Rapid Detection of *Klebsiella pneumoniae* Carbapenemase Genes in Enterobacteriaceae Directly From Blood Culture Bottles by Real-Time PCR

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**Key Words:** *Klebsiella pneumoniae;* Enterobacteriaceae; Carbapenemase; Bloodstream infection; Real-time polymerase chain reaction

**Abstract**

*Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae are endemic in New York City hospitals and have been associated with serious infections globally. A real-time polymerase chain reaction (RT-PCR) assay was developed to detect carbapenem resistance attributable to KPC from blood culture bottles positive for gram-negative bacilli. Culture confirmation of carbapenemase production included automated imipenem and meropenem susceptibility testing and ertapenem susceptibility testing by disk-diffusion. A total of 323 Enterobacteriaceae isolates were tested, of which 8.7% (n = 28) demonstrated carbapenem-resistance by automated and manual susceptibility testing methods or by RT-PCR. The sensitivity, specificity, and positive and negative predictive values of the RT-PCR assay when compared with the automated method were 92.9%, 99.3%, 92.9%, and 99.3%, respectively, and 96.4%, 99.7%, 96.4%, and 99.7%, respectively, when compared with the ertapenem disk-diffusion method. RT-PCR is a rapid and reliable means of detecting carbapenem resistance due to KPC-plasmids in Enterobacteriaceae directly from blood culture bottles.

Resistance to carbapenem among members of the Enterobacteriaceae family has become a major health care concern worldwide.1 While several mechanisms of carbapenem resistance have been reported among this group of pathogens, resistance due to *Klebsiella pneumoniae* carbapenemase (KPC) enzymes has spread since the initial report in 2001,2 causing hospital outbreaks in the northeastern United States and dissemination to at least 4 continents.3-5 The genes for the 10 known KPC variants (KPC 2-11) are carried on large plasmids2,6-15 within a transposon.16 Although KPCs are most often found in *K pneumoniae*, they have also been detected in *Klebsiella oxytoca*, *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus mirabilis*.17-22 These gram-negative pathogens are often highly resistant to multiple classes of antibiotics, including cephalosporins, fluoroquinolones, and aminoglycosides.23,24 In addition, outbreaks of infection with KPC-harboring pathogens have been associated with high mortality rates.15,23

The detection of KPC-producing bacteria can be challenging because of heterogeneous expression of β-lactam resistance. Automated and agar diffusion methods of susceptibility testing show some inconsistencies in reliably detecting KPC-mediated resistance, and this is influenced by the carbapenem that is used for testing.23 To address these issues, confirmatory tests such as the modified Hodge test25 have been used. In addition, several polymerase chain reaction (PCR)-based assays have been developed to detect KPC-mediated carbapenem resistance. Real-time PCR (RT-PCR) has been employed in the rapid detection of colonization/infection with KPC-producing Enterobacteriaceae in various types of samples and clinical isolates.26-28 These assays have demonstrated good sensitivity and specificity with favorable positive and negative predictive values.
An increasing number of antibiotic-resistant gram-negative bacilli are causing serious bloodstream infections. A recent study reported that 11% of K pneumoniae isolates that were associated with bloodstream infections due to central-line infections demonstrated carbapenem resistance. Successful treatment of bacteremia depends on prompt administration of appropriate antimicrobial agents. Rapid detection of bacterial targets directly from blood culture bottles using RT-PCR has been previously demonstrated. Using RT-PCR technology to detect KPC-producing gram-negative bacilli in blood culture bottles is expected to aid in rapid detection of carbapenem resistance. We describe a sensitive and specific RT-PCR assay for the rapid detection of KPC-mediated carbapenem resistance directly from positive blood culture bottles.

**Materials and Methods**

**Blood Culture Samples**

The study period was from January 2009 to February 2010. Aerobic and anaerobic blood culture bottles (Bactec Plus and Lytic, BD, Franklin Lakes, NJ) were incubated in the BACTEC Fx system (BD Diagnostic Systems, Sparks, MD) for up to 5 days, and Gram stains were performed when bottles were flagged as positive by the system. Bottles were subcultured onto 5% sheep blood, chocolate, and MacConkey agar plates (BBL, Sparks, MD) and incubated in 5% carbon dioxide for 18 to 24 hours.

