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Colistin Susceptibility Testing by Reference Broth Microdilution Method in Clinical Isolates of Carbapenem-Resistant Enterobacterales at a Tertiary Care Hospital in Dhaka, Bangladesh

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Abstract

Background: Colistin has re-emerged as an important antimicrobial agent in recent times despite its adverse effects for the treatment of patients infected with CRE (carbapenem-resistant Enterobacterales) isolates that were not responding to other antibiotics. So, this study was carried out to observe the susceptibility pattern of colistin in clinical isolates of CRE.

Materials and Methods: This cross-sectional study was conducted at the Department of Microbiology and Immunology of Bangladesh Medical University, Dhaka, Bangladesh from September 2018 to August 2019. One hundred and forty-five clinical isolates of CRE were subjected to test for colistin susceptibility by EUCAST and CLSI recommended broth microdilution method (BMD) and compared with the results of disc diffusion method (DDM).

Results: Forty-two (29.0%) out of 145 CRE isolates were resistant to colistin by BMD. The resistance rate of colistin among *Enterobacter spp, K. pneumoniae* and *E. coli were* 36.36%, 31.19% and 17.39% respectively. The highest MIC value (32µg /ml) was observed in 2 isolates of *Enterobacter* spp. MIC^{50} and MIC^{90} value of colistin among CRE isolates were 2µg/ml and 8µg/ml respectively. Colistin resistant CRE isolates exhibited higher MIC ^{50} and MIC^{90} value of 8µg/ml and 16µg/ml respectively. DDM remains unreliable due to high major errors (47.62% of VME and 13.59% of ME) and minimal agreement (k value 0.40) in comparison to BMD.

Conclusion: The findings of this study reflect an emerging burden of colistin resistant CRE in a tertiary care specialized hospital of Bangladesh and warrants implementation of reliable method for susceptibility testing of colistin.

Keywords: CRE, colistin, broth microdilution, disc diffusion, MIC^{50}, MIC^{90}, Bangladesh.

Introduction

Carbapenem-resistant Enterobacterales (CRE) is listed by the World Health Organization (WHO) as one of the critical priority pathogens for future research and development of new antibiotics [1]. As carbapenems are used for treating multidrug resistant bacteria, the emergence of CRE infections poses a particular threat in hospitals and nursing homes [1]. With the global increase of CRE and lack of new antibiotics, colistin has gained clinical value as a last resort to treating these severe and deadly infections due to its broad-spectrum activity against most species of Enterobacterales family [2]. Currently, there is an increased use of combination of colistin and

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carbapenem in clinical practice to treat CRE as it is associated with better outcomes [3]. In many countries, colistin is also used in livestock and agriculture and sometimes as an empirical therapy for the treatment of patients infected with CRE isolates [4,5]. Due to the high usage of colistin, there is an increased emergence of resistant colistin organisms which have become a serious threat for both humans and animals [5]. Accurate and reliable susceptibility testing of colistin is important to allow appropriate therapeutic decisions [6]. Though the DDM is simple, inexpensive and commonly used method for susceptibility testing of colistin in clinical microbiology laboratories, neither EUCAST nor CLSI recommend it for colistin [7]. Poor and slow diffusion of colistin into agar results in a smaller zone of inhibition and associated with high error rates compared to MIC based methods [8,9]. In 2016, a joint CLSI-EUCAST Polymyxin Breakpoints Working Group recommended that the ISO-20776 standard broth microdilution (BMD) method should be used as a standard format for susceptibility testing of colistin [10]. Thus, the present study has been carried out to observe the susceptibility of colistin by reference BMD in clinical isolates of CRE and to determine the discrepancies between the results of the BMD and DDM.

Materials and Methods

Isolate collection: An observational cross-sectional study was conducted at the Department of Microbiology and Immunology of Bangladesh Medical University (BSMMU), Dhaka, Bangladesh from September 2018 to August 2019. One hundred and forty-five non-duplicate clinical samples of CRE isolated from blood, urine, tracheal aspirate, wound swab, pus, sputum, throat swab, bile, drain tube fluid were studied. These clinical specimens were sent from different departments of BSMMU to the Department of Microbiology and Immunology for culture and susceptibility testing. The study was reviewed and approved by the Institutional Review Board (IRB) of Bangladesh Medical University, Dhaka, Bangladesh.

