Comparison of Colistin Susceptibility *via* Two Different Methods in Gram-Negative Extensive Drug-Resistance Isolates from ICU Patients

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ABSTRACT

Objective: To compare the susceptibility of colistin by two methods in extensive drug-resistant (XDR) Gram-negative isolates from ICU patients.

Study Design: Cross-sectional comparative analysis.

Place and Duration of the Study: Department of Microbiology, Combined Military Hospital Karachi, Pakistan, from August 2022 to February 2023.

Methodology: A total of 100 clinical specimens received from the intensive care unit yielded growth of extensively drug-resistant gram-negative bacteria, which were evaluated for polymyxin E susceptibility. The agar dilution method was compared with the reference broth microdilution (BMD) method. Minimum inhibitory concentration (MIC) was noted for both methods.

Results: Comparison of the MIC method by agar dilution showed a 90% correlation with the reference method of broth microdilution. With MICs within the acceptable range of the clinical and laboratory standards institute (CLSI) recommendations, 89 isolates were susceptible to colistin, whereas only 11 remained resistant. Polymyxin E's MIC 50 and MIC 90 were determined to be 1 and 2 µg/ml, respectively, with 97% susceptibility.

Conclusion: Agar dilution susceptibility method can be used for screening purposes for the susceptibility testing of polymyxin E. This method is reliable and can easily identify the heteroresistance.

Key Words: *Extensively drug-resistant, Broth microdilution, Multidrug-resistant, Agar dilution, Minimum inhibitory concentration, Colony forming unit.*

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INTRODUCTION

Antimicrobial resistance is a global health issue. Every year, the frequency of infections caused by drug-resistance bacteria increases in communities as well as hospitals. The duration of stay, morbidity, and mortality are reduced when multidrug-resistant bacterium-related serious diseases are treated quickly and efficiently. In intensive care units (ICU), the risk factors for getting this kind of infection are particularly important.[1] Currently, gram-negative rods (GNR) that produce extended-spectrum-lactamases are responsible for majority of infections.

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In addition to being essentially resistant to all beta-lactam antibiotics, these *enterobacteriaceae* also exhibit resistance to members of the other antimicrobial families, such as quinolones or aminoglycosides.[2]

Colistin (polymyxin E) and polymyxin B ensure a prompt bactericidal activity against a majority of gram-negative bacteria. Polymyxin has re-emerged as a vital line of defence against resistant gram-negative bacteria in this period of waning treatment alternatives.[3]

Colistin is also known as polymyxin E, that was first discovered in Japan in 1949. The agent has polycationic properties and is essentially a peptide in nature. *Bacillus polymyxa* is the true source of polymyxin E, and due to particular hydrophilic and lipophilic properties, it has an affinity for both water and lipid. A group of chemicals known as polymyxin is made-up of five separate chemicals: Polymyxin A, B, C, D, and E. From a clinical perspective, two of these polymyxins acquire great importance: Polymyxin B and polymyxin E (also called colistin).[4] The difference in structure between polymyxin B and E is that

leucine is present at position 6 in polymyxin E, but phenylalanine is present at the same location in polymyxin B.[5] Colistin and polymyxin B in particular should be used as the last-resort antimicrobials. Due to their harmful effects on the brain and ear, they ought to be given only under the strictest supervision. Polymyxin is currently essential for fighting life-threatening gram-negative infections.[6]

A quick and accurate approach for testing colistin's antimicrobial susceptibility is required due to the rise in multidrug-resistant gram-negative infections and the concurrent rise in colistin resistance. Although broth microdilution (BMD) is recommended as a reference method by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and the clinical and laboratory standards institute (CLSI), its use in standard laboratory practice is constrained due to time-consuming and challenging application of this process. It is now difficult to test for colistin susceptibility since several techniques, such as disc diffusion and the E-test, produce an increasing number of false positive results.[7] However, the Joint CLSI-EUCAST Polymyxin Breakpoints Working Group recommended the BMD method as a reference method to identify colistin susceptibility in March 2016. Despite EUCAST's warning to exclusively utilise the BMD, numerous techniques, such as semi-automated equipment, agar diffusion, and gradient experiments, are in use because BMD is labour-intensive.[8]

There are numerous reasons that testing colistin susceptibility has become a challenge. Colistin has an enormous molecular weight that inhibits its active diffusion in the agar, making it incompatible with agar diffusion. Colistin can interact with polystyrene, which is used in the synthesis of agar and microtiter plates, resulting in decreased and partial concentration of the active complex in media.[9]

The study's rationale derives from the increasing use of polymyxins in medicinal settings and the need for reliable methods of determining their susceptibility. The objective of this study was to compare the agar dilution (AD) and reference the BMD methods for evaluating polymyxin susceptibility.

