ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Occurrence of Multiple, Extensive and Pan Drug-Resistant *Pseudomonas aeruginosa* and Carbapenemase Production from Presumptive Isolates Stored in A Biobank at Ethiopian Public Health Institute

By; Tesfa Addis (GSR/6283/12)

Advisors: Kassu Desta (MSc, PhD candidate, Associate Professor)
Shambel Araya (MSc, Lecturer)

A research thesis submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, in partial fulfillment of Master of Science Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

June, 2021
Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by **Tesfa Addis**, entitled: **Occurrence of Multiple, Extensive and Pan Drug-Resistant *Pseudomonas aeruginosa* and Carbapenemase Production from Presumptive Isolates Stored in A Biobank at Ethiopian Public Health Institute** 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.

Signed by the Examining Committee:

External Examiner_____ Signature _____Date _____

Internal Examiner_____ Signature _____ Date _____

Advisor _____Signature _____ Date _____

Advisor _____Signature _____Date _____

_____

Chairman of the Department or Graduate Program Coordinator

ACKNOWLEDGEMENT

When we are doing this research, our country Ethiopia was experiencing political instability, internal conflict (war) in addition to locust swarm and global corona pandemic, because of this our citizens including me were under a great deal of stress. However, in the midst of all this, God gave me the stability and strength to do this research, thus first of all I would like to thank the almighty God again and again.

I would like to acknowledge Addis Ababa University, College of Health Science Department of Medical Laboratory Sciences to allow me to study and improve my professional career. Then, I extend my thanks to my advisors Mr. Kassu Desta and Mr. Shambel Araya for their professional advice for the successful completion of this research.

I am also grateful to all Ethiopian Public Health Institute, National Clinical Bacteriology, nd Mycology Reference Laboratory case-team staff members. You have been with me in advising and strengthen me during difficulties when I was doing this research, in addition to secured essential supplies needed for this project. Moreover, without your usual support, it would be nearly impossible to finish this work. I thank you all in advance!

I would like to extend my special thanks to Mr. Estifanos Tsige, Mr. Dawit Assefa, and Mr. Zelalem Tazu for your unforgettable and exceptional support.

Table of Contents

ABBREVIATIONS:

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BAP	Blood Agar Plat
BD	Becton and Dickinson
CLSI	Clinical and Laboratory Standard Institute
EPHI	Ethiopian Public Health Institute
ID	Identification
mCIM	Modified Carbapenem Inactivation Method
MDR	Multiple Drug Resistance
MIC	Minimum Inhibitory Concentration
NCBMRL	National Clinical Bacteriology and Mycology Reference Laboratory
PDR	Pan-Drug Resistant
QC	Quality Control
TSB	Tryptic Soy Broth
XDR	Extensive Drug Resistance

ABSTRACT

Background: *Pseudomonas aeruginosa* is a common cause of nosocomial infections with associated morbidity and mortality because the organism is unresponsive to commonly available antimicrobials. This study was undertaken to determine the Multiple Drug-Resistant (MDR), Extensive Drug-Resistant (XDR) and Pan Drug-Resistant (PDR) phenotype of *P.aeruginosa* and its carbapenemase production rate from presumptive isolates stored in the bio-bank at the Ethiopian Public Health Institute (EPHI).

Methods: A cross-sectional study was conducted at the EPHI laboratory, Addis Ababa, Ethiopia from March to June 2021. Stored isolates of *Pseudomonas* spp. which had been characterized by manual identification methods were further processed for species-level identification (ID) and antimicrobial susceptibility testing (AST) using a Becton Dickinson (BD) Phoenix automated system. The isolates were analyzed for carbapenemase enzyme production using the modified Carbapenem Inactivation Method (mCIM). The data analysis was done using SPSS version 20 software.

Results: In this study, 110 presumptive *Pseudomonas* isolates from a biobank were re-analyzed, 100 of them were found to be *Pseudomonas* and among these *P.aeruginosa* accounted for 98% and *P.putida* accounted for 2%. The majority of isolates were recovered from wound (46%) specimens followed by ear swabs (18%). The highest level of resistance was observed against Ceftazidime (35%) and the lowest level of resistance was observed against Amikacin (2%). Twenty-seven isolates were identified as candidates for carbapenemase enzyme production testing, of which only 3/27 (11%) isolates were detected as carbapenemase enzyme producers.

Conclusions: This study shows an increasing rate of MDR and XDR isolates and the appearance of PDR in *P.aeruginosa* strain, this is a serious problem in Ethiopia. The lack of newer *anti-pseudomonal* antibiotics adds to the problem. In order to alleviate this problem, Infection prevention activities should be promoted, and treatment of bacterial infections should be guided by antibiotic susceptibility test results.

Keywords*: P.aeruginosa,* Antimicrobial Resistance, Carbapenemase enzyme, Ethiopia

1. INTRODUCTION

1.1.Background

Pseudomonas species are gram-negative, rod-shaped, aerobic, non-spore-forming, non-fermenter, polar-flagellated organisms belonging to the family *Pseudomonadaceae,* which includes over 202 species in the current molecular classification. Among them, *Pseudomonas aeruginosa* is the most common medically important bacterial species that causes nosocomial infection in clinical settings; it is a ubiquitous microorganism found in the environment including water, soil, animals & plants (1-3).

P.aeruginosa produces different coloring substances like; **pyocyanin**, **pyoverdin, pyorubrin** or **pyomelanin** which are responsible for the formation of blue, yellow-brown/yellow-green, red, or brown pigmentations in different culture media`s respectively, in addition, some strains of *P.aeruginosa* can be able to produce both pyocyanin and pyoverdin coloring agents simultaneously resulting blue-green color development which makes unique this pathogen from other gram-negative bacteria (1). Moreover, *Pseudomonas* is non- saccharolytic, catalase and oxidase-positive organism having a fruity odder with greenish metallic β-hemolysis colonies on Blood Agar Plate (BAP) is the additional futures to recognize it faster (1).

Infection by *P.aeruginosa* is associated with mortality and morbidity, particularly in immunocompromised patients. It causes infections in wounds (especially in burn patients), the urinary tract, bloodstream, surgical sites, eye, external ear, and the respiratory tract (1, 4, 5) overall this pathogen could be identified from all clinical specimens. On the other hand, Antimicrobial resistance (AMR) by *P.aeruginosa* infections has become a great challenge because of its unresponsiveness to commonly available antimicrobials. To make matters worse, emergency of MDR, XDR, and PDR *P.aeruginosa* is reported here and there (6, 7) due to it has extraordinary adaptive mechanisms like; upregulation of efflux pumping genes, down regulation of outer membrane proteins, mutations in chromosomal genes, and horizontal acquisition of transferable resistant genes encoding; β-lactamases predominantly carbapenemases, 16S rRNA methylases, aminoglycoside-modifying enzymes (8, 9).

Furthermore, *P.aeruginosa* is intrinsically/inherently resistant to many antibiotics including; ampicillin, chloramphenicol, ceftriaxone/cefotaxime, ertapenem, tetracycline, tigecycline,

Trimethoprim-sulfamethoxazole, Amoxicillin-clavulanic acid, and other combination like Ampcilline-sulbactam (10). So that, in addition to its ability to acquired resistances having the above natural characteristics of *P.aeruginosa* can leads to therapeutic failures besides narrowing the antimicrobial options.

On the other hand, its ability to survive in limited nutrition, high rate of spreading through contaminated materials including; antiseptics, detergents, staff's clothing, computer keyboards, drains, taps, sinks, contaminated food, water, and other related resources in the hospital community makes its prevention strategy worse (11, 12). Regarding this, World Health Organization has currently categorized *P.aeruginosa* in the first list of ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) pathogens as a top priority (critical) organism for research, discovery, and new-drug development because of its higher level of resistant to carbapenems (13)

In developing counties like Ethiopia, this burden is expected to be higher and spreading faster because of poor health facilities, insufficient microbiology testing laboratories, widely practical empirical use of antibiotics, unregulated distribution of the drugs, poor sanitation, and infection prevention approaches.

Thus, to contain the spreading of nosocomial infection and to tackle such kind of problem, urgent focused interventions are required in addition to expanding microbiology laboratories to guide physicians for rational choices of antimicrobials. So that this study was aimed to determine the rate of multi-drug, extensive, and pan-drug resistant phenotype of *Pseudomonas* spp using BD Phoenix automated system, in addition, we have assessed the carbapenemase enzyme production rate from stored presumptive clinical isolates at the EPHI, furthermore to determine the prevalent species of *Pseudomonas* in Addis Ababa, Ethiopia.

