Whitepaper

DNA-Free Reagents and Materials for Molecular Analysis of Bacterial and Fungal Pathogens

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Summary

Microbial DNA is present at very low loads in clinical specimens. Molecular detection by amplification assays generally is a challenge because of a potentially multiple input of contaminating DNA from exogenous sources. Besides air-borne, handling and cross contamination, materials and reagents used in the molecular laboratory can contain microbial DNA which is a long underestimated potential source of false positive results. In this contribution decontamination procedures of material and reagents and the selection of certified microbial DNA-free components for sample collection, DNA extraction and PCR amplification are discussed with respect to the aim of building up a reliable molecular system for the analysis of bacterial and fungal organisms at the limit of detection.

Keywords: Fungal DNA, bacterial DNA, contamination, decontamination, DNA-free, PCR, pathogen DNA extraction, amplification assays, diagnosis, Next Generation Sequencing

Introduction

Microorganisms can be present at very low loads in clinical samples. For instance, Wain et al. [1] cultured *Salmonella typhi* from blood at a median load of 1 cfu/ml (range, <0.3 to 387 cfu/ml). In another, more broadly focused study Phillips and Bradley [2] plated blood samples from neonates on chocolate agar. Cell counts determined for Gram-positive bacteria, Gram-negative bacteria, yeasts of the genus *Candida* and *Malassezia* and mixed infections of *Candida* and Gram-positives ranged from 1 to >100 cfu/ml, 3 to 8 cfu/ml, 18 to 96 and 66 to >100 cfu/ml, respectively.

Low loads of microorganisms are challenging to be analyzed by molecular methods. For PCR or Real-Time PCR diagnosis, a highly sensitive assay is therefore necessary [3]. This holds true also for the analysis of low microbial load communities by Next Generation Sequencing (NGS; [4]). Clearly, analysis at the limit of detection can pose serious problems to accurate data collection as regards false positive results and wrong community structure by introduction of extraneous DNA. This can happen through carry-over inoculation from laboratory surfaces and equipment, cross-contamination from one sample to another during extraction, aerosols from previous amplifications and air-borne environmental sources, inappropriate handling, and contaminated reagents and consumables [5]. There are procedures described for clinical, forensic, ancient DNA and other applications to prevent sample contamination by DNA from exogenous sources [6-8] which will be not discussed here. This contribution focuses on the impact on the diagnosis of bacterial and fungal pathogens of contaminated reagents and consumables employed in the sampling, extraction and amplification of target DNA from clinical samples. Solutions for in-house DNA decontamination procedures and commercially available DNA-free products will be presented with relevance for bacterial and fungal diagnosis.

Sources of contamination in the course of sample collection, DNA extraction and molecular analysis

Reagents and consumables used in the molecular laboratory are generally not issued for the analysis of microbial sequences at very low concentration. Therefore, an important constraint of molecular analysis at the limit of detection is the potential presence of microbial DNA contaminating extraction chemicals, PCR reagents and consumables during the manufacturing process. Table 1 summarizes studies that indicated contamination of a variety of reagents and consumables by microbial DNA. The studies were conducted in the context of suitability of the material for sensitive molecular diagnosis. Contamination was found in the majority of reagents and consumables studied.

DNA of fungal organisms including *Aspergillus* spp., *Pneumocystis* spp., *Saccharomyces cerevisiae*, *Candida* spp. and *Acremonium* spp. were detected in collection tubes for samples

Table 1: *DNA contamination of materials and reagents employed in molecular analysis of pathogens in clinical samples* [a]

Process	% False positives (no. tests)	Origin	Reference
Sample collection			
Blood collection tubes	17 (185)	***Aspergillus* spp.**	[9]
Blood serum tubes	10 (160)	***Aspergillus* spp.**	[9]
	4 (50)	***Pneumocystis jirovecii***	[10]
Urine collection tubes	8 (25)	***Aspergillus* spp.**	[9]
Forceps for tissue preparation	57 (23)	*Escherichia* spp., *Propionibacterium* spp., *Stenotrophomonas* spp. *Pseudomonas* spp.	[11]
Nucleic acid extraction and processing			
Zymolyase	n.d.	***Saccharomyces cerevisiae***	[12]
Lyticase	n.d.	**unspecified fungus**	[12]
DNA extraction	100 (20)	*Burkholderia* spp., *Pseudomonas saccharophilia*, *Ralstonia* spp., *Alcaligenes* spp.	[13]
	20 (20)	*Legionella* spp., ***Aspergillus* spp.**	[14, 15]
	n.d.	***Aspergillus* spp., *Candida* spp.**	[16]
	n.d.	*Brucella* spp.	[17]
	<3 (36)	n.a.	[18]
Nucleic acid precipitation (glycogen)	22 (9)	*Acinetobacter lwoffii*	[19]
RNA stabilization reagent	5 (20)	***Aspergillus* spp.**	[9]
PCR amplification			
Taq polymerase	100 (4)	unspecified bacterium	[20]
	100 (4)	*Pseudomonas* spp.	[21]
	8 (24)	*Sphingomonas* spp., *Moraxella* spp.	[21]
	2 (41)	*Acinetobacter junii*	[21]
	n.d.	*Pseudomonas* spp., *Serratia marcescens*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp.	[22]
	10-30 (n.m.)	*Coxiella burnetii*	[23]
Primers	0-100 (18-66)	*Delftia tsuruhatensis*, *Klebsiella* spp., *Paenibacillus* sp.	[24]
PCR buffer	n.d.	***Acremonium* spp.**	[12]
Pipetting			
Pipette tips	18 (6/32)	unspecified bacterium	[25]

