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Ultrarapid Detection of $bla_{KPC1/2-12}$ from Perirectal and Nasal Swabs by Use of Real-Time PCR

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The novel real-time PCR assay developed as described here was able to detect $bla_{KPC1/2-12}$ ($bla_{KPC-1/2}$ to bla_{KPC-12}) from easily available clinical specimens in less than 2 h. The genotypic assay was highly sensitive (100%) and specific (98%). In some cases, it was able to detect bla_{KPC} 48 h before positive detection by standard phenotypic assay on patients who were monitored daily. The high sensitivity and rapidity of the molecular method make it the method of choice for KPC surveillance and, thus, containment purposes.

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Bacteria producing *Klebsiella pneumoniae* carbapenemases (KPCs) have emerged as a cause of multidrug-resistant nosocomial infections worldwide. KPC has been identified in several *Enterobacteriaceae* species, as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*; to date, 11 different variants (KPC-1/2 to KPC-12) have been reported (4) (www.lahey.org/Studies/other.asp#table1). Initially, carbapenem-resistant strains were found mainly in intensive care units, but unfortunately, they have now expanded to all hospital wards, probably leading to involvement of the nonhospitalized population, with subsequent dangerous community acquisition (1). Early identification of carbapenemase producers, also at the carriage state, is thus becoming mandatory for prevention and adequate management to contain the further spread of resistance.

Real-time PCR methods have been developed for the detection of bla_{KPC}; most of them need the clinical sample to be either cultured on agar plates to obtain pure cultures or inoculated to enrichment broth or blood culture bottles to maximize the recovery of KPC-positive organisms (2, 6), with a time delay from sample reception to diagnosis of at least 24 h.

We have developed an original ultrarapid assay based on fast real-time PCR for the detection of $bla_{KPC1/2-12}$ ($bla_{KPC-1/2}$ to bla_{KPC-12}) genes. The assay was performed on perirectal and nasal swabs which were processed for analysis without prior culturing of bacterial species, allowing the output of results less than 2 h from the reception of swabs.

All 216 clinical swabs (116 perirectal and 100 nasal) were obtained from 125 patients hospitalized in the Padua Teaching Hospital, Italy. The real-time PCR method was validated by comparison with standard diagnostic phenotypic analysis of cultured isolates. For phenotypic analysis, two swabs from the same patient were first streaked on nonselective enrichment agar plates (BBL blood agar or BBL chocolate II agar) and on differential and selective MacConkey II agar plates (all from Becton Dickinson Italia, Milan, Italy) and incubated at 35°C ± 2 for 16 to 18 h; if necessary, single colonies (usually, one or two) of each colony type were further streaked on enrichment agar plates to obtain pure cultures. In cases of noncorrespondence with the results of the molecular method, up to 15 single colonies of the Gram-negative species identified were restreaked on chocolate agar plates to obtain pure cultures and on Oxoid Brilliance CRE agar (Oxoid Ltd., United Kingdom) to check for the presence of KPC-producing species. Microbial identification and antimicrobial susceptibility testing on single colonies were performed by using the Vitek 2 automated system (bioMérieux, Marcy l'Etoile, France) and by disk diffusion and Etest according to CLSI guidelines. Carbapenem-resistant samples were further confirmed by the modified Hodge test.

For genotypic real-time PCR analysis, each swab previously streaked on agar plates was emulsified in 1 ml of 1× sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Total nucleic acid extraction was performed by using NucliSENS easyMAG (bioMérieux).

For design of real-time PCR primers and probes, reference gene sequences for KPC alleles from 1/2 through 12 were assembled from GenBank (http://www.ncbi.nlm.nih.gov/GenBank). Consensus primers (Fw, 5′-GCCGTGCAATACAGTGATAACG-3′; Rev, 5′-CGGGCCGCCCAACT-3′), which amplified a 60-bp fragment, and a novel TaqMan "minor groove binder" (MGB) probe (6FAM-CCGCCAATTTGTTGCT-MGBNFQ) (Applied Biosystems, Life Technologies, Carlsbad, CA) were designed to detect all KPC alleles. The β-globin gene, present in all human cells, was used as internal control to attest the presence of biological material on the clinical swab (7).

The real-time PCR assay was performed both in standard and fast conditions on a 7900HT fast real-time PCR system (Applied Biosystems). Each reaction mixture contained 10 μl of DNA extract solution, 15 μl of 2× TaqMan universal PCR master mix or 2× TaqMan fast universal PCR master mix, 0.75 μM each primer, 0.3 μM MGB probe, and sterile water to a final volume of 30 μl. The standard cycling conditions comprised two holding periods of 2 min at 50°C and 10 min at 95°C and 50 cycles, each of 15 s at 95°C and 1 min at 60°C. The fast conditions were 20 s at 95°C and 50 cycles, each of 1 s at 95°C and 20 s at 60°C. Real-time PCR data were analyzed with SDS 2.4 software. Each run contained a positive control for KPC amplification (KPC-2-encoding *K. pneumoniae*, ATCC BAA-

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1705) and at least one water blank as a negative control for DNA extraction and sample contamination.

The initial validation of the analytical method was performed on colonies of 15 previously identified KPC-positive samples, cultured on chocolate agar. These isolates were KPC-2 and KPC-3 (5 and 10 samples, respectively) and belonged to sequence type 147 (ST147), ST307, and ST437 and to ST37, ST258, ST510, ST512, and ST554, respectively. Real-time PCR was performed on heat-shocked cell lysates. All 15 samples were PCR positive.

