e-ISSN: 0976-822X, p-ISSN:2961-6042

Available online on http://www.ijcpr.com/

International Journal of Current Pharmaceutical Review and Research 2025; 17(10); 761-770

Original Research Article

Study of Colistin Sensitivity Among Clinical Isolates of Multidrug Resistant Gram-Negative Bacilli in A Tertiary Care Hospital in Bengaluru

Madhura CM[1], Sathyanarayan MS[2], Lavanya J[3], Kusuma GR[4]

[1]Assistant Professor, Department of Microbiology, Chikkamagaluru Institute of Medical Sciences, Chikkamagaluru, Karnataka, India

[2]Assistant Professor, Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru, Karnataka, India

[3]Associate Professor and Head, Department of Microbiology, Chikkamagaluru Institute of Medical Sciences, Chikkamagaluru, Karnataka, India

[4]Lecturer, Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru, Karnataka, India

Received: 20-08-2025 / Revised: 19-09-2025 / Accepted: 20-10-2025

Corresponding Author: Madhura CM

Conflict of interest: Nil

Abstract:

Aim: To study Colistin sensitivity among clinical isolates by:

1. Phenotypic method namely Broth Micro Dilution (BMD).
2. Genotypic method namely Polymerase Chain Reaction (PCR) to detect Colistin resistant genes in Gram negative bacilli (pmrA, pmrB, mgrB, phoP).

Methods: A cross-sectional study was conducted after collecting150 samples including pus, endotracheal aspirate, blood, sputum, urine samples from patients who have attended OPD and admitted in hospitals attached to Bangalore Medical College and Research Institute, processed in Infosys Central Laboratory, Department of Microbiology BMCRI, Bengaluru from February 2021 to January 2023, Colistin sensitivity was studied by BMD and PCR.

Results: 150 clinical isolates consisting of K. Pneumoniae 54(36%), 51 were resistant and 3 sensitive, E.coli 46 (31%), 33 were resistant and 13 sensitive ,A. baumannii 24(16%),16 were resistant and 8 sensitive, P. aeruginosa 22(14%),18 were resistant and 4 sensitive, E.cloacae 2(1%), E.aerogenes 1(1%), C.freundii 1(1%) all were resistant by BMD method, among 22 P. aeruginosa 15 found to be sensitive and 7 resistant, among 150 isolates pmrA detected in 22 isolates, pmrB in 19, mgrB in 13, phoP in 15 by PCR.

Conclusion: Due to increased usage there is rise in resistance, hence Colistin should be preserved by implementing antibiotic stewardship and appropriate infection control measures.

Keywords: Polymyxin; Broth Micro Dilution; Polymerase Chain Reaction.

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Introduction

Colistin belongs to the group of polymyxins, known as Colistin E.

Since 1959 Gram negative bacterial infections caused by P.aeruginosa, A. baumannii, E. coli, K. pneumoniae, Salmonella spp, Shigella spp, Enterobacter spp, H.i nfluenza were being treated by Colistin [1].

Due to its nephrotoxicity and neurotoxicity, Colistin use was discontinued around 1970, and cephalosporins, amikacin, and other potent antibiotics that were regarded as safer took its place. Multiple-drug resistant illnesses are on the rise. Due to the lack of effectiveness of the currently available antibiotics and the emergence of multi drug resistant Gram negative organisms (MDRO), interest in the last-resort medication Colistin has increased [2].

CMS is administered as prodrug which on hydrolysis in vivo produces Colistin [3].

Colistin is a cationic peptide that primarily affects Gram-negative bacilli by disrupting their outer membrane. Colistin is now being used more frequently in clinical settings to treat multidrug resistant Gram negative bacilli, particularly for Carbapenem. The danger of evolving resistance to Colistin has increased recently because of its broad clinical usage, due to chemical alteration or total deletion of the antibiotic's target lipopolysaccharide [4].

Pathophysiological effects of circulating endotoxin can be prevented by Colistin, resulting in increased interest in its usage [5].

Chromosomal mutations that affect lipopolysaccharide modification are mainly found in the genes mgrB, phoP, phoQ, pmrA, and pmrB that encode two-component systems. Colistin resistance is influenced by mcr1-5, a plasmid-mediated gene that encodes phosphoethanolamine transferase [5].

Colistin susceptibility testing is still considered challenging, though it is in clinical use for decades. Methods based on dilution are recommended to determine bacterial susceptibility to Colistin [4].

