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RESEARCH ARTICLE

PERFORMANCE OF RAPID POLYMYXIN NP TEST AMONG TWO SPECIES OF FAMILY ENTEROBACTERIACEAE

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Abstract

Objectives: Performing polymyxin susceptibility testing is a challenge in resource limited settings with broth microdilution as a standard method. Nordmann and Poirel introduced Rapid Polymyxin NP Test, qualitative calorimetric test for interpreting the susceptibility or resistance to colistin for Enterobacteriaceae. Due to limited data on the diagnostic accuracy of the test in central region of India, the current study was conducted to assess the diagnostic accuracy with ease of performance of Rapid polymyin NP test in comparison to the reference method, broth microdilution for Carbapenam resistant Escherichia coli and Klebsiella pneumoniae, the two prevalent species isolated across samples.

Methods: Single center tertiary care hospital based cross sectional study for a period of eight months. The Rapid polymyxin NP test and broth microdilution test was performed for 13 isolates of carbapenam resistant E.coli and 10 isolates of Klebsiella pneumoniae sub species pneumoniae, including only one isolate per patient.

Results: Rapid Polymyxin NP test showed 86.95% sensitivity and 100% specificity when compared to BMD test. Time to positivity by rapid polymyxin NP ST was 3 to 4 hrs when compared to BMD ST which was as almost 10-12hrs.

Conclusions: The specificity of 100% for the Polymyxin NP test indicates that it may be utilized as a confirmatory test. With the sensitivity as 86.95% Polymyxin NP test can be used as a very good screening test. The NPV was 40% hence whenever the test result is resistant by Polymyxin NP test one can only be 40% sure that it is a true resistant.

Introduction:-

Carbapenem antibiotics is one of the most potent group of antimicrobial agents which has been efficient in treating patients with severe Gram negative bacterial infections, including antimicrobial resistant strains to various

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antibiotics. Recent literature suggests increasing rates of carbapenem-resistant Enterobacteriaceae (CRE) among healthcare- associated isolates, in particular Klebsiella pneumonia. This gush in CRE is predominantly a result of emergence and spread of carbapenemases, a specific group of β- lactamases that has a power of hydrolyzing carbapenems.[1]There are three known group of carbapenemases – KPC (Klebsiella pneumonia carbapenemase), NDM (New Delhi metallo- β- lactamases), and OXA-48-which are major β- lactamases of clinical significance.[1] KPC is by far the most common carbapenemase produced by CRE,[2] but in US hospitals outbreaks of NDM – producing Enterobacteriaceae has been reported.[3]With emergence of MDR Gram negative bacteria and limited antimicrobials to be effective, led researchers, to explore antibiotics for long kept in shelf with new purpose of treating MDR; like polymyxins.[4] When β-lactams, aminoglycosides, or quinolones fail; the polymyxins especially colistin serves as a final alternative.[5]Colistin acts on bacterial cell membrane.[6-7]It binds to lipopolysacharides (LPS) and phospholipids part of outer cell membrane of Gram- negative bacteria. Colistin competitively displaces divalent cations (Ca2+ and Mg2+) from the phosphate groups of membrane lipids, leading to disruption of the cell membrane, leakage of intracellular contents and bacterial death.[8-11]Chromosomal and plasmid-mediated resistance to polymyxins is new threat to the society. Mutations that result in alterations of the target site of action i.e., lipopolysaccharide, result in elevated minimum inhibitoryconcentration (MICs) for these drugs.[12]The standard reference technique for determining susceptibility of Polymyxin stated by Clinical and Laboratory Standards Institute (CLSI) is broth microdilution, which requires repeated attention and a long time (24h) to perform.[13]Because of poor diffusion of polymyxin molecules in agar disk diffusion method; it is not reliable and the rates of false susceptibility is high. Acquired resistance to colistin in Enterobacteriaceae has been documented as a modification of lipopolysaccharides.[14]Plasmid mediated resistance species first documented was considered due to addition of phosphoethanolmine in LPS through the mcr – 1 gene.[15]As broth microdilution is very time taking and long process; new test was developed by Nordman/Poirel which is based on the phenomenon of carbohydrate metabolism and colour change in pH indicator.[16]This test is rapid, results detectable in maximum 4hrs and easy to perform.[17-18]This study aimed at evaluating the performance of Rapid polymyxin NP test to Broth microdilution test recommended for colistin for thetwo most prevalent species of Enterobacteriaceae.

Material and Methods:-

Study Setting:

Single centerhospital-based evaluation study conducted in the Microbiology Department at AIIMS Bhopal for a duration of 8months; first 2months for protocol preparation and ethical approval,six months simultaneous data extraction and analysis.