**Bacterial Identification and Antimicrobial Susceptibility Testing**

Isolated colonies were identified using the automated Vitek 2 (bioMérieux, Durham, NC). Imipenem and meropenem antimicrobial susceptibility results were determined by using the Vitek 2 AST card GN35. Ertapenem susceptibilities were determined by the Kirby-Bauer disk-diffusion method. The Clinical and Laboratory Standards Institute standard M100-S19 was used for interpretative criteria.

**DNA Extraction**

DNA was extracted from 393 positive blood culture bottles that were identified as having gram-negative bacilli. The BioRobot EZ1 automated system (Qiagen, Valencia, CA) was used for extracting DNA from 100-μL aliquots of positive blood culture media. Extracted bacterial DNA was stored at –20°C.

**KPC Real-Time PCR Assay**

RT-PCR primers and fluorescent probes were designed in house to amplify a 107-base-pair DNA fragment from positions 576 to 683 of the purported β-lactamase KPC (blaKPC) coding region (GenBank AF395881). As shown in Figure 1, the forward and reverse oligonucleotide amplification

![Figure 1](https://via.placeholder.com/150)

**KPC-2**

**KPC-3**

**KPC-4**

**KPC-5**

**KPC-6**

**KPC-7**

**KPC-8**

**KPC-9**

**KPC-10**

**KPC-11**

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**Figure 1** Klebsiella pneumoniae carbapenemase (KPC) real-time polymerase chain reaction (RT-PCR) assay to detect all known KPC variants (KPC 2-11). Colored boxes denote nucleotide (NT) positions (nucleotides 149, 308, 716, 814) at which single nucleotide changes have been detected in KPC variants. Dashed lines correspond to sequence regions that are preserved in all known variants. Arrows indicate the locations of the forward primer (nucleotide 576), reverse primer (nucleotide 683), and probe used for product amplification and detection. Primers and probes all correspond to a region of the blaKPC plasmid that is common to all known variants. A, adenine; C, cytosine; G, guanine; T, thymine.
primers and detection probe used in our assay were positioned such that all currently known KPC genes (KPC 2-11) would be detected. The forward primer (5'-CCTTCATGC-GCTCTATCG-3'), reverse primer (5'-TTTGTAAAGCTTTC-GCTCACG-3'), and the 6-carboxyfluorescein (FAM)-labeled blaKPC-specific probe (5'-CGCCATCCCAGGCGATGCG-GCG) sequences were found to be specific for blaKPC genes when the sequences were queried using the National Center for Biotechnology Information BLAST program. The pBK-CMV plasmid (Stratagene, La Jolla, CA) was used as an internal control with each sample. The forward primer (5'-CTTGGCTCACTGCGTAGGAGAGGCTATTCGGCTA-3'), reverse primer (5'-AGTAGGCGTAGGCATCACAGTTCATTACGGCAC-3'), and the CAL Fluor Red 590-labeled probe (5'-CGAGCGTGCTCTGATGCCGCC-GTGTTCCCCGCTG-3') (Biosearch Technologies, Novato, CA) detected the neomycin resistance gene in the plasmid. The SmartCycler (Cepheid, Sunnyvale, CA) was used for amplification and detection of the blaKPC gene. Each PCR run included a negative control and an external positive control from a known KPC-positive sample (verified by DNA sequencing). The amplification conditions were 95°C for 120 seconds followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 10 seconds.

**Data Analysis**

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 393 specimens analyzed, 323 isolates (82.2%) were Enterobacteriaceae and 70 (17.8%) were non-Enterobacteriaceae pathogens. Table I shows that the most common Enterobacteriaceae identified was *E. coli* (44.9%), followed by *K. pneumoniae* (25.4%), and *Enterobacter* spp (11.5%). Table 1 also summarizes the results of the automated (imipenem and meropenem) and disk-diffusion (ertapenem) susceptibility testing and the RT-PCR. The imipenem and meropenem susceptibility results agreed for all samples. Of the isolates, 8.7% were carbapenem-resistant by imipenem/meropenem testing (28/321), ertapenem testing (28/323), and RT-PCR (28/323). Meropenem/imipenem testing was not performed on the 2 *Salmonella* spp isolates, leaving a total of 321 samples that were tested by all study methods. There was agreement among the 3 testing methods for 98.8% (317/321) of the isolates. Among the samples that were confirmed positive for carbapenem resistance by all 3 methods, the rate of resistance for each organism was as follows: 23 (29%) of 80 for *K. pneumoniae*, 4 (2.8%) of 145 for *E. coli*, and 1 (3%) of 36 for *Enterobacter* spp.

