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Original Research Article

Detection of polymyxin B resistance in *Enterobacterales* by broth disk elution method

Aiswarya Lal[1], Harish Kumar K. S.[1]*, Jiju Janardhanan[2], Anila Gopal[1]

[1]Department of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kottayam, Kerala, India
[2]Department of Medical Microbiology, Co-operative Institute of Health Sciences Kerala, Co-operative Hospital Federation Ltd., Thalassery, Kannur, Kerala, India

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***Correspondence:**
Dr. Harish Kumar K. S.,
E-mail: drharishkumarks@gmail.com

ABSTRACT

Background: Antibiotic resistance being the critical issue faced by medical field, especially by Gram negative bacteria. It is a great threat in the case of both hospitals acquired and community acquired infections. They possess various mechanisms for their survival. The widespread resistance in Gram negative bacteria has necessitated evaluation of the use of older antimicrobials such as polymyxins. Polymyxins are disfavoured owing to their potential clinical toxicity, especially nephrotoxicity. Thus, they got abandoned in the sixties. But now they are re- emerged and used as last resort antibiotics.
Methods: 274 isolates of *Enterobacterales* including *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter spp*. *Enterobacter spp*. was collected from various diagnostic microbiology laboratories in Kerala. The polymyxin resistance among *Enterobacterales* by broth disk elution method recommended by CLSI.
Results: In this study prevalence of multi drug resistant is 37% and extensively drug resistant strains is 25%. And the threatening fact is that the colistin also shows resistance among *Enterobacterales* (9.2%).
Conclusions: Though the resistance to Polymyxin B is to the lesser side in the present study, increase in resistance to the agent is being documented globally elsewhere. So, rational use of Polymyxin B is warrantied as we could cherish polymyxin B as a "life - saving drug" to avoid no drug available. Knowledge of antimicrobial resistance and development of new antimicrobial agents and improved treatments are essential in the current situation.

Keywords: *Enterobacterales*, Extensively drug resistance, Life-saving drug, Multi-drug resistance, Polymyxin, Colistin,

INTRODUCTION

Antibiotic resistance is one of the greatest threats to human health. Many strains of gram-negative bacteria have developed resistance to antibiotics that are widely used. These strains spread globally and this may cause the reduction of therapeutic options. This rise in resistant strains such as multi-drug resistant (MDR), extensively drug resistant (XDR), pan drug resistant (PDR) are major emerging strains. These lead to decrease in number of antimicrobials available for the treatment of infection caused by these strains. The inexorable rise of antibiotic resistance and the paucity of new antimicrobials have led to a renewed interest in the use of the polymyxin group of antibiotics for the treatment of infections due to MDR bacteria.[1,2] Many strains such as *Acinetobacter baumanii, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* that exhibit resistance to almost all

available antibiotics except polymyxin have emerged as a common cause of hospital acquired infection in critically ill patient.[3,4] In the last few years carbapenem resistance among gram negative bacteria increased in prevalence, causing infections difficult to trea.[5,6]

Polymyxins are multicomponent polypeptide antibiotics that act primarily on the gram-negative cells, leading to rapid permeability changes in the cytoplasmic membrane and ultimately to cell death.[7] Colistin is a decapeptide administrated either as colistin methanesulfonate (CMS) where used intravenously.[8] There are five types of polymyxins, from A to E, but only colistin (polymyxin E) and polymyxin B is clinically used. Although the pharmacokinetics and pharmacodynamics are constantly questioned and the clinical use of colistin has been fought with challenges related to drug toxicity and it limited the clinical data and studies because of their nephrotoxic effect as well as neurotoxicity and better tolerated drugs were discovered. And for a clinician it is difficult to determine the dose of polymyxin especially in the case of patients who have kidney problems.[9]

The issues with antimicrobial susceptibility testing (AST) of the drug is due to the lack of clinical breakpoints for the *Enterobacterales* and the physiochemical properties of the drug, which render the in vitro AST challenging. All these reasons forced to keep the drug 'in shelf' as a last resort antibiotic.

