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Original article

Molecular detection of colistin resistant gene (*MCR-1*) of Gram-negative bacilli from clinical isolation in Khartoum State among patients under 15 years

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ABSTRACT

Background: Colistin resistant gene is uncommon, but recently several species of Gram negative bacteria have been MCR-1 such as *A.baumannii*, *K.pneumoniae* and *P.aeruginosa*. Many studies showed that the transfer of resistance genes is more effective than chromosomal mutation, the colistin resistant genes are located in a DNA sequence in plasmid which can be transferred from one plasmid to another. **Material and Methods:** In total, 100 consecutive non-repetitive Gram-negative bacilli isolates were obtained from different clinical specimens of patients less than 15 years. The isolates were reidentified by standard biochemical tests. The isolates were subjected to antimicrobial susceptibility testing. PCR was also used to detect the presence of *mcr1* gene in the isolates. **Results:** Totally, 50 (50%) isolates were resistant to colistin for all isolates, PCR technique was done for detection of *mcr1* gene, only (7) resistant samples carried *mcr1* gene. **Conclusions:** Number of *Escherichia coli* against colistin and carried *mcr1* gene was 2 (20%). *Klebsiella* spp carried *mcr1* gene was 2(8%). *Pseudomonas* spp against colistin and carried *mcr1* 2(15%). Finally, the number of *Proteus* spp against colistin and carried *mcr1* was 1 (25%).

Introduction

Antimicrobial Resistance (AMR) occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death. As a result of drug resistance, antibiotics and other antimicrobial medicines become ineffective and infections become increasingly difficult or impossible to treat [1].

Colistin also known as polymyxin E. Polymyxins are group of antibiotics (polymyxin A-E) different chemically, but only polymyxin B and polymyxin E (colistin) has been used clinically. Colistin was discovered in 1947 from a soil bacterium *Paenibacillus polymyxa* sub sp[2,3,4].

The use of polymyxin is increasing worldwide which resulted in increase in the percentage of resistance against bacteria, the prevalence of Colistin resistance gene in Sudan

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was not reported in published articles till now, the colistin resistance in *Enterobacterales* (with no intrinsic resistance) is around 0.67–1.6%, with *Escherichia coli* (0.2–0.6%), and a moderate rates in *Klebsiella pneumoniae* (1.5–6.8%), and much higher rates in *Enterobacter* spp. (13.9– 20.1%) [5].

One of the main problems of small and large hospitals in recent years has been outbreaks caused by Gram-negative bacilli in the creation of nosocomial infections, The multi-drug resistant Gram-negative bacilli (MDR-GNB) are responsible for many infections in various cases. Antibiotic resistance is growing and medical societies are fast running out of treatment options [6].

Early detection of these types of resistance genes such as mcr1, would be useful tool for the identification of infection thereby helping in controlling and prevention of their spread and choosing of appropriate antibiotics.

The aim of this study was to determine the prevalence of colstin resistance mcr1 gene isolated from clinical isolates by genotypic methods (PCR).

Material and method

Study design:

Experimental Cross-Sectional laboratory-based study.

Study area:

Gram-negative bacilli isolates were obtained from different hospitals in Khartoum State (Tabarak children Hospital, Omdurman Medical Military Hospital, Fadial Hospital and soba Hospital).

Study population:

Gram-negative bacilli isolates, from clinical specimens of children less than 15th years.

Inclusion criteria:

All gram-negative bacilli isolate from children less than 15th years included in this study.

Exclusion criteria:

All bacteria other than gram negative bacilli and gram-negative bacilli isolates from patient more than 15th years were excluded.

Sample size:

Hundred Gram-negative bacilli isolate from clinical specimens of child

under 15 years were collected during the study period. The duration of sample collection started on January 2022 and finished on March 2023.

Ethics approval and consent to participate

Ethical approval was obtained from the Gharb EL-Niel Ethical Research Committee in accordance with the Declaration of Helsinki Principles, and the agreement was taken from hospital administrations before sample and data collection.

Questionnaire was filled with the patient/or volunteer in their rest time without any interruption to their work (specify health worker), using protected cloth. Mask and gloves during collection of sample and questionnaire as protective issues from infection with covid 19.

Ethical clearance code number: GEN-RES/02-022-02

Date: 4/1/2022

Sample processing:

Sample collection:

Different Gram-negative bacilli isolates from clinical specimens of child under 15 years were collected in sterile test tube containing peptone water and then transported to the lab there after the subculture of all samples was made.

