Transfusion Medicine and Hemotherapy

Original Article · Originalarbeit

Transfus Med Hemother 2010;37:21–28
DOI: 10.1159/000265571

Received: October 9, 2008
Accepted: October 28, 2009
Published online: January 7, 2010

Obstacles of Multiplex Real-Time PCR for Bacterial 16S rDNA: Primer Specifity and DNA Decontamination of *Taq* Polymerase

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Key Words
Taq polymerase depleteted of bacterial DNA contamination · DNA decontamination methods

Summary
Background: The detection of a broad range of bacteria by PCR is applied for the screening of blood and blood products with special attention to platelet concentrates. For practical use it is desirable that detection systems include Gram-positive, Gram-negative and non-Gram-stainable bacteria. It is quite challenging to achieve high sensitivity along with a clear negative control with PCR reagents, because especially *Taq* polymerase is contaminated with traces of bacterial DNA. **Methods:** Bacterial DNA decontamination of *Taq* polymerase was attempted by two different methods using the restriction enzyme *Sau* 3A1 and microfiltration. Additionally a commercially available *Taq* polymerase depleted of bacterial DNA was included. A published real-time PCR specific for Gram-negative bacteria was adapted for Gram-positive bacteria, including certain *Staphylococcus* species and *Mycobacteria,* and was used to charge the three *Taq* polymerases depleted of bacterial DNA contamination **Results:** Despite published reports about successful DNA decontamination, all three approaches performed poorly in experiments done in this study. Sensitivity ranged at approximately 50–100 colony forming units (CFU) per PCR reaction for *Escherichia coli* and *Staphylococcus epidermidis*, corresponding to 1,250–2,500 CFU/ml sample material. **Conclusion:** It seems unsatisfying to accept detection limits that high for diagnostic bacterial PCR even if highly multiplexed. Reliable methods for DNA decontamination of *Taq* polymerase are needed and would present one important step towards bacterial DNA detection with high sensitivity.

Schlüsselwörter
Abreicherung bakterieller DNA Kontamination in Taq Polymerase · DNA-Dekontaminationsmethoden

Zusammenfassung
Hintergrund: Die Detektion eines breiten Spektrums verschiedener Bakterien mittels PCR wird bei der Untersuchung von Blut und Blutprodukten eingesetzt und ist bei der Untersuchung von Thrombozytenkonzentraten von besonderem Interesse. Aus praktischen Erwägungen erscheint ein Detektionssystem wünschenswert, das sowohl grampositive und gramnegative, als auch nichtgramfärbbare Bakterien erkennt. Der Wunsch nach hoher Sensitivität bei gleichzeitig eindeutigen Negativkontrollen stellt hierbei eine ganz besondere Herausforderung dar, weil die verwendeten PCR-Reagenzien und ganz besonders die Taq-Polymerase selbst, immer mit Spuren bakterieller DNA verunreinigt sind. **Methoden:** Die Abreicherung bakterieller DNA in *Taq*-Polymerase wurde durch zwei Methoden – den Einsatz des Restriktionsenzyms *Sau* 3A1 bzw. Mikrofiltration – versucht. Zusätzlich wurde eine kommerziell erhältliche *Taq*-Polymerase mit angeblich bereits reduziertem Bakterien-DNA-Gehalt in der Studie eingeschlossen. Eine publizierte «Real-time»-PCR-Methode, die vor allem für die Detektion gramnegativer Bakterien geeignet schien, wurde um die Erkennung grampositiver Bakterien erweitert und erlaubte so schlussendlich auch die Detektierung von Spezies der Gattungen *Staphylococcus* und *Mycobacteria*. Diese PCR wurde für die Beurteilung der drei verschiedenen *Taq*-Polymerasen und der Abreicherung bakterieller DNA eingesetzt. **Ergebnisse:** Trotz bereits publizierter Berichte über erfolgreiche DNA-Dekontamination enttäuschten alle drei in dieser Studie geprüften Ansätze. Die erreichte Sensitivität lag bei zirka 50–100 Colony Forming Units (CFU) pro PCR-Reaktion für *Escherichia coli* und *Staphylococcus epidermidis*, was 1,250–2,500 CFU/ml Probenmaterial entsprach. **Schlussfolgerung:** Trotz Mehrfachspezifität der verwendeten diagnostischen PCR erscheinen die erreichten Detektionslimits unbefriedigend hoch. Nach wie vor besteht ein Bedarf an verlässlichen Methoden für die DNA-Dekontamination von *Taq*-Polymerase, um eine hohe Sensitivität bei der Detektion bakterieller DNA zu erreichen.

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Introduction

The detection of a broad range of bacteria by PCR is usually applied for the screening of a variety of sample materials such as blood and blood products to diagnose bacteriemia and to detect the presence of a bacterial contamination, respectively [1–3].

