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Assessment and analysis of polymyxin sensitivity using the disc elution method on clinical isolates resistant to carbapenems

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Abstract

Elevated mortality rates have drawn the focus of researchers to multidrug-resistant Gram-negative *Enterobacteriaceae* over the last decade. In the case of multidrug-resistant Gram-negative bacteria, particularly carbapenem-resistant *Enterobacteriaceae* (Enterobacterales), polymyxin B stands as one of the last-resort antibiotics. This study aimed to evaluate clinical isolates of Gram-negative bacteria displaying resistance to carbapenems and subjected them to polymyxin B using the disc diffusion method. Clinical specimens were gathered from various anatomical sites, and bacterial isolates were obtained through culturing. After isolation, biochemical testing was conducted for bacterial identification. The identified isolates were then subjected to antibiotic susceptibility testing, employing the Kirby-Bauer Disc Diffusion technique. A comparative analysis was undertaken between the disc elution method and the disc diffusion method, with validation against the reference Broth Microdilution (BMD) technique. A total of one hundred and fifty-two (152) clinical specimens were obtained. The isolates primarily comprised *Klebsiella pneumoniae* (*n* = 49), *Escherichia coli* (*n* = 41), *Pseudomonas aeruginosa* (*n* = 31), *Acinetobacter baumannii* (*n* = 29), and *Proteus vulgaris* (*n* = 02). Diverse specimen types were collected from various patient sites, encompassing pus swabs (*n* = 82), urine (*n* = 36), tracheal tubes (*n* = 12), sputum (*n* = 11), blood cultures (*n* = 6), bronchial washings (*n* = 2), high vaginal swabs (*n* = 2), and cerebrospinal fluid (*n* = 1). The distribution of patients was categorized by gender, with 95 females and 57 males. The majority of the strains were identified in patients aged between 41 and 60 years. The disc elution method categorized 127 samples as susceptible, 13 as intermediate, and 12 as resistant to polymyxin B, while the disc diffusion method labeled 128 samples as susceptible and 24 as resistant. In routine antimicrobial susceptibility testing, the disc diffusion method is commonly used for polymyxin B, even though it is not recommended by CLSI. This study concludes that the disc elution method, recommended by CLSI, demonstrates superior accuracy, precision, and sensitivity in detecting susceptibility to polymyxin B compared to the disc diffusion method.

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Introduction

Infections caused by a resistant strain of *Enterobacteriaceae* have been increasingly challenging to treat during the past ten years. Due to the increasing variety of multidrug-resistant *Enterobacteriaceae* strains, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and other types, the situation has become quite challenging (Alanazi et al., 2018; Sendra et al., 2024). Gram-negative bacilli are a taxonomically diverse community of pathogens, like *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii,* and other types (Rana et al., 2025). Such pathogens are generally found to cause serious infections frequently, which significantly increases hospital mortality. Gram-negative bacilli that are nonfermenting are classified into different classes of pathogens, like *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are prominent (Buzilă et al., 2021).

The last line of defense for treating seriously unwell hospital patients is regarded to be carbapenems (Alhashem et al., 2017; Shahab et al., 2025). Polymyxins like polymyxin B and E are progressively used to treat patients who suffer from multidrug-resistant Gram-negative bacterial infections. Colistin (Polymyxin E) and B persist in the last line of antibiotics for Gram-negative bacteria with multidrug resistance (Simon et al., 2023), such as CRE. The assessment of colistin's minimal inhibitory concentration (MIC) using broth microdilution is required by the current joint criteria of the European Committee for Antimicrobial Susceptibility Assessment and the Clinical and Laboratory Standards Institute (Kazaz et al., 2025).

Polymyxin B broth-microdilution is the only effective procedure (Alhashem et al., 2017). The identification of carbapenemase producers from cultured isolates is possible using both phenotypic and genotypic molecular-based techniques (Vamsi et al., 2023). Carbapenem medications are recommended as the first line of treatment for infections caused by the most resilient bacteria, including *Serratia* spp., *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *A. baumannii*, *Enterobacter* spp., *Proteus* spp., and *Enterococcus faecium*. On a global level, drug resistance is seen as a serious healthcare concern (Aurilio et al., 2022). Thus, in the current study, the disc elution method was employed to determine and calculate the lowest inhibitory concentration of polymyxin B, and to appraise the superior accuracy, precision, and sensitivity in detecting susceptibility to polymyxin B compared to the disc diffusion method.