1.2.Statement of the problem

As it can be understood from the Global antimicrobial resistance surveillance report, antibiotic resistance is the rising global threat to public health and to the provision of health care worldwide (14) which leads to economic and lives loss. Globally, a minimum of 700,000 people is died each year because of AMR, if no action is taken this number will exceed up to 10 million people per year by 2050 (15). Nosocomial infections caused by MDR *P.aeruginosa* is one of the commonest rising problems particularly in health care settings, which accounts for 4%–60% in different parts of the world (16). In 2019, the center for disease control and prevention reported that MDR *P.aeruginosa* in the United States was recognized as a serious threat, which alone produced an estimated number of 2,700 deaths, 32,600 nosocomial infections, and an estimated 767 million dollar healthcare-related cost loss in 2017(17).

In 2015, the European antimicrobial resistance surveillance network reported that an estimated 671,689 infections were caused by antibiotic-resistant organisms in European countries, of which healthcare-associated infections were accounts 63.5%, in this year around 33,110 peoples loss their life and 874 541 disability-adjusted life-years were registered. In this report, carbapenem drug-resistant *P.aeruginosa* was the 3rd killer organism next to 3rd generation cephalosporin-resistant *E.coli* and Methicillin-resistant *S.aureus*, which alone produced around 4155 deaths and 61,892 infections (18).

Overall, physicians are enforced to prescribe the latest version of antibiotics (beta-lactams, carbapenems, and polymyxins) to treat MDR bacterial infections. In contrast enzymes like carbapenemases are emerged as an important cause of resistance to such drugs. That will escalate the emergency of MDR, XDR, and even PDR strains resulting in life and economic loss. Thus, all these reasons make *P.aeruginosa* has been noticeable as the highest priority for epidemiological research and surveillance (19).

On the other hand, species-level identification of *Pseudomonas* using manual techniques is very difficult in Ethiopia. Therefore, this study was intended for species-level identification of *Pseudomonas* using BD Phoenix automated system, to determine the situation of its drug-resistant feature, and to assess the prevalence of carbapenemase enzyme production rate by *Pseudomonas* spp in Ethiopia as per WHO recommendation.

1.3.Significance of the study

The finding of this work could be used to guide physicians in rational choices of antibiotics for empirical treatment of infections caused by *P.aeruginosa* in setups where immediate culture and susceptibility testing are difficult. This helps to avoid excessive use and misuse of antimicrobial drugs.

Bacteria can survive for thousands of years by their ability to adapt to antimicrobial agents. Thus this study was aimed to document the trends of MDR, XDR, and PDR nature of *Pseudomonas* in the study period in Ethiopia. More importantly, this study was also used to promote infection prevention and control strategies in health facilities; to encourage physicians for laboratory-based decisions rather than empirical treatment, to alert policymakers and other concerned bodies to financing and implementing other package`s in Ethiopia.

Ultimately, molecular techniques are needed to identify common and noble virulence genes and antimicrobial resistance determinant genes which help for the development of new antimicrobial drugs, vaccinations, and other prevention strategies. Therefore all those phenotypically characterized *Pseudomonas* isolates in this study will be used for further nucleic acid-base analysis like the whole-genome analysis.

2. LITERATURE REVIEW

In 2019, the **European** antimicrobial resistance surveillance network reported, *P.aeruginosa* isolates were (18.9%) weighted mean resistance rate for fluoroquinolones and 16.9%, 16.5%, 14.3%, and 11.5% for the piperacillin ± tazobactam, carbapenems, ceftazidime, and aminoglycosides antibiotics respectively, and the MDR and XDR trend was seen in (10.1%) and (6.2%) respectively and (3.4%) of *P.aeruginosa* isolates were resistance to five antimicrobial groups (20). The weighted mean carbapenems resistance in *P.aeruginosa* was almost comparable in the previous surveillance reported in 2018, which was 17.2% and the proportion of carbapenems resistance in between countries was higher in Hungary >50% and 1 to 5% in Scandinavians (21). This tells us carbapenems resistance is not equally distributed in European countries.

A retrospective study conducted in **Mexico** by 2019 showed among the total of 2,711 examined clinical samples 517 gram-negative bacteria were identified, of which *P.aeruginosa* was the predominant isolate accounts 30.2%(156/517), this isolate was mostly identified from bronchial secretions(65) followed by urine(21), blood(19), Catheter(13), wound (10) and the reaming 28 was from other samples, In this study, high resistance rate to Piperacillin12/17 (71%) followed by Piperacillin/tazobactam 71/155 (46%), Amikacin 90/155 (58%), Ceftazidime 100/154 (65%) Cefepime 83/155 (55%), Aztreonam 24/57 (42%), Imipenem 108/155 (70%), Meropenem 84/155 (54%), Ciprofloxacin 65/155 (41.93), Gentamicin 80/155 (52%) and Tobramycin 66/155 (43%) showed (22).

A six-year retrospective study to determine the AST trend of bacterial isolated in **Tehran, Iran** between 2013–2018 showed, *P.aeruginosa* was the predominant gram-negative bacterial which produced 100% resistant to amoxicillin-clavulanic acid and ticarcillin-clavulanic acid and 97.8%, 93.5%, 50.4%, 16.7%, 13% resistance to cefazolin, ceftriaxone, imipenem, ciprofloxacin, and levofloxacin respectively, in addition to having 29.8% (n=84/282) MDR rate (23).

The other retrospective cross-sectional study conducted in a tertiary care hospital in **India** using the Vitek-2 system to determine the pathogen burden and its antibiogram of *Pseudomonas* spp. showed that, a total of 2444 *Pseudomonas* spp were identified from the total isolated 16,210 bacteria which accounts for 15% and tracheal aspirate (23.3%) was the main specimen followed

by wound swab (22.7%), urine (20.9%), and the remaining (33%) was others specimens submitted between January 2012 to December 2016, *P.aeruginosa* was the most predominant one accounts 2331 (95%) and the remaining were 62 (2.5%) *P. putida*, 22 (1%) *P. luteola*, 15 (1%) *P. mendocina*, 12 (0.4%) *P. stutzeri* and 2 (0.1%) *P.pseudoalcaligenes*. When we look at the overall resistance pattern of *Pseudomonas* spp for different antibiotics; Tobramycin 1254 (51%) was the most potent *Anti-pseudomonal* drug followed by Imipenem 1301 (53%), Piperacillin 1363 (56%), Amikacin 1419 (58%), Meropenem 1533 (63%), Cefepime 1541 (63%), Ceftazidime 1623 (66%), Ciprofloxacin 1639 (67%), Gentamicin 1657 (68%), and Levofloxacin 1678 (69%) (24).

In the same country the other Laboratory-based prospective study conducted between 2014-2016 showed, from a total of 2261 clinical samples, 192 *P.aeruginosa* was identified, Of which no resistance was displayed by the antibiotics polymyxin B and colistin and a better susceptibility pattern was showed in imipenem (71.4%) followed by Meropenem and Piperacillin-tazobactam (64.5%), furthermore less sensitivity have been displayed in Ceftazidime (39.1%) and Cefepime (42.2%) (25).

In the study conducted in Egypt, 19 % (54) *P.aeruginosa* was isolated from 283 clinical specimens and the infections were common in (67%) males than (33%) female patients, the majority of the specimens were collected from burn unit and Intensive care unit, amikacin (80.%) was the most potent drug followed by imipenem (66.7%) and gentamicin (56.1%) in contrast cefepime piperacillin/tazobactam and ceftazidime were the least effective drugs having the resistance rate of (98%), (94.7%) and (91%) respectively (26).

The study conducted in Kampala Uganda used by BD Phoenix automated system showed from the total of 869 clinical specimens collected at the national hospital, 42 (5 %) *P.aeruginosa* isolates were recovered from tracheal aspirates (15), ear swabs (12), pus (7), and the remaining (8) isolates were from other specimens, 22 (52 %) of the participants were females and the remaining 20 (48 %) were males; most of the isolates were identified in the median age of 18.5 years of the participants, of those *P.aeruginosa* isolates 10 of them were carbapenem resistant and 73 % (52/71) isolates were possesses resistance to three or more classes of antimicrobials, meropenem and imipenem drugs were the most active having (14%) and (19%) resistance rate respectively, on the other hand aztreonam, cefepime, ciprofloxacin, gentamicin, and ceftazidime

antibiotics were showed higher resistance rate (40 %), (55 %), (64 %), (69 %), and (69 %) respectively, this study also showed 40 % (4/10) and 60% (6/10) carbapenemase activity from carbapenem-resistant *P.aeruginosa* using modified Hodge test and imipenem-EDTA tests respectively (27).

A cross-sectional study conducted in Kenyatta national hospital, **Kenya** by 2018 showed that from 188 *P.aeruginosa* isolates, 180 of them were identified from critical care unit and medical wards, all of the isolates were exhibited multidrug-resistant and of which (96%) Piperacillin-tazobactam, (54%) Meropenem, (54%) Aztreonam, (63.1%) Ceftazidime, (83%) Cefotaxime, (80%) ceftriaxone, (46.5%) Amikacin, (61%) Gentamycin, (71%) Tetracycline and (34%) of ciprofloxacin-resistant *P.aeruginosa* were identified, moreover the isolates gathered from critical care unit patients were a greater resistance rate (73%) than the isolates obtained from other wards (28).