As already described extensively, 16S rDNA is a highly homologous target sequence for practically all bacteria, that can be used for detection [1, 2]. Specificity and sensitivity are the classical limiting factors for all test systems. With respect to specificity, a correct choice of the amplified target sequence is essential. In order to obtain an optimal detection, DNA sequence which is most suitable for priming can be defined aligning a representative selection of possibly all bacterial rDNAs. With respect to sensitivity, the main problem of detection of bacterial DNA is the achievement of a high sensitivity along with a clear negative control. Up to this day this is an unresolved problem since the reagents used in the PCR process are contaminated with trace amounts of bacterial DNA, especially the *Taq* polymerase, where contamination originates from its production in bacterial cultures. No method for an absolute purification is known to date [4, 5]. A number of trials has been carried out to reduce the amount of contaminating DNA within the enzyme preparation with varying success [6–8]. However, only very recently successful decontamination of *Taq* polymerase from bacterial DNA by using DNAse approaches have been reported. [9]

Real-time PCR is the most advanced PCR method available for the detection purpose. In contrast to other PCR methods, it additionally produces quantitative results. Basically a certain fluorescence intensity is defined as a positive signal. The PCR cycle in which this intensity is reached for the first time is called C(t) value. The more target DNA, the sooner a positive signal will be achieved, which means a low C(t) value.

To decrease bacterial DNA contamination of *Taq* polymerase, we compared three different approaches: The elimination of DNA impurities by using the restriction enzyme *Sau* 3A1, filtration of the polymerase through a microfilter and the use of a 'Low-DNA-(LD)-*Taq* polymerase' which is described as a *Taq* polymerase with a very low concentration of contaminating bacterial DNA by the manufacturer (Ampli*Taq* Gold® DNA polymerase LD, Applied Biosystems). The rationale behind the usage of a restriction endonuclease for decontamination purposes is the cleavage of DNA contaminants in the *Taq* polymerase enzyme solution, which consequently should disable specific amplification of the degraded contaminating DNA later in the PCR. Microfiltration on the other hand relies on the molecular weight differences between the *Taq* polymerase enzyme and contaminating DNA. Using mild centrifugation, the polymerase is expected to pass, whereas contaminating DNA is thought to be withheld by a filter membrane of adequate size exclusion range [10].

The following article reports on our findings in the course of the de novo development of a real-time PCR method to detect bacterial 16S rDNA in anticoagulated whole blood samples. The report addresses two main performance characteristics: specificity improvement for a broad range of bacteria and sensitivity achievable in our hands.

Material and Methods

Extraction of DNA

We used the NucleoSpin® Blood L Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions , extracting 2 ml of whole blood. Blood samples were spiked with different concentrations of *Escherichia coli* and *Staphylococcus epidermidis*. For specificity extension experiments, *E. coli* and *S. epidermidis* were suspended in RPMI1640 (Biochrom AG, Berlin, Germany), instead of blood. The extracted DNA was eluted in 200 µl of elution buffer. In the case of *Mycobacterium tuberculosis* only, purified DNA was used, which has been kindly provided by Prof. W. Prodinger, Department of Hygiene, Microbiology and Social Medicine, Medical University Innsbruck, Austria.

To exclude further bacterial DNA contamination, all working steps must be carried out under sterile conditions.

PCR Mixture

12.5 µl Master Mix (*Taq*Man® Universal PCR Master Mix; Applied Biosystems, Branchburg, NJ, USA), 1 µl of all primers (5 pmol/µl, final concentration in the reaction of 200 nmol/l each), 0.5 µl of the probe (2.5 pmol/µl, final concentration in the reaction of 50 nmol/l), 4 µl of eluted DNA and water were mixed to a final volume of 25 µl per reaction. The following cycling profile was used: 50 cycles of 50 °C 2 min, 95 °C 10 min, (95 °C 15 s, 60 °C 1 min). *E. coli* and *S. epidermidis* were used for sensitivity testing. NucleoSpin® Blood L Kit elution buffer (Macherey-Nagel) was used as negative control. PCRs were run on an ABI Prism 7000 (Applied Biosystems). In our spiking experiments, PCR positivity versus negativity was defined as a difference of at least one C(t) value in the same PCR run.

Bacterial Preparations

Bacterial concentrations are given in colony forming units (CFU), e.g. 50 CFU/PCR means the use of an absolute number of 50 CFU in one PCR reaction, corresponding to a concentration of 1,250 CFU/ml of specimen. CFU were measured using a Spiral System (Spiral System Instruments, Bethesda, MD, USA) as described by the manufacturer. All spiking experiments were performed with bacteria (*E. coli* and *S. epidermidis*) from exponentially growing cultures which rather excludes that dead bacteria not counted by the spiral plater might contribute to the overall copy number. Direct conversions from CFU into bacterial genomes are hindered by the fact that the number of 16S rDNA genes per bacterial genome is variable, and exponentially growing bacteria may already include more than only one copy of their bacterial chromosome [11, 12].