Reference Broth Microdilution (BMD) method for Colistin: All the 145 CRE isolates were subjected to test for MIC of colistin by reference BMD which was performed according to CLSI document M07-A99 [11], the same methodology outlined in ISO 20776-1: 2006. MIC of colistin was done within a range of 0.125 to 64 µg/ml. Colistin sulphate as a dry powder with known potencies (1gram,19000U/mg) was purchased from Sisco Research Limited, India and Mueller Hinton Broth No.2 Control Cations (HiMedia, India) was used as Cation adjusted Mueller Hinton broth (CAMHB) medium. A 1024µg/ml stock solution of colistin sulphate was prepared freshly in sterile deionized water and stored at -20° C until the different test sessions were performed. The 96 well polystyrene plates (CELLSTAR) were bought from Greiner bio-one, Germany and were not treated in any way before use. For any working session, incremental dilutions of the stock solution were made in separate sterile test tube containing CAMHB to prepare ten working concentrations of colistin (ranging from 0.125 to 64 µg/ml in 2-fold dilutions). Hundred microliters of each concentration were dispensed into the wells of microwell plates. There was a sterility control well (100 µl of CAMHB without drug or inoculum suspension) in the first well and a growth control well (10 µl of inoculum suspension+ 100µl of CAMHB) in last well of the plate. Microdilution plates and CAMHB medium were freshly prepared for every test day. For each test isolate, a 0.5 McFarland standard bacterial suspension was prepared in distilled water which was further diluted to 1:20 dilution. After dilution, a ≤10 µl bacterial suspension was added to each well (to make final test concentration of bacteria approximately 5×10^5 CFU/ ml) within 15 minutes and incubated for 16 to 20 hours at 35°C in ambient air incubator. Results were interpreted according to EUCAST (2016) [12] colistin cut-offs for Enterobacterales where MIC value of ≤ 2µg/ml and >2µg/ml were regarded as susceptible and resistant respectively. The MICs were determined as the lowest concentration that completely inhibits the bacterial growth in the well and based on first well demonstrating growth inhibition by visual inspection. **(Figure-1)**

Figure1: Interpretation of colistin susceptibility by BMD

Columns: (From right to left) C1- Growth control well; C2-C11 (well containing colistin concentration 0.125 to 64 µg/ml); C12: Sterility control well

Rows: (Above to below) R1-R7-Test isolates: Susceptible- R2, R3, R4, R6, R7; Resistant- R1, R5] R8: Quality control strain *E. coli* ATCC 25922

Reference strain for quality control and colony counts of inoculum suspension

Quality was ensured using the reference strain *E. coli* ATCC 25922 (colistin-susceptible) in each microtiter plate along with test isolates and all results were considered accurate only when the MIC of the ATCC strain was within

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the acceptable range (0.25 to 2 µg/ml). Furthermore, to ensure the final inoculum concentration approximately 5 × 10^5 CFU/ml (for *E. coli* ATCC 25922 and test isolate), a 0.01-ml aliquot from the growth control well was diluted to 1:1000 and from the dilution, 0.1-ml aliquot was spread over the surface of MacConkey agar media. After incubation, the presence of approximately 50 colonies indicates an inoculum density of 5 × 10^5 CFU/ml. **(Figure-2)**

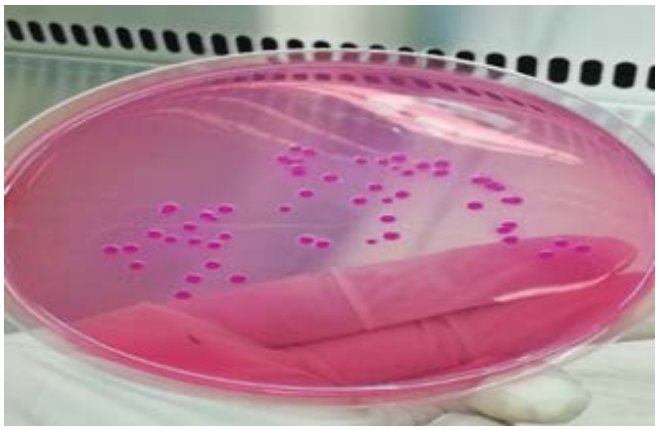

Figure 2: Quality control strain *E. coil* ATCC 25922. After 1:1000 dilution of final inoculum, presence of approximately 50 colonies indicates an inoculum density of 5 × 10^5 CFU/ml

Disc diffusion method for colistin: DDM was performed using 10 µg colistin disc (BioMaxima, Poland) and zone of inhibition was interpreted according to the CLSI 2007 guidelines [13] (resistant ≤10 mm and susceptible ≥11 mm) for colistin which was also used for Enterobacterales in a literature [14].

