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PREVALENCE AND ANTIBIOTIC RESISTANCE OF MBL-PRODUCING GRAM-NEGATIVE RODS IN A TERTIARY CARE HOSPITAL

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ABSTRACT
Antibiotic-resistant infections are becoming a global threat to public health as these are associated with higher morbidity and mortality rates. Various types of β-lactamases including metallo-beta lactamases (MBLs) are contributing to rapid dissemination of drug resistance among pathogens. This study was conducted to determine the antibiotic susceptibility profile of and prevalence of MBL producers among Gram-negative bacilli isolated from a Southern Punjab sub-population from January to June 2024. For this purpose, a total of 300 clinical specimens were cultured for bacterial isolation followed by Gram-staining. 100 isolates of Gram-negative rods were biochemically identified in a tertiary care hospital, followed by antibiotic susceptibility testing using Kirby-Bauer disc diffusion method and MBL detection using double disk synergy and combined disk tests. The isolates included Pseudomonas spp. (N=48), E. coli (N=25), Klebsiella spp. (N=12), Proteus spp. (N=8), and Acinetobacter spp. (N=7). Colistin was the most effective antibiotic as none of the isolates was resistant to it. Tigecycline was also highly effective with a resistance rate of 30%. Amikacin, Ampicillin, Cefixime, and Ceftriaxone were the most ineffective antibiotics as all of the isolates were resistant to these. Ceftazidime, Gentamicin, and Sulfamethoxazole/Trimethoprim were also largely ineffective because ≥80% of the isolates manifested resistance to these. Overall, 61 isolates were likely MBL-producers and 39 were not. The highest prevalence of MBL was observed among Acinetobacter spp. (86%) followed by E. coli (68.0%), Proteus spp., (63%), Pseudomonas spp. (60%), and Klebsiella spp. (33.3%), respectively. A high prevalence of antibiotic resistance and MBL production among Gram-negative rods is highly concerning. Hence, implementing and maintaining stringent infection control measures and mitigating the spread of antibiotic resistance is the need of the hour.
Keywords: *Gram-negative bacilli; MBL; Pseudomonas; E. coli; Antibiotic resistance*

INTRODUCTION

The worldwide increase in resistance to antibiotics poses a danger to antimicrobial therapy. This is particularly relevant to Gram-negative bacteria, where β-lactamases play a critical role in mediating resistance to β-lactams (Walsh et al., 2005; WHO, 2015). Approximately, 340 types of β-lactamases exist which mainly consist of ESBLs, AmpC, and MBLs. As plasmids encode the extended-spectrum-beta-

lactamases (ESBLs), these can rapidly spread between species, and become a threat. In recent years, metallo-beta lactamase (MBL)-induced antibiotic resistance has been more often observed in clinical isolates from all over the world. MBLs are chromosomal β-lactamases that have been recovered from a variety of bacteria (Bush and Bradford, 2020). One major global public health concern is the Enterobacteriaceae's rapidly rising rate of MBL production, which primarily affects E. coli and K. pneumoniae, the two most frequently encountered human pathogens (Bora et al., 2014).
Numerous community, medical, and industrial environments have been found to harbor bacteria with MBLs. Not much research has been done on Pakistan's MBLs' resistance pattern. Prior to 2000, imipenem resistance was only infrequently available in Pakistan (Khan et al., 2004). Later, P. aeruginosa in Lahore and Karachi was found to be primarily resistant to carbapenem (Irfan et al., 2011; Khan et al., 2014). According to a recent report in Rawalpindi, 78% of the isolates produced MBL, with Acinetobacter baumannii and Pseudomonas aeruginosa showing the highest frequency of MBL production (Mudasir et al., 2021). Even though MBL-producing strains provide a significant therapeutic challenge, there is surprisingly little clinical information to date identifying the best course of action for infections caused by these strains.
There are reasonable concerns regarding the lack of effectiveness of available medicines because several MBL producers show widespread resistance in vitro. But, as susceptibility testing is usually conducted in media like cation-adjusted Mueller-Hinton broth with high zinc concentrations and because the host immune system imposes a state of zinc deprivation during infection, there is evidence that in vivo resistance to carbapenems may be less than it appears in vitro (Gammoh and Rink, 2017). In addition to impeding MBLs' ability to catalyze reactions, this zinc deficiency may also disrupt the folding of their proteins and encourage the periplasmic destruction of the enzyme (González et al., 2016; Asempa et al., 2020).
Among the available antibiotics, Aztreonam is stable against MBLs, but it loses its effectiveness against organisms that additionally produce AmpC or ESBLs (Crandon and Nicolau, 2013). There isn't much clinical evidence of aztreonam as monotherapy for MBL producers; however, there was a modest success when aztreonam was paired with ceftazidime-avibactam, which inhibits ESBLs (Davido et al., 2017). Currently, the cornerstone of treatment for infections caused by MBL producers is Colistin. Fosfomycin frequently maintains its complete in vitro action against Enterobacterales that produce MBL and has recently shown promise in intravenous treatment of chronic ulcerative reflux disease (Kaye et al., 2019). Although it may be useful against Enterobacterales that produce MBL, especially E. coli, its major recommendation is to use it in combination because of worries about resistance emergence, especially in Klebsiella spp. (Demir and Buyukguclu, 2017). Fosfomycin and Meropenem exhibit in vitro synergy against MBL-producing P. aeruginosa strains (Albiero et al., 2019). Keeping in view the necessity of gaining knowledge regarding the antibiotic susceptibility pattern of MBL producers, the present study aimed at determining the prevalence and antibiotic susceptibility profile of MBL producers among Gram-negative bacteria isolated from the local population of South Punjab, Pakistan.