Several methods have been developed to detect colistin resistance, such as BMD, colistin broth disk elution method, CHROM agar COL, Resa Colistin NP test, rapid Colistin NP test, and the colistin agar method [6]

Materials and Methods

After obtaining approval and clearance from the Institutional Ethics Committee, the patients fulfilling the inclusion criteria enrolled for the study after obtaining informed consent. Prospective cross-sectional analytical study was carried out in Department of Microbiology, Infosys Central Laboratory, BMCRI from February 2021 to January 2023 by collecting 150 samples including pus, endotracheal aspirate, blood, sputum, urine samples collected from patients who have attended OPD and admitted in hospitals attached to BMCRI.

Inclusion Criteria: All Gram-negative clinical isolates identified as multi drug resistant.

Exclusion Criteria:

1. All clinical isolates sensitive to most of the antibiotics tested.
2. Gram positive clinical isolates
3. Burkolderia species, Proteus species, Providencia species, Serratia species, Morganella (intrinsically-resistant).

Isolation and identification: The specimens were inoculated on MacConkey agar and incubated at 37°C aerobically for 24 hours. Pure isolated colonies on MacConkey agar were processed according to standard microbiological [7] and CLSI guidelines [8]

Identification and antibiotic sensitivity test were done by automated ID and AST system (Vitek-2 compact, Biomeriux), 150 multidrug resistant Gram negative isolates were further subjected to Colistin susceptibility test by

1. **Broth Micro Dilution Method:** Susceptibility of MDR Gram negative isolates to Colistin was studied by microbroth dilution method using ready to use broth microdilution micro-titre trays according to CLSI guideline.

Preparation of inoculums: 150 MDR Gram negative clinical isolates and E. coli ATCC25922 and P. aeruginosa ATCC 27853 were taken and plated on a MacConkey agar, pure isolated colony was picked and emulsified in about 50µL of normal saline and transferred to inoculum broth and adjusted to 0.5 Mc Farland opacity standard using densitometer. BMD strips were placed in a Test Panel Tray and strips were marked with the patient ID and 200µl of inoculum broth was loaded to all the wells using 200µl pipette. Test Panel was covered with tray cover and incubated at 37°C for overnight.

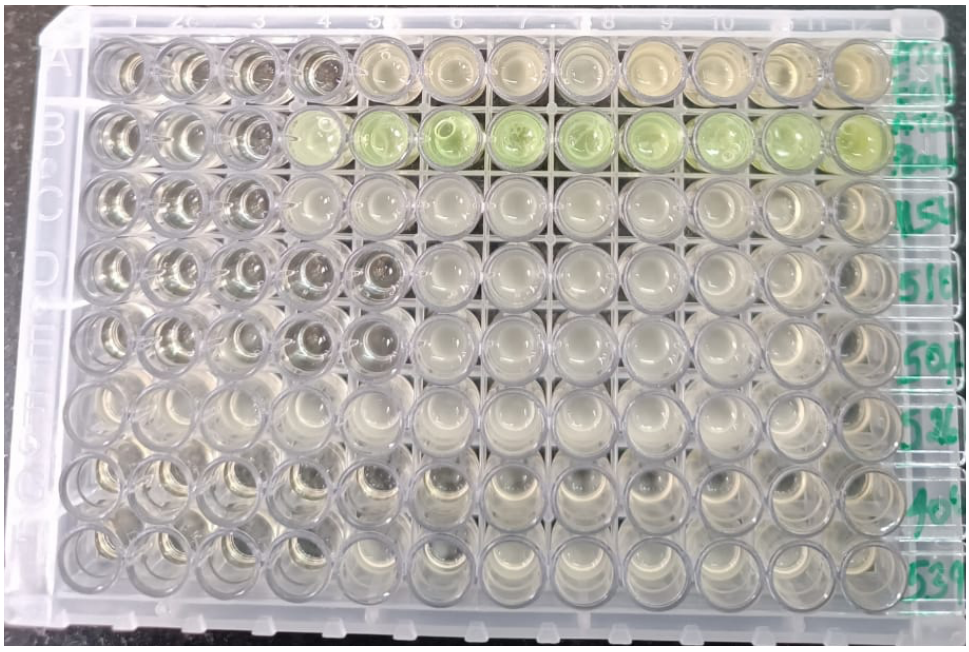

Figure 1:

Microtitre Tray For Colistin Broth Micro Dilution

Interpretation of Results

Results were interpreted according to EUCAST guidelines [9]

Susceptibility value for Colistin is as follows:

For Enterobacterales and Acinetobacter spp susceptibility is ≤2 µg/ mL, resistant ≥2 µg/ mL.

