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Phenotypic and genotypic detection of colistin resistance from clinical isolates of *Escherichia coli* in a tertiary care hospital.

Saranya, Kalyani
Department of Microbiology, Saveetha medical college, Chennai.

Abstract:

Back ground and objective: Colistin resistant *Escherichia coli* have emerged in humans, animals and in hospital sector. The aim of this study was to assess the occurrence of MCR-1 and MCR-2 gene producers among *Escherichia coli* isolated from Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of May 2019 to October 2019.

Methods: The present study is to check the prevalence, demonstrate the virulence factors and study the antibiotic susceptibility pattern of *Escherichia coli*in our clinical settings. Phenotypic colistin resistance was screened for by the antibiotic broth microdilution method. Multiplex PCR was carried out to determine mcr 1 and mcr 2 genes.

Results: From 105 clinical *E. coli* isolates, we identified 4 isolates that exceeded the colistin resistance breakpoint (>2 mg/mL) using the broth dilution method. After the concentration -were taken for DNA extraction and amplification. Of these, 4 mcr-positive isolates were identified using the mcr-1,2–specific primers and were clearly colistin resistant. Full gene sequencing confirmed that all these 2 strains encoded MCR-1. The other 2 colistin-resistant isolates were found to have mcr-2 genes using specific primers. We observed an unexpectedly high prevalence of mcr-1 than mcr -2 in patients attending a tertiary care hospital by PCR with estimated of 9.0% (95% confidence interval). The mcr-1 and mcr-2 gene was detected predominantly in Escherichia coli.

Conclusion: Studies on the prevalence of drug resistant *Escherichia coli*has been done in our hospital. The data emerging out of this study helps in understanding the dynamics of this infection and provide inputs for antibiotic policy in the treatment of such infections.

Keywords: Colistin; resistance; *Escherichia coli*; Polymerase chain reaction; microdilution method.

INTRODUCTION:

Urinary tract infection (UTI) can be defined as a spectrum of diseases caused by microbial invasion of the genitourinary tract that extends from the renal cortex of the kidney to the urethral meatus. [1,2] It is the second most common infection,next to respiratory infection.

Gram-negative rods, especially Enterobacteriaceae, are considered as the main organisms that cause nosocomial infections [2] *E. coli* was among the main strains associated with such infections. *E. coli* causes some major infections, including bacteremia, neonatal meningitis, urinary tract infections, intra-abdominal infections, sepsis and gastroenteritis in developing countries.

Recently, the emergence of multidrug-resistant (MDR) phenotype as the consequence of antibiotics overconsumption in the treatment of human and animal diseases caused a global challenge to health systems. MDR was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories [3]

Carbapenems are the choice drugs to treat such infections. However, with increasing global incidence of carbapenem resistance, colistin is now widely used as the last resort antibiotic for the treatment of carbapenem-resistant Enterobacteriaceae. However, a gradual increase in the prevalence of colistin resistance has been noted in the last few years, and elucidation of underlying resistance mechanisms is critical.

Structural modifications of bacterial lipopolysaccharide are the main routes of colistin resistance in gram-negative bacteria. The phosphoethanolamine transferase *mcr-1*, a recently identified horizontally transferable plasmid-mediated colistin resistance gene, is also worrisome, as it has been detected in over 20 countries within 3 months of its identification [4], including in Europe, Asia, South America, North America, and Africa [4]. In China, *mcr-1* was detected in as many as 20% of animal strains and 1% of human strains. Colistin resistance is most frequently observed in *E. coli*, but is present in various genera, including *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, and *Enterobacter*.

Subsequently, Wang et al. [5] described several other MCR homologs (*MCR-2*, *MCR-3*, *MCR-4*, and *MCR-5*. Two MCR homologs (MCR-6 and MCR-7) were placed into GenBank, and very recently, the mcr-7.1 gene was found in *E.coli* of chicken origin in China. *mcr-2*, which has about 76.7% nucleotide and 81% amino acid identity to *mcr-1*, the archetypal form. Hence, *mcr-2* is a similar threat to public health as *mcr-1*, although its transfer, origin, and mechanism of resistance are not fully understood [6]. We have now evaluated the rates of colistin resistance in clinical enterobacterial infectious isolates from tertiary Cancer Hospital in India, europe to assess the presence of *mcr-1* and *mcr-2*, as well as of mutations in *mgrB*.

The aim of this study was to find out the prevalence of plasmid-mediated colistin resistance in a collection of colistin resistant *E.coli* isolates from clinical samples.