Study population:

CarbapenamResistant Enterobacteriaceae (CRE) isolates of Escherichia coli and Klebsiella pneumoniae sub species pneumoniaefrom various patient samples for a period of 6 months April to September 2018. Only the non-duplicate Carbapenam resistant isolates belonging to Enterobacteriaceae per patients for this study period regardless of body site and susceptibility profile were considered. Isolates from environmental surveillance sampling were excluded. All samples were processed as per the laboratory guidelines. Isolates were confirmed as Enterobacteriaceae, to genus species level by routine phenotypictests. Following the isolate confirmation, antimicrobial susceptibility testing (AST) was carried by techniques as recommended for each antibiotic by CLSI M100-S28 guidelines. CRE was identified by disk diffusion interpretative guidelines as per CLSI M100-S28imipenam or meropenam zone diameters ≤ 19mm and ertapenam ≤ 18mm. For all the CRE isolates as per inclusion criteria; ST for colistin was done by standard Broth microdilution (BMD) test as recommended by CLSI (M07-A10). Only wild type and nonwild type epidemiological cut off values was provided by CLSI M100-S27& S28 for colistin testing in Enterobacteriaceae without anyinterpretative breakpoints; hence European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretation guideline was followed. CLSI includes E.coli ATCC 25922 with MIC range of 0.25-2 and Pseudomonas aeruginosa ATCC 27853 with MIC range 0.5-4.

Aim and Objectives:-

Study aimed to evaluate diagnostic accuracy of rapid Polymyxin NP test (Qualitative test) for colistin with recommended standard broth micro dilution susceptibility testing for colistin.

The objectives were, firstly, to know the specificity and sensitivity of the test; secondly to monitor the turnaround time to final result and, finally, to assess the ease of performance on a routine basis.

Ethical clearance:
This study was approved by Institutional Human Ethical committee (IHEC AIIMS Bhopal) with LOP no. STS0143. This was STS project under ICMR with Reference ID: 2018-00124

Method of Rapid Polymyxin NP test:
All CRE isolates with ST for colistin by broth microdilution were tested in duplicates for Rapid Polymyxin NP test. The steps included

Preparation of Stock solution of colistin sulphate powder:
Colistin sulphate powder (Sigma Aldrich) was diluted into CA-MHB medium to obtain a concentration of 0.2 mg/ml. Colistin sulphate powder was stored at 40^0C and the stock solution at -200C for around 2 four months.

Preparation of Rapid Polymyxin NP Solution (Ref. Method protocol Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in Enterobacteriaceae. Emerg Infect Dis 6:1038–1043.)

For a 250 ml Polymyxin NP solution: 6.25 g of CA-MHB powder, 0.0125 g of phenol red (Sigma Aldrich) and 225 ml of distilled water were mixed. The pH adjusted to 6.7. Then sterilized. At room temperature, 25 ml of 10% anhydrous d (+)- glucose sterilized by filtration was added. This solution was prewarmed at 37^0C before use to prevent growth delay and therefore, a delayed colour change.Just before starting the test; colistin solution to the rapid polymyxin NP solution added and mixed in sterile glass tubes to obtain a rapid polymyxin NP solutioncontaining a final colistin concentration of 5µg/ml, c. Preparation of Bacterial Inoculum, first a standardized inoculum for each test and control species by using freshly obtained overnight bacterial colonies grown on Muller-Hinton plates prepared. Bacterial colonies resuspended into 10 ml of sterile Nacl (0.85%) to obtain a 3.0-3.5 McFarland optical density (approx. 10^9CFU/ml) bacterial suspension for the colistin-susceptible (ATCC E. coli 25922) and colistin resistant Proteus mirabilisobtained from patient isolates were used as standard strains. 96well polystyrene micro test plate sterile,with round base was used. For each of the 25 isolates isolate, bacterial suspension was inoculated in parallel 2 wells, with and without colistin. One isolate ATCC Escherichia coli 25922 and one Proteus mirabilis routine isolate was tested. The test result was considered on the basis of following observation: Positive (Colistin resistance) the colistin- resistant isolate grew in presence of colistin as indicated by orange to yellow color change indicating glucose metabolism. Negative (Colistin susceptibility) the colistin susceptible isolates did not grow in presence of colistin which gave exactly same colour as the well without colistin which remains orange. Results were interpreted at 2 hrs and 4 hours.

