Rapid Detection of \( \text{bla}_{\text{KPC}} \) Carbapenemase Genes by Real-Time PCR

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Carbapenem resistance among \textit{Enterobacteriaceae} is an emerging problem worldwide. \textit{Klebsiella pneumoniae} carbapenemase (\( \text{bla}_{\text{KPC}} \)) enzymes are among the most common \( \beta \)-lactamases described. In this study, we report the development and validation of a real-time PCR (q-PCR) assay for the detection of \( \text{bla}_{\text{KPC}} \) genes using TaqMan chemistry. The q-PCR amplification of \( \text{bla}_{\text{KPC}} \) DNA was linear over 7 log dilutions (\( r^2 = 0.999 \); slope, 3.54), and the amplification efficiency was 91.6%. The q-PCR detection limit was 1 CFU, and there was no cross-reaction with DNA extracted from several multidrug-resistant bacteria. Perianal/rectal swabs (\( n = 187 \)) collected in duplicate from 128 patients admitted to Sheba Medical Center surgical intensive care units were evaluated for the presence of carbapenem-resistant bacteria by culturing on MacConkey agar-plus-carbapenem disks and for \( \text{bla}_{\text{KPC}} \) genes by q-PCR. Carbapenem-resistant organisms, all \textit{K. pneumoniae}, were isolated from 47 (25.1%) of the 187 samples collected, while \( \text{bla}_{\text{KPC}} \) genes were detected in 54 (28.9%) of the patient samples extracted by the NucliSENS easyMAG system. Of these, seven samples were positive for \( \text{bla}_{\text{KPC}} \) genes by q-PCR but negative for carbapenem resistance by culture, while all samples in which no carbapenem-resistant bacteria were detected by culture also tested negative by q-PCR. Thus, the sensitivity and specificity of the q-PCR assay after extraction by the NucliSENS easyMAG system were 100% and 95%, respectively. Similar values were obtained after DNA extraction by the Roche MagNA Pure LC instrument: 97.9% sensitivity and 96.4% specificity. Overall, the \( \text{bla}_{\text{KPC}} \) q-PCR assay appears to be highly sensitive and specific. The utilization of q-PCR will shorten the time to \( \text{bla}_{\text{KPC}} \) detection from 24 h to 4 h and will help in rapidly isolating colonized or infected patients and assigning them to cohorts.

Carbapenem resistance among \textit{Enterobacteriaceae}, in particular among \textit{Klebsiella pneumoniae} and \textit{Escherichia coli}, is an emerging problem worldwide (14, 16, 20, 22, 25, 26). Several resistance mechanisms have been reported to be involved in the spread of carbapenem-resistant bacteria. Carbapenemases (carbapenem-hydrolyzing \( \beta \)-lactamases) are the most prominent enzymes that neutralize carbapenems (17, 18). Class A carbapenemases, which include \( \text{bla}_{\text{KPC}} \)-NMC, SME-1 to -3, IMI-1, and GES, have been characterized in several genera of the family \textit{Enterobacteriaceae} (17). Other carbapenem resistance mechanisms, including porin changes and changes in penicillin-binding proteins, have also been implicated in carbapenem resistance (23, 27, 28). \( \text{bla}_{\text{KPC}} \) enzymes, so called because they have been identified mainly in \textit{K. pneumoniae}, have been reported in \textit{Klebsiella oxytoca}, \textit{Serratia} spp., \textit{Enterobacter} spp., \textit{Salmonella} spp., \textit{Citrobacter freundii}, and \textit{Pseudomonas aeruginosa} (3, 8, 10, 11, 15, 24, 28). The genes encoding the \( \text{bla}_{\text{KPC}} \) enzymes are usually flanked by transposon-related sequences that have the potential to disseminate rapidly. Three different \( \text{bla}_{\text{KPC}} \) genes have been reported to date: \( \text{bla}_{\text{KPC-2}} \), \( \text{bla}_{\text{KPC-3}} \), and \( \text{bla}_{\text{KPC-4}} \); \( \text{bla}_{\text{KPC-1}} \) and \( \text{bla}_{\text{KPC-2}} \) are identical (27). \( \text{bla}_{\text{KPC}} \)-producing bacteria are usually resistant to virtually all classes of antibiotics—\( \beta \)-lactam agents, including penicillins, cephalosporins, monobactams, and carbapenems (1, 27, 28)—leaving physicians with limited antibiotic choices for treating infected patients.

