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Occurrence of Fungal DNA Contamination in PCR Reagents: Approaches to Control and Decontamination

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Nucleic acid amplification techniques permitting sensitive and rapid screening in patients at risk for invasive fungal infections are an important addition to conventional fungal diagnostic methods. However, contamination with fungal DNA may be a serious threat to the validity of fungal amplification-based assays. Besides rigorous handling procedures to avoid false-positive test results from exogenous sources, we have implemented protocols for comprehensive assessment of fungal contamination in all materials involved in the analytical process. Traces of fungal DNA were found in different commercially available PCR reagents, including lyophilized primers, TaqMan probes, and master mix solutions. These contaminants resulted in a considerable rate of false-positive tests in panfungal real-time PCR analysis. To address this problem, we have established a decontamination protocol based on the activity of a double-strand specific DNase. Using this approach, we have significantly reduced the frequency of false-positive test results attributable to contaminated reagents. On the basis of our findings, we strongly recommend routine monitoring of all reagents used in fungal PCR assays for the presence of relevant contaminants. As long as fungal-grade reagents are not readily available, pretreatment methods facilitating elimination of fungal DNA are critical for reducing the risk of false-positive results in highly sensitive molecular fungal detection assays.

Nucleic acid amplification-based techniques can detect minute quantities of fungal DNA and can enable fast and sensitive screening in patients at risk for invasive fungal infections (IFI). They can overcome the limitations of conventional diagnostic methods that are slow or have inadequate sensitivities (1–3). However, a major drawback of highly sensitive PCR applications is that even low-level contaminations can lead to false-positive results (4). This is particularly evident when broad-range screening methods are applied because their ability to detect contaminating DNA from many different fungal species renders them more vulnerable to trace amounts of nucleic acid contamination than species-specific assays (1). This problem is already well described for universal bacterial assays, where PCR testing based on 16S rRNA gene amplification can be compromised by DNA contamination of reagents derived from bacterial sources such as *Taq* DNA polymerase or uracil-N-glycosylase (5, 6).

Problems with fungal contamination during PCR-based assays have been previously reported. Airborne conidia or spores and trace amounts of fungal nucleic acids in commercially available enzymes used for fungal cell lysis and in components of DNA extraction kits were identified as possible sources of contamination (7–10). Here we provide the first evidence for the presence of fungal DNA contaminants in lyophilized primers and TaqMan probes as well as in master mix solutions for real-time PCR analysis purchased at renowned biotechnology companies. Using a TaqMan-based panfungal PCR assay (11) established for sensitive screening of immunocompromised patients at risk for IFI, we detected traces of fungal DNA in several batches of the indicated reagents. To overcome the risk of false-positive PCR test results attributable to this issue, we have successfully applied a decontamination method based on the activity of a double-strand specific DNase (dsDNase).

MATERIALS AND METHODS

Measures to prevent carryover contamination. Rigorous handling conditions were implemented to avoid false-positive test results from exogenous sources. All working steps were performed in laminar airflow (LAF) systems that were UV decontaminated on a daily basis. Laboratory coats, fresh gloves, and sterile arm sleeves were worn and changed frequently. In addition, the LAF surfaces, pipettes, and all reagents transferred into the LAF workbench were regularly cleaned with hypochlorite solutions. Handling of the PCR reagents, including the preparation of primer and probe and master mix aliquots as well as all pipetting steps, was performed using a LAF workbench that was separated (in a different room) from the area where fungal template DNA was added to the PCR. Lyophilized primers were reconstituted in DNA-free water. DNA-free disposables were used throughout. Master mix solutions contained uracil-N-glycosylase (UNG) to avoid carryover contamination from PCR amplicons.

DNase-mediated digestion of spiked fungal DNA. Digestion of quantified *Candida albicans* DNA was performed in 30-μl reaction mixtures containing 0.75 μl (5 U/μl) of dsDNase (DNA decontamination kit; ArcticZymes, Tromsø, Norway), 0.75 μl of 1 mM dithiothreitol (DTT), filter-sterilized Tris buffer (20 mM Tris-HCl, 5 mM $MgCl_2$ [pH 8]), and different amounts of fungal DNA ranging from 100 fg to 10 pg. The samples were subjected to vortex mixing and briefly centrifuged to remove liquid from the lid. Digestion of genomic DNA was performed at 40°C for 30 min followed by an enzyme deactivation step at 65°C for 15 min.

