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"Molecular detection of colistin resistance among Multidrug Resistant (MDR) Pseudomonas aeruginosa"

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ABSTRACT: P.aeruginosa is a common cause of hospital-acquired infections, and it's especially troublesome in intensive care units. Pseudomonas aeruginosa shows intrinsic resistance to a number of antibiotics. Treatment of infection has become a challenge as number of antibiotic resistant strains is on the rise. In general, Pseudomonas aeruginosa resist antibiotic attack by intrinsic, adaptive, and acquired resistance mechanisms. Resistance can be acquired by transfer of genes coding resistance carried on plasmids, transposons, integrons or prophages or through mutational changes. Now, worldwide emergence of colistin resistance has also occurred. Colistin resistance in Pseudomonas aeruginosa can be caused by chromosomal mutations as well as by the acquisition of plasmid-carrying determinants, primarily mcr-1. Hence, this current study was aimed to detect the presence of mcr genes among MDR P.aeruginosa isolated from various clinical specimens.
Keywords: P.aeruginosa, intrinsic resistance, colistin resistance, mcr genes

INTRODUCTION:

Pseudomonas aeruginosa is an aerobic, Gram negative, non-fermenting bacillus belonging to the family Pseudomonadaceae. It infects both immunocompetent and immunocompromised hosts, causing a wide range of infections. It commonly resides in the environment. (1) Pseudomonas aeruginosa causes a broad spectrum of infections in humans. Infections acquired from community include otitis externa, septic arthritis in intravenous drug users, hot foot syndromes, folliculitis, or green nail. (2)

P.aeruginosa is a common cause of hospital-acquired infections, and it's especially troublesome in intensive care units. Pseudomonas aeruginosa shows intrinsic resistance to a number of antibiotics. Treatment of infection has become a challenge as number of antibiotic resistant strains is on the rise. (3) Pseudomonas aeruginosa and Acinetobacter baumannii that are resistant to carbapenems have been added to the list of the potential pathogens against which development of new therapeutics is necessary. (4)

Colistin (polymyxin E) is a bactericidal polycationic peptide from the polymyxin family. It specifically targets the negative charges in bacterial lipopolysaccharide (LPS), which constitutes the major part of gram-negative outer membrane. (6) Colistin was introduced in the 1950s and was effective in

treating infections caused by GNB. Around 1970, it was discovered that colistin had nephrotoxicity and neurotoxicity; as a result, its use was discontinued and replaced by other effective and safer drugs. [7] Since Pseudomonas aeruginosa is acquiring resistance to most of the currently used antibiotics, WHO has recommended colistin as a last resort antibiotic to be given against this organism. The re-introduction of colistin as a last-line treatment option for multidrug resistant (MDR) gram negative bacterial infections has provided some relief to clinicians around the world. [8] Now, worldwide emergence of colistin resistance has also occurred. Colistin resistance in Pseudomonas aeruginosa can be caused by chromosomal mutations as well as by the acquisition of plasmid-carrying determinants, primarily mcr-1. [6] The report of first mcr gene (mcr-1) came out in 2016. Succeeding years witnessed the discovery of other mcr genes (mcr-2 to mcr-10) in Enterobacteriaceae and few have also been discovered in Pseudomonas spp and Acinetobacter spp. [10] Hence, this study was aimed to detect the presence of mcr genes among MDR P.aeruginosa isolated from various clinical specimens.

MATERIALS AND METHODS:

A laboratory based prospective study was conducted in the Department of Microbiology for a period of one year. Clinical isolates of MDR P.aeruginosa were included for the study. The study included 100 clinical isolates of multidrug resistant P.aeruginosa. The various clinical samples such as pus, blood, ear swabs, endotracheal aspirate, sputum, urine which were received in the laboratory were processed according to the standard protocol. Identification and AST of isolates were done by automated VITEK-2 system. MDR Pseudomonas aeruginosa isolates were further subjected to the colistin broth disk elution test and Colistin agar test to determine colistin susceptibility, furthermore those isolates that showed resistant to Colistin from either of two tests among CBDE, CAT and Vitek 2 were subjected to DNA extraction and amplification using PCR to detect the presence of mcr genes. Brief methodology of CBDE, CAT and PCR is as follows:

Colistin broth disk elution test:

The test was carried out using cation adjusted Mueller Hinton broth (CAMHB) and 10µg colistin sulphate disks. For an isolate, 4 test tubes labelled 0µg/mL (control), 1µg/mL, 2µg/mL and 4µg/mL were taken, each of which contained 10mL of CAMHB. Aseptically, one, two and four colistin disks were added to the test tubes labelled 1µg/mL, 2µg/mL and 4µg/mL respectively. The test tubes were kept for 30 minutes at room temperature for elution of the antibiotic from the disks. Colonies from a fresh non-selective agar plate were picked and suspended in sterile saline (5mL) and matched to 0.5 McF turbidity standards. 50 µL of inoculum was added to each of the four test tubes so that an end inoculum concentration of approximately 7.5×10^5 CFU/mL was obtained. Capped tubes were gently vortexed and incubated for 16 hours at 35°C in presence of air. Lowest concentration of colistin that fully inhibited the isolate's growth was taken as the MIC value and result was interpreted according to CLSI guidelines (≤ 2 µg/mL = intermediate, ≥ 4 µg/mL = resistant).

Colistin agar test:

Mueller Hinton agar (MHA) plates containing 0µg/mL (control), 1µg/mL, 2µg/mL and 4µg/mL of colistin were prepared. Each agar plate were divided and labelled with appropriate isolate number. Colonies from a fresh non-selective agar plate were picked and suspended in sterile normal saline (5mL), matched to 0.5 McF turbidity standards and diluted 1:10 in saline. Using a pipette, 10µL of

the diluted inoculum was added to specific parts of MHA plates. Plates were incubated for sixteen hours at 35°C in presence of air. The plates were observed for colony or thin film of growth using transmitted light. Lowest concentration of colistin that fully inhibited the isolate's growth was taken as the MIC value and results were interpreted according to CLSI guidelines (≤ 2 µg/mL = intermediate, ≥ 4 µg/mL = resistant).

PCR

DNA extraction and amplification: 0.5mL of overnight culture suspended in saline was centrifuged and supernatant was discarded. The pellet obtained was resuspended in 180µL of digestion buffer and 20µL of lysozyme and incubated for 15 minutes at 37°C. 200µL of binding buffer and 20µL of proteinase K was added and incubated at 56°C for 15 minutes. 200µL of 100% absolute ethanol was added, mixed well and centrifuged at 8000rpm for one minute. The contents were then washed with wash buffer 1 followed by wash buffer 2. 60µL of elution buffer was added and centrifuged at 130000 rpm for 2 minutes. The purified DNA was stored at -20°C. The target genes their sequence, base pair size and thermal cycler conditions are mentioned in Table 1 and 2. Extracted DNA was subjected to Multiplex PCR according to the protocol mentioned for detection of mcr genes.

RESULTS:

Over the course of the study period, 100 MDR Pseudomonas aeruginosa isolates were collected for this study. Most of the isolates (41%) were obtained from patients above the age of 60 and the least (6%) were from patients under the age of 20. 77% of the total isolates were obtained from males and 23% were obtained from females. MDR Pseudomonas aeruginosa isolated were from following samples: pus samples (34%), urine samples (33%), endotracheal aspirates (17%), ear samples (6%), sputum samples (5%), blood samples (4%), and central line tip (1%). Majority of isolates belonged to patients from neurology and general medicine ward (17% each) and 2% each from nephrology, covid ward and RICU. Out of 100 MDR P. aeruginosa isolated, more than 90% of them showed resistance to levofloxacin and ciprofloxacin. 83% of the isolates showed intermediate susceptibility to colistin and highest rate of sensitivity was seen towards amikacin (20%) , according to Vitek 2 results (Table3). CBDE test was carried out following CLSI guidelines to find out colistin susceptibility for the 100 MDR Pseudomonas aeruginosa isolated. Out of this, seventy seven isolates (77%) showed intermediate results and twenty three isolates (23%) were resistant to colistin. CAT was carried out following CLSI guidelines to check for colistin susceptibility of the 100 MDR Pseudomonas aeruginosa isolated, in which 76 isolates (76%) showed intermediate results and 24 isolates (24%) showed resistance to colistin. The resistance pattern of the isolates to colistin as shown in VITEK 2, CAT and CBDE test were compared and it was found that more number of isolates showed resistance to colistin in CAT test (24%) followed by CBDE test (23%) and VITEK 2 (17%). Out of the 100 test isolates, 22 MDR P.aeruginosa isolates showed resistant to colistin by two of the three tests (VITEK 2 and CBDE or VITEK 2 and CAT or CBDE and CAT), and 7 isolates were resistant in all three tests. These 29 isolates were chosen to perform Multiplex PCR to detect mcr genes. Out of the 29 colistin resistant test isolates which were subjected to multiplex PCR, mcr 1 gene was detected in 4 isolates

(13.8%), mcr 3 was detected in 3 isolates (10.3%) and no mcr genes were detected in the remaining isolates (Table 4).

Demographic, clinical characteristics and colistin susceptibility pattern of isolates that detected the presence of mcr genes were compared and analysed further. Of six isolates which were positive for mcr genes (mcr-1 and mcr-3), three were obtained from pus samples and the remaining ones were from urine sample, ear swab and central line tip (1 each). 2 test isolates were from males and remaining 4 were from females. 2 test isolates were obtained from patients admitted to ICU and rest were from patients in surgery, ENT, general medicine and neurology ward (1 each). 4 of the mcr positive test isolates were resistant to colistin by CAT, CBDE test and VITEK 2. The remaining 2 isolates showed resistance to colistin by CAT and CBDE test and intermediate result was shown by VITEK 2 (Table 5).

DISCUSSION:

Among various phenotypic and genotypic mechanisms of colistin resistance, mcr genes play a major role as they are easily transmitted among different bacteria. mcr genes code for phosphoethanolamine transferase enzyme which on attachment to the outer membrane of GNB confers resistance to colistin. Hence our study focused on detection of mcr genes in colistin resistant MDR P. aeruginosa. Our research included 100 MDR P. aeruginosa most of which were isolated from patients above sixty years of age (41%). Most of the MDR P. aeruginosa included in our study were obtained from pus samples (34%) and urine samples (33%). It was also noted that MDR P.aeruginosa in our study showed high rate of resistance towards levofloxacin and ciprofloxacin (91% and 90% respectively) and majority of them (20%) showed sensitivity towards amikacin. CAT and CBDE tests were accepted by CLSI for determining colistin susceptibility in Pseudomonas aeruginosa stating that it gave reproducible results when compared with broth microdilution method and are more user-friendly for laboratory purposes. Hence we performed CAT and CBDE tests for 100 MDR Pseudomonas aeruginosa and the results were compared with VITEK 2 compact system. Out of the 100 MDR P.aeruginosa, 24 isolates were resistant to colistin by CAT, 23 isolates were resistant by CBDE test and 17 isolates were resistant by VITEK2. Discrepancies were observed in the results which might be due to technical errors. In our study, 29 isolates (29%) among 100 MDR P.aeruginosa were resistant to colistin (concluded by comparing the results obtained from CAT, CBDE, VITEK 2) and among these, mcr 1 gene was detected in 4 isolates (13.8%) and mcr 3 gene was identified in 3 isolates (10.3%) by PCR. Whereas, a study conducted by Fareeha Hameed et al., reported that colistin resistance was found in 11.9% (10/84) of P. aeruginosa by broth micro dilution and agar dilution method and among these isolates, mcr-1 gene was identified in one strain by PCR. Three of the colistin resistant MDR P.aeruginosa carrying mcr-3 gene were isolated in our current study. On the contrary, mcr-3 in P.aeruginosa isolated from human samples were rarely reported in previous researches. Out of the 29 colistin resistant MDR P.aeruginosa, 23 isolates did not show the presence of any mcr genes which confer colistin resistance. Colistin resistance in these isolates may be due to other chromosomal point mutations or due to other mcr genes (mcr-6 to mcr-10) which were not included in our study. In coclusion, Discrepancies in the findings of the phenotypic tests done for colistin susceptibility emphasise the fact that a single phenotypic test cannot be relied on entirely for detecting colistin resistance. For a comprehensive understanding of colistin resistance, new

