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Detection of *mcr*-1 gene of plasmid-mediated colistin resistance in extended spectrum beta lactamase (ESβL) producing *Escherichia coli* in Khartoum State, Sudan

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ABSTRACT

Background: Multidrug resistant *Escherichia coli* (MDR-*E.coli*) is becoming a major public health concern worldwide causing obstruction of disease control and increased the cost of treatment because pathogens have become resistant to commercially available drugs, necessitating the use of more expensive therapies. **Objectives:** This study aimed to detect the presence of plasmid mediated colistin resistant *mcr*-1 gene in extended spectrum β-lactamase (ESβL) producing *Escherichia coli* from clinical samples. **Methods:** In this cross-sectional study a total of 88 multidrug resistant *E. coli* isolates were collected from different hospitals in Khartoum state. The isolates were cultured on MacConkey agar media at 37º C for 24 hours subsequently routine bacterial identification was done by performing colonial morphology, Gram staining, and biochemical tests. Antimicrobial susceptibility testing was performed following the modified Kirby-Bauer disc diffusion method, then phenotypic confirmatory test for ESβL producers was done by using the double disc synergy test. **Results:** A total of 88 (100%) *E. coli* isolates were multi drug resistant, 7(8%) of which were phenotypically resistant to colistin. After performance of the double disc synergy test for detection of extended spectrum β lactamase, 29 (33%) isolates were positive. PCR technique was done to detect the presence of *mcr*-1 gene in 7 MDR isolates, the result showed that 4 (57,1%) were carriers for *mcr*-1 gene. **Conclusion:** This study confirms the presence of *mcr*-1 gene in *E. coli* in clinical isolates and all *E. coli* isolates that were carriers of *mcr*-1 gene were confirmed to be producers of ESβL.

Introduction

Escherichia coli is the predominant facultative anaerobe of the human colonic flora. *E. coli* is the species of the genus *Escherichia*, which contains mostly motile Gram-negative bacilli within the family Enterobacteriaceae. *E. coli* grows at 37°C [1].

In 1944, **Kauffman** proposed a scheme for the serologic classification of *E. coli* which is

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still used in modified form today. According to the modified Kauffman scheme, *E. coli* is serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles [2].

Due to the genetic variability some *E. coli* strains are different from their commensal counterparts and encode specific virulence traits that render them capable of causing disease in a variety of animals. Pathogenic *E. coli* is broadly divided into two groups, extra intestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC) [3].

During the last few decades, the incidence of microbial infections has increased dramatically. Continuous deployment of antimicrobial drugs in treating infections has led to the emergence of resistance among the various strains of microorganisms. Multidrug resistance (MDR) is defined as resistance of a microorganism to the administered antimicrobial medicines (which are structurally unrelated and have different molecular targets) despite earlier sensitivity to it.

According to WHO, these resistant microorganisms (like bacteria, fungi, viruses, and parasites) are able to combat attack by antimicrobial drugs, which leads to ineffective treatment resulting in persistence and spreading of infections [4].

MDR provokes obstruction in disease control by intensifying the possibility of spreading of resistant pathogens, thus, declining efficacy of treatment and, hence, resulting in prolonged time of infection in patient [5].

Extended-spectrum β-lactamases (ESβLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. Typically, they derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases.

Plasmids responsible for ESβL production frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides). Therefore, antibiotic options in the treatment of ESβL-producing organisms are extremely limited [6].

Colistin is a polymyxin antibiotic that has been used for many years in veterinary medicine. Nowadays, a need for using colistin in human medicine has evolved as the last resort drug for the treatment of infections caused by multidrug-resistant bacteria, especially carbapenem-resistant Enterobacteriaceae [7].

The mechanism of the resistance of mobilized colistin resistant gene is a phosphatidylethanolamine transferase. The enzyme transfers a phosphoethanolamine residue to the lipid (A) present in the cell membrane of Gram-negative bacteria. The altered lipid A has much lower affinity for colistin and related polymyxins resulting in reduced activity of the antimicrobial. This type of resistance is known as target modification [8].

This study was conducted to investigate antibiotic resistance and molecular epidemiology of *E. coli* strains producing ESβLs *mcr*-1 gene. The presence of ESβL-producing *E. coli* in clinical settings has become a serious concern owing to the fact that these strains exhibit a wide range of resistances not only to β-lactam, but frequently to other classes of antibiotics.

