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Multidrug Resistance and Carbapenemase Producing *Enterobacteriaceae* on Clinical Samples Referred to Wudassie Advanced Medical Laboratory, Addis Ababa, Ethiopia.

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This is to certify that the thesis prepared by Yordanos Getachew, entitled Multidrug Resistance and Carbapenemase Producing Enterobacteriaceae on Clinical Samples Referred to Wudassie Advanced Medical Laboratory and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (diagnostic and public health microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

AR-	Antibiotic resistance
ATCC-	American Type Culture Collection
CLSI-	Clinical Laboratory standards Institute
CPE-	Carbapenemase producing *Enterobacteriaceae*
CRE-	Carbapenem resistant *Enterobacteriaceae*
CRKP-	Carbapenem resistant *Klebsiella pneumonia*
ESBL-	Extended spectrum β-lactamases
IRB -	Internal Review Board
KPC-	*Klebsiella pneumoniae* carbapenemase
MALDI-TOF-	Matrix-Assisted Laser Desorption & Ionization- Time of Flight.
MBLs-	Metallo-beta-lactamases
MDR-	Multidrug-resistant
MDRE-	Multidrug resistant *Enterobacteriaceae*
mCIM	Modified carbapenem inactivation method
NDM-	New Delhi metallo-beta-lactamase
SOP-	Standard operating procedure
Spp-	Species
SPSS -	Statistical Package for social sciences statistical software
VIM-	Verona integron-encoded metallo-beta-lactamase
WAML-	Wudassie advanced medical laboratory

Abstract

Background: One of the largest risks to world health is the spread of microorganisms that are resistant to drugs. According to estimates, the mortality rate from Carbapenem-resistant *Klebsiella pneumonia* (CRKP) infections ranges from 37.2% to 42.1%. In 2019, Ethiopia reported 21,200 deaths directly attributable to Anti-microbial resistance.

Objectives: To assess multidrug resistance and carbapenemase producing Enterobacteriaceae on clinical samples referred to Wudassie Advanced Medical Laboratory.

Method: A cross-sectional study was conducted from December 2024 to March 2025. A total of 1105 patients from which 439 were suspected of sepsis, 110 suspected of wound infection, 45 suspected of meningitis, 353 suspected of urinary tract infection and 153 suspected of other infections, whose specimens were sent to wudassie advanced medical laboratory during the study period were included in this study. Isolates were identified using Matrix-Assisted Laser Desorption & Ionization- Time of Flight (MALDI-TOF) (EXS 3000, Zybio Inc., China). Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion technique and carbapenemase production was confirmed by modified carbapenem inactivation technique. Data entered using epi data version 4.6 and analyzed using Statistical Package for social sciences statistical software version 26 and the result was displayed with graphs, tables and words and compared with previous studies.

Result: Out of 1105 eligible patients, 288 of them had microbial growth. Of this,156 of them were *Enterobacteriaceae*. The predominant isolate was *E. coli* followed by *K. pneumoniae* and *P. mirabilis*. From 156 *Enterobacteriaceae* isolated, 78.8% of them were multidrug resistant and 19.5% of them were carbapenemase producing *Enterobacteriaceae* (CPE). *K. pneumoniae* was the most abundant CPE isolate. Most of CPE producing isolates were from urine specimen followed by wound and sputum specimen.

Conclusion: The findings highlights a high prevalence of multidrug-resistance and a rise in the prevalence of carbapenemase-producing *Enterobacteriaceae,* Thus, it is important to review current treatment guidelines for better patient management and outcome.

Key words: *Enterobacteriaceae*, Multidrug resistance, Carbapenemase production

1.0 Introduction

1.1 Background

The introduction of antibiotics marked a major advancement in medicine, but their misuse has significantly contributed to the rapid development of multidrug-resistant pathogens. The increase of antimicrobial resistance contributes to higher rates of illness, prolonged hospital stays, and increased healthcare expenses and death [1].

Infections caused by multidrug-resistant (MDR) bacteria have become a major global health concern. MDR *Enterobacteriaceae* are becoming increasingly prevalent in hospital and community settings around the world. Carbapenem antibiotics are effective against multidrug-resistant bacilli, particularly those producing extended-spectrum beta-lactamases (ESBL) as well as a broad range of Gram-positive bacteria. However, their utility is jeopardized by the introduction and proliferation of bacteria that produce carbapenemase enzymes [2].

Due to their broad-spectrum activity, carbapenems are commonly used to treat life-threatening infections. Unfortunately, overprescribing these medications has led to an increase in carbapenem resistance [3]. Although carbapenemase enzymes are the most common mechanism for carbapenem resistance, resistance arises through various mechanisms, including the acquisition of genes that produce beta-lactamases which break down the antibiotic, the action of efflux pumps that remove the drug from the bacterial cell before it can reach its target, and alterations of binding sites that prevent the antibiotic from achieving effective concentrations needed to eliminate the entire polymicrobial community [4,5]. Carbapenem resistance has been recognized in *Enterobacteriaceae*, primarily in *Klebsiella pneumoniae* compared to *Escherichia coli* or other Enterobacterial species [6].

Since 1993, several forms of carbapenemase have been characterized as corresponding to three molecular classes: the Ambler classes A, B, and D beta-lactamases [7]. *Klebsiella pneumoniae* carbapenemase (KPC) is found in class A carbapenemase group and this class can break down monobactams, carbapenems, cephalosporins, and penicillins. New Delhi metallo-beta-lactamases (NDM), Verona integron-encoded metallo-beta-lactamase (VIM), and Imipenemase (IMP) type enzymes are examples of class B, metallo-beta-lactamases (MBLs); these enzymes have distinct hydrolysis activity against all beta-lactam antibiotic, except monobactam (aztreonam) [8]. The

OXA-48 enzyme is found in class D and it exhibits significant activity against penicillin and limited activity against third and fourth generation cephalosporins but beta lactam antibiotics are rarely used as a treatment since other beta-lactamases, including ESBL, are frequently associated with this enzyme [9].

Despite the growing burden, the best course of treatment for CRE infections is mostly unknown. There is little evidence of the effectiveness of the few therapeutic alternatives that are still available to support their use in therapy. Although earlier drugs like aminoglycosides, polymyxins and fosfomycin, have been used seldom due to efficiency and safety issues, they are still part of the current therapy options. Other treatment approaches being investigated include combination therapy and dosing regimen optimization [10].

1.2 Statement of the problem

Despite big advances in antimicrobial treatments and infection control strategies, the rise of antibiotic resistance remains a critical threat, particularly for individuals with weakened immune systems. Infections caused by multidrug-resistant gram-negative pathogens are associated with increased mortality, higher morbidity, and escalating healthcare expenses [11, 12].

In 2019 there were an estimated 4.95 million deaths associated with bacterial AMR globally. At the regional level, death rate attributable to resistance estimated to be highest in western sub-Saharan Africa, at 27.3 deaths per 100 000, and lowest in Australasia, at 6.5 deaths per 100 000 hospitalizations [13].

Antibiotic resistance is a serious global concern with an estimated 2.8 million antibiotic resistant infections occurring annually in the United States alone. These infections are responsible for roughly 35,000 deaths each year. In 2017, healthcare costs associated with carbapenem-resistant *Enterobacteriaceae* in the U.S. were estimated at around 130 million dollars. Antibiotic resistance has contributed to an overall increase in U.S. healthcare expenses, by 55 billion dollars annually when lost productivity taken into account. Hospital costs alone are approximately 29 % higher for patients infected with multidrug-resistant (MDR) pathogens, due to longer average hospital stays and greater resource utilization [14]. According to estimates, the mortality rate from Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections ranges from 37.2% to 42.1% [15].

A nationwide meta-analysis in Africa found the pooled prevalence of CPE to be 5.44% Variability by region: highest in Central Ethiopia (~6.45%), lowest in SNNPR (~1.65%). Highest prevalence observed in studies from 2017–2018 (~17.4%), with lower rates (~2.2%) in earlier periods (2015–2016) [16]. The current burden is significantly higher because of the recent indiscriminate use of carbapenem medications and the quick spread of the carbapenemase gene discovered on plasmids between various *Enterobacteriaceae* groups.

Thus, conducting this research updates the prevalence of MDR and CP-CRE as there is no recent study regarding this in Ethiopia especially from private health care sectors. The aim of this study is to determine MDR and CP-CRE from clinical samples from private and public hospitals in Addis Ababa.

1.3 Significance of the study

By identifying the prevalence and resistance patterns of *Enterobacteriaceae*, this research will help clinician to implement evidence-based antibiotic selection, thereby improving clinical outcomes and reducing treatment failures. Moreover, it provides data for Policy makers for implementing effective infection prevention and control strategies in healthcare settings, which is essential to limit the spread of these resistant strains. The findings also contribute to antimicrobial stewardship by promoting rational antibiotic use and preserving the efficacy of last-resort drugs like carbapenems. The study can also be used by other researches to conduct further research on carbapenemase producer *Enterobacteriaceae*.