The readout was performed by the use of a yeast-specific PCR of a panfungal real-time assay described earlier (11). Three independent experiments were performed. Negative controls (DNA-free water as the template) as well as positive controls (100 fg of completely untreated *C. albicans* DNA) were included on the same PCR plate.

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TABLE 1 DNase-mediated decontamination of oligonucleotides

Oligonucleotide batch[a]	Master mix (batch 1)[b]			
	Untreated		Treated	
	% false positives	DNA amt (fg)	% false positives	DNA amt (fg)
1	26.7 ($n = 60$)	22.29 ± 10.21 [$n = 16$]	1.7 ($n = 60$)	26.11 [$n = 1$]
2	12.5 ($n = 40$)	24.69 ± 9.34 [$n = 5$]	0.0 ($n = 40$)	

[a] The oligonucleotides include the primers and the TaqMan probe.
[b] DNA amounts were estimated using a standard curve obtained from *C. albicans* DNA. Mean DNA amounts and standard deviations are displayed. The numbers of specimens analyzed are indicated in parentheses and the numbers of contaminated reactions in brackets.

Decontamination of reagents. DNase-mediated decontamination of complete PCR mixtures, including PCR primers, TaqMan probes, and master mix solutions, performed according to the manufacturer's instructions (ArcticZymes) was not successful, thus requiring separate decontamination of individual PCR components. On the basis of the experience from the experiments performed with spiked fungal DNA, decontamination conditions were optimized for each reagent. Lyophilized primers and probes were reconstituted in DNA-free water at 100 μM. A separate reaction mixture was prepared for each primer and probe. Primers and probes were diluted in filter-sterilized Tris buffer to concentrations of 10 μM (for each primer) and 2.5 μM (probe). Reactions were preheated at 40°C for 5 min to allow partial denaturation of double-stranded hairpins or primer dimers followed by addition of 12.5 U DNase and 2.5 mM DTT. Digestion was performed at 40°C for 30 min followed by an enzyme deactivation step at 65°C for 15 min. Decontamination reactions were usually performed in a total volume of 100 μl. Decontaminated primer and probe solutions were stored in aliquots at −20°C. For independent decontamination of master mix solutions, 2.5 μl DNase (5 U/μl) and 2.5 μl of 1 mM DTT were added per 100 μl. DNase treatment was performed at 37°C for 20 min followed by an enzyme deactivation step at 60°C for 20 min. Decontaminated master mixes were used immediately. Decontamination with the heat-labile version of the dsDNase (ArcticZymes) was performed in the presence of 0.1 U/μl DNase and 1 mM DTT. DNase treatment was performed at 37°C for 20 min followed by an enzyme deactivation step at 55°C for 15 min. Additionally, experiments were performed under less aggressive conditions at 25°C for 30 min (incubation) and 50°C for 20 min (enzyme inactivation) as reported earlier (4). To monitor residual DNase activity after heat inactivation, control amplification of 100 fg *C. albicans* DNA was performed in the presence of both treated and untreated reagents.

Real-time PCR amplification. PCRs were performed in 25-μl reaction mixtures that included primers, TaqMan probe, and master mix as previously described (11). Untreated PCR reagents, DNase-treated PCR reagents, and combinations of treated and untreated PCR reagents were employed. Amplification was performed on a TaqMan 7500 Fast instrument (Life Technologies, Carlsbad, CA, USA) using the following parameters: 2 min at 50°C (uracil N-glycosylase step) and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. For negative-control reactions, 5 μl of DNA-free water (Molzym, Bremen, Germany) was used. Positive-control reaction mixtures contained 100 fg of DNA from *C. albicans* (DSM 3567).