The study aimed to detect *mcr*-1 gene of plasmid-mediated colistin resistance among Extended spectrum lactamase (ESβL) producing *Escherichia coli* in Khartoum state/ Sudan.

Material and methods

This was cross-sectional, hospital-based study. It was conducted in Khartoum state, (Soba University Hospital, Fedail Hospital, Royal Care Hospital, Military Hospital).

Bacterial isolates (88 = isolates) were collected from clinical samples from different hospitals in Khartoum state.

Sample size

The sample size was calculated according to the equation:

$$N = \frac{(Z)^2 PQ}{D^2}$$

N = Sample size.

Z= Normal standard deviation (1.96).

P= Frequency of occurrence of an event ‘prevalence’

Q= 100-P Frequency of non occurance of an event.

D= Degree of precision (5%)

$$N = \frac{(1.96)^2 X 6.1 X 93.9}{5^2} = 88$$

Exclusion criteria

Non multidrug resistant *Escherichia coli* isolates.

Sample collection

A total of 88 *E. coli* isolates from clinical samples were collected by subculturing on MacConkey agar by streaking method.

Identification of bacterial isolates

Gram staining technique

Air dried was flooded, heat fixed smear of cells for 1 minute with crystal violet staining reagent. The quality of the smear (too heavy or too light cell concentration) was affected the Gram stain results. Slide washed in a gentle and indirect stream of tap water for 2 seconds. Slide flooded with the mordant: Gram's iodine. Wait 1 minute. Slide washed in a gentle and indirect stream of tap water for 2 seconds. Slide flooded with decolorizing agent. Wait 15 seconds or added drop by drop to slide until decolorizing agent running from the slide runs clear (see Comments and Tips section). Slide flooded with counterstain, safranin. Wait 30 seconds to 1 minute. Slide washed in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper. Results was observed of the staining procedure under oil immersion using a bright field microscope. At the completion of the Gram Stain, Gram-negative bacteria stained pink/red and Gram-positive bacteria stained blue/purple.

In a smear that has been stained using the Gram Stain protocol, the shape, arrangement and Gram reaction of culture will be revealed, shows Gram negative (pink/red) rods [9].

Colonial morphology

The colonies on MacConkey agar, were lactose fermenting, medium in size, round, smooth, low convex and pink in color.

Biochemical tests

Kligler's iron agar (KIA)

This is a differential medium. It tests for organisms' abilities to ferment glucose and lactose to acid plus gas end products. It also allows for identification of sulfur reducers. If the organism is capable of using neither glucose nor lactose, the organism uses solely amino acids / proteins. *Pseudomonas aeruginosa* is an example of a non fermenter. The tube incubated at 37^{o} C for 24 hrs [10].

Simmon's citrate agar

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of Enterobacteriaceae. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaoloacetic acid and acetic acid. The tube incubated at 37℃for 24 hrs. The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result [11].

Indole production test

The organism breaks down the treptophan and produce Indole which can be detected by Kovac's reagent (4 (p) – dimethyl - aminobenzaldehyde. Incubate the tube at 37℃ for 24 hrs,0.5 ml of Kovac's reagent added , shaked gently and examined for red colour ring within 10 mins[12].

Urease test

This test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. The hydrolysis of urea forms the weak base, ammonia, as one of its products. This weak base raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink. The tube incubated at 37℃ for 24 hrs [13].

Motility test

It is a differential medium used to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark. The tube incubated at 37℃ for 24 hrs. The results of motility agar are often difficult to interpret. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab [14].

