each other in a close zigzag pattern, rotating the swab during sampling to ensure that the entire surface of the swab was used according to the guidelines [40]. Sewage samples were collected by spot sampling methods using a wide-mouth container directly from the manholes. A total of 111 surface swabs and 42 sewage samples were collected. Using a single proportion formula, 192 housefly samples from both dry and liquid waste disposal sites of the hospital were included in this study. The sample size was calculated considering a 2.5% margin of error, a 95% confidence level, and a 3.3% prevalence of ESBL-producing *E. coli* isolated from fly samples reported in a previous study [41]. The houseflies were captured using a sweeping net and dumped in one milliliter of sterile normal saline in separate sterile glass test tubes. All samples were transported to the Core Research Laboratory of Jimma University for analysis.

4.4. Bacteria Isolation

In the core research laboratory, the housefly external flora was collected by dipping the housefly into a tube containing 1 mL of normal saline. Then, the housefly was briefly vortexed inside the tube to detach the bacterial flora, and all the houseflies were discarded thereafter. After this, 100 µL of the sample was inoculated on MacConkey agar. Similarly, surface swabs and 100 µL sewage samples were also inoculated on MacConkey agar. All the plates were then incubated aerobically at 37 °C for 16–18 h. After an overnight incubation, the plates were inspected and if there was growth, separate colonies were selected and sub-cultured again on MacConkey agar and incubated at the same environmental conditions to get pure cultures. For the sewage samples, to purify them easily, different individual colonies were selected from the third or fourth quadrant of the inoculated plate. These selected colonies were then subcultured under similar environmental conditions. Once the pure colony was obtained, they were saved with storage media containing skimmed milk, glucose, glycerol, tryptone soya, and distilled water at −81 °C.

4.5. Bacterial Identification

All stored isolates were transported to the Medical Microbiology Laboratory in Munich, Germany, and identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Ettlingen, Germany).

4.6. Antibiotics Susceptibility Test

The antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method for 16 antibiotics, namely ampicillin (10 µg), amoxicillin-clavulanic acid (30 µg), amikacin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), cefuroxime (30 µg), cefepime (30 µg), cefoxitin (30 µg), gentamicin (10 µg), meropenem (10 µg), moxifloxacin (5 µg), piperacillin (100 µg), trimethoprim-sulfamethoxazole (1.25 + 23.75 µg), tobramycin (10 µg), and piperacillin-tazobactam (10 µg) (Bio-Rad, Feldkirchen, Germany), and read using the ADAGIO 93400 automated system (Bio-Rad, Feldkirchen, Germany). The readings were interpreted as resistant, intermediate (susceptible with increased exposure), or susceptible according to the respective breakpoints for every organism in the European Committee on Antimicrobial Susceptibility Testing [42].

4.7. Extended Spectrum β-Lactamase Detection

The phenotypic detection of ESBL production was performed for all Gram-negative isolates by a double disc synergy test (DDST) using ceftazidime and cefotaxime with amoxicillin-clavulanic acid (10 µg) on Mueller–Hinton agar [43].

4.8. DNA Extraction

All *E. coli* strains that showed ESBL features from DDST and/or were resistant to cefotaxime, cefepime, cefoxitin, piperacillin-tazobactam, or meropenem in the Kirby–Bauer disc diffusion antibiotic susceptibility tests were selected for genotyping. After overnight

aerobic incubation on blood agar (Oxoid, Cambridge, UK) at 37 °C, three to five pure colonies were taken with an inoculating loop and suspended in nuclease-free water and extracted using a High Pure PCR template preparation kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The quantity, purity, and concentration of extracted DNA was measured by NanoDrop ND-100 (Thermo Fisher Scientific, Wilmington, NC, USA).

4.9. Molecular Characterization of E. coli Strains

Check-MDR CT103XL DNA microarray kits (Wageningen, The Netherlands) were used to detect and identify encoding genes for carbapenemase (IMP, VIM, KPC, NDM-1, SPM, OXA-23 like, OXA-24 like, OXA-48 like, and OXA-58 like), AmpC-type β–lactamase (ACC, ACT, CMY, DHA, FOX, MIR, and MOX), ESBL (cefotaximase-Munich (CTX-M type)), GES, VER, PER, BEL, Temoneira β-lactamase (TEM), and sulfhydryl (SHV) variant encoding genes using the DNA microarray technique [44].

4.10. Data Quality Assurance

To ensure the reliability of the data, quality control (QC) measures were implemented throughout the entire laboratory process. Standard operating procedures (SOPs) were followed during the pre-analytical, analytical, and post-analytical stages to ensure the quality of the test results, thereby maintaining a high level of accuracy. Using DensiCHEK plus (BioMérieux, Craponne, France), the inoculum density of bacterial suspensions was standardized to 0.5 McFarland for all phenotypic antibiotic susceptibility tests. The Mueller–Hinton agar plates (Bio-Rad, Feldkirchen, Germany) were evenly streaked and loaded with antibiotic discs (Bio-Rad, Feldkirchen, Germany) according to the EUCAST guidelines [42]. Control strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were utilized to monitor the performance of antibiotic susceptibility tests.

