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Aspergillus DNA contamination in blood collection tubes

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Abstract

Fungal PCR-based diagnostic methods are at risk of contamination. Sample collection containers were investigated for fungal DNA contamination using real-time PCR assays. Up to 18% of blood collection tubes were contaminated with fungal DNA, probably *A. fumigatus*. Lower proportions of contamination in other vessels were observed.

The incidence of invasive aspergillosis (IA) is increasing amongst immunocompromised individuals with an estimated one million deaths worldwide (Maschmeyer and Haas 2008). The need for a universally applicable rapid and sensitive diagnostic method is pressing (Denning 1998). PCR based detection of *Aspergillus* nucleic acids provides a well explored diagnostic technique that could fulfil these criteria (Chen *et al* 2002, Perlin and Zhao 2009). However, *Aspergillus* is ubiquitous in the environment and DNA contamination in a diagnostic assay can arise from dead or fragmented fungi. Given the costs and side effects of the preferred antifungal therapeutics (Menzin *et al* 2009), even very low levels of false positive results or contamination have serious consequences. The most convenient source of material for diagnosis is blood. An 8% contamination rate in over 3,000 fungal PCR assays performed on whole blood over 2 years has been reported (Loeffler *et al* 1999). Contaminating DNA from 12 different *Aspergillus* species caused a false positive rate of 19% with sources of contamination including DNA extraction and PCR reagents (Palmer *et al* 2001).

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We investigated the prevalence of fungal DNA contamination originating from blood collection tubes and a variety of other clinically relevant vessels from a range of manufacturers using a DNA extraction kit that has been proven free from fungal DNA (data not shown) (MycXtra™, Myconostica, Manchester, UK). Extracted DNA was tested using a quality controlled commercial molecular beacon (MB) real-time PCR assay that detects *Aspergillus* and *Penicillium* spp. (FXG:Resp (Asp+), Myconostica). Positive samples were screened using a second PCR assay with a TaqMan (TM) probe specific for *Aspergillus fumigatus* only.

Collection vessels tested and rates of contamination detected are listed in table 1. To test for microbiological contamination, sterile PBS-Tween 80 (1mL) was added to each vessel and agitated on a vortex mixer for 1 minute. An aliquot (100μL) was then spread onto Sabouraud dextrose agar plates and incubated at 30°C for 2 weeks. To test for fungal DNA contamination, molecular grade sterile water (1mL) was added to each vessel and agitated on a vortex mixer for 1 minute followed by DNA extraction with the MycXtra™ kit. Tubes containing liquid additives had 1mL of water added and 1mL total liquid removed for DNA extraction. DNA extraction was performed in a laboratory that is environmentally monitored for fungal contamination. Sabouraud dextrose agar plates were left open on the laboratory bench top during extraction to measure the local airborne fungal flora and incubated at 30°C for 2 weeks. After DNA extraction, 5μl of the extract was tested in an *Aspergillus* MB real-time PCR assay (Myconostica) using an ABI 7500 (Applied Biosystems, CA, USA) which has a detection limit of 50 target 18S rRNA copies, equating to approximately 1 genome (Herrera *et al* 2009,Tyagi and Kramer 1996). Each multiplex PCR included primers and molecular beacons to detect *Aspergillus* DNA with an internal amplification control to assess the occurrence of inhibition. PCR positive samples and controls were tested with an *Aspergillus fumigatus* TM assay described elsewhere (Challier *et al* 2004). PCR set-up was performed under sterile laminar flow using a dedicated hood, equipment and lab coats. The area was regularly disinfected and monitored for fungal spores using settle plates and an air sampler. *Aspergillus* DNA-free sterile supplies of water, pipettes, pipette tips and plasticware were maintained throughout.

No fungal colonies grew from the collection vessels tested. All DNA extraction controls were culture and PCR negative. The frequency of MB PCR positive results are listed in table 1. Ct values ranged from 32.5 to the limit of detection (LOD) of 38.1. The TM assay showed 96% (48/50) agreement with samples that were MB positive.