approaches must be coupled, particularly for strains bearing mcr genes, so that doctors may quickly change therapies or segregate the carrier patients in hospitals.

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Table 1: Target genes, Sequence and base pair size

TARGET GENE	PRIMER NAME	SEQUENCE (5'-3')	SIZE (bp)
mcr-1	mcr1_320bp_fw	AGTCCGTTTGTTCTTGTGGC	320
	mcr1_320bp_rev	AGATCCTTGGTCTCGGCTTG	
mcr-2	mcr2_700bp_fw	CAAGTGTGTTGGTCGCAGTT	715
	mcr2_700bp_rev	TCTAGCCCGACAAGCATACC	
mcr-3	mcr3_900bp_fw	AAATAAAAATTGTTCCGCTTATG	929
	mcr3_900bp_rev	AATGGAGATCCCCGTTTTT	
mcr-4	mcr4_1100bp_fw	TCACTTTCATCACTGCGTTG	1,116
	mcr4_1100bp_rev	TTGGTCCATGACTACCAATG	
mcr-5	MCR5_fw	ATGCGGTTGTCTGCATTTATC	1,644
	MCR5_rev	TCATTGTGGTTGTCCTTTTCTG	

Table 2: Thermal cycler conditions

Cycle	Cycle Point
Hold 1	Hold @ 95°C, 5min 0s
Cycling (55 repeats)	Step 1: Hold @ 95°C, 15s
	Step 2: Hold @ 55°C, 35s
	Step 3: Hold @ 72°C, 20s, acquiring to Cycling Green
Hold 2	Hold @ 72°C, 5min 0s
Melt	Ramp from 65°C to 95°C
	Hold for 90s on the 1st step
	Hold for 2s on next steps, Melt A

Table 3: Antimicrobial susceptibility profile of MDR Pseudomonas from Vitek 2

ANTIBIOTICS	SENSITIVE	INTERMEDIATE	RESISTANT
CEFEPIME	10	14	76
CEFOPERAZONE/SULBACTAM	9	7	84
CEFTAZIDIME	11	4	85
PIPERACILLIN/ TAZOBACTAM	11	8	81

IMIPENEM	7	9	84
MEROPENEM	12	5	83
GENTAMICIN	12	5	83
AMIKACIN	20	8	72
LEVOFLOXACIN	8	1	91
CIPROFLOXACIN	7	3	90
COLISTIN	-	83	17

Table 4: Distribution of mcr genes among MDR P.aeruginosa isolates

mcr GENE	**FREQUENCY**	**PERCENTAGE**
mcr 1	4	13.79
mcr 2	0	0
mcr 3	3	10.34
mcr 4	0	0
mcr 5	0	0

Table 5: DEMOGRAPHIC, CLINICAL CHARACTERISTICS AND COLISTIN SUSCEPTIBILITY PATTERN OF mcr POSITIVE TEST ISOLATES

Sl. no	mcr gene	Sample	Patient details		Diagnosis	Ward	CAT result	CBDE result	VITEK 2 result
			Age	Sex					
1	mcr 1	pus	87	male	ulcer	surgery	R	R	I
2	mcr 3	pus	76	female	diabetic foot	general medicine	R	R	R
3	mcr 1, mcr 3	ear swab	31	female	ear infection	ENT	R	R	R
4	mcr 1	urine	54	female	Head injury	neurology	R	R	R
5	mcr 1	pus	60	female	burns	ICU	R	R	I
6	mcr 1	central line tip	66	male	COPD	ICU	R	R	R