Kirby-Bauer disk diffusion susceptibility test method

Six mm filter paper disk impregnated with a known concentration of an antimicrobial compound was placed on a Mueller-Hinton (MH) agar plate, immediately water was absorbed into the disk from the agar. The antimicrobial began to diffuse into the surrounding agar. The rate of diffusion through the agar is not as rapid as the rate of extraction of the antimicrobial out of the disk, therefore the concentration of antimicrobial is highest closest to the disk and a logarithmic reduction in concentration occurs as the distance from the disk increases. The rate of diffusion of the

antimicrobial through the agar is dependent on the diffusion and solubility properties of the drug in MH agar and the molecular weight of the antimicrobial compound. Larger molecules are diffuse at a slower rate than lower molecular weight compounds. These factors, in combination, result in each antimicrobial having a unique breakpoint zone size indicating susceptibility to that antimicrobial compound. Growth occurs in the presence of an antimicrobial 3 compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The estimated time of a bacterial suspension to reach critical mass is 4 to 10 hours for most commonly recovered pathogens, but is characteristic of each species, and influenced by the media and incubation temperature. The size of the zone of inhibition of growth is influenced by the depth of the agar, since the antimicrobial diffuses in three dimensions, thus a shallow layer of agar will produce a larger zone of inhibition than a deeper layer [15].

DNA extraction

Ten mL transferred of mid-to late-log-phase culture (0.5 – 0.7 at OD600) to a falcon tube and pellet the cells through centrifugation at 7,500 rpm for 10 minutes.

Pellet resuspend with 467 µL RNase A in Buffer P1 and transferred to a 1.5-mL microcentrifuge tube. Eight µL lysozyme and 5 µL achromopeptidase, mixed and incubated at 37°C for 60 minutes.

Thirty µL 10% SDS (sodium dodecyl sulfate) and 3 µL proteinase K, inverted and incubated at 50°C for 60 minutes.

525 µL PCI (Phenol:Chloroform:Isoamyl) solution and mixed for 10 minutes by gentle inversion. Centrifuged at 12,000 rpm for 15 minutes.

The upper aqueous phase transferred to a sterile 1.5-mL microcentrifuge tube, taking care not to disturb the bilayer, an equal volume of −20°C 100% ethanol and mixed by inversion. Centrifuged at 12,000 rpm for 20 minutes. Carefully decanted the supernatant and thoroughly dried pellet at room temperature or in a 50°C incubator.

Over drying result in making the DNA pellet more difficult to dissolve back into solution. The pellet may or may not be visible to the naked eye.

The pellet resuspended in 50 µL TE (Tris-EDTA) buffer and allowed pellet to sit overnight at 4°C.

Presence and concentration of bacterial DNA was confirmed by running 5 µL of product on a 1.5% agarose gel. Purified DNA appeared as a defined band when visualized under UV light [16].

PCR detection of the *mcr*-1 gene for the identification of *E. coli*

PCR amplification of total DNA [obtained using the heat method] was used to identify all Gram-negative isolates, 25 µl reactions were achieved by mixing 10 µmol/µl [1 µl] of each forward and reverse primer **(Table 1)**, 12.5 µl of 2 x GoTaq Green master mix (Promega), 5.5 µl of molecular grade distilled water and 5 µl of total bacterial DNA. Negative controls were analyzed with each set of amplifications; forward and reverse primers and master mix were added as previously described, the final volume of 25 µl was achieved using molecular grade distilled water. Gene expression was visualized using gel electrophoresis within a 1% agarose gel. The template DNA was amplified on the Stratagene Robocycler, using the following program:

Denaturation at 95°C for 3 minutes,

Denaturation at 95°C for 45 Seconds,

Annealing at 60°C for 45 Seconds x 35 cycles,

Extension at 72°C for 60 Seconds,

Extension at 72°C for 10 minutes.

Data analysis

Statistical analysis of the data was performed using chi-square test (level of significance was 0.05) with SPSS software version.

Ethical considerations

This study was approved by ethical committee board of National University. Written informed consent obtained from each participant or individual informed were completed. Research purpose and objectives were explained to the participant in clear simple words. A participant has the right to voluntary informed consent. A participant has the right to withdraw at any time without any deprivations. A participant has right to no harm (privacy and confidentiality by using coded questionnaire). A participant has right to benefit from the researcher knowledge and skills.

Results

A total 88 multi-drug resistant (MDR) *Escherichia coli* isolates were collected from different clinical specimens collected from different hospitals in Khartoum State.

The *E. coli* was confirmed by colonial morphology, indirect Gram's staining technique and different biochemical tests.

Antibiotic susceptibility testing by using disc diffusion test (Kirby-Bauer method) on Muller Hinton agar for colistin (10 µg), ceftriaxone (10 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and imipenem (10 µg).

