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Original Article

Detection of Colistin Resistance in Gram Negative Pathogens: A One Year Cross-sectional Study in a Tertiary Care Centre in Northeast India

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ABSTRACT

Background: Indiscriminate use of colistin for gram-negative infections has led to increase incidence of colistin resistance. The problem of nosocomial infections especially caused by multi-drug-resistant gram-negative bacteria (MDR-GNB), particularly *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* is a matter of great concern. This study was conducted to find out the prevalence of colistin resistant gram-negative isolates from patients attending outdoor patient department (OPD), those admitted in wards and Intensive care unit from a Tertiary care centre in North-East India. **Materials and Methods:** Clinical samples obtained were processed using standard microbiological methods. The gram-negative isolates showing colistin resistance by Kirby- Bauer's disc diffusion method were included and further subjected for MIC (minimum inhibitory concentration) testing by VITEK-2 system followed by confirmation by Broth microdilution method. **Results:** Colistin resistance was observed in 26 isolates out of 1040 gram-negative isolates using Broth microdilution method. The MIC values varied from 8 to ≥ 32 µg/ml. Majority of them belong to *Pseudomonas species* followed by *Acinetobacter species* and were highly resistant to ß-lactams, aminoglycosides, fluoroquinolones. **Conclusion:** This study highlights an increasing trend of colistin resistance amongst multidrug resistant (MDR) gram-negative isolates warranting routine screening for colistin resistance to guide appropriate therapy for future use.

Keywords: Broth microdilution, Colistin, Disc diffusion, Multidrug resistant organisms, VITEK-2.

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INTRODUCTION

The increasing trend of resistance to various classes of antimicrobial agents has become a major public health problem worldwide. Limited options in the pipeline for antibiotic therapy especially for the treatment of gram-negative infections has further compounded the issue.[1] Hospital acquired multi drug resistance (MDR) gram-negative infections, especially those caused by *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae is* a major concern worldwide. The increasing antibiotic resistance amongst gram-negative pathogens to the commonly prescribed group of antibiotics, including the last resort carbapenem group, has forced the re-introduction of colistin as an alternative therapeutic modality.[2] In 1950s colistin (Polymyxin E) was used for the first time in Japan.[3] Unfortunately indiscriminate use of this antibiotic especially for MDR gram-negative infections has contributed to the surge of colistin resistance in most of the countries.[2]

Colistin resistance is attributed to various mechanisms like specific modification of outer membrane porins, reduction in the overall negative charge of the lipopolysaccharide, overexpression of efflux pump systems and overproduction of capsule polysaccharide.[4] In the recent times plasmid mediated colistin resistance due to *mcr-1* gene has also been reported which may enhance the resistance further through horizontal spread.[5]

Colistin remains the last resort antimicrobial agent against MDR *Enterobacteriaceae* infections including those producing carbapenemase and New Delhi metallo-β-lactamase enzymes. Though resistance to colistin presently is low and varies amongst the gram-negative isolates, the discovery of *mcr-1* gene in *Escherichia coli* in China is alarming, as this limits the use of colistin as a last therapeutic option for these infections.[5]

In Northeast India there is paucity of information regarding colistin resistance for gram-negative infections. This may be attributed to lack of standardized laboratory diagnostic facilities. Hence this study was planned to detect colistin resistance among gram- negative isolates using screening and confirmatory phenotypic methods.

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MATERIALS AND METHODS

Study Design: This was a hospital based one-year prevalence study from January 2018 to December 2018 in the Department of Microbiology, North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS), a tertiary health care centre in Northeast India. All gram-negative isolates from clinical specimens obtained from patients attending outdoor patient department (OPD) and admitted in Intensive care unit (ICU) which fitted into the inclusion criteria were enrolled in the study.

Inclusion Criteria: Clinical gram-negative isolates obtained from different samples (blood, pleural fluid, ascitic fluid, peritoneal fluid, cerebrospinal fluid, sputum, tracheal secretion, bronchial secretion, exudates and urine) showing colistin resistance by disc diffusion method[6] were included in the study.