For Pseudomonas spp susceptibility is ≤4 µg/ mL, resistant ≥4 µg/ mL(34).

Genotypic Method: Detection of Colistin resistant genes- pmrA gene, pmrB gene, mgrB gene and phoP gene by conventional PCR.

All 150 MDR Gram negative clinical isolates were subjected to molecular detection of targeted genes.

Steps are as follows

DNA extraction [10]: DNA extraction was done by boiling method.

Polymerase Chain Reaction (PCR): The multiplex PCR done for Colistin resistant genes namely pmrA, pmrB, mgrB and uniplex PCR was done for phoP gene. The primers used were obtained from Barcode BioSciences and are as described below.

Table 1: Primer sequence for Colistin resistant genes [10]

Genes	Forward primer	Reverse primer	Base pair
pmrA	ATGACAAAAATCTTGATGATTGAA	TTATGATTGCCCCAAACGGTA	675
pmrB	GACTGATTTGGGGCACCTC	TGTTTCATGTAAATGTAAAACTTTAGG	1304
mgrB	CGC GGT TTA AGA AGG TCA TG	AGG CGT TTA TTC TAC CAC CC	1196
phoP	ATA CCC ACA GGA CGT CAT CA	CAG GTG TCT GAC AGG GAT TA	2800

Multiplex PCR Reaction mixture: (For pmrA, pmrB, mgrB)

(For One Reaction of 25ul)

Red TaqMan Mastermix = 12.5ul

Forward Primer = 2.25ul (0.75ul of each primer)

Reverse Primer = 2.25ul (0.75ul of each primer)

Nuclease Free Water = 6 ul

DNA template = 2ul

Total Reaction Volume = 25ul

Uniplex PCR Reaction mixture: (For Phos P)

(For One Reaction of 25ul)

Red TaqMan Mastermix = 12.5ul

Forward Primer = 2.25ul

Reverse Primer = 2.25ul

Nuclease Free Water = 6 ul

DNA template = 2ul

Total Reaction Volume = 25ul

The above reaction mixture was spinned in a microcentrifuge and amplified in a PCR thermocycler. Cycling conditions are followed as mentioned below

- Initial denaturation at 95°C for 10 minutes
- Denaturation at 95°C for one minute
- Annealing 60°C for 45 seconds, } **30 cycles**
- Extension at 72°C for one minute
- Final extension at 72°C for 10 minutes.

Finally, the Amplified DNA Product was run on 1% agarose gel, and the prevalence of each Colistin resistant gene determined using agarose gel electrophoresis. The size of the amplified DNA assessed comparing with the bands of DNA ladder [11]. The target DNA molecule was visualized under Ultra-violet Trans-illuminator at 265nm and photographic image was obtained in a Gel Documentation System.