4.11. Data Analysis

The data generated in the laboratory were entered into Epi-Data software version 4.6 and then analyzed using Microsoft Office 2016 Excel sheets and GraphPad Prism version 8.4.3. The findings were presented using descriptive measures, including tables, figures, and percentages.

4.12. Ethical Consideration

Ethical clearance was obtained from the Ethical Review Board of Jimma University, Institute of Health (protocol numbers: IHRPGO/495/2018 and IHRPGO/1087/21), and the Ethics Committee of the Medical Faculty of Ludwig-Maximilians-Universität of Munich, Germany (Opinion No: 21-0157).

5. Conclusions

The present study revealed a high rate of ESBL-producing Gram-negative bacteria originating from patient surroundings and the hospital environment, including houseflies caught in the hospital vicinity, as well as sewage samples. Moreover, the detection of carbapenemase- and beta-lactamase-encoding genes was observed in *E. coli* strains, with a predominant presence of *bla*NDM and *bla*CTX-M group 1, respectively. The isolation rate of MDR bacteria from the houseflies was remarkable. Therefore, the implementation of rigorous waste management and housefly control practices in and around healthcare facilities is crucial to minimize the transmission of these resistant bacteria to patients and the community at large. This includes the regular and thorough cleaning of surfaces and medical devices, along with the proper segregation, handling, and disposal/inactivation of hospital waste, particularly those with the potential for bacterial contamination. There is also a dire need for proper sewage treatment, given the total absence, especially for hospital wastewater.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13040374/s1, Figure S1: Solid waste disposal practice at Jimma Medical Center, a tertiary referral hospital in Ethiopia; Table S1: The proportion of antibiotic resistant Gram-negative bacterial species obtained from surface swabs, housefly, and sewage samples at a tertiary hospital in Ethiopia.

Author Contributions: M.G., E.K.G., G.F., S.A., A.K. and A.W. conceptualized the study. M.G., E.K.G., W.T. and A.W. conducted the formal analysis. M.G. and W.T. carried out the laboratory work. The methodology was developed by M.G. and A.W., while supervision was provided by M.G. and A.W. The validation of this study was performed by M.G. and A.W. The original draft of this manuscript was written by M.G., and the review and editing were performed by M.G., E.K.G., W.T., G.F., S.A., T.S., A.K. and A.W. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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Appendix A: Paper III

OPEN

Neonatal Sepsis Due to Multidrug-resistant Bacteria at a Tertiary Teaching Hospital in Ethiopia

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Background: The burden of multidrug-resistant bacterial infections in low-income countries is alarming. This study aimed to identify the bacterial etiologies and antibiotic resistance patterns among neonates in Jimma, Ethiopia.

Methods: An observational longitudinal study was conducted among 238 presumptive neonatal sepsis cases tested with blood and/or cerebrospinal fluid culture. The bacterial etiologies were confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry. The antibiotic resistance patterns were determined using the automated disc diffusion method (Bio-Rad) and the results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing 2021 breakpoints. Extended-spectrum β-lactamases were detected using a double disc synergy test and confirmed by Mast discs (Mast Diagnostica GmbH).

Results: A total of 152 pathogens were identified. Of these, *Staphylococcus aureus* (18.4%) was the predominant isolate followed by *Klebsiella pneumoniae* (15.1%) and *Escherichia coli* (10.5%). All the isolates exhibited a high rate of resistance to first- and second-line antibiotics ranging from 73.3% for gentamicin to 93.3% for ampicillin. Furthermore, 74.4% of the Gram-negative isolates were extended-spectrum β-lactamase producers and 57.1% of *S. aureus* strains were methicillin resistant. The case fatality rate was 10.1% and 66.7% of the deaths were attributable to infections by multidrug-resistant pathogens.

Conclusions: The study revealed a high rate of infections with multidrug-resistant pathogens. This poses a significant challenge to the current global and national target to reduce neonatal mortality rates. To address these challenges, it is important to employ robust infection prevention practices and continuous antibiotic resistance testing to allow targeted therapy.