The other cross-sectional study conducted in **Southern Ethiopia**, undertaken between 2015 to 2016, shows from 216 samples collected from the delivery room, intensive care unit, and operation room, 24 *P.aeruginosa* isolates were identified to the maximum from intensive care unit with the profile of Trimethoprim-Sulfamethoxazole 21(88), Ciprofloxacin 20 (83%), Ceftriaxone and Gentamicin equally 19(79%), Cefotaxime 17(71%), Cefepime and Aztreonam 14 (58%), Meropenem and Imipenem equally 10(42%), Ceftazidime 7(29%) and Amikacin was relatively the good one which accounts 6 (25%) resistance to the isolates (29).

The above study was almost similar to the cross-sectional study conducted in **Northwest Ethiopia** which showed that from a total of 238 patients, the prevalence of *P.aeruginosa* was 11 (4.6%) and the rate of this nosocomial infection 2 (3.3%), 2 (6.3%), 7 (4.8%) were isolated from the urinary tract, surgical site infections, and bloodstream infection respectively. All *P.aeruginosa* isolates were 100% resistant to piperacillin and ampicillin and also 36.4% and 45.5% resistance against ciprofloxacin and meropenem respectively (30). In the other study at **Jimma Ethiopia**, from the total of 73 urine samples obtained from catheterized patients 36/73 (49 %) of *P.aeruginosa* was identified from 17 males and 19 females patients with Antimicrobial susceptibility patterns of 100% for Ciprofloxacin and Norfloxacin and 86% for Gentamicin (31).

A study done by Bitew A in **Addis Ababa Ethiopia** between 2016 – 2017 using VITEK 2 compact system showed *Pseudomonas* spp was the dominant pathogen accounts 78 (57.8%) followed by *Acinetobacter* 47 (34.8%) among 135 gram-negative non-lactose fermenters which were identified from 996 clinical samples, this study revealed (27.9%) of ceftazidime, (23.0%) of levofloxacin, (19.7%) of ciprofloxacin and cefepime, (16.4%) of Piperacillin/tazobactam, (13.1%) of gentamicin and (6.6%) of tobramycin drugs resistance rate with 83.3% (65/78) of MDR, 9%(7/78) of XDR and 5%(4/78) PDR rate despite not followed the standard definition of those words, In this study, *P.aeruginosa* 61 (78%) was the dominate one followed by P. *luteola* 7 (10%), P. *fluorescens* 4 (5%) and P. *putida* 6 (8%) (32).

The other cross-sectional study conducted by Asres GS et al. in 2017 showed, from postoperative wound infections at tikur anbessa specialized hospital, Addis Ababa, Ethiopia *Pseudomonas* was the second most common gram-negative organism (33) Similar results was reported from the meta-analysis done in Ethiopia by 2019, in which *Pseudomonas* was the second gram-negative organism causing wound infection in Ethiopia having (87%) resistance rate for amoxicillin, (77%) for ampicillin, (95%) Amoxicillin-clavulanic acid, (58%) for ceftriaxone, (78%) for Cotrimoxazole, in contrast, gentamicin and ciprofloxacin were the most potent *Anti-pseudomonal* drugs have the resistance rate of (18%) and (16%) respectively (34).

3. OBJECTIVES

3.1.General Objective

To determine the occurrence of Multiple, Extensive and Pan Drug-Resistant *Pseudomonas aeruginosa* and carbapenemase production from presumptive isolates stored in a biobank at Ethiopian public health institute

3.2. Specific objectives

- To assess the distribution of MDR, XDR, and PDR *Pseudomonas* isolates and changes overtime period
- To determine the prevalence of carbapenemase-producing *Pseudomonas* spp

4. MATERIALS AND METHODS

4.1.Study area

This study was performed on clinical specimens referred from different referring health facilities such as; Black lion specialized hospital, Yekatite-12 hospital medical college, St. Peter hospital, St. Paulo's hospital millennium medical college, Addis Ababa burn emergency and trauma hospital, Ras Desta Damtew memorial hospital, Menelik-II hospital, Zewditu memorial hospital, Tirunesh Beijing hospital and from other health facilities in Addis Ababa, Ethiopia and all parts of the county.

Referral specimens were examined at EPHI, National Clinical Bacteriology and Mycology Reference Laboratory (NCBMRL), which is located at Gulele sub-city, Addis Ababa, Ethiopia. EPHI is recognized as a key wing of a minister of health for covering most aspects of public health-related issues. The NCBMRL is one of the full-scope accredited laboratories by the Ethiopian national accreditation office since 2016 after fulfillment of ISO/IEC15189:12 requirements by providing bacterial culture and sensitivity testing from different clinical samples.

4.2. Study design and Period

A laboratory-based cross-sectional study was conducted using stored presumptive *Pseudomonas* isolates at the NCBMRL bio-bank, the isolates have been manually identified and stored between January-2017 up to March-2021 and its demographic data was assessed retrospectively and the laboratory procedures were performed between March to June – 2021.

4.3.Sampling Population

4.3.1. Source Population/ Isolates

All patient specimens which was referred to EPHI, NCBMRL for routine culture and sensitivity test during the study period

4.3.2. Study Population/Isolates

All presumptive *Pseudomonas* isolates, which were manually identified and stored between January-2017 up to March -2021 and fulfill the inclusion criteria.

4.4.Inclusion and Exclusion criteria

4.4.1. Inclusion criteria

- All consecutive non-duplicated presumptive *Pseudomonas* isolates which have all important information's like; age, sex, type of specimen submitted, and date & year

4.4.2. Exclusion criteria

- Isolates from contaminated and mislabeled samples
- Isolates with incomplete information were excluded

4.5. Study Variables

4.5.1. Dependent variables

- Antimicrobial resistance pattern of each *Pseudomonas* isolates
- The extent of carbapenemase enzyme production
- Species of *Pseudomonas* isolates

4.5.2. Independent variables

- Age
- Sex
- Source of specimen
- Year of bacterial isolation

4.6.Measurement and Data collection

4.6.1. Sample size determination

We have included all presumptive *Pseudomonas* isolates stored at the EPHI biobank started January-2017 up to March-2021.

4.6.2. Sampling method

Non-probability consecutive sampling method was employed to consider all manually identified presumptive isolates

4.6.3. Stored clinical isolates and sub-culturing

As per the NCBMRL protocol, all pathogenic bacteria which have been isolated by using manual identification method from all clinical samples were stored at -80^0c using Tryptic

Soy Broth (TSB) with 5% glycerol, after properly labeled with the name of the organism, type of the specimen originated, date of identification and its unique laboratory identification number. For this study, only presumptive *Pseudomonas* isolates were used by sub-cultured into Blood agar plate, Cetrimide agar, and MacConkey Agar pates.

4.6.4. Bacterial Identification

The bacterial identification was achieved by using the BD Phoenix M50 automated system, which utilizes a series of conventional, fluorogenic, and chromogenic biochemical tests to determine species-level identification of the organism. The Phoenix identification panel has 51 wells with dried biochemical substrates and 2-fluorescent control wells.(35)

Both enzymatic and growth-based substrates are employed to shows a variety of reactivity in the range of taxa. Therefore bacterial identification is based on microbial utilization and degradation of specific substrates detected by various color indicator systems. When the testing organism utilized the carbohydrate substrate, the PH drops because of acid formation which is indicated by the phenol red indicator, in addition upon enzymatic hydrolysis of either p-nitroanilide or p-nitrophenyl compounds the chromogenic substrate forms a yellow color (35).

There were also additional tests that can detect the ability of the organism to degrade, hydrolyze, reduce, or otherwise utilize a substrate. Finally, results from each substrate displayed as +, -, V, or X for each reaction, then the organism identification was reported with the probability percentage, compared from the Phoenix updated database version V6.81A (35).

4.6.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was also performed simultaneously by BD Phoenix M50 automated system using NMIC/ID-431 combination panels according to the manufacturer's instructions. The AST panels have 84 wells with dried antimicrobial agents and 1 growth control well. Principally, the system is a broth-based micro-dilution using a redox indicator solution for the detection of testing bacterial growth in the existence of an antimicrobial agent (35, 36). Side by side, once the organism

identification is done, the MIC values of each antibiotic were generated and interpreted as Susceptible, Intermediate, or Resistant based on the most recent CLSI M100 performance standard guideline for possible phenotypes for microorganism antimicrobial agent combination with the internal database (35) (10).

NMIC/ID-431 panels were intended for use with Phoenix M50 automated system to determine the susceptibility of the clinically relevant aerobic gram-negative rod to the antimicrobial agents, in addition, indicates resistance markers like carbapenemase. NMIC/ID-431 panels are composed of all recommended antimicrobial agents for *Pseudomonas* spp like; Amikacin, Cefepime, Ceftazidime, Ceftolozane-Tazobactam, Ciprofloxacin, Colistin, Gentamycin, Imipenem, Levofloxacin, Meropenem, Piperacillin-Tazobactam, and other intrinsically resistant antimicrobials for *Pseudomonas* spp (10).