Supplemental Primer Design for Detection of Bacterial 16S rRNA Genes

For optimal positioning of the primers, an alignment containing a total of 110 different bacterial 16S rRNA genes (table 1) and homologous regions of the human mitochondrial DNA was elaborated using GeneRunner Software (Version 3.05, Hastings Software Inc., Westwood, NJ, USA, 1994). We used Gram-positive, Gram-negative and non-Gram-stainable bacteria for our alignment and compared already published primers in the alignment for their detection ability of bacterial DNA [4]. Supplemental primer pairs for the additional detection of *M. tuberculosis* and *Staphylococcus* ssp. were added and tested for their specificity. All forward primers used showed at least 52% (11 of 21 bases) and all reverse primers at least 32% (6 of 19 bases) mismatching to the most homologous

Table 1. List of bacterial species along with their NCBI accession numbers used for alignment and in silico analysis of the 16S rRNA genes

Bacterium	Access-No.	Bacterium	Access-No.
Homo sapiens mitochondrial DNA	NC_012920.1	*Morganella morganii*	AJ301681
Bartonella bacilliformis	M65249	*Mycobacterium bovis*	AY360331
Bartonella grahamii	Z31349	*Mycobacterium tuberculosis*	AJ536031
Bartonella henselae	AJ223778	*Mycobacterium paratuberculosis*	M61680
Bartonella quintana	AJ250247	*Neisseria gonorrhoeae*	X07714
Bartonella taylorii	Z31350	*Proteus mirabilis*	AF128840
Bartonella vinsonii	U26258	*Proteus vulgaris*	AJ233425
Brucella abortus	X13695	*Pseudomonas aeruginosa*	M34133
Brucella canis	L37584	*Pseudomonas cepacia*	M22518
Brucella melitensis	L26166	*Pseudomonas diminuta*	M59064
Brucella neotomae	L26167	*Pseudomonas flavescens*	U01916
Brucella ovis	L26168	*Pseudomonas mendocina*	M59154
Brucella sius	L26169	*Pseudomonas putida*	L28676
Campylobacter coli	M59073	*Pseudomonas testosteroni*	M11224
Campylobacter concisus	L06977	*Rickettsia conorii* strain Malish 7	AF541999
Campylobacter curvus	L06976	*Rickettsia prowazekii*	M21789
Campylobacter fetus	L14633	*Rickettsia rickettsii*	U11021
Campylobacter fetus fetus	M65012	*Rickettsia sibirica*	D38628
Campylobacter fetus veneralis	M65011	*Rickettsia* sp. *(Kytorhinus sharpianus symbiont)*	AB021128
Campylobacter hyointestinalis	M65009	*Rickettsia* sp. *Bar29-like isolate 249*	AF487650
Campylobacter jejuni	M59298	*Rickettsia* sp. *Chad*	AF510102
Campylobacter rectus	L06973	*Rickettsia typhi*	M20499
Campylobacter showae	L06975	*Salmonella enterica* subsp. *enterica (serovar Shomron)*	X80678
Candidatus Rickettsia tarasevichiae	AF503168	*Salmonella enteritidis*	U90318
Citrobacter farmeri	AF025371	*Salmonella give*	X80683
Citrobacter freundii	M59291	*Salmonella paratyphi A*	X80682
Citrobacter rodentium	AF025363	*Salmonella paratyphi B*	U88547
Citrobacter sedlakii	AF025364	*Salmonella paratyphi C*	U88548
Citrobacter werkmanii	AF025373	*Salmonella sofia*	X80677
Chlamydophila pneumoniae	L06108	*Salmonella typhi*	U88545
Chlamydophila psittaci	E17342	*Salmonella typhimurium*	X80681
Chlamydophila trachomatis	D85719	*Serratia entomophila*	AJ233427
Eikenella corrodens (strain FDC 1073)	M22515	*Serratia ficaria*	AJ233428
Enterobacter aerogenes	AF395913	*Serratia fonticola*	AJ233429
Enterobacter agglomerans	AF024613	*Serratia grimesii*	AJ233430
Enterobacter cancerogenus	Z96078	*Serratia marcescens*	M59160
Enterobacter cloacae	AF157695	*Serratia odorifera*	AJ233432
Enterobacter dissolvens	Z96079	*Serratia plymuthica*	AJ233433
Enterobacter nimipressuralis	Z96077	*Serratia proteamaculans*	AJ233435
Escherichia coli	J01859	*Serratia rubidaea*	AJ233436
Francisella philomiragia	Z21933	*Shigella boydii*	X96965
Francisella tularensis	Z21932	*Shigella dysenteriae*	X80680
Francisella tularensis var. novicida	L26084	*Shigella dysenteriae*	X80680
Helicobacter pylori	AY366424	*Shigella flexneri*	X80679
Helicobacter pylori	U00679	*Shigella sonnei*	X80726
Klebsiella oxytoca	AB053117	*Staphylococcus aureus*	L37597
Klebsiella pneumoniae	AY369139	*Staphylococcus epidermidis*	L37605
Klebsiella pneumoniae	X80684	*Streptococcus sanguis*	AF003928
Klebsiella pneumoniae strain CC-88170	AY315447	*Streptococcus pyogenes*	X59029
Klebsiella pneumoniae subsp. *ozaenae*	AF228919	*Streptococcus.oralis*	X58308
Klebsiella rhinoscleromatis	AF009169	*Vibrio aspartigenicus*	M98446
Klebsiella sp. *CC-88168*	AY315448	*Vibrio cholerae*	L05178
Leptospira biflexa	Z12821	*Vibrio parahaemolyticus*	M59161
Leptospira illini	M88719	*Yersinia enterocolitica*	AF366378
Leptospira interrogans	M71241	*Yersinia pseudotuberculosis*	Z21939
Leptospira kirschneri	Z21628		

Table 2. Sequences of the primers and the *Taq*Man-probe[a].