However, interest in systemic polymyxins has recently reignited owing to the growing incidence of infection caused by MDR gram negative bacterium such as *Acinetobacter baumanii, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli*.[10] Unfortunately, the extensive use of colistin as a livestock food addictive along with its inappropriate use in clinical medicine has led to reservoirs of high levels of resistance in gram negative bacteria.[11] This makes the need of susceptibility data clinically because of the increased need of polymyxin treatment in critically ill patients in the intensive care unit. The in vitro susceptibility testing of polymyxin group of antimicrobials is hampered by several different factors. As the diffusion rate of polymyxin is very low into agar the accuracy of disk diffusion is unsatisfactory as a result there is no reliable correlation of zone diameters and minimum inhibitory concentration (MICs) and there is no any standard base among nations on this matter.[12]

So, recently newer dilution techniques were followed such as colistin broth disk elution method (CBDE) to determine colistin susceptibility against *Enterobacterales*. So the aim of this study is to detect the polymyxin resistance among members of *Enterobacterales* that are available by CBDE and also access the resistance status among them.

METHODS

The present cross-sectional study was conducted at School of Medical Education (SME), Kottayam, Kerala from January 2022 to November 2022. 274 isolates of *Enterobacterales* including *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter spp*. and *Enterobacter spp*. were collected from various diagnostic microbiology laboratories in Kerala and was re-identified by routine biochemical reactions.

Antimicrobial susceptibility testing of Enterobacterales

Antimicrobial susceptibility testing by disc diffusion as prescribed by Clinical Laboratory Standards Institute (CLSI) guidelines M02-A13.[13,14] The following antibiotics were tested; Gentamicin (10 µg), Amikacin (10 µg), Imipenem (10 µg), Cefuroxime (30µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Aztreonam (30 µg), Ampicillin (10 µg), Amoxyclav (20/10 µg), Tetracycline (30 µg), Cefixime (5 µg), Ceftazidime (30 µg), Ceftazidime/Clavulanic Acid, Cefotaxime (30 µg), Cefotaxime/Clavulanic Acid. Based on the recommendations of the Centre for Disease control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), isolates were termed as MDR which are non-susceptible to at least one agent in three or more antimicrobial categories. XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories). PDR is defined as non-susceptibility to all agents in all antimicrobial categories (i.e., no agents tested as susceptible for that organism). ESBL production was detected phenotypically by disc combination method as recommended by guidelines of CLSI by using ceftazidime (30 µg) – ceftazidime/ clavulanic acid and cefotaxime (30 µg) – cefotaxime/clavulanic acid. A difference of ≥5 mm between cephalosporin disks and their respective cephalosporin/clavulanic acid disk is taken as ESBL producing strain.[14]

Detection of polymyxin B resistance in Enterobacterales by broth disk elution method

Colistin and polymyxin B are considered equivalent agents. MIC obtained from testing colistin predict MIC to polymyxin B and vice versa. Using a loop picked 3 – 5 colonies from a fresh (18 to 24 hours) non selective agar plate and transfer to peptone water. Adjust turbidity to equivalent of 0.5 McFarland turbidity standards. 10 ml cation adjusted – Mueller Hinton broth (CA-MHB) was added to test tubes and using all the aseptic techniques colistin disks (10µg) was added as follows: 1 colistin disk to the tube labelled "1 µg/ml"; 2 colistin disks to the tube labelled "2 µg/ml"; 4 colistin disks to the tube labelled "4 µg/ml".

One tube is kept as control which contains only CA-MHB and inoculum. Gently vortex the tubes with the added disk and let the colistin elute from the disk for 30 minutes. Add 50 µg standardized inoculum to the control and all the tubes with disks to attain a final inoculum concentration of approximately 7.5×105 CFU/ml. Incubate the tubes at 35°C for 16 to 20 hours. On the very next day the incubated tubes are examine for growth and turbidity in the control tube. Read the MIC as the lowest concentration that completely inhibits the growth of the test isolates such as ≤2 µg/ml =intermediate, ≥4 µg/ml =resistant.[14]

This study was approved by the institutional ethical committee (IEC) at the School of Medical Education, Kerala. The data was analysed using Microsoft excel 2019 and statistical package for the social sciences (SPSS) 16.

RESULTS

In the present study, a total of 270 isolates of *Enterobacterales* belonging to four genera were obtained from various clinical samples. The isolates obtained were *K. pneumoniae* (n=120), *E. coli* (n=100), *Citrobacter spp*. (n=25), *Enterobacter spp*. (n=25).