Materials and Methods

Between September 2021 and March 2022, a total of 152 samples were obtained from the Fauji Foundation Hospital, Rawalpindi, Pakistan. All samples, including blood, pus, nasal swab, urine, tracheal tube, bronchial washing, sputum, and stool, adhered to standard collection procedures, ensuring anonymity by excluding patient names from the study. The study received approval from the Ethical Committee at the University of Haripur.

Identification of isolates

Samples were inoculated onto various media and sub-cultured to attain pure cultures, which were then identified using biochemical tests through the Analytical Profile Index (API) 20E kit. The manufacturer's guidelines were strictly adhered to during the implementation of the essential tests. Identification of isolated strains relied on the API-profile record, where a valid identification was confirmed by recording the strain's profile number, determined by its responses, in the register following Holmes et al. (1978). Gram-staining was also done following the instructions, and the stained slides were examined using a microscope at various magnifications, specifically 4X, 10X, 40X, and 100X, with the application of oil emulsion at the 100X resolution. Gram-negative bacteria were characterized by a distinctive pink/red coloration (Moyes et al., 2009).

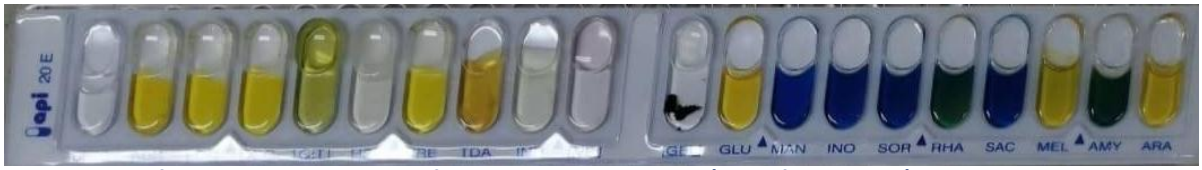

Figure. 1: API for microorganism identification using the 20E kit (BioMérieux, USA).

When the bacterial suspension was added to the API kit, it was incubated in an incubator at 37 ºC for 24 hours. The next day, API reagents were added to the API cupules, and recorded the results.

The result number then corresponds to the organism guidebook in the API. The results of the Gram-negative *Enterobacteriaceae* obtained using the API kit are shown in Figures 1 & 2.

Figure 2: API 20E kit results for Gram-negative *Enterobacteriaceae* (BioMérieux, USA).

Antibiotic sensitivity testing

Antibiogram testing using the Kirby-Bauer disc diffusion technique involved creating a bacterial suspension in normal saline, adjusting the turbidity with a McFarland turbidity standard, and spreading it on Muller-Hinton agar. After allowing the suspension to dry, antimicrobial discs were applied using sterile forceps or a semi-automated disc puncture device. Incubation at 37 ºC for 24 hours was followed by observation, where the presence or absence of bacterial growth around the discs determined antibiotic susceptibility. Measurement of cleared zones was conducted, and antibiotic sensitivity or resistance was determined based on CLSI criteria (Table 1).

Polymyxin B disc elution method

The procedure involved preparing six glass test tubes with varying volumes of Mueller-Hinton broth, each receiving a disc of polymyxin B (30 µg) to achieve final concentrations ranging from 1 µg mL^{-1} to 16 µg mL^{-1}, as shown in Table 1. After incubation at 37 ºC, the eluted polymyxin B antibiotic solutions were obtained and transferred to the labeled test tubes. The bacterial suspension, prepared using the McFarland turbidity standard, was added to these tubes and incubated for 18 to 22 h at 37 ºC. The results were visually observed the next day, with turbidity indicating resistance and a clear solution suggesting sensitivity. For cationic adjustment of the Mueller-Hinton broth, 38 g of the broth was dissolved in 1000 mL of distilled water, boiled, and autoclaved for sterilization. After cooling, magnesium (10 mg L^{-1}) and calcium (20 mg L^{-1}) solutions were added, creating cationic Mueller-Hinton broth, as per CLSI guidelines (Wayne, 2020).

Table 1: Tube distribution with CA-MHB

Tube No.	Polymyxin B (30 µg) - disc	CA-MHB(mL)	Antibiotic conc. (µg mL^{-1})
1st tube	1	30	1
2nd tube	1	15	2
3rd tube	1	10	3
4th tube	1	07	4
5th tube	1	3.75	8
6th tube	1	1.8	16
7th tube	1	0.95l	32

Results

Sample size and distribution

Among 152 samples, isolation from pus swab accounted for the highest proportion at 54% (*n* = 82), followed by urine samples at 24% (*n* = 36). Tracheal infections contributed 7.8% (*n* = 12), sputum samples accounted for 7.2% (*n* = 11), and blood infections constituted 3.9% (*n* = 6). Additionally, there were 1.3% (*n* = 2) each of bronchial wash and vaginal swab samples, and 0.6% (*n* = 1) of cerebrospinal fluid (CSF) samples Table 2). During the study, female patients were found to be more dominant than male patients. The distribution of gender is illustrated in Figure 3.