Method of Broth Microdilution test:-

Colistin Stock Solution according to CLSI, reference powder 30,000 units/mg should be used;but the colistin powder which we used is colistin sulphate salt (Sigma Aldrich) which had 15000 units/mg. Therefore, we prepared colistin of double strength. We added 2mg of colistin in 1ml of distilled water which was considered as 1mg/ml and as we had to add 4X drug to the well because only 25µl of drug added in the well so for making 4µg/ml we had to add 16µg/ml of colistin stock solution in 984ml of distilled water. By this working solution we did 2-fold dilutions for further concentration up to 0.25µg/ml and 25µl of this working solution added in wells for each isolate,Organism Suspension Preparation as per CLSI; organism suspension should be 5 x 10^5 CFU/ml. We first made 0.5 Mc Farland turbidity for all 20 isolates but 0.5 Mc Farland is equal to 1.5 x 10^8CFU/ml. Therefore, to calculate dilution of organism in well 1.5 x 10^8equal to300 times. As the organism in the well woud get diluted by 4X, we needed to prepare 75 times dilution of 0.5 McFarland. So we took 10µl of 0.5McF organism and added 740µl distilled water to it. Then 25 µl of this solution was added in well. Cation adjusted Muller Hinton Broth was the media used and 50µl of this solution was added in the well. The final concentration of the organism in the well was 5x10^5CFU/ml

In 96 well microtitre plate; 25µl of colistin added in the well. 25 µl of organism suspensionadded to the well. 50 µl of MHB media (2X strength) was added. One column was bacterial control i.e., inoculum with media. Another column was sterility control i.e., drug with media. Two rows constituted QC strain E.coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 with media and drug. After adding media, drug and test organism to the plate, it was incubated overnight at 37^oC.Proteus mirabilis also tested.MIC was interpreted next day by comparing with the controls. The data analysed for sensitivity and specificity of Rapid polymyxin NP test with BMD for colistin as standard.

Results:-

Total 23CRE fulfilled the inclusion criteria in a duration of six months. Both Rapid Polymixin NP and Broth Microdilution tests were performed for 25 isolates.

Comparison of carbapenem resistant Escherichia coli by BMD and Rapid Polymyxin NP test shown in Table 1

Out of 13 tested carbapenem resistant Escherichia coli one isolate was resistant by both the methods and one isolate was resistant by Rapid polymyxin test while susceptible by BMD method. The categorical agreement of polymyxin NP test with BMD for carbapenem resistant E coliwas, very major error (false susceptibility) was zero and major error (false resistant) was 1 out of 13 (7.69%). Percentage Concordance for Rapid polymyxin NP test was the number of isolates reporting identically by both methods divided by the number of isolates tested; 13/14 (92.85%) concordance for rapid polymyxin NP test and % discordance for Rapid polymyxin NP test was the number of isolates reported nonidentical by these two methods divided by the number of isolates tested; 1/14 (7.1%) discordance for Rapid polymyxin NP test.

Comparison of carbapenem resistant Klebsiella pneumoniae sub spp. pneumoniae by BMD and Rapid Polymyxin NP test shown in Table 2

Out of 10 tested carbapenem resistant Klebsiella pneumoniae sub spp. pneumoniae 2 isolates were resistant by the Rapid polymyxin test while susceptible by BMD method. The categorical agreement of polymyxin NP test with BMD for carbapenem resistant Klebsiella pneumoniae sub spp. pneumoniae was, very major error (false susceptibility) was zero and major error (false resistant) was 2 out of 10 (20 %). Percentage Concordance for Rapid polymyxin NP test was 08/10 (80 %) and % discordance was 2/10 (20 %) discordance for Rapid polymyxin NP test.

Diagnostic accuracy of Rapid polymyxin NP test shown in Table 1, 2 and 3 with Fig 1 &2: The test was assumed to be true Positive when test result was susceptible in both BMD and Polymyxin NP test; n= 20; the test result was taken to be true Negative when the gold standard BMD test result and polymyxin NP test result was Resistant, n=2 ; the test was called to be false Negative when BMD test result was Susceptible but was resistant by Polymyxin NP test result, n=3 and the test was considered false positive when BMD result was resistant and Polymyxin NP test was susceptible, n=0. Specificity of polymyxin NP test was 100%, sensitivity 86.95%, and the negative predictive value was 40%. Overall Errors defined in this study defined as a minor error (mE) , when the result was intermediate in one system and susceptible or resistant in the other; a major error (ME) indicated a false-resistant result; and a very major error (VME) indicated a false-susceptible result. When calculating the rates of error, we applied the following denominators in respective species for estimation: The number of resistant isolates by Polymyxin NP test but susceptible by BMD n=3, Major error 3; the number of susceptible isolates by Polymyxin NP test resistant by BMD, n=0, VME =0.

Figure 1:- Rapid Polymyxin NP test for colistin for Enterobacteriaceae. Orange- Colistin Susceptible; Yellow- Colistin Resistant.

Figure 2:- BMD ST for colistin for Enterobacteriaceae. Button - Colistin resistantYellow- due to pigmentation of Pseudomonas aeruginosa.

The turnaround time and ease of test performance shown in Table 4-5.