Primer name	Origin	Sequence
Primer F (forward)	Corless et al., 2000 [4]	5'-CCATGAAGTCGGAATCGCTAG-3'
Primer T (reverse)	Corless et al., 2000 [4]	3'-ACTCCCATGGTGTGACGG-3'
Primer 16S-Mtb-F (forward)	This work	5'-CCGTGAAGTCGGAGTCGCTAG-3'
Primer 16S-Mtb-R (reverse)	This work	5'-CCACTTTCATGACGTGACGG-3'
Primer 16S-Staph-F (forward)	This work	5'-CCATGAAGCTGGAATCGCTAG-3'
Primer 16S-Staph-R (reverse)	This work	5'-CACTCTCGTGGTGTGACGG-3'
Probe	Corless et al., 2000 [4]	FAM-5'-CGGTGAATACGTTCCCGGGCCTTGTAC-3'-TAMRA

[a]The *Taq*Man probe was marked with 6-carboxyfluorescein (FAM) at the 5'-terminus and with 6-carboxytetramethylrhodamine (TAMRA) at the 3'-terminus.

a

Escherichia coli	CTCGACT	CCATGAAGTC	GGAATCGCTA	GTAATCGTGG	ATCAG
Homo sapiens mitochondrial	ACTT-AG	GGTC----GT	---T-TAGC-	----A-TAA-	-GT--
Mycobacterium tuberculosis	------C	--G-------	---G------	-------CA-	-----
Mycobacterium bovis	------C	--G-------	---G------	-------CA-	-----
Mycobacterium intracellulare	------C	----------	---G------	-------CA-	-----
Staphylococcus haemolyticus	------A	--------**CT**	----------	--------A-	-----
Staphylococcus aureus	------A	--------**CT**	----------	--------A-	-----
Staphylococcus epidermidis	------A	--------**CT**	----------	--------A-	-----
Primer F		CCATGAAGTC	GGAATCGCTA	G – 3'	
Primer 16S-Mtb-F		CCGTGAAGTC	GGAGTCGCTA	G – 3'	
Primer 16S-Staph-F		CCATGAAG**CT**	GGAATCGCTA	G – 3'	

b

Escherichia coli	TCTTTTGCAA	CCCACTCCCA	TGGTGTGACG	GGCGGTGTGT
Homo sapiens mitochondrial	--C----A-G	TAT---TGAG	GA-G------	----------
Mycobaterium tuberculosis	--GGG--TT-	--G---TT--	--AC------	----------
Mycobacterium bovis				
Mycobacterium intracellulare	----------	----------	----------	----------
Staphylococcus hämolyticus	--GGG--TT-	-A**A**----**T**-**G**	----------	----------
Staphylococcus aureus	--GGG--TT-	-A**A**----**T**-**G**	----------	----------
Staphylococcus epidermidis	--GGG--TT-	-A**A**----**T**-**G**	----------	----------
Primer T (rev. comp)		ACTCCCA	TGGTGTGACG	G – 3´
Primer 16S-Mtb-R (rev. comp)		CCACTTTCA	TGACGTGACG	G – 3´
Primer 16S-Staph-R (rev. comp)		**C**ACTC**T**C**G**	TGGTGTGACG	G – 3´

Fig. 1. Positioning of **a** the forward- and **b** reverse primers: The sequence of parts of the 16S rRNA gene of *E. coli* is shown. Genetic differences to *Mycobacteriae* (gray) and *Staphylococci* (bold) are shown. Homologous regions of human mitochondrial DNA are displayed for illustration. Modifications of primers T lead to an improved detection of mycobacterial DNA (primer 16S-Mtb-F and -R, gray) and gram-positive bacteria (primer 16S-Staph-F and -R, bold).

human mitochondrial DNA sequence. Therefore, combined specificities were thought to be restrictive of all homologous human mitochondrial DNA sequences. This assumption was not tested independently. Sequences of the primers used and the probe are shown in table 2. The position of the different primers are given in figure 1.

DNA Decontamination Using the Restriction Enzyme Sau *3A1*

Since definitive *Taq* polymerase concentration in the *Taq*Man Universal PCR Master Mix was not provided by the manufacturer, we estimated that 20 µl of Master Mix contained approximately 1 U of *Taq* polymerase. PCR reaction mixtures containing 1 U, 3 U and 6 U of *Sau* 3A1 (Roche Diagnostics, Mannheim, Germany) per 1 U of *Taq* polymerase were prepared. The complete mixture, containing the primers, the *Taq*Man probe, the water and the *Taq*Man Universal PCR Master Mix were incubated at 37 °C for 30 min. Inactivation of the restriction enzyme was done by heating at 96 °C for 2 min, following the protocol of Carroll et al. [7]. After inactivation, 4 µl of eluted bacterial test DNA were added.