4.6.6. Carbapenemase production

After AST testing candidate (non-susceptible isolates having ≥ 4 µg/ml MIC for Imipenem and/or meropenem) were selected for further carbapenemase production testing, then carbapenemase production test was performed according to CLSI 2020 recommendation by using modified Carbapenem Inactivation Method (10).

For each tested isolate, a 10-µL loopful of colonies was emulsified in 2 ml TSB and a 10 µg Meropenem disk added, then incubated for a minimum of 4 h at 35-37 ºC. Following completion of incubation, 0.5 McFarland suspension of American Type Culture Collection (ATCC) 25922 *E. coli* was prepared in saline. Then a Mueller Hinton Agar (MHA) plate was inoculated with the prepared *E. coli* ATCC 25922 suspensions as the routine disk diffusion procedure and the Meropenem disk taken from the TSB-meropenem suspension was placed in it, then after labeling this was incubated at 35-37 ºC in ambient air for 18-24 h. (10).

Following incubation, the measured inhibition zone diameter of 6-15 mm or pinpoint colonies within 16–18 mm was determined as positive for carbapenemase enzyme production, and a zone of inhibition ≥19 mm was considered to be negative for carbapenemase enzyme production according to CLSI guideline. The procedure was monitored using Quality Control (QC) strains; *K. pneumoniae* ATCC BAA-1706 as negative and *K. pneumoniae* ATCC BAA-1705 as the positive control (10).

4.7.Data Quality Assurance:

Pre-analytic phase; All stored presumptive *Pseudomonas* isolates were checked for the proper labeling with the name of the organism, type of the specimen originated, date of identification, and its unique laboratory identification number before processing. All culture media were prepared according to the manufacturer instruction, then the sterility of prepared media`s was verified by overnight incubation at 35-37 $^{o}$c and checked the absence of visible microbial colonies, then the performance of culture media`s was checked using ATCC *P.aeruginosa* 27853, *E.coli* ATCC 25922 and *S.aureus* ATCC 25923, then stored properly at 2-8 ^{O}c.

Analytic phase*;* The phoenix system was verified using standard strains like; *P.aeruginosa* ATCC 27853, *E.coli* ATCC 25922, and *Klebsiella pneumonia* ATCC 700603 as per the manufacturer's instructions. Each new lot ID and AST panel also verified using the above QC strains and the obtained results were compared against the given expected results of each QC organism on the package inserts, to ensure the appropriate setup procedure and acceptable performance of the system.

Post analytic phase*;* All captured results including dependent variables, independent variables were checked at a time of enrolment for their completeness during data entry and during result analysis. The double entrance was used to check the correctness of the information.

4.8. Data analysis and interpretation

The data entry and analysis were done using SPSS version 20. The analysis of descriptive statistics was used to see the relationship between the dependent variable and independent variables. Then the determined frequencies of different variables were compared. Lastly, the results were presented in words, tables, and figures.

4.9. Ethical Considerations

The study was carried out after the approval of the Institutional Review Board of the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University (DRERC/566/20/MLS), and permission were taken from EPHI institutional

review board and NCBMRL. All the information gathered from the study subjects was coded to maintain confidentiality.

4.10. Dissemination of Results

The finding of this study will be presented to the college of health sciences, department of medical laboratory sciences, Addis Ababa University as the partial fulfillment of the Master's program, then accessible for health care communities, clinicians, for policymakers, and at local and international conferences. Moreover, the result of the study will be disseminated to the international scientific community through publication in high-profile peer-reviewed clinical research journals.

4.11. Operational Definitions

- **Clinical Isolate;** a population of microorganisms that have been obtained in pure culture from a clinical specimen.
- **Multiple Drug Resistance (MDR);** indicates resistance *Pseudomonas* species to at least one antimicrobial drug in three or more antimicrobial categories
- **Extensive Drug Resistance (XDR);** is the non-susceptibility of *Pseudomonas* species to all antimicrobial agents except in two or less antimicrobial categories
- **Pan Drug Resistance (PDR);** indicates *Pseudomonas* species resistance to all antimicrobial agents in all antimicrobial categories except polymyxin B (colistin)
- **Carbapenemase;** an enzyme produced by the bacteria, which can hydrolyze penicillin's, cephalosporins, monobactams, and carbapenem antimicrobial categories
- **Antimicrobial Resistance;** is the ability of *Pseudomonas* species to resist the effects of antibiotics to which they were once sensitive

5. RESULTS

5.1.General Description of the *Pseudomonas* isolates

Altogether 110 presumptive *Pseudomonas spp.* isolates were tested, and 100 isolates were found to be *Pseudomonas spp.* The remaining isolates belonged to other gram-negative species. Among the 100 isolates 98% were *P.aeruginosa*, and *P. putida* was found in 2%, with the mean confidence value of 97% according to the BD Phoenix identification system. The maximum numbers of isolates were detected among 21-30 years age groups and male study subjects were most commonly affected by *Pseudomonas* infection with a rate of 63%. The detail of the sex and age characteristics of the study subjects has been shown in Table 1 below.

Table1. Sex and Age distribution of patients with *Pseudomonas* infection at national reference laboratory Ethiopia, January 2017- March 2021

Age Groups	Sex		
	Female	**Male**	**Total**
≤10	2	7	9
11-20	5	7	12
21-30	10	20	**30**
31-40	8	7	15
41-50	9	10	19
≥60	3	12	15
Total	**37**	**63**	**100**

Most *Pseudomonas* spp were predominantly isolated from the clinical specimen wound (46%), followed by ear swab (18%), urine (15%), sputum (8%), tracheal aspirate (6%), blood (5%), CSF and other body fluid (2%). MDR and XDR isolates were recovered from almost in all specimens and two PDR isolates were particularly identified from tracheal aspirate and urine samples. From the total number of isolates to the highest (34%) were identified in 2018 and the lowest (7%) were identified in the first three months of 2021. The total number of isolates per year has been shown in Figure1 below.

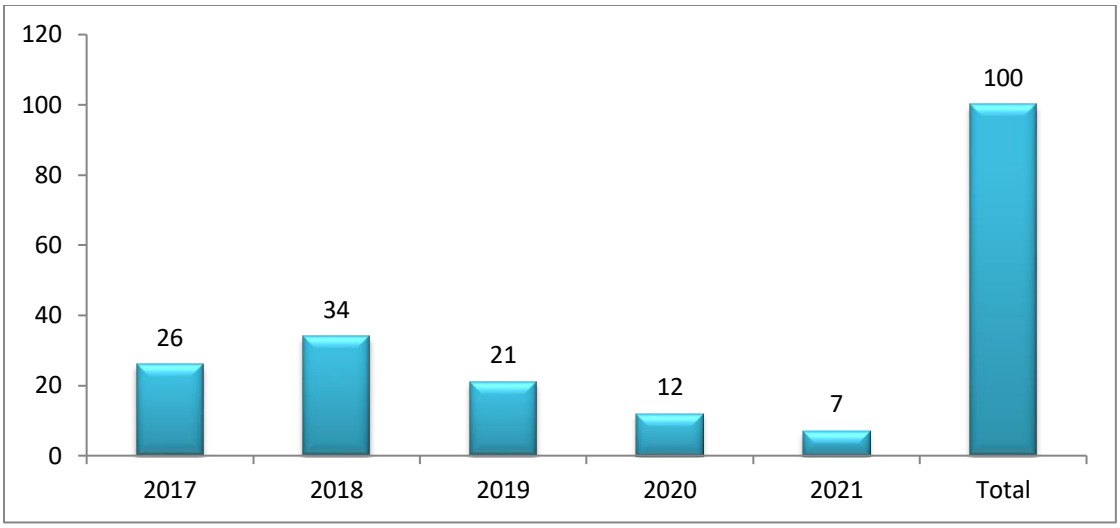

Figure1. Number of *Pseudomonas* recovered from different clinical samples at national reference laboratory Ethiopia, January 2017- March 2021

5.2.Antimicrobial susceptibility profile

The overall resistance rate of *P.aeruginosa* and *P. putida* was higher against Cephalosporins i.e. Ceftazidime (35%) and Cefepime (31%) followed by Fluoroquinolones i.e. Levofloxacin (24%) and Ciprofloxacin (18%), then Carbapenems i.e. Imipenem (18%) and Meropenem (13%). Relatively combination antimicrobials were more active than the above antibiotics; the resistance rate was Ceftolozane-tazobactam (9%) and Piperacillin-tazobactam (16%).