2.0 Literature review

A meta-analysis on the epidemiology of carbapenem-resistant *Enterobacteriaceae* (CRE) was carried out in Asia from 2000 to 2012. During this time, the overall prevalence of CRE remained low, with average resistance rates of 0.6% for imipenem (95% CI: 0.6–0.8%) and 0.9% for meropenem (95% CI: 0.7–1.2%). Despite this, resistance to both antibiotics showed a consistent increasing trend. *Klebsiella* species made up the majority of imipenem-resistant isolates, followed by *Escherichia coli* and *Serratia* species. [17].

A systematic review was conducted on MDR bacteria in Ethiopia from 2008–2020. The review comprises 6856 bacteria, of which 4949 isolates were MDR. The overall pooled prevalence of MDR was 70.5%. Sidama (81.7%) had the highest MDR and Tigray (51.1%) the lowest. The highest proportion of multidrug resistance was observed in infections originating from multiple sites (76.8%), while the lowest was found in bloodstream infections (62.9%). MDR prevalence was greater in studies focusing solely on hospital-acquired infections (72.1%) compared to those examining both hospital- and community-acquired infections (69.8%) [18].

To estimate the global incidence of carbapenem-resistant Enterobacteriaceae (CRE), a total of 81,781 Enterobacteriaceae isolates were collected between 2012 and 2017 from patients in 39 countries across five geographic regions. Among these, 2,666 isolates (3.3%) were found to be non-susceptible to meropenem, with regional variation ranging from 1.4% in North America to 5.3% in Latin America. These CRE isolates originated from various infection sites, including the lower respiratory tract (n=778), urinary tract (n=631), skin and soft tissue (n=581), intra-abdominal infections (n=408), bloodstream (n=266), and other sources (n=2). The predominant resistant species was *Klebsiella pneumoniae* (n=2,046; 76.7%), followed by *Enterobacter cloacae* (n=177; 6.6%) and *Escherichia coli* (n=136; 5.1%). The remaining isolates (12%) included 101 *Klebsiella* spp., 79 *Citrobacter* spp., 70 *Proteus*, 31 *Serratia marcescens*, 18 *Enterobacter* spp., and 8 *Raoultella* spp. The prevalence of meropenem non-susceptibility increased from 2.7% in 2012–2014 to 3.8% in 2015–2017 [19].

In a cross-sectional study conducted in 2022 in an impoverished area of Punjab, Pakistan, Mustafai MM *et al*. tested 1206 patients for potential bacterial infections, and 384 of them tested positive for different bacterial infections. Of these, 100 of them (26%) were *Enterobacteriaceae*. From this

E. coli was the most abundant isolate, followed by *K. pneumoniae.* From 28 isolates that were resistant to carbapenem, 24 have CP genes by PCR. Carbapenem resistance was higher in blood than in the other samples [20].

A prospective study was conducted by Baran I *et al*. in Turkey from January 31, 2013, to February 14, 2014, 181 carbapenem-resistant Enterobacteriaceae (CRE) were identified from a total of 6,426 Enterobacteriaceae isolates. *Klebsiella pneumoniae* was the most frequently isolated species (n=69; 38.12%), followed by *Serratia marcescens* (n=33; 18.23%), *Morganella morganii* and *Proteus mirabilis* (each n=17; 9.39%), *Escherichia coli* and *Enterobacter cloacae* (each n=13; 7.18%), *Enterobacter aerogenes* (n=10; 5.52%), and less commonly *Citrobacter freundii*, *Raoultella planticola*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii*, *Cronobacter sakazakii*, and *Klebsiella oxytoca* (each comprising 0.55–1.1%). The majority of CRE isolates were recovered from blood samples (n=59; 32.6%), followed by urine (n=49; 27.07%) and wound specimens (n=31; 17.13%). Other sources included tracheal aspirates (n=21; 11.6%), abscess cultures (n=9; 4.97%), catheters (n=4; 2.21%), pleural fluid (n=3; 1.66%), and single isolates from peritoneal fluid, bile, sputum, rectal swab, and cerebrospinal fluid (each n=1; 0.55%) [21].

A cross-sectional study conducted by Mirzaei B *et al.* in northern Iran between 2017 and 2019. From 2,645 clinical specimens analyzed, 297 (11.2%) Enterobacteriaceae isolates were identified using the Analytical Profile Index (API 20E) system. The isolates included *Escherichia coli* (n=93; 31%), *Citrobacter freundii* (n=65; 21.9%), *Klebsiella pneumoniae* (n=48; 16.2%), *Enterobacter* spp. (n=43; 14.5%), and *Proteus* spp. (n=23; 7.7%). High resistance rates were observed for ampicillin (81.1%) and cephalexin (80.9%), whereas nalidixic acid (65%) and amikacin (59.2%) were the most effective antibiotics. Notably, multidrug-resistant strains were more frequently isolated from patients in burn units and the burn intensive care unit (BICU) compared to other hospital wards [22].

A cross-sectional study conducted by Bandy A *et al.* from January 1 to December 31, 2019, at a referral hospital in the Aljouf region of Saudi Arabia. 617 Enterobacteriaceae isolates were identified from clinical specimens. *Escherichia coli* was the most frequently isolated species, followed by *Klebsiella pneumoniae* and *Proteus mirabilis*. *E. coli* and *K. pneumoniae* showed susceptibility rates of 96.5% and 74.9%, respectively, to amikacin. In contrast, cephalothin, a first-generation cephalosporin, was largely ineffective, with resistance rates exceeding 80%. All studied

isolates displayed over 50% resistance to second- through fourth-generation cephalosporins, except for cefoxitin. Among the isolates, 432 (70%) were identified as multidrug-resistant (MDR), including 167 (38.6%) *E. coli*, 170 (39.3%) *K. pneumoniae*, and 95 (21.9%) *P. mirabilis* [23].

A descriptive cross-sectional study conducted by Aminu A et al. between January and December 2018 at two tertiary hospitals in Kano State, Nigeria, investigated Enterobacteriaceae isolated from 190 ICU patients. 76 samples yielded clinical isolates, including *Escherichia coli* (n=34; 44.7%), *Klebsiella pneumoniae* (n=19; 25%), *Enterobacter aerogenes* (n=4; 5.3%), *Proteus mirabilis* and *Proteus vulgaris* (n=3 each; 3.9%), and several less frequent species such as *Citrobacter freundii*, *Klebsiella ozaenae*, *K. oxytoca*, *Salmonella* subsp. 3b, *Enterobacter agglomerans*, *E. cloacae* (n=2 each; 2.6%), and *Serratia odorifera* (n=1; 1.3%). High resistance rates to ceftriaxone were observed in *E. coli* (88.5%) and *K. pneumoniae* (63.2%). All *Proteus* species exhibited complete resistance to both ampicillin and ceftriaxone. Resistance to ciprofloxacin was also notable, affecting 88.4% of *E. coli* and 73.7% of *K. pneumoniae*. Interestingly, *K. oxytoca* remained fully susceptible to ampicillin. Gentamicin showed the lowest resistance rates, with 41.2% resistance in *E. coli* and 21.1% in *K. pneumoniae*. Ertapenem disc screening identified 8 isolates (10.5%) as carbapenem-resistant Enterobacteriaceae (CRE), and modified Hodge test confirmed 6 (7.9%) as carbapenemase-producing Enterobacteriaceae (CPE) [24].

A cross-sectional hospital-based study was conducted by Almugadam BS *et al* in Kosti city, Sudan, from October 2016 to August 2017. A total of 100 Enterobacteriaceae isolates were obtained from patients with wound infections, including 33 *Proteus mirabilis*, 25 *Klebsiella pneumoniae*, 23 *Escherichia coli*, 11 *Enterobacter aerogenes*, and 8 *Citrobacter freundii*. The overall prevalence of carbapenem-resistant Enterobacteriaceae among these isolates was 6%, with a higher rate observed in females (8.3%, 2 out of 24) compared to males (5.2%, 4 out of 76). Among the species, *K. pneumoniae* showed the highest carbapenem resistance at 12% (3/25), followed by *E. aerogenes* at 9% (1/11), and *P. mirabilis* at 6% (2/33), while no resistance was detected in *E. coli* and *C. freundii* [25].

A cross-sectional study conducted by Tekele SG *et al.* from January to April 2018 in Addis Ababa, Ethiopia, 312 Enterobacteriaceae isolates were identified from various clinical specimens the most common bacterial isolates were *E. coli* at 72.4% (226/312), followed by *K. pneumoniae* at 13.8%

(43/312). 68.6% of the isolates were multidrug-resistance and 2.6% of them were carbapenemase-producing Enterobacteriaceae representing different Ambler classes. Antibiotic resistance rates were higher in CPE isolates compared to other MDR Enterobacteriaceae, with resistance observed at 100% versus 77.6% for ampicillin, 75% versus 20.6% for cefoxitin, and 50% versus 13.1% for piperacillin/tazobactam [26].

A hospital-based cross-sectional study was conducted by Seman A. *et al.* at Tikur Anbessa Specialized Hospital (TASH) between September 2018 and January 2019, on patients suspected of bloodstream infections. Out of 2,397 suspected cases, 104 (4.3%) Enterobacteriaceae were isolated. The predominant species identified were *Klebsiella pneumoniae* (52%, 55/104), *Escherichia coli* (19.2%, 20/104), and *Klebsiella oxytoca* (17.3%, 18/104). Among the isolates, 18 (17.3%) were resistant to carbapenems and were subsequently tested for carbapenemase production using the modified carbapenem inactivation method (mCIM). Of these, 8 isolates (44.4%) were confirmed to be carbapenemase-producing Enterobacteriaceae (CPE), with *K. pneumoniae* accounting for the majority (62.5%, 5/8) [27].