METHODOLOGY

After receiving approval from the Institutional Ethical Review Committee of CMH Malir, the study was conducted in the Department of Microbiology at CMH Hospital, Malir, from August 2022 to February 2023.

The 5% sheep blood agar (SBA) and MacConkey agar were used to inoculate all clinical specimens from the hospital's ICU. Gram-negative *bacilli* growth was identified and standard laboratory protocols were utilised for further identification of such isolates. The organisms were confirmed by API 20E.[10]

The standard quantity of polymyxin E sulphate salt powder (Biosynth Germany) was dissolved in sterile distilled water to yield the stock solution (2.56mg/ml). This stock solution was then stored at -70°C. Molten Mueller-Hinton agar (Oxoid, UK) was added with the drug to create two-fold serial dilutions ranging from 0.5 to 32 ug/ml. Before each susceptibility test, an aliquot of the drug was thawed and diluted to the required concentration. By mixing the drug with molten Mueller-Hinton agar, two-fold serial concentrations were obtained, ranging from 0.5 to 32 ug/ml. The medium was then put into 90mm standard marked petri dishes with a depth of 3-4 mm. The pH of the medium was kept between 7.2 and 7.4. Agar plates were allowed to solidify at room temperature before being packed in a plastic bag for storage. After proper labelling and dating, plates were kept between 2 to 5°C. For the most significant results, plates were used within five days of preparation. Before inoculation, these plates acclimatised to an ambient temperature. A 0.5 McFarland was taken as standard to prepare bacterial suspension and inoculation of each agar plate was done by using 10ul pipette to obtain the final inoculum having 104 CFU per spot. The inoculum was utilised within 15 minutes after preparation.[7,14] Results were obtained following an incubation period of 16–20 hours at 35–37°C, and they were interpreted in accordance with the standards outlined in the CLSI recommendations.[10]

BMD, the primary reference method was performed using the cation-adjusted mueller hinton broth (BBL-Becton Dickinson) in 96-well microtiter plates in accordance with the CLSI recommendations.[7] From 0.5 ug/ml to 32 ug/ml of Polymyxin E Sulphate salt (Biosynth Germany) was evaluated. The initial bacterial suspension inoculum, which was produced from an overnight culture 0.5 McFarland turbidity, was subsequently diluted to produce a final inoculum with approximately $5x10^5$ CFU/ml. Subsequently within 15 minutes, each well of the designated row of 96 well microtiter plate was filled with the diluted bacterial inoculum suspension. A growth-control well was kept drug-free and sterility control well-kept inoculum-free. Incubation was done for 18 hours at 35 ± 2°C aerobically. To check the correct inoculum density and purity of the test isolate, SBA plate was streaked by taking 10µl suspension from the growth control well. An inoculum density of $5x10^5$ CFU/mL would be indicated by the presence of about 50 colonies. The MIC was defined as the lowest concentration of Polymyxin E at which there was no visible growth. According to the CLSI 2022 standards, a colistin MIC of ≤2 and ≥4 µg/ml was taken as the breakpoint for susceptibility.[10]

Quality control testing: *E. coli* ATCC 27853 was used as the CLSI-recommended quality-control (QC) strain. The strain was tested by both agar and broth microdilution methods. The MICs were within the accepted QC range of 0.5 to 2 µg/ml using both test methods.

