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# Optimization of Real-Time PCR Assay for Rapid and Sensitive Detection of Eubacterial 16S Ribosomal DNA in Platelet Concentrates

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**A real-time PCR assay was developed for rapid detection of eubacterial 16S ribosomal DNA in platelet concentrates. The sensitivity of this assay can be hampered by contaminating DNA in the PCR reagents. Digestion of the PCR reagents with *Sau*3AI prior to PCR amplification was effective in eliminating this contaminating DNA without affecting the sensitivity of the assay.**

Bacterial contamination of blood products is a major cause of transfusion-related morbidity and mortality. Due to their storage at 20 to 24°C, platelets are responsible for most of the cases of blood component-associated sepsis (1, 6, 11). Recently, Nadkarni et al. (12) have reported the use of universal primers and probes to estimate the total bacterial load in clinical samples. To detect bacterial contamination in platelet concentrates (PCs), the DNA must be extracted as efficiently as possible and the PCR mixture must not be contaminated by DNA present in the PCR reagents. Poor efficiency of DNA extraction may restrict the sensitivity of the assay, while DNA that is contaminating PCR reagents can serve as a template in PCRs, producing false-positive results. Attempts to reduce the amount of contaminating DNA from, e.g., *Taq* polymerases (3, 4, 5, 7) in real-time PCR have been described previously (9, 10, 13, 15). None of these methods, however, proved very effective on low copy numbers of bacterial ribosomal DNA in the PCR reagents. In addition, these strategies affect the sensitivity of the real-time PCR. Here, a rapid and sensitive PCR assay based on TaqMan technology to detect bacterial contamination in PCs is described.

Two different isolation methods were used to prepare template DNA from PCs: a fully automated method with the MagNA Pure LC instrument (Total Nucleic Acid isolation kit, Roche Diagnostics) (8) and a manual extraction procedure with the NucliSens extraction kit (bioMérieux) (2, 14). Two strategies to reduce contaminating DNA in real-time PCR amplification were evaluated and found to be suited to meet the requirements of the PCR system to detect bacteria in PCs without affecting the high sensitivity of the assay.

To determine the detection limit of the assay, 1 ml of PCs was spiked with 100 μl of serial dilutions of *Escherichia coli*. The number of bacteria added to the PCs ranged from 10 to 20,000 CFU/ml. DNA was subsequently extracted from these spiked PCs using both the MagNA Pure LC instrument and the NucliSens extraction manual method.

In the MagNA Pure method, DNA was extracted from a 200-μl aliquot of spiked material and eluted in a final volume of 50 μl. In the NucliSens method, DNA was purified from a 2,000-μl aliquot of spiked PCs and eluted in a final volume of 50 μl. In addition, DNA was extracted from serial dilutions of a pure culture of *E. coli* by the same MagNA Pure extraction procedure.

A 466-bp fragment of the bacterial 16S ribosomal DNA was amplified using the forward primer 5′-TCCTACGGGAGGC AGCAGT-3′, the reverse primer 5′-GGACTACCAGGGTA TCTAATCCTGTT-3′, and the probe (6-FAM)-5′-CGTATT ACCGCGGCTGCTGGCAC-3′-(TAMRA) (12).

The PCRs were performed in a total volume of 25 μl using the TaqMan Universal PCR Master Mix on the ABI 7700 sequence detection system (Applied Biosystems). The reactions comprised 900 nM (each) forward and reverse primers, 200 nM probe, and 5 μl of template DNA. Negative controls (unspiked PCs) were included throughout the procedure. No-template controls (NTC) with water instead of template DNA were incorporated in each run under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 min and 60°C for 1 min.

The result of each PCR is indicated by a threshold cycle ($C_T$) value. The detection limit of the assay was 1 CFU equivalent/

TABLE 1. Detection limit of real-time PCR assay performed with DNA isolated from PCs (spiked with *E. coli*) using either MagNA Pure or NucliSens extraction procedure

| No. of PCs spiked with *E. coli* (CFU equivalent/PCR) | DNA extraction method | $C_T$[a] |
|---|---|---|
| $8 \times 10^1$ | NucliSens | 27.84 ± 0.43 |
| $8 \times 10^0$ | NucliSens | 31.58 ± 0.13 |
| $4 \times 10^0$ | NucliSens | 32.80 ± 0.21 |
| $2 \times 10^0$ | NucliSens | 33.40 ± 0.26 |
| $1 \times 10^0$ | NucliSens | 34.73 ± 0.70 |
| $2 \times 10^2$ | MagNa Pure | 31.11 ± 0.42 |
| $4 \times 10^1$ | MagNA Pure | 33.42 ± 0.10 |
| $4 \times 10^0$ | MagNA Pure | 34.83 ± 0.83 |
| $1 \times 10^0$ | MagNA Pure | 35.42 ± 0.45 |

[a] Mean ± standard deviation of three independent experiments.