Amikacin (98%) was the most sensitive antimicrobial for *Pseudomonas* infections followed by Colistin (94%) and Gentamicin (93%). Levofloxacin and Imipenem drugs showed higher resistance than Ciprofloxacin and Meropenem drugs respectively. Among the total of 18 imipenem-resistant isolates, 16 (89%) of them were susceptible to Amikacin, 15 (83%), 12(67%), and 11(61%) were susceptible to Gentamicin, Ceftolozane-tazobactam, and Piperacillin-Tazobactam drugs respectively. The proportional level of resistance was increased from year to year in all antimicrobials except Colistin. In contrast, from the total of 100 *Pseudomonas* isolates 34% of them were susceptible to all tested antibiotics (Table 2).

Table2. Antibiotic susceptibility pattern of *Pseudomonas* spp at national reference laboratory Ethiopia, January 2017 to March 2021

Antimicrobial Category	Antibiotics	2017 N=26			2018 N=34			2019 N=21			2020 N=12			2021 N=7			Total N=100		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	%S	%I	%R
Aminoglycosides	Amikacin	26	0	0	34	0	0	20	1	0	11	0	1	6	0	1	97	1	2
	Gentamicin	25	0	1	31	2	1	19	0	2	11	0	1	5	0	2	91	2	7
Cephalosporins	Cefepime	21	0	5	25	0	9	12	0	9	7	0	5	4	0	3	69	0	31
	Ceftazidime	19	1	6	24	0	10	9	0	12	8	1	3	3	0	4	63	2	35
Fluoroquinolones	Ciprofloxacin	23	2	1	29	0	5	12	3	6	9	0	3	4	0	3	77	5	18
	Levofloxacin	22	1	3	23	3	8	11	3	7	9	0	3	4	0	3	69	7	24
Carbapenems	Imipenem	24	2	0	26	2	6	15	0	6	6	2	4	4	1	2	75	7	18
	Meropenem	26	0	0	27	4	3	15	0	6	8	1	3	5	1	1	81	6	13
Polymyxin B	Colistin	20	4	2	33	0	1	18	0	3	10	2	0	6	1	0	87	7	6
β-Lactam combinations	Ceftolozane-tazobactam	25	0	1	32	0	2	18	0	3	10	0	2	5	1	1	90	1	9
	Piperacillin-tazobactam	20	2	4	26	2	6	12	7	2	9	1	2	4	1	2	71	13	16

S=sensitive, **I**=intermediate, **R**=Resistance

The overall MDR, XDR, and PDR rate of *P.aeruginosa* was found to be 23%, 9%, and 2% respectively from the total tested isolates. The proportions of MDR isolates per year showed an increasing pattern for five consecutive years except in 2020. Likewise, the proportion of XDR isolates increased from 4% to 17% from the year 2017 to 2020 rising to 14% in the first three months of 2021. In the latter years, one isolate from 2020 and one another isolate from 2021 proportionally 8% and 14% of PDR isolates were identified (Figure 2).

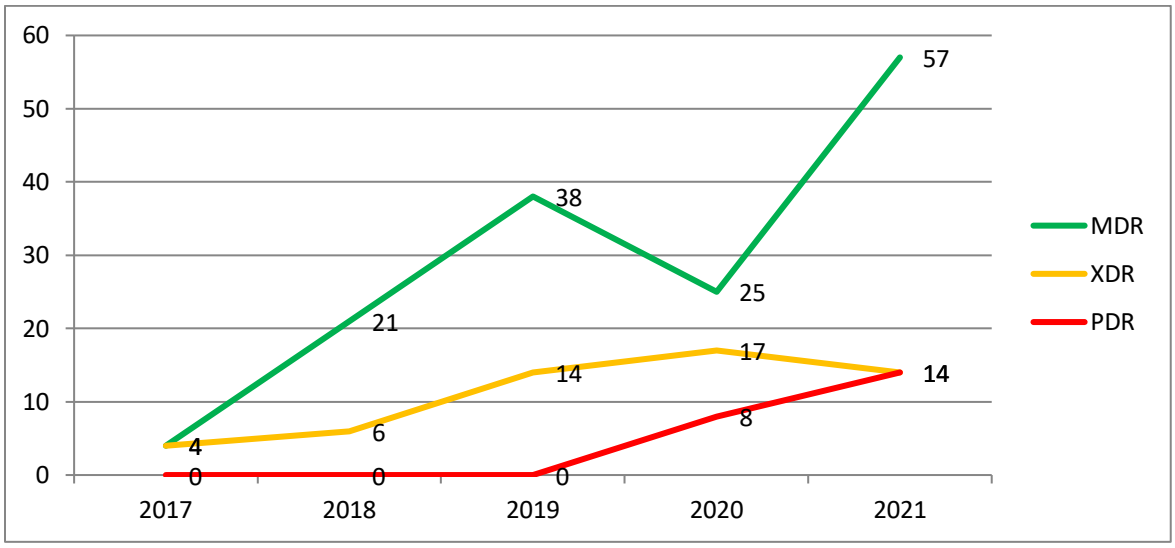

Figure2. Proportions of MDR, XDR, and PDR *P.aeruginosa* and *P.putida* at national reference laboratory Ethiopia, January 2017 to March 2021

Among the total of nine XDR isolates, 6 (67%) of them were resistant to both carbapenem drugs and all 9 were resistant to both cephalosporin drugs. On the other hand, of the 23 MDR isolates, 10 (43%) were resistant to both carbapenem and cephalosporin drugs, and 18 (78%) of them were resistant to both cephalosporin drugs.

5.3.Carbapenemase producing *Pseudomonas* spp

Among 100 identified isolates, 27 isolates were identified as a candidate (non-susceptible to Imipenem and/or Meropenem) for carbapenemase enzyme production testing. Of these 10/27 (37%) were detected as carbapenemase producers by the BD Phoenix automated system from those isolates identified as carbapenemase producers by the system, one isolate was susceptible to both carbapenem drugs, which means that it was not a candidate for carbapenemase testing, Therefore, as per the manufacturer's recommendation, an additional

confirmatory test (mCIM) was performed. Of the 27 isolates tested with the mCIM only 3/27 (11%) were positive, they were also positive by the BD Phoenix system, and the remaining seven isolates were negative by this method. Hence, the reason for the carbapenem resistance phenotype of *P.aeruginosa* might not always be a production of carbapenemase enzymes. One of the three carbapenemase positive isolates by mCIM was PDR and the remaining two isolates were XDR. The mCIM results are shown below in (Figure 3).

Figure3. Modified carbapenem inactivation method results for tested *P.aeruginosa* at national reference laboratory Ethiopia.

In this study, the most commonly used/prescribed antimicrobials such as Amoxicillin-clavulanate, Ampicillin, Cefazolin, Ceftriaxone, Cefuroxime, Ertapenem, Tigecycline, and Trimethoprim-sulfamethoxazole were 100% confirmed as resistant.

6. DISCUSSION

In this study, we have described the antimicrobial resistance profile and carbapenemase production rate of *P.aeruginosa* isolated from referral samples in the EPHI. The isolates were characterized to species level using a BD Phoenix automated system. In this study, 9% (10 out of 110) isolates had been misidentified as *Pseudomonas spp*.by the manual method. They were identified as; *Stenotrophomonas maltophilia (2), Burkholderia cepacia, Alcaligenes feacalis (2), Pantoea agglomerans, Serratia marcescens, Achromobacter sp., Citrobacter farmer, and Providencia rustigianii.* This misidentification could be due to the limited biochemical tests available in the laboratory and the overlapping phenotypic nature of the isolates.

In this study, *P.aeruginosa* (98%) was the most prevalent species and the remaining was *P.putida* (2%) identified as the common cause of human pathogen among the genus *Pseudomonas*. Similar results were reported in Ethiopia and elsewhere in the world (24, 32, 37); these reports included a few uncommon species such as *P. luteola, P. stutzeri, P.mendocina, P. pseudoalcaligenes, and P.fluoresens* which were not seen in this study.

P.aeruginosa is an important cause of nosocomial infection and may be isolated from any clinical specimen. In this study the majority of the isolates were recovered from wound and ear infections (46% and 18%, respectively); this finding is similar to that of another study conducted in Ethiopia (38). This might be due to the environmental spread of *P.aeruginosa* in the health facilities, and contaminated surgical instruments. In addition, the prevalence of this organism in ear infection might be due to its ability to survive in competition over other bacteria(11) (12).

Currently, treatment of *P.aeruginosa* infections is challenging because of its intrinsical resistance to the majority of antibiotics, in addition to diverse acquired resistance mechanisms to adapt the remaining *Anti-pseudomonal* drugs. In the present study, both *Pseudomonas* spp were showed higher resistance to cephalosporins particularly for ceftazidime (35%) and cefepime (31%). This is almost in harmony with the findings done by Adane B in Ethiopia (28% and 20%) (32) and in Iran (35% and 38%) (39) for both drugs respectively. In contrast, our finding is lower than the reports in Uganda (69% and 55%)

(27), in Egypt (91% and 98%) (26), in Mexico (65% and 55%) (22), and in India (66% & 63%) (24) for ceftazidime and cefepime respectively. Unnecessary prescription of cephalosporins leads to genetic alteration of the pathogen; particularly over-production of β – lactamases could be the possible reason in those countries.