Methods

Place and duration of study

The present study was performed at the Microbiology lab of Nishtar Hospital, Multan, Pakistan from January to June 2024. Clinical samples for this microbiological study were collected from patients at indoor/outdoor departments and burn wards of Nishtar Hospital. A total of 300 patients were included belonging to the age group of 20-60 years and both genders. Various types of samples were collected, including urine, wound swabs, blood, sputum, tracheal secretions, pus, stool and high vaginal swab. Informed consent was obtained from each patient (or guardian) before sample collection.

Isolation and characterization of isolates

These clinical specimens were immediately transferred to the Microbiology lab for further processing. Blood and MacConkey agar were used to isolate bacteria from these specimens. The plates were incubated at 37°C overnight. Subsequently, Gram-staining was performed and a total of 100 Gram-negative rods were selected for biochemical characterization. No more than one isolate was selected for each specimen. Biochemical characterization was done following Bergey's manual of systematic bacteriology. These included catalase, oxidase, coagulase, indole, citrate utilization, TSI, and SIM tests.

Antibiotic susceptibility profiling

The Kirby-Bauer disc diffusion method was used to determine the antibiotic susceptibility profile of isolates. A fresh bacterial suspension was inoculated onto Mueller-Hinton agar plates. Antibiotic discs were aseptically placed in the wells of Petri plates at a 1.5cm distance. The plates were incubated at 37°C overnight. The ruler was used to measure the zones of inhibition as sensitive (S), intermediate sensitive (IS), and resistant (R) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Commercially available antibiotic discs (Oxoid™) were used including Amikacin (AK), Ampicillin (AMP) Aztreonam (ATM), Cefixime (CFM), Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Colistin (CT), Gentamicin (CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MRP), Moxifloxacin (MXF), Piperacillin/Tazobactam (TZP), Sulfamethoxazole/Trimethoprim (SXT), and Tigecycline (TGC).

MBL detection test

MBL detection was accomplished using double disk synergy (DDS) and combined disk (CD) tests. For the DDS test, a bacterial suspension equivalent to 0.5 McFarland standard was inoculated on the Mueller-Hinton agar plate. Discs containing Imipenem (30 µg) and Ceftazidime (30 µg) were placed around wells containing inhibitor solution that was added to a blank disk. EDTA was used as an inhibitor. If the zone of inhibition was found to be enhanced between antimicrobial and inhibitor disks, it was considered a positive result indicative of MBL production. For the CD test, inhibitor solution was directly added to the discs containing Imipenem (30 µg) and Ceftazidime (30 µg) which were aseptically placed in the wells of Mueller-Hinton agar plates with 0.5 McFarland bacterial suspension. The size of the inhibition zone produced by the antibiotic and inhibitor was compared with the size of the antibiotic alone to establish a positive result.

