Detection of colon flora in peritoneal drain fluid after colorectal surgery: can RT-PCR play a role in diagnosing anastomotic leakage?

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Received: July 2009, Accepted: October 2009.

ABSTRACT

Background and objectives: A semi-quantitative Real-Time PCR strategy was developed to identify indicator organisms for an indication on anastomotic leakage in peritoneal drainage fluid, Escherichia coli and Enterococcus faecalis.

Materials and Methods: The analytical performance of the amplification method was validated with 10 culture-positive and 7 culture negative peritoneal drain fluid samples, obtained from 9 different patients with a colorectal anastomosis.

Results: Real-Time PCR results were fully concordant with the microbiological culture results. However, among the culture negative samples, four false-positive RT-PCR results were found. All false-positives originated from a single patient with a surgical site infection. This may indicate an elevated sensitivity of the RT-PCR method.

Conclusion: The results showed that the semi-quantitative RT-PCR method has the clear potential to be useful as a powerful tool in early detection of anastomotic leakage.

Keyword: Peritoneal drain fluid, anastomic leakage, colon flora. Real Time PCR.

INTRODUCTION

Anastomotic leakage is the most feared complication after colorectal surgery, with leakage rates varying between 2 and 24 % (1-4). Anastomatic leakage (AL) is defined as intestinal content leaking into the peritoneal cavity through an anastomotic defect. Consequently colonic bacteria like Escherichia coli and Enterococcus faecalis spread throughout the peritoneal cavity possibly leading to peritonitis. This, in turn, may lead to sepsis and mortality.

Current diagnostic methods for AL include observation of clinical parameters such as symptoms of AL, wound aspect, change in vital signs and imaging. However, observation of clinical parameters is not specific and can mimic less severe surgical site infections. Imaging modalities like CT-scan or conventional X-ray are not routinely performed but only used for confirmation. A high index of suspicion remains necessary. With these diagnostic methods the interval between operation and the diagnosis of AL varies between 8 and 13 day (1, 5, 6). By this time, the patient will already be severely affected.

Prophylactic drainage is an accepted and widely used method to evacuate blood and wound fluid in the postoperative phase (7, 8). However, regarding AL, controversy remains on whether it has a beneficial effect on leakage rates (9, 10).

In case of AL, the bacterial load in the drainage fluid will increase over time. Detection of this trend could be diagnostic for anastomotic leakage and when detected in the early postoperative phase, morbidity and mortality rates may be reduced. To achieve this goal, a fast and sensitive method like real time polymerase chain reaction (RT-PCR) is required. Cur-
rently, no RT-PCR tests are available to detect colon flora in drainage fluid. Therefore, we have proposed to develop a diagnostic test based on the detection of micro-organisms indicative for AL in peritoneal drain fluid using a quantitative real-time PCR approach. The indicative micro-organisms included *E. coli* and *E. faecalis*, which are both commonly present in the colon. The analytical performance of this strategy has been validated in a pilot study.

**MATERIALS AND METHODS**

**Patient samples.** Seventeen peritoneal drain fluid samples from a culture-positive (n=10) and a culture-negative pool (n=7) were obtained from nine patients enrolled in the pilot study preceding the APPEAL-study (Analysis of Predictive Parameters for Evident Anastomotic Leakage). This is a prospective observational multicenter study on the subject of potential biomarkers for AL in drainage fluid, which is registered in the Dutch Trial Register (http://www.trialregister.nl/trialreg/admin/trtview.asp?TC=1258), study number NTR 1258.

These selected patients underwent colorectal surgery with construction of an anastomosis and they received a closed, passive drainage system. Preoperatively they received prophylactic antibiotics intravenously, cefazoline 2 grams and metronidazole 1, 5 grams.

Postoperatively, the drains’ reservoir was emptied twice daily, at 22.00 h and 9.00 h for 5 days after the intervention. Only the morning collection was drained in a sterile Falcon tube, immediately transported to the laboratory and used for analysis. Cultures were performed directly by inoculating drainage fluid with a 10 µl inoculation loop on two plates, one Columbia Blood agar and one MacConkey agar. Aferwards, the drainage fluid was centrifuged for 10 minutes, 2800 g at 4 degrees Celsius. Supernatant was separated from the cell-pellet and frozen in – 80 degrees Celsius until analysis.