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TABLE 2. Detection limit of assay with DNA extracted from a pure culture of *E. coli* and serially diluted[a]

| Sample type | $C_T$ for template *E. coli* DNA in the following units (CFU equivalents/PCR)[b]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NTC | $7.5 \times 10^{5}$[a] | $7.5 \times 10^{4}$ | $7.5 \times 10^{3}$ | $7.5 \times 10^{2}$ | $7.5 \times 10^{1}$ | $7.5 \times 10^{0}$ | $10^{0}$ |
| Untreated | 34.56 ± 0.08 | 21.39 ± 0.40 | 25.73 ± 0.24 | 29.9 ± 0.21 | 33.37 ± 0.7 | 34.8 ± 0.27 | 35.07 ± 1.04 | 34.58 ± 0.34 |
| +*Sau*3A1 | 37.46 ± 0.09 | 22.62 ± 1.32 | 25.83 ± 0.01 | 30.2 ± 0.06 | 33.77 ± 1.69 | 35.97 ± 1.44 | 36.61 ± 2.39 | 36.03 ± 2.80 |
| +Filter | 40.00 ± 0 | 22.15 ± 0.03 | 26.11 ± 0.81 | 30.51 ± 0.03 | 34.59 ± 0.11 | 37.6 ± 0.08 | 40.00 ± 0 | 40.00 ± 0 |
| +DNase I | 24.53 ± 0.08 | 26.20 ± 0.78 | 21.95 ± 1.03 | 23.47 ± 0.20 | 23.89 ± 0.55 | 24.84 ± 0.02 | 24.25 ± 0.19 | 25.04 ± 0.06 |

[a] DNA extraction was done with the MagNA Pure LC instrument. The results of different pretreatments (digestion with *Sau*3AI, filtration, and treatment with DNase I) are also presented.
[b] Mean ± standard deviation of three independent runs.

PCR (Table 1). This corresponds to 100 CFU equivalents/ml of spiked PCs by the MagNA Pure method and to 10 CFU equivalents/ml with the NucliSens extraction kit. This difference is due to the starting volumes of the clinical samples used to extract DNA.

The initial PCR assays showed a high background, as can be deduced from the threshold value of the NTC (Table 2); this was probably due to the presence of traces of bacterial DNA in the enzymes used in the PCR. Three pretreatment procedures were used on the PCR mixtures for the reduction of this source of contamination: digestion with *Sau*3AI, ultrafiltration, and DNase I.

**Digestion with *Sau*3AI.** *Sau*3AI recognizes GATC sequences and is active in the PCR mixture (to digest 1 μg of substrate DNA in 16 h, a minimum of 0.50 U is needed). Prior to the addition of template DNA, the PCR mixture was subjected to digestion with the enzyme *Sau*3AI (1 U/PCR; New England Biolabs). After incubation at 37°C for 30 min, the solution was heat inactivated at 65°C for 20 min. PCRs were subsequently performed as described above.

**Ultrafiltration.** Before the addition of template DNA, the PCR mixture was filtered with an Amicon Microcon YM-100 centrifugal filter device (Millipore Corp.) as described by Yang et al. (16). The PCR mixture was passed through the YM-100 filter unit at 100 × *g* for 30 min.

**DNase I treatment.** DNase I (Amersham Pharmacia Biotech) (1 U of activity of this nonspecific endonuclease causes an increase in absorbance at 260 nm of 0.001/min/ml at 25°C with DNA as a substrate) was added to the PCR mixture (0.2 U/PCR) without template DNA. The solution was incubated at 37°C for 10 min, followed by heat denaturation at 65°C for 15 min. After this treatment, template DNA was added and the PCR was carried out.

The PCR performed on DNA obtained by either of the two isolation methods described in this paper was very sensitive, and as little as 1 CFU equivalent/reaction mixture could be detected. DNA extraction by the NucliSens method, however, requires a 10-fold larger volume of PCs than the MagNA Pure method: 2,000 instead of 200 μl. The extraction methods also differ in operating time: with the MagNA Pure LC instrument, results can be obtained within 4 h, while the NucliSens extraction kit requires 6 h.