MATERIALS AND METHODS:

METHODOLOGY AND RESEARCH DESIGN:

This is a cross sectional study conducted in the department of microbiology, Saveetha Medical College and Hospital, Thandalam, Chennai.

SAMPLE SIZE AND SAMPLING TECHNIQUES: [7]
Continuous sampling method was used in the study, samples received in the clinical microbiology laboratory during the period of three months (May2019 –October 2019) were included in the study.
Clinical samples were cultured on blood agar and MacConkey agar. Isolates were identified by standard microbiological techniques (Colonial morphology, Gram stain, oxidase and the use of several biochemical tests) andVITEK-2 Compact system (bioMerieux, Marcy l'E´toile, France), using *E. coli* ATCC 25922 as control strain.

ANTIBIOTIC SENSITIVITY TESTING
Antimicrobial susceptibility testing by disk diffusion was performed as a part of the routine testing and interpretation was done according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [8]. Isolates were tested for susceptibility to Ampicillin (10µg), Gentamicin (10µg), Cefuroxime (30µg), Cefotaxime (30µg), Norfloxacin (5µg), Amikacin (30µg), Nitrofurantoin (30µg), Ceftazidime/Clavulinic acid (30µg), Ceftazidime (30µg), Co-trimoxazole (1.25/23.75mcg), Polymyxin B (300µg), Colistin (10µg) by Kirby-Bauer disk diffusion method [9].

MINIMUM INHIBITORY CONCENTRATION - Broth dilution method (MIC):
For colistin susceptibility testing, the isolates (n=105) were subjected to the broth microdilution (BMD) method, with susceptible *E. coli* ATCC 259226. The antibiotic pure substance, colistin sulphate powder, was obtained from Sigma-Aldrich. The uses of MIC are in the determination of antibiotic sensitivities of organisms from patients with serious infections, e.g. *Mycobacterium tuberculosis*.
16µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL dilutions of colistin were prepared.

DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION (MBC):
The minimum bactericidal concentration (MBC) is the amount of agent that will prevent growth after subculture of the organism to antibiotic free medium.
An aliquot from each wells of microtitre plate was inoculated and streaked on to nutrient agar plate. The plates were incubated at 37ºC for 24 hours and the minimum concentration at which the bactericidal activity occurred were determined.

PCR (Polymerase Chain Reaction):
PureFast® Bacterial DNA minispin purification kit (Kit contains Lysozyme, Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1,Wash Buffer-2,Spin columns with collection tube and elution buffer.HELINI 2X ReDdye PCR Master Mix,Agarose gel electrophoresis consumables and MCR1 & MCR2 Primers are from HELINI Biomolecules, Chennai, India.

COMPONENTS	QUANTITY
HELINI RedDye PCR Mater mix	10µl
HELINIRedDye to use– Primer Mix	5µl
Purified Bacterial DNA	10µl
Total volume	25µl

PCR Procedure: [20]
1. Reactions set up as follows;
2. Mixed gently and spin down briefly.
3. Place into PCR machine and program it as follows.
Initial Denaturation: 95˚C for 5 min.
Denaturation: 94˚C for 30sec
Annealing : 58˚C for 30sec
Extension : 72˚C for 30sec
Final extension: 72˚C for 5 min

LOADING:
1. Prepared 2% agarose gel.[2gm of agarose in 100ml of 1X TAE buffer].
2. Run electrophoresis at 50V till the dye reaches three fourth distances and observe the bands in UV Transilluminator.

Agarose Gel Electrophoresis:
Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven). When the agarose gel temperature was around 60˚C, added 5µl of Ethidium bromide. Poured warm agarose solution slowly into the gel platform. Kept the gel set undisturbed till the agarose was solidifies. Poured IXTAE buffer into submarine gel tank. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above than the gel. PCR Samples are loaded after mixed with gel loading dye along with 10µlHELINI100bpDNAladder.[100bp, 200bp, 300bp, 400bp, 500bp, 600bp,700bp, 800bp, 900b, 1000bp and 1500bp]. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel. Gel viewed in UV Transilluminator and observed the bands pattern.

Data Analysis: [Descriptive analysis and pivot function]
We calculated the frequency of identification of *mcr* genes and their antibiotic resistance pattern for *mcr*-positive bacteria. Pivot table function of Microsoft Excel 2016 was used to calculate the descriptive analysis (as a percentage), and the prevalence of *mcr*-harboring strains among the total strains including 95% confidence intervals (CIs), the total number of resistant isolates (number of resistant isolates/total number of positive isolates from same species) to each individual antimicrobial drug.
In addition, student's *t*-test calculations were performed to identify the significance of Mcr 1 and mcr 2 genes.