One *E. cloacae* isolate was susceptible by imipenem/meropenem testing and resistant by ertapenem testing. This isolate was KPC-positive by RT-PCR, in agreement with ertapenem testing results. Two samples with discordant results were identified among the *K. pneumoniae* isolates. One isolate was resistant by imipenem/meropenem testing, susceptible by ertapenem, and negative by RT-PCR. The second isolate was resistant by imipenem/meropenem and ertapenem and negative by RT-PCR. The DNA internal control included

### Table 1

| Organism                  | Total Isolates | Vitek Imipenem |  | Vitek Meropenem |  | Vitek Ertapenem |  | KPC RT-PCR |  |
|---------------------------|----------------|----------------|---------------------------------|----------------|----------------|---------------------------------|----------------|----------------|---------------------------------
|                           |                | Susceptible    | Resistant                        | Susceptible    | Resistant                        | Susceptible    | Resistant                        | Negative | Positive |                      |
| *Citrobacter* spp         | 4              | 4              | 0                                | 4              | 0                                | 4              | 0                                | 4         | 0         |                      |
| *Enterobacter gergoviae*  | 1              | 1              | 0                                | 1              | 0                                | 1              | 0                                | 1         | 0         |                      |
| *Enterobacter aerogenes*  | 8              | 8              | 0                                | 8              | 0                                | 8              | 0                                | 8         | 0         |                      |
| *Enterobacter asburiae*   | 3              | 3              | 0                                | 3              | 0                                | 3              | 0                                | 3         | 0         |                      |
| *Escherichia coli*        | 145            | 142            | 3                                | 142            | 3                                | 142            | 3                                | 141       | 4         |                      |
| *Klebsiella oxytoca*      | 7              | 7              | 0                                | 7              | 0                                | 7              | 0                                | 7         | 0         |                      |
| *Klebsiella pneumoniae*   | 82             | 57             | 25                               | 57             | 25                               | 58             | 24                               | 59        | 23        |                      |
| *Morganella morgani*      | 5              | 5              | 0                                | 5              | 0                                | 5              | 0                                | 5         | 0         |                      |
| *Pantoea agglomerans*     | 6              | 6              | 0                                | 6              | 0                                | 6              | 0                                | 6         | 0         |                      |
| *Proteus mirabilis*       | 16             | 16             | 0                                | 16             | 0                                | 16             | 0                                | 16        | 0         |                      |
| *Serratia marcescens*     | 19             | 19             | 0                                | 19             | 0                                | 19             | 0                                | 19        | 0         |                      |
| *Salmonella* spp          | 2              | ND             | ND                               | ND             | ND                               | ND             | ND                               | 2         | 0         |                      |
| Total                     | 323            | 293            | 28                               | 293            | 28                               | 296            | 28                               | 295       | 28        |                      |

KPC, *Klebsiella pneumoniae* carbapenemase; ND, not done; RT-PCR, real-time polymerase chain reaction.

* Testing for resistance to carbapenem medications was performed by automated testing on the Vitek 2 using imipenem and meropenem and disk-diffusion method with ertapenem. Imipenem and meropenem susceptibility were not evaluated for the *Salmonella* spp isolates. RT-PCR was for detection of the blaKPC plasmid.
in the RT-PCR reactions amplified as expected for both of these samples.

One *E. coli* isolate was positive by RT-PCR, while culture-based testing (imipenem/meropenem and ertapenem) demonstrated carbapenem susceptibility. Subsequent cultures obtained from this patient during the next month were carbapenem-resistant by RT-PCR and culture. A carbapenem-resistant *E. cloacae* strain was also subsequently isolated from this patient.

**Table 2** and **Table 3** summarize the results of the RT-PCR assay compared with the imipenem/meropenem and ertapenem susceptibility testing, respectively. The sensitivity, specificity, PPV, and NPV of the RT-PCR when compared with automated susceptibility testing with imipenem/meropenem were 92.9%, 99.3%, 92.9%, and 99.3%, respectively. The sensitivity, specificity, PPV, and NPV of RT-PCR when compared with ertapenem disk-diffusion susceptibility testing were 96.4%, 99.7%, 96.4%, and 99.7%, respectively.

