Open Access

Original Article

Diagnostic accuracy of resazurin based rapid test for Colistin susceptibility in Carbapenem resistant *Acinetobacter baumanii* isolated form ICU settings

Sidra Muqarrab, Irfan Ali Mirza, Sakeenah Hussain Naqvi, Adeel Gardezi, Muhammad Roshan, Rabia Sajjad, Anam Imtiaz

Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi/National University of Medical Sciences (NUMS) Pakistan

ABSTRACT

Objective: To determine diagnostic accuracy of rapid resazurin based test for Colistin susceptibility testing in Carbapenem resistant *Acinetobacter baumannii* isolated from ICU patients.

Study Design: Validation study.

Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Jul to Dec 2022.

Methodology: A resazurin based rapid test for Colistin susceptibility testing in *Acinetobacter baumannii* was assessed using a total of 100 clinical isolates from ICU patients. This test detects the presence of viable bacterial cells in Mueller-Hinton broth containing a predefined concentration of Colistin sulfate. Bacterial suspensions of the isolates are added to Colistin containing Mueller-Hinton broth on a 96-well polystyrene microtiter plate and incubated for 3 hours at 35° ± 2°C. Resazurin (a viability colorant) is then added to the wells and results are interpreted on basis of color change (from blue to pink in resistant isolates) after a total of 4 hours.

Results: The test accurately detected resistance in all 12 resistant isolates and had a sensitivity and specificity of 100% and 97.7% respectively. False positive results were seen in 2 out of 88 sensitive isolates. The overall diagnostic accuracy of the test was 98%.

Conclusion: This resazurin based test is in-expensive, easy to perform, accurate and provides earlier results in just 4 hours as compared to 16-24 hours with the routine lab methods. This test can serve as a good alternate to conventional methods in resource limited settings and for infection control purposes.

Keywords: *Acinetobacter Baumannii*, Colistin Susceptibility, Rapid Resazurin Test.

How to Cite This Article: *Muqarrab S, Mirza IA, Naqv SHi, Gardezi A, Roshan M, Sajjad R, Imtiaz A. Diagnostic accuracy of resazurin based rapid test for Colistin susceptibility in Carbapenem resistant Acinetobacter baumanii isolated form ICU setting. Pak Armed Forces Med J 2025; 75(Suppl-2): S360-S364. DOI: https://doi.org/10.51253/pafmj.v75iSUPPL-2.10171*

INTRODUCTION

Acinetobacter baumannii belongs to Gram-negative group of cocco-bacilli and has also been included in ESKAPE group of microorganisms which cause nosocomial infections with high mortality rates especially in ICU settings. They are strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, and oxidase-negative bacteria. Multi drug resistance in this organism is on the rise since past few years due to abundant and injudicious use of broad spectrum antibiotics.[1,2] Some of the infections caused by *A. baumannii* are; bloodstream infections, lower respiratory tract, urinary tract, and wound infections, burn infections, skin and soft tissue infections, meningitis, osteomyelitis and endocarditis.[3] World health organization in 2017 has published a global priority list of organisms for which new antibiotics and treatment options are required due to increasing resistance and *Acinetobacter baumannii* is included in highest priority category.[4]

Carbapenems have been an important treatment option in patients infected with Multi-drug resistant Gram negative bacteria including *Acinetobacter baumannii*.[5] Number of patients in ICUs getting infected with Carbapenem resistant, XDR (extensively drug resistant) or PDR (Pan Drug resistant) *A. baumannii* is now on the rise.[6,7] Carbapenem resistance in *Acinetobacter baumannii* exceeds 90% in various parts of the world and according to published data it is around 85-100% in Pakistan.[8–10] In such cases, Colistin and Tigecycline are options for therapy with variable activity against Carbapenem resistant strains.[11] Polymyxins were first discovered in the 1950s but their use was discontinued due to their nephrotoxicity and availability of other treatment options but they are currently being used as first line antimicrobials for Carbapenem resistant *Acinetobacter baumannii* either alone or in combination with other drugs.[12] But recently due to its increased use in the clinical settings, the rate of resistance against Colistin is also on the rise

Correspondence: Dr Sidra Muqarrab, Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi Pakistan

Received: 01 Apr 2023; revision received: 08 Jun 2023; accepted: 15 Jun 2023

and there is a need for rapid and accurate antimicrobial susceptibility testing methods for Colistin.[13]

Susceptibility testing of *Acinetobacter baumannii* against colistin is an arduous task in the laboratory as Colistin, a cationic substance, attaches to the polymers used to make most testing plates, leading to inconsistent results. Moreover, Colistin has poor diffusion in agar because of its high molecular weight, which leads to inaccurate results when using diffusion-based techniques like E-test or disc diffusion.[14] Currently, broth microdilution (BMD) is the approach advised by EUCAST and CLSI for the detection of Colistin susceptibility.[15] It is challenging to incorporate this technique into clinical laboratories' standard testing for Gram-negative isolates. Also, all these above mentioned tests are time consuming and take a minimum of 16-24 hours in final reading of susceptibility results. To determine Colistin susceptibility, simple, quick, and accurate procedures are needed.