Key Words: sepsis, neonate, multidrug resistant, extended-spectrum β-lactamases, methicillin-resistant *Staphylococcus* aureus, low-income countries, Ethiopia

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Neonatal sepsis is a life-threatening condition characterized by a systemic response to infection that can affect newborns less than 1 month of age.[1] Neonates that are born prematurely or with low birth weight are particularly vulnerable.[2] Infections with multidrug-resistant (MDR) bacteria are difficult to treat because they have developed resistance to multiple clinically used antibiotics.[3–5] This significantly limits the treatment options and thus despite antibiotic treatment, neonatal sepsis continues to be one of the leading causes of morbidity and mortality in neonates.[6] According to a report by Waters et al, neonatal mortality accounts for over one million deaths each year, with low-income countries contributing to 99% of these deaths, which are largely caused by infections with MDR bacteria.[2,7,8]

In low-income countries, treating infections with MDR bacteria can be even more challenging due to limited availability of effective antibiotics.[9] Combination therapy with multiple antibiotics including carbapenems may be required in some cases to achieve acceptable therapeutic outcomes.[10] However, carbapenems are not widely available in low-income countries, and their widespread and inappropriate use would increase the risk of bacterial resistance against these antibiotics.[11] Therefore, preventing neonatal infections caused by MDR bacteria is crucial.[12] To achieve this, practicing good hygiene and proper infection control measures in health care settings are essential. Additionally, judicious and targeted use of antibiotics, early diagnosis and proper sanitation in neonatal units can help to reduce the risk of these infections.[13,14]

In neonates, bacterial infections with extended-spectrum β-lactamases (ESBL)-producing *Enterobacterales*,[9,15,16] methicillin-resistant *Staphylococcus aureus* (MRSA)[9,17] and extremely drug-resistant *Acinetobacter baumannii*[18] are more common and frequently fatal.[7,16,19] The problem is even worse, particularly, in low-income countries where there are inadequate diagnostic facilities, limited antimicrobial susceptibility tests, lack of reliable diagnostic markers, a shortage of skilled health workers and limited antibiotic options to treat these infections.[9,17–19] In addition, surveillance data about infections with MDR bacteria and the outcome of those cases are scarce in low-income countries. Therefore, this study aimed to determine the extent of MDR bacteria causing sepsis in neonates admitted to a tertiary teaching hospital in Ethiopia and the fate of those cases.

MATERIALS AND METHODS

Setting

Jimma Medical Center is the largest university hospital in southwest Ethiopia with over 800 beds and a catchment population of over 20 million. The study was conducted at the neonatal intensive care unit (NICU) of the hospital, which is a second-level NICU with a total bed capacity of 50 and an annual admission of 1800–2400 neonates.

Study Design

An observational longitudinal study was carried out between April and October 2018. All neonates admitted to the NICU with

a clinical diagnosis of sepsis and a request for blood culture and/or cerebrospinal fluid (CSF) cultures during the study period were invited to this study. Case definitions, participant recruitment, data collection procedures, and other clinical profiles of the study participants as well as risk factors for infections have been published elsewhere.[20] The participants were recruited after obtaining consent from their parents or caregivers and were followed until discharge or death. During their recruitment and follow-up, different variables including demographic information, risk factors (neonatal and maternal), clinical presentations, laboratory results, microbiologic data, and antimicrobial treatment were collected using case report forms (CRF) (Questionnaire, Supplemental Digital Content 1, http://links.lww.com/INF/F530).

Study Population

In this study, a total of 352 neonates were recruited. Of these, we obtained completed CRF in 309 cases. Blood and/or CSF cultures were performed on 195 neonates with complete CRF and 43 neonates with incomplete CRF who had a presumptive diagnosis of sepsis (Figure, Supplemental Digital Content 2, http://links.lww.com/INF/F531).

Sample Collection

As a part of a routine workup, one sample of 1–3 mL venous blood was collected from the neonates by trained nurses. Additionally, 2–3 mL of CSF was also collected by resident physicians from the neonates with suspected meningitis. The specimens were collected aseptically and were immediately transported to the microbiology laboratory of Jimma Medical Center for processing and analysis.

Isolation and Identification of Pathogens

The blood specimens were inoculated into a BD BACTEC Peds Plus/F bottle (Becton Dickinson, Sparks, MD). The bottles were then incubated in the BD BACTEC FX40 (Becton Dickinson, Sparks, MD) automated culture machine for up to 5 days until they were flagged "negative" or "positive" for growth. Positively flagged bottles were subcultured on Blood, Chocolate, and MacConkey agar (Oxoid, Cambridge, England). The CSF specimens were directly inoculated on Blood, Chocolate and MacConkey agar (Oxoid, Cambridge, England) plates within 30 minutes of collection.