Results

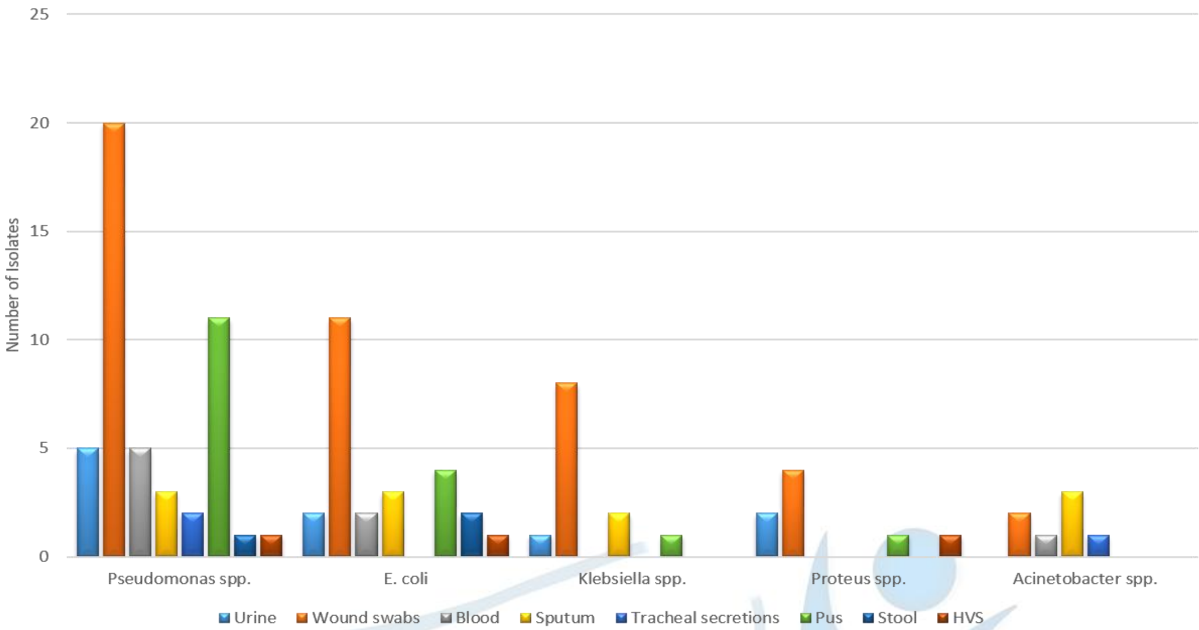
Age and gender distribution

This study provides a comprehensive analysis of Gram-negative bacterial infections and their antibiotic resistance profile in a diverse patient population from January to June 2024. The dataset contains comprehensive demographic data, sample types, bacterial species, and the outcomes of antibiotic sensitivity tests for males and females between the ages of 20 and 60 years. Out of 100 samples that were selected, 69 were males and 31 were females. In terms of age, 33 were 20-29 years old, 34 were 30-39 years old, 22 were 40-49 years old, and 11 were 50-59 years old.

Prevalence of bacterial isolates

A total of 100 Gram-negative rods were identified based on biochemical tests and these included Pseudomonas spp. (N=48), E. coli (N=25), Klebsiella spp. (N=12), Proteus spp. (N=8), and Acinetobacter spp. (N=7). Most commonly, bacteria were isolated from wound swabs (N=45), followed by pus (N=17), sputum (N=11), urine (N=10), blood (N=8), tracheal secretions (N=3), stool (N=3), and high vaginal swab (N=3), respectively. As shown in **Figure 1**, Pseudomonas spp. was the most commonly found in wound swabs followed by pus, urine, blood, sputum, tracheal secretions, stool, and high vaginal swabs, respectively. E. coli was mostly isolated from wound swabs, followed by pus, sputum, blood, urine, stool, and high vaginal swabs respectively. Klebsiella spp. was found in wound swabs, sputum, pus, and urine, in decreasing order of prevalence. Proteus spp. occurred mostly in wound swabs, and less commonly in urine, pus, and high vaginal swab. Acinetobacter spp. was more common in sputum and wound swabs, but less frequent in blood and tracheal secretions.

