ORIGINAL ARTICLE

Evaluation of Real Time PCR as a Diagnostic Method for Early Detection *of Klebsiella pneumoniae* Carbapenemase-producing Enterobacteriaceae Infections from Positive Blood Culture

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ABSTRACT

Background*: Rapid identification of infection caused by Klebsiella pneumoniae carbapenemase producing bacteria, is critical for the beginning of appropriate antimicrobial treatment and ending their spread.* ***Objectives****: to study and evaluate RT-PCR technology as a rapid method to directly detect KPC-producing Enterobacteriaceae in positive blood culture bottles.* ***Methodology was****: cross sectional study, conducted at Benha University Hospital. 253 blood culture bottles were incubated in the BD BACTEC™ 9050 Blood Culture System for up to 5 days, and Gram staining was done when the bottles were identified as being positive by the Bactec system. All Gram-negative pathogens were subcultured and identified Antimicrobial susceptibilities to meropenem and imepinem were tested by disc diffusion and interpreted according to The Clinical and Laboratory Standards Institute guidelines 2014. Klebsiella pneumoniae carbapenemase producing enterobacteriaceae in positive blood culture bottles was identified by Real time PCR.* ***Results****: out of 253 specimens,235 were positive, 186 (79.1%) were Enterobacteriaceae, 57 isolates (30.6%) of all Enterobacteriaceae isolates were resistant by disc diffusion test, and real-time Polymerase chain reaction detected 55 isolates (29.6%) as positive for the presence of Klebsiella pneumoniae carbapenemase gene. The sensitivity of PCR was 96.5%, specificity was 100%, PPV=100%. NPV= 98.5%, and diagnostic accuracy was 98.9%, in comparison with disc diffusion as a gold standard test.* ***Conclusion****: real time Polymerase chain reaction is a useful, rapid, sensitive, and specific tool to detect Klebsiella pneumoniae carbapenemase directly in positive blood culture bottle.*

INTRODUCTION

Bacterial resistance to antibiotics has become a most important community health problem. Both Community-acquired and hospital-acquired infections caused by Enterobacteriaceae are among the most common human infections.[1]

Most reported Carbapenem-resistant Enterobacteriaceae in hospital-associated infections are related to more than 50% mortality, which poses a great challenge in patient care units. [2]

In 1993 (NmcA) was identified as the first carbapenemase producer in Enterobacteriaceae. After that, a large variety carbapenemase producer in Enterobacteriaceae belonging to Ambler class A, B, and D β-lactamases have been identified. Also a chromosome encoded cephalosporinases that belong to Ambler class C produced by Enterobacteriaceae may have slight extended activity toward carbapenems. [3]

Klebsiella pneumoniae carbapenemases (KPCs) are the most clinically common enzymes in the molecular class A carbapenemases. They are serine carbapenemases belongs to Bush's subgroup 2f, effectively hydrolyze carbapenems and can be inhibited better by tazobactam than by clavulanic acid. KPCs are plasmid encoded, harbored on a Tn3-like transposon, called Tn4401, a highly mobile genetic element.[4]

KPCs causes resistance to all beta lactam agents such as cephalosporins, penicillins, monobactams, and carbapenems. In addition, KPC-producing strains often exhibit multidrug resistance phenotypes, leaving few therapeutic options for treatment. This characteristic, along with taking a high possibility for propagation due to its plasmid position, has been a goal for concern in hospitals and healthcare institutions in all over the world. [5]

KPCs are predominantly detected in Enterobacteriaceae (mostly in *K. Pneumonie*, as well with other species such as *Escherichia coli*, Enterobacter spp. *Proteus mirabilis, Klebsiella oxytoca, Citrobacter freundii*, Acinetobacter spp. and *Pseudomonas aeruginosa*. [6,7]

Infections due to these microorganisms are associated with worse outcomes, more time of hospital

stay, and increase rate of morbidity and mortality. Mortality rates, up to 66%, were associated with bacteremia due to blaKPC-positive bacteria.[8]

The quick identification of these enzymes, the stoppage of extent of carbapenemase-producing bacteria and the improvement of novel drugs unaffected by carbapenemase hydrolysis were recommended .[9] Rapid identification of KPC positive bacteria is critical for the beginning of the appropriate antimicrobial treatment and ending their spread. [10]