Recently a rapid resazurin based test was developed by Lescat *et al*., for determination of Colistin susceptibility in *Acinetobacter baumannii* within a time span of 4 hours instead of 16-24 hours by conventional methods.[16] The principle of this test is based on reduction of resazurin (PrestoBlue), a cell viability reagent, by the cells which are metabolically active, which produces a color change from blue to pink.[17] Viability of bacteria in medium containing a certain concentration of Colistin is tested in the presence of this dye and results are interpreted on the basis of color change.[16] This study was aimed to evaluate the performance of this rapid resazurin based test for detection of Colistin susceptibility in *Acinetobacter baumannii* when compared with the reference broth microdilution method (BMD).

METHODOLOGY

It was a validation study carried out at Armed Forces Institute of Pathology, Rawalpindi Pakistan, from July 2022 to December 2022 after obtaining approval from the Institutional Review Board and the Ethical committee (FC-MIC21-16/READ-IRB/23/1673, Dated: 29-03-2023). The sample size was calculated by sensitivity-specificity calculator considering resazurin test sensitivity 96% and prevalence of *Acinetobacter baumannii* to be 17.8% in ICU patients, confidence level 95% and margin of error 5%.[18,19] The sample size was taken to be 100. The sampling technique was non-probability consecutive sampling.

Inclusion criteria: Carbapenem resistant *Acinetobacter baumannii* isolates from different clinical samples of ICU patients.

Exclusion criteria: Duplicate isolates from same patient were excluded.

One hundred (n=100) isolates of Carbapenem resistant *Acinetobacter baumannii* from different clinical specimens were tested for Colistin susceptibility (Table-I). Carbapenem resistance was detected by Modified Kirby-Bauer method using CLSI-M100-2022 guidelines.20The isolates were identified to species level using API 20NE. ATCC 25922 E.coli which is a Colistin-susceptible strain and the NCTC 13846 E.coli which is a Colistin-resistant strain were used as negative and positive controls for the determination of MIC of Colistin, respectively. Additionally, two separate isolates of *A. baumannii*, one Colistin resistant and the other Colistin susceptible (institutional controls) were also used as controls for rapid Resazurin based test.

Table-I: No. of *Acinetobacter baumannii* Isolates from Different Clinical Specimens Included in the Study (n=100)

Specimen type	No. of isolates
Bronchioalveolar lavage (BAL)	29
Endobronchial washings (EBW)	16
Non-directed bronchial lavage (NBL)	10
Sputum	10
Tissue	2
Cerebrospinal fluid (CSF)	8
Pus	2
Pus swab	2
CVP tip	2
Pleural fluid	4
Blood	15
TOTAL (n)	100

Colistin susceptibility of all isolates was checked twice using in-house Broth Microdilution method (BMD) using 96-well polystyrene plate. Colistin sulfate (Sigma-Aldrich, France) in two-fold serial dilutions ranging from 0.125µg/ml to 32µg/ml was used to determine the MIC of test and control isolates. Results of BMD were interpreted according to CLSI-M100 (2022) and EUCAST guidelines (resistance being at MIC >2mg/L).[15,20]

Rapid resazurin based test: This test was done using a 96-well polystyrene plate. A Colistin stock solution in MHB was prepared to a final concentration of 3.75µg/ml according to instructions published by

the developers of the test. For each isolate to be tested a standardized bacterial suspension was made (3.5 McFarland standard) from colonies not older than 24 hours grown on Blood agar plates.[16] Institutional controls of Colistin susceptible and Colistin resistant *A. baumannii* were used with each isolate / batch tested. To perform the test on the 96-well polystyrene plate, following steps were followed:

1. 180µL of MHB (ThermoFisher scientific) without Colistin sulfate was pipetted in well A1, A2, A3 and 180µL of MHB with Colistin sulfate (3.75µg/ml) was inoculated in wells B1, B2, B3.
2. Wells A1 and B1 were inoculated with 20µL of 0.85% normal saline acting as control for sterility of the broths.
3. 20µL of prepared bacterial suspension (3.5 McFarland) was pipetted in parallel in two wells (one without Colistin acting as growth control for that particular isolate and one with Colistin). Wells A2 and B2 were inoculated with 20µL of institutional control of Colistin resistant *A. baumannii*.
4. Wells A3 and B3 were inoculated with 20µL of institutional control of Colistin susceptible *A. baumannii*.
5. Test isolates were inoculated in the same manner in successive wells on the tray.
6. The tray was incubated for 3 hours at 35°C (± 2°C) in ambient air. After 3 hours of incubation 22µL of resazurin reagent (PrestoBlue) was added (10%V/V) and pipetted up and down in each well.