A cross-sectional study done by Shibabaw A. et al on patients suspected of sepsis and admitted between January and June 2021 at Debre Berhan Comprehensive Specialized Hospital (DBCSH), Amhara area. A total of 384 samples were inoculated and 164 of them has a growth of *Enterobacteriaceae. E.coli* was the predominant isolate followed by *K.pneumonia, K.oxitoca, C.divresus, E. aerogenes, M.morganii, K. ozaenae*. From fourteen antibiotics from different classes the highest level of susceptibility was observed to meropenem (77.4%) followed by imipenem (70.4%), the highest level of resistance was observed to ampicillin (100%) followed by tetracycline (97.6%). 10.97% (18/164) of the isolates were CP-CRE. *K. pneumoniae* (n=8, 4.9%), and *E. coli* (n=7, 4.9%) were the most common CP-CRE isolates followed by *Klebsiella oxytoca* (n=3, 1.8%). The most frequent site of CP-CRE infection was bloodstream (n=11/18, 61.1%), followed by infections of the skin and soft tissues (n=5/18, 27.8%) and the gastrointestinal tract (n=2/18, 11.1%) [28].

The prevalence of multi drug resistance and carbapenemase production has shown an increasing trend over past few years, thus determining the current prevalence and resistance pattern is essential to review current treatment guidelines and intervene in spread of drug resistance.

3.0 Objectives

3.1 General objective

- To assess multidrug resistance and carbapenemase producing Enterobacteriaceae from clinical samples referred to Wudassie Advanced Medical Laboratory.

3.2 Specific objectives

- To determine the profiles of Enterobacteriaceae from clinical samples referred to WAML.
- To determine the antimicrobial susceptibility pattern of Enterobacteriaceae isolates from clinical samples referred to WAML.
- To determine the prevalence of CPE among Enterobacteriaceae isolates from clinical samples referred to WAML.

4.0 Hypothesis

H0 = The prevalence of multi drug resistant and carbapenemase producing *Enterobacteriaceae* is similar to study conducted in Addis Ababa Ethiopia with 68.6% MDR, and 2.6% of CP-CRE [26].

5.0 Materials and methods

5.1 Study area

The study was conducted at Wudassie advanced medical laboratory (WAML) located in central Addis Ababa, Ethiopia; in the Arada sub-city. The laboratory was founded in 2020. And it has three main sections, Hematology, clinical chemistry, and Microbiology. The microbiology laboratory is accredited by Ethiopia accreditation service (EAS) with accreditation number M0085. On average 360 urine sample,152 wound samples, 353 blood culture, and 102 CSF and other body fluids are referred to wudassie advanced medical laboratory in 3 months from different clinics, health centers, public and private hospitals found in Addis Ababa.

5.2. Study design and period

A cross-sectional study was conducted from December 2024 to March 2025.

5.3. Population

5.3.1. Source population

All patients, whose specimens were referred to WAML for diagnosis purpose during the study period, were the source population.

5.3.2. Study population

All patients who were suspected of bacterial infections and whose specimen were referred to WAML for culture were the study population.

5.4 Inclusion and exclusion criteria

5.4.1 Inclusion criteria

- Specimens from all age group patients who were suspected of having different bacterial infections.

5.4.2 Exclusion criteria

- Patients who took antibiotics within 7 days of specimen collection

5.5 Study variables

5.5.1 Dependent variable

- Profile of Enterobacteriaceae
- Antimicrobial resistance pattern
- Prevalence of carbapenemase producers

5.5.2 Independent variable

- Age
- Sex
- Specimen type
- Health facility types
- Inpatient/outpatient

5.6 Sample size calculation

The sample size was calculated using a single population proportion formula by taking the prevalence (p) of 68.6% from a study done in 2018 on clinical samples in Addis Ababa Ethiopia [26]. Considering 95% confidence interval (Zα/2), 5% margin of error (d), the sample size was calculated using the following standard formula.

$$n = \frac{(z_{\alpha/2})^2 \cdot pq}{d^2}$$

Where

n= minimum sample size needed

p= proportion (68.6%)

q= 1-p

Z= significance level at confidence interval of 95%

d= margin of error (0.05)

Z α/2= value of standard normal distribution corresponding to significant level of alpha (α) 0.05 which is 1. 96

$$n = \frac{(1.96)^2 * 0.686(1-0.686)}{(0.05)^2} = 331$$

Despite this a total of 1105 patients were enrolled in this study in order to maximize growth and get a representative data.

5.7 Sampling Technique

Convenient sampling technique was used.

5.8 Data collection tools and technique

5.8.1 Data collection

Socio demographic data and data regarding admission status and facility type were collected by trained laboratory technicians through pre-structured data collection tool.

5.8.2. Sample collection techniques

Urine sample collection

Midstream clean-catch urine specimens were collected into sterile, wide-mouth, screw-cap containers. Patients were instructed to clean the urethral area with a sterile wipe, begin voiding, and collect midstream urine. In catheterized patients, urine was obtained directly from the catheter using a sterile needle and syringe. The sample were processed within 2 hours or stored at 4°C for up to 24 hours.

Discharge sample collection

Wound specimens were collected after cleaning the wound with sterile saline to remove surface contaminants. Deep tissue or pus were collected using a sterile swab and syringe. Swabs were placed in stuart transport media to maintain the viability of the microorganism during transport to laboratory.

Blood sample collection

Blood sample was collected using prepared blood culture bottles. Two bottles were collected for adults with 8-10ml blood in each to increase the sensitivity of detection and to rule of contaminants growth from single bottle. In pediatric,1-2ml of blood in a single or pair of bottles and incubated overnight.

Body fluids sample collection

Pleural fluid, pericardial fluid, peritoneal fluid and CSF were collected by trained physician and were transported to laboratory using sterile red top test tube.

Stool sample collection

Stool is collected in a clean, sterile, wide-mouthed, leak-proof container. Patients were told to avoid contamination with urine, water, and toilet paper. For infants, stool can be collected from a clean diaper using a sterile spatula.

Sputum sample collection

Sputum sample were collected from deep coughs into a sterile, leak-proof container. The patient was instructed to rinse the mouth with water and avoid eating or brushing teeth before sample collection to reduce contamination.

5.8.3 Inoculation and identification

Gram stain findings of sterile specimens were reported as a preliminary result. All specimens were inoculated on MacConkey agar, Blood agar, XLD agar and Chocolate agar plates according to their specific growth requirement then incubated at 37°C for up to 72 hours. All isolates were identified by matrix-assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI TOF) (EXS 3000, Zybio Inc., China).

5.8.3.1 Principle of MALDI-TOF

The sample is prepared by mixing or coating it with a solution containing an energy-absorbing organic compound known as a matrix. As the matrix dries and crystallizes, the sample becomes co-crystallized within it. During analysis, a laser beam automatically ionizes the sample embedded

in the matrix and produces singly protonated ions from the analytes present in the sample. The protonated ions are accelerated under a constant electric potential, causing them to separate based on their mass-to-charge ratio (m/z). These charged analytes are then detected and measured using a time-of-flight (TOF) analyzer. In MALDI-TOF analysis, the m/z ratio is determined by measuring the time it takes for each ion to travel through the flight tube. This time-based data is used to generate a unique spectrum known as a peptide mass fingerprint (PMF), which is then compared to a reference library to identify the bacterial species present in the sample [30].

5.8.4 Antimicrobial susceptibility testing

Enterobacteriaceae isolates that has been identified by MALDI-TOF were tested for in vitro antimicrobial susceptibility using the Kirby–Bauer disk diffusion method, following the procedures outlined in Clinical and laboratory Standards Institute (CLSI) guidelines, version 2024 and zone of inhibition were interpreted based on the standard. [30]. The antibiotic discs used in this study were ampicillin: (AMP:10 µg), amoxicillin-clavulanic acid (AMC: 20/10 µg), ceftriaxone (CRO:30 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), cefepime (FEP: 30 µg), meropenem (MER: 10 µg), gentamicin (GEN:10 µg), amikacin (30 µg) ciprofloxacin (CIP: 5 µg:), nitrofurantoin (NI:300µg) sulfamethoxazole-trimethoprim (SXT: 3.75/1.25 µg). and chloramphenicol (C), (30 µg), (using commercially available Oxoid, Basingstoke, England).

Bacterial isolates that have showed resistance to meropenem were considered as potential carbapenemase producer and were subjected to confirmatory test using modified Carbapenem inactivation method (mCIM).

5.8.5 Confirmatory test

All Enterobacteriaceae that showed resistance to meropenem in vitro was processed again to check the production of carbapenemase enzyme by modified carbapenem inactivation method. A fresh bacterial colony was taken using a 1 µL loop and suspended in 2 mL of tryptic soy broth, and then meropenem disk was placed into the suspension, and incubated at 35°C for 4 hours ± 15 minutes.