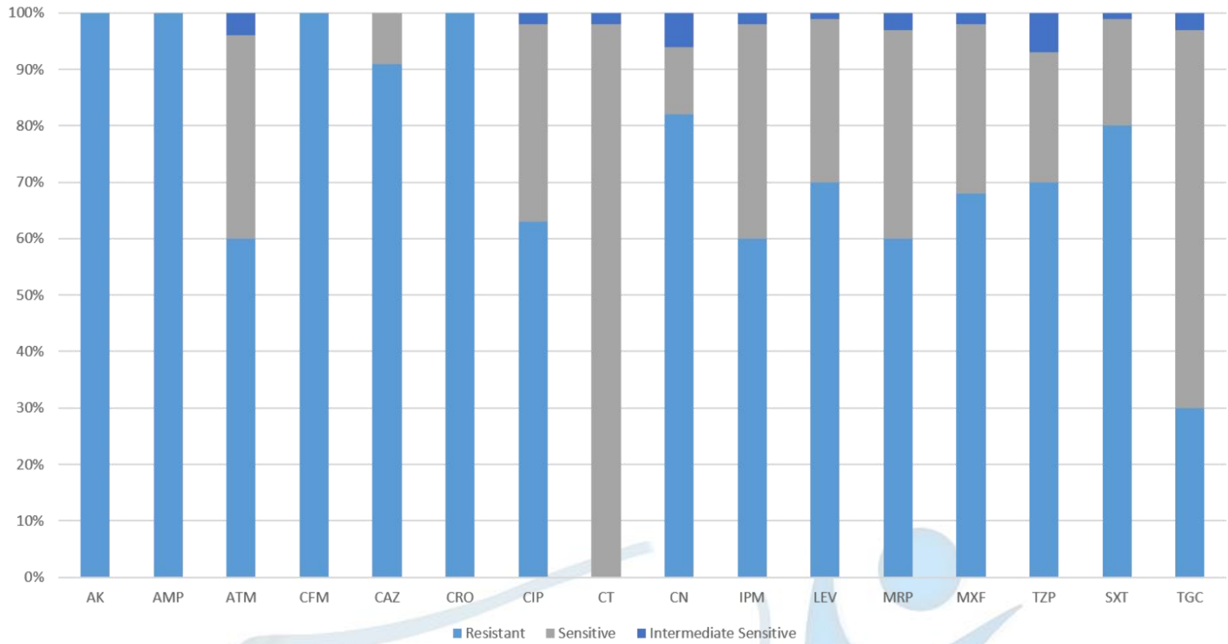
Figure 1: Distribution of bacterial isolates across different clinical specimens



Antibiotic sensitivity profiling of isolates

Antibiotic sensitivity test reveals critical information about the effectiveness of different antibiotics against bacterial isolates. As shown in **Figure** 2, the isolates showed 100% resistance to AK, AMP, CFM, and CRO, indicating that these antibiotics had become completely ineffective against pathogens. On the contrary, CT was the most effective drug as all of the isolates were completely susceptible to it. In terms of efficacy, TGC followed CT as 70% of the isolates were either sensitive or intermediate sensitive to it. ATM, IPM, and MRP were equally effective as manifested by a resistance rate of 60%. Compared to these three antibiotics, CIP showed a slightly higher rate of resistance followed by MXF, LEV, and TZP, in ascending order. CN and SXT showed comparable rate of resistance and were largely ineffective. Finally, CAZ was also nearly ineffective against a vast majority of the isolates.

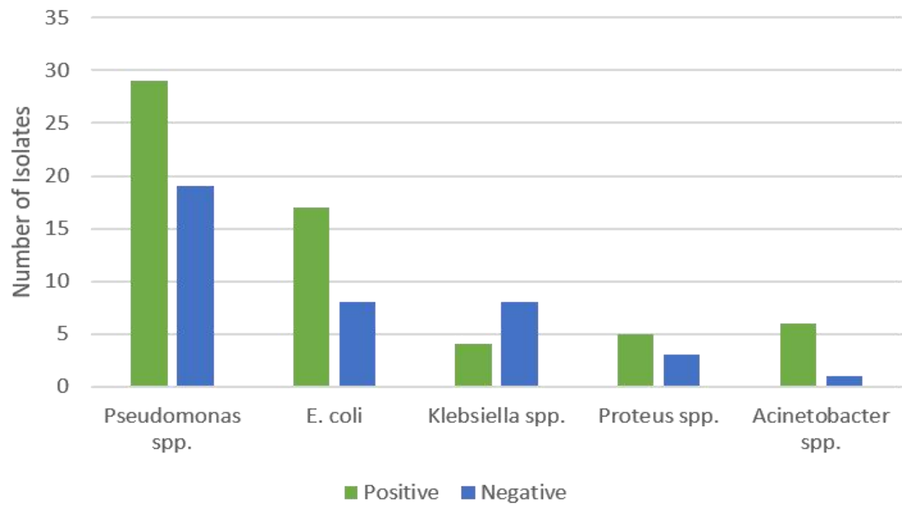
Figure 2: Antibiotic susceptibility test result for bacterial isolates



Prevalence of MBL

MBL detection was done by the DDS and CD tests to identify the production of MBL by Gram-negative bacilli. Among males, 59.4% (N=41) of the isolates yielded positive result for MBL while 40.6% (N=28) were MBL-negative. In females, 64.5% (N=20) of the isolates were MBL-positive while 35.5% (N=11) were negative for MBL. The prevalence of MBL among various bacterial isolates has been shown in **Figure 3**. Among the isolates of Pseudomonas spp., 60.4% were MBL-positive and 39.6% were MBL-negative. In terms of E. coli isolates, 68.0% were MBL-positive and 32.0% were MBL-negative. Furthermore, 33.3% of the Klebsiella spp. were MBL-positive and 66.7% were MBL-negative. With regard to Proteus spp., 62.5% were MBL-positive and 37.5% were MBL-negative. Finally, 85.7% of the isolates of Acinetobacter spp. were MBL-positive and 14.3% were MBL-negative.