Subculture:

All the samples were inoculated in MacConkey agar and Nutrient's agar (NA), incubated overnight at 37C.

Identification procedure:

Indirect Gram stain:

Smears from the growth were prepared and stain by Gram stain as follow: fixed by heat, after cooling covered by crystal violet stain for 30-60 seconds, washed off stain by clean water, covered with iodine for 30-60 seconds, washed with cleaned water, covered with safranine stain for 2 minutes, then washed and let to air dry and microscopically examined using oil immersion objective (100X) to observe morphological appearance, Gram positive reaction and Gram negative. The results of Gram stain were reported.

Biochemical test:

KIA test:

A small part of the tested colony was picked off using a straight loop and inoculated in KIA medium. First stabbing the butt, then streaking the slope in zigzag pattern, and then incubated at 37oC aerobically overnight.

Indole test:

Procedure: by sterile wire loop inoculate an organism on peptone water, incubate at 37C for 18-24 hrs., on second day add 2-3 drops of Kovacs reagent and then observe and record the result.

Urease test:

By using sterile straight wire take inoculums from organism and inoculate by stabbing. Incubate at 37 C overnight. Observe and record the result.

Citrate utilization test:

By using sterile straight wire take inoculums from organism, Streak in a zigzag form in slope area, incubate at 37 C overnight and observe the result.

Oxidase test:

A filter paper was soaked with the substrate tetramethyl-p-phenylenediamine di hydrochloride Moisten the paper with sterile distilled water. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Antimicrobial susceptibility testing:

Disc diffusion method (Kirby –Bauer method):

By using sterile wire loop 3-5 colonies of similar appearance were selected and emulsified in 3-4 ml of sterile physiological saline and compared with McFarland's turbidity standard. By sterile swab suspension was inoculated in Mueller Hinton agar plate and by sterile forceps an antibiotic disc was placed in the inoculated plate, incubated aerobically at 370C for overnight, then the inhibition zones measured.

Interpretation of the result was done by aid of interpretative chart. By using the chart, the organism was characterized as being resistant, intermediate, or susceptible to the specific antibiotic.

The following antimicrobial agents were tested: colistin(10mcg), Imipenem(10mcg) (30mcg), Meropenem (10mcg) (30mcg), Ciprofloxacin (5mcg), levofloxacin (5 mcg).

Molecular Technique:

DNA Extraction:

DNA was extracted from isolated culture media of Enterobacteriaceae by using boiling method.

To prepare the suspension add 200 micro liters of water for injection in Eppendorf tube and then mixing with three to five colonies, vortex the suspension for 30-40 second, the sample boiling in water bath at100 Co for 15-30min, freezing the sample for 10 min, vortex the suspension 30-40 seconds, centrifuged at 3000 rpm for 5 min, carefully remove upper clear layer to new Eppendorf tube without disrupting the precipitate and stored at -20c till use.

Polymerase chain reaction (PCR):

PCR was carried out for all isolates by using specific primer of mcr1 gene:

Mcr1 F(5-ATGATGCAGCATACTTCTGTG-3)

mcr-1R(5-TCAGCGGATGAATGCGGTG-3)

Amplicon size = 320 bp.

Amplification was performed at final volume 25 µl (5µl Mater mix, 5µl from DNA, 2µl primer and 13µl D.W). The PCR cycling conditions were as follows: an initial denaturation step of 10 min at 94℃ followed by 35 cycles

of 30 s at 94℃, 30 s at 55℃ and 30s at 72℃, with a final extension of 10 min, (Tc-412, UK) thermo cycler machine was used. PCR products was separated on 2% agarose gel by electrophoresis to detect specific amplified product by comparing with standard molecular weight marker 100 base pair (DNA ladder) 5µl of PCR product was loaded into the well. Electrophoresis was carried out at 100 Volts, 60 AMP and 30 minutes.

The amplified products of the study samples were visualized by Trans -illuminator. Photographed by a digital camera and transferred to computer data for labeling and storage.

Preparation of master mix for PCR:

Master Mix prepared according to protocol of manufacturing instruction (Master mix for PCR Primers - Santa Cruz Biotechnology Dallas, TX, USA).