Drain fluid samples were indicated with a letter A through E, each referring to the consecutive postoperative day (A for the collection on postoperative day one, etc.).

**Standard curves.** The semi-quantitative inoculum of indicator organisms potentially present in the peritoneal drain fluid at the time of anastomotic leakage, has been determined by using a reference dilution series of *E. coli* and *E. faecalis* inocula. The reference series were produced by spiking 500 µl of culture-negative drain fluid with a 10 log serial dilution (0, 10 through 10⁵ CFU) of both *E. coli* and *E. faecalis*. The standard curve was generated by comparing the real-time PCR results (threshold cycle or Ct-value) to the inoculum sizes. The approximate inoculum size of the query patient sample was determined after interpolation of its Ct-value within the standard curve. Patient samples were analyzed in duplicate.

**DNA isolation.** Prior to DNA isolation, 500 µl of drain fluid was spiked with 15 µl Phocine Herpes Virus (PhHV, supplied by the Department of Virology, Erasmus MC Rotterdam, The Netherlands) as an internal control. Drain fluid samples used for generating the standard curve were not spiked with PhHV. Subsequently, each sample was centrifuged at room temperature for 5 minutes at 100 g. Supernatant was diluted 10 times in a total volume of 250 µl and centrifuged at room temperature for 5 minutes at 8000 g. The resulting pellet was resuspended in 180 µl buffer containing 20 mMTris, 2mM EDTA, 1% Tween 80 and lysozyme (50 mg/ml) and incubated for 30 minutes at 37°C on a shaking device at 600 rpm (Sanyo Orbital Shaker, München, Germany). DNA extraction was continued using the Macherey-Nagel NucleoSpin® Tissue Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). First, 25 µl protease was added to the sample, followed by incubation at 56°C for 2 hours at 700 rpm in a shaking device (Thermomixer Compact, Eppendorf, Hamburg, Germany). The protocol was executed according to the manufacturer’s instructions. Finally, template DNA was eluted in nuclease-free water in a total volume of 100 µl.

**Semi-quantitative Real-Time PCR.** All PCR reactions were performed in a total volume of 25µl. The PCR-mix for detection of *E. coli* consisted of 12.5µl 2× DyNAmo™ HS SYBR® Green mix (Finnzymes Oy, Espoo, Finland), 0.25 µl forward primer (50pmol/µl), 0.25µl reverse primer (50pmol/µl), 0.25µl 100nM Fluorescein Calibration Dye (Bio-Rad, Hercules, CA, USA), 5 µl template DNA and 6.75µl water. In order to detect *E. faecalis*, a PCR-mix containing 12.5µl 2× DyNAmo™ HS SYBR® Green mix (Finnzymes Oy), 0.45µl forward primer (50pmol/µl), 0.15µl reverse primer (50pmol/µl), 0.25µl 100nM Fluorescein Calibration Dye (Bio-Rad), 5µl template DNA and 6.65µl water was used. Detection of the internal control, PhHV was performed with a PCR-mix consisting of 12.5µl 2× DyNAmo™ HS SYBR® Green mix (Finnzymes Oy), 0.2 µl forward primer (50pmol / µl), 0.25 µl reverse primer (50pmol / µl),
0.25 µl 100 nM Fluorescein Calibration Dye (Bio-Rad), 5µl template DNA and 6.75µl water.

The following primers were used for real-time quantitative PCR: *E. coli uidA* gene forward primer 5'-GGC.TTC.TGT.CAA.CGC.TGT.TT-3', *E. coli uidA* gene reverse primer 5'-CCC.ATG.GAA.GAG.AAA.TGG.AA-3', *E. faecalis* 23S rRNA gene forward primer 5'-AGA.AAT.TCC.AAA.CGA.ACT.TG-3', *E. faecalis* 23S rRNA gene reverse primer 5'-CAG.TGC.TCT.ACC.TCC.ATT-3', PhHV forward primer 5'-GGG.CGA.ATC.ACA.GAT.TGA.ATC-3', PhHV reverse primer 5'-GCC.GTT.CCA.AAC.GTA.CCA.A-3'. The Bio-Rad IQ5 I Cycler (Bio-Rad, Veenendaal, The Netherlands) was used as real-time PCR platform and the PCR conditions for *E. coli*, *E. faecalis* and PhHV were as follows: a single predenaturation step of 15 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 59°C. Finally, the sample temperature was gradually increased to 95°C in order to generate dissociation curves. These curves were used to assess the specificity of the PCR product. The dissociation temperature was 76.0°C for the *E. coli*-specific PCR product and 77.0°C for the *E. faecalis*-specific product. The PCR efficiency was calculated using the slope of the standard curve (efficiency = $10^{-\text{slope}}-1$).