Exclusion Criteria: Any repeat isolate from the same patient was excluded to avoid duplication. Gram negative bacteria having known intrinsic colistin resistance including *Edwardsiella* spp, *Morganella morganii, Proteus* spp, *Providencia* spp and *Serratia* spp (100% of these organisms are intrinsically resistant to colistin) were also excluded.

Study Procedure: Specimens like blood, pleural fluid, ascitic fluid, peritoneal fluid, cerebrospinal fluid, sputum, tracheal secretion, bronchial secretion, exudates and urine obtained from patients were cultured on routine bacteriological media. Biochemical identification and Antimicrobial susceptibility testing (by Kirby-Bauer's method) of the isolates were performed according to the standard laboratory procedures.[7] Gram negative identified isolates showing colistin resistance by disc diffusion method[6] were included in the study and further analysed.

Phenotypic Methods for Detecting Colistin Resistance

Screening by Disc Diffusion Method: 10 μg Colistin (Methane Sulphonate) disks (HiMedia Laboratories Pvt. Ltd.[6] were put on the Muller Hinton agar (MHA) plate for screening of colistin resistance according to Kirby-Bauer's disc-diffusion method following standard laboratory protocols.[7]

Automated Bacterial Culture System: Identification of all the screened isolates were further analysed for Minimum Inhibitory Concentration (MIC) by automated methods (VITEK 2). Antimicrobial susceptibility testing for a panel of antibiotics including colistin was performed using AST-N280 card for determination of resistance pattern and the MIC. The standard procedures recommended by the manufacturer were followed for Identification and Antimicrobial susceptibility testing (AST).[8] MIC values were interpreted as per CLSI 2018 guidelines.[9] Isolates having MIC of ≥ 4 μg/ml were categorized as colistin resistant.[9]

Colistin Microbroth Dilution: Colistin powder obtained from Sigma Aldrich, having an assay potency of 15,000 units/mg was used for determining the minimum inhibitory concentration (MIC) by Microbroth dilution.

The required potency as per CLSI is 30,000 units/mg. Hence, colistin of double strength was used. For media MHB (Muller Hinton Broth) of strength 21g/L was used. 100ml of 2X strength MHB per use was prepared. The concentration of colistin tested ranged from 0.25 to 32 μg/ml. Organism strength added in the well was $5x10^5$ CFU/ml as per CLSI. After adding media, drug and organism to the well of the microtiter plate, it was incubated overnight at 37°C. The results were noted on the next day. ATCC strain *Escherichia coli* 25922 and *Pseudomonas aeruginosa* 27853 were used as quality control.[9]

Independent Variables: The data collected was classified and analysed in terms of the following independent variables: age, gender, in-patient/out-patient, icu/non-icu, site of infection, clinical sample, date of collection, organism, multidrug-resistant (MDR)/non-MDR and previous administration of colistin (if yes, then duration).

Outcome Variables: The sensitivity pattern of colistin (sensitive/resistant) in different phenotypic methods and the MIC were the measures of outcome in this study.

Data Analysis

The data from the isolate proforma was compiled using Microsoft Excel v2007 for Windows. Demographic and clinical parameters were tabulated and graphed using the same. Significance of statistical association of the colistin resistant isolates in comparison to the colistin sensitive isolates has been calculated from standard probability (*p*-value) using Chi- Square test. The observation was considered statistically significant if the *p*-value was less than 0.05. The arithmetic mean of continuous variable such as age was calculated using MedCalc for Windows, Version 19.1 (MedCalc Software, Ostend, Belgium). The statistical analysis was done using MedCalc for Windows version 19.1 (Ostend, Belgium).

RESULTS

A total of 1040 Non-duplicate, consecutive patient-specific gram-negative isolates were included in the study. All these isolates were screened for colistin resistance by Kirby Bauer's disc-diffusion method. Out of the 1040 isolates, 97 were found to be resistant to colistin by disc diffusion. On further susceptibility testing by VITEK -2 system, 43 out of the 97 isolates showed colistin resistance having MIC of ≥16 μg/ml. Broth-microdilution method was performed for the 43 resistant isolates (by VITEK) following which only 26 (2.5%) isolates showed colistin resistance.