Out of 88 *E. coli* isolates 7 (7,9%) were phenotypically resistant to colistin **(Table 2)** and **(Figure 1).**

The colistin resistant *E. coli* isolates were positive for ESβL test **(Figure 2).**

Four (57,1%) of *E. coli* were tested by using PCR technique to detect the gene, and the isolates were carriers of the *mcr*-1 gene **(Figure 2).**

Table 1. Primer sequences used for detection of the *mcr*-1 gene by PCR technique.

Primer Name	Sequence 5' to 3'	Tm	Amplicon Size (bp)
mcr-1 Forward	AGTCCGTTTGT	55	320
mcr-1 Reverse	AGSTCCTTGGTC	60	320

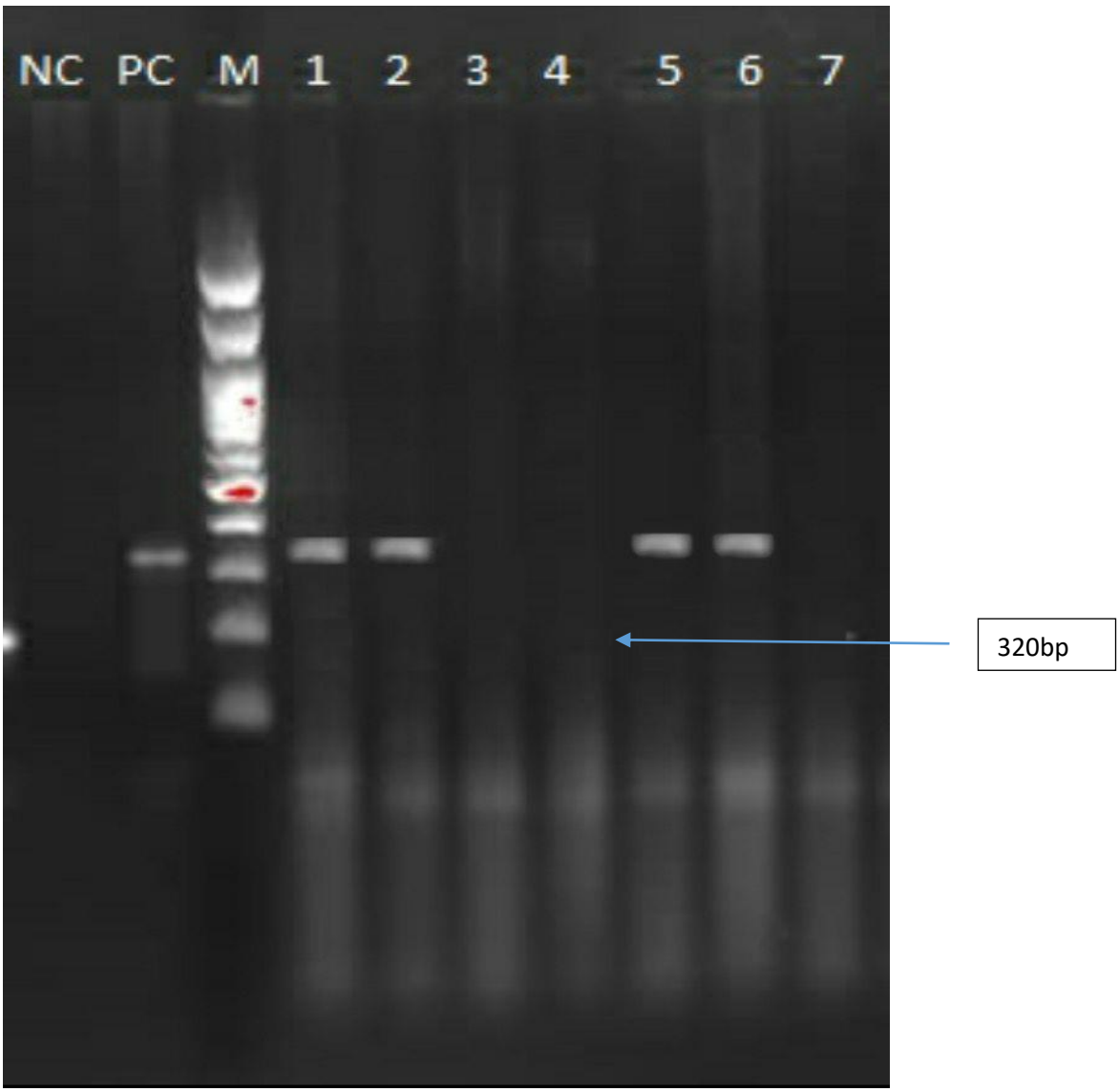
Table 2. Results of resistance in antibiotic susceptibility testing.