Carbapenem sensitive E. coli ATCC 25922 was used to make suspension of 0.5 McFarland and inoculated on MHA plate as standard antimicrobial sensitivity testing, and within 15 minutes of

inoculation, meropenem disk that has been incubated with the test organism was taken out and placed on to the inoculated MHA plate, and incubated over night at 37^0c.The next day, a zone of inhibition between 6 to 15 mm was interpreted as carbapenemase producer, zone of inhibition >19mm as non carbapenemase producers. A zone of inhibition between 16 and 18 mm around a disk with distinct colonies inside zone of inhibition was considered as positive test thus carbapenemase producer but a clear zone of inhibition between 16 and 18 mm around a disk was considered indeterminate thus the purity of the *E. coli* ATCC 25922 and integrity of the meropenem disks was verified and the test was repeated [31].

5.9. Data quality assurance

To ensure data quality, the data collection form was pre-tested and the data that that has been collected was thoroughly checked on spot and daily for their completeness, accuracy, and clarity. All of the pre-analytical, analytical and post analytical steps were carried out in strict adherence to the Standard Operating Procedures (SOPs) of the microbiology laboratory at WAML to assure the quality of laboratory results.

5.9.1. Pre analytical phase

A Socio-demographic characteristic of patients was collected using pre-structured data collection formats. All specimens were collected by trained personnel in accordance with standard operating procedures. Upon arrival at the laboratory, specimens were inspected for proper labeling and assessed for acceptance based on set criteria.

5.9.2. Analytical phase

All Procedures, equipment's and materials were adequately controlled. All reagents were clearly labeled, dated, and stored appropriately. The processes of media preparation, identification and AST were conducted in accordance with the SOPs of the WAML and CLSI version 2024. New batch of culture media was tested for performance and sterility before use. At each test, a quality control was performed by using *K. pneumoniae* ATCC BAA-1705 as positive and *K. pneumoniae* ATCC BAA-1706 as negative control isolates.

5.9.3. Post Analytical Phase

In the post-analytical phase, results were recorded using the patients' identification numbers. All reports were clear, concise, and well-organized. Prior to leaving the microbiology laboratory, each

report was thoroughly reviewed for accuracy. Purified bacterial isolates were preserved in tryptic soy broth containing 20% glycerol at –70°C and were sub cultured monthly to maintain viability.

5.10 Data analysis and interpretation

Data was entered using Epidata version 4.6 and analyzed using Statistical Package for the Social Sciences (SPSS) software, version 26. Descriptive statistics were applied to evaluate dependent and independent variables through frequencies and cross-tabulations. The final results were presented in narration form, as well as through graphs and tables.

5.11. Ethical consideration

Ethical clearance was obtained from the Departmental Research and Ethics Review Committee (DRERC) of Addis Ababa University College of Health Sciences, Department of Laboratory Sciences; protocol number DRERC/782/24/MLS and permission was obtained from wudassie advanced medical laboratory. To ensure confidentiality, patient's data was secured by password and unauthorized persons had no access to the data. Only the principal investigator and advisors of the principal investigator could access the data.

5.12. Result dissemination

The findings of this study will be forwarded to the Department of Medical Laboratory Sciences, College of Health Science, Addis Ababa University. Attempt will be made to present the findings in various conferences and will be sent for publication in peer-reviewed journals.

5.13 Operational and definition of terms

Multi Drug Resistance: MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories [32, 33].

Carbapenemase production: is defined as bacteria that have resisted carbapenem groups of drugs and the isolate is Positive for carbapenemase production by a phenotypic method [34].

6.0 Results

6.1 Socio-demographic characteristics of the patients

A total of 1105 patients who met the eligibility criterion were included in this study. Among this 55.4% (612/1105) were male. The median and standard deviation of age were 44 and 25.5 respectively. About 28.3% (313/1105) of the patients were above the age of 60 and 76.1% (841/1105) of the patients were from private health facility while 78.9% (872/1105) of them were admitted patients. Blood specimen holds majority of the specimen 39.7% (439/1105) followed by urine specimen 31.9% (353/1105) (Table 1).

Table 1: Socio-demographic data of the patients

Variables	Category	Frequency	Percent
Age (years)	<1	84	7.6
	1-15	122	11
	16-30	167	15.1
	31-45	208	18.8
	46-60	211	19.1
	>60	313	28.3
Sex	Female	493	44.6
	Male	612	55.4
Facility type	Private	841	76.1
	Public	264	23.9
Ward	Inpatient	872	78.9
	Outpatient	233	21.1
Specimen type	Blood	439	39.7
	Urine	353	31.9
	Wound	110	10.0
	CSF	45	4.1
	Body fluid	89	8.1

	Sputum	34	3.1
	Stool	35	3.2

6.2 Bacteria isolates from different clinical samples

From 1105 different clinical specimens that were inoculated, 26.1% (288/1105) of them had growth and of this 54.2% (156/288) of them were *Enterobacteriaceae,* 13.54% (39/288) of them were non fermentative gram-negative rods, 31.25% (90/288) of them were gram positive bacteria and the remaining 1.04% (3/288) isolates were fungal. From *Enterobacteriaceae* isolates, 50.6% (79/156) of them were from females and 35.3% (55/156) of isolates were from patients above the age of 60. *E. coli,* 57.1% (89/156) was the most abundant bacteria isolated followed by *K. pneumonia*, 30.1% (47/156) *P. mirabilis*, 3.2% (5/156), *K. oxytoca*, 1.9% (3/156), *C. freundii* (1.9%) 3/156, *E. cloacae*, 1.9% (3/156), *M. morganii*, 1.3% (2/156), *Salmonella spp*, 1.3% (2/156), *S. marcences*, 0.64% (1/156), and *P. vulgaris*, 0.64% (1/156). Majority of the *Enterobacteriaceae* were isolated from urine 60.9% (95/156) followed by wound 16% (25/156) and sputum 8.3% (13/156) (Table 2).

Table 2: Frequency (percentage) distribution of isolates from different specimen

Isolate (number)	**Blood n (%)**	**Urine n (%)**	**Wound n (%)**	**CSF n (%)**	**Body fluid n (%)**	**Sputum n (%)**	**Stool n (%)**	**Total n (%)**
C.freundii (n=3)	0(0)	2(66.7)	1(33.3)	0(0)	0(0)	0(0)	0(0)	3(1.92)
*E. coli (*n=89)	3(3.37)	70(78.7)	9(10.1)	0(0)	6(6.74)	1(1.12)	0(0)	89(57.1)
E. cloacae (n=3)	0(0)	0(0)	3(100)	0(0)	0(0)	0(0)	0(0)	3(1.92)
K. oxytoca (n=3)	0(0)	1(33.3)	1(33.3)	0(0)	0(0)	1(33.3)	0(0)	3(1.92)
K.pneumonia e (n=47)	7(14.9)	18(38.3)	8(17)	1(2.1)	3(6.4)	10(21.3)	0(0)	47(30.1)
M. morganii (n=2)	0(0)	1(50)	1(50)	0(0)	0(0)	0(0)	0(0)	2(1.28)
P. mirabilis (n=5)	0(0)	3(60)	1(20)	0(0)	0(0)	1(20)	0(0)	5(3.2)
P. vulgaris (n=1)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	1(0.64)
Salmonella spp (n=2)	0(0)	0(0)	0(0)	0(0)	1(50)	0(0)	1(50)	2(1.28)
S. marcescens (n=1)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(0.64)

Total (n=156)	11(7.05)	95(60.9)	25(16)	1(0.64)	10(6.4)	13(8.3)	1(0.64)	156(100)

Body fluid: Pleural fluid, Peritoneal fluid, Synovial fluid, Amniotic fluid

6.3 Antimicrobial Resistance of different isolates

From 89 *E. coli* isolated 95.5% of them were resistant to ampicillin, 69.7% of them were resistance to ciprofloxacin and only 4.5% of the isolates were meropenem resistant. From 47 *K. pneumoniae* isolated 93.6% of them were resistance to co-trimoxazole, 78.7% of them were resistance to ciprofloxacin and gentamycin and 59.6% of them were resistance to meropenem antibiotic. Ampicillin have an overall resistance rate of 96.1 % followed by amoxicillin–clavulanic acid 95.5%, ciprofloxacin has overall resistance of 71.8% while co-trimoxazole have an overall resistance rate of 84%. (Table 3)

Table 3: Frequency (percentage) distribution of antimicrobial resistance pattern of isolates to different antibiotics