Identification of fungal contamination. In reagents with fungal contamination identified by panfungal real-time PCR (11), species identification was performed by PCR amplification of the polymorphic internal transcribed spacer 2 (ITS2) region as described previously (12) followed by Sanger sequencing of the amplicons. The master mix, primer, and probe suspected to contain fungal contaminants were used as the template in 5-μl solutions in independent PCRs. The presence of PCR products in the expected range of 277 to 507 bp was confirmed by agarose gel electrophoresis. Amplicons were purified (QIAquick PCR purification kit; Qiagen, Hilden, Germany) and subjected to Sanger sequencing (Microsynth, Balgach, Switzerland). Sequence alignment was performed using the GenBank sequence database.

Statistical analysis. GraphPad Prism for Windows was used for statistical calculations. The Wilcoxon signed-rank test was applied for testing the significance of differences in threshold cycle (C_T) values between DNase-treated and untreated PCR reagents.

RESULTS

Identification of fungal DNA contamination in PCR reagents. Despite the rigorous measures for the prevention of fungal contamination described above, false-positive results of panfungal PCR analysis were revealed by negative controls in a high proportion of samples tested within individual series (Table 1). The rate of false-positive test results was evaluated by performing nine independent series of negative-control reactions ($n = 40$ to 92 per series) in which DNA-free water replaced the template (no-template controls [NTC]), and the entire sample processing was performed using the stringent precautions outlined above. Within individual series of NTC specimens tested by panfungal PCR analysis, false-positive results were observed in up to 26.7% of samples analyzed (Table 1). The frequency of false-positive results remained constant as long as the same batches of master mix and primer and probe preparations were tested. Possible contamination with fungal DNA was determined by screening every new lot of individual reagents in PCR assays with components previously shown to be free of contaminants. Test results from two different batches of commercial master mix preparations are displayed (see Table 2). Batch 1 of the master mix was apparently free of fungal contamination as indicated by the consistently very low rate of false-positive signals in the presence of DNase-treated primers and probes. In contrast, batch 2 displayed an elevated level of false-positive signals in testing with treated oligonucleotides, thereby indicating the presence of traces of fungal DNA in the product. Nine independent test series, including a total of 532 NTC reactions, indicated that (i) both the oligonucleotides and master mixes provided the sources of contamination rather than other materials used and (ii) the levels of contamination with fungal DNA differed considerably between different batches of master mix and primer and probe solutions (Tables 1 and 2). Absence of any contamination with traces of fungal DNA, as determined by a highly sensitive panfungal PCR assay (11), was rarely observed in individual batches of these reagents obtained from different commercial providers. The panfungal PCR assay used includes two separate reactions primarily covering a broad spectrum of molds (reaction I) or yeasts and zygomycetes (reaction II) (11), and the majority of relevant contaminations were discovered by reaction II. To identify the fungal species involved, individual reagent components, including the primers, the TaqMan probe, and the master mix, were used as the template for amplification of the highly variable ITS2 region followed by

TABLE 2 Documentation of DNA contamination in master mix solutions

DNase-treated oligonucleotide batch[a]	Master mix[b]			
	Batch 1		Batch 2	
	% false positives	DNA amt (fg)	% false positives	DNA amt (fg)
1	1.7 ($n = 60$)	26.11 [$n = 1$]	5.4 ($n = 92$)	25.40 ± 18.70 [$n = 5$]
2	0.0 ($n = 40$)	-	7.5 ($n = 80$)	36.96 ± 27.61 [$n = 6$]

[a] The oligonucleotides include the primers and the TaqMan probe.
[b] DNA amounts were estimated using a standard curve obtained from *C. albicans* DNA. Mean DNA amounts and standard deviations are displayed. The numbers of specimens analyzed are indicated in parentheses and the numbers of contaminated reactions in brackets.

Sanger sequencing analysis. The nucleotide sequences obtained showed high sequence similarities with the following fungal species derived from the GenBank database: *Cryptococcus victoriae* (99% identity within an overlap region of 195 bp), *Malassezia restricta* (99% identity within an overlap region of 407 bp), and *Phlebia radiata* (99% identity within an overlap region of 198 bp).