Antimicrobial susceptibility pattern of Enterobacterales

In the current study, *K. pneumoniae* exhibited 66.6% sensitivity and 33.33% resistance to gentamicin, 59.16% sensitive, 5.83% intermediate and 35% resistance to amikacin, 52.5% sensitive, 13.33% intermediate and 34.16% resistance against imipenem, 13.33% sensitive, 23.33% intermediate and 64.16% resistance to cefuroxime, 35% sensitive, 5.83%intermediate and 59.16% resistance against cefoxitin, 29.16% sensitive, 7.5% intermediate and 62.5% resistance to ciprofloxacin, 40.83% sensitive, 2.5% intermediate and 56.66% resistance to aztreonam, 26.66% sensitive, 10% intermediate and 63.33% resistance to amoxyclav, 45.83% sensitive, 6.66% intermediate and 47.5% resistance to tetracycline, 38.33% sensitive, 1.66% intermediate and 60% resistance to cefixime, 21.66% sensitive, 14.16% intermediate and 64.16% resistance to ceftazidime, 24.16% sensitive, 10.83 % intermediate and 65% resistance to cefotaxime. E.coli exhibited 79% sensitivity, 2% intermediate and 19% resistance to gentamicin, 77% sensitive, 5% intermediate and 19% resistance to amikacin, 77% sensitive, 11% intermediate and 11% resistance against imipenem, 31% sensitive, 8% intermediate and 61% resistance to cefuroxime, 59% sensitive, 2% intermediate and 39% resistance against cefoxitin, 41% sensitive, 9% intermediate and 50% resistance to ciprofloxacin, 42% sensitive, 13% intermediate and 45% resistance to aztreonam, 25% sensitive, 2% intermediate and 73% resistant to ampicillin, 42% sensitive, 13% intermediate and 45% resistance to amoxyclav, 62% sensitive, 6% intermediate and 325% resistance to tetracycline, 29% sensitive, 4% intermediate and 67% resistance to cefixime, 17% sensitive, 28% intermediate and 55% resistance to ceftazidime, 13% sensitive, 13% intermediate and 74% resistance to cefotaxime, *Citrobacter spp*. exhibited 100% sensitivity to gentamicin, 88% sensitive, 12% intermediate to amikacin, 88% sensitive, 8% intermediate and 4% resistance against imipenem, 100% sensitive, to cefuroxime and cefoxitin, 96% sensitive and 4% intermediate to ciprofloxacin, 100% sensitive to aztreonam, 96% sensitive, 4% intermediate to amoxyclav, 100% sensitive to tetracycline and cefixime, 84% sensitive and 16% intermediate to ceftazidime, 68% sensitive, 28% intermediate and 4% resistance to cefotaxime.

Table 1: Antibiogram of *Enterobacterales*.

Antibiogram	*K. pneumoniae*			*E. coli*			*Citrobacter spp.*			*Enterobacter spp.*		
	S	I	R	S	I	R	S	I	R	S	I	R
Gentamicin	66.66	0	33.33	79	2	19	100	0	0	88	0	12
Amikacin	59.16	5.83	35	77	5	19	88	12	0	72	20	8
Imipenem	52.5	13.33	34.16	77	11	11	88	8	4	40	12	48
Cefuroxime	13.33	23.33	64.16	31	8	61	100	0	0	-	-	-
Cefoxitin	35	5.83	59.16	59	2	39	100	0	0	-	-	-
Ciprofloxacin	29.16	7.5	62.5	41	9	50	96	4	0	84	16	4
Aztreonam	40.83	2.5	56.66	42	13	45	100	0	0	84	0	16
Ampicillin	-	-	-	25	2	73	-	-		-	-	-
Amoxyclav	26.66	10	63.33	42	13	45	96	4	0	-	-	-
Tetracycline	45.83	6.66	47.5	62	6	32	100	0	0	80	0	20
Cefixime	38.33	1.66	60	29	4	67	100	0	0	80	0	16
Ceftazidime	21.66	14.16	64.16	17	28	55	84	16	0	72	4	24
Cefotaxime	24.16	10.83	65	13	13	74	68	28	4	80	8	12

Enterobacter spp. exhibited 88 sensitivity and 12% resistance to gentamicin, 72% sensitive, 20% intermediate and 8% resistance to amikacin, 40% sensitive, 12% intermediate and 48% resistance against imipenem, 84% sensitive, 16% intermediate and 4% resistance to ciprofloxacin, 84% sensitive and 16% resistance to aztreonam, 80% sensitive and 20% resistance to tetracycline, 80% sensitive and 16% resistance to cefixime, 72% sensitive , 4% intermediate and 24% resistance to ceftazidime, 80% sensitive, 8% intermediate and 12% resistance to cefotaxime. The antibiogram of all the isolates are given in the Table 1.