Isolate	**AMP n (%)**	**AMC n (%)**	**CRO n (%)**	**CTZ n (%)**	**FEP n (%)**	**SXT n (%)**	**CIP n (%)**	**AK n (%)**	**CN n (%)**	**NI n (%)**	**C n (%)**	**MEM n (%)**
C.freundii n=3	3 (100)	3 (100)	2 (66.7)	2 (66.7)	2 (66.7)	3 (100)	3 (100)	1 (33.3)	1 (33.3)	0 (0)	1 (100)	1 (33.3)
E. coli n=89	85 (95.5)	84 (94.4)	60 (67.4)	55 (61.8)	41 (46.1)	71 (79.8)	62 (69.7)	14 (15.7)	30 (33.7)	11 (15.7)	13 (68.4)	4 (4.5)
E. cloacae n=3	3 (100)	3 (100)	2 (66.7)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	1 (33.3)	2 (66.7)	0 (0)	2 (66.7)	0 (0)
K. oxytoca n=3	3 (100)	3 (100)	1 (33.3)	1 (33.3)	1 (33.3)	2 (66.7)	2 (66.7)	0 (0)	1 (33.3)	1 (100)	1 (50)	0 (0)
K. pneumoniae n= 47	47 (100)	46 (97.9)	41 (87.2)	41 (87.2)	39 (83)	44 (93.6)	37 (78.7)	30 (63.8)	37 (78.7)	13 (72.2)	20 (69)	28 (59.6)
M. morganii	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (1.28)	1 (50)	2 (100)	1 (100)	1 (100)	0 (0)

n=2												
P. mirabilis n=5	4 (80)	4 (80)	3 (60)	2 (40)	2 (40)	5 (100)	3 (60)	2 (40)	3 (60)	3 (100)	1 (50)	1 (20)
P. vulgaris n=1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)
Salmonella spp n=1(stool)	1 (100)	NA	NA	NA	NA	0 (0)	0 (0)	NA	NA	NA	NA	NA
Salmonella spp n=1(body fluid)	1 (100)	NA	0 (0)	NA	NA	0 (0)	0 (0)	NA	NA	NA	NA	NA
S. marcescens n=1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	NA	1 (100)	0 (0)
Total	150 (96.1)	147 (95.5)	113 (72.9)	107 (69.5)	90 (58.4)	131 (84)	112 (71.8)	50 (32.5)	78 (50.6)	30 (31.6)	41 (73.2)	35 (22.7)

Abbreviations: AMP: ampicillin; AMC: amoxacillin/clavulanate;; CN: gentamicin; SXT: trimethoprim-sulfamethoxazole; CIP: ciprofloxacin; CRO: ceftriaxone; NI: nitrofurantoin; CTZ: ceftazidime; FEP: cefepime; AK: amikacin; C: chloramphenicol; MEM: meropenem.

6.4 Multi drug resistant *Enterobacteriaceae*

From *Enterobacteriaceae* isolated, 78.8% (123/156) of them were multidrug resistant bacteria. All isolate of *C. freundii* (3/3), *S. marcescens* (1/1), *M. morganii* (2/2*) and P. vulgaris* (1/1) were MDR. Out of 89 *E. coli* isolates 67(75%) of them were MDR. Out of 47 *K. pneumoniae* isolates 42(89%) of them were MDR. *E. coli* was the most abundant MDR isolate 54.5% (67/123) followed by *K. pneumoniae* 34% (42/123). As depicted below in Table 4, only 2.56% (4/156) isolates have no resistance to any antibiotic category and 16% (25/156) of the isolates were resistant to seven antibiotic categories and 80% (20/25) of them were *K. pneumoniae* isolates.

Table 4: Frequency (percentage) distribution of drug resistance to different clinical isolates

Isolate (number)	**R0 n(%)**	**R1 n(%)**	**R2 n(%)**	**R3 n(%)**	**R4 n(%)**	**R5 n(%)**	**R6 n(%)**	**R7 n(%)**	**MDR (R≥3) n(%)**
C. freundii (3)	0(0)	0(0)	0(0)	1(33.3)	1(33.3)	0(0)	0(0)	1(33.3)	3(100)
E. coli (89)	3(3.4)	9((10.1)	10(11.2)	13(14.6)	30(33.7)	18(20.2)	5((5.6)	1(1.1)	67(75.3)
E. cloacae (3)	0(0)	1(33.3)	0(0)	0(0)	0(0)	1(33.3)	1(33.3)	0(0)	2(66.7)
K. oxytoca (3)	0(0)	1(33.3)	0(0)	0(0)	1(33.3)	1(33.3)	0(0)	0(0)	2(66.7)
K. pneumoniae(47)	0(0)	2(4.3)	3(6.4)	2(4.3)	5(10.6)	4(8.5)	11(23.4)	20(42.5)	42(89.4)
M. morganii (2)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	1(50)	0(0)	2(100)
P. mirabilis (5)	0(0)	1(20)	1(20)	0(0)	1(20)	0(0)	0(0)	2(40)	3(60)
P. vulgaris (1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)
Salmonella Spp(2)	1(50)	1(50)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. marcescens (1)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	1(100)
Total	4(2.56)	15(9.61)	14(8.97)	16(10.26)	38(24.4)	26(16.7)	18(11.54)	25(16)	123(78.8)

R0-Absence of resistance to any antibiotic category, R1- resistance to one antibiotic category, R2- resistance to two antibiotic categories, R3- resistance to three antibiotic categories, R4- resistance to four antibiotics category, R5- resistance to five antibiotic categories, R6 -resistance to six antibiotic category and R7- is resistance to seven antibiotic categories used for treatment.

Majority of MDR bacteria were isolated from urine specimen, 47.4% (74/156) followed by wound, 12.2% (19/156), blood, 7.1% (11/156), sputum, 7.1% (11/156). And 84.5% (104/123) of the MDRE were from admitted patients (Figure 1).

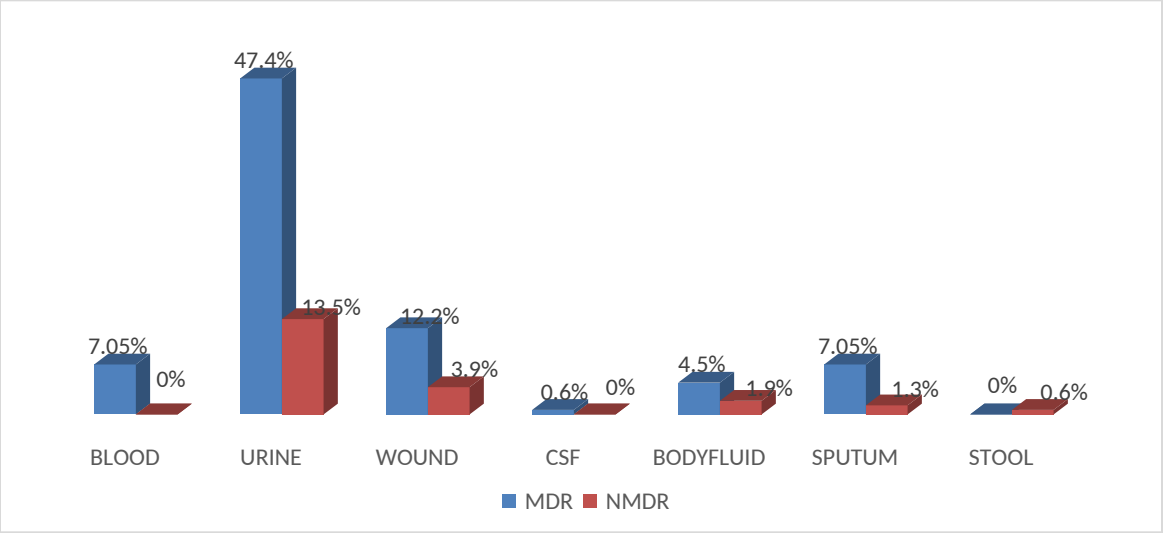

Figure 1: Multi drug resistance from different specimens.

6.5 Carbapenemase producing *Enterobacteriaceae*

From *Enterobacteriaceae* isolated, 22.7% (35/154) of them were carbapenem resistant. Those isolates that has resisted carbapenem antibiotic were further checked for carbapenemase production by mCIM and 85.7% (30/35) of them were confirmed to be carbapenemase producers. *K.pneumoniae,* 80% (24/30) accounts majority of the isolates that produces carbapenemase enzyme (Figure 2).

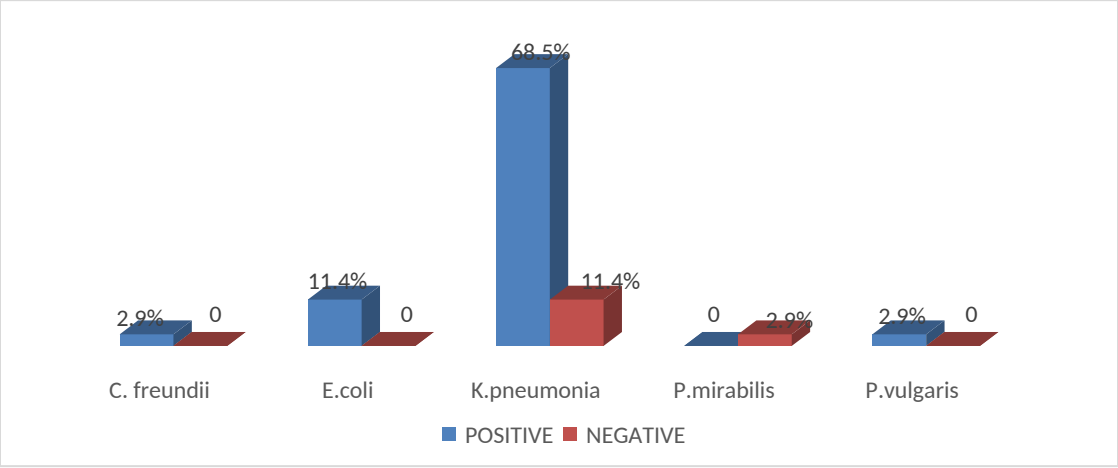

Figure 2: Carbapenemase production for different clinical isolates.