Table 2: The sample distribution

Sample name	Sample size
Pus swab	54% (*n* = 82)
Urine	24% (*n* = 36)
Tracheal tube specimen	7.8% (*n* = 12)
Sputum	7.2% (*n* = 11)
Blood culture sensitivity	3.9% (*n* = 6)
Bronchial washing	1.3% (*n* = 2)
High vaginal swab	1.3% (*n* = 2)
Cerebrospinal fluid specimen	0.6% (*n* = 1)

Identification of the isolates

Gram-negative bacterial strains were identified using the API kit method, as demonstrated in Figure 4. As observed, *K. pneumoniae* accounted for 49 (32.2%), *E. coli* for 41 (27.0%), *P. aeruginosa* for 31 (20.3%), *A. baumannii* for 29 (19.0%), and *Proteus vulgaris* for 2 (1.1%) (Figure 4). Most of the strains displayed resistance against commonly used antibiotics that were prescribed by physicians.

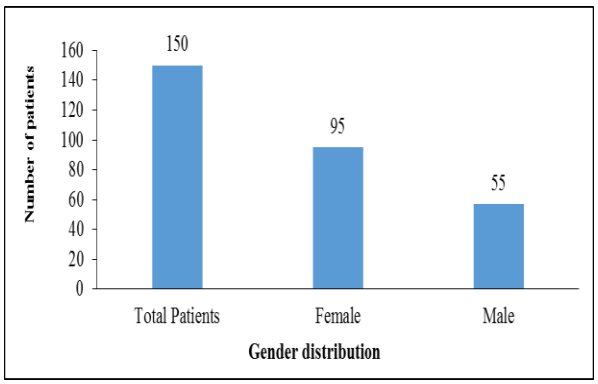

Figure 3: Distribution of patients according to gender

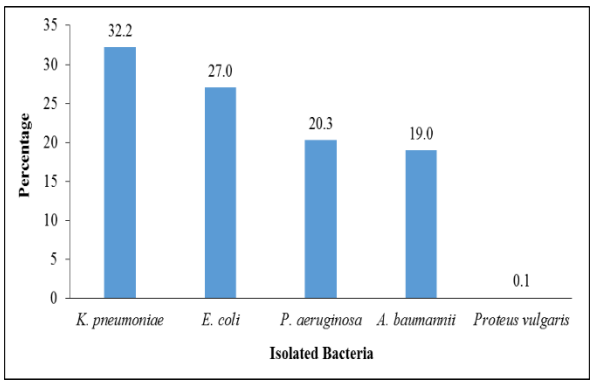

Figure 4: Percentage of isolated Gram-negative bacteria from clinical samples

Antibiotic susceptibility

Disc diffusion technique by the Kirby-Bauer method

After identifying the Gram nature of the organism, suitable antibiotic discs were applied to the sensitivity medium plates containing bacterial suspensions. Incubation at 37 ºC for 24 h was followed by visual observation to determine resistance, intermediate, or sensitivity based on the area around antibiotic discs. Various antibiotics, including Imipenem, Meropenem, Amikacin, Gentamicin, Ceftazidime, Aztreonam, Doxycycline, Co-trimoxazole, Cefotaxime, Nalidixic acid, Ampicillin, Ciprofloxacin, and Sulfamethoxazole, were used, and inhibitory regions were measured in millimeters using a scale. The interpretation of antimicrobial susceptibility, based on CLSI criteria, was presented in Table 3. The widely used disc diffusion Kirby-Bauer technique was employed for antimicrobial susceptibility testing, with the Mueller-Hinton agar as the recommended medium.

Comparison of the disc diffusion and disc elution techniques by assessing the antibiotic susceptibility

Among 152 clinical isolates resistant to carbapenem, the disc elution method revealed 127 samples as susceptible, 13 as intermediate, and 12 as resistant to polymyxin B. In contrast, the disc diffusion method identified 24 samples as resistant and 128 as sensitive, with no intermediate criteria. Detailed results of the susceptibility tests using the disc elution and disc diffusion techniques are presented in Table 3.