RESULTS

The present cross sectional study was conducted at Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of May 2019 to October 2019.Ethical clearance was obtained.

Out of 3420, *Escherichia coli* were isolated from 105 samples. 76 (72.4%) were from urine, 10 (9.5%) were from wound swab, 7(6.6%)were from high vaginal swab, 9 (8.5%) were from pus, 3 (2.8%)were from blood.

Out of 105 samples yielding *Escherichia coli*, 62 (59%) were from female patients and 43 (40.95%) were from male patients.

Out of 105 samples from which *Escherichia coli* was isolated, majority of the samples 56 (53.33%) were from the age group 21- 40. The least number of samples were from the age group >81 the percentage of which was 2.47%.Among 105 samples of *Escherichia coli*, majority samples were from OBG (40%), followed by Urology (31%) and Female surgery ward (11.42%)which was statistically significant* (p=0.005).

The ABST pattern is debrited in*Escherichia coli* were shown in figure 1.

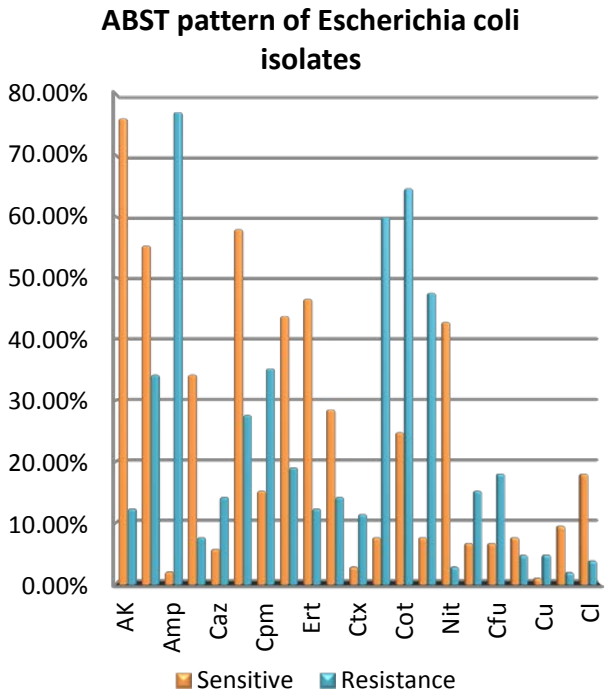


Figure 1: Antibiotic Susceptibility Pattern of *Escherichia coli*

The higher rate of resistance were found to be for the following drugs Amikiacin (78%), Piperacillin tazobactum (69%), Gentamicin (66%).

MIC and MBC for Colistin :

On treating with different serial dilutions 16µg/mL,8µg/mL,4µg/mL,2µg/mL,1µg/mL, 0.5µg/mL and 0.25µg/mL of colistin. *Escherichia coli* showed different inhibitory [Figure :2] and bactericidal patterns.

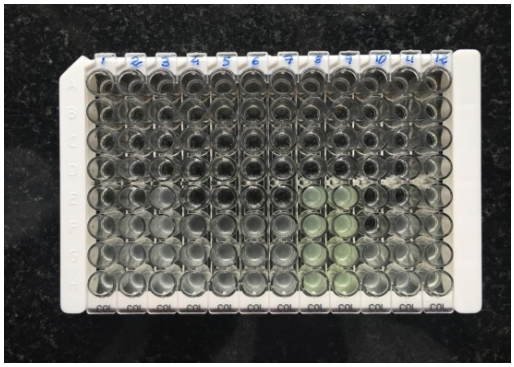


Figure : 2 Minimum inhibitory concentration by broth dilution method

From the 105 *Escherichia coli* strains 4 (3.80%) were found to be resistant to the Colistin by broth dilution method, according to the CLSI guidelines.

Out of 105 *Escherichia coli*, 2 isolates were in the MIC range of 0.25µg, 15 isolates were in the MIC range of 0.5µg, 10 isolates were in the range of 1µg, 3 isolates were in the MIC range of 2µg, 4 isolates were in the MIC range of 4µg, 1 isolates were in the MIC range of 8µg, 0 isolates were in the MIC range of 16µg.

