Rectal Swabs Are Suitable for Quantifying the Carriage Load of KPC-Producing Carbapenem-Resistant Enterobacteriaceae

A. Lerner, J. Romano,* I. Chmelnitsky, S. Navon-Venezia, R. Edgar, Y. Carmeli
Molecular Epidemiology and Antimicrobial Resistance Laboratory, Division of Epidemiology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

It is more convenient and practical to collect rectal swabs than stool specimens to study carriage of colon pathogens. In this study, we examined the ability to use rectal swabs rather than stool specimens to quantify Klebsiella pneumoniae carbapenemase (KPC)-producing carbapenem-resistant Enterobacteriaceae (CRE). We used a quantitative real-time PCR (qPCR) assay to determine the concentration of the bla<sub>KPC</sub> gene relative to the concentration of 16S rRNA genes and a quantitative culture-based method to quantify CRE relative to total aerobic bacteria. Our results demonstrated that rectal swabs are suitable for quantifying the concentration of KPC-producing CRE and that qPCR showed higher correlation between rectal swabs and stool specimens than the culture-based method.

Klebsiella pneumoniae carbapenemase (KPC)-type enzymes are β-lactamases, capable of hydrolyzing all known β-lactam antibiotics (1). The spread of the bla<sub>KPC</sub> genes has led to the emergence of carbapenem-resistant Enterobacteriaceae (CRE), mostly K. pneumoniae, as important nosocomial pathogens causing outbreaks in various parts of the world (2). Infections by these pathogens are severe, with an estimated case fatality rate of 35%, and thus considered a clinical and public health threat (3).

Active surveillance of high-risk patients has been advocated in areas where CRE are endemic or where there are ongoing outbreaks (4). Although stool samples are considered the “gold standard” specimen for studying gut bacteria in general (5, 6) and for detecting CRE in particular (7), hospital epidemiologists, clinical microbiologists, and researchers frequently use rectal swabs due to practical considerations, such as ease of collection, handling, and processing (8–11). Several studies compared the qualitative sensitivity of rectal swabs versus stool specimens for the recovery and detection of various pathogens, such as Campylobacter fetus subsp. jejuni, vancomycin-resistant Enterococcus faecium (VRE), and Lawsonia intracellularis, by culturing and/or molecular quantification methods (12–14). These studies had mixed results, with some studies reporting comparable test sensitivity with either sample type (12–14) and others reporting significantly lower sensitivity with rectal swabs (15, 16). For quantitative testing, rectal swabs are believed to be inadequate due to the high variation in the quantity of fecal material on each swab and the difficulties in determining this quantity (17–20).

As the human colon flora is a diverse ecosystem, which is populated by both anaerobic and aerobic microorganisms (21), development of quantitative methods, such as culture- and quantitative real-time PCR (qPCR)-based techniques is important for studying the bacterial composition of this complex ecosystem. The use of these methods can lead to a thorough knowledge about gastrointestinal community composition, the effects of antibiotics, and their roles in health and disease.

Here, we suggest an alternate methodology for quantifying pathogens that would overcome the problem of the unknown quantity of fecal material on the rectal swab by assessing the relative concentration of the target bacteria compared to total bacteria. We used two quantification methods: a culture-based method comparing the number of CRE to total aerobic culturable bacterial growth and a quantitative real-time PCR method for detection and quantification of bla<sub>KPC</sub> genes and 16S rRNA genes as a total bacterial reference gene. We evaluated whether, by using these methods, rectal swabs are adequate substitute for stool samples to quantify KPC-producing CRE (KPC-CRE).

MATERIALS AND METHODS

Sample collection. Specimens were collected from 37 hospitalized patients known to be KPC-producing CRE carriers; they were identified as carriers by bla<sub>KPC</sub> PCR-based testing (22). Stool samples and rectal swabs (perirectal swabs were not allowed) were simultaneously collected from each CRE carrier and immediately transported to the lab. Rectal samples were collected using eSwab (Copan, Brescia, Italy) by inserting the swab 1 cm into the rectum while rotating the swab. Following sampling, the swab was placed in the transport tube (tube supplied by the manufacturer) containing 1 ml sterile Amies transport medium and was vortexed for 1 min at maximum speed upon arrival to the lab. In parallel, the weight of the stool sample (wet weight) was determined, diluted 1:10 (wt/vol) with 0.9% saline, and vortexed for 5 min at maximum speed to generate a stool suspension. Thirty-seven stool and rectal swab specimens were subjected to analysis; 22 pairs were analyzed in parallel using both culture-based and qPCR-based analysis. The remaining 15 pairs were analyzed only by one method due to insufficient material submitted (in most cases, the stool sample, not the rectal swab, was the specimen type with insufficient quantity). In addition, a known CRE carrier patient was sampled 5 times over a 1-month period, and the specimens were analyzed for temporal changes.