In order to control the spread of \( \text{bla}_{\text{KPC}} \)-containing bacteria in hospitalized patients, effective infection control measures and controlled antibiotic usage must be complemented by the utilization of rapid and sensitive \( \text{bla}_{\text{KPC}} \) diagnostic assays (13). The utilization of such diagnostic tools will help in rapidly isolating colonized or infected patients and assigning them to cohorts.

In this report, we describe the development and validation of a real-time PCR (q-PCR) assay for the detection of \( \text{bla}_{\text{KPC}} \) genes. The assay was validated by comparing q-PCR with regular bacterial culturing on MacConkey agar-plus-carbapenem disks of routine surveillance perianal/rectal swabs obtained from the Sheba Medical Center intensive care units during an outbreak of one strain of \textit{K. pneumoniae} harboring \( \text{bla}_{\text{KPC-3}} \). Moreover, two automated bacterial DNA extraction methodologies were compared: the bioMérieux NucliSENS easyMAG system and Roche MagNA Pure LC DNA isolation kit III (bacteria, fungi).

MATERIALS AND METHODS

Collection of surveillance samples. Sheba Medical Center is a 1,200-bed tertiary teaching medical center in the center of Israel. As a result of an increasing rate of detection of carbapenem-resistant \textit{Enterobacteriaceae} (CRE), from less than 1% in 2005 to 18% in 2006 and 30% in the first half of 2007, surveillance perianal/rectal swabs were routinely collected from all patients admitted to the

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16-bed surgical intensive care unit and the 4-bed neurosurgical intensive care unit in order to control the spread of CRE. Perianal/rectal swabs were collected upon a patient’s admission to the unit and once weekly until the patient was discharged. During the study period from 1 April to 31 July 2007, 187 perianal/rectal swabs were randomly collected in duplicate from 128 patients. One sample was collected on a Copan Amies sterile transport swab (Copan Diagnostics, Corona, CA) and transported to the microbiology laboratory for the detection of CRE. The other sample was collected on a rayon swab, submerged in 1 ml saline, and stored at −20°C pending q-PCR analysis. The study was approved by the Sheba Medical Center Ethics Review Board.

**Bacterial stocks.** The following reference bacterial strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and evaluated for the presence of *bla*KPC genes: *K. pneumoniae* ATCC 13883, extended-spectrum-β-lactamase-positive *K. pneumoniae* ATCC 700603, *E. coli* ATCC 25922, and β-lactamase-positive *E. coli* ATCC 35218. In addition, clinical isolates of *bla*KPC-3-positive *K. pneumoniae*, AmpC-positive *K. pneumoniae*, AmpC-positive *Escherichia coli*, Citrobacter koseri, Enterobacter species, Serratia marcescens, Salmonella species, Shigella species, Proteus mirabilis, multidrug-resistant (MDR) *Pseudomonas aeruginosa*, Morganella morgani, Providencia species, MDR Acinetobacter baumannii, *Aeromonas hydrophila*, methicillin-resistant *Staphylococcus aureus*, methicillin-susceptible *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Enterococcus* species recovered at Sheba Medical Center during the year 2007 were evaluated in the study. A *bla*KPC-5-positive *K. pneumoniae* strain was generously provided by Shiri Navon-Venezia, Tel-Aviv Sourasky Medical Center.