Out of 105 *Escherichia coli*, 2 isolates were in the MBC range of 0.25µg, 1 isolates were in the MBC range of 0.5µg, 3 isolates were in the MBC range of 1µg, 8 isolates were in the MBC range of 2µg, 7 isolates were in the MBC range of 4µg, 11 isolates were in the MBC range of 8µg, 6 isolates were in the MBC range of 16µg. [Figure : 3]

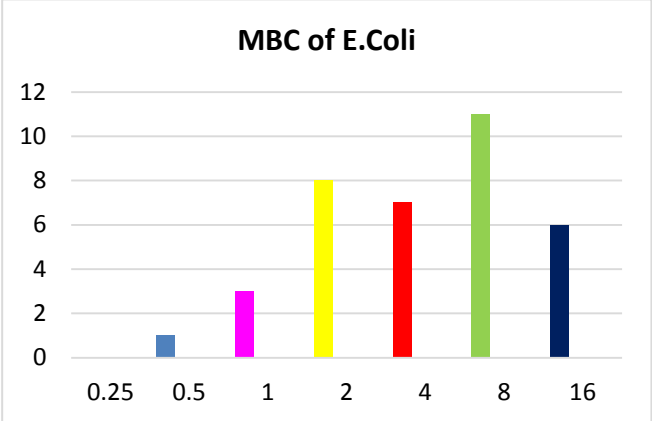


Figure 3: Minimum Bactericidal Concentration of *Escherichia coli*

MOLECULAR DETECTION OF mcr-1 and mcr-2 gene :

From 105 clinical *E. coli* isolates, we identified 4 isolates thatexceeded the colistin resistance breakpoint (>2 mg/mL) usingthe agar dilution method. Of these, 4 mcr-positive isolates were identified using the mcr-1,2–specific primers and were clearly colistin resistant. Full gene sequencing confirmed that all these 2 strains encoded MCR-1. The other 2 colistin-resistant isolates were found to have mcr-2 genes using specific primers. [Figure 4a,4b]

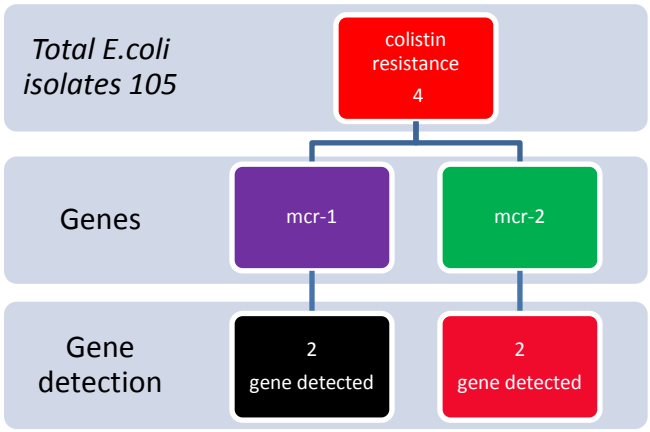
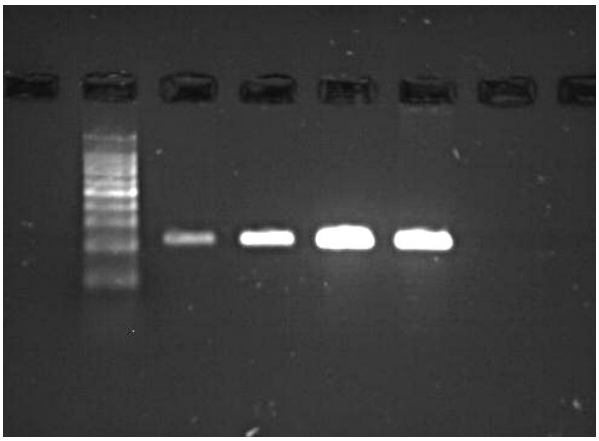
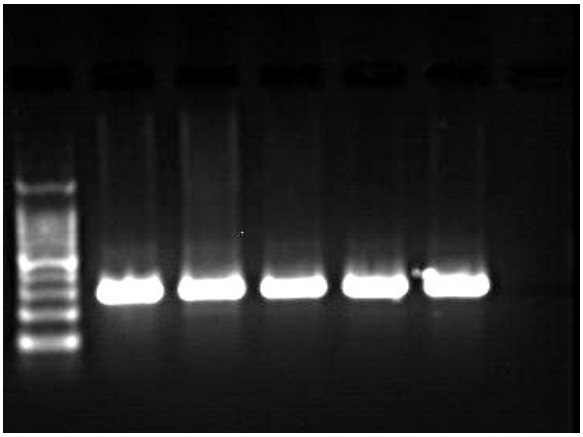


Figure:4a. Result for molecular detection.



