Association of Carbapenem and Colistin Resistance in Pathogenic Gram Negative Bacteria

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Abstract

Diseases caused by multidrug-resistant (MDR) bacteria continue to challenge physicians and endanger their patients’ lives. Polymyxins, including colistin, are the last resort antibiotics to treat serious infections caused by carbapenem-resistant bacteria. The aim of this study is to explore the resistance of Gram negative isolates recovered from 200 clinical specimens to carbapenem and colistin antibiotics, and the prevalence of plasmid-mediated mcr-1 gene in the resistant isolates. Clinical specimens were collected from two teaching hospitals and two private clinical laboratories in Cairo, Egypt. We identified one hundred and thirty isolates as Gram negative. These isolates were screened for their susceptibility to β-lactams antibiotics, carbapenems, colistin, polymyxin B, levofloxacin and amikacin. Thirty isolates were found to be resistant to the tested carbapenems. Of these, five isolates were found to be resistant to both carbapenem and colistin. They were tested for the presence of mcr-1, pmrB and pmrA genes; known to be among the reasons for colistin resistance. One isolate showed the presence of pmrA while three isolates showed the presence of pmrA and pmrB. Only one isolate showed the presence of mcr-1, pmrA and pmrB. This was tested by real time PCR to ensure the activity of this plasmid-mediated gene. Using 16S rRNA sequencing, the isolate showed 100% similarity to Escherichia coli strain K12 (MG1655). Here, we report a carbapenem-resistant and colistin-resistant Escherichia coli strain producing mcr-1 gene that is the first to be reported in Egypt between human.

Keywords: carbapenem resistance, colistin resistance, mcr-1, pmrB, pmrA.
INTRODUCTION

Carbapenem-resistant parts of the family Enterobacteriaceae have disseminated all over the world\(^1\). Carbapenem-resistant Enterobacteriaceae are co-resistant for many classes of antimicrobial agents\(^2\). This means poor treatment outcomes can be a considerable threat to public health. Colistin has been recently considered clinically important, because of being frequently successful in managing infections caused by multidrug resistant (MDR) Pseudomonas, Escherichia and Klebsiella pathogens\(^4\). It should be administered intravenously and by inhalation in combination with other drugs to manage infections with Pseudomonas aeruginosa in children and adult patients with cystic fibrosis\(^5\). Colistin is a poly-peptide anti-microbial which acts through electrostatic interactions. Its cationic moieties disturb the bacterial external membrane, leading to leakage of cell contents and cellular death\(^6\). Resistance for Colistin generally due to inefficient binding of polymyxins to the lipid A group of lipopolysaccharide. This can be basically due to the 4’-phosphoethanolamine (PEA) modification of the lipid A on the LPS\(^7\,8\). This type of chemical modification on the bacterial lipid A can be ascribed to either the chromosome as in Klebsiella pneumonia\(^9\), or the plasmid-transferred mobilized colistin resistance (mcr-1) gene in Enterobacteriaceae like Escherichia coli \(^10\). In previous years, scientists have recorded infections that showed resistance to both carbapenems and colistin-resistant globally as in, Greece \(^11\) and Israel \(^12\). Plasmid-mediated colistin resistance encoded by mcr-1 was discovered in China \(^10\). Reports followed in several nations, among which France\(^13\), Denmark\(^14\), Venezuela\(^15\) and USA\(^16\). Recently, Wang and his coworkers at 2018 assembled the largest data set of mcr-1-positive sequenced isolates\(^17\). They found that the nations having the largest numbers of mcr-1-positive samples were China, Vietnam and Germany\(^17\). Interestingly, more mcr genes have been recently reported to be included in colistin resistance such as mcr-2 and mcr-4\(^18,19\).

In this study, the author screened two hundred isolates, recovered from clinical specimens in Egypt, for susceptibility to selected carbapenems and colistin. Further, the mechanism of resistance was explored.

MATERIALS AND METHODS

Isolation and biochemical identification of bacteria

Two hundred clinical specimens were collected from two teaching hospitals (Sayed Jalal and Al Hussein hospitals) and two private clinical laboratories in Cairo, Egypt. These were isolated from urine samples, sputum, pleural fluid, endotracheal secretion and swabs. The samples were collected at November 2016-October 2017. Isolates were collected from patients admitted in ICU, PICU as well as outpatients. Their ages ranged from 6 – 50 years. They suffered from respiratory infection, UTI or Meningitis. Gram negative isolates were identified using standard microbiological tests; Gram stain, motility test, catalase test, triple sugar iron, citrate agar test, oxidase test and witek test.

Antimicrobial susceptibility testing

The antibiotic susceptibility testing was performed by Kirby Bauer disk diffusion method\(^2\). Gram negative isolates were screened for susceptibility to six β-lactam antibiotics; meropenem, imipenem, amoxicillin-clavulanic, cefotaxime, amoxicillin and ceftazidime. Resistant isolates were identified using standard microbiological tests; Gram stain, motility test, catalase test, triple sugar iron, citrate agar test, oxidase test and witek test.