Preparation of agarose gel:

Amount of 2 g of agarose powder dissolved by boiling in 100 ml 1X TBE buffer (2%), then was cooled to 55°C in a water bath, then, 2 µl of (10

mg\ml) Ethidium bromides were added, mixed well, and poured on to the casting tray that has been taped up appropriately and was equipped with a suitable comb to form well in place. Any bubbles were removed and the gel was allowed to

sit at room temperature. After solidification, the comb was gently removed and the space from the opened sides was removed.

Electrophoresis:

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface,10 µl of PCR products from each sample was added to wells of electrophoreses, 5 µl of 100-bp DNA ladder, was added to the well in each run. The gel electrophoresis apparatus was connected to the power supply (100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands (With different specific base pair (bp) for MCR1) by Ultraviolet (U.V) transilluminator and photographed (Uvitec – UK).

Results

A total of one-hundred-gram negative clinical isolates from different clinical specimens collected from patient (child under 15th years) different hospitals in Khartoum State.

Biochemical results of gram-negative bacilli isolates:

According to colonial morphology on MacConkey agar and nutrients agar, indirect gram stain and routine biochemical test (Table 1&2) re-isolation and identification the isolated microorganism.

The frequency of isolated gram-negative bacilli:

Escherichia Coli 28(28%), *Klebsiellaspp* 32(32%), *Pseudomonas spp* 30(30%), *Proteus* spp8(8%), *Acinetobacter*spp 2(2%) (Table 3).

Antibiotic susceptibility test:

Antibiotic susceptibility test by disc diffusion method on meuellerhinton agar for Ciprofloxacin 5mcg, colistin 10mcg and Levofloxacin5mcg, Imepenem 10 mcg and meropenem 10 mcg.

The frequency of resistant organisms was: Ciprofloxacin 76(76%), Levofloxacin 53 (53%), colistin 50 (50%), Impenem 44(44%) and meropenem 34 (34%).

The frequency of Sensitive organisms was: Colistin 50(50%) andLevofloxacin 35(35%) and ciprofloxacin20(20%) and Impenem 50 (50%) and Meropenem 60(60%).

The frequency of intermediate organisms was: Levofloxacin 12(12%) and ciprofloxacin 4(4%) and Imepenem 6(6%) and Meropenem 6(6%). (Table 4).

Interpretation of antibiotic susceptibility test:

Results of disc diffusion test for antibiotics (ciprofloxacin, Imepenem, Colistin, Levofloxacin, Meropenem.) to *Escherichia Coli*, *Klebsiellaspp*, *Pseudomonas* spp, *Proteus* spp, *Acinetobacter*spp.

Results of colistin antibiotic disc patterns:

In *Escherichia Coli* 10 (35.71%) were resistant, and 18 (64.28%) were sensitive, in *Klebsiella*spp 23(72.87%) were resistant, and 9(28.12%) were sensitive, In *Pseudomonas* spp 13(43.33%) were resistant, 17 (56.66%) were sensitive, and in *Proteus* spp 4(50%) were resistant and 4(50%) were sensitive, in *Acinetobacter* spp 2 (100%) sensitive. Frequency of mcr1 resistance gene:

A total of 100 of Gram-negative bacilli Isolates that produce Colistin resistance gene mcr1 were 7 (7%) bacteria (Figure 1).

Frequency of organisms carrying mcr1:

Resistant to Colistin 50(50%) isolates were sensitive to Colistin and 50(50%) isolates for all colistin -resistant and sensitive PCR technique was

done for detection of mcr1 gene, only 7(7%) resistance samples were carried for mcr1 gene.

Number of *Escherichia coli* that resist to Colistin and carried mcr1 gene is 2 (20%). And *Klebsiella*spp resist to Colistin and carried mcr1 gene is 2(8%). And *Pseudomonas* spp that resist to colistin and carried mcr1 2(15%).

Finally, the number of *Proteus* spp that resist to Colistin and carried mcr1 is 1 (25%). The results of detection mcr1gene showed in (table 6).

Data analysis:

By using excel 2010. The categorical data were presented as numbers andPercentages.

Table 1. Result of biochemical test of Gram-negative bacilli

Biochemical Test	***E. coli***	***Klebsiellaspp***	***Pseudomonas spp***	***Proteusspp***	***Acinetobacterspp***
Indole	+ve	-ve	-ve	6+ve/2-ve	+ve
Urease	-ve	+ve	-ve	+ve	+ve
Citrate	-ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	+ve	-ve	-ve

Table 2. Result of kligler iron agar (KIA).