The fluoroquinolones; ciprofloxacin (18%), and levofloxacin (24%) were better active than cephalosporins, which was also consistent with the above finding in Ethiopia reported (19.7% and 23%) to ciprofloxacin and levofloxacin respectively (32) and 19% average resistance for both drugs were reported in 2019 Europe AMR surveillance (20). In contrast, Ethiopian scholars reported (35-61%)(30, 38, 40), Uganda (64%)(27), India (67%) (24) resistant rate for ciprofloxacin. The reason for this discrepancy could be due to merged analysis of intermediate and resistant categories, lower isolates or variation in sample size, different study settings & patient conditions, and considerable geographical differences.

In this study, the resistance rate for carbapenems; imipenem (18%) and meropenem (13%) were lower than the above cephalosporins and fluoroquinolones. Thus, those drugs are considered to be good *Anti-pseudomonal* drugs. Even though carbapenemase-producing strains threatened their use (27) and keep in mind that, those drugs are the last resort of drugs for gram-negative infections. Our finding was consistent with the other reports done in Ethiopia (40), Uganda (27), and Europe (20). Despite that higher reported (70%) & (54%) in Mexico (22) and (53%) & (63%) in India (24) for imipenem and meropenem respectively. Comparatively lower resistance rate for those carbapenems in this study might be because of lower prescription practice in addition to higher cost to buy those drugs in Ethiopia.
In this study, Imipenem resistant rate was higher than the meropenem. This could be because of molecular structure variability between the two drugs; meropenem is more potent against *P.aeruginosa* because it passes more quickly through the outer membrane porin-D (*OprD*) (8) In contrast, imipenem has been less active because of associated with a higher risk of membrane selection(41). This finding was similar to the other study conducted in Uganda (27) which reported 19% and14% resistance for imipenem and meropenem respectively.

This study revealed better antimicrobial activities in combination drugs; ceftolozane-tazobactam (9%) and piperacillin-tazobactam (16%) than cephalosporins, fluoroquinolones, and carbapenems alone. Among 18% of imipenem-resistant strains tested for combinations, 67% and 61% of them were susceptible to ceftolozane-tazobactam and piperacillin-tazobactam combinations respectively. Therefore, combination drugs are very helpful therapeutics in case of infections by carbapenem resistance *Pseudomonal* infections. In the same way, utilization of those combinations in clinical practices could reserve carbapenems. While inherent side effects from usage of multiple agents should be considered (41).
Experimental research demonstrated using rabbits showed that, ceftolozane-tazobactam was a highly potent *Anti-pseudomonal* drug that can eradicate genetically distinctive *Pseudomonas* strains having excellent host survival with effective clearance from tissue (42). Various surveillance-based studies also showed greater than 90% susceptibility results on this drug (43) which was in harmony with our finding. Nowadays, this drug is approved by United States Food and Drug Administration as it is confirmed a novel *Anti-pseudomonal* β-lactam/β-lactamase inhibitor combination (44). In Ethiopia, there are no available data regarding combination drugs particularly on ceftolozane-tazobactam.

Aminoglycosides were the most potent *Anti-pseudomonal* drugs in this study having only a 2% resistance rate for amikacin and 7% resistance rate for gentamicin. Those drugs are remaining the most powerful therapeutic options for carbapenem-resistant *Pseudomonas* strains, this study showed among 18 imipenem-resistant strains tested for aminoglycosides; 16 (89%) and 15 (83%) of the isolates were susceptible for amikacin and gentamicin respectively. Our finding on aminoglycosides was comparable with the European surveillance report (20) and relatively lower than the reports in Ethiopia (13-28%) for gentamycin (32, 40), Uganda (31% and 69%) (27), India (58% and 68%) (24), Mexico (58% and 52%) (22) resistant rate for both amikacin and gentamicin respectively. Amikacin is the highly reserved drug used in specialized centers for the treatment of multidrug-resistant tuberculosis in Ethiopia (45) and physicians are not preferred to prescribe this drug since it's not accessible in local pharmacies, this might be the reason for the lower resistance rate reported in this study. Moreover, relatively the report done by Ethiopian scholars seems

higher this could be because of the lower sample size and merged analysis of intermediate and resistant results.

In this study, Colistin was the only treatment of choice for PDR *P.aeruginosa*, despite having a 6% overall resistant rate. This drug was previously not used by clinicians, due to its nephrotoxicity and neurotoxicity effect but nowadays it is being prescribed, and International consensus is that the optimal use of this drug would be for the treatment of infections with MDR gram-negative bacilli, particularly for *P.aeruginosa* and *Acinetobacter* spp (46, 47). As of 2020, Colistin is on the Ethiopian essential medicines list; it is reserved for the treatment of confirmed or suspected infections caused by MDR organisms (45). However, it's not accessible in pharmacies. Our finding supports the introduction of colistin into clinical practice for the treatment of infections caused by PDR *P.aeruginosa*. This has also been seen with clinical research studies, with the clinical outcome of patients. This is needed in the Ethiopian context.

Our study on colistin-resistant rate was nearly similar to the other study conducted by Saderi and Owlia in Iran (39) which was reported 9%, and higher reported in Egypt (23%) (48). The disparity might be because of method variability, and/or prevalence of colistin-resistant strains due to misuse of this drug in veterinary medicine since it has been widely used for the growth promotion of food-producing animals (49). The emergence of colistin-resistant strains of *P.aeruginosa* is highly alarming and it is a serious global problem because of no other choices of drugs at all, in fact, colistin is unsafe for human medicine. In Ethiopia, there is no research previously conducted in this area, thus advanced molecular researches should be encouraged to identify the sequence type, resistance determinants and to have detailed information on such strains.

The overall proportion of MDR, XDR, and PDR isolates of *Pseudomonas* spp in this study was 23%, 9%, and 2% respectively. The definition of those abbreviated words was done according to the international standard document for *P.aeruginosa* (50), using all categories of *Anti-pseudomonal* drugs in Table-2, exception of colistin is not considered in the case of PDR isolates because of the above-stated reasons.

Published literature in Ethiopia regarding *P.aeruginosa* has not followed the above definition; this makes it difficult to compare our findings to the local setting. While our findings on MDR, XDR, and PDR *Pseudomonas* isolates were compared with the five years European antimicrobial resistance surveillance report(20) which was (10%) MDR, (6.2%) XDR, and (3.4%) resistance to five antimicrobial groups. Whereas lower from (55%) MDR, (33%) XDR, and 0% PDR reported in Iran (39). The proportion of MDR and XDR isolates were increased from year to year in our finding, this showed that antimicrobial resistance is progressive and also the occurrence of PDR isolates from the latter two years is highly alarming, and thus prompt actions are needed.

P.aeruginosa has different acquired resistance mechanisms (8, 9), the production of carbapenemase enzyme is the most serious one b/c this enzyme can hydrolyze all penicillin, cephalosporin, and carbapenem drugs. In our finding the rate of carbapenemase enzyme was found to be 10% from all isolates and 37% (10/27) from candidate isolates by the BD phoenix system, while it was 3% from all isolates and 11% (3/27) from candidates using mCIM, this method was investigated by CLSI and demonstrated 100% specificity and >97% sensitivity for the detection of carbapenemases among *P.aeruginosa* (10). Therefore, even though automated systems are efficient for bacterial ID and AST testing, the software in this phoenix system will not always precisely inferring the presence of this enzyme, similar results were reported in Uganda (27). In this regard, we can conclude that majority of carbapenem-resistant *Pseudomonas* isolates in this study were not b/c of the production of carbapenemase enzyme.

7. STRENGTH AND LIMITATION

7.1.Strengths

- The study was conducted in ISO 15189 accredited laboratory since 2016
- Good isolate storage condition in bio-bank and good data archival system at EPHI
- Accurate identification of bacteria to the species level by using an automated technique which has up to date software version
- Employed standard technique for carbapenemase detection using the latest CLSI guideline

7.2.Limitations

- Because of inconsistent result (except resistant one) in the BD Phoenix system, the MIC value of colistin was interpreted manually referring to the most recent CLSI guideline
- Two *Anti-pseudomonal* drugs; Piperacillin and Aztreonam were not tested because those drugs are not incorporated in the AST panel.
- Total referral samples and the number of *Pseudomonas* isolates were relatively declined from the latter years of the study period because of the covid-19 pandemic, and only three month's data was taken from 2021, which makes it difficult to do trend/association between the study period and resistance pattern of this organism.