Out of the total 26 colistin resistant isolates, 14 (53.8%) were obtained from males and 12 (46.2%) from females. Maximum resistant isolates were detected in 40-49 years age group (42.3%) (Figure 1). 12 out of the 26 resistant isolates (46.2%) were detected from patients with bloodstream infections (Figure 2) admitted in the ICU (Figure 3). The colistin resistant isolates were mostly obtained from blood as shown

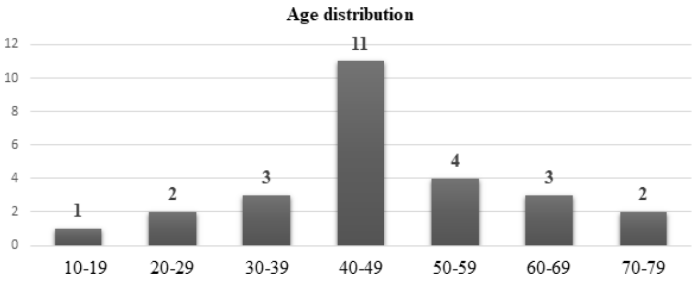

Figure 1: Age distribution of study subjects.

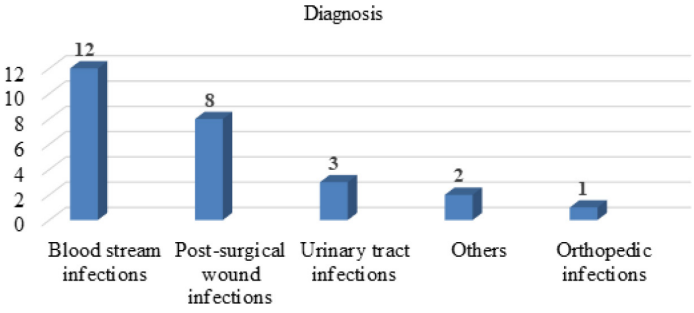

Figure 2: Clinical spectrum of patients showing colistin resistant Gram-negative isolate.

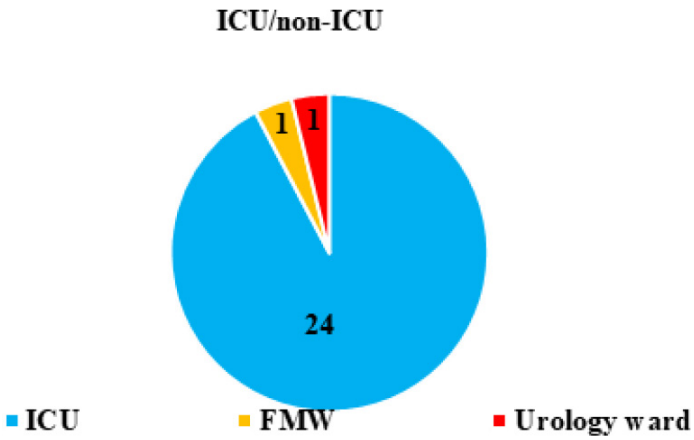

Figure 3: Department wise distribution of the patients.

Table 1: Source of the isolates showing colistin resistance by Broth microdilution method.

Sample Distribution	Total	Colistin resistant isolates by Broth microdilution	'p' value
Blood	697 (67.01%)	21 (80.7%)	0.1417
Tracheal aspirate	159 (15.28%)	2 (7.6%)	0.2801
Urine	126 (12.11%)	2 (7.6%)	0.4848
PCN	58 (5.57%)	1 (3.8%)	0.6966
Grand Total	**1040**	**26**	

Table 2: Gram-negative isolates showing colistin resistance by Broth-microdilution method.