The Chocolate and Blood agar plates were incubated at 5%–10% CO_2. All the plates were incubated at 35–37 °C aerobically for 18–24 hours for first inspection. Growth was monitored for a total of 72 hours to detect also fastidious organisms. After overnight incubation, all the inoculated plates were inspected, and organisms grown on the plates were identified according to the standard microbiologic identification techniques and stored in storage media at −80 °C.[21] These isolates were then transported to the Munich research laboratory at the Max von Pettenkofer Institute, Hospital Hygiene and Medical Microbiology (Ludwig-Maximilians-Universität, Munich, Germany), on dry ice. Here, detailed resistance testing and identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker, Germany) were performed.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was carried out by standardized Kirby-Bauer disc diffusion technique to 25 antibiotics in total for Gram-negative and Gram-positive bacterial pathogens (Table, Supplemental Digital Content 3, http://links.lww.com/INF/F532). The results were read using the ADAGIO 93400 automated system (Bio-Rad, Feldkirchen, Germany), and interpreted as resistant, intermediate or susceptible according to the European Committee on Antimicrobial Susceptibility Testing (2021) guideline.[22]

Extended-spectrum β-lactamase Detection

Phenotypic detection of ESBL production by double disc synergy test using ceftazidime and cefotaxime with amoxicillin-clavulanic acid (10 μg) was performed on all Gram-negative isolates that showed resistance to selected β-lactam antibiotics such as cefotaxime (30 μg), cefepime (30 μg), cefoxitin (30 μg), piperacillin-tazobactam (30 μg) or meropenem (10 μg). Results were interpreted automatically by the ADAGIO (Bio-Rad, Feldkirchen, Germany) system. Furthermore, the ESBL phenotypes were confirmed using mast discs (Mast Diagnostica GmbH, Reinfeld, Germany) for all resistant isolates. The results were interpreted with the Mast discs combi D68C ESBL/AmpC calculator spreadsheet (Mast Diagnostica GmbH, Reinfeld, Germany) and reported as negative or positive for ESBL and/or AmpC phenotypes.

Quality Control

All the laboratory activities were carried out according to the laboratory's standardized operating procedures. Using DensiCHEK plus (BioMérieux, Deutschland GmbH, Nürtingen, Germany), the inoculum density of bacterial suspensions was standardized to 0.5 McFarland for all phenotypic susceptibility tests. The Mueller-Hinton agar plates (Bio-Rad, Feldkirchen, Germany) were evenly streaked and loaded with antibiotic discs (Bio-Rad, Feldkirchen, Germany) and mast discs (MAST Diagnostica GmbH, Reinfeld, Germany) according to the European Committee on Antimicrobial Susceptibility Testing guideline.[22]

Statistical Analysis

The data were entered and analyzed using Microsoft Office 2016 Excel. Tables and graphs were used to display the frequency of bacterial pathogens, as well as their antimicrobial resistance (AMR) patterns.

Ethical Considerations

Ethical approval was obtained from both Jimma University Institute of Health Institutional Review Board, Ethiopia (protocol number: IHRPGD/274/2018) and the Ethics Committee at the Medical Faculty of Ludwig-Maximilians-Universität of Munich, Germany (opinion No: 21-0157). Written informed consent was obtained from the families or guardians of each neonate before they were recruited into the study. All the data were collected prospectively and anonymized after data merging. The microbiology results obtained during routine workup were provided to the treating physician to ensure that the respective neonates received the required medical attention based on the findings.

RESULTS

A total of 352 neonates were included in the study. Among them, 68.0% (204/352) were male, and 87.8% (309/352) had CRF completed. More than 85.0% (301/352) of the neonates were recruited within the first week of life. Of the neonates who had their birth weight determined, more than half of them (75/146) had a low birth weight. Specifically, 19.2% (28/146) weighing less than 1500 g and the remaining 32.2% (47/146) fell within the weight range of 1500–2499 g. Moreover, 41.7% (83/199) of the neonates were born preterm, while 20.7% (63/304) were resuscitated at birth. Regarding maternal factors, 95.7% (291/304) of the mothers had at least one antenatal care follow-up, and 94.3% (284/304) gave birth in health care facilities. Among neonates treated at the Jimma Medical Center, most frequently observed signs and symptoms of sepsis were rapid breathing, fever, and changes in feeding patterns, accounting for 64.6% (122/195), 48.1% (91/195) and 39.0% (76/195), respectively, as detailed in a previously published article.[20]

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Microbiologic analyses were performed for 313 clinical specimens (211 blood and 102 CSF) obtained from 238 neonates with presumptive diagnosis of sepsis. Both blood and CSF cultures were done for 75 of the neonates. Overall, 63.5% (134/211) and 3.9% (4/102) of the blood and CSF cultures, respectively, were positive with a total of 152 isolated pathogens. Of all, 59.2% (90) of the pathogens were Gram-negative rods, 35.5% (54) were Gram-positive cocci and 5.3% (8) were fungal pathogens. The most predominant isolates were *S. aureus* 18.4% (28) followed by *Klebsiella pneumoniae* 15.1% (23), *Escherichia coli* 10.5% (16) and *A. baumannii* 9.2% (14) (Table 1).