DNA extraction and manipulation

DNA extraction for double resistant (Colistin/Carbapenem) isolates consisted of boiling lysates prepared from the strains; a loopful of culture was suspended in 1 mL of sterile PBS buffer, centrifuged 5 min at 20000 g and the supernatant rejected. Then, the pellet was resuspended in 100 µL TE buffer (10:1) and boiled for 10 min at 100°C. For use as template in the polymerase chain reaction, this DNA was further diluted at 1:10 in TE buffer. The DNA extract was checked by running on 0.8 % agarose gel, stained with ethidium bromide (Thermo Fischer Scientific, USA) using gel electrophoresis (Labnet, USA). The gel was illuminated under Whitman U.V Trans-illuminator (Biometra, Germany) according to Sambrook et al. \(^20\).

PCR amplification of specific sequences of some genes encoding for colistin and polymyxin resistance

Three genes contributing to colistin and polymyxin resistance were selected in this study and amplified by PCR; mcr-1 primers {(
CLRr/CLRf (5’CGGTCAGTCCGTTTGTTC’3) CLR/r (5’CTTGGTCGGTCTGTAGGG’3) 94°C for 3 min + 25°C (94°C for 30 sec + 58°C for 60 sec) + 72°C for 10 min, pmrA primers LT_PMRA/REV (F (5’CAT TTC GCA CTG TCT GC’3) R (5’CAG CTT GTA GCA AAC AG’3)) 95°C for 10 s, 52°C for 30 s and 72°C for 1 min. Amplification was performed using thermocycler in total volume of 25 µL containing 1 µL DNA extract, 1 µL of forward primer (10 µM), 1 µL reverse primer (10 µM), 12.5 µL tag master mix (Qiagen, Germany) and 9.5 µL of nuclease-free water. Amplification using Gene-AMP-PCR-system-9700 thermocycler (Applied bio system). After amplification, 10 µL of the PCR mixture was analyzed by gel electrophoresis (1.5% agarose in tris-EDTA stained with ethidium bromide). As mcr-1 mostly carried on plasmid and pmr-A, pmr-B are chromosomal genes so mcr-1 dissemination is highly predicted that lead to increase occurrence of colistin resistance globally, so SYBR Green-based real-time PCR assay was designed for specific detection of the mcr-1 gene. Cell lysates (3 µL), prepared as described above, were used as DNA template, MCR-F: 5’-ACGGCGTATTCTGTGCCGTGTAT-3’ and MCR-R: 5’-GCTGTTCTTTTGGTGCAAAGGCATTT-3’ were used as primers for PCR analysis of mcr-1 real-time PCR were performed using QuantiNova SYBR Green mixture and Qiagen Rotor Gene system (Qiagen, Hilden, Germany). A typical 20 µL of PCR reaction includes 0.7 µM of each primer, 3 µL of lystate and SYBR Green mixture (1x). The PCR conditions used are: 95°C, 10 minute; 30 cycles including 95°C, 15 seconds, 60°C, 1 min; and 72°C, 30 seconds. Confirmation of the identity of isolate harboring mcr-1 by 16S rRNA gene sequence analysis

Identification was confirmed by sequencing of partially amplified 16S rRNA gene of the bacterial isolate that harbored mcr-1 gene. Molecular characterization of the selected strain was carried out according to modified Sambrook et al. 20. The 16S rRNA gene was amplified using universal primers pair: the forward primer sequence was 5-AGAGTTTGATCCTGAGCGTCA-3 and the reverse primer sequence was 5-ACGAGCTGACGACGCTCAT-3 (IDT, USA). PCR was carried out in a total volume of 50 µL using 25 µL of My Taq PCR Master Mix (2X) (Bio line, Germany), containing 1 µL (10 µM) of each forward and reverse primers together with equivalent micro liters containing 50 µg of the genomic DNA and finally completed to 50 µL by nuclease free water. Thermo cycling was carried out using thermocycler (Primus 25 Advanced, Germany). It was carried out as follow: first step was denaturation for 3 minutes at 94°C, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C C for 30 sec and extension at 72°C for 2 min. Finally, extension was done at 72°C for 10 min. The amplified sequence was verified by gel electrophoresis. PCR products were purified by Gene Jet Gel Extraction Kit (Thermo Fisher Scientific, USA). The purified products were subjected to sequencing. Nucleotide sequence similarities were determined using other known sequences found in the GenBank database using BLAST program of National Center for Biotechnology Information (NCBI) databases.

