Real-Time Detection of *bla*_{KPC} in Clinical Samples and Surveillance Specimens[▽]

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A real-time PCR assay was developed targeting the *bla*_{KPC} responsible for *Klebsiella pneumoniae* carbapenemase (KPC)-mediated carbapenem resistance and was validated for testing colonies or enrichment broth cultures. The assay accurately detects KPC-containing strains with high analytical specificity and sensitivity.

Broad-spectrum β -lactamases, such as *Klebsiella pneumoniae* carbapenemases (KPCs), are spreading widely within the United States and cause resistance to virtually all beta-lactam antimicrobials (4, 5). Rapid identification of KPC-containing *Enterobacteriaceae* is critical to controlling the spread of these pathogens and can be accomplished by molecular detection (6). We have developed and validated a real-time PCR assay for *bla*_{KPC} detection, including an internal control, and applied it to various clinical specimens, including isolated colonies and surveillance swabs from intensive care unit (ICU) patients after broth enrichment, as a tool for infection control of KPC resistance.

For analytical validation, 46 KPC-positive isolates were cultured on MacConkey agar (Remel, Thermo Fisher Scientific, Lenexa, KS) plates containing 4 μ g/ml meropenem (AstraZeneca Pharmaceuticals, Wilmington, DE) at 37°C for 24 to 48 h. Twenty-eight isolates (including *K. pneumoniae*, *Klebsiella oxytoca*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, and *Staphylococcus epidermidis*) with no known resistance were included as negative controls. The clinical validation included 41 isolates from 34 patients and surveillance swabs (nasal or other respiratory site, axilla, and perirectal) from 379 ICU patients at four NorthShore hospitals; results were compared to phenotypic KPC activity assessed by modified Hodge test (2). Screening and confirmatory tests for suspected carbapenemase production were performed according to CLSI performance standards in M100-S19 on clinical isolates (2).

Colonies from plated cultures were resuspended in 200 μ l lysis buffer (7). Suspensions were heated for 10 min at 99°C and microcentrifuged, and 2 μ l of supernatant was used for real-time PCR. Surveillance swabs were inoculated to enrichment broth (5 ml tryptic soy broth medium [Remel] containing one BD Sensi-Disc 30- μ g ceftazidime disc [Becton Dickinson, Franklin Lakes, NJ]) and incubated overnight at 37°C to maximize recovery of KPC organisms and reduce the growth of

other bacteria. DNA was obtained by adding 250 μ l of broth to 200 μ l of lysis buffer and processed as described above.

After aligning GenBank sequences for *bla*_{KPC} gene isoforms, consensus primers and a 5' exonuclease probe labeled with 6-carboxyfluorescein (FAM) were designed to detect KPC-1/2 through KPC-11 (Table 1, Fig. 1). The 16S rRNA gene found in all bacteria was used as an internal control (Table 1). Optimized reaction mixtures contained 1 $\times$ LightCycler FastStart DNA hybridization probe reagent (Roche Diagnostics, Indianapolis, IN), 5 mM MgCl₂ (final concentration), and primers and probes (final concentrations in Table 1). To remove trace microbial DNA (1), master mix was predigested with HpaII restriction enzyme (New England BioLabs, Ipswich, MA; 5U per 100 μ l of master mix). After predigestion, 2 μ l of lysis buffer supernatant (or water for the blank) was added to 18 μ l master mix. Amplification was performed on LightCycler 2.0 instruments (Roche Diagnostics): one cycle at 95°C for 10 min, 50 cycles of 10 s at 95°C (ramp, 20°C/s; no acquisition), 30 s at 56°C (ramp, 20°C/s; no acquisition), and 20 s at 72°C (ramp, 2°C/s; single acquisition). The *bla*_{KPC} gene was detected at 530 nm, while the internal control was detected at 610 nm, using color compensation.