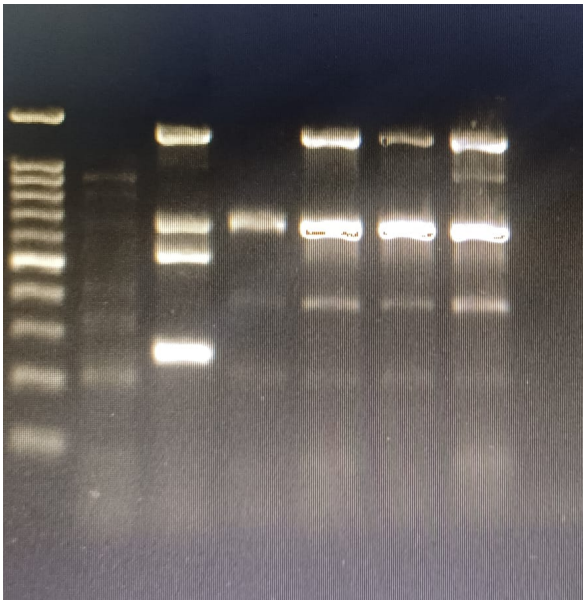

Figure 2: Gel electrophoresis image of pmrA, pmrB, mgrB GENES

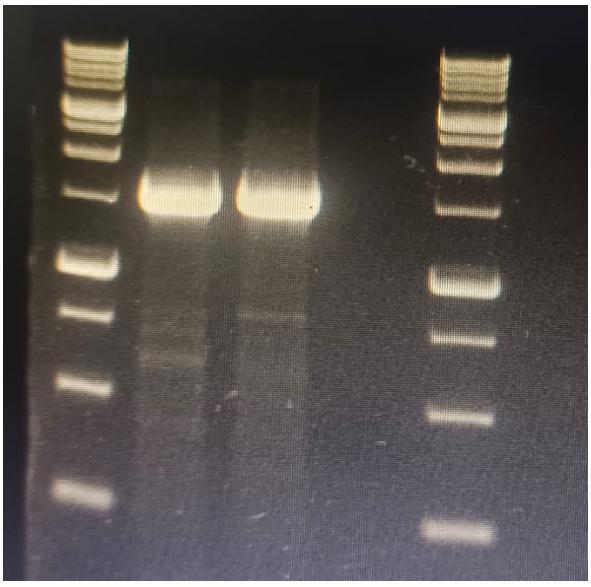

Figure 3: Gel electrophoresis image of phoP gene

Results

Age distribution

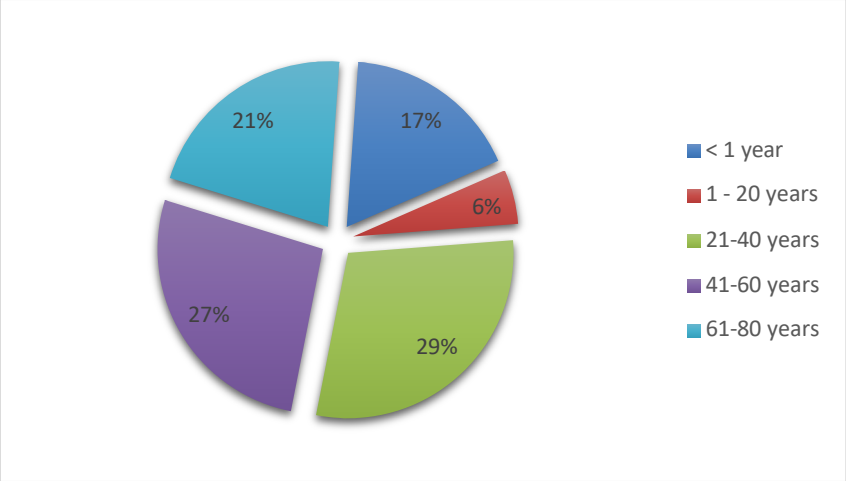

Figure 4: Age wise distribution of 150 MDR Gram negative clinical isolates

Gender distribution

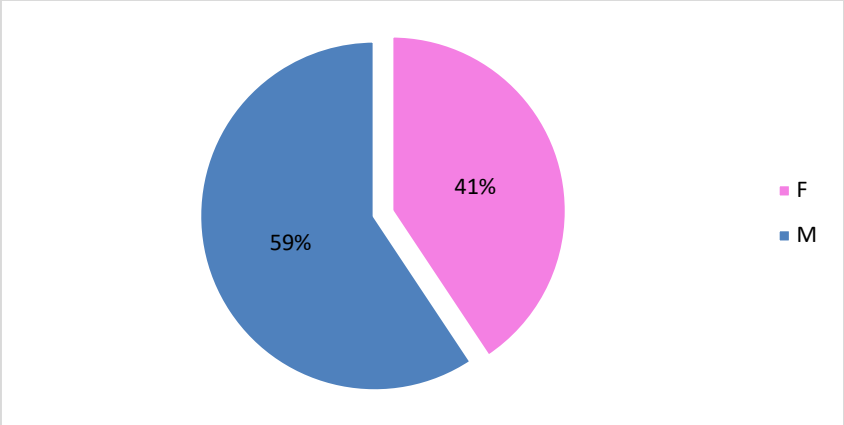

Figure 5: Gender wise distribution of 150 MDR Gram negative clinical isolates

Ward distribution

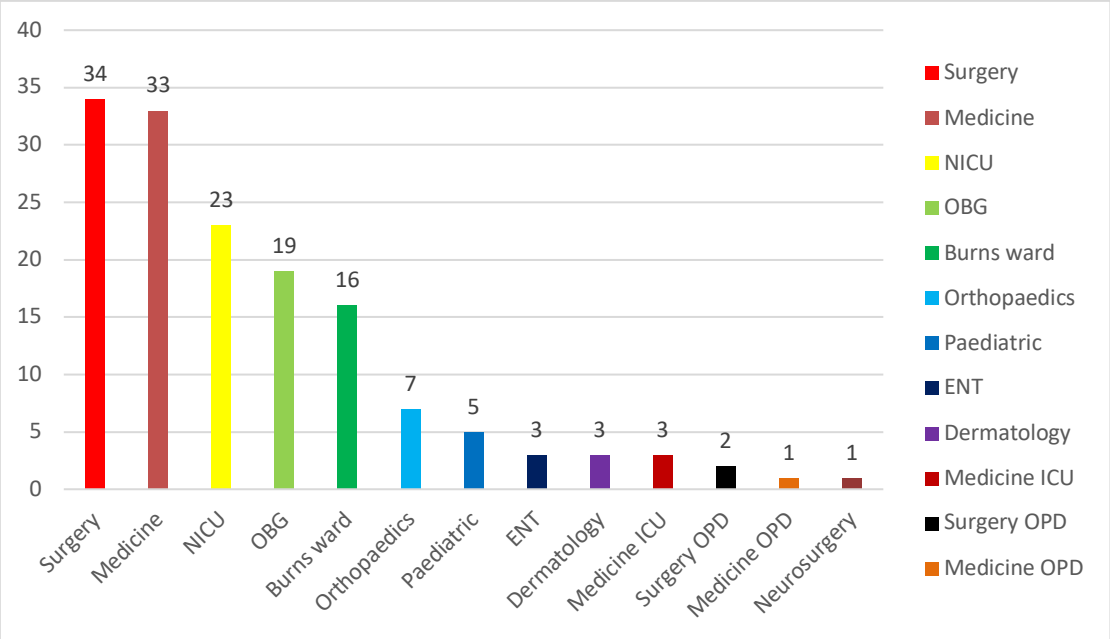

Figure 6: Ward wise distribution of 150 MDR Gram negative clinical isolates

Sample wise distribution: Among 150 MDR Gram negative clinical isolates most of them were from pus sample followed by blood, urine and others.

Table 2: Number and percentage of clinical samples

Clinical sample	Number	Percentage (%)
Pus	75	50
Blood	31	21
Urine	18	12
Sputum	11	7
Endotracheal aspirate	6	4
Vaginal swab	4	2
Ascitic fluid	3	2
Pleural fluid	1	<1
CSF	1	<1

Of the 150 clinical isolates, K. pneumoniae was 54 followed by E. coli 46, A. baumannii 24, P. aeruginosa 22, E. cloacae 2, E. aerogenes 1, C. freundii 1as shown in table

Table 3: Number and percentage of clinical isolates

Isolated Organism	Number	Percentage (%)
Klebsiella pneumoniae	54	36
Escherichia coli	46	31
Acinetobacter baumannii	24	16
Pseudomonas aeruginosa	22	14
Enterobacter cloacae	2	1
Enterobacter aerogenes	1	<1
Citrobacter freundii	1	<1

Phenotypic test

1. **Broth Micro Dilution**

Among 54 K. pneumoniae 51 were resistant and 3 were sensitive, among 46 E. coli 33 are resistant and 13 were sensitive, among 24 A. baumannii 16 were resistant and 8 were sensitive, among 22 P. aeruginosa 18 were resistant and 4 were sensitive,