RESULTS

Isolation and identification of bacteria

Of the two hundred isolates collected from clinical specimens of various sources distributed (Fig.1). One hundred and thirty isolates were identified as Gram negative bacteria, as presented in Fig.2. The other isolates were excluded from this study. Antimicrobial susceptibility testing

A hundred and thirty Gram negative isolates were tested for susceptibility to six B-lactam antibiotics. Results were interpreted as resistant (R), intermediate (I) or susceptible (S) based on the criteria listed in CLSI (2016). Thirty isolates were found to be resistant to the tested carbapenems meropenem and imipenem. These were tested for susceptibility to colistin in addition to polymyxin B, levofloxacin and amikacin. As demonstrated in Table (1), five isolates were found to be resistant to both carbapenems and colistin. Of the latter isolates, three were susceptible to levofloxacin, while two isolates were resistant to all antibiotics used in this study, and only one sample was harboring the mcr-1 gene. The identity of the mcr-1 harboring double resistant (Colistin/Carbapenem) bacterium was confirmed by 16S rRNA genetic analysis.

**PCR screening of selected genes associated with colistin resistance**

The five double-resistant isolates (Carbapenem/Colistin) showed variable patterns upon analysis using specific primers of mcr-1, pmrA and pmrB genes. One isolate showed the presence of pmrA, while three isolates showed the presence of pmrA and pmrB. Only one isolate (isolate 35) showed the presence of mcr-1(300bp), pmrA(170bp) and pmrB(400bp). Fig. 3. Identification of mcr-1 carrying isolate by 16S rRNA

The PCR product of 16S rRNA of the isolate which has showed mcr-1 was confirmed by sequencing. Using BLAST program of National Center for Biotechnology Information (NCBI) database the sequence showed 100% similarity to *Escherichia coli* strain K-12 substr. MG1655.

**Confirmation of mcr-1 gene expression**

A SYBR Green-based real-time PCR assay was designed for detection of the mcr-1 gene expression in the isolate. The experiment showed positive mcr-1 gene expression and show CT point after 36 cycles as illustrated at Fig. 4.

**DISCUSSION**

The intense overuse and misuse of antibiotics has led to emergence of multi-drug resistant strains which is difficult to treat. In this study, approximately a quarter of the tested isolates were found to be carbapenem resistant, while five isolates were resistant to both carbapenems and colistin. Only one of them was confirmed to harbor mcr-1 and was identified to be *Escherichia coli* strain k12 substr MG1655. The results obtained from the real-time PCR showed obvious presence of mcr-1 which was

Fig. 3. Using 1000 pb ladder, isolate no 35 shows the presence of mcr-1(300bp), pmrA(170bp) and pmrB(400bp). In figure 3(a), primer 1519/1520 was used to detect the presence of pmr-B gene. In figure 3(b), primers LT2PMRA/REV and CLRf/CLRr were used to detect the presence of pmrA and mcr-1, respectively.

Fig. 4. Real time PCR show mcr-1 gene expression, presented ct at 36 cycles.

also confirmed by conventional PCR, suggesting a probability that mcr genes may be currently prevalent in EGYPT. This study is the first to detect a multi-resistant Escherichia coli strain which is resistant to both carbapenem and colistin in Egypt. A previous work has reported an mcr-1 mediated Escherichia coli isolate detected in Egypt. This had phenotypic resistance to colistin, but was susceptible to carbapenems. Worthy to note, the SENTRY antimicrobial surveillance program carried out a worldwide survey in 2009 and reported that rates of resistance to polymyxins among Gram-negative pathogens ranged between 0.1% to 1.5%. However, a trend of rise of resistance followed worldwide in later years to reach 3.2%in 2016.

This study showed that the carbapenem-colistin double resistant Escherichia coli was sensitive to levofloxacin. Therefore, colistin might be combined with levofloxacin in treatment regimen, as it seems to produce a synergistic or additive effect. The use of colistin in combination with other antibiotics that are typically active against Gram-positive bacteria has been also explored by others and confirmed to be effective.
for highly drug-resistant Gram-negative pathogens expressing mcr-1. Most commonly rifampicin\textsuperscript{25} and carbapenems\textsuperscript{26} but also macrolides\textsuperscript{27}, minocycline\textsuperscript{28}, tigecycline\textsuperscript{29}, and glycopeptides\textsuperscript{30}. The coexistence of mcr-1 resistance in the carbapenem-resistant strains might be explained by the fact that mcr-1 has been observed on plasmids containing other antimicrobial resistance genes such as carbapenemases\textsuperscript{31,32} and extended-spectrum $\beta$-lactamases\textsuperscript{13,33}. This suggests a significant clinical concern that world is approaching a pan drug-resistant era for which the use of colistin and other antimicrobials might become ineffective.

**CONCLUSION**

The spread of carbapenem-resistant Enterobacteriaceae has become a serious problem worldwide that represents a marked threat to public health. To the best of our knowledge, this study constitutes the first to report in Egypt of mcr-1 positive Escherichia coli that is carbapenem-resistant and colistin-resistant. That gives an alarming sign for the dissemination of resistance in the area, which can considerably limits treatment options. Our finding from this study stresses how important to monitor the use of colistin in treatment of both human and animal infections and emphasizes the need for tightened infection control practices to restrict further dissemination of multi-resistant isolates.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

**DATA AVAILABILITY**

All datasets generated or analyzed during this study are included in the manuscript.

**ETHICS STATEMENT**

This article does not contain any studies with human participants or animals performed by any of the authors.

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