A number of non-molecular-based tests have been accessible for discovery and detection of carbapenemases, on the other hand none of them have 100% specificity or sensitivity. So, the use of routine susceptibility tests to identify carbapenem resistance would be followed by phenotypic and genotypic confirmation. [9]

Numerous molecular methods such as simplex and multiplex PCRs, DNA hybridization and sequencing are considered as the reference for identification of carbapenemase genes.[11,12] Numerous polymerase chain reaction (PCR)-based assays have been established to detect KPC mediated carbapenem resistance. Real-time PCR (RT-PCR) has been used as a rapid methods in detection of infection with KPC-producing Enterobacteriaceae in several types of samples and clinical isolates. [11]

The aim of this work was to study and assess RT-PCR technology as a rapid method to directly detect KPC-producing Enterobacteriaceae in positive blood culture bottles.

METHODOLOGY

***Study design*: Cross sectional study**

***Study setting*:** The study was conducted at Benha University Hospital, during the period from June 2016 to September 2016, the practical part of the study was done at Microbiology and Immunology Department, Benha Faculty of Medicine. The design of the research was approve by the ethical committee, Faulty of Medicine, Benha University.

Two hundred fifty -three blood culture bottles were incubated during this period

Blood Culture Samples:

The blood culture bottles (BACTEC™ Plus Aerobic/F culture vials BD, Franklin Lakes, NJ) were incubated in the BD BACTEC™ 9050 Blood Culture System (Becton Dickinson, Sparks,MD) for up to 5 days, and Gram staining were done for the bottles flagged as being positive by the Bactec system

Identification of Bacteria and Antimicrobial Susceptibility Testing

All Gram-negative pathogens were subcultured onto MacConkey agar plates (Oxide, UK) and incubated for 18 to 24 hours. Isolated colonies were identified by API 20E (BioMérieux,France). Meropenem (10 ug) and imepinem (10ug) (Oxoid, UK) antimicrobial susceptibility testing of the identified Enterobacteriaceae was performed using Kirby–Bauer disc diffusion method and interpreted according to CLSI guidelines 2014.[13]

Real time PCR for detection of KPC gene:

Bacterial DNA was extracted from positive blood culture bottles that were identified as having gram-negative bacilli using the DNA extraction kit (QIAamp DNA Blood Mini Kit, Qiagen Corporation, Chatsworth, Calif. Germany) according to the manufacturer's instructions, the purified DNA was put in storage at -20°C until used in amplification step.

The specific primers used were as follows: the forward primer sequence (5' TTGTTGATTGGCTAAAGGG-3') and reverse primer sequence (5'- CCATACACTCCGCAGGTT-3') were designed in the conservative region of several blaKPC types (blaKPC −2 to blaKPC-13) (Biosearch technologies, USA).[14]

The real time PCR was done in a total volume of 20 µl consisting of 10 µl Super Real Pre Mix Plus SYBR Green, master mix (TIANGEN Biotech, Beijing), 2 µl of Rox dye, 0.6 µl of each primer, 2 µl of template DNA and water (nuclease-free) was added to achieve a reaction volume of 20 µl. The real time PCR instrument ABI7900HT (Applied Biosystems, Foster City, CA, USA) was used with the following instrument settings: 95°C for 10 min (initial denaturation) followed by 40 amplification cycles (95°C for 15 Sec. and 60°C for 30 sec.) for denaturation and annealing / elongation respectively. Negative control that contains 2 µL nuclease free water instead of sample DNA was included in the run. Amplification specificity was checked by melting-curve analysis.

Melting curve acquisitions were done directly after the final amplification step by heating at 96°C for 5 Sec, cooling to 55°C for 1 min, and heating slowly at 0.11°C per second to 96°C with continuous fluorescence recording.

Melting curves were recorded by plotting the fluorescence signal intensity versus temperature. Amplicon melting temperatures(Tm) were determined according to the RQ manager program 1.2 ABI SDS software (ABI 7900HT).The results were visualized by plotting the negative derivative against temperature.