**Detection of carbapenem-resistant bacteria by culture.** Perianal/rectal swabs were streaked onto MacConkey agar plates (Hy-Lab, Rehovot, Israel), ensuring that all sides of the swab touched the initial quadrant. The initial quadrant was plated as a lawn, and a meropenem (10 µg) and an ertapenem (10 µg) disk were placed in this area using sterile forceps (5). The plates were incubated overnight at 35°C in ambient air. The presence of bacterial colonies in the area surrounding either disk was worked up microbiologically. Classic bacteriological methods were used to identify the organisms according to routine protocols based on the 2nd edition of the Clinical Microbiology Procedures Handbook, and susceptibility testing was performed according to the CLSI guidelines (7, 12). During the study period, carbapenem resistance was confirmed by the Kirby-Bauer disk diffusion method on Mueller-Hinton plates (BD Diagnostics, Heidelberg, Germany) using meropenem (10 µg) and anertapenem (10 µg) disk and ertapenem (10 µg) (Oxoid, United Kingdom).

**DNA extraction.** (i) DNA extraction from cultured bacterial colonies. Fresh well-isolated colonies were used for DNA extraction with a QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany) according to the protocol suggested by the manufacturer. Brieﬂy, a 2-McFarland-standard bacterial suspension was prepared in saline, and bacterial DNA was extracted from 200 µl (1.2 × 10⁸ CFU) of the suspension. Extracted bacterial DNA was eluted from the columns in 100 µl elution buffer and stored at −20°C.

(ii) DNA extraction from perianal/rectal swabs using the Roche MagNA Pure LC instrument. The MagNA Pure LC DNA isolation kit III (bacteria, fungi) was used to extract bacterial DNA from well-vascularized perianal/rectal swabs in 1 ml saline according to the manufacturer’s suggestions. Brieﬂy, external lysis was performed on the maximum aliquot volume allowed (100 µl) to inactivate the bacteria. This was followed by DNA extraction using the MagNa Pure LC extractor. Extracted bacterial DNA was eluted in 100 µl elution buffer and stored at −20°C.

(iii) DNA extraction from perianal/rectal swabs using the bioMérieux NucliSENS easyMAG system. Bacterial DNA was extracted from well-vascularized perianal/rectal swabs in 1 ml saline according to the manufacturer’s suggestions. Brieﬂy, external lysis was performed on the maximum aliquot volume allowed (200 µl) to inactivate the bacteria. This was followed by DNA extraction using the easyMAG extractor. Extracted bacterial DNA was eluted in 110 µl elution buffer and stored at −20°C.

**blaKPC detection by q-PCR.** The ABI Prism 7700, 7500, and 7000 sequence detection systems (Applied Biosystems, Foster City, CA) were used for the amplification and detection of the *blaKPC* amplicon (246 bp) by TaqMan technology. The forward primer sequence (5′-GAT ACC AGC TCT CGT G-3′) specific for the detection of all *blaKPC* types was designed in-house. The reverse primer sequence (5′-GCA GTC TCT GGT TTT GTC TC-3′) was previously reported by Tenover et al. (22). In this study, the *blaKPC*-specific probe (6-carboxyfluorescein-5′-AGC GGC AGC AGC AOT TGG TGT AIT G-3′; 6-carboxyfluorescein at the 5′ end and the 6-carboxytetramethylrhodamine quencher at the 3′ end) was used. The sensitivity of the TaqMan assay was optimized by evaluating different concentrations of the primers (200, 300, 600, and 900 nM) and probe (100, 200, and 300 nM). The concentrations of the primers and probe used in this