Organisms	Total	Colistin resistant isolates by Broth microdilution	'p' value
Pseudomonas aeruginosa	447 (42.98%)	12 (46.2%)	0.7434
Acinetobacter baumannii	301 (28.94%)	8 (30.7%)	0.8452
Klebsiella pneumoniae	93 (8.94%)	2 (7.6%)	0.8128
Enterobacter spp.	85 (8.17%)	2 (7.6%)	0.9165
Klebsiella oxytoca	61 (5.86%)	1 (3.8%)	0.6575
Escherichia coli	53 (5.09%)	1 (3.8%)	0.7670
Grand Total	**1040**	**26**	

in (Table 1). The predominant resistant isolate was *Pseudomonas aeruginosa* isolate followed by *Acinetobacter baumannii* as shown in (Table 2). MIC value of ≥ 32 µg/ml (57.7%) were observed in majority of the study isolates using Broth microdilution method followed by ≥ 16 µg/ml (34.7%). Seasonal peak for these isolates was observed in July-August (34.6%) which can be explained due to the maximum number of admissions during the summer season.

The ROC (receiver operating characteristic) curve for VITEK 2 test as compared to the Broth microdilution test is shown in (Figure 4). All the colistin resistant *Pseudomonas aeruginosa* isolates showed resistance to piperacillin, fluoroquinolones, ceftazidime, cefepime and (piperacillin + tazobactum). Low sensitivity rates were observed for the colistin resistant isolates against amikacin (16.66%), gentamicin (17%) and imipenem

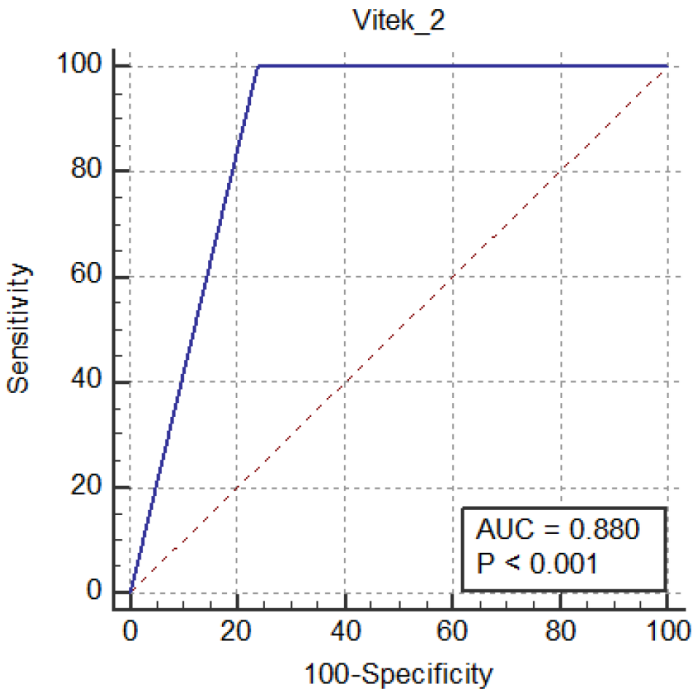

Figure 4: ROC curve for Vitek 2 test.

Area under the curve	0.880
Standard error	0.0255
Significance level 'p'	< 0.001
95% Confidence Interval	0.799 to 0.937
Sensitivity	100.00
Specificity	76.06

(16.66%). Highest sensitivity was seen with meropenem (41.66%) for the colistin resistant *Pseudomonas* isolates.

All the colistin resistant *Acinetobacter baumannii* isolates were resistant to ofloxacin, piperacillin, gentamicin, cefoperazone, cefotaxime and ceftriaxone. High resistance rates were observed against ampicillin + salbactum (62.5%), ciprofloxacin (87.5%), amikacin (75%), cotrimoxazole (87.5%) and cefepime (87.5%). Most of the isolates (75%) were sensitive to meropenem.

All the colistin resistant *Klebsiella* species, *Enterobacter* spp. and the *Escherichia coli* isolate were resistant to ampicillin + salbactum, fluoroquinolones, piperacillin, gentamicin, cefoperazone, cefotaxime, ceftriaxone and cefepime. The *Klebsiella* isolates were sensitive to meropenem and intermediately sensitive to imipenem. All *Enterobacter* spp. isolates were intermediately sensitive to amikacin. The *Escherichia coli* had intermediate sensitivity to ampicillin + salbactum, amikacin, piperacillin + tazobactum and imipenem. The isolate was sensitive to meropenem.

Overall Meropenem showed the highest sensitivity (57.69%) amongst the colistin resistant isolates followed by piperacillin + tazobactum (23.07%) and imipenem (19.23%). Other antibiotics had less sensitivity for the study isolates ranging from 0% to 16.66%.