Antibiotic Resistance Patterns of the Isolates

All isolated Gram-negative bacteria were resistant against cefuroxime. High rate of resistance was also observed against first- and second-line antibiotics for the treatment of neonatal sepsis in the study area. This includes ampicillin (93.3%), cefotaxime (83.3%), ceftazidime (76%) and gentamicin (73.3%). On the other hand, a lower proportion of resistance was detected against meropenem (12.2%) and amikacin (13.3%) (Fig. 1). Over 80% of *Klebsiella* species, the second most frequently isolated bacteria, were resistant to the tested β-lactam antibiotics excluding meropenem. Furthermore, all *Serratia marcescens*, *Pseudomonas aeruginosa, Acinetobacter* and *Enterobacter* species were nonsusceptible to ampicillin, amoxicillin-clavulanic acid, cefoxitin and cefuroxime (Table 2).

Among cultured Gram-positive bacterial pathogens, most *S. aureus* strains were penicillinase producers with a high level of resistance to penicillin/ampicillin (89.3% each), and amoxicillin-clavulanic acid (53.6%). Of *Staphylococcus haemolyticus* isolates, the second most frequent Gram-positive bacteria, all showed complete resistance against penicillin and ampicillin. Overall, 38.9% of Gram-positive isolates and 57.1% of *S. aureus* strains were methicillin resistant based on the phenotypic result of cefoxitin testing (Table 3). Half of the Gram-positive isolates were resistant to erythromycin and nearly 29.6% of the Gram-positive isolates showed inducible clindamycin resistance. In general, the most efficient treatment options left were amikacin and meropenem for Gram-negative isolates (Table 2), and linezolid, vancomycin and tigecycline for Gram-positive isolates (Table 3).

Detection of Extended-spectrum β-lactamase Phenotypes

In the current study, both the double disc synergy test and the ESBL phenotype analysis with mast discs were performed for 83.3% (75) of Gram-negative bacterial isolates due to detected resistance. The analysis revealed that 53.3% (48), 11.1% (10) and 10% (9) of the Gram-negative isolates showed ESBL, AmpC, and ESBL + AmpC phenotypes, respectively. All of *Enterobacter cloacae*, *Klebsiella oxytoca* and *Acinetobacter seifertii*; and 87% of *K. pneumoniae* strains showed ESBL and/or AmpC phenotypes. Carbapenem resistance was detected in 78.6% of *A. baumannii* isolates (Table 4).

In our study, a total of 24 (10.1%) neonates included in this study unfortunately passed away during their admission. It is important to note that all these neonates were born prematurely. In terms of their birth weight distribution, 45.8% (11) of the cases weighed less than 1500 g, 37.5% (9) fell within the weight range of 1500–2499 g and the remaining 16.7% (4) had a weight greater than 2500 g. Among these unfortunate cases, 66.7% (16/24) had culture-confirmed sepsis and all the culture-confirmed cases were attributed to MDR Gram-negative pathogens such as *Klebsiella* species (7), *A. baumannii* (5), *P. aeruginosa* (2) and *E. cloacae* (2).

DISCUSSION

In this study, blood culture was able to identify etiologic agents in 63.5% of neonates with presumptive diagnosis of sepsis. The detection rate is higher than in previous studies conducted in different parts of Ethiopia; 46.6% in Gondar,[23] 44.7% in Addis Ababa[24] and 29.4% in Asella.[25] It is also higher than studies done in other low- and middle-income countries; 49.7% in Tanzania,[26] 43.4% in India,[27] 16.9% in Nepal[28] and 12.2% in Iran.[29] The high rate of culture positivity in our study may be explained by various reasons. First, the participants in the current study were neonates admitted to NICU only, excluding neonates in relatively stable medical conditions admitted to other pediatric wards. Second, we used a highly sensitive automated blood culture system (BD BACTEC Blood Culture) unlike in most of the other studies mentioned above where manual systems were used.[30] Third, since we used only one blood specimen for culture, substantial isolation

TABLE 1. Organisms Isolated From Neonates Admitted With Sepsis to Neonatal Intensive Care Unit at Jimma Medical Center, Ethiopia

Type of Microorganism	Bacteria	Number	Percent
Gram-positive bacteria (n = 54)	*Staphylococcus aureus*	28	18.4
	Staphylococcus haemolyticus	12	7.9
	Staphylococcus epidermidis	4	2.6
	Staphylococcus xylosus	3	2.0
	Staphylococcus sciuri	3	2.0
	Staphylococcus cohnii	2	1.3
	Staphylococcus hominis	1	0.7
	Staphylococcus lugdunensis	1	0.7
Gram-negative bacteria (n = 90)	*Klebsiella pneumoniae*	23	15.1
	Escherichia coli	16	10.5
	Acinetobacter baumannii	14	9.2
	Klebsiella variicola	10	6.6
	Serratia marcescens	10	6.6
	Enterobacter xiangfangensis	6	3.9
	Enterobacter cloacae	4	2.6
	Klebsiella oxytoca	3	2.0
	Pseudomonas aeruginosa	2	1.3
	Acinetobacter seifertii	1	0.7
	Enterobacter bugandensis	1	0.7
Fungal infections (n = 8)	*Candida* species	8	5.3