Culture-based quantification of KPC-CRE. After preliminary testing to ascertain the optimal conditions, the stool suspension (500 μl) and the
TABLE 1 qPCR primers and probe used in this study

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Group specificity</th>
<th>Sequence (5′→3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primersa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>515F</td>
<td>Eubacteria</td>
<td>GTGCCAGACGGCGGTAA</td>
<td>23</td>
</tr>
<tr>
<td>685R</td>
<td>Eubacteria</td>
<td>TCTACGCGTACCCCATAC</td>
<td>24</td>
</tr>
<tr>
<td>blaKPC gene</td>
<td>Gut KPC producers</td>
<td>CGCTCAGTGTCGTGTCG</td>
<td>This study</td>
</tr>
<tr>
<td>blaKPC</td>
<td>Gut KPC producers</td>
<td>GATACTACGCTGCGCTGAG</td>
<td>This study</td>
</tr>
<tr>
<td>blaKPC</td>
<td>Gut KPC producers</td>
<td>CTCGACCACTGCTG</td>
<td>This study</td>
</tr>
</tbody>
</table>

a The direction of the primer is indicated at the end of the primer name as follows: F for forward and R for reverse.

eSwab Amies transport medium containing bacteria (100 μl) were used for performing viable bacterial counts by serial 10-fold dilutions in 0.9% saline. Bacteria were plated on tryptic soy agar plates supplemented with 5% sheep blood (HyLabs, Rehovot, Israel) to determine the total culturable aerobic bacteria (TAB), and bacteria were plated on CHROMagar KPC plates (HyLabs, Rehovot, Israel) to determine the CRE bacteria. Viable bacterial counts were determined after 18 h of growth at 37°C, and the ratio of CFU/ml of CRE to TAB was determined and expressed as CRE/TAB.

Molecular biology-based quantification of KPC-CRE. (i) DNA extraction from pure cultures, rectal swabs, and stool samples. The reference bacterial strains used for molecular quantification are listed in Table S1 in the supplemental material. These strains were used to evaluate the performance (sensitivity and specificity) of the PCR primer-probe sets.

A bacterial colony from each of these strains was suspended in 100 μl molecular biology-grade water (Bio-Lab, Jerusalem, Israel) to determine the CRE bacteria. Viable bacterial counts were determined after 18 h of growth at 37°C, and the ratio of CFU/ml of CRE to TAB was determined and expressed as CRE/TAB or log CRE/TAB.

(ii) Design of primers and probes. The primers and probe used in this study are listed in Table 1. An alternative set of primers and probe for blaKPC has been reported (25); however, since the primer and probe set listed in Table 1 had already been confirmed in our laboratory (see below), we elected to continue using them. The blaKPC primers and probe set were designed for conserved regions based on alignment of blaKPC sequences deposited at the NCBI site (http://www.ncbi.nlm.nih.gov/), and their specificities were confirmed in silico using the BLAST network service (26; http://blast.ncbi.nlm.nih.gov/).

(iii) Conventional PCR. For specificity testing, conventional PCR was used on bacterial lysates and fecal DNA as mentioned above. The PCR mixtures (25 μl) contained 0.125 μl of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 2.5 μl of 10X buffer (Qiagen, Hilden, Germany), 2.25 mM MgCl2, (Qiagen, Hilden, Germany), 0.8 μM of each primer, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP) (Qiagen, Hilden, Germany), and 1 μl of template DNA. Amplifications were performed with an initial denaturation step of 15 min at 95°C, followed by 35 cycles, with each cycle consisting of denaturation for 30 s at 94°C, annealing for 20 s at 60°C, and elongation for 20 s at 72°C. A final extension step was performed at 72°C for 5 min. The PCR product was purified, cloned into pDrive cloning vector (Qiagen, Hilden, Germany) and sequenced (Macrogen Inc., Seoul, South Korea). The identities of the cloned fragments were determined using the BLAST network service.

