

Opportunistic *Aspergillus* pathogens measured in home and hospital tap water by quantitative PCR (QPCR)

S. J. Vesper, M. E. Rogers, A. N. Neely and R. A. Haugland

ABSTRACT

Opportunistic fungal pathogens are a concern because of the increasing number of immunocompromised patients. The goal of this research was to test a simple extraction method and rapid quantitative PCR (QPCR) measurement of the occurrence of potential pathogens, *Aspergillus fumigatus*, *A. flavus*, *A. terreus* and *A. niger*, in home tap water and a hospital water supply.

Water samples were taken from the kitchen tap in the homes of 60 patients who were diagnosed with legionellosis. Water samples were also taken from three locations in a hospital that generated all of its hot water by flash heating. Opportunistic infectious agents *Aspergillus fumigatus*, *A. flavus*, *A. terreus* and *A. niger* were measured using QPCR. *Aspergillus terreus* DNA was found in 16.7% and *A. fumigatus* DNA in 1.7% of the samples taken from the kitchen tap. None of the *Aspergillus* species were found in any of the hospital water samples.

The development of a simple DNA extraction method along with QPCR analysis is suitable for rapid screening of tap water for opportunistic fungal pathogens. This simple method can be used to obtain pathogen occurrence results in about 3 h, instead of waiting days to weeks for culture data. Obtaining pathogen occurrence data in a timely manner could promote the elimination of the pathogens from the water supply of immunocompromised patients.

Key words | *Aspergillus*, immunocompromised, quantitative PCR, tap water

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INTRODUCTION

Invasive aspergillosis is an important cause of morbidity and mortality in the immunocompromised (Walsh *et al.* 2003). Aspergillosis is an opportunistic disease primarily caused by *Aspergillus fumigatus*, followed by *A. flavus*, *A. terreus*, and *A. niger* (Richardson & Warnock 2003). Aspergillosis increased about 8-fold between 1976 and 1996, i.e. from 4.8 to 38 cases per million population, and fatality rates for these invasive fungal infections range from 50–100% (Richardson & Warnock 2003). Therefore prevention of these diseases is paramount. The sources of these pathogens remain elusive.

Air is often considered the source of *Aspergillus*, but the home and hospital water supply have been largely overlooked. Yet, earlier studies have shown that many fungi can

survive in the potable water system, including species of *Aspergillus* (Rosenzweig *et al.* 1986; Arvanitidou *et al.* 1999; Doggett 2000; Pryor *et al.* 2004). *Aspergillus fumigatus* from a hospital water system was genotypically linked to a case of aspergillosis (Anaissie *et al.* 2003). However, there is growing evidence that cases of aspergillosis may be acquired outside of the hospital (Hajjeh & Warnock 2001). In a two-year period of surveillance, up to 70% of aspergillosis cases were apparently acquired outside the hospital.

With many immunocompromised patients recuperating at home, a simple, rapid method is needed to test their home water supply for potential opportunistic pathogens before the patient returns home from the hospital. Culture-based methods can take days to weeks to complete. This paper

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describes the measurement of four pathogenic *Aspergillus* species in water samples using a simple extraction and QPCR analysis.

MATERIALS AND METHODS

Selection of homes and hospital

All human subject activities were approved by the VA Hospital of Pittsburgh's Institutional Review Board, the participating hospitals and health departments. Cases of pneumonia reported by participating study hospitals or health departments were confirmed by using routine *Legionella* urinary antigen testing and/or culturing respiratory specimens (Stout *et al.* 1992).

As a control, water samples were obtained from Shriners Burns Hospital in Cincinnati, Ohio because this hospital uses a flash water heater system (Aerco B Plus™, Northvale, NJ). The potable cold water goes through a water softener and then was flash heated to 74°C and immediately put into circulation. There was no storage tank. Depending upon where the water was used, it can be tempered down by use of a bimetal switch valve which allowed cold water to mix with the hot. The hot water delivered to the patients' rooms was about 44°C. Three locations in the hospital were selected for sampling: the sink in the hydrotherapy room, the sink in the operating room and a patient's shower.