Table 1:- CRE Escherichia coli n= 14, (13, Carbapenam resistant E.coli and one ATCC E.coli 25922).

S.No	Test Performed	BMD for Colistin		Rapid Polymyxin NP test	
	Organism	**MIC Value**	**Result**	**Colour Observed**	**Result**
1.	E. coli	MIC-1	Susceptible	Orange	Susceptible
2.	E. coli	MIC-0.25	Susceptible	Orange	Susceptible
3.	E. coli	MIC-1	Susceptible	Orange	Susceptible
4.	E. coli	MIC-0.5	Susceptible	Orange	Susceptible
5.	E. coli	MIC-0.25	Susceptible	Orange	Susceptible
6.	E. coli	MIC-1	Susceptible	Orange	Susceptible
7.	E. coli	MIC-0.25	Susceptible	Orange	Susceptible
8.	E. coli	MIC-2	Susceptible	Orange	Susceptible
9.	E. coli	MIC-0.5	Susceptible	Orange	Susceptible
10.	E. coli	MIC-1	Susceptible	Orange	Susceptible
11.	**E. coli**	**MIC-4**	**Resistant**	**Yellow (TN)**	**Resistant**
12.	E. coli	MIC-0.5	Susceptible	Orange	Susceptible
13.	E. coli ATCC 25922	MIC-1	Susceptible	Orange	Susceptible
14.	**E. coli**	**MIC-2**	**Susceptible**	**Yellow (FN)**	**Resistant**

Table 2:- Carbapenam ResistantKlebsiella Pneumoniae sub spp. pneumoniae n= 10

S.No	Test Performed	Broth Mico dilution		Rapid Polymyxin Test	
	Organism	**MIC Value**	**Result**	**Colour Observed**	**Result**
1.	K. pneumoniae	MIC-1	Susceptible	Orange	Susceptible
2.	K. pneumoniae	MIC-0.50	Susceptible	Orange	Susceptible
3.	K. pneumoniae	MIC-2	Susceptible	Orange	Susceptible
4.	K. pneumoniae	MIC-1	Susceptible	Orange	Susceptible
5.	K. pneumoniae	MIC-2	Susceptible	Orange	Susceptible
6.	K. pneumoniae	MIC-0.25	Susceptible	Orange	Susceptible
7.	K. pneumoniae	MIC-0.5	Susceptible	Orange	Susceptible
8.	K. pneumoniae	MIC-1	Susceptible	Orange	Susceptible
9.	**K. pneumoniae**	**MIC-1**	**Susceptible**	**Yellow (FN)**	**Resistant**
10.	**K. pneumoniae**	**MIC-2**	**Susceptible**	**Yellow (FN)**	**Resistant**

Table 3:- Intrinsically resistant to colistin.

Test performed	Broth Mico dilution		Rapid Polymyxin Test	
Organism	**MIC Value**	**Result**	**Colour Observed**	**Result**
Proteus mirabilis	MIC-4	Resistant	Yellow **(TN)**	Resistant

Table 4:- Turnaround time of the Rapid polymyxin NP test and Broth Microdilution for colistin from completed identification of organism to ST completion

Variables	Report of results from laboratory in hours	No of organisms tested
Rapid Polymyxin test	3-4	20
Broth microdilution	10-12	20

Table 5:- Ease of Performance of the Rapid polymyxin NP test and Broth Microdilution for colistin.

Variables	No of rounds of test for 20 test organisms	Microtiter plates required for 20 test organisms
Rapid Polymyxin test	1	1
Broth microdilution	4	4

Discussion:-

The rapid polymyxin NP test showed 100% specificity for the two species of family Enterobacteriaceae, Escherichia coli and Klebsiella pneumoniae. The control strains also gave similar results confirming the process. The sensitivity was around 87 % in our study. Similar result was documented by an Indian study done in 2016 by Yamuna et.al with sensitivity and specificity of 100%

The study by Nordmann P, Jayol A, Poirel L in 2016 showed sensitivity and specificity of 99.3% and 95.4%, respectively as they used acidifying media for some isolates which gave false positives.

In a study by Yamuna et.al majority of their tested isolates showed positive interpretable result in rapid polymyxin NP test in ≤3 hours which was also seen in our study. All studies have showed the rapidity and ease of performing rapid polymyxin NP test (Table 4 &5) and suggested it's use as a preliminary screening test which can be confirmed by BMD.

Conclusion:-

Rapid Polymyxin NP Test is a good qualitative test for susceptibility testing for Colistin and is a technique which can complement to the BMD ST which can give MICs. It is a test with nominal requirements, minimal skill hence a good screening test. Itonly mandates more than one observer to avoid false results documentation being a calorimetric test with naked eye viewing.

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