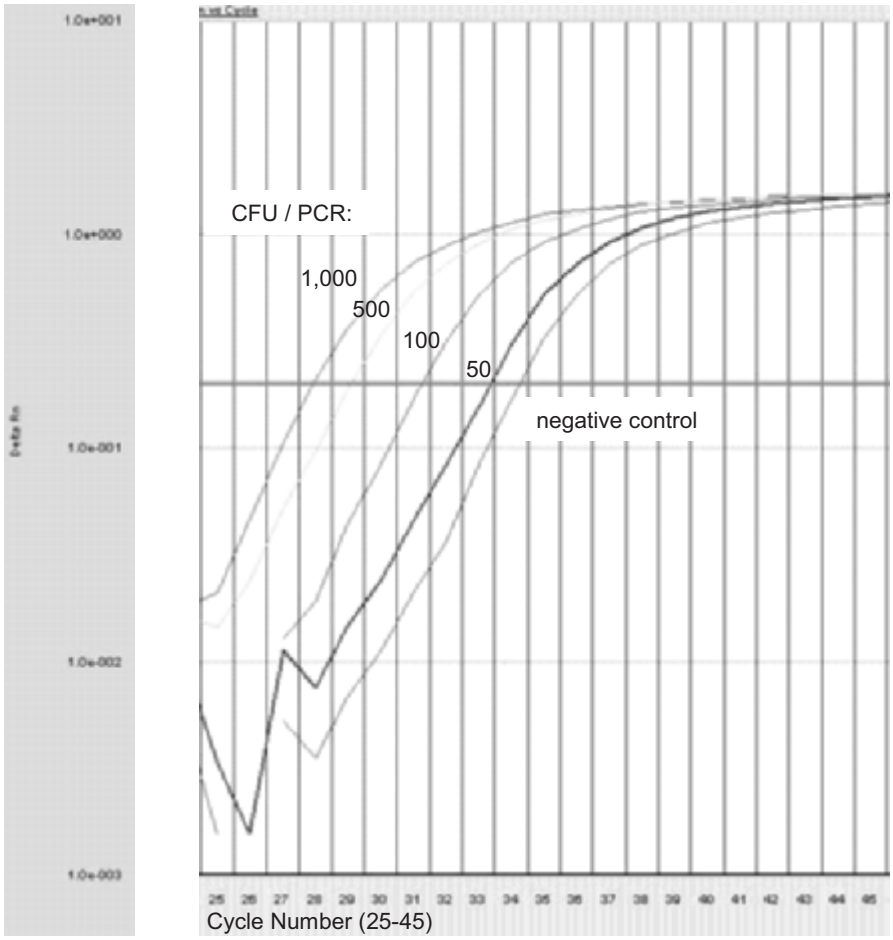

Fig. 2. Exemplary amplification plot of different *E. coli* dilutions. Amplification plots of 1,000, 500, 100, 50 CFU per PCR and negative control (no template control) for PCR cycles 25 to 45 are shown. C(t) = 27.90, C(t) = 29.08, C(t) = 31.21 and C(t) = 33.30 represent 1,000, 500, 100, and 50 CFU/PCR, respectively. C(t) = 34.21 represents the negative control.

Usage of a Commercially Available Taq *Polymerase Depleted of Bacterial DNA*

We also used Ampli*Taq* Gold® DNA polymerase LD (Low DNA, 250 U with gold buffer and $MgCl_2$ solution; Applied Biosystems). This lot, according to the manufacturer, contains a maximum of 10 copies of 16S rDNA per 5 U of polymerase.

DNA Decontamination Using a Microfilter

Furthermore, a microfilter with a cut-off molecular size of about 100 kDa (Amicon Microcon YM-100 Centrifigal Filter Unit; Millipore Corporation, Bedford, MA, USA) was used to diminish the amount of contaminating DNA of the polymerase. Using a centrifugal device, the polymerase and the water were filtered through the filter's membrane at 100 *g* for 30 min according to the protocol of Yang et al. [10].

Results

Detection of Gram-Positive Bacterial and Mycobacterial 16S rDNA

According to our in silico analysis, primers used by Corless et al. [4] were well suitable for the detection of Gram-negative bacteria but less qualified to detect Gram-positive bacteria, especially certain *Staphylococcus* spp. Additionally – judging from our alignment – Corless' primers also seemed to be inappropriate for the detection of mycobacterial DNA. Therefore, we considered to use Corless' primers for the detection of Gram-negative bacteria only and added two additional primer pairs to increase the detection range to Gram-positive, non-Gram-stainable *M. tuberculosis* and especially certain *Staphylococcus* spp. The primer sequences for the detection of Gram-positive bacteria (primer 16S-Staph-F and primer 16S-Staph-R), Gram-negative bacteria (primer F and primer T from the publication of Corless et al. [4]) and non-Gram-stainable bacteria (primer 16S-Mtb-F and primer 16S-Mtb-R) are shown in figure 1.