Statistical analysis: Statistical analysis was performed using the SPSS™ software, version 23.0 (IBM Crop. New York, NY). The qualitative data were presented as frequency, percentages and diagrams. The results from DDM were compared with those obtained by the reference BMD, considering BMD as gold standard. A false-susceptible result by the disc diffusion test was denoted as a very major error (VME), whereas a false-resistance result by disc diffusion was denoted as a major error (ME). Intermediate result by disc diffusion and a susceptible or resistant category by broth microdilution was considered as minor errors (mE). Acceptable levels were < 1.5% for VME, < 3% for ME and < 10% for mE as recommended in CLSI document M23-A2. [15,16,17] Cohen's kappa (k) statistics were calculated to observe the agreement between BMD and DDM.

Results

The CRE isolates of this study comprise *K. pneumoniae* (109), *K. oxytoca* (2), *E. coli* (23) and *Enterobacter* spp (11)

Colistin susceptibility of CRE isolates by BMD: Colistin MIC was ≥ 4 µg / ml in 42 out of 145 (29.0%) isolates which were identified as colistin resistant CRE. In 103 (71.0 %) isolates, the MIC value was ≤ 2µg/ml which were considered as colistin susceptible CRE. The susceptibility pattern of colistin in CRE isolates was shown in **Table-1**.

Table 1: Colistin susceptibility patterns of CRE isolates according to MIC of colistin by BMD

CRE isolates	Colistin susceptibility	
	Susceptible n (%)	Resistant n (%)
***K. pneumoniae*, n=109**	75 (68.8)	34 (31.2)
***K. oxytoca*, n=2**	2 (100.0)	0 (0.0)
***E. coli*, n=23**	19 (82.6)	4 (17.4)
***Enterobacter* spp, n=11**	7 (63.6)	4 (36.4)
Total CRE, n=145	**103 (71.0)**	**42 (29.0)**

The highest number of colistin resistant CRE were isolated from tracheal aspirates and wound swabs (26.19% from each specimen) followed by urine (19.05%), blood (14.29%), pus (9.52%), sputum and bile (2.38% of each).

MIC, MIC^{50}, MIC^{90} value of colistin among CRE isolates: The distribution of the MIC values of colistin in CRE isolates were tabulated in **Table-2**. MIC^{50} and MIC^{90} value of colistin in case of colistin sensitive CRE isolates were 1µg/ml and 2µg/ml respectively. On the other hand, colistin resistant CRE isolates exhibited higher MIC^{50} and MIC^{90} value of 8µg/ml and 16µg/ml respectively.

Agreement between BMD and DDM for susceptibility testing of colistin:

We observed the distribution of MIC value of colistin according to the zone diameter of DDM. For DDM and BMD calculated kappa (k) statistics was 0.40 which indicates slight agreement between these two methods for susceptibility testing of colistin in CRE **(Table-3).**

When disc zone diameters were interpreted according to CLSI 2007 guidelines, 47.62 % (20) of VME and 13.59 % (14) of ME were observed. **(Figure-3)**.

Discussion

Colistin (group E polymyxin), is a popular drug not only for animals (as growth promoters and protective agents) but also for the treatment of infections with drug-resistant bacteria in humans [18]. Despite potential adverse effects (nephrotoxicity and neurotoxicity), colistin is frequently used to treat patients infected with CRE due to strong antimicrobial activity [19]. The increased use leads to the emergence of colistin resistant CRE across the globe including in South Asian Countries [18,19]. In this study, the resistant rate of colistin in clinical CRE isolates was 29% by BMD. Colistin resistance rate among *K. pneumoniae*, *E. coli* and *Enterobacter* spp was 31.19%, 17.39% and 36.36%

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Table 2: Distribution of MIC values of colistin ranging from 0.5 to 32 µg /ml by BMD among CRE isolates