Decontamination capacity of dsDNase treatment. After evaluation of different approaches to the elimination of double-stranded DNA (4), we selected a protocol based on the enzymatic activity of dsDNase which had been previously employed for the decontamination of PCR reagents in hypersensitive PCR applications (4, 13). Due to its double-strand specific activity, single-stranded DNA molecules such as primers and TaqMan probes remain undigested as long as there are no double-stranded structures, including internally formed hairpins or primer dimers (14). After the decontamination process, the enzyme can be heat inactivated at 65°C in the presence of DTT prior to implementing the reagents in PCR assays. The contamination levels of untreated PCR reagents with fungal DNA were found to reach a maximum range of approximately 50 to 100 fg per PCR, as determined by C_T values of real-time PCR analyses. We have therefore tested the capacity of the dsDNase to digest contaminating fungal DNA in the range between 100 fg and 10 pg by incubating different concentrations of genomic DNA derived from *C. albicans* (Table 3). The effect of decontamination by dsDNase in the presence of 100 fg ($n = 6$) and 1 pg ($n = 6$) of fungal DNA resulted in reductions of approximately 50-fold and more than 200-fold, respectively. Amplification of the residual undigested DNA by panfungal PCR provided very high C_T values beyond the standardized threshold for positive test results; such amplification results would therefore be regarded as negative (Table 3). Digestion of 10 pg fungal DNA ($n = 6$) resulted in a more-than-400-fold reduction of amplifiable nucleic acid levels, but the remaining undigested material provided clearly positive results upon PCR analysis. These observations indicate that 100 fg to 1 pg of fungal DNA is digested by dsDNase with adequate efficacy for subsequent PCR testing but that larger amounts may exceed the capacity of the enzyme for sufficient depletion of contaminating fungal DNA (Table 3).

TABLE 3 Efficacy of dsDNase treatment using different amounts of *C. albicans* DNA

Sample type	C_T values for indicated volume of *C. albicans* DNA[a]		
	100 fg[b]	1 pg[c]	10 pg[d]
dsDNase treated	39.46 ± 0.77 ($n = 6$)	38.18 ± 0.65 ($n = 6$)	35.71 ± 0.53 ($n = 6$)
Untreated	34.00 ± 0.21 ($n = 6$)	30.40 ± 0.11 ($n = 6$)	27.06 ± 0.06 ($n = 6$)

[a] Mean C_T values and standard deviations are shown. Samples displaying C_T values of >37.5 are considered negative according to the standardized protocol for panfungal PCR analysis. Significant differences in C_T values between treated and untreated reagents were observed ($P < 0.05$). The numbers of specimens analyzed are indicated in parentheses.
[b] Δ C_T for treated versus untreated samples, 5.46.
[c] Δ C_T for treated versus untreated samples, 7.78.
[d] Δ C_T for treated versus untreated samples, 8.65.

Decontamination of reagents. Two different batches of contaminated primers and probes were selected for assessing the efficacy of DNase treatment. From batch 1, 120 PCRs lacking template DNA and containing either untreated ($n = 60$) or DNase-treated ($n = 60$) oligonucleotide preparations were performed. Of the PCRs with untreated oligonucleotides, 26.7% revealed false-positive results. The rate of false positivity apparently reflected the stochastic distribution of traces of amplifiable fungal DNA in individual reactions. In contrast, in PCRs with oligonucleotides pretreated by DNase, a significantly lower proportion of tests (1.7%) revealed false-positive results ($P < 0.01$) (Table 1). Similar findings were obtained with batch 2 upon performing 80 PCRs with untreated ($n = 40$) and treated ($n = 40$) reagents. The frequency of false-positive results decreased significantly from 12.5% to 0% ($P < 0.01$) when decontaminated oligonucleotides were employed (Table 1). No adverse effects on PCR assay performance attributable to residual DNase activity were observed, as indicated by stable C_T values obtained after amplification of 100 fg *C. albicans* DNA in the presence of treated and untreated reagents (data not shown).