Prevalence of ESBL production in Enterobacterales

In this study, the ESBL production in *Enterobacterales* were detected as 54% (n=146). *K. pneumoniae* manifests 61.6% (n=74), *E. coli* shows 69%, (69) *Enterobacter spp*. showed 12% (n=3) ESBL production and no ESBL producing strains were obtained in case of *Citrobacter spp*. in this study as shown in the Figure 1.

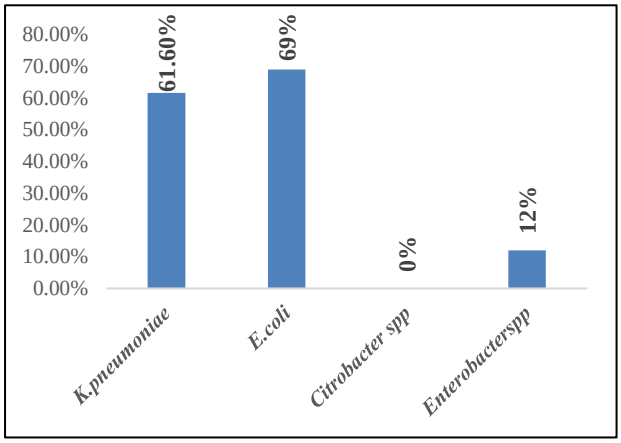

Figure 1: Prevalence of ESBL producing strains among *Enterobacterales*.

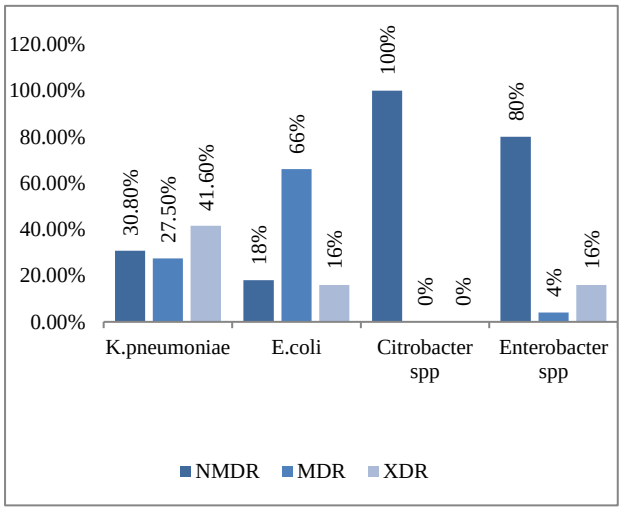

Figure 2: Distribution of NMDR, MDR and XDR among *Enterobacterales*.

Prevalence of NMDR, MDR, XDR strains in Enterobacterales

In this study, the prevalence of NMDR and MDR strains in *Enterobacterales* were detected as 37% (n=100) and 25.9% (n=70) in case of XDR. *K. pneumoniae* revealed, 30.8% (n=37) NMDR, 27.5% (n=33 MDR) and 41.66% (n=50) XDR. *E. coli* exhibited 18% (n=18) NMDR, 66% (n=66) MDR and 16% (n=16) XDR. *Citrobacter spp*. showed 100% (n=25) NMDR. *Enterobacter spp*. shows 80% (n=20) NMDR), 4% (n=1) MDR and 16% (n=4) XDR as shown in the Figure 2.

Colistin susceptibility pattern

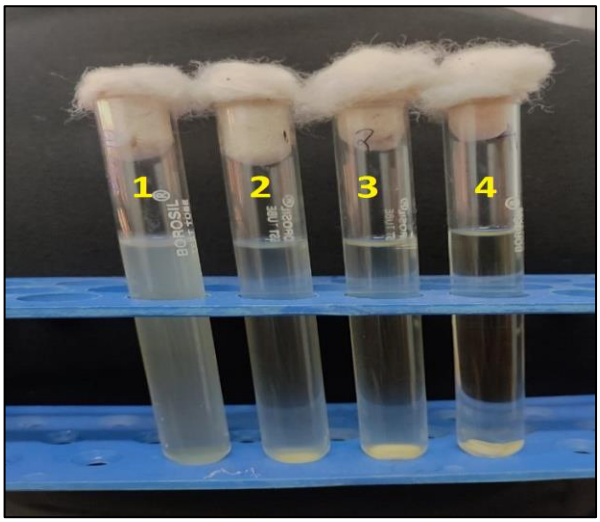

Figure 3. Broth disk elution method- *E. coli* isolate sensitive to polymyxin B, tube 1 - control with no colistin disk exhibiting growth, tube 2 - 1 µg/ml – no growth, tube 3 -2 µg/ml – no growth and Tube 4 – 4 µg/ml – no growth.

