Prevalence of carbapenem resistance among multidrug-resistant Gram-negative uropathogens

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ABSTRACT

Multidrug-resistant (MDR) uropathogens have become a public health threat, especially in developing countries. Carbapenems are a class of antimicrobial agents often reserved for infections caused by MDR microorganisms. The aim of this study was to determine the prevalence and genotypic basis of plasmid-mediated carbapenem resistance among MDR uropathogens from one of the major clinical settings in Cairo, Egypt. A total of 150 bacterial isolates from patients suffering from urinary tract infections were collected from the Microbiology lab of El-Demerdash Hospital, Cairo, Egypt. All isolates were identified using standard methods. Antimicrobial susceptibility testing was carried out by Kirby Bauer's disk diffusion method following the CLSI guidelines. Plasmids were extracted from MDR uropathogens that also showed carbapenem resistance to be used as templates for PCR amplification. The resulting amplicons were subjected to DNA sequencing. The extracted plasmids were also transformed into Escherichia coli DH5α to compare the phenotypic resistance of the transformants with that of the clinical isolates from which the plasmids were extracted. Of the 150 collected isolates, 116 (77.3%) were Gram-negative, 51 of which (44%) were MDR. Carbapenem resistance was observed in 16/51 (31.4%) of the MDR isolates, 12 of which harbored plasmids. The blaOXA gene was detected in the plasmids of only 9 MDR carbapenem-resistant isolates.

From this study, it can be concluded that Gram-negative uropathogens show high rates of multidrug resistance. The prevalence of MDR uropathogens that are also carbapenem-resistant has increased greatly over the past few years, and this resistance can be easily acquired by horizontal transfer.

Keywords: urinary tract infection; carbapenem resistance; multidrug-resistance; MDR; acquired resistance; plasmid-mediated resistance.

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1. INTRODUCTION

Urinary tract infections (UTIs) are among the most common infections worldwide¹. Such infections are caused by Gram-negative or Gram-positive bacteria, as well as by some fungi. The most common uropathogen is *Escherichia (E.) coli*. Antimicrobial resistance (AMR) is growing at a distressing rate, perhaps even more rapidly in developing countries⁴. There has been a steady increase in AMR to the agents commonly used in the treatment of UTIs⁵ due to the misuse and abuse of antimicrobials⁶. The emergence and spread of multidrug-resistant (MDR) pathogens keep increasing over time, and UTI cases that
require intravenous treatment due to the lack of effective oral therapy have become a challenge for clinicians, complicating formerly simple-to-treat infections.

β-lactams are diverse antimicrobial agents used for the treatment of a wide range of infections. Carbenems such as imipenem, meropenem, ertapenem, and doripenem are the latest developed β-lactams possessing a broad spectrum of activity and are usually reserved for infections caused by MDR pathogens. Lately, the dissemination of community-acquired E. coli isolates capable of producing extended-spectrum β-lactamases (ESBLs) that can hydrolyze almost all β-lactams except for carbenems has been reported worldwide; consequently, the use of carbenems has increased greatly and the emergence of carbenem resistance has become a serious cause for concern. Carbenem resistance may arise from the acquisition of plasmid or chromosomal resistance genes encoding serine carbenemases or Metallo-β-lactamases and efflux pumps, or from modification of porin expression in association with an ESBL. The nature of the resistance determinants can affect the dynamics of its spread. Acquired class A (KPC), class B (VIM, IMP, NDM), or class D (OXA) carbenemases, are the most common determinants imparting carbenem resistance.

The aim of this study was to reveal the prevalence and molecular bases of acquired carbenem resistance in MDR bacteria causing UTIs from one of the major clinical settings in Cairo, Egypt.

2. MATERIALS AND METHODS

2.1. Specimen Collection

Starting October 2015 to May 2016, 150 bacterial isolates from patients suffering from UTIs were collected from the Microbiology lab of El-Demerdash Hospital, Cairo, Egypt. This study was approved by the ethics committee of the Faculty of Pharmacy, Ain Shams University (Nr. 212), and informed consent was obtained from patients after explaining the study purpose.

2.2. Identification of the Recovered Bacterial Isolates

Isolates were categorized based on their Gram reactions. Gram-negative isolates were selected for further studying. Culture characteristics on nutrient agar, MacConkey’s agar, cetrimide agar, and eosin methylene blue (EMB) agar were recorded. Biochemical tests including urease test, oxidase test, and citrate utilization test were performed. Identification was confirmed using API® 20E kits (BioMérieux, France) for isolates that proved to be MDR according to antimicrobial susceptibility tests that were performed later in this study.