![FIG. 1. Linear limits of detection of the *blaKPC* q-PCR assay. Serial (10-fold) dilutions of *K. pneumoniae* DNA harboring a *blaKPC*-plasmid were prepared and tested by the q-PCR assay. *C*ₐ values were obtained for each dilution and plotted against the number of CFU per reaction.](image-url)
were evaluated by the BLAST search program, available at www.ncbi.nlm.nih.gov. No matches to the primers and probe sequences were found other than those for the \textit{bla}_{KPC} genes. In addition, the \textit{bla}_{KPC} q-PCR assay was negative with DNA extracted from the following bacterial pathogens: \textit{K. pneumoniae} ATCC 13883, extended-spectrum-beta-lactamase-positive \textit{K. pneumoniae} ATCC 700603, \textit{E. coli} ATCC 25922, beta-lactamase-positive \textit{E. coli} ATCC 55218, AmpC-positive \textit{K. pneumoniae}, AmpC-positive \textit{E. coli}, Citrobacter koseri, Enterobacter species, Serratia marcescens, Salmonella species, Shigella species, Proteus mirabilis, MDR Pseudomonas aeruginosa, Morganella morgani, Providencia species, MDR Acinetobacter baumannii, Aeromonas hydrophila, methicillin-resistant \textit{Staphylococcus aureus}, methicillin-susceptible \textit{Staphylococcus aureus}, \textit{Streptococcus agalactiae}, and \textit{Enterococcus} spp. Likewise, the assay was negative for an MDR \textit{Proteus mirabilis} clinical isolate that was resistant to all carbenapenem, was positive by the modified Hodge test, and did not encode the \textit{bla}_{KPC} genes.

## Detection of carbenapenem-resistant organisms by culture and by q-PCR

Carbenapenem-resistant organisms, all \textit{K. pneumoniae} (\textit{bla}_{KPC}-), were cultured from 35 (27.3\%) of the 128 patients evaluated during the study period, while \textit{bla}_{KPC} genes were detected in 40 (31.3\%) and 38 (29.7\%) of the patient samples extracted with the NucliSENS easyMAG and Roche MagNA Pure LC extractors, respectively. No statistically significant difference between the two extraction methods was noted (\textit{P} = 0.25 by McNemar’s test). None of the patient samples grew more than one type of carbenapenem-resistant bacteria.

Upon stratification of the results by the total number of samples analyzed, which in some cases included more than one sample from the same patient depending on the duration of the hospital stay, carbenapenem-resistant organisms, all the same strain of \textit{K. pneumoniae}, were isolated from 47 (25.1\%) of the 187 swabs collected, while \textit{bla}_{KPC} genes were detected by q-PCR from 54 (28.9\%) of the patient samples extracted by the NucliSENS easyMAG system. Seven samples were positive for \textit{bla}_{KPC} by q-PCR and negative for carbenapenem resistance by culture, while all samples for which no carbenapenem-resistant bacteria were detected by culture also tested negative by q-PCR. Thus, the sensitivity of the q-PCR assay after extraction by the NucliSENS easyMAG system was 100\% (95\% confidence interval, 92.4 to 100\%), while the specificity was 96.4\% (95\% confidence interval, 91.9 to 98.5\%) (Table 1). The difference between the detection of carbenapenem-resistant bacteria by culture and that by q-PCR after extraction by the Roche MagNA Pure LC kit was not statistically significant (\textit{P} = 0.22 by McNemar’s test). Of the positive samples analyzed by q-PCR after Roche MagNA Pure LC extraction, 20 (39.2\%) gave \textit{C}_{T} readings less than 30, 30 (58.8\%) had \textit{C}_{S} between 30 and 40, and 1 (2.0\%) had a \textit{C}_{T} between 40 and 45.

The discouragencies for the seven samples positive for \textit{bla}_{KPC} by q-PCR but negative for carbenapenem-resistant bacteria by culture were resolved by repeating the extraction from the original tubes using both extraction systems and repeating the q-PCR assay for both new and old extractions. All samples that were initially \textit{bla}_{KPC} positive by q-PCR tested positive upon repetition, and the negative samples remained negative. In addition, q-PCR-positive samples (from both extraction methods) were also confirmed positive by agarose gel electrophoresis, and PCR amplicons of appropriate sizes were sequenced using the ABI Prism Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) as previously described (19). Sequence analysis revealed that all seven positive samples extracted by the NucliSENS easyMAG system and five positive samples extracted by the Roche MagNA Pure LC instrument were \textit{bla}_{KPC} genes. The sample that was positive by culture for carbenapenem-resistant bacteria but negative by q-PCR for \textit{bla}_{KPC} genes after Roche MagNA Pure LC extraction was low positive (\textit{C}_{T}, 41) for \textit{bla}_{KPC} after extraction with the NucliSENS easyMAG system. This sample was considered false negative after the Roche MagNA Pure LC extraction.