Interpretation of results: The color change was observed every 15 mins for 1st one hour and all the tests were interpreted within a maximum duration of 4 hours since incubation. The results of the tests were considered valid if both the wells A1 and B1 remained blue (no color change) at the end of 4 hours indicating sterility of broths used, wells A2 and B2 turned pink (growth of Colistin resistant isolate in both growth control well and the well with Colistin) indicating valid result for Colistin resistant institutional control, and the well A3 turned pink (growth control for negative control) while B3 remained blue (indicating Colistin susceptibility of negative control) (Figure-1).

The test showing any discrepancy with the reference broth microdilution method was repeated in triplicate for final result.

Sensitivity (number of resistant isolates correctly identified as resistant), specificity (number of sensitive isolates correctly identified as sensitive), positive predictive value, negative predictive value and diagnostic accuracy were calculated.

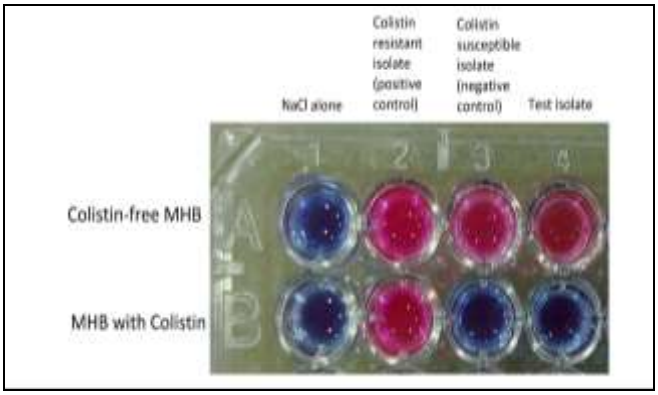

Figure-1: Representative Results of Rapid Resazurin Based Test for Colistin Susceptibility Testing in *Acinetobacter baumannii*

RESULTS

A total of 100 isolates were tested for Colistin susceptibility from different clinical samples. Maximum number of isolates (29%) were retrieved from bronchoalveolar lavage (BAL) specimens and 2% each from pus, tissue, pus swab and CVP tip. Out of these 88 were Colistin sensitive (MICs ranging from <0.125 mg/L to 1mg/L) and 12 were found to be colistin resistant (MICs ranging from 4mg/L to >32 mg/L) on reference BMD method. Two out of 88 colistin susceptible isolates (MICs 1 mg/L and 0.125 mg/L) were found to be resistant on rapid resazurin based test. None out of 12 Colistin resistant isolates on BMD method none was found to be susceptible to Colistin on resazurin based test (Table-II). Results of rapid resazurin based test were compared with reference Broth microdilution method to obtain the diagnostic accuracy of the test.

Table-II: Diagnostic Accuracy of Resazurin Based Test for Colistin Susceptibility Testing in *Acinetobacter baumannii*

	Resistant on BMD	Sensitive on BMD
Resistant on rapid-resazurin test	12 (True positive, TP)	2 (False positive, FP)
Sensitive on rapid-resazurin test	0 (False negative, FN)	86 (True negative, TN)

Sensitivity of the test (number of resistant isolates correctly identified as resistant) was calculated to be 100%. The specificity of the test (number of sensitive isolates correctly identified as sensitive) was calculated to be 97.7%. Positive predictive value of the

test was 85.7% and negative predictive value was 100%. The overall diagnostic accuracy of the test was calculated to be 98%. (Table-III). Out of the 12 Colistin resistant isolates 6 showed color change within 15 minutes (50%), 3 within 30 minutes (25%) and 3 within 45 minutes. None of the wells with Colistin resistant isolate remained blue for > 45minutes and the results were interpreted within one hour of addition of resazurin. One out the two false positive isolates turned pink within 15 minutes and the other at 45 minutes. These false positive tests were repeated in triplicate but the results remained the same.