(iv) qPCR. Quantitative real-time PCR was performed in two singleplex assays, one for assessing the total bacteria using the 16S rRNA gene primers set 515F and 685R (SYBR green reaction; Table 1) and the other for assessing colonic KPC producers with the newly designed KPC primers and TaqMan MGB probe (Table 1). For standards for each singleplex reaction, we used the pDrive cloning vector containing the 16S rRNA gene or the blaKPC gene. The standards were diluted to generate samples ranging from approximately 1 to 1×10^6 copies per PCR. Each singleplex reaction tube contained 4 μl of DNA, 0.25 μM each blaKPC primer or 0.2 μM each 16S rRNA gene primer (Table 1), 10 μl of 2× Absolute blue QPCR ROX mix (Thermo Fisher Scientific, Epsom, United Kingdom) (for the TaqMan reaction) or 2× Absolute blue SYBR green ROX mix (Thermo Fisher Scientific, Epsom, United Kingdom) (for the SYBR green reaction), 0.2 μM TaqMan probe (Table 1), and molecular biology-grade water up to 20 μl. Cycling conditions consisted of one holding period at 95°C for 15 min, followed by 50 cycles, with each cycle consisting of 10 s at 95°C, 15 s (for the SYBR green reaction) or 60 s (for the TaqMan reaction) at 60°C, and 15 s at 72°C for the SYBR green reaction only. All reactions were carried out in a Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia) in duplicate samples. The results were expressed as a ratio of the number of copies of the blaKPC gene/number of copies of 16S rRNA genes (blaKPC/16S rRNA genes) or log blaKPC/16S rRNA genes.

(v) Specificity, sensitivity, and inhibition of the qPCR assays. Suspensions (in molecular biology-grade water [Bio-Lab, Jerusalem, Israel]) of overnight colonies of Klebsiella pneumoniae carrying the blaKPC gene (KPC-Kpn), Klebsiella oxytoca, Salmonella sp., Proteus sp., Citrobacter koseri, Enterobacter cloacae, and Enterococcus gallinarum were normalized to an optical density at 600 nm (OD600) of 1 using a spectrophotometer (UVmini-1024; Shimadzu, MD), approximately 1×10^8 CFU/ml, and bacterial lysates were prepared as mentioned above. The specificity of the real-time TaqMan assay was determined on KPC-Kpn bacterial lysate as the target and on a bacterial mixture containing the target and nontarget microorganisms. The specificity of the 16S rRNA gene qPCR SYBR reaction was determined using the bacterial lysates of K. oxytoca, Proteus sp., Enterobacter cloacae, and Enterococcus gallinarum separately and in combination. To determine the effect of fecal material on the sensitivities of our assays, DNA extracted from rectal swabs of KPC carriers was diluted 10-fold from 10^9 to 10^2, spiked with lysate of the target bacteria KPC-Kpn (TaqMan assay) or with K. oxytoca (16S rRNA gene qPCR SYBR reaction) and with the mixtures mentioned above. The copy numbers obtained were compared to those obtained for the target bacteria alone in water.

Statistical analysis. To identify correlations between the values for rectal swabs and stool samples, a linear regression analysis was performed with the use of Microsoft Excel (Microsoft Corporation, Redmond, WA) and the Analyze-It version 2.26 software (Analyze-it Software, Ltd., Leeds, United Kingdom). Three outliers out of the 60 samples in the correlation graphs, two in the microbiological (culture) graph and one in the molecular quantification graph were not included in the final statistical analysis. The outliers were not specific to the stool sample or to the swab sample, and the reasons for these outliers are unknown. As they divert from the general behavior of all the other samples, they were excluded from the correlation analysis. Nevertheless, the correlations with these outliers are indicated in the Results section, and they did not change the conclusions.

Statistical analysis of the regression plots was done using the JMP IN v 3.2.1 software (SAS Institute Inc.). All tests were two-sided, and a P value of <0.05 was considered significant.