Antibiotic Disk	Resistant		P- Value
	Frequency	Percentage	
Ceftriaxone (10 µg)	57	64%	0.0046
Ciprofloxacin (5 µg)	55	62.5%	
Ceftazidime (30 µg)	73	82.9%	
Gentamicin (10 µg)	68	77.3%	
Imipenem (10 µg).	20	22.7	
Colistin (10 µg)	7	8.0%	

P value = 0.0046 (not significant)

Figure 1. shows positive for phenotypical ESβL test.

Figure 2. shows positive PCR for detection of *mcr*-1 gene on gel electrophoresis.



Discussion

Antibiotic resistance genes are spreading in *Escherichia coli*, complicating the treatment of serious nosocomial infections. Resistance to third generation cephalosporins due to the presence of plasmids that contain genes that encode for extended spectrum beta lactamases (ESβLs) and these plasmids often carry other resistance genes.

Polymyxin serves as the last line of defense against multidrug-resistant Gram-negative bacteria infections. This common belief has been substantially challenged by the recent finding of the mobile colistin resistance gene (*mcr*-1) in food animals and humans worldwide. Most crucially, the *mcr*-1 gene was discovered to be co-localized with other antibiotic resistance genes, raising the likelihood of the emergence of superbugs with pan-drug resistance.

The mobilized colistin resistance (*mcr-1*) gene confers plasmid mediated resistance to colistin. The mechanism of the resistance of MCR gene is a phosphatidylethanolamine transferase. The enzyme transfers a phosphoethanolamine residue to the lipid A present in the cell membrane of Gram-negative bacteria. The altered lipid A has much lower affinity for colistin and related polymyxins resulting in reduced activity of the antimicrobial. This type of resistance is known as target modification [17].

This study aimed to detect the presence of plasmid mediated colistin resistant *mcr*-1 gene in

extended spectrum β-lactamase (ESβL) producing *Escherichia coli* from clinical samples.

The isolates of Multi drug resistance *Escherichia coli* was confirmed by colonial morphology on MacConkey agar and it was confirmed by different biochemical tests.

Antimicrobial sensitivity testing was done in this study for 88 isolates of *E. coli* by using different types of antibiotics disc. The results obtained were 88 (100%) of isolates were multi drug resistant these findings agree with previous study, in which 75% of *E. coli* isolates were MDR, and disagree with another study revealed that 67% from 100 *E. coli* isolates were MDR [18,19].

ESβL have become a widespread and serious issue. Many pathogenic bacterial strains are increasingly expressing these enzymes, which has the potential to spread. The presence of ESBL impairs the efficacy of broad-spectrum antibiotics, resulting in considerable treatment challenges and a negative influence on patient outcomes.

In this study the percentage of ESβL producing *E. coli* was 29 (33%), where the percentage of ESBL producing *E. coli* was also 33%. They were also consistent with the results of another study [14] where the percentage of ESβL *E. coli* was 32% [20,21].

The percentage of the mcr-1 gene in this study from the *E. coli* isolates that were phenotypically resistant to colistin was 4 (57%), these findings are similar to previous report were the percentage of *mcr*-1 in *E. coli* was 40% and all *mcr*-1 carriers were positive for ESβL. These findings contradict the results of another study were the percentage of *mcr*-1 was 1.4% and not all *mcr*-1 carriers were positive for ESβL [22,23].

Previous report studied the prevalence of AMR genes in MDR bacterial strains in-silico and its impact on antimicrobial resistance patterns [24,25].

Conclusion

This study confirms the presence of *mcr*-1 gene in *E. coli* clinical isolates. All *E. coli* isolates that were carriers of *mcr* -1 gene were confirmed to be producers of ESβL.

The study recommends that antibiotics should only be used when needed, the right drug, right dose, right route and in the right time. Other studies should be done, with large sample size, longer duration and covering another region in Sudan.

Conflicts of intereset: None to be declared.
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