DISCUSSION

Colistin remains as a last option for treating multi drug and pan dug resistant gram-negative infections.[10] Indiscriminate and irrational antibiotic use has paved the way to increased cases of colistin resistance.

The true burden of colistin resistance is undermined because of lack of routine testing in the Microbiology laboratory. This study was an endeavour to determine the prevalence of colistin resistance in gram-negative infections.

In this study 2.5% colistin resistance was observed by broth microdilution method amongst the gram-negative isolates which shows concordance to the study by Falagas *et al*. where colistin resistance was seen in 1.9% -3.3% isolates.[11] In contrast to our findings Pawar *et al*. reported 9.98% colistin resistance amongst gram-negative bacilli.[12]

In our study, majority of the colistin resistant isolates obtained were from males (53.8%). This is not statistically significant as higher number of males were admitted (p =0.1938). Patients aged between 40 to 49 years comprised the largest group in our study which was statistically significant (p = 0.0252). The mean age of the study group was 46.11 years. Most (80.7%) of the isolates were obtained from blood samples. This may be explained on the basis of multisystem involvement of ICU patients by the nosocomial pathogens. In our study, though colistin resistance was observed mostly in ICU patients, however, there was no previous treatment with this antibiotic.

Majority of the colistin resistant isolates were *Pseudomonas aeruginosa* (46.2%) followed by *Acinetobacter baumannii* (30.7%) and *Klebsiella species* (11.5%). Similar findings were seen in a study at a tertiary care rural hospital from western India[12] and in a study by Maspi *et al*. in Iran.[13] In contrast to our study Goli *et al*. reported only 4.8% *Pseudomonas* isolates resistant to colistin.[14] This discrepancy can be due to the misuse of drugs, dissimilar policies of hospitals for controlling the infection, sanitation, and topographical distribution.

The colistin resistant study isolates were highly resistant to ß-lactams including cefepime, ampicillin-salbactum, piperacillin-tazobactum, and also to aminoglycosides (92.30%), fluoroquinolones (96.15%) and cotrimoxazole (78.57%). The isolates showed maximum sensitivity against meropenem (57.69%). Similar resistance pattern was also reported in a study by Arjun *et al*. especially with respect to amikacin and cotrimoxazole[15] and also from a study in western India with respect to piperacillin-tazobactum, fluroquinolones and imipenem.[12]

In our study, disc diffusion was used for screening of colistin resistance which is not a recommended method, as colistin has poor diffusion on agar culture medium. This may lead to erroneous results in different environmental conditions.[16] Moreover, hetero-resistance which has been defined as a phenomenon in which a pre-existing subpopulation of resistant cells can rapidly replicate in the presence of a given antibiotic, whereas the majority population of susceptible cells are killed[17] was also not addressed in our study. Among the 26 cases of colistin resistance, there was no history of previous treatment with this drug, a known risk factor for acquiring resistance. However, exposure to environmental or animal colistin was not addressed in this study.

The present study highlights an increasing prevalence of colistin resistance in Gram-negative infections especially in the ICU settings harbouring multidrug resistant organisms. The detection and surveillance of colistin resistant organisms is of utmost importance in the present day scenario where antimicrobial resistance is on the rise. This will enable formulation of appropriate antibiotic policies. Mere study of resistance profile to other antibiotics will not help in predicting colistin resistance. Hence a standard protocol must be formulated so that suspected clinical isolates are subjected to screening for colistin resistance. Phenotypic and/or genotypic tests should be adopted for confirmation of colistin resistance. Antibiotic stewardship programmes to reduce the antibiotic resistance besides improving the patient care with reduced treatment failure is the need of the hour.

CONCLUSION

This study highlights an increasing trend of colistin resistance amongst multidrug resistant (MDR) gram-negative isolates warranting routine screening for colistin resistance to guide appropriate therapy for future use.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

MDR: Multidrug resistance; **OPD:** Outpatient department; **ICU:** Intensive care unit; **MHA:** Mueller hinton agar; **AST:** Antimicrobial susceptibility test.

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