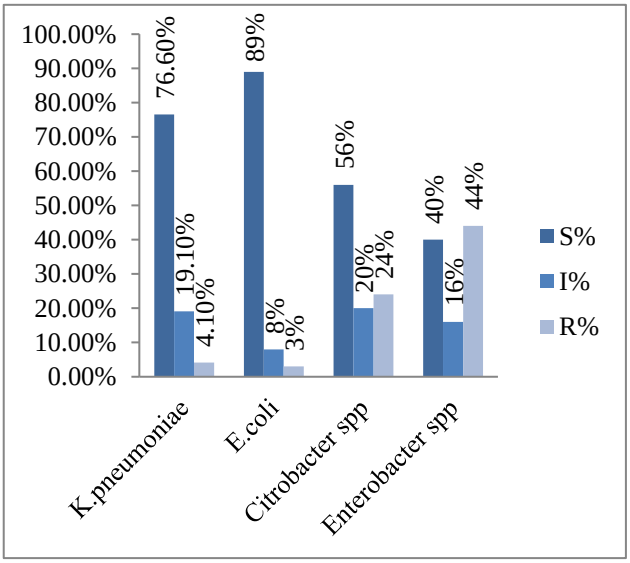

Figure 4: Colistin susceptibility pattern of *Enterobacterales*.

In the study *Enterobacterales* exhibited 75.9% sensitive, 14.8% intermediate and 9.2% resistance to colistin, Figure 3. Among this *K. pneumoniae* showed 76.6% (n=92) sensitive, 19.1% (n=23) and 4.1% (n=5) resistance. *E. coli* exhibited 89% (n=89) sensitive, 8% (n=8) intermediate and 3% (n=3) resistance. *Citrobacter spp*. exhibited 56% (n= 14) sensitive, 20% (n= 5) intermediate and 24% (n=6) resistance. *Enterobacter spp*. exhibited 40% (n= 10) sensitive, 16% (n= 4) intermediate and 44% (n=11) resistance, as shown in the Figure 4.

DISCUSSION

The emerging multi drug resistance in nosocomial GNB has necessitated use of parenteral polymyxins for the treatment of life threatening infections. The susceptibility test for polymyxin is very necessary to predict clinical responds. In this study we categories the isolated organisms into MDR, NMDR and XDR. For this the general susceptibility pattern is analysed this is compared with the study done by Ramakrishna et al. and Harish et al.[15,16]

In the case of general susceptibility test done, highest susceptibility is shown against gentamicin, 76.2% and the lowest against cefuroxime 57.7% and it is imipenem with 84.2%. and lowest is Amikacin, 60.2%. In the study conducted by Harish Kumar et al. the highest susceptibility is shown by amikacin, 92.7% and lowest by cefuroxime, 50%. *Citrobacter spp*. shows highest sensitivity against Gentamicin, 100% and *K. pneumoniae* shows most resistance against cefuroxime, 64.16%.[16]

The MDR and XDR strains isolated were 37% (n= 100). Of this *K. pneumoniae* contribute highest percentage of XDR strains that is 41.66% and *E. coli* and *Enterobacter* reveals 16% and it is comparable with the study done by Ramakrishna et al, it is 38.1% MDR and 14.88% XDR, in which *E. coli* exhibits 21% XDR and 55% MDR and *K. pneumoniae* presents 33.66% XDR and 38.77% MDR.[15]

In our study, the ESBL producing strains in total *Enterobacterales* is, 54% (n=146), while it is 40% (by combination disk test of cephalosporin/clavulanic acid) in study done by Ramakrishna et al and it is 64% in the study done by Harish et al, *K. pneumoniae* contribute 61.6% and *E. coli* contribute 69% in our study but other reveals, 37.31% *K. pneumoniae* and 35.42% by *E. coli*.[15,16] Colistin resistance in our study reveals 9.2% in total *Enterobacterales* and it is 8.5% in the study done by Behera et al of this Enterobacter contributes highest colistin resistance, 44% and it is 8% in the above study. In the study done by Bir et al 15% shows colistin resistance.[17,18]

CONCLUSION

In conclusion with the emergence of multi drug resistance among *Enterobacterales* as led to the revival in use of Polymyxins as last resort antibiotics despite severe nephrotoxicity and neurotoxicity limits their use by the early 1970s. Though the resistance to polymyxin B is to the lesser side in the present study, increase in resistance to the agent is being documented globally elsewhere. So, rational use of polymyxin B is warrantied as we could cherish polymyxin B as a "life - saving drug" to avoid no drug available.

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