Digestion of the PCR mixture with *Sau*3AI reduced the amplification signal of the NTC by two PCR cycles (Table 2). Amplification of the reaction mixtures containing template DNA increased accordingly, with one or two PCR cycles, while 1 CFU equivalent/reaction was still detectable.

Filtration of the PCR mixture through a YM-100 filtration unit resulted in an increase of the $C_T$ of the NTC to 40, which indicated that contaminating DNA was eliminated. Filtration of the PCR mixture before the addition of template DNA, however, also reduced the sensitivity of the assay (Table 2).

Pretreatment of the PCR mixtures with DNase I led to an unexpected decrease in the $C_T$ values of all PCRs, including that of the NTC (Table 2).

Similar results were found when PCR assays were performed with DNA isolated from spiked PCs (Table 3).

The problem of contamination present in the reagents used in the PCR was solved by two of the three tested methods: digestion with *Sau*3AI or removal of DNA by ultrafiltration. Removal of any trace of DNA from the reaction mixtures allows the achievement of maximal sensitivity of the real-time PCR assay.

Digestion with *Sau*3AI proved to be effective in reducing contamination of the PCR mixture, as shown by the increase of the mean $C_T$ of the NTC. In the case of pretreatment with *Sau*3AI, the detection limit remained 1 CFU equivalent/PCR.

Following the prefiltration procedure, a mean $C_T$ of 40 was generated for the NTC. However, the sensitivity of the assay performed with DNA isolated from spiked PCs was reduced to 30 CFU equivalents/PCR. Thus, *Sau*3AI seems to be effective, especially when detection of only a few molecules of a target

TABLE 3. Detection limit of assay with DNA extracted from PCs spiked with *E. coli* DNA without treatment and pretreated by *Sau*3AI, ultrafiltration, and DNase I

| Sample type | $C_T$ for template DNA isolated from PCs spiked with *E. coli* in the following amts (CFU equivalent/PCR)[a]: | | | | |
|---|---|---|---|---|---|
| | NTC | $3 \times 10^{3}$ | $3 \times 10^{2}$ | $3 \times 10^{1}$ | $3 \times 10^{0}$ |
| Untreated | 34.56 ± 0.08 | 29.59 ± 1.26 | 33.40 ± 0.31 | 34.93 ± 0.66 | 34.72 ± 0.30 |
| +*Sau*3A1 | 37.46 ± 0.09 | 29.82 ± 1.68 | 33.8 ± 0.36 | 35.84 ± 0.43 | 36.01 ± 0.74 |
| +Filter | 40.00 ± 0 | 30.55 ± 0.05 | 34.01 ± 1.11 | 39.37 ± 0.40 | 40.00 ± 0 |
| +DNase I | 24.53 ± 0.08 | 23.63 ± 0.72 | 23.00 ± 0.36 | 23.15 ± 1.08 | 25.40 ± 0.43 |

[a] Mean ± standard deviation of three independent runs.

DNA sequence is required. When a detection limit of <10 CFU equivalents/reaction is not needed, prefiltration of the PCR mixture seems to be more appropriate.

Both methods can be simply integrated into the PCR procedure to overcome the problem of contaminating DNA. Pretreatment with *Sau*3AI extends the assay by 50 min, whereas the prefiltration step prolongs the PCR by 30 min. Pretreatment with DNase I resulted in an unexplainable decrease in the $C_T$ values of all PCRs. This nonspecific endonuclease cleaves both DNA strands. During single-strand digestions, DNase I causes gaps and nicks that can be repaired by DNA polymerase. Possibly, this process makes the target sequence more accessible and the amplification of contaminating DNA more efficient. As a result a decrease in the $C_T$ values of all PCRs occurred. Since the results of this pretreatment were not consistent, the use of this enzyme to reduce contaminating DNA is not favorable.

In summary, real-time PCR assay in combination with the automated MagNA Pure DNA extraction method meets the requirements for pretransfusion screening of PCs: the assay takes only 4 h to be completed and has the capability to detect very small numbers of bacteria.

When contaminating DNA is a limitation, digestion of the PCR mixture with *Sau*3AI can be used to remove contaminating DNA without affecting the sensitivity of the assay.

Before this assay can be implemented as a tool to screen PCs for bacterial contamination, further validation studies are needed.

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