In order to validate our in silico findings, the primers and *Taq*Man probe were tested for their specificity with DNA preparations of *E. coli*, *S. epidermidis* and *M. tuberculosis* suspended in RPMI1640. Primers used by Corless et al. [4] showed pooror even negative signals for *M. tuberculosis*, and Gram-positive bacteria were only detectable in very high concentrations (>10,000 CFU), as exemplified by *S. epidermidis* when compared to *E. coli*. After addition of our newly designed two primer pairs (fig. 1), specificity for Gram-positive bacteria increased clearly as exemplified using DNA of *S. epidermidis*. Even mycobacterial DNA was now detectable as exemplified by *M. tuberculosis*. C(t) values before adaptation of the system were 32.2 for *E. coli* (50 CFU) and 32.1 for *S. epidermidis* (1,000 CFU); *M. tuberculosis* showed comparable values to the negative control (C(t) = 33.5. After adaptation

C(t) values for 50 CFU of *E. coli* were still 32.2, but the detection limit for *S. epidermidis* was improved to a value of 50 CFU (C(t) = 32.2), and DNA of *M. tuberculosis* was now detectable in several concentrations like 2 ng (C(t) = 22.3), 0.2 ng (C(t) = 26.1) and 0.02 ng (C(t) = 29.5). Whereas 0.02 ng of DNA of *M. tuberculosis* corresponds to approximately 4,000 single bacterial genomes, the low C(t) value of only 29.5 still indicated a higher potential with respect to sensitivity [13].

Detection Limit without Taq *Polymerase Decontamination Procedures*

Each experiment included NucleoSpin elution buffer as a negative control at least in duplicates, and mean values of them were used for calculations. C(t) values of these reagent controls were remarkably reproducible and amount to 33.1–35.0 for all controls in all 6 experiments performed (n = 15 single controls in total; mean C(t) = 33.9; standard deviation C(t) = 0.64). Due to the presence of contaminating bacterial DNA, as expected, we never observed a completely negative amplification result, even in our planned negative controls. Therefore and in order to avoid general sensitivity reduction by competitive internal controls, we also refrained from considering a separate positive amplification control in our experiments.

Positive samples were 8-step serial dilutions of *E coli* and *S. epirdermidis* in whole blood in all experiments calculated to a final concentration of 5,000, 1,000, 500, 250, 100, 50, 10 and 1 CFU/PCR. For illustration, an experimental PCR run of *E. coli* with 1,000, 500, 100, 50 CFU/PCR and a negative control is shown in figure 2. CFU concentrates of 1 CFU/PCR were identical to our negative control in all experiments performed, with C(t) values of 33.1–34.7 (n = 6 single tests with 1 CFU/PCR in total; mean C(t) = 33.6; standard deviation C(t) = 0.32). The 1 CFU/sample setup can be regarded as negative control also for whole blood and as a clear indication that the PCR does not cross-react with human mitochondrial DNA.

Defining a difference of at least 1 C(t) as threshold between PCR positivity and negativity, 3 out of 6 (50%) *E. coli* and 4 out of 6 (67%) *S. epidermidis* dilutions would have been typed positive at concentrations as low as 50 CFU/PCR, and all 6 (100%) *E. coli* and 6 (100%) *S. epidermidis* dilutions of 100 CFU/PCR would have been found correctly positive. These data would correspond to a detection limit of our method of 1,250 and 2,500 bacterial CFU/ml sample material for *E. coli* and *S. epidermidis*, respectively.

DNA Decontamination of Taq Polymerase Using the Restriction Enzyme Sau *3A1*

At a mixing ratio of *Taq*Man Universal PCR Master Mix to *Sau* 3A1 of 1 unit : 1 unit restriction enzyme, no raise of C(t) values, was observed compared to the tests without *Sau* 3A1, indicating a successful removal of contaminating bacterial DNA in the negative controls. When using *E.coli* (10,000 CFU) as a positive control, increasing the PCR Master Mix to *Sau* 3A1 ratio from 1:1 to 1:3 or 1:6, C(t) values raised, at first view indicating a decrease of contaminating bacterial DNA. However, the C(t) values of the negative control were also raised accordingly (table 3). Furthermore, the C(t) value difference between positive and negative control also decreased (table 3), indicating a desensitization of the detection system in general. Comparable data were observed using *S. epidermidis* as a positive control. The above mentioned observations indicate that the treatment of the *Taq*Man Universal PCR Master Mix with the restriction enzyme *Sau* 3A1 in our protocol seemed to deteriorate *Taq* polymerase activity only and did not have any beneficial effect on the general test system sensitivity.

Table 3. Bacterial DNA decontamination trial of *Taq* polymerase using the restriction enzyme *Sau* 3A1[a]

	Untreated	*Sau* 3A1	
		1:3	1:6
E. coli (10,000 CFU)	24.51	30.95	33.26
Negative control (elution buffer)	32.96	38.62	39.68
Delta C(t) negative control – *E. coli*	8.45	7.67	6.42

[a]C(t) values of untreated and mixing ratios of *Taq*Man Universal PCR Master Mix to *Sau* 3A1 of 1 unit : 3 units restriction enzyme and 1:6 ratio are shown.

Testing the Commercially Available Taq *Polymerase Depleted of Bacterial DNA*

We were unable to observe a pronounced difference between the mean C(t) values for the negative control duplicate performed with Ampli*Taq* Gold DNA polymerase LD (C(t) = 32.5) and the 'regular' *Taq*Man Universal PCR Master Mix (C(t) = 33.1). The same observations were made for the 8-step serial dilution (5,000, 1,000, 500, 250, 100, 50, 10 and 1 CFU/PCR) of both positive controls, *E. coli* and *S. epidermidis*. Therefore, we could not confirm a lower bacterial DNA contamination in the depleted DNA polymerase compared to those commonly used in real-time PCR master mixes.