Drug resistance pattern of CP-CRE isolates were much higher than other MDR isolates with ampicillin (100% versus 94.1%), ceftriaxone (100% versus 65%), ciprofloxacin (96.6% versus 64.5%) and amikacin (80% versus,18.5%). For CPE isolates majority of the antibiotics were inefficient (Figure 3).

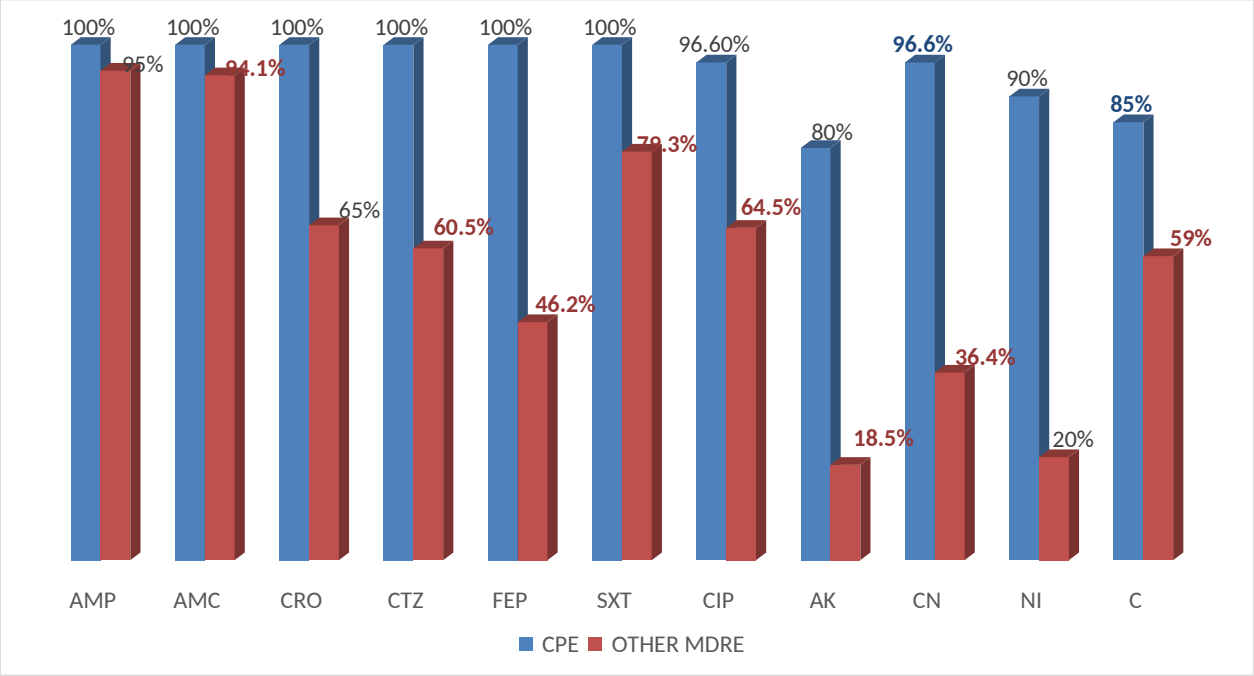

Figure 3: Antibiotics resistance profile for CPE and Other MDRE

Abbreviations: AMP: ampicillin; AMC: amoxicillin/clavulanate; CN: gentamicin; SXT: trimethoprim-sulfamethoxazole; CIP: ciprofloxacin; CRO: ceftriaxone; NI: nitrofurantoin; CTZ: ceftazidime; FEP: cefepime; AK: amikacin; C: chloramphenicol; MEM: meropenem.

All isolates from blood sample were MDR (11/11) and 36.4% (4/11) of them were CPE. 77.9% (74/95) of urine isolates were MDR and 10.5% (10/95) of them were CPE. 84.6% (11/13) of Sputum isolates were MDR and 53.8% (7/13) of them were CPE (Table 5).

Table 5: Summary of MDR and CPE frequency (percentage) from different clinical specimens

Specimens with Number of Isolates	MDR N (%)	CRE N (%)	CPE N (%)
Blood (n=11)	11 (100)	5(45.5)	4(36.4)
Urine (n=95)	74 (77.9)	13(13.7)	10(10.5)
Wound (n=25)	19(76)	7(28)	7(28)
CSF (n=1)	1 (100)	0(0)	0(0)
Body fluid (n=10)	7 (70)	2(20)	2(20)
Sputum (n=13)	11 (84.6)	8(61.5)	7(53.8)
Stool (n=1)	0 (0)	0(0)	0(0)
Total (n=156)	123(78.8)	35(22.7)	30(19.5)

7.0 Discussion

The global rise of carbapenemase-producing Enterobacteriaceae (CPE) represents a significant threat to public health and clinical medicine. These pathogens produce enzymes capable of hydrolyzing carbapenems, a class of β-lactam antibiotics typically reserved for the treatment of multidrug-resistant bacterial infections. As a result, therapeutic options are increasingly limited, contributing to higher morbidity, mortality, and healthcare burdens.

In this study *E. coli* was the most abundant isolate followed by *K. pneumoniae*, this finding was in line with the study conducted in Saudi Arabia [23], Iran [22], Nigeria [24], Debre birhan [28] Addis Ababa [26]. This finding was different from the study done in Sudan [25], where the predominant isolate were *P. mirabilis* followed by *K. pneumoniae* the reason for this variation might be due to difference in geographical location and difference in the type of specimen the study was conducted on.

Multi drug resistance in this study was 78.8%. This finding was in line with the study done in Bahir Dar (80.0%) [35], (81.1%) [39], Saudi Arabia (70%) [23], Iran (78.31%) [22].The prevalence was lower than a study done in Addis Ababa (94.5%) [37], Gondar, Dessie, Debre Markos collectively (85.8%) [41], the reason for this variation might be due to difference in sample size. The finding of current study was higher than study done in Addis Ababa (68.6%) [26], (68.3%) [36], (45.2%) [38], Nepal 36.0%, [43] this variation might be due to time difference between those studies.

The predominant MDR isolates in this study were *E. coli* and *K. pneumoniae* and this finding was in line with the study finding in Bahir Dar [35], Saudi Arabia [23], Addis Ababa [37, 38]. The finding was different from a study done in Nepal [43], where the predominant MDR isolates were *E. coli* and *Citrobacter spp* and from study done in Addis Ababa [26], where, Enterobacter *spp*. and *Citrobacter spp* were the predominant MDR isolates.

From 156 *Enterobacteriaceae* isolated in this study, 30(19.5%) of them were carbapenemase producers. This finding was in line with a study done in Bahir Dar (16.2%) [35], Gondar, Dessie, Debre Markos collectively (15.7%) [41].This finding was higher than the findings in study done in Addis Ababa (7.7%) [27], (2%) [37], (5.4%) [38], Bahir Dar (5.2%) [39], (6%), [40], Sidama (9%) [42], Nigeria (7.9%) [24] and Debre birhan (10.97%) [28].The reason for this variation might be due to time difference and difference in methods used for confirmation of enzyme production.

And the finding of this study was lower than the finding in a study done in Pakistan 24%, [20] this difference might be due to the difference in economic status of the participants together with factors in the implementation of infection control polices of the country.

The predominant CPE isolate of present study are *K. pneumoniae* and *E. coli.* This finding is in line with the study done in Addis Ababa [27, 37, 38] and Debre Birhan [28]. The findings were different from a study done in Bahir Dar [35, 39, and 40] where the predominant isolates were *K. pneumoniae* and E. *cloacae*.

In this study the antimicrobial resistance pattern of CPE isolates indicates 100% resistance to ampicillin, amoxicillin-clavulanic acid, ceftriaxone, cefepime, co-trimoxazole, 96.6% resistance to ciprofloxacin and 80% to Amikacin. This was different from a study done in Addis Ababa with 100% resistance to ampicillin, amoxicillin with clavulanic acid, 87.5% resistance to, sulfamethoxazole-trimethoprim and 75.0% resistance to ceftriaxone and cefepime [26]. This difference might be due to presence of other beta lactamase like ESBL and the presence of time difference between those studies.

There are limited treatment options for CPE isolates with Amikacin being the least resistant drug with resistance of 80%. This finding was much higher compared to the study done in Addis Ababa with no resistance to amikacin for CPE isolates [26]. This might be due to the acquisition of resistance gene over time.

8.0 Strength of the study

- MALDI-TOF was used for bacterial identification. This advanced diagnostic method enabled accurate and rapid identification of *Enterobacteriaceae* isolates.

9.0 Limitation of the study

- Due to the constraints of the MALDI-TOF MS database, it was not possible to differentiate Salmonella isolates to the species level.
- This study lacks detail information about participants due to lack of availability of the data.

10.0 Conclusion

Escherichia coli and *Klebsiella pneumoniae* were the predominant *Enterobacteriaceae* isolates responsible for various bacterial infections. A high proportion of these isolates showed multidrug resistance, highlighting the growing challenge of antimicrobial resistance in clinical settings. Furthermore detection of increased prevalence carbapenemase-producing Enterobacteriaceae is concerning, which further limits therapeutic options and patient outcome. *Klebsiella pneumoniae* was the most common carbapenemase producer. Highest resistance was noted against ampicillin, amoxicillin-clavulanic acid, co-trimoxazole, and ciprofloxacin. These findings underscore the urgent need for robust antimicrobial stewardship, early detection strategies, and reinforced infection control.