RESULTS
Establishment of a qPCR for the blaKPC gene and the 16S rRNA genes. In this study, we chose to use quantitative PCR as a molecular biology-based method to compare two sampling methods, rectal swabs versus stool samples, using two singleplex assays. We first evaluated the performance of the two qPCR assays (blaKPC gene and 16S rRNA genes) by following guidelines presented by Espy et al. (27) and Bustin et al. (28).

qPCR evaluation. (i) Specificity of the blaKPC and 16S rRNA gene qPCRs. The performance of the 16S rRNA gene PCR assay
and the newly designed \textit{bla}_{KPC} qPCR assay was evaluated by PCR on 35 DNA samples purified from target strains (i.e., carrying the \textit{bla}_{KPC} gene; \(n = 5\)) and nontarget strains (not carrying the \textit{bla}_{KPC} gene; \(n = 30\)) (see Table S1 in the supplemental material).

PCR amplifications revealed a single band of the expected size for the \textit{bla}_{KPC} gene and the 16S rRNA genes, 160 bp and 180 bp, respectively. Using the \textit{bla}_{KPC} primers, only DNA from bacteria that were known to contain the \textit{bla}_{KPC} gene (target bacteria) were amplified (data not shown). We further tested the specificity on DNA extracted from rectal swabs of 2 known KPC carriers. Sequencing of the PCR products showed 100% identity to known KPC or 16S rRNA gene sequences (data not shown). These results show that the primers and the assay conditions for the detection of \textit{bla}_{KPC} and 16S rRNA genes are highly specific.

(iii) Sensitivity of the \textit{bla}_{KPC} and 16S rRNA gene qPCRs. We analyzed the sensitivity and precision of the reaction by serial dilutions of known concentrations of plasmids carrying the \textit{bla}_{KPC}\textsubscript{3} gene or 16S rRNA genes. The amplification was linear over 6 log dilutions for the \textit{bla}_{KPC} (\(r^2 = 0.996\); slope, \(-3.4299\); efficiency = 96%; \(P < 0.0001\); TaqMan qPCR) and over 7 log dilutions for the 16S rRNA gene (\(r^2 = 0.999\); slope, \(-3.350\); efficiency = 98%; \(P < 0.0001\); SYBR green-based qPCR). The detection limit was 10 and 40 plasmid molecules/PCR for the \textit{bla}_{KPC} and 16S rRNA gene assays, respectively. The performance of the assays on purified plasmid DNA (10\(^5\) copies) over 10 runs indicated that the qPCR was highly stable and precise with a mean threshold cycle (\(CT\)) of 18.34 \(\pm\) 0.13 or 19.08 \(\pm\) 0.18 for \textit{bla}_{KPC} and 16S rRNA gene qPCR, respectively.

(iii) Inhibition. DNA extracted from fecal specimens often contains PCR inhibitors that can reduce the sensitivity of the qPCRs (15). Therefore, we evaluated the effect of fecal DNA on the sensitivity of the reaction. We found inhibition only in an undiluted fecal DNA sample, whereas further 10-fold serial dilutions showed no inhibition. Spiking of KPC-Kpn or \textit{K. oxytoca} alone or preprepared in an artificial mix with fecal DNA extracts did not affect the sensitivity of the assay (data not shown).
The CRE/TAB ratio was the value for the culture method, and the number of copies of the KPC gene/number of copies of the 16S rDNA gene was the value for the molecular quantification method. The CRE/TAB ratio was higher in 17/30 (57%) samples, while in 17/30 (57%) samples, they were lower. However, with the use of qPCR-based method, the stool ratios were lower in 15/29 samples (52%), higher in 13/29 samples (45%), and equal in 1/29 samples (3%; Table 2). These results indicate that rectal swabs are as efficient and accurate as stool samples in detecting CRE by either method.

**Enumeration of bla<sub>KPC</sub> populations over time.** bla<sub>KPC</sub>-encoding populations in stool samples and rectal swabs were monitored longitudinally in a single KPC carrier, over 1 month (at 5 time points) using the qPCR method. We calculated the ratios of bla<sub>KPC</sub>/16S rRNA genes (see above) at each time point. The results obtained for stool specimens and rectal swabs were highly similar at all time points (Fig. 3).