B.Mcr 1



C. Mcr 2

Figure - 4b and 4c: MOLECULAR DETECTION OF mcr-1 and mcr-2 GENE FOR COLISTIN RESISTANCE BY PCR:

Statistical analysis:

Overall, By descriptive analysis method, mcr [1 and2] genes prevalence determined by both culture and PCR methods was estimated to be 9.0% (95% confidence interval of 5.7%–13.7%, Wilson score interval). This was statistically significant* (p=0.005).By students t test, t-stat reported in the output is higher than 1.96 and coefficient is significant at 5% significance level.

DISCUSSION

In this study, Out of 3420, 105 *Escherichia coli* strains were collected. 76 (72.4%) were from urine, 10 (9.5%) from wound swab, 7 (6.6%) from high vaginal swab, 6(5.7%) from pus, 3 (2.8%) from blood, 3 (2.8%) were from rectal swab. A study done by N Prim et al,[in Barcelona, Spain] were isolated 3011 clinical isolatesbetween January 2012 to May 2012 66 (62.85%) were from urine, 36 (34.28%)were from pus,10 (9.5%)were from wound swab. A study in Quan J et al [9,10] were isolated 1,270 *Escherichia coli* between August 2017 to October 2018.

In our study, there was a slight increase in incidence of urinary tract infection in Female as 62 (59%), Male was 43 (40.95%). This correlates to a study done by J Rodríguez-Navarro et al., [11]states that female gender is affected more for urinary tract infection[12,22] . Like Magiorakos AP et al., majority of samples fall under the age groups 21- 60years. In our study, majority of the samples belong to middle age group, which is also similar to our study Spencer, J., et al. (2016) [14] The average age of the patients was 68.5 (range, 53 to 93) years, and six of them were male.

In this study, there was an increase in incidence of all various samples from Urology unit 28%, followed by OBG ward 38%, Male Medicine and 6.66%, Female Medicine ward 6.66%, Special ward 5.71%, Dermatology 1.90%, SICU 4.76%, ER 1.90%, Male surgery 7.61%, Female surgery 11.42%. In the study well with that of lin cao1 *et al* [16], there was an increase in incidence of all various samples from Nephrology unit 11.42%, Pediatric ward 17.14%, Dermatology unit 22.85%, Male surgery ward 8.57%, Female surgery ward 19.04%, which was similar wards of our study and H Hasman 1 *et al,* [15,21] were from Surgical ICU 24%, which is also similar to our study.

The highest Resistance was noted for Ampicillin 78%. The resistance to Cotrimoxazole was 64% and resistance to Ciprofloxacin 60%. In the study of lin cao1 et al, [16] The antimicrobial agents tested included meropenem, imipenem, tigecycline, colistin, aztreonam, amikacin, levofloxacin, cefoperazone-sulbactam, cefotaxime, cefepime, and trimethoprim/sulfamethoxazole. The highest susceptibility were found to be towards the Cefoperazone-sulbactam 48% and the highest rate of resistance were found to be towards Amikiacin 82% which is least similar to our study.

When we talk about colistin resistance exclusively in our study *Escherichia coli* isolates were 1% resistance towards colistin by antibiotic susceptibility testing. The MIC showed 1% of the strains were resistance to colistin.

Out of 105 *Escherichia coli* isolates,four(4%) were resistance in MIC to colistin by Broth dilution method.

Among 105 isolates four (3.8%) isolates which were showing resistance to colistin by MIC method were sent for molecular detection for mcr-1 and mcr-2 gene all 4 isolates were positive for both the genes. This study correlates lin cao1 et al, [17,18] the mcr-1 gene was detected by PCR. This study correlates Quan J et al, 13 mcr-positive colistin-resistant isolates and 8 mcr-negative

colistin-resistant isolates were selected as similar to our study.

CONCLUSION:

To conclude, Since Disk diffusion and Vitek interpretation for colistin is not recommended, Minimum inhibitory concentration is the only method by which the susceptibility of colistin can be reported. Incidence of colistin resistance could be higher among MDR isolates for which colistin can be used in treatment. Hence, judicial use of this drug will help in preserving this drug usage in infections with multi drug resistant strains. Misuse and overuse of antibiotics can be prevented by constant monitoring of the antibiotic susceptibility testing for the bacterial isolates in the hospital and by framing antibiotic policy and initiating antibiotic stewardship program.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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