2.3. Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion susceptibility test was performed on Gram-negative isolates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines using commercially available antimicrobial disks (Oxoid, UK). Isolates were considered MDR if they showed resistance to three or more classes of antimicrobial agents. Minimum inhibitory concentration (MIC) values of meropenem against MDR isolates were determined by the broth microdilution method according to CLSI guidelines. The reference strain E. coli ATCC® 25922 was used for quality control of the disk diffusion method and MIC determination.

2.4. Phenotypic Carbenemase Detection

Modified Hodge Test (MHT) was used to detect potential carbenemase production in multidrug-resistant Enterobacteriaceae (MDRE)
isolates that showed resistance to imipenem in disk diffusion test and/or meropenem in MIC broth microdilution following CLSI guidelines\textsuperscript{14}.

2.5. Plasmid Extraction from MDR Isolates

Zyppy\textsuperscript{TM} Plasmid Miniprep Kit (Zymo Research, USA) was used for the extraction of plasmid DNA from the MDR isolates according to the manufacturer's instructions. The extracted plasmids were analyzed using agarose gel electrophoresis\textsuperscript{17} and visualized via UV transilluminator.

2.6. Amplification of Some Plasmid-Encoded Carbapenem Resistance Genes

Amplification of some carbapenem resistance genes was carried out by PCR using the proper primers (Table 1); and the plasmid DNA of the MDR isolates that showed carbapenem resistance as templates. Primers were manufactured by LGC Biosearch Technologies, USA. The amplicons were analyzed by agarose gel electrophoresis, and the expected DNA product size was determined by comparing to a 100 bp DNA ladder (New England Biolabs, UK).

Table 1. Primers sequences, expected product sizes, and annealing temperatures ($T_a$) of the tested genes

| Gene    | Primer | Primer sequence ($5' \rightarrow 3'$) | Expected product size (bp) | $T_a$ (°C) | References |
|---------|--------|--------------------------------------|---------------------------|-----------|------------|
| $bla_{KPC}$ | $P_f$  | GTCACGTGTATCGCCGTC                   | 900                      | 51        | Doyle et al.\textsuperscript{25} |
|         | $P_r$  | CTCAGTGGCTCTACAGAAAACC                |                          |           |            |
| $bla_{IMP}$ | $P_f$  | CTACCGCAGCAGAGTCTTTTG                 | 587                      | 50        | Woodford et al.\textsuperscript{26} |
|         | $P_r$  | AACCAGTTTGGCTTTACCAT                 |                          |           |            |
| $bla_{VIM}$ | $P_f$  | TCTACATGACCAGGTCTGTC                 | 748                      | 50        | Poirel et al.\textsuperscript{27} |
|         | $P_r$  | TGTGCTTTGACAACGTTCGC               |                          |           |            |
| $bla_{NDM}$ | $P_f$  | GGTTTGGCGATCTGGTTTTC               | 621                      | 50        | Nordmann et al.\textsuperscript{28} |
|         | $P_r$  | CGGAATGGCTCATCAGAT                     |                          |           |            |
| $bla_{OXA}$ | $P_f$  | GCGTGGTAAAGGATGAACAC               | 438                      | 52        | Doyle et al.\textsuperscript{25} |
|         | $P_r$  | CATCAAGTCCAACCCACCG               |                          |           |            |

Notes: $bla_{KPC}$, $bla_{IMP}$, $bla_{VIM}$, $bla_{NDM}$, and $bla_{OXA}$ genes code for KPC, IMP, VIM, NDM, and OXA carbapenemases, respectively. $T_a$, calculated annealing temperature.

2.7. Sequencing of Selected PCR Products

GeneJET\textsuperscript{TM} purification kit was used for purification of PCR products at Sigma Scientific Services Company, Egypt. Selected PCR products of the amplified genes were sent for sequencing at GATC, Germany using ABI 3730xl DNA Sequencer. The alignment and assembly of the obtained forward and reverse sequences into the final consensus was done using BioEdit v7.2.5 software.