E. cloacae being 2 in no both were resistant, E. aerogenes and

C. freundii being single were resistant to Colistin.

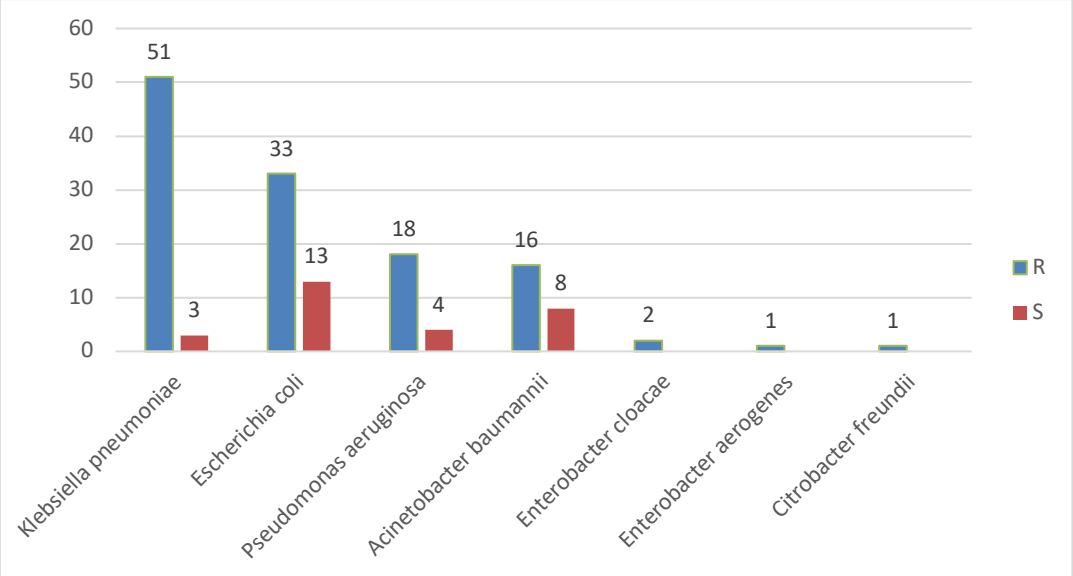

Figure 7: Distribution of number of resistant and sensitive clinical isolates

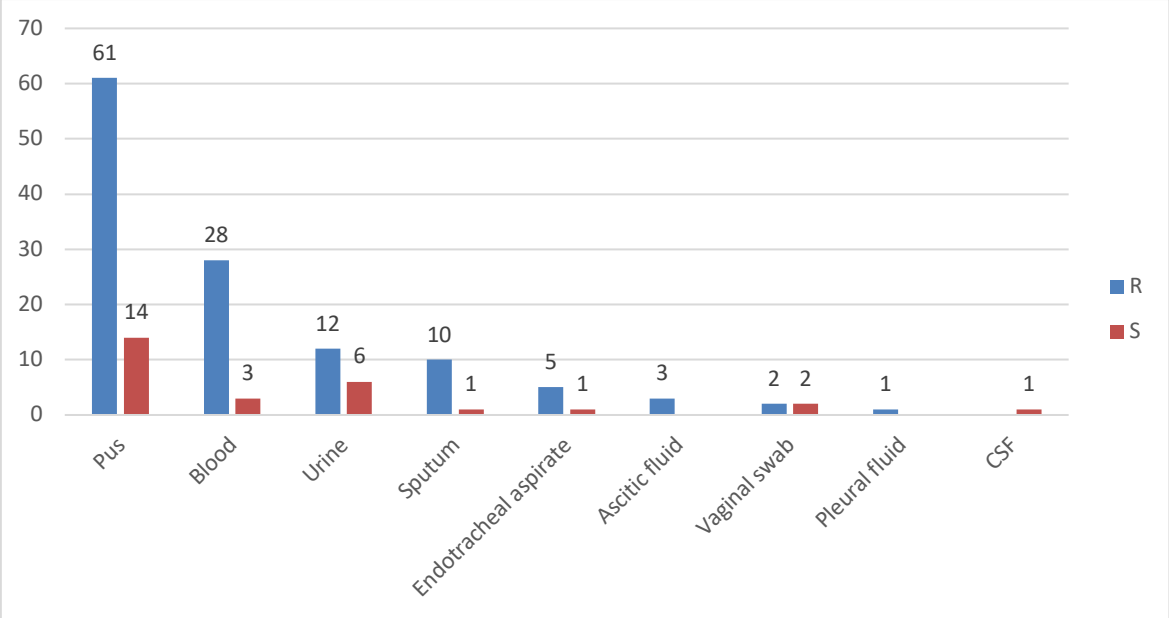

Figure 8: Sample wise distribution of number of resistant and sensitive isolates

Genotypic Method

Polymerase Chain Reaction

Among 150 MDR Gram negative clinical isolates, pmrA was detected in 22 isolates, pmrB in 19 isolates, phosP in 15 isolates, mgrB in 13 isolates.