Table-III: Summary of Diagnostic Parameters of Rapid Resazurin Based Test for Colistin Susceptibility Testing in *Acinetobacter baumanii*

Diagnostic parameters	Values
Sensitivity = TP/ (TP+FN)	100%
Specificity = TN/(TN+FP)	97.7%
Positive Predictive Value = TP/ (TP + FP)	85.7%
Negative Predictive Value = TN / (TN + FN)	100%
Diagnostic Accuracy = (TP +TN)/ Total isolates	98%

DISCUSSION

In 2016, Nordmann *et al.,* developed a rapid test for Colistin susceptibility testing in Enterobacterales.[21] However, this test could not be used for non-fermenters. More recently Lescat *et al.,* have developed a rapid resazurin based method for Colistin susceptibility testing in *Acinetobacter baumannii* which is now also available in form of commercial kits as Rapid Resa Polymyxin *Acinetobacter* NP® test (Liofilchem, Italy).[16,18] To the best of our knowledge this is the first study conducted in Pakistan on rapid in-house Resazurin based test for Colistin susceptibility in *Acinetobacter baumannii.*

The Carbapenem resistance amongst *Acinetobacter* isolates in Pakistan has been estimated to be around 85% by Khalid, Fizza *et al*.[9] This is also supported by another study conducted by Ahsan, Umaira *et al.,* in 2022 where out of 150 isolates collected from five different hospitals of Pakistan 84% were resistant to Carbapenems and 7.3% were resistant to Colistin as well.[10] This warrants for development of rapid sensitivity detection methods especially for detection of Colistin susceptibility in *A. baumannii* isolated from patients admitted in ICU settings. Our study was aimed at establishing the diagnostic accuracy of this rapid test to facilitate early availability of susceptibility results and also to provide a cheaper alternative in resource limited settings. In our study rapid resazurin based test was found to be very accurate with an excellent sensitivity of 100% and specificity of 97.7% and an overall diagnostic accuracy of 98%.

The results of our study were similar to a study published by Jia, Huaiyu *et al.,* in 2020 where they found the sensitivity of the in-house resazurin based test to be 100% and specificity to be 96%.[22] A study was conducted by Bouvier *et al.,* in 2021 on 62 different *A. baumannii* isolates where they evaluated the industrial version of this test, Rapid Resa-Polymyxin *Acinetobacter* NP® test (Liofilchem, Italy). The sensitivity of this test was calculated to be 97% and the specificity was calculated to be 96%.[18] One contrasting result was found in a study conducted by Kon *et al.,* where they compared results of the commercial Rapid Resa-Polymyxin *Acinetobacter* NP® test with BMD method for 87 *A. baumannii* isolates and the commercial kit had a sensitivity of 78.9% and a specificity of 98.2%.[23] Germ *et al.,* in their study also concluded that this test could be used as an effective screening test (sensitivity and specificity of 93.3%) for Colistin susceptibility in regions with a high prevalence of Carbapenem resistant *A. baumannii.*[24]

LIMITATIONS OF STUDY

A major limitation of this test is that it does not give us MICs for the sensitive or resistant isolates. The small sample size of our study and even lesser number of Colistin resistant isolates tested may also be considered a limitation of our study and more studies with a greater number of Colistin resistant isolates may help validate this rapid test even further.

CONCLUSION

This test is cheap, rapid and easy to perform and can act as a reliable alternative to BMD in resource limited settings. The rapid provision of susceptibility results could be really helpful for early initiation of optimal treatment strategy in critically ill patients. It may also be helpful in adapting good clinical practices in light of Antimicrobial stewardship guidelines and in implementing infection control measures.

Conflict of Interest: None.

Funding Source: None.

Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

SM& IAM: Data acquisition, data analysis, critical review, approval of the final version to be published.