### TABLE 1. Comparison between detection of carbenapenem-resistant bacteria in perianal/rectal swabs by culture and detection of \textit{bla}_{KPC} genes by q-PCR after extraction with the BioMérieux NucliSENS easyMAG or the Roche MagNA Pure LC automated extractor

<table>
<thead>
<tr>
<th>Result by culture</th>
<th>BioMérieux NucliSENS easyMAG</th>
<th>Roche MagNA Pure LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>133</td>
</tr>
<tr>
<td>Negative</td>
<td>133</td>
<td>3</td>
</tr>
</tbody>
</table>

\(a\) The percent sensitivity (95\% confidence interval) was 100\% (92.4 to 100\%), and the percent specificity was 95\% (90 to 97.6\%).

\(b\) The percent sensitivity (95\% confidence interval) was 97.9\% (88.9 to 99.6\%), and the percent specificity was 96.4\% (91.9 to 98.5\%).

## DISCUSSION

Rapid detection of \textit{bla}_{KPC} genes is of the utmost importance, since these MDR organisms have the potential to spread rapidly in hospital environments and cause nosocomial infections with high mortality rates (4, 20). During a recent \textit{K. pneumoniae} \textit{bla}_{KPC}- outbreak at Sheba Medical Center, the mortality rate of bacteremic patients was 40\% (unpublished data). A similar mortality rate (47\%) was reported by Bratu et al. for bacteremic patients from New York City (4).

Detection of carbenapenem-resistant organisms has been problematic, because some isolates express low levels of resis-
tance that may not be detected by conventional automated and nonautomated methods (2, 6, 13). To circumvent this problem, it has been recommended to use ertapenem to screen for these organisms. Ertapenem has been shown to be the most sensitive indicator of the presence of \( \text{bla}_{KPC} \) enzymes (2, 5). In our experience, ertapenem has also proved to be the most sensitive antibiotic for the detection of \( \text{bla}_{KPC} \)-positive organisms. Currently, ertapenem is not on the panel of most conventional automated and nonautomated methods.

Molecular techniques have been used to rapidly detect \( \text{bla}_{KPC} \)-resistance genes from patients’ original samples (22). In addition, in an outbreak setting similar to that witnessed at Sheba Medical Center, screening for colonized but asymptomatic patients by molecular techniques was critical for controlling the outbreak. Indeed, after Israel’s Ministry of Health established guidelines, based on CDC recommendations, for assigning patients colonized or infected with \( \text{bla}_{KPC} \)-positive organisms to cohorts, the incidence of \( \text{bla}_{KPC} \) strains declined from 0.8/1,000 patient days to 0.4/1,000 patient days (21).

In this study we validated a rapid, sensitive, and specific q-PCR assay for the detection of \( \text{bla}_{KPC} \) genes from perianal/rectal surveillance samples after extraction by the NucliSENS easyMAG or the Roche MagNA Pure LC automated extractor. Regardless of the automated extraction system utilized, the assay can be performed in less than 4 h, which will allow for rapid assignment of colonized patients to cohorts, thus reducing the chance of spreading the organism in the hospital setting. This was in contrast to the time-consuming, less sensitive, and nonstandardized bacterial culture method, which can take more than 24 h to detect carbapenem-resistant bacteria (13). q-PCR eliminates both the chance of post-PCR contamination and all the time-consuming post-PCR processes such as gel electrophoresis (9). In addition, we have shown that the assay was highly stable and precise, as evidenced by the performance of the aliquoted \( \text{bla}_{KPC} \) DNA-positive control, which was stable over 20 consecutive runs (mean \( C_{T} \), 29.6; standard deviation, 0.7; coefficient of variation, 2.2%). The performance of the \( \text{bla}_{KPC} \)-positive control was not affected by the type of ABI instrument utilized (model 7700, 7500, or 7000); all gave similar results. However, we predict that the utilization of a real-time PCR instrument that allows for analysis of bacterial DNA extracted from patient samples upon receipt (i.e., SmartCycler) will be an asset to the laboratory, eliminating dependence on batching patient samples. Such instrumentation would improve the efficiency of assignment of patients colonized with \( \text{bla}_{KPC} \)-containing bacteria to cohorts.