8. CONCLUSION AND RECOMMENDATION

8.1.Conclusions

In this study, the proportionally increasing rate of MDR, XDR isolates, and the appearance of the PDR *Pseudomonas* strain in Ethiopia is a serious problem. The lack of newer alternative *Anti-pseudomonal* drugs is making things worrisome. Utilization of colistin in clinical practice in case of infection by PDR *Pseudomonas* strains has a life salvage advantage. In the same way, aminoglycosides and combination drugs particularly ceftolozane-tazobactam were very helpful therapeutics in case of infection by carbapenem resistance *Pseudomonas* strains.

8.2.Recommendations

- Antimicrobials have different efficacy in various geographical locations; therefore updating the treatment guidelines in the local setting using such kinds of studies is very crucial.
- Treatment of bacterial infections should be guided by antibiotic susceptibility test results. Thus, expansion of microbiology laboratory is vital for accurate antimicrobial choice.
- Maximizing the number of biochemical tests and other supplies or utilization of automated systems in the microbiology laboratory is very essential for the accurate identification of pathogens
- Isolation of patients in case of XDR and PDR bacterial infection is advantageous to control the spread of serious nosocomial infection
- Advocacy and maximizing awareness to the community level, strengthening infection prevention team, and antimicrobial stewardship committee in the health facilities are very important.
- Continuous nationwide surveillances are essential; to combating, tracing, and for early detection of emerging resistance strains and to characterize the national spread.

9. REFERENCES

1. Moore NM, Flaws ML. Introduction: Pseudomonas aeruginosa. Clinical laboratory science. 2011;24(1):41.
2. Tindall BJ, Kämpfer P, Euzéby JP, Oren A. Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. International Journal of Systematic and Evolutionary Microbiology. 2006;56(11):2715-20.
3. Özen AI, Ussery DW. Defining the Pseudomonas genus: where do we draw the line with Azotobacter? Microbial ecology. 2012;63(2):239-48.
4. Fujitani S, Moffett KS, Yu V. Pseudomonas aeruginosa. Antimicrobe: Infectious Disease & Antimicrobial Agents http://www antimicrobe org/new/b112 asp. 2017.
5. Karami P, Mohajeri P, Mashouf RY, Karami M, Yaghoobi MH, Dastan D, et al. Molecular characterization of clinical and environmental Pseudomonas aeruginosa isolated in a burn center. Saudi journal of biological sciences. 2019;26(7):1731-6.
6. Safaei HG, Moghim S, Isfahani BN, Fazeli H, Poursina F, Yadegari S, et al. Distribution of the strains of multidrug-resistant, extensively drug-resistant, and pandrug-resistant Pseudomonas aeruginosa isolates from burn patients. Advanced biomedical research. 2017;6.
7. Horcajada JP, Montero M, Oliver A, Sorlí L, Luque S, Gómez-Zorrilla S, et al. Epidemiology and treatment of multidrug-resistant and extensively drug-resistant Pseudomonas aeruginosa infections. Clinical microbiology reviews. 2019;32(4):e00031-19.
8. Meletis G, Exindari M, Vavatsi N, Sofianou D, Diza E. Mechanisms responsible for the emergence of carbapenem resistance in Pseudomonas aeruginosa. Hippokratia. 2012;16(4):303.
9. Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk clones. Drug Resistance Updates. 2015;21:41-59.
10. CLSI. *Performance standards for antimicrobial susceptibility testing*.30th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standard Institute; 2020. .
11. Davane M, Suryawanshi N, Pichare A, Nagoba BS. Pseudomonas aeruginosa from hospital environment. Journal of Microbiology and Infectious Diseases. 2014;4(01).
12. de Abreu PM, Farias PG, Paiva GS, Almeida AM, Morais PV. Persistence of microbial communities including Pseudomonas aeruginosa in a hospital environment: a potential health hazard. BMC microbiology. 2014;14(1):118.

13. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. Geneva: World Health Organization; (WHO/EMP/IAU/2017.12). (Licence: CC BY-NC-SA 3.0 IGO.. 2017; Available from: https://www.who.int/medicines/areas/rational_use/PPLreport_2017_09_19.pdf?ua=1.

14. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2020. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO. Available from: https://apps.who.int/iris/bitstream/handle/10665/332081/9789240005587-eng.pdf?ua=1.

15. Interagency Coordination Group on Antimicrobial Resistance; No Time to Wait: Securing the Future from Drug-Resistant Infections Report to the Secretary-General of the United Nations; April. 2019 Available from: https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG_final_report_EN.pdf?ua=1.

16. Morales E, Cots F, Sala M, Comas M, Belvis F, Riu M, et al. Hospital costs of nosocomial multi-drug resistant Pseudomonas aeruginosa acquisition. BMC Health Services Research. 2012;12(1):122.

17. CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC;. 2019.; Available from: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf.

18. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. The Lancet infectious diseases. 2019;19(1):56-66.

19. Balabanova Y, Gilsdorf A, Buda S, Burger R, Eckmanns T, Gärtner B, et al. Communicable diseases prioritized for surveillance and epidemiological research: results of a standardized prioritization procedure in Germany, 2011. PloS one. 2011;6(10):e25691.

20. European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2019. Stockholm: ECDC; 2020.; Available from: https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2019.pdf.

21. European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe 2018. Stockholm: ECDC; 2019.; Available from:

https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2018.pdf.

22. Uc-Cachón AH, Gracida-Osorno C, Luna-Chi IG, Jiménez-Guillermo JG, Molina-Salinas GM. High prevalence of antimicrobial resistance among gram-negative isolated Bacilli in intensive care units at a tertiary-care hospital in Yucatan Mexico. Medicina. 2019;55(9):588.

23. Azimi T, Maham S, Fallah F, Azimi L, Gholinejad Z. Evaluating the antimicrobial resistance patterns among major bacterial pathogens isolated from clinical specimens taken from patients in Mofid Children's Hospital, Tehran, Iran: 2013–2018. Infection and drug resistance. 2019;12:2089.

24. Kumari M, Khurana S, Bhardwaj N, Malhotra R, Mathur P. Pathogen burden & associated antibiogram of Pseudomonas spp. in a tertiary care hospital of India. The Indian Journal of Medical Research. 2019;149(2):295.

25. Kaur A, Singh S. Prevalence of Extended Spectrum Betalactamase (ESBL) and Metallobetalactamase (MBL) producing Pseudomonas aeruginosa and acinetobacter baumannii isolated from various clinical samples. Journal of pathogens. 2018;2018.

26. Mahmoud AB, Zahran WA, Hindawi GR, Labib AZ, Galal R. Prevalence of multidrug-resistant Pseudomonas aeruginosa in patients with nosocomial infections at a university hospital in Egypt, with special reference to typing methods. J Virol Microbiol. 2013;13:165-59.

27. Kateete DP, Nakanjako R, Namugenyi J, Erume J, Joloba ML, Najjuka CF. Carbapenem resistant Pseudomonas aeruginosa and Acinetobacter baumannii at Mulago Hospital in Kampala, Uganda (2007–2009). Springerplus. 2016;5(1):1-11.

28. Kilivwa J.S. Mukaya SMN, ohn Maina, B.A. Museve, A.K. Nyerere and J.N. Kiiru. Prevalence of Multidrug-Resistant Pseudomonas aeruginosa at Kenyatta National Hospital. Asian Journal of Medical Sciences. 2018;9(1):6-9. Epub 2018

29. Solomon FB, Wadilo F, Tufa EG, Mitiku M. Extended spectrum and metalo beta-lactamase producing airborne Pseudomonas aeruginosa and Acinetobacter baumanii in restricted settings of a referral hospital: a neglected condition. Antimicrobial Resistance & Infection Control. 2017;6(1):106.

30. Motbainor H, Bereded F, Mulu W. Multi-drug resistance of blood stream, urinary tract and surgical site nosocomial infections of Acinetobacter baumannii and Pseudomonas aeruginosa

among patients hospitalized at Felegehiwot referral hospital, Northwest Ethiopia: a cross-sectional study. BMC Infectious Diseases. 2020;20(1):92.

31. Bekele T, Tesfaye A, Sewunet T, Waktola HD. Pseudomonas aeruginosa isolates and their antimicrobial susceptibility pattern among catheterized patients at Jimma University Teaching Hospital, Jimma, Ethiopia. BMC research notes. 2015;8(1):488.

32. Bitew A. High Prevalence of Multi-Drug Resistance and Extended Spectrum Beta Lactamase Production in Non-Fermenting Gram-Negative Bacilli in Ethiopia. Infectious Diseases: Research and Treatment. 2019;12:1178633719884951.

33. Asres G, Legese M, Woldearegay G. Prevalence of multidrug resistant Bacteria in postoperative wound infections at Tikur Anbessa specialized hospital, Addis Ababa, Ethiopia. Arch Med. 2017;9(4):12.

34. Sisay M, Worku T, Edessa D. Microbial epidemiology and antimicrobial resistance patterns of wound infection in Ethiopia: a meta-analysis of laboratory-based cross-sectional studies. BMC Pharmacology and Toxicology. 2019;20(1):35.