11.0 Recommendation

- Efficiency of combination antibiotics should be studied and be incorporated for the treatment of CPE isolate
- Factors associated with increment of resistance should be studied and be used for the control of this rapid rate of drug resistance increment.

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Annexes

Annex 1 Media preparation

1. Sop for preparation of MacConkey Agar

MacConkey agar is selective and differential medium to distinguish gram negative Enterobacteriaceae and lactose fermenting bacteria from non-lactose fermenters. Crystal violet is included in the medium to inhibit the growth of gram positive bacteria, especially enterococci and staphylococci. Differentiation of enteric microorganisms is achieved by the combination of lactose and the neutral red indicator. Colorless or pink to red colonies are produced depending upon the ability of the isolate to ferment the carbohydrate.

Procedure for preparation:

Prepare as instructed by the manufacturer.

1. Suspend 51.1g of powder in 1 liter of distilled or deionized water.
2. Heat and boil until completely dissolved with frequent agitation.
3. Sterilize in autoclave at 1210C for 15 minutes
4. Cool to 45-50 0C
5. Mix well and dispense by dispenser (15-20 ml) aseptically into sterile petri dishes.
6. Leave standing for thirty minutes to solidify.
7. Perform sterility testing as described before.
8. Label the bottom of each plate with date of preparation and batch number.

Store the culture media plates upside down at 2-8^{0}C sealed in plastic bags to reduce chances of contamination. Test Samples for performance, using stable, typical control cultures.

2. SOP for preparation of Blood agar plate (BAP)

Blood agar plate is a non-selective medium for the isolation and cultivation of many pathogenic microorganisms. Organisms can be differentiated by their hemolysis on this agar.

Blood Agar Base formulation has been used as a base for preparation of blood agar and to support good growth of a wide variety of fastidious microorganisms. Because it is a highly nutritious medium it can be used as a general purpose growth media without adding blood.

Procedure for Preparation BAP

Measure 500ml of distilled water using a measuring cylinder.

1. Transfer the distilled water into a 1litre capacity conical flask.
2. Weigh 20g of Blood Agar Base powder using a weighing balance.
3. And then add into the 500ml of distilled water and mix thoroughly.
4. Boil until completely dissolved
5. Autoclave at 121°C for 15 minutes.
6. Allow to cool to 45-50°C in a water bath.
7. Once the medium has been melted and cooled to 45-50 ºC
8. Add 5-10% of defibrinated sterile sheep blood, in this case you can recuperate
9. Rotate the flask or bottle slowly to create a homogeneous solution. \
10. Aseptically add 25 ml of sterile defibrinated sheep blood with constant shaking.
11. Label on the bottom top of the blood agar plates the batch number & date prepared.
12. Store the culture media plates upside down at 2-80C sealed in plastic bags to reduce chances of contamination. Shelf life: up to sixteen weeks provided there is no change in appearance.

3. Sop of Xylose Lysine Desoxycholate Agar [XLD]

It is a selective differential medium suitable for isolation of Shigella and Salmonella from stool specimens and other clinical specimens. Differentiation of these two species from non-pathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulfide production. Shigella colonies on XLD agar are transparent pink or red smooth colonies 1–2-mm in diameter. S. dysenteriae1 colonies on XLD agar are frequently very tiny; unlike other Shigella spp. Coliforms appear yellow. Salmonella colonies are usually red with black centers but can also be yellow with black centers.

Procedure for Preparation of XLD

Prepare according to manufacturer's instructions.

1. Weigh 56.68gm XLD powder.
2. Mix with 1000ml distilled water thoroughly
3. Heat with agitation just until the medium boils. Do not overheat; overheating when

boiling XLD or allowing the medium to cool too long may cause the medium to precipitate.

4. Cool flask under running water until just cool enough to pour; avoid cooling the medium too long.
5. Pour the XLD into Petri plates, leaving the lids ajar for about 20 minutes so that the surface of the agar will dry. Plates can be stored at 4°C for up to a week.

4. Preparation of Mueller Hinton Agar (MHA)

It used for antibiotic susceptibility testing of non-fastidious organisms.

Procedure for preparation of MHA:

1. Weigh, dissolve by heat the medium as instructed by the manufacturer.

2. Adjust the pH of the medium to 7.2–7.4.

3. Autoclave at 121°C at 15 psi for 20 minutes. Do not over heat.

4. Pour into 150 mm or 100 mm diameter sterile petri dishes to a depth of 4 mm (about 25 ml per plate).

It should be dispensed into plates on a level surface so that the depth of the medium is uniform because too thin medium may give falsely large inhibition zones and too thick medium may give falsely small zones.

Annex 2. Application of Antimicrobial Discs into MHA plates

Application the discs onto MHA plate should be done within 15 minutes of inoculation. The selected antimicrobial discs was dispensed evenly using forceps/sterile needle/surgical blade. Each disc must be pressed down gently to ensure complete contact with the agar surface.

1. A maximum of 12 discs on a 150 mm plate or 5 discs on a 100 mm plate should be applied, keeping at least a distance of 24 mm between discs. Since some of the drugs could diffuse

instantaneously, a disc should not be relocated once it has come in contact with the agar surface.

2. Place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.

3. Disc containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.

4. Incubation should be within 15 minutes after discs are applied, the plates was inverted and incubated at 35º ± 2ºC in ambient air.

Each plate was examined after overnight incubation (16-18 hours), for confluent growth and circular zones of inhibition according to the recommendation of CLSI 2018.

Annex 3: Laboratory procedure for Gram staining technique

1. Labeling the slides clearly with patient code number.

2. Making of smears by spread evenly covering an area about 15-20mm diameter on a slide.

3. Drying of smears after making smears, the slide should be left in a safe place to air-dry, protected from flies and dust.

4. Fix the dried smear by using heat or chemicals (methanol).

5. Cover the fixed smear with crystal violet stain for 30-60 seconds.

6. Rapidly wash off the stain with clean water. If the tap water is not clean, use filtered water or clean boiled rainwater.

7. Tip off all the water, and cover the smear with lugol's iodine for 30-60 seconds.

8. Wash off the iodine with clean water.

9. Decolorize rapidly (few seconds) with acetone alcohol. Wash immediately with clean water.

10. Cover the smear with neutral red or safranine stain for 2 minutes.

11. Wash off the stain with clean water.

12. Wipe the back of the slide clean, and place in a draining rack for the smear to air-dry.

13. Examine the smear microscopically, first with the 40 X objective to check the staining and to see the distribution of materials and then with the oil-immersion objective to look for bacteria and cells.

Result

• Gram positive bacteria ----------dark purple

• Gram -negative bacteria ----------pale to dark red

Annex 4: SOP for MALDI-TOF (EXS3000 Series)

The automatic microbial mass spectrometry detection system is based on the matrix assisted laser desorption ionization (MALDI) method to ionize sample molecules into ions, and then detect samples with different m/z through the Time of Flight (TOF) The time when the ion arrives at the detector is converted into a peptide fingerprint, which is matched and searched with the pre-stored database for identification of microorganisms.

Detection Principle

The matrix and sample on the sample target are transformed from molecules to ions under the trigger of laser energy. At the same time, a high voltage is applied to the sample target to form a high-voltage electric field in the ion source. The sample ions and the matrix ions accelerate under the action of the electric field. When the ions fly out of the ion source, they fly in the flight tube at a constant speed until it reaches the detector, the time for ions with different mass-to-charge ratios to reach the detector is different. The ions finally generate electronic signals on the detector, and then undergo processing such as digital converters to obtain digital signals on the computer, and finally through the computer Processing and analysis can obtain the mass spectrum of the

corresponding sample. Search the sample's mass spectrum and microbial protein fingerprint library to realize the search and identification of the sample.

Instrument And Reagents

1. Microbe Sample Pretreatment Kit

2. Fresh bacteria colony

3. Centrifuge tube（1.5ml）

4. Pipette and tips (1ul, 200ul, 1000μL)

5. Ultra-pure water or sterile water

6. Absolute alcohol

7. Vortex mixer

8. Centrifuge machine

Procedure

1. Transfer the appropriate amount of bacteria isolates (without transferring any medium) into a 1.5 ml centrifuge tube containing 300 μL ultrapure water, fully suspend;

2. Add 900 μL ethanol and mix well;

3. Centrifuge at 12, 000 rpm for 2-3 min, remove and discard the supernatant. Centrifuge at the same speed for another 1 min and remove the residual supernatant. Let the sediment dry at room temperature for 5 min;

4. Add 20 μL microorganism lysate I to the pellet and mix well with pipette (for gram-positive bacteria and some fungi, let the solution stand at room temperature for 5 min);

5. Add 20 μL microorganism lysate II, mix well, and centrifuge at 12, 000 rpm for 2 min;

6. Add 1 μL supernatant to the target plate and allow it to dry. Cover with 1 μL matrix solution and allow it to dry.

7. Put the target plate into the Mass Spectrometry System for analysis

Normal working condition

• Ambient temperature: 10 °℃~30℃;

• Relative humidity: ≤85%;

• Power condition: 100-240 V, 50/60 Hz, 500 VA;

• There is no strong mechanical vibration and electromagnetic interference source around;

• Avoid direct exposure to strong light;

• There should be a good grounding environment;

• The ground is level and the inclination is less than 1/200;

• For indoor use, the room should be clean and dust-free, with good ventilation.