[a] Samples of the same or different lots or samples from different manufacturers; signals were observed in negative PCR controls using DNA-free water; best match species were identified by sequencing of the amplicons and BLASTn search; n.d., not determined; n.a., not applicable; n.m., not mentioned.

like blood, serum and urine, DNA and RNA extraction reagents as well as PCR buffer (Table 1, species in bold). The origin of the contamination could also be assigned to eubacteria, among them environmental organisms like *Alcaligenes* spp. and *Pseudomonas* spp., skin colonizers and opportunistic pathogens like *Propionibacterium* spp., *Serratia marcescens* and *Sphingomonas* spp. as well as potential pathogens, including *Brucella* spp., *Coxiella burnetii*, *Escherichia coli* and *Legionella* spp. The rate of contamination was found to be high in some materials (>50% false positives), including forceps for tissue preparation, DNA extraction reagents and Taq polymerases (Table 1). Evidence for low and tolerable loads of contaminating microbial DNA (2 and 8% false positive rate) was given for some Taq polymerases (Table 1, ref. [21]). The list of evidence of DNA contamination stresses the importance of taking care in the selection of material and reagents specially treated by elaborated decontamination procedures or, as far as available, low contamination commercial products manufactured under quality-controlled conditions for use for molecular microbial diagnosis.

Consumables and reagents for pathogen DNA analysis

The problem of false positive results in molecular analysis of pathogens through contaminated consumables, buffers and reagents has been addressed by a number of approaches. Millar et al. [8] propose a risk assessment model detailing the manipulations, contamination haz-

ards and risks, and corrective action involved in the broad-range 16S rRNA gene PCR diagnosis of bacterial blood stream pathogens which may also serve as a guideline for fungal diagnostics by broad-range rRNA gene PCR and other assays. The model divides the analytical process into three parts, sample collection, DNA extraction and amplification, all of which demand careful handling and the availability of molecular-grade, in particular DNA-free consumables and reagents. In the following sections ways of decontamination of materials and reagents and employment of commercially available DNA-free materials are discussed as regards the setup of a reliable, highly sensitive system for the direct detection and identification of bacterial and fungal organisms in clinical and other sample materials with low pathogen loads.

Sample collection

Consumables for the collection and handling of samples are in line with analytical processes other than molecular diagnosis of microbial DNA present at very low concentrations. Material for the collection and processing of blood to plasma and serum, stabilization of blood cells and preparation of tissue biopsies has been shown to be a potential source of exogenous DNA of bacterial and fungal organisms (Table 1). So far, material routinely tested from lot to lot for the absence of microbial DNA does not seem to be available from commercial sources. Millar et al. [8] proposed to prepare lots of sterile DNA-free collection tubes, EDTA solution and water for blood drawing. Reduction of amplifiable bacterial sequences and cells over up to 4 orders of magnitude to below the limit of detection was observed when surfaces of plastic ware were experimentally contaminated with DNA or microorganisms and treated with methanol radicals or ethylene oxide [26, 27]. Radical-based treatment was regarded superior to UV or gamma irradiation which tends to have a negative influence on the plastic consumables.

DNA decontamination of water and buffers can be achieved by UV or Gamma irradiation [7]. Water and buffers are also commercially available as molecular biology grade and certified human DNA-free products. Absence of microbial DNA, however, is mostly not indicated and should be tested by the user by PCR negative control runs. Other sources of certified bacterial and fungal DNA-free water exist although available only as small volume products provided for PCR analysis (see Table 2, amplification reagents).