**DISCUSSION**

Quantitative studies of colon flora are of great interest; however, performing a quantitative study is complicated due to the need to obtain a stool specimen and to determine its weight (dry or wet weight) for normalization of the data. Rectal swabs have been considered inappropriate for quantifying the gut flora because the total amount of stool represented on each swab is unknown; thus, the lack of a common denominator makes comparison between different samples impossible.

Here we chose an alternative approach for quantifying resistant bacteria in the gut flora. Rather than measuring the amount of the target organisms per unit of stool weight, we determined the concentration of target organism relative to other members of the gut flora. We hypothesized that by using this approach, we would be able to use rectal swabs rather than stool specimens to perform quantitative analysis of the gut flora. To test our hypothesis, we chose to study the KPC-producing CRE.

Our results show that rectal swabs are an appropriate alternative to stool specimens for quantifying KPC-producing CRE load using our newly designed bla<sub>KPC</sub> TaqMan assay combined with the 16S rRNA gene assay. In our proposed methodology, calculating the ratio of carbapenem-resistant bacterial CFU or carbapenem resistance gene copies to an appropriate reference, representing other gut flora, enables determination of the relative CRE load. We found that the results obtained for rectal swabs had better similarity to the results for stool samples when using molecular quantification rather than the culture-based method.

We used two quantification methods to determine bacterial populations in the human colon. There are several limitations to these methods. First, the molecular quantification method accounts for all gut bacteria, while the culture-based method does not account for anaerobic, unculturable, and nonviable bacteria. Second, we assumed that there is only one copy of the bla<sub>KPC</sub> gene.

**Table 2**

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>Total no. of samples analyzed</th>
<th>Stool value =</th>
<th>Stool value &gt;</th>
<th>Stool value &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>30</td>
<td>17 (57)</td>
<td>13 (43)</td>
<td></td>
</tr>
<tr>
<td>Molecular quantification</td>
<td>29</td>
<td>13 (45)</td>
<td>15 (52)</td>
<td></td>
</tr>
</tbody>
</table>

* The CRE/TAB ratio was the value for the culture method, and the number of copies of the bla<sub>KPC</sub> gene/number of copies of the 16S rRNA gene was the value for the molecular quantification method.
but not for stool samples (P method offers a practical reliable alternative, especially in studies which showed a significant culture-based method versus molecular quantification method, tions. This notion was strengthened with the comparison of the relative concentration of a target bacterium to other reference rRNA genes are a good alternative to stool weight to assess the on the 16S rRNA gene for bacterial quantification suffers from IMP, VIM, SPM, and others (29), are also involved. Last, relying the ratio of tient, using quantitative PCR for

Third, we assumed that the resistance to carbapenem is related to the blKPC gene, but it might be that other mechanisms, such as IMP, VIM, SPM, and others (29), are also involved. Last, relying on the 16S rRNA gene for bacterial quantification suffers from bias, as bacteria can harbor different numbers of copies of this gene in their genome (30–32).

In summary, the results of our study demonstrate that 16S rRNA genes are a good alternative to stool weight to assess the relative concentration of a target bacterium to other reference strains. In addition, we show that rectal swabs are as good as stool samples in quantifying CRE relative to other bacterial populations. This notion was strengthened with the comparison of the culture-based method versus molecular quantification method, which showed a significant P value for rectal swabs (P = 0.0461) but not for stool samples (P = 0.1664). We believe that this method offers a practical reliable alternative, especially in studies with a large number of individuals.

ACKNOWLEDGMENTS

We are grateful to Y. Paitan (Meir Medical Center, Israel) and O. Peleg for technical assistance and to L. Temkin for editing the language of the man-

This work was supported by the European Commission FP7: SATURN-Impact of Specific Antibiotic Therapies on the Prevalence of Human Host Resistant Bacteria research grant 241796 and by the European Commission FP7 AIDA project (preserving old antibiotics for the future: assessment of clinical efficacy by a pharmacokinetic/pharmacodynamic approach to optimize effectiveness and reduce resistance for off-patent antibiotics) research grant 278348.

REFERENCES

cation of intestinal bacterial populations by real-time PCR with a uni-

Downloaded from http://aac.asm.org on July 5, 2017 by guest