	MIC of colistin µg /ml								
CRE	**0.5 n (%)**	**1 n (%)**	**2 n (%)**	**4 n (%)**	**8 n (%)**	**16 n (%)**	**32 n (%)**	**MIC^{50} µg/ml**	**MIC^{90} µg/ml**
***K. pneumoniae* (n=109)**	0 (0)	42(38.5)	33(30.3)	13(12.0)	14(12.8)	7(6.4)	0(0)	2	8
***K. oxytoca* (n=2)**	0 (0)	1 (50.0)	1(50.0)	0 (0)	0 (0)	0 (0)	0 (0)	-	-
E. coli (n=23)	1(4.3)	8 (34.8)	10(43.5)	3 (13.0)	1 (4.3)	0 (0)	0(0)	2	4
***Enterobacter spp* (n=11)**	1(9.1)	2 (18.2)	4(36.4)	2 (18.2)	0 (0)	0 (0)	2(18.2)	-	-
Total CRE (N=145)	2 (1.4)	53(36.6)	48(33.1)	18(12.4)	15(10.3)	7(4.8)	2(1.4)	2	8

Table 3: Agreement between DDM and BMD for colistin susceptibility testing of CRE isolates (N=145)

DDM	**MIC by BMD**				
	Susceptible n	**Resistant n**	**Total**	**k value**	***P* value**
Susceptible	89	20	109	**0.4**	**< .001**
Resistant	14	22	36		
Total	103	42	145		

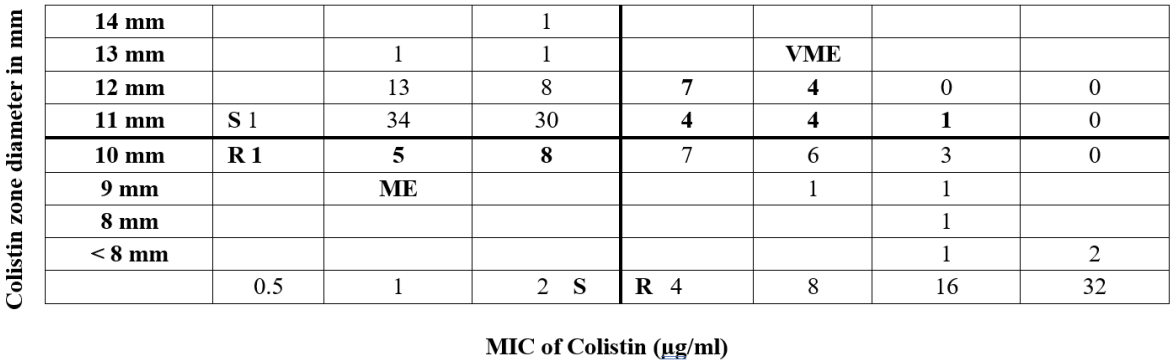

Figure 3: Scattergram comparing the broth microdilution method and 10 µg disc zone diameters for colistin assessed against 145 CRE isolates. The vertical solid line represents the EUCAST susceptibility breakpoints for colistin MIC (Susceptible ≤ 2µg/ml, Resistant > 2 µg/ml). The horizontal solid line represents the susceptibility breakpoints for colistin disk diffusion according to the CLSI 2007 guidelines (resistant ≤10 mm and susceptible ≥11 mm)