The collected data were input into the SPSS programme (version 25) for statistical analysis. Both qualitative and quantitative variables were subjected to the descriptive statistics calculations. MICs of polymyxin E were determined. The mean for numerical variables was calculated. A 0.05 p-value was considered significant. To measure acceptable performance criteria, standards developed by the International Organisation for Standardisation were followed: ≥90% for categorical or essential agreements, ≤3% for very major or major errors. The percentage of MICs within log2 dilution of the MIC measured by BMD was referred as essential agreement (EA). The percentage of isolates categorised by BMD and the method under review in the same susceptibility group are known as categorical agreement (CA). Major errors (MEs) stood for a false-resistant result, whereas very major errors (VMEs) stood for a false-susceptible result. Minor errors (ME) that are intermediate either resistant or susceptible, are labelled as intermediate results.[10]

Table I: Demographical data and clinical sources of XDR (n = 100) isolates.

Demographical data		Clinical samples (n = 100)	MIC Range (≤0.5-32µg/ml)
Age	Gender	Urine 31	
		Pus 22	
8 - 45 years = 50 isolates	Males = 61	Blood 15	
46 - 83 years = 50	Females = 39	Respiratory samples (Sputum+NBL) = 19	MIC 50 = 1
Mean age = 46 ± 17 years		Tissue 6	MIC 90 = 2
		Intravenous catheter device 7	

Table II: Polymyxin's MIC by susceptibility testing method (AD) and its categorical agreement with reference method (BMD) (n = 100).

Test method	No. of isolates and MICs				No. of isolates and results	% category errors			% CA with BMD				
	≤5	1	2	≥4	Intermediate	Resistant	V. Major	Major	≤0.5 ≤16	1 ≤16	2 ≤16	≥4 ≤16	Overall
Broth	16	53	20	11	89	11	3	3	88%	71%	63%	60%	90%
Agar	20	50	18	1	89	11							

RESULTS

Among 100 XDR isolates, the majority of the isolates were observed from urine samples. A mean age of 46 ± 17 years (Figure 1) and a male preponderance were observed (Table I). *K. pneumonia* was predominated among the isolates (n = 32). MIC 50 and MIC 90 were found 1 ug/ml and 2 ug/ml, respectively, according to the CLSI-recommended range (Table I).

Between AD and BMD, there was an overall 90% categorical agreement. In the research, 95% accuracy was attained with three very major and two major errors. While evaluating different BMD and AD ranges, there was 60% agreement for values greater than 4ug/ml and 63% for values greater than 2ug/ml. By finding 90% essential agreement (EA) Table II, the results were within acceptable limits. *E. coli, A. baumani and K. oxytoca* showed one VME while, *K. pneumoniae*, *A. baumani* and *E. cloacea*, each showed one ME that are also in an acceptable range.

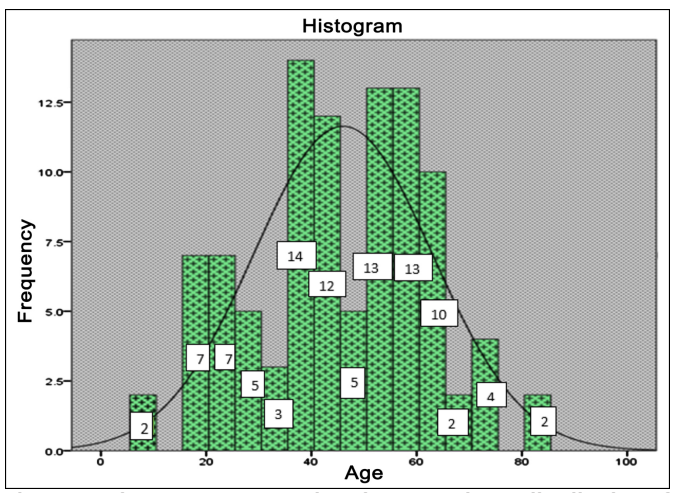

Figure 1: Histogram representing the general age distribution of males and females.