TABLE 1. Sequence information for each oligonucleotide used in the real-time assay, the final concentration in the reaction, and the size of the amplified product for both the *bla*_{KPC} gene and the 16S rRNA gene used as an internal control

Oligonucleotide ^a	Sequence ^b	Concn (μ M)	Size (bp)
KPC78U20	CAACCTCGTCGCGGAACCAT	0.2	236
KPC294L20	ACCACGGAACAGCGCATTT	0.2	236
KPC220TAQ22	FAM-TTTCCTTGCTGCCGCTGT GCTGG-BBQ	0.2	236
16S793U22	GTAGTCCACGCGTAAAC GATG	0.3	292
16S1062L22	AACCAACATYTCACRAC ACGA	0.3	292
16STAQ2	TexasRed-CGAWGCAACGCGA AGAACCTTACCT-BBQ	0.2	292

^a The 5' nucleotide position for each oligonucleotide is indicated in the middle of each name and corresponds to EU784136 for *bla*_{KPC} and JF513979 for the 16S rRNA gene.

^b BBQ, BlackBerry quencher.

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FIG. 1. Amplification curves for analytical validation specimens after color compensation calculations have been applied. The top panel shows the fluorescence measured at 530 nm to detect FAM, indicating the presence of the *bla*_{KPC} gene. Note that only the bacteria with KPC activity show positive amplification curves; all bacteria lacking any carbapenemase activity show no fluorescence similar to the water blank reaction. The bottom panel shows fluorescence measured at 610 nm to detect Texas Red, indicating amplification of the 16S rRNA gene as an internal control. The curves in this panel that do not have the typical sigmoid curve and lower 610-nm fluorescence are the KPC-positive bacteria that have strong FAM fluorescence at 530 nm, affecting the amount of Texas Red fluorescence measured for these samples. All bacterial specimens show positive amplification curves, indicating acceptable amplification. The water blank has no fluorescence, indicating no background amplification.

Limit of detection experiments utilized four of the original isolates grown to 0.5 McFarland standard concentrations (2) and diluted serially. One to 100 bacterial genome copies per reaction were detectable by PCR; variable plasmid numbers are likely in the individual isolates studied.

In contrast to previously published assays, an internal control was multiplexed into each reaction to monitor adequate amplification of bacterial DNA. The internal control was detected in all specimens tested, and no signal was detected in the water blanks.

Of the 46 KPC-positive isolates (identified as KPC-2, KPC-3, KPC-4, KPC-7, or uncharacterized *bla*_{KPC}) received from outside hospitals, 45 were positive for the *bla*_{KPC} gene. The single discrepant sample was also negative using an alternative real-time assay (3) targeting a different region of the *bla*_{KPC} gene, confirming that the gene was not present in the specimen. The modified Hodge test was negative for this sample when grown at NorthShore; the probable explanation is that the bacterial strain had lost the plasmid containing the *bla*_{KPC} gene after initial characterization.

All 24 clinical isolates of *Enterobacteriaceae* that were multidrug resistant and showed modified Hodge positivity were PCR positive for the *bla*_{KPC} gene. All specimens phenotypically negative for carbapenemase were PCR negative for *bla*_{KPC} (sensitivity of 100%, 95% confidence interval [CI] of 90% to 100%; specificity of 100%, 95% CI of 85% to 100%). Testing of isolated colonies for *bla*_{KPC} was completed within 2 h of receipt into the laboratory.

Four of 379 surveillance specimens tested were KPC positive by PCR, three of which were also identified by conventional phenotypic testing. Real-time PCR testing of the fourth surveillance specimen was reproducible and supported by positive PCR results using the confirmatory assay. Although both *K. pneumoniae* and *E. coli* were cultured from this specimen, neither was PCR positive for *bla*_{KPC}, leaving this discrepant result unresolved. PCR analysis of surveillance swabs could be completed within 24 h, compared to 48 to 96 h or longer, using a modified Hodge test on colonies grown on meropenem-containing agar plates. The PCR assay was 100% (95% CI of 91% to 100%) sensitive and 99.8% (95% CI of 98% to 100%) specific for the combined clinical isolates and surveillance specimens.

In summary, we have developed and validated a real-time PCR assay for detection of *bla*_{KPC}, complete with an internal control. We have successfully utilized this testing to identify KPC-producing *Enterobacteriaceae*, both as clinical isolates and on surveillance swabs obtained from patients at risk, with excellent sensitivity, specificity, and turnaround time. This assay now is part of a comprehensive infection control program that aims to reduce the spread of these and other antibiotic-resistant organisms.

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