2.8. Transformation

The plasmids extracted from carbapenem-resistant MDR isolates were transformed into competent E. coli DH5$\alpha$ according to Sambrook and Russel\textsuperscript{17} to test the phenotypic properties of the transformants compared to those of the corresponding clinical isolates from which the plasmids were obtained. Transformants were cultured on LB/meropenem and LB/ampicillin agar plates at concentrations of 25 $\mu$g/mL and 100 $\mu$g/mL respectively. Transformants that showed growth on LB/meropenem plates were further subjected to plasmid DNA extraction, and the extracted plasmids from transformants were compared to those of the corresponding clinical isolates via agarose gel electrophoresis.
3. RESULTS

3.1. Identification of the Bacterial Isolates

150 bacterial isolates were collected; 116 (77.3%) of which were Gram-negative bacilli (GNB), while 34 (22.7%) were Gram-positive cocci (GPC). Of the 116 GNB isolates; 107 (92.2%) were identified as members of Enterobacteriaceae, 7 (6%) were Pseudomonas (P.) spp., and 2 (1.7%) were Acinetobacter (A.) spp.

3.2. Antimicrobial Susceptibility Testing

Out of the 116 GNB isolates; 51 (44%) isolates showed resistance to three or more classes of antimicrobial agents and were thus categorized as MDR isolates; including E. coli (24/51; 47.1%), Klebsiella (K.) pneumoniae (15/51; 29.4%), K. terrigena (4/51; 7.8%), Proteus mirabilis (5/51; 9.8%), A. baumannii (2/51; 3.9%), and P. aeruginosa (1/51; 2%). Carbapenem resistance was observed in 16/51 (31.4%) of the MDR isolates which were selected for further studying.

3.3. Phenotypic Carbapenemase Detection

Fourteen of the 16 carbapenem-resistant MDR isolates were members of Enterobacteriaceae, they were tested for potential carbapenemase production using Modified Hodge Test (MHT) following CLSI guidelines. The results of this test revealed that 10 (71.4%) out of the 14 tested MDRE showed enhanced growth of E. coli ATCC® 25922 around the test organism in the form of indentation of the inhibition zone, indicating potential carbapenemase production. Fig. 1 shows MHT of 3 of the tested isolates.

3.4. Plasmid Extraction from MDR GNB Isolates

Plasmids were successfully extracted from 12 (75%) of the 16 carbapenem-resistant MDR isolates. The extracted plasmids were analyzed using agarose gel electrophoresis, and the band sizes were compared to a 1 kb DNA ladder (New England Biolabs, UK).

Fig. 1. Modified Hodge Test (MHT) of 3 carbapenem-resistant MDR isolates. 1, E. coli (58E) showing positive test indicated by indentation of inhibition zone in the form of a clover leaf; 2, E. coli (55E) showing negative test; 3, K. pneumoniae (79K) showing positive test; MEM, meropenem (10 μg).

3.5. Amplification of Some Plasmid-Encoded Carbapenem Resistance Genes

Amplification of some carbapenem resistance genes was carried out by PCR using the proper primers, and the plasmids of the 12 carbapenem-resistant MDR isolates as PCR templates. Out of the 12 isolates, 9 (75%) carried the blaoXA gene. The rest of the tested genes were not detected in any of the tested carbapenem-resistant MDR isolates. The results of agarose gel electrophoresis are shown in Fig. 2.

Fig. 2. Agarose gel electrophoresis of PCR products of blaoXA gene (438 bp) from carbapenem-resistant MDR isolates. Lanes: 1, K. pneumoniae (3K); 2, K. pneumoniae (14K); 3, K. terrigena (39K); 4, E. coli (55E); 5, E. coli (58E); 6, K. pneumoniae (79K); 7, K. pneumoniae (89K); 8, K. pneumoniae (92K); 9, E. coli (99E); 10, K. terrigena (105K); 11, K. pneumoniae (124K); 12, K. pneumoniae (132K); and M, 100bp size marker.
3.6. Transformation

The plasmids of the 9 carbapenem-resistant MDR isolates harboring \textit{blaOXA} gene were transformed into competent \textit{E. coli} DH5α prepared according to the modified Hanahan method\textsuperscript{18} to test the phenotypic resistance of the transformants. Transformants were cultured on plates containing LB/meropenem and LB/ampicillin at concentrations of 25 µg/mL and 100 µg/ml, respectively. Two transformants (coded: trans-92 and trans-132, respectively) showed growth on both media, indicating successful transformation. One of the transformants (trans-92) failed to grow upon further subculturing on LB/meropenem. The other transformant (trans-132) was successfully subcultured three successive times on LB/meropenem. The plasmid DNA was extracted from this transformant (trans-132) and compared to that of the corresponding carbapenem-resistant MDR isolates (132K), from which the plasmids were originally obtained, by agarose gel electrophoresis. Results showed that plasmids from the transformant and the corresponding isolate had identical band sizes in the gel when visualized against a 1 kb DNA ladder (Fig. 3).