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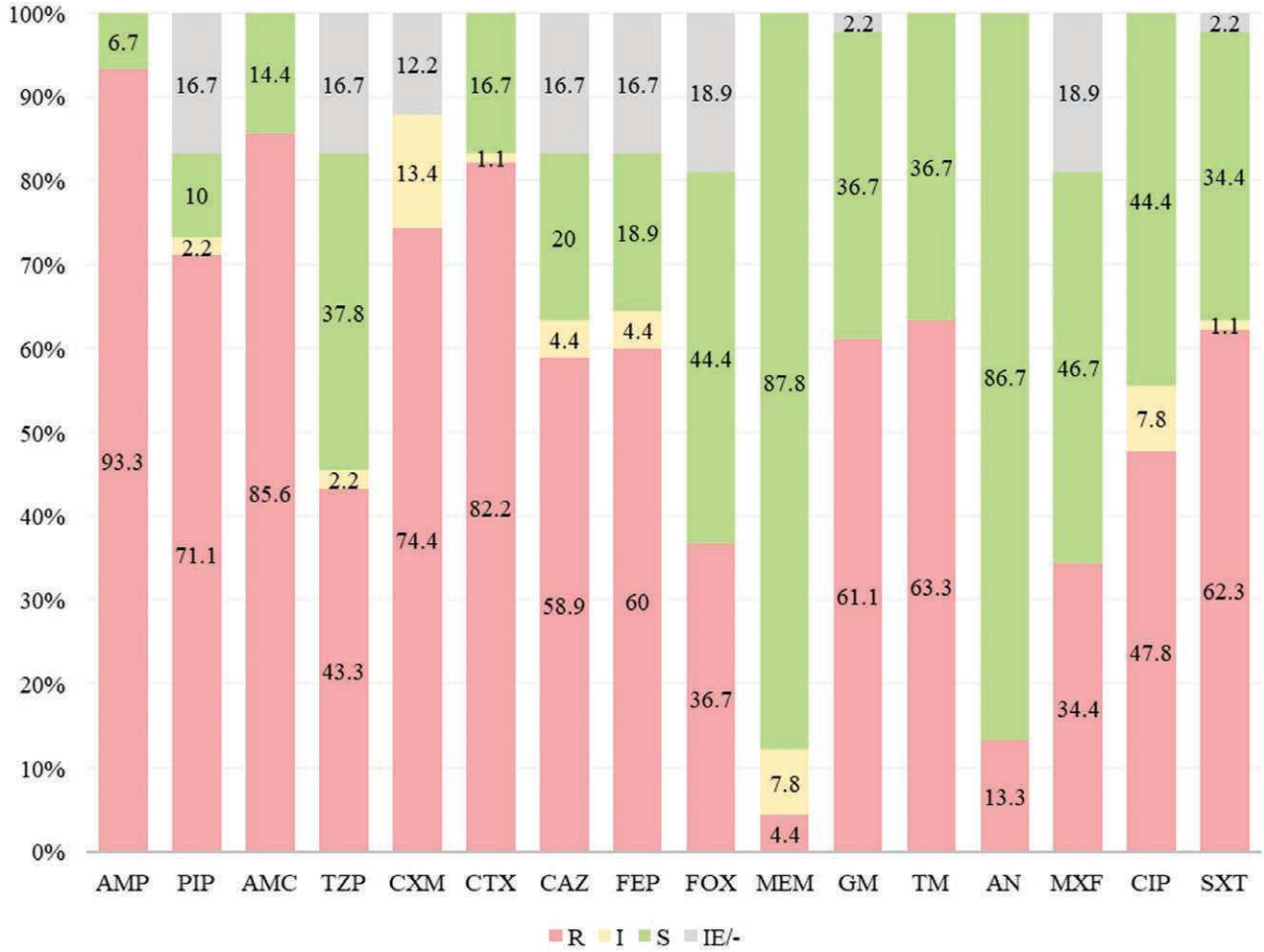

FIGURE 1. Antibiotic resistance patterns of Gram-negative bacterial pathogens."-" indicates no breakpoints; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; AN, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; GM, gentamicin; IE, insufficient evidence; MEM, meropenem; MXF, moxifloxacin; PIP, piperacillin; SXT, sulfamethoxazole-trimethoprim; TM, tobramycin; TZP, piperacillin-tazobactam. full color online

of coagulase-negative *Staphylococci* (15.1%) with a suspicion of representing residential flora contaminants might have contributed to higher culture positivity rates. However, the presence of low birth weight in over half of the neonates included in our study highlights the importance of considering the role of coagulase-negative *Staphylococci* in causing endogenous nosocomial bloodstream infections among these vulnerable neonates.[31] Nevertheless, in such a low-income setting where studies on neonatal infections are limited, such a finding also reflects the reality on the ground.