Decontamination by dsDNase is also feasible in master mix preparations suspected or known to contain traces of fungal DNA. However, in contrast to the use of decontaminated oligonucleotides in subsequent PCR assays, DNase treatment of master mixes has affected the performance of PCR analysis. The negative effect was documented in PCR analyses of positive controls ($n = 12$) which revealed higher C_T values (mean C_T value increase of 1.66; range, 0.98 to 2.34) when dsDNase-treated master mixes were employed. Polymerase contained in commercial master mix preparations may be prematurely activated and exhausted by exposure to higher temperatures prior to PCR (4). To assess the role of temperature during the decontamination process, we have therefore tested a heat-labile version of the dsDNase (14) that can be deactivated at lower temperatures. However, lower temperatures during decontamination and inactivation using the heat-labile dsDNase severely affected PCR performance. Analysis of samples ($n = 16$) under different conditions revealed a mean C_T value increase of 2.51 (range, 1.46 to 3.56) during digestion at 37°C and deactivation at 55°C, and a similar mean C_T increase of 2.50 (range, 1.35 to 3.65) was detected under less-stringent conditions that included digestion at 25°C and deactivation at 50°C.

TABLE 4 DNA-free consumables and reagents used in this study[a]

Consumable or reagent	Product line (manufacturer, location)
Pipetting filter tip	Biosphere (Sarstedt, Germany)
Microcentrifuge tube	Biopure (Eppendorf, Germany)
Optical 96-well plate	MicroAmp (Life Technologies, USA)
DNA-free water	MolYsis (Molzym, Germany)

[a] DNA-free materials from other manufacturers may also be available.

DISCUSSION

In this work, we identified fungal DNA contamination in critical, commercially available reagents required for nucleic acid amplification assays. Awareness of this problem is important because it can affect the interpretation of positive test results in highly sensitive PCR tests for fungal detection. The presence of traces of contaminating fungal DNA in commercial PCR reagents is particularly problematic when molecular fungal screening is performed in clinical specimens generally displaying very low fungal loads, such as peripheral blood, where highly sensitive assays must be employed (12, 15–18). Fungal DNA contamination was found in lyophilized oligonucleotides, including both primers and probes, and in master mix solutions purchased at renowned biotech companies. Various batches were tested, and different grades of contamination with traces of fungal nucleic acids were identified. Contaminating DNA from three fungal species, including *C. victoriae*, *M. restricta*, and *P. radiata*, was identified by ITS2 amplicon sequencing and alignment analysis using the GenBank database. *P. radiata*, a lignin-degrading white-rot fungus that colonizes dead wood in Eurasian and North American forests (19), and *C. victoriae*, a psychrophilic yeast that was first isolated in the Antarctica (20) but has also been found in samples retrieved in Europe, North and South America, and Asia (21), seem to be widely distributed environmental fungi and may therefore represent common contaminating organisms. In contrast, the presence of *M. restricta* DNA indicates an anthropogenic origin since this species is associated with the human skin (22). These observations strongly suggest that PCR reagents implemented in fungal diagnostic testing should be routinely tested for the presence of contaminating fungal DNA and that certified DNA-free materials should be used, whenever available (Table 4). In addition to rigorous handling procedures for sample processing that prevent exogenous contamination by fungal spores or components, careful control of every new batch of reagents for fungal PCR analysis is essential. Here we present data indicating that treatment of contaminated reagents by a dsDNase prior to their employment in fungal testing by sensitive PCR assays can significantly reduce the risk of false-positive test results. This approach was successful in decontaminating lyophilized oligonucleotides and can also be applied to master mixes for PCR analysis. However, treatment of master mixes containing DNA polymerases can result in decreased performance of the ensuing PCR assay, which bears the risk of false-negative results. We therefore carefully select commercial providers of master mix products and other reagents, because major differences between individual manufacturers have been observed, but every new batch is routinely subjected to internal control. Batches found to contain traces of fungal DNA may still be used for other PCR applications, but highly sensitive molecular screening for fungal infections requires the use of master mixes demonstrated to be free of relevant contaminations.

The availability of fungal-grade materials and reagents for molecular diagnostics is highly desirable, and ensuring their availability poses an important challenge for commercial providers. However, as long as products guaranteed to be free of fungal DNA are not readily available, methods for controlling and eliminating contaminations will remain of great importance.

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