Table 3: Evaluation of antibiotic susceptibility in clinical isolates of carbapenem-resistant *Enterobacteriaceae* (CRE) using both disc elution and disc diffusion methods for polymyxin B

Method	Susceptible	Intermediate	Resistant
Disk diffusion	128	---	24
Disk elution	127	13	12

Validation of findings using the broth microdilution technique

This microliter-volume method, resembling the disc elution technique, employed 96-well plates with microliter-scale volumes (100 µL). Initially, 100 µL of the Mueller-Hinton broth was added to each well of the 96-well plate. Subsequently, the eluted antibiotic solution was introduced into the 96-well microtiter plates. These plates, each filled with 10 µL of the test organism, underwent incubation

Table 4: Breakpoint and interpretive category for polymyxin by CLSI

Definitional categories	Breakpoint by two methods	
	MIC method (µg mL^{-1})	Zones area(mm)
Susceptible value	≤2	-----
Intermediate value	4	-----
Resistant value	≥8	-----

(MIC: Minimal inhibitory concentration) (CLSI, 2020).

at 35 °C for 16 to 20 h. The following morning, turbidity in the incubated plates was assessed either manually or with automatic readers. Organism growth indicated antibiotic resistance, while inhibited growth signified sensitivity to the tested antibiotic. The interpretation of polymyxin Minimum Inhibitory Concentration (MIC) values aligns with the CLSI criteria: ≤ 2 µg mL^{-1} is sensitive, 4 µg mL^{-1} is intermediate, and ≥ 8 µg mL^{-1} is resistant, as detailed in Table 4. Notably, the results obtained from the broth microdilution testing mirror those acquired using the disc elusion method.

Discussion

Polymyxin drugs serve as a crucial option, either in monotherapy or in conjunction with other antimicrobials, particularly as the last-resort treatments for clinical isolate infections caused by *Enterobacteriacea*e (Perez et al., 2019). This is especially prevalent in regions with a high prevalence of carbapenem-resistant *Enterobacteriaceae*, such as Italy, China, Greece, and Taiwan, as highlighted by Dafopoulou et al. (2019). Potent antibiotics like Cefiderocol and meropenem-tazobactam are recommended for severe Gram-negative infections (Wang et al., 2022). However, the availability of these expensive options is limited in many nations. Consequently, polymyxins remain the primary treatment for infections induced by carbapenem-resistant isolates in regions where these advanced antibiotics are not accessible (Salvaterra Pasquini et al., 2024; Yang et al., 2024).

The recommended approach by CLSI and EUCAST for identifying polymyxin resistance through broth microdilution is acknowledged to be intricate and poses challenges for integration into routine microbiology laboratory procedures (Hindler and Humphries, 2013; Bakthavatchalam et al., 2017; Bellerose et al., 2019). The broth microdilution method stands as the standard for determining Minimum Inhibitory Concentrations (MIC) for polymyxins (Rocha et al., 2023). However, it comes with drawbacks, including the potential for polymyxins to adhere to the plastic surface of polystyrene plates. Moreover, this method is deemed costly, time-consuming, and labor-intensive, as highlighted by Dalmolin et al. (2020). A more cost-effective and user-friendly alternative is the Polymyxin B broth disk elution technique. This approach, found to be precise and equally reliable as broth microdilution, was assessed for polymyxin B susceptibility in Enterobacterales (Cielo et al., 2020). In our study, we tested 152 isolates with polymyxin B using the disc elution method, which is known for its simplicity compared to the broth microdilution. The results indicated 84% sensitivity of polymyxin B to all isolated Gram-negative bacterial strains, with prominent clinical isolates being *Klebsiella pneumoniae*, *E. coli, Pseudomonas aeruginosa*, and *Acinetobacter baumannii.*

The consistent sensitivity testing across the methods underscores the reliability of the elution techniques, emphasizing the need for affordable and rapid methods for polymyxin B susceptibility testing. The elution techniques demonstrated simplicity and cost-effectiveness, utilizing readily available components in standard laboratories.

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Conceptualization: MS, HIS, MRA. Experimentation: MRA, HIS, MS, NN. Data analysis: MRA, MS, HIS, SK, MA, FZ. Preparation of Figures and Tables: MRA, MS, HIS, NN, SK. Writing the first draft: MRA, HIS, MS, NN, MA, FZ, SK. Revision and proofreading: All authors.

Permissions and ethical compliance
This study was approved by the research committee of the University of Haripur (Reg No. F20-2009-MIC-MPHIL/UOH).

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with great care during collection and experimental procedures. After completion of the study, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher/Journal management. These are available with the corresponding author (s) and/or with other author(s) as declared by the corresponding author (s) of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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