KIA	**Butt**	**Slope**	**Gas**	**H2S**
E. coli	Yellow	Yellow	+ve	-ve
Klebsiellaspp	Yellow	Yellow	+ve	-ve
Pseudomonas spp	Yellow	Red	-ve	-ve
Proteusspp	Black	Red	+ve	+ve
Acinetobacterspp	Yellow	Red	-ve	-ve

Table 3. Frequency of isolated gram-negative bacilli.

Organsim	**Frequency**	**Percentage %**
E. coli	28	28%
Klebsiellaspp	32	32%
Pseudomonas spp	30	30%
Proteusspp	8	8%
Acinetobacterspp	2	2%
Total	**100**	**100%**

Table 4. Result of antibiotic susceptibility.

Antibiotic	**Sensitive**	**Resistance**	**Intermediate**
Colistin	50	50	-
Meropenem	60	34	6
Levofloxacin	35	53	12
Imepenem	50	44	6
ciprofloxacin	20	76	4

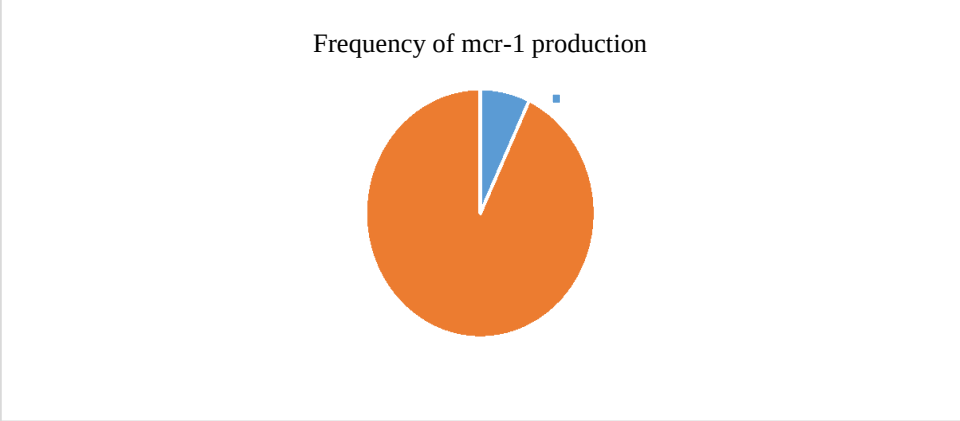
Table 5. Frequency of colistin susceptibility.

Organsim	**Number**	**Sensitive**	**Resistance**
E. coli	28	18	10
Klebsiellaspp	32	9	23
Pseudomonas spp	30	17	13
Proteusspp	8	4	4
Acinetobacterspp	2	2	-

Table 6: Frequency of organism carrying MCR-1.

Organism	MCR-1	Total resistant to colistin	MCR1% within these resistance
E. coli	2	10	20
Klebsiellaspp	2	23	8
Pseudomonas spp	2	13	15
Proteusspp	1	4	25
Acinetobacterspp	0	0	**0**
Total	**7**	**50**	**68**

Figure 1: Frequency of MCR-1 showed 50 isolates were resistant to colistin, 7 MRC-1 isolates within 50 isolates.



Discussion

Antibiotic resistance now is considered as one of major global crisis. In the last years the ability of rapid transmission of plasmid-mediated colistin resistant gene decrease the useful of colistin (polymyxins E) antibiotic as an important and last resort therapy against carbapenem-resistant and other multidrug resistant infection, in addition there were nearly to 22 genetic variations of mcr-1 have been reported [7]. However, the resistance of Klebsiella pneumoniae with moderate range (1.5–6.8%) and other *Enterobacterales* to polymyxins is increasing worldwide. Colistin resistance gene (mcr-1) was first described in *Enterobacteriaceae* isolated from animals, food and human in 2015 China, then it disseminated in Asia, Europe and North America, Egypt and Italy [8.9,10].

In this study mcr-1 was detected in a total of (100) gram negative isolates were collected from child less than 15th years from various hospitals at Khartoum State from January 2022 to March 2023, the results report the presence of mcr-1 gene in seven sample (7%).