Table 4: Number of isolates detected pmrA gene with respect to clinical samples

Clinical samples	K. pneumoniae	A. baumannii	P. aeruginosa
Pus	1	6	5
Blood	1	4	-
Endotracheal aspirate	1	**2**	-
Sputum	2	-	-

Table 5: Number of isolates detected pmrB gene with respect to clinical samples

Clinical samples	K. pneumoniae	A. baumannii
Blood	5	3
Pus	4	2
Sputum	2	1
Ascitic fluid	1	-
Pleural fluid	1	-

Table 6: Number of isolates detected phoP gene with respect to clinical samples

Clinical samples	K. pneumoniae	E. aerogenes
Pus	4	1
Sputum	4	-
Blood	3	-
Endotracheal aspirate	2	-
Ascitic fluid	1	-

Table 7: Number of isolates detected mgrB gene with respect to clinical samples

Clinical samples	K. pneumoniae	A. baumannii	P. aeruginosa	E. coli
Pus	2	2	2	1
Blood	1	2	-	-
Endotracheal aspirate	1	1	-	-
Sputum	1	-	-	-

Discussion

Colistin has been mostly abandoned in the clinical treatment of bacterial infections since 1970s because of its neurotoxicity and low renal clearance. The rise

of multidrug-resistant bacteria brought on by antibiotic overuse has, however, altered the situation [11]

Limited therapeutic options have forced clinicians and microbiologists to reappraise the clinical application of Colistin for treating MDR Gram negative, especially Carbapenem resistant GNB [12].

Table 8: Comparison of results of present study with other studies

Place	Author	Sample size	Isolates	Methods	Results	Remarks
Vellore	Ramesh N et al [13]	94	E.coli-48 K.pneumoniae-9 P.aeruginosa- 10 A.baumannii- 2 E.cloacae- 3	Identification was done by 16sRNA AST by BMD	28.7% resistant to colistin	Present study has high percentage of resistant isolates
	Present study	150	K.pneumoniae-54 E coli-46 E cloacae- 2 A baumannii-24 P. aeruginosa-22	Identification was done by Vitek 2 AST by BMD	81.3% resistant to colistin	
Iran	Khosbhayan et al [14]	293	K.pneumoniae-195 E coli- 98	AST by BMD	10.7% of K.pneumoniae resistant to colistin Among E. coli no resistance was detected	Present study has high percentage of resistant isolates
Iran	Haeli M et al [4]	109	K.pneumoniae-34 E.coli- 20 P.aeruginosa- 38 Acinetobacter-17	AST by BMD	19.2% of total isolates resistant to colistin All E.coli are sensitive 12.8% consists of K.pneumoniae,1% consists of Acinetobacter,2.6%consists ofP.aeruginosa	
North East India	Biswas et al [15]	1040	P.aeruginosa-447 A baumannii-301 K.pneumoniae-93 Enterobacter spp- 85 K.oxytoca-61 E coli-53	AST by DD, Vitek 2 and BMD	97 were resistant by DD 43 out of 97 were resistant by Vitek 2 26(2.5%) out of 43 were resistant by BMD	
	Present study	150	K.pneumoniae-54 E coli-46	AST by BMD	94.4 % of K.pneumoniae resistant to colistin 5 isolates had mgrB gene, 13 had pmrB gene	
Dhaka	Mostafa et al [10]	204		AST by DD PCR to detect colistin resistant genes	10.78% were resistant to colistin pmrA detected in 50% of ColR isolates phoP in 27.27% pmrB in 22.72% mgrB in 13.63%	

	Present study	150	K.pneumoniae-54 E coli-46	AST by BMD PCR to detect colistin resistant genes	81.3% resistant to colistin pmrA detected in 18% ColR isolates pmrB in 15.57% mgrB in 10.65% phoP in 12.29	
Tunisia	Jaidane N et al [16]	13 Age group(16-79)	K.pneumoniae-13	WGS	All 13 isolates had mutated mgrB gene	
	Present study	150 Age group(0-80)	K.pneumoniae-54	PCR	5 isolates had mgrB among which 4 are resistant and 1 sensitive to colistin	

Conclusion

Micro broth dilution is the gold standard reference method for testing susceptibility of Colistin. Other than microbroth reference method, automated Colistin susceptibility testing methods such as vitek compact 2 play a role in detecting the resistance and sensitivity.

Colistin is used as last resort drug to treat MDR Gram negative infections, increase in Colistin resistance pattern has been noted, rise in resistance pattern is multifactorial, one of the reasons may be due to increased usage, necessitating the need for routine screening for colistin resistance to guide appropriate therapy.

Colistin being the last resort drug should be preserved by implementing stringent antibiotic stewardship and appropriate infection prevention and control measures.

Acknowledgement

Dr Ambica R, Professor, Department of Microbiology, BMCRI.

Miss Himabindu, Technologist, Department of Microbiology, BMCRI.

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