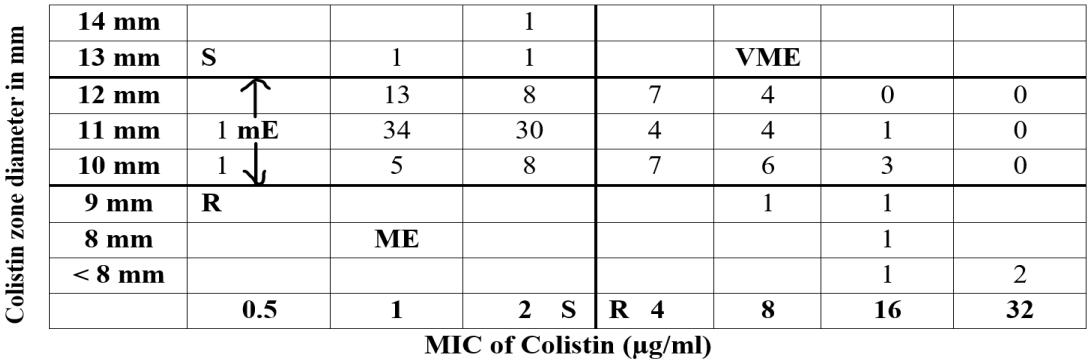

Figure 4: Scattergram comparing the broth-microdilution method and 10 µg disc zone diameters for colistin tested against 145 CRE isolates. The vertical solid line represents the EUCAST susceptibility breakpoints for colistin MIC (Susceptible ≤ 2µg/ml, Resistant > 2 µg/ml). The horizontal solid line represents the proposed breakpoints (≤9 mm as resistant and ≥13 mm as susceptible zone) according to this study, considering acceptable level of VME and ME.

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respectively. All (2) *K. oxytoca* isolates were susceptible to colistin. Study of Pakistan and India report 10.46% and 5.6% of colistin resistant CRE and *K. pneumoniae* respectively [20,21]. Fifteen percent of colistin resistant CRE, in which 16% *E. coli* and 14.6% *K. pneumoniae* were reported by another study of India [22]. Higher percentages of colistin resistance were also observed in meropenem and imipenem resistant Enterobacterales (61.0 and 54.2% respectively) [23]. Like ours, a study of Pakistan reported urine (34%), blood (27%), tracheal aspirates (20%), pus (13%) sputum (3%) as the source of colistin resistant isolates [24]. MIC^{50} and MIC^{90} value of colistin in CRE isolates was 2µg/ml and 8 µg/ml respectively. In colistin susceptible strains, higher $MIC^{50/90}$ (1µg/ml and 2 µg/ml) value of colistin was observed in comparison to another study (both MIC^{50} and MIC^{90} 0.5 µg/ml) [22]. Colistin susceptible isolate with high MIC^{90} (16 µg/ml) was observed in another study [25]. A study of India reports MIC^{50} of 8 µg/ml and MIC^{90} of 16 µg/ml in colistin resistant isolates which supports our study. Similar MIC^{90} value for *E. coli* (4 µg/ml) and *K. pneumoniae* (8 µg/ml) and a lower MIC^{50} (0.5 µg/ml for both isolates) were also observed [22]. A higher $MIC^{50/90}$ value (1/64 mg/ ml) were also reported for *K. pneumoniae* [26]. Highest MIC value (32 µg /ml) was observed in 2 isolates of *Enterobacter* spp and another study also reports *Enterobacter* with higher MIC value [25]. In many facilities of our country, colistin is used as an empiric therapy to treat CRE infections and MIC values of colistin of this study may provide important data regarding antimicrobial stewardship in hospital settings. Despite of interpretive challenges, DDM remains as mainstay method for colistin in clinical laboratories. Reference BMD provides higher precision but time consuming and laborious method [7, 27]. During our study period, there is no breakpoint of colistin for Enterobacterales in CLSI guideline and we used breakpoints defined by EUCAST as susceptible and resistant for CRE isolates. High VME and ME of DDM compared to BMD of this study also reflect the unreliability of DDM for colistin susceptibility testing of clinical isolates of CRE. Colistin resistance was only detected using BMD reference method for determination of MIC but no underlying molecular resistance mechanism was studied. We recommend a multi-center large scale study to see the prevalence of colistin resistance in Enterobacterales with detection of molecular resistance mechanism. As susceptibility testing of colistin by BMD reference method is difficult to perform and different organizations follow different methods (DDM, MIC by VITEC), other methods should be evaluated considering BMD as gold standard that can be easily incorporated in the clinical Microbiology laboratories of our country.

Conclusions

Colistin resistance is a very serious issue as it is mainly used in critically ill patients and accurate susceptibility report is essential for the better outcome of the patients. The high percentage of colistin resistance in CRE isolates of this study is alarming and high major errors of DDM demand the implementation of a reliable method for susceptibility testing of colistin.

Data Availability: All data are contained in the submitted text and tables.

Conflict of Interests: The authors declare no conflict of interest in this work.

Author's Contribution: Conception and study design: AAS, SA and FA; Data collection and laboratory work: FA; Results and statistical analysis: FA; Validation of results: AAS, SA, TM; Writing -first draft: FA; Writing- editing and critical revision: SA, TM, FA; Supervision: AAS and SA. All the authors met ICMJE authorship criteria.

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