All the Gram-negative isolates in the current study showed a high rate of resistance against commonly used antibiotics such as ampicillin (93.3%), cefotaxime (83.3), ceftazidime (76%) and gentamicin (73.3%). Similarly, recent studies in Ethiopia, India, Nepal and China also showed high levels of resistance against ampicillin (85%, 78%, 100% and 80% respectively) and ceftriaxone (57%, 100%, 100% and 50% respectively) in Gram-negative organisms.[23,27,28,32] Despite this high rate of resistance, 90% of the neonates in the current study were treated with the combination of ampicillin and gentamicin as first-line treatment.[20] Of all *K. pneumoniae* isolates on the other hand, more than 80% were resistant against all tested β-lactam antibiotics excluding meropenem. Likewise, *S. aureus*, the most common causative agent of neonatal sepsis, showed a high rate of resistance to penicillin (89.3%), ampicillin (89.3%) and amoxicillin-clavulanic acid (53.6%).

In the present study, the prevalence of ESBL phenotypes among Gram-negative bacteria was 74.4%. This is higher than previous reports in other low- and middle-income countries such as Nepal (20.6%),[33] Tanzania (10.5%)[34] and India (67.3%).[9] In our data, 87% of *K. pneumoniae* isolates were confirmed phenotypically as ESBL producers. Comparably high prevalence was reported in previous studies from Yemen[35] and Tanzania[34] in which 100% and 65.6% of *K. pneumoniae* were ESBL producers, respectively. Additionally, the prevalence of MRSA in the current study was 57.1%, which was comparable with a recent multisite study done in Asian and African countries (61.1%),[36] and another study from India where 56.6% were methicillin resistant.[9] In our NICU, despite the routine use of gloves and gowns, due to a shortage of handwashing facilities and hand sanitizers, health care personnel and parents/caregivers do not wash or rub their hands before and after dealing with neonates infected with MDR pathogens consistently. Additionally, the ward is not restricted so that the traffic flow is high and facilitates the spread of MDR pathogens. These poor infection prevention and control practices, irrational antibiotic use, and the transfer of neonates with severe infections from

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TABLE 2. Antibiotic Resistance Patterns Including Intrinsic Resistance Among Gram-negative Bacterial Pathogens in Neonates Admitted With Sepsis to Neonatal Intensive Care Unit of Jimma Medical Center, Ethiopia

Antibiotics	*Klebsiella* spp. n (%)	*Escherichia coli* n (%)	*Acinetobacter* spp. n (%)	*Enterobacter* spp. n (%)	*Serratia marcescens* n (%)	*Pseudomonas aeruginosa* n*
AMP	36 (100)	10 (62.5)	15 (100)	11 (100)	10 (100)	2
PIP	36 (100)	10 (62.5)	IE	11 (100)	7 (70)	2
AMC	29 (80.6)	10 (62.5)	15 (100)	11 (100)	10 (100)	2
TZP	23 (63.9)	6 (37.5)	IE	10 (90.9)	0	2
CXM	36 (100)	16 (100)	15 (100)	-	10 (100)	2
CTX	32 (88.9)	9 (56.3)	15 (100)	11 (100)	6 (60)	2
CAZ	31 (86.1)	7 (43.8)	-	11 (100)	6 (60)	2
FEP	32 (88.9)	7 (43.8)	-	11 (100)	6 (60)	2
FOX	7 (19.4)	5 (31.3)	-	11 (100)	10 (100)	-
MEM	0	0	11 (73.3)	0	0	0
GM	29 (80.6)	5 (31.3)	13 (86.7)	8 (72.7)	0	IE
TM	29 (80.6)	6 (37.5)	13 (86.7)	7 (63.6)	1 (10)	1
AN	0	0	11 (73.3)	0	0	1
MXF	16 (44.4)	8 (50.0)	-	7 (63.6)	0	-
CIP	17 (47.2)	8 (50.0)	15 (100)	6 (54.5)	2 (20)	2
SXT	26 (72.2)	7 (43.8)	14 (93.3)	9 (81.8)	1 (10)	-

*We did not calculate the percentage (%) if the total number of isolates was less than 10.

"-" indicates no breakpoints; AM, ampicillin; AMC, amoxicillin + clavulanic acid; AN, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; GM, gentamicin; IE, insufficient evidence; MEM, meropenem; MXF, moxifloxacin; PIP, piperacillin; SXT, sulfamethoxazole + trimethoprim; TM, tobramycin; TZP, piperacillin + tazobactam.

other low-level facilities could all explain the high prevalence of MDR bacteria, including MRSA and ESBL. Many infections are suspected to be health care related. For example, the *Acinetobacter* strains are known to live within the clinics and infect also patients with burn wounds and surgical patients.[37]

In our study, the inpatient neonatal mortality rate was 10.1%, which is comparable with a recent multisite study conducted in low- and middle-income countries of Asia and Africa, which reported an overall neonatal mortality rate of 11.3%.[36] It is noteworthy that a significant proportion of these deaths were attributed to infections caused by MDR bacteria. In addition to the devastating impact on mortality, infections with MDR bacteria such as ESBL producers and MRSA can have several other adverse consequences. These infections often lead to prolonged hospital stays, increasing health care costs and placing an additional burden on already scarcely available health care facilities. This may be improved using proper antibiotic treatment strategies and implementing an antibiotic stewardship program. Also, early sample taking and resistance testing would be essential to switch treatment to effective substances early during the septic episode.