### RESULTS

Characteristics of the patients from which drain fluid was retrieved are depicted in Table 1. None of the patients suffered from clinical anastomotic leakage. The microbiological culture results and semi-quantitative RT-PCR data obtained from the clinical drain fluid samples are summarized in Table 2. Results from both diagnostic techniques were fully concordant for patients 1, 2, 4, 5, 10 and 17. The first drain fluid sample after intervention obtained from patient 8 revealed *E. faecalis*-positive PCR and culture negative result. A similar result was obtained from the first drain fluid sample from patient 9 of which *E. coli*-specific RT-PCR was positive, while no bacterial growth was observed in the microbiological culture. From patient 11 all consecutively sampled drain fluids were analyzed. *E. faecalis* could be cultured 5 days after surgical intervention. With semi-quantitative RT-PCR approximately $10^2 - 10^3$ genome copies (Ct-value 32.4) could be detected in the first sample. The *E. faecalis* genome copy size remained stable for 4 days (Ct-values of 33.9, 31.4 and 33.1 for samples B, C and D respectively) and increased to $10^4$ genome copies on day 5 (Ct-value 28.1 for sample E). All *E. coli* PCR results were negative for patient 11. The PCR product melting or dissociation temperature confirmed the product-specificity, which was 76°C (+/- 0.1°C) for *E. coli* and 77°C (+/- 0.1°C).
Table 2. Comparative analysis of microbiological culture and Real-Time PCR results for the colon flora indicator organisms <i>E. coli</i>, <i>E. faecalis</i> and the internal control.

<table>
<thead>
<tr>
<th>Code</th>
<th>PO day</th>
<th>Culture</th>
<th>&lt;i&gt;E. coli&lt;/i&gt; RT-PCR</th>
<th>&lt;i&gt;E. faecalis&lt;/i&gt; RT-PCR</th>
<th>IC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ct-value</td>
<td>copy number</td>
<td>T melt</td>
</tr>
<tr>
<td>1B</td>
<td>2</td>
<td>&lt;i&gt;S. oralis, S. aureus&lt;/i&gt;</td>
<td>33.8</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>1E</td>
<td>5</td>
<td>&lt;i&gt;E. coli&lt;/i&gt;</td>
<td>37.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>2D</td>
<td>4</td>
<td>&lt;i&gt;E. coli&lt;/i&gt;</td>
<td>38.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>4A</td>
<td>1</td>
<td>&lt;i&gt;S. aureus, B. melaninogenicus&lt;/i&gt;, non-identified anaerobic Gram-pos rod</td>
<td>34.5</td>
<td>-</td>
<td>70-71</td>
</tr>
<tr>
<td>5A</td>
<td>1</td>
<td>&lt;i&gt;E. coli&lt;/i&gt;</td>
<td>37.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>5C</td>
<td>3</td>
<td>&lt;i&gt;E. coli&lt;/i&gt;</td>
<td>36.4</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>8A</td>
<td>1</td>
<td>-</td>
<td>34.2</td>
<td>-</td>
<td>70-71</td>
</tr>
<tr>
<td>9A</td>
<td>1</td>
<td>-</td>
<td>36.4</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>9B</td>
<td>2</td>
<td>-</td>
<td>34.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>10A</td>
<td>1</td>
<td>Mixed skinflora</td>
<td>34.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>10E</td>
<td>5</td>
<td>-</td>
<td>34.1</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>11A</td>
<td>1</td>
<td>-</td>
<td>32.9</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>11B</td>
<td>2</td>
<td>-</td>
<td>34.5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>11C</td>
<td>3</td>
<td>-</td>
<td>37.5</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>11D</td>
<td>4</td>
<td>&lt;i&gt;S. aureus&lt;/i&gt;</td>
<td>38.3</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>11E</td>
<td>5</td>
<td>&lt;i&gt;E. faecalis&lt;/i&gt;</td>
<td>37.1</td>
<td>10^4</td>
<td>76</td>
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<tr>
<td>11C</td>
<td>3</td>
<td>&lt;i&gt;E. faecalis&lt;/i&gt;</td>
<td>38.3</td>
<td>10^4</td>
<td>76</td>
</tr>
<tr>
<td>11E</td>
<td>5</td>
<td>&lt;i&gt;E. faecalis&lt;/i&gt;</td>
<td>37.1</td>
<td>10^4</td>
<td>76</td>
</tr>
</tbody>
</table>

for <i>E. faecalis</i>.