The analytical sensitivity of the \( \text{bla}_{KPC} \) q-PCR assay was 1 CFU, with a linear dynamic range from 1 CFU to \( 1 \times 10^{6} \) CFU, regardless of the imipenem MIC (8 to \( \geq 32 \) \( \mu g/ml \)) for the carbapenem-resistant \( K. pneumoniae \) strains evaluated. This analytical sensitivity was better than that reported by Landman et al., who showed that as little as 2.7 CFU/ml of \( K. pneumoniae \) could be detected for isolates with high imipenem MICs (\( \geq 32 \) \( \mu g/ml \)) by culture. The q-PCR sensitivity was even higher, by about 6 log units, when a carbapenem-resistant strain with a low imipenem MIC (1 to \( 8 \) \( \mu g/ml \)) was evaluated (13). A drawback of the protocols described by Landman et al. is that they are time-consuming, requiring on average 48 h to confirm an isolate as \( \text{bla}_{KPC} \)-positive (13).

The excellent analytical sensitivity of the q-PCR assay described was complemented by excellent clinical sensitivity after extraction with the NucliSENS easyMAG (100%) or Roche MagNA Pure LC (97.9%) automated extractor. Overall, the results from the NucliSENS easyMAG system were 2 to 3 \( C_{T} \)s better than those from the Roche MagNA Pure LC extractor. The difference is likely related to the maximum sample volume allowed by the extraction method: 100 \( \mu l \) for the MagNA Pure LC compared to 200 \( \mu l \) for the NucliSENS easyMAG extractor. The MagNA Pure LC extraction of bacterial DNA required longer sample manipulation (20 to 30 min) before loading than the NucliSENS easyMAG method (10 min). One of the drawbacks of utilizing either automated extraction system is that every eight samples must be batched in order to reduce sample extraction costs. However, this downside is minimized in large laboratories, where large numbers of samples are extracted.

As a result of the detection of seven \( \text{bla}_{KPC} \)-positive samples by q-PCR that were negative for carbapenem-resistant bacteria by culture, enrichment broth with a meropenem disk (10 \( \mu g \)) was added to the \( \text{bla}_{KPC} \) culture protocol as previously described (13). The addition of enrichment broth has been reported to increase the sensitivity of the culture-based detection of carbapenem-resistant bacteria; however, an extra 24 h are needed for the detection of carbapenem-resistant bacteria, thus increasing the chance of the spread of these highly drug-resistant organisms in a hospital setting (2, 13).

One of the drawbacks of the q-PCR assay described is that it detects only \( \text{bla}_{KPC} \)-positive bacteria, while bacterial culture can detect bacterial isolates with both \( \text{bla}_{KPC} \) and non-\( \text{bla}_{KPC} \) mechanisms of carbapenem resistance (17). In addition, we have not evaluated \( \text{bla}_{KPC} \)-positive organisms, since we had no access to these strains. Based on the BLAST search program analysis, we predict that the \( \text{bla}_{KPC} \) q-PCR assay described will be as sensitive for the detection of \( \text{bla}_{KPC} \)-positive since no reported mutations are present in the primers or probe sites.

Our data support the utilization of q-PCR for screening patients upon admission and routinely during hospitalization in areas with high prevalences of \( \text{bla}_{KPC} \)-positive Enterobacteriaceae. Efforts should be invested in developing rapid and sensitive diagnostic techniques for laboratories where q-PCR technology is not available.

REFERENCES

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