DNA Decontamination by the Use of a Microfilter

We were unable to observe a pronounced difference between the mean C(t) values for the negative control duplicate performed with filtered (C(t) = 34.6) and unfiltered *Taq* polymerase (C(t) = 33.7). The same observations were made for a 8-step serial dilution (5,000, 1,000, 500, 250, 100, 50, 10 and 1 CFU) of both positive controls, *E. coli* and *S. epidermidis*. Thus, we were unable to proof a pronounced effect of the DNA decontamination by microfiltration.

Discussion

Even if real-time PCR is used as a tool, detection of microbial DNA is difficult. Current PCR reagents – especially the *Taq* polymerase itself – is contaminated with bacterial DNA origi-

nating from its bacterial production host, and the *Taq* polymerase showed a high natural affinity to DNA, both complicating the enzyme purification process.

One possibility to improve the sensitivity may be an optimization of the bacterial DNA preparation and PCR setup. An increase of DNA concentration in the final PCR may be achieved by rising the quantity of sample material for one single DNA preparation, reducing the elution volume of e.g. column-based DNA preparation procedures and increasing the absolute volume of DNA eluate per PCR.

Another way to improve the system's general detection sensitivity would be to reduce the contaminating bacterial DNA input mainly thought to be found in *Taq* polymerase enzyme preparations. Therefore, in this study two methods for DNA decontamination of *Taq* polymerase and a commercially available *Taq* polymerase depleted of bacterial DNA were tested to increase sensitivity of bacterial DNA detection using 16S rRNA gene real-time PCR. However, treatment of PCR master mix with a *Sau* 3AI restriction enzyme in order to degrade contaminating DNA only reduced *Taq* polymerase activity. This has been also found by others [7]. Neither did microfiltration of the PCR master mix lower the 'false' positivity of the negative control in our hands. Other reports on this method are controversial: Yang et al. [10] described an improvement, whereas Mohammadi et al. [14] found deterioration of sensitivity after filtration. With respect to commercially available *Taq* polymerase depleted of bacterial DNA, as already suggested by Rand et al. [5], again in our tests this approach did not show any influence on the total sensitivity. Additionally variations in bacterial DNA contamination found in different batches of *Taq* polymerase have been reported by Petershofen et al. [15]. Therefore, our discouraging findings may be due to the fact that only one batch of one specific distributor has been tested. Other *Taq* polymerases depleted of bacterial DNA are currently available, but we did not aim to perform a market survey of changing qualities of *Taq* polymerase suppliers. If users wish to become independent of batch-dependent bacterial DNA contamination in *Taq* polymerase, our preliminary conclusion is that other methods will be needed to address this problem. Currently, at least two other methods are reported to be effective: one of them uses ethidium monoazide [16], the other DNAse I to decontaminate *Taq* polymerase before its use [17].

Another approach could be to develop 'multiplex' PCRs specific for all bacterial species of interest but the *Taq* polymerase-producing host. However, since *E. coli* is both a pathogen which has to be detected and an organism widely used for *Taq* polymerase production, this seems illogical. Still, if *Taq* polymerase could be produced in a bacterial host, non pathogenic for humans and with unique 16S rDNA genes, this would offer an 'all but host' priming specificity for diagnostic test systems. An additional problem is that the pharmaceutical companies do not seem to be eager to reveal the species (and its 16S rDNA) of the *Taq* polymerase-producing host. Declaration of such 16S rRNA gene sequences of production bacteria would certainly help to develop more specific and contamination-independent PCR assays.

Although earlier descriptions of bacterial DNA decontamination of *Taq* polymerase were described as effective, taken together none of them proved functional in our hands. We were unable to achieve an improvement in sensitivity lower than for a total of 100 CFU per PCR reaction, which corresponds to a concentration of 2,500 CFU/ml sample material. Comparable detection limits, e.g. 238 fg corresponding to 48 *E. coli* cells per PCR, which is comparable to 50–100 CFU/PCR in our study, have already been described elsewhere [18]. However, even much lower detection limits, e.g. 175 CFU/ml specimen of *Propionibacterium* spp. [14] and 150 *E. coli* and 20 *S. epidermidis* CFU/ml whole blood [19], have been reported.

We are unable to explain the sensitivity reached by Mohammadi et al. [14].

However, highly variable number of rRNA operon numbers in the genomes of different bacterial species and possible aggregation of bacteria might offer some explanations for variable sensitivity reports [11, 12]. To compare different protocols, standardized bacterial reference material with known copy numbers of rRNA operons would be needed. Sensitivity reached by Mühl et al. [19] may partially be explained by the usage of an optimized DNA preparation strategy as concentration of starting specimens improved their sensitivity by a calculative factor of 6.25 compared to our method. The capacity of this potential improvement was not tested during our experiments.

In other settings, e.g. single cells in preimplantation diagnosis, PCR has proven its enormous potential already decades ago [20]. It is therefore unsatisfying to accept detection limits as high as 100 copy numbers/PCR in the course of bacterial PCR.