The efficiencies of both the <i>E. coli</i>-specific and <i>E. faecalis</i>-specific RT-PCR protocol were 97%, which can be considered as very good. The non-specific Tm-values could all be explained by primer-dimer formation.

**DISCUSSION**

<i>E. coli</i> and <i>E. faecalis</i> are facultative aerobic colon bacteria, ubiquitously present in faecal samples. Because of these traits, these bacteria are well suited to serve as indicator organisms for diagnosis of anastomotic leakage. Therefore, we have developed a tool for detecting contamination of drainage fluid with intestinal bacteria.

A Real-Time PCR protocol was used to semi-quantitatively detect and to identify species-specific target DNA in purified peritoneal drain fluid samples.

The results obtained from the multiplex molecular-based detection of both <i>E. coli</i> and <i>E. faecalis</i> were concordant to the results obtained with conventional microbiological culture techniques, which are considered to be the gold standard.

This means that with this technique it is possible to reliably determine bacterial contamination of peritoneal drain fluid. The RT-PCR showed several false positive results; <i>E. coli</i> was detected twice with RT-PCR, while it was not found in culture. <i>E. faecalis</i> was detected five times without culture confirmation. This discrepancy may be explained by the preoperative use of antibiotic prophylaxis, cefazoline and metronidazol. Bacteria that are killed by the antibiotics will not grow on culture media, rendering these cultures negative. However, the bacterial genome will remain
intact allowing detection with RT-PCR and generating test (false) positive. This indicates superior sensitivity of RT-PCR to detect bacterial contamination of drain fluid compared to conventional culture. In addition, the detection of the bacterial contamination can be performed more or less independent of differences in drain fluid transport conditions such as temperature and time. Therefore this would allow more flexibility in logistical matters.

The semiquantitative aspect of this RT-PCR is important in the detection of anastomotic leakage. Initial positive samples could be positive for *E. coli* and *E. faecalis* due to intraoperative spill, which occurs when the intestine is opened. However, in case of anastomotic leakage bacterial load will increase over time.

Besides the clinically evident anastomotic leakage, subclinical anastomotic leakage can occur as well. This means leakage of intestinal content occurs but the patient is not affected by it. This condition does not require treatment and is often not even detected.

Both intraoperative spill and subclinical AL may be responsible for positive test results on drainage fluid in this study while no patient developed AL. However, it is expected that clinically relevant AL can be differentiated from subclinical AL and spill by the bacterial load which is probably vastly lower in the latter two conditions.

Drain fluid retrieval is prone to sample contamination through the person retrieving the fluid. However, *E. coli* and *E. faecalis* were selected as indicator bacteria since they are less likely to be brought into the drain fluid another way than through anastomotic leakage or intraoperative spill. The specificity of species-specific DNA targets by Real-Time PCR amplification is guaranteed by the determination of the PCR product dissociation temperature, which is highly specific for each target DNA, with minor tolerance (+/- 0.1°C).

We noticed that optimal results (highest sensitivity) could be retrieved by application of a short centrifugation step and subsequent DNA extraction from the supernatant of the drain fluid.

We have developed a RT-PCR test that allows detection of *E. coli* and *E. faecalis* in peritoneal drain fluid equally sensitive as the gold standard, potentially more sensitive. Whether this test can be considered as a potential test for anastomotic leakage is currently being studied in the APPEAL-study.

**Note.** PO day, postoperative day and refers to the day of sampling; copy number, approximate number of bacteria after interpolation to the standard curve; T melt, melting temperature. Patient samples were analyzed in duplicate; indicated Ct-values, copy numbers and Tm-values are average data within CI 99.0%.

**ACKNOWLEDGEMENTS**

This work was supported by a grant from the “Stichting Technische Wetenschappen (STW)".

**REFERENCES**