DISCUSSION

Colistin is used as a last-resort to treat serious infections caused by multidrug-resistant *enterobacteriaceae*, hence consistent and precise testing of its susceptibility has become crucial for clinical laboratories across the world. Compared to many other antimicrobial agents, it is crucial that labs report accurate results and acceptable essential agreement.[11] Global trends towards higher colistin MICs have been observed, highlighting the significance of accurate colistin susceptibility results.[12]

Regarding its reliability, reproducibility and potential for automation, BMD has been referred as the gold standard as suggested by both CLSI and EUCAST. However, since it is done manually and is a time-consuming approach, there could be major inaccuracies. The AD method is capable of detecting heterogeneous population and multiple isolates could be tested at a time on the same plate. However, this procedure is extremely time-consuming, the prepared plates cannot be utilised after a week. As colistin could not diffuse completely in agar, false positive results in the AD method may be anticipated.[13]

The objective of the current investigation was to analyse 100 gram-negative, XDR isolates, with a notable prevalence in urine samples of ICU patients. The study revealed a male predominance among the patient population, with a mean age of 46 ± 17 years, aligning with previously observed findings of Vincent *et al*.[14]

The study by Qamar *et al*. in Pakistan also showed male predominance (61%) with mean age of 46 ± 17, the findings were similar to the present study.[15] The most common isolate found in this study was *K. pneumoniae* in urine, similar to the study of Arjun *et al*.[16]

According to the study by Paczosa *et al.*, *Klebsiella pneumoniae (K.p)* was the most common organism, highlighting its significance in healthcare-associated infections.[17] However, contrary to this *E. coli* was found to be prominent in isolates according to a research in India.[18]

The ability of the agar dilution method to effectively detect heteroresistance is a noteworthy finding. Heteroresistance, wherein subpopulations within a bacterial isolate exhibit varying degrees of susceptibility to an antibiotic can significantly impact treatment outcomes. The study of Mashaly *et al*. also suggested agar dilution as a tool for detecting

heteroresistance, highlights its clinical relevance in guiding treatment decisions, and preventing treatment failures.[19]

The highest percentage of *K. pneumoniae* (32%), followed by *E. coli* (23) and 11% XDR were detected among 100 isolates in the current investigation, which was equivalent to Furqan *et al*.'s study, which reported 18% CRE among 176 isolates utilising 2ug and 4ug/ml concentration.[20]

Results of this study demonstrated a comparison between AD and BMD that was consistent with the research of Sana *et al*., whereby major and minor errors were limited and the categorical agreement between AD and BMD was considered within the acceptable range.[13] The significance of this level of agreement was further validated by the study's establishment of 95% accuracy, with three very major errors, and two major errors noted. These findings collectively underscore the utility and reliability of the AD method as a feasible alternative to the reference BMD method, in line with other research findings of Kareem *et al*. [21]

Colistin resistance was 11% in the present research. The VME rates of AD and broth are acceptable according to CLSI standards (less than 3% VME rate is acceptable), in contrast to Kar *et al*., who reported 13.5% of colistin resistance with 11% VME rates exhibited by AD.[22]

The MIC 50 and MIC 90 values of 1 µg/ml and 2 µg/ml, respectively, for polymyxin E, further substantiate its potential as an effective treatment option against extensively drug-resistant gram-negative rods. These values are in line with those reported by Lee *et al*., reinforcing the consistency of colistin susceptibility profiles across different settings and populations.[23]

The study examined varying MIC ranges, finding lower agreement percentages for concentrations above 2 µg/ml (63%) and 4 µg/ml (60%). However, a substantial 90% essential agreement (EA) was observed, indicating the acceptable results. This emphasises the significance of MIC ranges in assessing method agreement and enhancing study reliability as found in the study of Sader *et al*.[24] Small sample size, short duration of study, and single-centric research are the main limitations of the present study.

CONCLUSION

The implications of this study are crucial in resource-constrained regions. It highlights the reliability of the agar dilution for determining colistin susceptibility in economically underdeveloped nations like Pakistan. By providing a reliable screening tool, this technique can improve management tactics again XRD infections in critically ill patients, leading to better healthcare outcomes.

ETHICAL APPROVAL:

The protocol has been reviewed by the Ethical Review Committee of the CMH Hospital, Malir, Karachi. After consulting the IRB, a formal ethical review approval was obtained.

PATIENT'S CONSENT:

Written informed consent from the patients was not required. Identity numbers were used for identification of isolates instead of patient's name etc.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

ST: Conception of the work, acquisition, analysis, and interpretation of the data for the work.
TA, FS: Drafting the work and critical revision for important intellectual content.
AA, SFR, WH: Final approval of the version to be published.
All authors approved the final version of the manuscript to be published.

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