The software has the dual functions of spectrum collection and microbial identification. After the mass spectrum is collected, the obtained mass spectrum is matched and searched with the standard library to complete the microbial identification of the sample. The software can directly display the identification results of the strains, and display the confidence level of the identification results of each sample site in color. The software uses a three-point system for scoring rules. When the score value is ≥2.0, the identification result is displayed as green, which represents the possible species level identification. The higher the score value, the higher the confidence of the species level; when the score value is ≥1.7 and <2.0, the identification result is displayed as yellow, which represents the possibility For genus level identification, the higher the score, the higher the confidence of the genus level; when the score value is less than 1.7, the identification result is displayed in red, indicating that the identification result is unbelievable, that is, an unreliable identification.

Limitations

Limitations of the application of automatic microbial mass spectrometry detection system:

(1) Analyze patient samples directly;

(2) Analyze mixed cultures;

(3) Identify the microorganisms in the liquid culture medium;

(4) Identify highly pathogenic microorganisms;

(5) Identify microorganisms of the types of bacteria not included in clinical trials;

(7) Identify the serotype.

(8) Identify some microorganisms to species level

Annex :5 Procedure for mCIM

1. Preparation of isolates to test and controls
    - Prepare and label the number of tubes necessary depending on number of isolates to test (1 tube per isolate, plus 2 tubes for positive and negative controls).
    - Add 2ml of tryptic soy broth using sterile technique to each tube.
    - From a pure culture on blood agar of less than 24h growth, prepare a suspension using a 1 µL sterile loop of the isolate to test in the correctly labelled tube. Centrifuge the suspension.
    - Using sterile technique, add to each tube a 10 µg meropenem disk. Ensure that the disk is well immersed.
    - Incubate each tube for 4 hours (+/- 15 minutes) in aerobic conditions at 35ºC.

2. Preparation of agar for plating
    - The Mueller-Hinton agars must be taken out of the refrigerator and left at room temperature for about 1 hour prior to use.
    - In addition, if there is condensation on the cover or on the agar, the boxes may be placed for 10 to 30 minutes in a biosafety cabinet with their covers half-open. Prepare a suspension of 0.5 McFarland of the *Escherichia coli* ATCC 25922 isolate in Mueller-Hinton broth, tryptic soy agar or saline and plate on Mueller-Hinton agars as per usual protocol for disk diffusion testing. Ensure that the suspension is plated within 15 minutes.

- Replace the cover and let dry for 3 to 10 minutes to permit absorption of the inoculum.
- After incubating the tubes containing meropenem disks for 4 hours, recuperate the disks, using a 10 µL loop while making sure to eliminate the surplus of liquid by pressing the disk to the side of the tube. Deposit the meropenem disks on the Mueller-Hinton agars plated with *Escherichia coli* ATCC 25922. Press down gently in the center of the disk to ensure good contact to the agar.
- Ensure the above step is performed within 15 minutes.
- Up to 4 disks can be placed per Mueller-Hinton agar of 100mm (small) and 8 disks per Mueller-Hinton agar of 150mm (large).
- Label the back of the agar plate the identifier for each disk as well as for the positive and negative controls.
- Incubate the Mueller-Hinton agar aerobically at 35^{o}C for 18-24 hours (overnight incubation).

3. Reading
    - Place the Mueller-Hinton agar on a black background and lift the cover to perform reading.
    - Using the caliper, measure the zone of inhibition around each meropenem disk.
    - Note the results for each clinical isolate and the controls.
    - Ensure that the control isolates give the expected results before interpreting the clinical isolates.

4.Interpretation
    - Positive result:
        - A zone of inhibition of 6 to 15 mm around a disk is considered as a positive result.
        - A zone of inhibition of 16 to 18 mm around a disk including the presence of colonies in the zone of inhibition is considered as a positive result.
        - A positive result is strongly suggestive of the presence of a carbapenemase-producing *Enterobacteriaceae.*
    - Negative result:

    - A zone of inhibition ≥ 19 mm around a disk is considered a negative result.
    - A negative result suggests that the isolate is resistant to carbapenems by a mechanism other than production of carbapenemase.
- Indeterminate result
    - A zone of inhibition between 16 and 18 mm around a disk is considered indeterminate.
    - Verify the purity of the *E. coli* ATCC 25922 isolate.
    - Verify the integrity of the meropenem disks used by performing a quality control with control isolates used for routine disk diffusion.
    - Repeat the test for the isolate giving an indeterminate result.

Annex 6: Data collection format

I. Patient identification

Date of specimen collection_____

Sample ID._____

Age (years) _____

Gender: Male ☐ Female ☐

Inpatient ☐ Out patient ☐

Health facility type _____

Sample type _____

II. Laboratory Data

1. Gram stains result _____

2. Organism isolated _____

3. Drug susceptibility pattern

3.1. Sensitive to _____

3.2. Intermediate to _____

3.3 Resistance to _____

4. Carbapenemase production with mCIM _____

Name of principal investigator _____

Signature _____ Date _____

Annex 7: Profiles of non-fermenter gram negative rod, Gram positive bacteria and fungal isolate

From a total of 288 microbial growths, 156 were *Enterobacteriaceae*, 39 were non fermenting gram negative rods, 90 were gram positive bacteria and 3 of them were fungal. Isolates other than *Enterobacteriaceae* were not included in the thesis as this study focuses on only Enterobacteriaceae isolates.

Table 6: Frequency of non-fermenter gram negative rod, Gram positive bacteria and fungal isolate

Name of isolate	BLOOD	URINE	WOUND	CSF	BODYFLUID	SPUTUM	Total
Acinetobacter baumannii	4	2	3	3	1	2	15
Acinetobacter lwoffii	1	0	0	0	0	1	2
Acinetobacter pitti	0	1	0	0	0	0	1
Achromobacter xylosoxidans	2	0	0	0	0	0	2
Burkholderia cepacia	0	1	0	0	0	0	1
Candida albicans	0	2	0	0	0	0	2
Candida krusi	0	1	0	0	0	0	1
Enterococcus faecalis	3	7	1	1	1	0	13
Enterococcus faecium	9	12	3	0	0	0	24
Enterococcus raffinosus	0	0	0	0	1	0	1

Pseudomonas aeuroginosa	2	4	6	0	1	4	17

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Date: 27/12/24

Ref.No. MLS/ /24

Departmental Research and Ethics Review Committee (DRERC) decision

Meeting No: 028/2024

Protocol number: DRERC/782/24/ MLS/

Protocol title: MultiDrug Resistance nd Carbapenemsase Producing Enterobacteriaceae From Clinical Samples Referred to Wudassie Advanced Medical Laboratory, Addis Ababa, Ethiopia

Principal investigators: Yordanos Getachew

Institute: AAU-CHS CLS

Elements reviewed [√] Attached [ ] Not attached

Review of revised application [ ] Yes [√] No

Date of previous review: _____

Decision of the meeting: [√] **Approved** [ ] Approved with recommendation

[ ] Approved on Condition (Major revision) [ ] Disapproved

Obligation of the PI:

1. Should comply with the standard international and national scientific and ethical guidelines
2. All the amendments and changes made in protocol and consent form needs DRERC approval
3. The PI should report any updates to DRERC within 10 days of the event.
4. End of the study, including manuscripts and thesis works should be reported to the DRERC/ Department

Departmental Research and Ethics Review Committee (DRERC) Approval period: Jan 1, 2025 to November 1 2025.

Follow up report expected in

2 months √ 6 months_____ 9 months_____ one year_____

Chairperson, DRERC: Mikias Negash, Chair of the day **Chair Person for DMLS:** Melatwork Tibebeu

Signature: _____ **Signature:** _____

Date: 27/12/2024 **Date:** 27/12/2024

Annex 8: Ethical clearance letter

Annex 9: Institutional consent form

Wudassie and Family Business PLC

Wudassie Diagnostic Center

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OUR REF. NO. WDC|K|02|264|17

DATE DEC 5|2024

To Whom It May Concern,

Subject: Institutional Consent for Academic Research

This letter is to confirm that Wudassie Advanced Medical Laboratory has granted permission to Ms. Yordanos Getachew, a postgraduate student from Addis Ababa University, to conduct research titled: Multidrug Resistance and Carbapenemase Producing Enterobacteriaceae on Clinical Samples Referred to Wudassie Advanced Medical Laboratory, Addis Ababa, Ethiopia.

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#6 - SIDIST KILO, INFRONT OF MINILIK HOSPITAL

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been fully acknowledged.

Yordanos Getachew [Msc candidate]

Signature_____ Date of submission_____

This proposal has been submitted with our approval as advisors

Kassu Desta (PhD) Signature_____ date _____

Regassa Diriba (M;LSc) Signature_____ date _____

Place: Addis Ababa, Ethiopia