**Discussion**

The rapid detection of KPC-producing Enterobacteriaceae is of great importance as some studies have indicated that mortality may be higher in patients who are infected with KPC-mediated carbapenem-resistant strains in comparison with carbapenem-susceptible strains. Therefore, rapid detection of carbapenem resistance may lead to more timely treatment with appropriate antimicrobials and better outcomes for patients.

KPC-mediated carbapenem resistance was detected in *K. pneumoniae*, *E. coli*, and *E. cloacae* in our study. The frequency of carbapenem resistance in 29% of *K. pneumoniae* isolates is similar to what has been previously reported in the literature for hospitals in New York City. It is important to note that KPC enzymes have also been identified in *Pseudomonas aeruginosa*, *C. freundii*, *Salmonella cubana*, *P. mirabilis*, *K. oxytoca*, *Serratia marcescens*, and *Acinetobacter*. These pathogens were identified among our positive blood cultures; however, no KPC-producing isolates were detected.

While overall the results between automated susceptibility testing with imipenem/meropenem and disk-diffusion testing with ertapenem were in agreement, there were 2 samples that had discordant results on automated and disk-diffusion testing. The detection of KPC-producing pathogens using conventional culture-based methods has sometimes proven difficult owing to heterogeneous expression of resistance. The ertapenem disk-diffusion test has been shown to be a reliable screening method for KPC-mediated resistance. Therefore, results for culture-based susceptibility to ertapenem are often used for determining carbapenem resistance in routine clinical microbiology laboratories. However, resistance to ertapenem alone is not a marker for KPC expression; it has been shown that most ertapenem resistance is related to factors such as an extended-spectrum β-lactamase (ESBL) or AmpC production in association with outer membrane porin mutations.

When comparing the RT-PCR and culture susceptibility, we identified 2 samples that were negative for KPC by RT-PCR and carbapenem-resistant by culture. These results were not due to inhibition of the RT-PCR reactions because the internal control target was successfully amplified. It is possible that the carbapenem resistance was due to one of several other mechanisms, including changes in outer membrane permeability, increased activity of antibiotic efflux systems, or the production of AmpC β-lactamases, ESBLs, or non-KPC carbapenemases. In addition, it is also possible that the amount of template DNA for these samples was inadequate or that sequence alterations in the *bla*~KPC~ gene affected the binding of the primers or probes used in the assay.

Previous studies have suggested that the analytic sensitivity of an RT-PCR assay for detecting carbapenem resistance due to KPC is greater than culture. Results from the present study are consistent with this, especially when considering the patient whose serial samples were positive by RT-PCR before resistance was detected by culture.
The identification of KPC-positive, carbapenem-resistant *E. coli* and *E. cloacae* isolates from 1 patient may have resulted from infection with gram-negative bacteria that independently harbored a KPC plasmid. It is also possible, however, that the KPC plasmid might have been transferred between the bacteria, as previously documented in the literature.37 Plasmid analysis studies would be necessary to investigate the latter possibility.

Overall, this RT-PCR assay is sensitive and specific compared with culture-based methods of detecting carbapenem resistance attributable to KPC. Results demonstrated by this investigation are consistent with other studies evaluating the performance of RT-PCR assays for detecting KPC genes in clinical samples, including blood culture bottles.28,32

A limitation of the RT-PCR assay is that it is designed to detect only carbapenem resistance that is mediated by KPC, while culture-based methods of susceptibility testing can identify KPC- and non-KPC-mediated resistance to carbapenems. Therefore, it will be important to use the PCR assay in conjunction with culture, which provides identification of the pathogen in question and detection of carbapenem resistance due to various mechanisms. There are several KPC subtypes reported in the literature, and each subtype represents a unique nucleotide mutation compared with KPC subtype 2 on the KPC-carrying plasmid. In this study, we did not determine the specific KPC subtypes (KPC 2-11); however, from the location of the primers and probe used in the assay, all known KPC plasmids are expected to be detected (Figure 1).

The data from this study support the use of RT-PCR for the rapid detection of carbapenem-resistant *Enterobacteriaceae* directly from blood culture bottles. Using this assay, the time from identification of a positive blood culture bottle to determining carbapenem resistance can be reliably reduced from greater than 48 hours to approximately 3 hours, particularly in areas where carbapenem resistance in *Enterobacteriaceae* is due to KPC plasmids.

**References**