4. DISCUSSION

Multidrug-resistant \textit{Enterobacteriaceae} (MDRE), particularly \textit{E. coli}, and other GNB that produce ESBLs have become a widespread cause of UTIs both in the community and the hospital setting. Carbapenems are now considered to be the drugs of choice for the treatment of severe infections caused by ESBL-producing \textit{Enterobacteriaceae}\textsuperscript{10}. Unfortunately, the increased reliance on such antimicrobial agents has led to the emergence and spread of carbapenem resistance, especially among \textit{Enterobacteriaceae}\textsuperscript{19}. Although this used to be a problem encountered only in \textit{P. aeruginosa} and \textit{A. baumannii}, lately, carbapenem resistance has escalated in other species including \textit{K. pneumoniae} and \textit{E. coli}\textsuperscript{20}. The rising trend in \textit{E. coli} is of particular concern, as this may result in untreatable community-acquired infections\textsuperscript{21}.

In this study, out of the 116 collected GNB isolates, 51 (44%) were resistant to three or more classes of antimicrobial agents; and were thereby deemed MDR isolates. Carbapenem resistance was observed in 16/51 (31.4%) of the MDR isolates. Contrary to our results, a study conducted in 2014 by Eshetie \textit{et al.} showed much higher prevalence rates of MDR GNB uropathogens (87.4%), but only 2.73% of them were carbapenem-resistant\textsuperscript{22}. This indicates that carbapenem resistance in the current study is relatively high.

Phenotypic detection of carbapenemase production was performed using MHT, which revealed that 10/14 (71.4%) tested MDRE were potential carbapenemase producers. Genotypic
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Detection of some carbapenem resistance genes was used to confirm the results of phenotypic detection. The current study was focused on plasmid-mediated carbapenem resistance, as this type is an acquired resistance that can easily be transferred horizontally among various bacterial species. The 16 carbapenem-resistant MDR isolates were subjected to plasmid extraction. Plasmids were successfully extracted from 12 isolates, the remaining 4 isolates appeared to have been intrinsically carbapenem-resistant as they lacked plasmids. These extracted plasmids were used as templates for PCR amplification of some plasmid-encoded carbapenem resistance genes mentioned in Table 1. Only 9/12 (75%) carried the blaOXA gene. The rest of the tested genes were not detected in any of the tested carbapenem-resistant MDR isolates.

In order to confirm that the blaOXA gene conferred carbapenem resistance, plasmids of the 9 carbapenem-resistant MDR isolates harboring the gene were transformed into competent E. coli DH5α, the resulting transformants were cultured on plates containing LB/meropenem and LB/ampicillin at previously mentioned concentrations. Two transformants (coded: trans-92 and trans-132, respectively) showed growth on both media, indicating the success of the transformation process, and that indeed the plasmid-encoded blaOXA gene is capable of conferring resistance not only to carbapenems but also to penicillins. A study by Poirel et al. revealed that the OXA-48 β-lactamase had a narrow-spectrum hydrolysis profile that included penicillins and imipenem, which backs up our results.

For even further confirmation that the acquired resistance of transformants was due to the plasmid-encoded gene and not due to random mutation which might have occurred during subculturing, the plasmid DNA was extracted from the transformant (trans-132) and was compared to that of the corresponding carbapenem-resistant MDR isolate (132K), from which the plasmid was originally obtained, by agarose gel electrophoresis. Results showed that plasmids from the transformant (trans-132) and the corresponding isolate (132K) had identical band sizes in the gel when visualized against a 1 kb DNA ladder (Fig. 3). This confirms that the resistance gene that was harbored on the plasmid was the reason for the newfound resistance to carbapenems and penicillins of the transformant, thereby confirming that horizontal transfer of carbapenem resistance is a serious threat. In accordance to our findings, a study performed by Götting et al. verified the in vivo intergenus gene transfer of OXA-48 in the gut of an infected patient, which showed even higher transmission frequencies when compared to in vitro conditions.

Based on the obtained results, future research directions include studying various antibiotic combinations to evaluate their efficacy on carbapenem-resistant MDR uropathogens. Furthermore, alternative approaches for treating UTIs should be considered; such as studying the effect of bacteriophages or probiotics.

5. CONCLUSION

High rates of multidrug-resistance were observed among Gram-negative uropathogens. The prevalence of MDR uropathogens that are also carbapenem-resistant has increased greatly over the past few years. Carbapenem resistance can be easily transferred horizontally among various bacterial species. The use of carbapenems as the treatment of choice for infections caused by MDR pathogens might still be effective but has become questionable. It has become clear that new clinical guidelines should be implemented in Egypt to avoid the misuse of antimicrobial agents.
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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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