Consumables for handling in molecular analysis

The selection of suitable plastic consumables employed for DNA extraction and molecular analysis, including pipette tips, sample tubes, centrifugation vials and PCR or Real-Time PCR tubes and plates is crucial for the avoidance of false positive results by contaminating DNA. Sterility and absence of nucleases as characters for molecular-grade articles are not a guarantee for the absence of exogenous DNA as evidenced by our own experience (Table 1). Therefore, as part of the setup of a system for low load pathogen DNA analysis, testing of products from different suppliers for the absence of bacterial and fungal DNA is recommended.

Radical gas treatment of plastics is a common procedure for the destruction of amplifiable sequences (see previous section). There are some suppliers of consumables which declare their products bacterial DNA-free (Table 2). Only one among the three suppliers listed in Table 2, however, files testing for bacterial and fungal DNA. Nevertheless, it seems that absence of bacterial DNA is a good indicator of the absence of fungal DNA as well from the point of view of our experience (see comment in Table 2). Nonetheless, to be sure as indicated above, consumables not explicitly labelled as tested for the absence of fungal DNA should be subjected to negative control run analysis in the laboratory.

DNA extraction

As with other materials used for molecular biology, DNA extraction products are generally not designed for the purpose of ultra-sensitive detection of microorganisms at very low loads in clinical and other specimens. In fact, they generally contain exogenous DNA of bacterial and fungal origin (Table 1). Systematic studies have been performed to eliminate contaminating microbial DNA from extraction buffers by binding the DNA to silica-based membrane columns in a procedure employing filtration washing [13]. Ethylene oxide treatment of plastic consumables, including mini spin columns, was successfully employed for the destruction of DNA contaminants [27].

Industry has reacted to the increasing demand for microbial DNA-free reagents and consumables by the supply of ultra-clean products for DNA extraction from clinical samples. Table 2 lists certified bacterial and fungal DNA-free products dedicated to the extraction of microbial DNA for the analysis of low loads of microorganisms. The products address the manual, semi-automated and fully automated extraction of small and large sample volumes

Table 2: *Materials and reagents for the analysis of low microbial loads*

Component	Brand	Manufacturer	Absence of DNA tested [a]		Comment
			Bacterial	Fungal	
Consumables					
Filter tips, tubes, centrifuge vials	MGrade®	Greiner Bio-one (Kremsmünster, Austria)	+	+	
	Biopur®	Eppendorf (Hamburg, Germany)	+	-	
	Biosphere® Plus	Sarstedt (Nümbrecht, Germany)	+	-	tested for absence of fungal DNA [b]
Extraction kits					
Manual protocols	QIAamp® UCP PurePathogen Blood	Qiagen (Hilden, Germany)	+	+	manual DNA extraction; up to 8 ml blood
	MolYsis™	Molzym (Bremen, Germany)	+	+	manual DNA extraction; up to 10 ml clinical samples
Automated systems	Blood Pathogen Kit™	Seegene (Seoul, South Korea)	+	+	semi-automated; 1 ml blood; Seeprep12™ instrument (Seegene)
	SelectNA™ Blood Pathogen Kit	Molzym (Bremen, Germany)	+	+	semi-automated; up to 10 ml clinical samples; Liaison® IXT instrument (Diasorin, Saluggia, Italy)
	MagNA Pure® LC Microbiology kit	Roche Diagnostics (Penzberg, Germany)	+	+	automated DNA extraction; 0.1 ml samples; MagNA Pure LC 2.0 instrument (Roche)
	MolYsis SelectNA™*plus*	Molzym (Bremen, Germany)	+	+	automated DNA extraction; 1 ml liquid samples, tissue biopsies; SelectNA™*plus* instrument (Molzym)
	EasyMag®	bioMerieux (Marcy-l'Étoile, France)	-	-	modified automated protocol for 5 ml blood [29]; see text

Table 2: *Materials and reagents for the analysis of low microbial loads (continued)*

Component	Brand	Manufacturer	Absence of DNA tested [a]		Comment
			Bacterial	Fungal	
Amplification reagents					
Taq DNA polymerase, master mix, water	Taq DNA Polymerase, DNA-free	Applichem (Darmstadt, Germany)	+	+	
	MolTaq 16S/18S	Molzym (Bremen, Germany)	+	+	
	innuTaq UltraPure DNA Polymerase	Analytic Jena (Jena, Germany)	+	-	
	MTP™ Taq DNA Polymerase	Sigma-Aldrich (St. Louis, MO, USA)	+	-	
	DFS-Taq DNA Polymerase	Bioron (Ludwigshafen, Germany)	+	-	
	Taq DNA Polymerase	Amresco (Solon, OH, USA)	+	-	
	DNA free-Taq DNA Polymerase	Xpress Bio (Frederick, MD, USA)	+	-	
	DF Taq Polymerase E (DNA-free)	Genaxxon (Ulm, Germany)	+	-	
	Mastermix 16S/18S Basic	Molzym (Bremen, Germany)	+	+	Master mix for assays with custom primers
	Mastermix 16S Complete	Molzym (Bremen, Germany)	+	+	Broad-range 16S rRNA gene PCR assay for detection of bacterial DNA
	Mastermix 18S Complete	Molzym (Bremen, Germany)	+	+	Broad-range 18S rRNA gene PCR assay for detection of fungal DNA
	Microbial DNA-free Water	Qiagen (Hilden, Germany)	+	+	
	DNA-free Water	Molzym (Bremen, Germany)	+	+	
	PCR Water, DNA-free	Applichem (Darmstadt, Germany)	+	+	