Figure 3: Results for MBL detection in bacterial



isolates

Discussion

Multidrug resistance in Gram-negative bacteria is an emerging global threat in the field of medicine, and is important in the management of growing morbidity and mortality due to infections (Garcia-Bustos et al., 2022; Giovagnorio et al., 2023). This work thus offers significant information on the frequency and antibiotic resistance trend of MBL-producing Gram-negative rods, especially among isolates from a tertiary setting. The results are also in accord with the global pattern of growing resistance, thus underscoring the imperative for targeted interventions (Kaur, 2024).

The demographic analysis revealed that there were more infected male persons (69%) than female persons (31%). Additionally, the persons in the age bracket of 30-39 years were more commonly infected (34%). These trends imply further that being of a certain age and gender may make one more susceptible to developing these conditions. The distribution of bacterial isolates shows that the most common organisms are Pseudomonas spp. (48%), E. coli (25%), Klebsiella spp. (12%), Proteus spp. (8%), and Acinetobacter spp. (7%). The largest proportion of the isolates originated from wound swabs (45%); this could be due to the high incidence of wound infection in the target group.

The resistance patterns obtained in this study are quite worrisome, as all the isolates showed resistance to aminoglycosides (AK), ampicillin (AMP), cefixime (CFM), and ceftriaxone (CRO). These consequences are due to the reduced effectiveness of first-generation antibiotics caused by irrational consumption and poor antibiotic management. On the other hand, CT had a 100% efficacy rate; it is now the only option for treating MDR-Gram-negative bacterial infections. Among all the medicines, the highest percentage of susceptibility was observed for Tigecycline (TGC, 70%) which shows that it can be used as an effective alternative. Carbapenems showed moderate levels of resistance ranging from 58%-61%, Aztreonam was nearly in mid-range at 56% while Cipro- and Levofloxacin had a high level of resistance at 75% and 76%, respectively. These results share the same trend as data from across the world that shows carbapenem resistance to have emerged rather quickly, especially in hospital-acquired infections (Mancuso et al., 2023; Wise et al., 2024).

The study further confirmed high percentages of MBL production at 68% among E. coli and 85.7% among Acinetobacter spp. These results suggest how MBLs are involved in resistance to beta-lactams and carbapenems (Garcia-Bustos et al., 2022). The results stress the imperative for species-specific and targeted antimicrobial therapy according to susceptibility (Bassetti et al., 2024). Infections related to MBL-producing organisms require proper use of effective antibiotics like Colistin and Tigecycline as well as avoiding the use of ineffective antibiotics to reduce compromise of resistance (Bandić Pavlović et al., 2024). Further, there is a requirement for effective measures of disease control such as widespread and strict precaution measures including proper washing, isolation of patients with contagious diseases, and actual measures in cleaning the environment from contagious diseases to minimize nosocomial infections.

By applying two-way ANOVA analysis, a significant (p=<0.05) prevalence of antibiotic-resistant bacteria was observed among isolates from different sites. The observed resistance patterns in this study align with the current global trend, especially in terms of cases arising from hospital-associated infections, where the main isolated bacteria are Pseudomonas spp. E. coli, and Acinetobacter spp. (Garcia-Bustos et al., 2022; Giovagnorio et al., 2023). Carbapenems and beta-lactams are now facing increasing levels of resistance and globally coordinated programs for surveillance of resistance, education on the prudent use of antibiotics, as well as support for the innovation of new drugs are needed (Tadesse et al., 2023). Furthering understanding of the molecular basis of resistance, identifying potential non-conventional therapeutic approaches, and identifying biomarkers for quick identification of patients for targeted treatments must be the focus of research in the future. Specific measures pursued in health promotion campaigns as well as modifications in health policies are crucial to calm unnecessary and improper use of antibiotics contributing to the prevention of dissemination of resistance among bacteria.

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Competing Interests
The authors have no relevant financial or non-financial interests to disclose.

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Data Availability Statement
All data generated or analyzed during this study have been included in this article.

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