For limit-of-detection analysis, colonies were picked up with a sterile loop, resuspended in Phoenix ID broth to 0.2 McFarland standard concentration (PhoenixSpec Nephelometer; Becton Dickinson Italia), which corresponded to roughly 1×10^8 CFU/ml, and pelleted. Cell pellets were resuspended in 200 μl of sterile water and lysed at 99°C for 10 min. Cellular debris was eliminated by centrifugation, and supernatants were serially diluted. Each dilution was loaded in 8 replicates to assess the analytical sensitivity of the assay. Both in fast and standard real-time PCR conditions, the amplification was linear over 6 log dilutions ($r^2 = 0.98$, slope $= -3.31$) and the detection limit was 1 CFU.

Of the 216 samples, 41 and 158 were KPC positive and negative, respectively, as agreed by both real-time PCR and phenotypic analysis. All positive isolates were *K. pneumoniae*. For positive samples, the range of cycle threshold (C_T) values representing positivity was 14 to 38 C_T, both in fast and standard conditions. The remaining 17 samples were positive by real-time PCR but negative by the phenotypic method, attesting to a sensitivity of 100% (95% to 100%, 95% confidence intervals [95%CI]) and a specificity of 90% (85% to 96%, 95%CI).

However, of these 17 apparently false-positive samples, 9 samples were from 6 patients whose perirectal or nasal swabs were subsequently (within 48 h) found to be positive by phenotypic analysis also. Even though in principle the phenotypic method is as sensitive as the genotypic one on pure cultures, on mixed samples, the genotypic method performs better, probably due to the difficulty of detecting only a few colonies of the suspected pathogen among hundreds of normally resident noninfectious organisms. In fact, in our case, the real-time PCR analysis was able to detect the presence of the bla_{KPC} gene before the standard phenotypic method did.

On the other hand, 4 apparently false-positive samples were from 2 patients whose body fluids (urine, sputum, or perirectal swabs) had been previously (7 to 20 days) diagnosed as KPC positive. In this case, the molecular method probably identified KPC-positive bacteria that had been killed by antibiotic treatment and which did not grow on culture plates, therefore preventing phenotypic detection.

All of these 13 samples displayed a high C_T count (>30), indicating small bla_{KPC} amounts. Therefore, even when assayed by standard PCR with two primers that amplified the complete bla_{KPC} gene (5), they tested negative, due to the remarkably lower sensitivity of the standard PCR method.

Finally, 4 of the apparently false-positive samples were from 4 patients who, being discharged, did not provide any further clinical samples. These samples were subjected to standard PCR analysis. Only one sample, which displayed the larger gene amount in real-time PCR analysis (15 C_T), was found to be positive. The other three samples contained amounts of bla_{KPC} (34, 38, and 39 C_T) that were too small to be detected either by standard PCR or phenotypic analysis. Thus, due to these technical problems (patients being discharged and small amounts of bla_{KPC}), these three samples had to be considered false positive. The resulting sensitivity of the real-time PCR method was 100% and the specificity 98% (96% to 100%, 95%CI).

To avoid the risk of false-negative detection by the real-time PCR method due to a small KPC-positive bacterial load on the swab, 47 representative negative samples were also grown on enrichment broth for 24 to 72 h. Swabs were resuspended in BBL Trypticase soy broth (BD) for 24, 48, and 72 h and streaked and incubated on enrichment chocolate or MacConkey agar plates for 24 h. No sample revealed a subsequent presence of KPC-positive colonies, confirming no detection of false-negative samples by the molecular method.

It is important to note that the method did not show cross-reactivity with other pathogenic or resident microbial species on the swab. The pathogen species characterized by phenotypic identification included both Gram positive (mainly *Staphylococcus* spp. and *Streptococcus* spp.) and Gram negative (*Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter* spp., *Haemophilus influenzae*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Moraxella chatarralis*, *Morganella morganii*, and *Serratia marcescens*) bacteria and fungi, such as *Candida* spp.

In summary, we have developed and validated an original real-time PCR assay for the detection of $bla_{KPC1/2-12}$. The clinical specimens chosen were perirectal and nasal swabs, which are easily available and appropriate for the detection of both ongoing infection and colonization by KPC-encoding bacteria. The major novelties of the method with respect to previous methods (3) are (i) the use of an innovative minor groove binding probe and a nonfluorescence quencher which results in lower fluorescence background, extending the limit of detection of the molecular method, (ii) the use of β-globin as an internal control to assess the integrity of the clinical sample, and (iii) the amplification of a short bla_{KPC} sequence, which enables the use of the fast PCR system, without conferring cross-reactivity with other microbial species. Therefore, by avoiding the bacterial culture step, we were able to output results in less than 2 h or 4 h for the fast and standard amplification, respectively (1 h for swab resuspension and DNA extraction and 45 min or 2.15 h for fast or standard real-time PCR, respectively). To our knowledge, this is the first report of the detection of all circulating KPC types in such a short time.

In conclusion, no new antibiotics are currently available (nor will be in the near future) to treat Gram-negative multiresistant infections; therefore, it becomes of the utmost importance to restrict the rate of KPC dissemination in hospitals by early diagnosis and isolation of KPC carriers. This can be achieved by screening newly admitted patients and periodically monitoring infected or pathogen-carrying individuals, as well as hospital personnel. The improved rapidity and sensitivity of the molecular technique presented here make it the method of choice for surveillance purposes and may help restrict the diffusion of carbapenem resistance. For cases where other carbapenemases, such as NDM, VIM, IMP, and OXA-48, are also implicated, a multiplex–real-time PCR method is advisable.

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