Of the whole blood collection tubes, 12 of 85 (14%) spray dried and 19 of 100 (19%) liquid tubes were contaminated. Of the serum blood collection tubes, 5 of 85 (6%) containing a spray dried clot activator and 11 of 75 (15%) without additives were contaminated. One of 20 (5%) RNA*Later* reagent aliquots and 2 of 50 (4%) of the urine collection containers were contaminated. No Cell Preparation Tube™, PaxGene RNA tube, BAL collection tube, cryovial or pipette tip was contaminated.

The results presented highlight a problem in DNA based detection methods for rapid diagnosis of fungal infection. PCR is highly sensitive and appears to reveal the presence of fragments of fungi containing DNA in microbiologically sterile containers. The fill volume of each tube is typically larger than the 1mL of sterile water used here and the results probably overestimate the problem in the clinic. However, blood tubes are often under filled and small volumes are the norm in paediatric practice. Five of the 50 (10%) positive PCR signals were strong (Ct values <34), suggesting that the assay would still be positive after dilution with larger volumes.

The MB assay detects both *Aspergillus* spp. and some *Penicillium* spp. and does not discriminate between specific species (unpublished data). The TM assay is specific for *A. fumigatus* targeting the 28S rDNA region. Agreement between assays on 96% of positive samples reinforces the conclusion that the results are robust (the 2 samples that were TM negative were close to the LOD with the MB assay). It is likely that most of the contaminated tubes contained *A. fumigatus* DNA. This is problematic in the clinical setting.

Another explanation for contamination is introduction of DNA during extraction or PCR set-up. In this study, the DNA extraction and PCR set up procedures were rigorously controlled using settle plates, negative extraction controls and no-template PCR controls. All controls were culture and PCR negative and it is most likely that the contamination arose from the collection vessels.

This data suggests that between 0 and 18% of blood collection tubes are contaminated with *Aspergillus* species DNA with possible contamination of urine collection vessels and RNA stabilization reagents. We conclude that development of fungal real-time PCR will require consideration of the ubiquitous nature of fungal flora and DNA and the impact of any contamination on the sensitivity of the technique. This result has serious implications for the development of PCR diagnostics for fungal disease using standard collection vessels.

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Table 1

Collection vessels tested, manufacturer, number tested and frequency of *Aspergillus* PCR positivity

Type of vessel	Additive	Manufacturer	Lots tested	Tested per lot (total tested)	MB PCR positive (%)
Whole blood collection tube 2mL	K_2EDTA (Spray Dried)	BD Vacutainer	1	10 (10)	0 (0)
Whole blood collection tube 6mL	K_2EDTA (Spray Dried)	BD Vacutainer	3	25 (75)	12 (16)
Whole blood collection tube 6mL	K_3EDTA (Liquid)	BD Vacutainer	1	25 (25)	0 (0)
Whole blood collection tube 7mL	K_3EDTA (Liquid)	BD Vacutainer	3	25 (75)	19 (15)
Serum blood collection tube 3mL	Clot Activator (Spray Dried)	BD Vacutainer	1	10 (10)	0 (0)
Serum blood collection tube 6mL	Clot Activator (Spray Dried)	BD Vacutainer	3	25 (75)	5 (7)
Serum blood collection tube 6mL	None	BD Vacutainer	2	25 (50)	11 (18)
Serum blood collection tube 10 mL	None	BD Vacutainer	1	25 (25)	0 (0)
Cell preparation tube (CPT)	Sodium citrate and Ficoll™	BD Vacutainer	3	6 (18)	0 (0)
PaxGeneRNA	Multiple†	PreAnalytiX GmbH	2	6 (12)	0 (0)
RNA *later*	RNA stabilization reagent	Ambion	2	10 (20)	1 (5)
BAL collection container 40cc	-	Busse	2	12 (24)	0 (0)
Urine collection container (sterile cup only)	-	Medline	1	25 (25)	2 (8)
Urine collection container (sterile mid stream kit)	-	Medline	1	25 (25)	0 (0)
Cryovial container 2mL	-	Simport	1	10 (10)	0 (0)
Cryovial container 3mL	-	Simport	2	10 (20)	0 (0)
Pipette tips 1000μL	-	Eppendorf	2	5 (10)	0 (0)

†Paxgene additives: Tetradecyltrimethylammonium oxalate solution, tartaric acid and undisclosed non-hazardous substances