Data analysis:

The data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 20. Qualitative data were expressed in numbers and percent. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the RT-PCR assay were calculated using culture based susceptibility testing for comparison.

RESULTS

Of the studied 253 specimens, 235 were positive, 186 (79.1%) were positive for Enterobacteriaceae and 49 (20.9%) were non-Enterobacteriaceae. Of the isolated Enterobacteriaceae, 57 (30.6%) were resistant by the disc diffusion test and 129 (69.4%) were sensitive. The results of susceptibility to imipenem and meropenem agreed for all isolates. By real-time PCR, 55 isolates (29.6%) of all Enterobactericea isolates were positive for the presence of KPC gene and 131 (70.4%) were negative (table 1)

Table 1: Results of Carbapenem susceptibility of clinical isolates by disc diffusion methods and real time PCR

Organism	Total isolates	Disk diffusion				RT-PCR KPC	
		Imipenem		Meropenem			
		S	R	S	R	N	P
Escherichia coli	67(36%)	51	16	51	16	52	15
Klebsiella pneumoniae	64 (34.4%)	35	29	35	29	35	29
Enterobacter spp	39 (21.1%)	28	11	28	11	29	10
Proteus mirabilis	7(3.7%)	7	0	7	0	7	0
Citrobacter spp	8 (4.3%)	7	1	7	1	7	1
Serratia marcescens	1 (0.5%)	1	0	1	0	1	0
Total	186 (100%)	129 (69.4%)	57 (30.6%)	129 (69.6%)	57 (30.6%)	131 (70.4%)	55 (29.6%)

S: sensitive, R: resistant N: negetive, P: positive

The resistant rate between the samples that were confirmed positive for carbapenem resistance by both disc diffusion and RT-PCR, was as follows: 29 (45.3%) of 64 for *K pneumoniae*, 10 (25.6%) of 39 for *Enterobacter spp.*, 15 (22.4%) of 67 for *E. coli* and one (12.5%) of 8 for *Citrobacter spp.*.

By Real time PCR 55 (96.5%) of carbapenem resistant isolates by the disc diffusion test were positive, thus the PCR sensitivity was 96.5%, specificity was 100%, PPV=100%. NPV= 98.5%, and diagnostic accuracy was 98.9%, in comparison with disc diffusion test as the gold standard test (Table 2).

Table 2: Comparison of imipenem and meropenem disk diffusion susceptibility testing and KPC RT-PCR results

disk diffusion susceptibility testing (imipenem, meropenem)	KPC RT-PCR		Total
	Positive	Negative	
Resistant	55	2	57
Susceptible	0	129	129
Total	55	131	186
Sensitivity = 96.5% Specificity = 100% PPV=100% NPV= 98.5% Diagnostic accuracy=98.9%			

PPV: positive predictive value, NPV: positive predictive value

DISCUSSION

Rapid and early recognition of carbapenemases is crucial for prevention of spreading, detect outbreak and for an effective antibiotic policy to improve the consequence in infected patients.[15] Recognition of carbapenemases in Enterobacteriaceae is challenging particularly in the existence of carbapenemase producing Klebsiella pneumoniae strains with low carbapenem MICs.[16]

As the rapid detection of bacterial resistance mechanisms assists the physician in both patient management and infection control; several studies were done to evaluate the usefulness of molecular methods to detect KPC-producing carbapenem-resistant bacteria directly in various clinical samples.[17,18]

When positive blood cultures are tested directly for the presence of carbapenem resistance, this can diminish the time from more than 48 hours to few hours.[15,19]

This study compared between RT-PCR for direct detection of KPC-producing Enterobacteriaceae in positive blood culture bottles and the routinely used carbapenem (meropenem& imipenem) disk diffusion method following Clinical Laboratory Standards Institute recommendations (CLSI) guidelines 2014.