35. Phoenix BD. Laboratory procedure. latest released user manual. 2020-03:13-9.

36. Lancaster MV, Fields RD. Antibiotic and cytotoxic drug susceptibility assays using resazurin and poising agents. Google Patents; 1996.

37. England PH. Identification of Pseudomonas species and other NonGlucose Fermenters. UK Standards for Microbiology Investigations. ID 17 Issue 3. 2015; Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/422699/ID_17i3.pdf.

38. Mulu W, Abera B, Yimer M, Hailu T, Ayele H, Abate D. Bacterial agents and antibiotic resistance profiles of infections from different sites that occurred among patients at Debre Markos Referral Hospital, Ethiopia: a cross-sectional study. BMC research notes. 2017;10(1):1-9.

39. Saderi H, Owlia P. Detection of multidrug resistant (MDR) and extremely drug resistant (XDR) P. aeruginosa isolated from patients in Tehran, Iran. Iranian journal of pathology. 2015;10(4):265.

40. Mekonnen H, Seid A, Molla G, Gebrecherkos T. Antimicrobial Resistance Profiles and associated factors of Acinetobacter and Pseudomonas aeruginosa nosocomial infection among

patients admitted at Dessie comprehensive specialized Hospital, North-East Ethiopia. A cross sectional study. 2021.

41. Kanj SS, Kanafani ZA, editors. Current concepts in antimicrobial therapy against resistant gram-negative organisms: extended-spectrum β-lactamase–producing enterobacteriaceae, carbapenem-resistant enterobacteriaceae, and multidrug-resistant Pseudomonas aeruginosa. Mayo Clinic Proceedings; 2011: Elsevier.

42. Petraitis V, Petraitiene R, Naing E, Aung T, Thi WP, Kavaliauskas P, et al. Ceftolozane-tazobactam in the treatment of experimental Pseudomonas aeruginosa pneumonia in persistently neutropenic rabbits: impact on strains with genetically defined mechanisms of resistance. Antimicrobial agents and chemotherapy. 2019;63(9).

43. Farrell DJ, Flamm RK, Sader HS, Jones RN. Antimicrobial activity of ceftolozane-tazobactam tested against Enterobacteriaceae and Pseudomonas aeruginosa with various resistance patterns isolated in US hospitals (2011-2012). Antimicrobial agents and chemotherapy. 2013;57(12):6305.

44. Cho JC, Fiorenza MA, Estrada SJ. Ceftolozane/Tazobactam: A Novel Cephalosporin/β-Lactamase Inhibitor Combination. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2015;35(7):701-15.

45. Authority MoHEFaD. Ethiopian Essential Medicines List. 2020; Available from: http://www.fmhaca.gov.et/wp-content/uploads/2020/12/EML-sixth-edition.pdf.

46. Dijkmans AC, Wilms EB, Kamerling IM, Birkhoff W, Ortiz-Zacarías NV, van Nieuwkoop C, et al. Colistin: revival of an old polymyxin antibiotic. Therapeutic drug monitoring. 2015;37(4):419-27.

47. Tsuji BT, Pogue JM, Zavascki AP, Paul M, Daikos GL, Forrest A, et al. International Consensus Guidelines for the Optimal Use of the Polymyxins: Endorsed by the American College of Clinical Pharmacy (ACCP), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Infectious Diseases Society of America (IDSA), International Society for Anti-infective Pharmacology (ISAP), Society of Critical Care Medicine (SCCM), and Society of Infectious Diseases Pharmacists (SIDP). Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2019;39(1):10-39.

48. Abd El-Baky RM, Masoud SM, Mohamed DS, Waly NG, Shafik EA, Mohareb DA, et al. Prevalence and some possible mechanisms of colistin resistance among multidrug-resistant and extensively drug-resistant Pseudomonas aeruginosa. Infection and drug resistance. 2020;13:323.
49. Organization WH. The detection and reporting of colistin resistance. . 2018; Available from: https://apps.who.int/iris/bitstream/handle/10665/277175/WHO-WSI-AMR-2018.4-eng.pdf?sequence=1&isAllowed=y.
50. Magiorakos A-P, Srinivasan A, Carey Rt, Carmeli Y, Falagas Mt, Giske Ct, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection. 2012;18(3):268-81.

10. ANNEXES

Annexe-1: Strain Refreshment Log sheet

No	Laboratory Id number	Date Stored	Specimen Type	Name of organism	Refrigerator No	Rack No	Box No	Cell No	Remarks
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									

Annexe-2: Laboratory procedure for identification and AST testing by BD phoenix system

1. Confirm the Gram stain reaction and select the appropriate Phoenix panel for inoculation.
2. Place the panel on the inoculation station with ports at the top and pad on the bottom.
3. Label a Phoenix ID Broth tube with the patient's specimen number. Using aseptic technique, pick colonies of the same morphology with the tip of a sterile cotton swab
4. Suspend the colonies in the Phoenix ID Broth (4.5 mL).
5. Cap the tube and vortex for 5 seconds.
6. Allow approximately ten seconds for air bubbles to surface.
7. Insert the tube into the BBL CrystalSpec or BD PhoenixSpec Nephelometer.
8. If the inoculum density is set to 0.5 McFarland for the panel type being run, then a range of 0.50-0.60 is acceptable. If the inoculum density is set to 0.25 for the panel type being run, then a range of 0.20-0.30 is acceptable.
9. Label a Phoenix AST Broth tube (8.0 mL) with the patient's specimen number. Holding the AST Indicator Solution bottle vertically, add one free-falling drop of AST indicator solution to the AST broth tube. Invert to mix. DO NOT VORTEX.
10. If an inoculum density of 0.50 – 0.60 was used, transfer 25 µL or If an inoculum density of 0.20 – 0.30 was used transfer 50 µL of the bacterial suspension from the ID tube into the AST broth tube.
11. Cap the AST tube and invert several times to mix. Do not vortex.
12. Wait a few seconds for air bubbles to surface. Tap the tube gently to aid in eliminating bubbles.
13. Pour the ID tube inoculum into the fill port on the ID side of the panel (51-well side).
14. Pour the AST tube inoculum into the fill port on the AST side of the panel (85-well side).
15. Snap on the panel closure. Make sure that the closure is fully seated.
16. Loaded the Panels into the instrument within 30 minutes.

Annexe-3: Laboratory testing procedure for modified Carbapenemase Inactivation Method (mCIM)

1. Emulsify a 10 µL loop full of candidate *P.aeruginosa* from an overnight incubated from BAP and emulsified in 2 ml TSB.
2. Vortex for 10-15 seconds
3. Then add 10µg meropenem disk to each tube using sterile forceps. Ensure the entire disk is immersed in the suspension
4. Then incubated at 35-37 ^{o}C in ambient air for 4 hours +15 minutes.
5. Just before or immediately following completion of the TSB-meropenem disk suspension incubation, prepare 0.5 McFarland suspension (using the direct colony suspension method) of *E.coli* ATCC 25922 in nutrient broth or saline
6. Then inoculate MHA plate with *E.coli* ATCC 25922 as the routine disk diffusion procedure make sure the inoculum suspension preparation and MHA plate inoculation steps are each completed within 15 minutes .Allow the plates to dry for 3-10 minutes before adding the meropenem disks
7. Remove the meropenem disk from each TSB-meropenem disk suspension using a 10 µL loop by placing the flate side of the loop aginst the flat edge of the dick and using surface tension to pull the disk out the liquid. Carefully drag and press the loop along the inside edge of the tube to expel excess liquid from the disk. Contiue using the loop to remove the disk from the tube and then place it on the MHA plate previously incoulated with meropenum-suspension *E.coli* ATCC 25922 indicator strain. Disk capacity ; 4 disks on a 100 mm MHA plate ; 8 disks on a 150 mm MHA
8. Invert and inculate the MHA plates at 35-37 ^{o}C in ambinet air for 18-24 hours
9. followinf incubation, measure the zone size of inhibition as for the routin disk diffusion method

Interpretation

a. Carbapenemase positive

- Zone diameter of 6-15 mm or pinpoint colonies within a 16–18 mm zone
- If the test isolate produce a carbapenemase, the meropenem in the disk will be hydrolyzed and there will be no inhibition or limited growth of the meropenem-suspension *E.coli* ATCC 25922

b. Carbapenemase Negative

- Zone diameter of ≥19 mm (clear zone)
- If the test isolate does not produce a carbapenemase, the meropenem in the disk will be hydrolyzed and there will not be not inhibition or limited growth of the meropenem-suspension *E.coli* ATCC 25922

c. Carbapenemase indeterminate

- Zone diameter of 16-18 mm
- Zone diameter of ≥19 mm and the presence of pinpoint colonies within the zone
- The presence or absence of a carbapenemase cannot confirmed

11. 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 duly acknowledged.

M.Sc. candidate: Tesfa Addis (B.Sc.)

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: Kassu Desta (MSc, PhD candidate)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: Shambel Araya (MSc)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.