Alternatively, instead of bacterial DNA detection, direct microbiological methods, such as Scansystem™ (Hemosystem, Marseille, France) and BacT/ALERT® (bio-Mérieux, Nürtingen, Germany), can be taken into consideration for routine testing of leukoreduced platelet-rich plasma-derived platelets and apheresis platelets [21]. Last but not least, blood and blood products void of bacterial contamination may also be granted, not only by means of diagnosis but also by pathogen inactivation applications [22, 23]. Hence, detection of bacterial contamination of blood and blood products is not only a technical challenge, but a rather multifactorial topic in transfusion medicine, also dependent on medical, scientific and organizational parameters, best managed by experts in the field.

Acknowledgments

DNA of *M. tuberculosis* was kindly provided by Prof. Dr. W. Prodinger, Institute of Microbiology and Social Medicine, Medical University Innsbruck, Austria.

Disclosure

The authors declared no conflict of interest.

References

1 Mothershed EA, Whitney AM: Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. Clin Chim Acta 2006;363(1–2):206–20.

2 Barken KB, Haagensen JA, Tolker-Nielsen T: Advances in nucleic acid-based diagnostics of bacterial infections. Clin Chim Acta 2007;384(1–2):1–11.

3 Zhang T, Fang HH: Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Appl Microbiol Biotechnol 2006;70(3):281–9.

4 Corless CE, Guiver M, Borrow R, Edwards-Jones V, Kaczmarski EB, Fox AJ: Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. J Clin Microbiol 2000;38(5):1747–52.

5 Rand KH, Houck H: *Taq* polymerase contains bacterial DNA of unknown origin. Mol Cell Probes 1990;4(6):445–50.

6 Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB: Simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 2001;39(4):1553–8.

7 Carroll NM, Adamson P, Okhravi N: Elimination of bacterial DNA from *Taq* DNA polymerases by restriction endonuclease digestion. J Clin Microbiol 1999;37(10):3402–4.

8 Klaschik S, Lehmann LE, Raadts A, Hoeft A, Stuber F: Comparison of different decontamination methods for reagents to detect low concentrations of bacterial 16S DNA by real-time-PCR. Mol Biotechnol 2002;22(3):231–42.

9 Silkie SS, Tolcher MP, Nelson KL: Reagent decontamination to eliminate false-positives in *Escherichia coli* qPCR. J Microbiol Methods 2008; 72(3):275–82.

10 Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE: Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J Clin Microbiol 2002;40(9):3449–54.

11 Bremer H, Dennis PP: *Escherichia coli* and *Salmonella typhimurium;* in Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, et al. (eds)*:* Cellular and Molecular Biology. Washington, DC, American Society for Microbiology, 1996, pp 1553–1569.

12 Klappenbach JA, Saxman PR, Cole JR, Schmidt TM: rrndb: the Ribosomal RNA Operon Copy Number Database. Nucleic Acids Res 2001;29(1):181–4.

13 Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al: Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 1998;393(6685):537–44.

14 Mohammadi T, Pietersz RN, Vandenbroucke-Grauls CM, Savelkoul PH, Reesink HW: Detection of bacteria in platelet concentrates: comparison of broad-range real-time 16S rDNA polymerase chain reaction and automated culturing. Transfusion 2005;45(5):731–6.

15 Petershofen EK, Fislage R, Faber R, Schmidt H, Roth WK, Seifried E: Detection of nucleic acid sequences from bacterial species with molecular genetic methods. Transfus Sci 2000;23(1):21–7.

16 Rueckert A, Morgan HW: Removal of contaminating DNA from polymerase chain reaction using ethidium monoazide. J Microbiol Methods 2007; 68(3):596–600.

17 Tondeur S, Agbulut O, Menot ML, Larghero J, Paulin D, et al: Overcoming bacterial DNA contamination in real-time PCR and RT-PCR reactions for LacZ detection in cell therapy monitoring. Mol Cell Probes 2004;18(6):437–41.

18 Nadkarni MA, Martin FE, Jacques NA, Hunter N: Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. 2002;148(Pt 1):257–66.

19 Mühl H, Kochem AJ, Disqué C, Sakka SG: Activity and DNA contamination of commercial polymerase chain reaction reagents for the universal 16S rDNA real-time polymerase chain reaction detection of bacterial pathogens in blood. Diagn Microbiol Infect Dis 2008; *doi:10.1016/j.diagmicrobio.2008.07.011.*

20 Erlich HA, Gelfand D, Sninsky JJ: Recent advances in the polymerase chain reaction. Science 1991; 252(5013):1643–51.

21 Schmidt M, Hourfar MK, Heck J, Weis C, Montag T, Nicol SB, Seifried E: Scansystem™ enables rapid and sensitive bacterial detection in platelets stored in additive solution with implementation of standard positive control capsules. Transfus Med Hemother 2006;33(3):274–8.

22 Corash L: Inactivation of viruses, bacteria, protozoa and leukocytes in platelet and red cell concentrates. Dev Biol (Basel) 2000;102:115–23.

23 van Rhenen D, Gulliksson H, Cazenave JP, et al: Transfusion of pooled buffy coat platelet components prepared with photochemical pathogen inactivation treatment: the euroSPRITE trial. Blood 2003;101:2426–33.