Those findings indicate the spread of colistin resistance in Sudan mainly in *Enterobacteriaceae*, mostly in *Klebsiella*, *E. coli*, *pseudomonas spps* with low occurrence in proteus and *Acinetobacter spp* .

Most of the isolated organisms in this study were highly resistant to ciprofloxacin (76%). The finding of report showed that the plasmid mediated colistin resistance (mcr-1) gene was detected in gram negative that resistance colistin by disc diffusion method, this result indicates presence

of express gene. Other studies show that many of bacteria known as mcr-1 positive are known to exhibit low level of resistance to colistin. The result expects that silent dissemination can increase risk of colistin resistant in which plasmid transfer is one of common mechanism of antibiotic resistance. In addition, it suggests that the unknown dissemination of antibiotic resistance gene in general and plasmid mediated colistin resistance gene may explain the presence of large number of untreatable infections specially that causes by multi drug resistant *enterobacteriaceae* in case of colistin resistance in Sudan [11.12.13.14,15,16].

Protonotariou *et al*., mentioned that the specific mcr-1.1 allele was located in a 32.722 bp plasmid belonging to the IncX4 group with no additional resistance genes. A potential spread of mcr-1 in Greece is concerning because of the existing high rates of carbapenem resistance and colistin usage as a last resort regimen [17, 18].

Wu and his colleagues reported that Colistin is regarded as one of the last-resort antimicrobials for severe infections. The potential risk of spread of MCR-1-carrying, ESBL-producing E. coli in the community is observed. The genome sequence of E. coli LX13 will facilitate the understanding of colistin resistance mechanisms and genomic features of clinically isolated colistin-resistant E. coli [19].

Antimicrobial resistance against colistin has emerged worldwide and is threatening the efficacy of colistin treatment of multi-resistant Gram-negative bacteria. In this study, PCRs were used to detect mcr genes (mcr-1, mcr-2, mcr-3) in 213 anal and 1,339 nasal swabs from pigs (n = 1,454) in nine provinces of China, and 1,696 cloacal and 1,647 oropharyngeal samples from poultry (n = 1,836) at live-bird markets in 24 provinces. The mcr-1 prevalence in pigs (79.2%) and geese (71.7%) were significantly higher than in chickens (31.8%), ducks (34.6%) and pigeons (13.1%) [20].

Yassin et al., reported that antimicrobial resistance to colistin threatening the efficacy of one of the last-resort antimicrobials used for the treatment of multidrug-resistant Enterobacteriaceae infection in humans. In this study, we investigated the presence of colistin resistance genes (mcr-1, mcr-2, mcr-3) in Escherichia coli strains isolated from poultry and livestock collected between 2004 and 2012 in China. Furthermore, we studied the maintenance and transfer of the mcr-1 gene in E. coli after serial passages. Overall, 2.7% (17/624) of the E. coli isolates were positive for the mcr-1 gene while none were positive for the mcr-2 and mcr-3 genes. The prevalence of mcr-1 was similar in E. coli isolates from chickens (3.2%; 13/404), pigs (0.9%; 1/113) and ducks (6.8%; 3/44) but were absent in isolates from cattle (0/63). The mcr-1 gene was maintained in the E. coli after six passages (equivalent to 60 generations). In vitro transfer of mcr-1 was evident even without colistin selection. Our data indicate the presence of mcr-1 in extraintestinal E. coli from food-producing animals in China, and suggest that high numbers of the mcr-1-positive bacteria in poultry and livestock do not appear to be readily lost after withdrawal of colistin as a food additive [21].

In summary, this study revealed that all mcr1 gene detect in bacteria resistant to Colistin that indicate absences of silent gene (un expression gene). Our results expect that many of antibiotic resistance gene difficult to be detected and also cannot be detected by most laboratory tests as different types of sensitivity test and other phenotypic tests, Consequent of this, more wide dissemination of the resistant gene will occur, additionally to unwariness with the real scale of problems which lead to minimize of suitable controlling.

Conclusion

The study concluded that the sensitivity of gram-negative isolates varies in the tested different antibiotics. The mcr-1 gene was detected by conventional PCR in gram negative isolates in child less than 15th years with 7%from total isolates gram negative (50 colistin resistant).

The present study recommends use of large sample size should be performed in other studies for more information of colistin resistance. Other type of mcr-1 and sub types mcr-1 must be included in studies for detection of different type of plasmid mediated colistin resistance in Sudan.

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