[a] +, Information (homepage) on testing provided by manufacturer; -, no information available
[b] Own results, absence of fungal DNA shown at 40 cycles PCR employing Mastermix 18S Complete (Molzym)

in the range 0.1 to 10 ml as well as tissue biopsies. Two products, MagNA Pure® (Roche) and easyMag® (bioMérieux), extract total nucleic acids, while all others aim at the preparation of microbial DNA with low loads of human DNA. A variation of the standard easyMag® protocol was described by Wiesinger-Mayr et al. [28] by which bacterial DNA preparations were greatly depleted of human DNA. Excess host DNA can be a factor of false positive results and loss of assay sensitivity as a consequence of unspecific primer binding and amplification of non-target sequences [29].

Five of the six products are declared by the manufacturers to be routinely tested for the absence of bacterial and fungal DNA during the manufacturing process. As regards the product without information about testing for microbial DNA contamination, easyMag®, there is evidence that also this system is suitable for sensitive analysis of pathogens, although demonstrated only for bacteria. By developing a modified easyMag® protocol for the extraction of bacterial DNA from 5 ml EDTA blood, Wiesinger-Mayr et al. [28] could detect a variety of Gram-positive and Gram-negative bacteria at low loads (10^1-10^2 cfu/ml) while extracts from negative extraction controls were negative over 40 cycles in the broad-range 16S rRNA gene assay employed which indicates that bacterial DNA contamination was below the detection level. The authors regarded the protocol as a promising system for bacterial DNA extraction. As indicated above, contaminating fungal DNA is likely to be absent, but should be proven before using the system for the development of a protocol for fungal DNA extraction.

Molecular analysis

The record of references regarding contamination of amplification reagents comprises mainly bacterial DNA (Table 1). As regards fungal DNA contamination, literature is scarce. Loeffler et al. [12] systematically studied contamination of reagents involved in the whole diagnostic process, including amplification. They found one component, the 10x PCR buffer of a certain lot of the product to be contaminated by fungal DNA which by sequence analysis of the amplicon could be assigned to *Acremonium* spp. This shows that care has to be taken in the selection of amplification reagents as was discussed before regarding sample collection and extraction.

Champlot et al. [7] conducted a systematic evaluation of various methods for the decontamination of PCR components. The authors provide protocols involving Gamma-irradiation of water and short UV irradiation by which PCR buffers and other liquids can be decontaminated efficiently and rather easily from exogenous DNA. They point out that sensitive components like Taq DNA polymerase, primers and dNTPs are inactivated by UV and therefore need a different treatment. Employment of a protocol using a heat-labile endonuclease resulted in 99.5% degradation of double-stranded DNA while the efficiency and sensitivity of the PCR assay was comparable to the control indicating that primers were not affected by the nucleolytic treatment. Although focused on the removal of bovine DNA contamination, the study provides valuable guidelines for the setup of a decontamination protocol for PCR components as regards fungal DNA.

When screening the information provided by manufacturers, several PCR components, including Taq DNA polymerases, master mixes and molecular grade water are available which are certified bacterial and, in many cases, also fungal DNA-free (Table 2). The use of commercial products may be helpful in the reduction of microbial DNA contamination and the standardization of sensitive bacterial and fungal target assays among laboratories. Importantly, when designing an analytical assay for the detection of minute amounts of fungal DNA, DNA-free products should also guarantee a high amplification activity [21].

Even though buffers and reagents of the amplification reaction are available DNA-free, either as single components or as master mixes (Table 2), primers designed for specific targets are generally produced under conditions which introduce considerable levels of microbial DNA into the primer preparation (Table 1). This is a frequently experienced problem which demands special care regarding decontamination (see above).

Conclusions

There are procedures described for in-house decontamination from microbial DNA of consumables and reagents used on the way from sample collection over extraction to molecular analysis. In the last years, manufacturers of molecular grade articles have become aware of the problem and now supply materials and reagents with very low loads of endogenous microbial DNA. In terms of standardization, DNA-free products enable the exact comparative NGS analysis of metagenomic structures and the rapid diagnosis of pathogens in clinical routine.

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