Our study is one of only few available studies on etiologies and AMR patterns in neonatal sepsis in Ethiopia. We believe that the findings in this study could provide important data for the policy level to eventually facilitate interventions to tackle neonatal mortality and AMR. However, since our study is limited to one facility and that only neonates admitted to the NICU were included may limit the generalizability of the findings. However, it will be representative of the prevalence of severe neonatal infections in low-income settings, as all those cases ultimately end up in NICU.

TABLE 3. Antibiotic Resistance Pattern of Isolated Gram-positive Bacterial Strains in Neonates Admitted With Sepsis to Neonatal Intensive Care Unit of Jimma Medical Center, Ethiopia

Antibiotics	*Staphylococcus aureus* n (%)	*Staphylococcus haemolyticus* n (%)	Others n (%)	Total N (%)
Benzylpenicillin	25 (89.3)	12 (100)	14 (100)	51 (94.4)
Ampicillin	25 (89.3)	12 (100)	14 (100)	51 (94.4)
Amoxicillin-clavulanic acid	15 (53.6)	8 (66.7)	5 (35.7)	28 (51.9)
Cefoxitin	16 (57.1)	1 (8.3)	4 (28.6)	21 (38.9)
Meropenem	1 (3.6)	3 (25)	2 (14.3)	6 (11.1)
Ciprofloxacin	3 (10.7)	11 (91.7)	6 (42.9)	20 (37)
Moxifloxacin	2 (7.1)	11 (91.7)	6 (42.9)	19 (35.2)
Clindamycin	5 (17.9)	6 (50)	5 (35.7)	16 (29.6)
Erythromycin	11 (39.3)	9 (75)	7 (50.0)	27 (50.0)
Gentamicin	11 (39.3)	9 (75)	6 (42.9)	26 (48.1)
Linezolid	1 (3.6)	0	0	1 (1.9)
Mupirocin	0	0	0	0
Rifampicin	5 (17.9)	5 (41.7)	3 (21.4)	13 (24.1)
Trimethoprim-Sulfamethoxazole	3 (10.7)	11 (91.7)	6 (42.9)	20 (37.0)
Tigecycline	0	0	0	0
Vancomycin	0	0	0	0

Others: *Staphylococcus epidermidis* (4), *Staphylococcus xylosus* (3), *Staphylococcus sciuri* (3), *Staphylococcus cohnii* (2), *Staphylococcus hominis* (1) and *Staphylococcus lugdunensis* (1).

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TABLE 4. Prevalence of Extended-spectrum β-lactamases Phenotypes and Carbapenem-resistant Gram-negative Rods (n = 90)

	ESBL Phenotypes				Carbapenemase Resistance	
Bacterial Isolates	Negative or Inconclusive	ESBL	AmpC	ESBL and AmpC	Yes	No
Klebsiella pneumoniae (n = 23)	3	20	0	0	0	23
Escherichia coli (n = 16)	7	7	2	0	0	16
Acinetobacter baumannii (n = 14)	6*	0	6	2	11	3
Enterobacter cloacae (n = 11)	0	6	2	3	0	11
Klebsiella variicola (n = 10)	2	7	0	1	0	10
Serratia marcescens (n = 10)	3	4	0	3	0	10
Klebsiella oxytoca (n = 3)	0	3	0	0	0	3
Pseudomonas aeruginosa (n = 2)	2	0	0	0	0	2
Acinetobacter seifertii (n = 1)	0	1	0	0	0	1
Total (n = 90)	23 (25.6)	48 (53.3)	10 (11.1)	9 (10)	11 (12.2)	79 (87.8)

*Isolates showed inconclusive result in the ESBL phenotype analysis (equivocal further work required, consider presence of carbapenemase using D73C).

CONCLUSIONS

The finding of our study demonstrates a high rate of infections caused by MDR pathogens including ESBL producers and MRSA among neonates. This poses a significant challenge to the national and eventually also global target to reduce neonatal mortality rates. Therefore, it is crucial to implement policies and interventions to address this issue effectively. This highlights the importance of developing locally acceptable and applicable guidelines, adhering to evidence-based practices, committing to rational antimicrobial use, improving diagnostic facilities including routine antimicrobial susceptibility testing and implementing regular AMR surveillance in neonatal units to reduce the burden of infections with MDR pathogens and improve patient outcome.

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