Our results reported that 55 (96.5%) of carbapenem resistant isolates by using the disk diffusion method, were KPC positive by RT-PCR. The sensitivity of PCR was 96.5%, specificity was 100%, PPV of 100%, NPP of 98.5% and diagnostic accuracy was 98.9%, all in relation to disk diffusion test as the gold standard test. These results are consistent with those of a similar study that compared between KPC detection by RT-PCR assay and carbapenem susceptibility by an automated method, the sensitivity, specificity, positive and negative predictive values of the RT-PCR assay were 92.9%, 99.3%, 92.9%, and 99.3%, respectively.[19] In another study that also aimed at direct PCR based detection of KPC in positive blood culture bottles, the sensitivity, specificity, positive predictive value and negative predictive value of the PCR assay compared to the culture based results were all 100%.[20]

In the present study, PCR detected 2 cases negative for KPC by PCR but were resistant by disk diffusion. The other mechanism for carbapenem resistance may be outer membrane permeability variations, increased activity of antibiotic efflux systems, or non-KPC carbapenemases.[21]

Carbapenemases of the KPC family have the most extensive global distribution of all carbapenemases associated with Enterobacteriaceae. KPC is the most prevalent Ambler class A carbapenemase worldwide and has the biggest clinical significance. [22] In our study, 55 out of 186 (29.6%) had KPC gene by real time PCR. In the eastern United States, the prevalence rates of KPC was more than 30% of isolates have been recorded.[23] A study done in Brooklyn hospitals stated 38% prevalence of blaKPC by real time PCR.[24] A laboratory surveillance program found a high frequency (89.3%) of KPC-type enzymes among carbapenemase producers between 2010 and 2012. [25] Also A study was done in Egypt reported that 91 out of 150 (60.6%) had KPC gene by real- time PCR.[26]

In the current study the occurrence of KPC carbapenem resistance was 45.3% in *K. pneumoniae*, 25.6 % in Enterobacter spp, 22.4% in E coli and 12.5% in Citrobacter spp, these findings are in agreement with Al Hindi et al.[26] who found that 46.1% of *K. pneumoniae* isolates contain the KPC gene. These results also are in hand with those reported by Al Tamimi et al. [10] who found that *Klebsiella pneumoniae* was the most common bacterial isolates that had KPC gene (34.8%), Enterobacter species (17.4%), Escherichia coli (17.4%) and Acinetobacter baumannii was the least common species (8.7%). In a New York-based multicenter survey, the prevalence of *bla* KPC within *K. pneumoniae* isolates peaked at 36% in 2006. Of great interest, a notable decline has since been recorded to 25% in 2009 and 13% in 2013–2014.[27] These results also are in accordance with those of [24,28] where 95% and 100% of clinical isolates of *K. pneumoniae* were confirmed as KPC producers by PCR.

However, Marschall et al.[29] and Francis et al.[19] reported that the most common KPC positive enterobacteriaceae was *Escherichia coli* (44.9% and 37% respectively). This can be clarified by presence of highly effective Infection control programs, strict and efficient antibiotic policies, lesser hospital stay days, and other significant health care measures that reduce the probabilities of acquiring and spread of KPC gene.

Of the total 186 enterobacteriaceae isolated from blood culture bottles, 57 (30.6%) were resistant to carbapenems by disk diffusion method. This is similar to a study conducted in Saudi Arabia to compare between phenotypic and PCR methods for detection of carbapenemase resistant Enterobacteriaceae which reported that out of the 60 isolates 26 (43%) were positive for carbapenemase production.[10]. A similar resistance rate was detected by Fattouh et al.[30] who reported that 27.4% of isolates were resistant to carbapenem in Sohag University Hospital. Our results are in agreement with studies from Europe and Israel [31,32] which reported that the carbapenem-resistance by disc diffusion was 26% and 25.1% respectively.

Although resistance rate stated in Menoufia University hospitals, Egypt was 55.3% to one or more carbapenems by disk diffusion method[26]. Also, a study conducted in New York, reported that 61.5% of lactose fermenting gram-negative bacilli were imipenem resistant by disc diffusion.[33] This variability in resistance rates can be attributed to the difference in geographical areas, antibiotic cut offs, guidelines and different techniques being used for detection of CRE and infection control measure. So early detection of resistance genes limiting the dispersal of these organisms and achieved good control for its spreading.[34]

CONCLUSION

The data from this study support the use of real time PCR as a useful, rapid, sensitive, and specific tool to detect KPC directly from positive blood culture bottle which will reduce the chance of spreading the resistant organism in the hospital which is an important first step in controlling its spread.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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