SHM& AG: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

MR & RS & AI: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

1. Vázquez-López R, Solano-Gálvez SG, Juárez Vignon-Whaley JJ, Abello Vaamonde JA, Padró Alonzo LA, Rivera Reséndiz A, et al. Acinetobacter baumannii Resistance: A Real Challenge for Clinicians. Antibiotics 2020; 9(4).
2. Kyriakidis I, Vasileiou E, Pana ZD, Tragiannidis A. Acinetobacter baumannii Antibiotic Resistance Mechanisms. Pathogens 2021; 10(3).
3. Ramirez MS, Bonomo RA, Tolmasky ME. Carbapenemases: Transforming Acinetobacter baumannii into a Yet More Dangerous Menace. Biomolecules 2020; 10(5).
4. WHO publishes list of bacteria for which new antibiotics are urgently needed [Internet]. [cited 2023 Mar 17]. Available from: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed
5. Nguyen M, Joshi SG. Carbapenem resistance in Acinetobacter baumannii, and their importance in hospital-acquired infections: a scientific review. J Appl Microbiol 2021; 131(6): 2715–2738.
6. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018; 18(3): 318–327.
7. Hamidian M, Nigro SJ. Emergence, molecular mechanisms and global spread of carbapenem-resistant Acinetobacter baumannii. Microb Genomics [Internet]. 2019 Oct 1 [cited 2023 Mar 20];5(10). Available from: https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000306
8. Mohd Sazlly Lim S, Zainal Abidin A, Liew SM, Roberts JA, Sime FB. The global prevalence of multidrug-resistance among Acinetobacter baumannii causing hospital-acquired and ventilator-associated pneumonia and its associated mortality: A systematic review and meta-analysis. J Infect 2019; 79(6): 593–600.
9. Khalid F, Saleem S, Ahmad I. High prevalence of carbapenem resistant Acinetobacter baumannii associated respiratory tract infections in Pakistani hospitals. J Pak Med Assoc 2020; (0): 1.
10. Ahsan U, Mushtaq F, Saleem S, Malik A, Sarfaraz H, Shahzad M, et al. Emergence of high colistin resistance in carbapenem resistant Acinetobacter baumannii in Pakistan and its potential management through immunomodulatory effect of an extract from Saussurea lappa. Front Pharmacol 2022; 13: 986802.
11. Piperaki ET, Tzouvelekis LS, Miriagou V, Daikos GL. Carbapenem-resistant Acinetobacter baumannii: in pursuit of an effective treatment. Clin Microbiol Infect 2019; 25(8): 951–957.
12. Isler Burcu, Doi Yohei, Bonomo Robert A., Paterson David L. New Treatment Options against Carbapenem-Resistant Acinetobacter baumannii Infections. Antimicrob Agents Chemother 2018; 63(1): e01110-18.
13. Jung H, Pitout JDD, Mitton BC, Strydom KA, Kingsburgh C, Coetzee J, et al. Evaluation of the rapid ResaPolymyxin Acinetobacter/Pseudomonas NP test for rapid colistin resistance detection in lactose non-fermenting Gram-negative bacteria [Internet]. Vol. 70, Journal of Medical Microbiology. Microbiology Society; 2021. Available from: https://www.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.001373
14. Turlej-Rogacka A, Xavier BB, Janssens L, Lammens C, Zarkotou O, Pournaras S, et al. Evaluation of colistin stability in agar and comparison of four methods for MIC testing of colistin. Eur J Clin Microbiol Infect Dis 2018; 37: 345–353.
15. EUCAST. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group [Internet]. EUCAST; 2016 Mar [cited 2023 Mar 23]. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf
16. Lescat Mathilde, Poirel Laurent, Tinguely Camille, Nordmann Patrice. A Resazurin Reduction-Based Assay for Rapid Detection of Polymyxin Resistance in Acinetobacter baumannii and Pseudomonas aeruginosa. J Clin Microbiol 2019; 57(3): e01563-18.
17. O'brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267(17): 5421–5426.
18. Bouvier M, Sadek M, Pomponio S, D'Emidio F, Poirel L, Nordmann P. RapidResa Polymyxin Acinetobacter NP® Test for Rapid Detection of Polymyxin Resistance in Acinetobacter baumannii. Antibiotics 2021; 10(5).
19. Tahseen U, Talib MT. Acinetobacter infections as an emerging threat in intensive care units. J Ayub Med Coll Abbottabad 2015; 27(1): 113–116.
20. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 32rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2022. M100-Ed32.
21. Nordmann P, Jayol A, Poirel L. Rapid Detection of Polymyxin Resistance in Enterobacteriaceae. Emerg Infect Dis 2016; 22(6): 1038–1043.
22. Jia H, Fang R, Lin J, Tian X, Zhao Y, Chen L, et al. Evaluation of resazurin-based assay for rapid detection of polymyxin-resistant gram-negative bacteria. BMC Microbiol 2020; 20(1): 7.
23. Kon H, Abramov S, Amar Ben Dalak M, Elmaliach N, Schwartz D, Carmeli Y, et al. Performance of Rapid PolymyxinTM NP and Rapid PolymyxinTM Acinetobacter for the detection of polymyxin resistance in carbapenem-resistant Acinetobacter baumannii and Enterobacterales. J Antimicrob Chemother. 2020; 75(6): 1484–1490.
24. Germ J, Poirel L, Kisek TC, Spik VC, Seme K, Premru MM, et al. Evaluation of resazurin-based rapid test to detect colistin resistance in Acinetobacter baumannii isolates. Eur J Clin Microbiol Infect Dis 2019; 38(11): 2159–2162