Collection, filtering and extraction of water samples

Tap water samples were obtained from each of 60 homes of patients who were diagnosed with legionellosis. The kitchen tap water was allowed to run for 1 min and then a 500 ml water sample was obtained. The hospital water samples (500 ml) were taken once a week for four weeks (in the summer) at the three locations (described above) after allowing the water to run for 1 min. Each sample was filtered through polycarbonate filter, 0.2 µm pore × 45 mm diameter (Osmonics, Inc., Minnetonka, MN). The entire filter was then folded onto itself and placed a 2.0 ml conical bottom, screw-cap tube (PGC Scientifics, Gaithersburg, MD) and then frozen at –20°C until extracted and analyzed (Brinkman *et al.* 2003).

Calibrator and reference stock preparation

Aspergillus species and *Geotrichum candidum* cultures were grown for one to two weeks on potato dextrose agar (PDA) (Becton and Dickinson, Fairfax, VA) at room temperature. Cells were harvested using a moistened, sterile cotton swab and resuspended in sterile water containing 0.05% Tween 80. Suspended cell stock concentrations were determined by counting in a hemocytometer chamber at 400 × magnification as previously described (Haugland *et al.* 2004) and 50 to 100 ml aliquots were stored at –80°C. Aliquots of *G. candidum* stocks, containing 2×10^8 cells per ml, and aliquots of different *Aspergillus* species, containing between 10^6 and 10^7 cells per ml, were used as the reference and calibrators, respectively.

QPCR analysis of *Aspergillus* species

The cell suspensions used as calibrators for determining assay specificity, amplification efficiency and cell detection limits were extracted by a rapid bead-milling method (Haugland *et al.* 2002). Briefly, 10 ml aliquots of both the target *Aspergillus* species and *G. candidum* reference cell stocks were combined with 200 ml of AE buffer (Qiagen, Valencia, CA) in a 2.0 ml conical bottom, screw-cap tube (PGC Scientifics, Gaithersburg, MD) containing 0.3 g acid-washed glass beads (Sigma, St. Louis, MO). The tubes were shaken in a mini bead-beater (Biospec Products, Bartlesville, OK) for one min at the maximum rate, then centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 3 min. The genomic DNA in the supernatant above the beads was transferred to a sterile microfuge tube and stored at –80°C. In some cases, the extracts were further purified by use of a Qiagen purification kit procedure. This procedure was performed by adding 300 µl of binding buffer from an Elu-Quik® DNA purification kit (Schleicher and Schuell, Keene, NH) to 100 µl of the supernatant indicated above and purifying on a DNeasy™ glass filter column (Qiagen, Valencia, CA, USA), as previously described (Haugland *et al.* 2002).

QPCR reactions

All primer and probe sequences in the assays for *Aspergillus fumigatus*, *A. terreus*, *A. flavus* and *A. niger* have been

previously reported (Haugland et al. 2004). The theoretical detection limits for these four *Aspergillus* species are 2 cell equivalents or less (Haugland et al. 2004). Primers and probes were synthesized commercially (Applied Biosystems, Foster City, CA; Integrated DNA Technologies, Coralville IA; Sigma Genosys, Woodlands, TX).

Reactions were performed in 0.5 ml thin-walled, optical grade PCR tubes (Applied Biosystems, Foster City, CA) by addition of the following components: 12.5 ml of TaqMan Universal Master Mix, a 2 × concentrated, proprietary mixture of AmpliTaq Gold™ DNA polymerase, AmpErase® UNG, dNTPs with UTP, passive reference dye and optimized buffer components; 5 ml of a mixture of forward and reverse primers (5 mM each) and 400 nM TaqMan probe; 2.5 ml of 2 mg/ml bovine serum albumin (fraction V, Sigma Chemical, St. Louis, MO) and 5 ml of DNA template. The reactions were monitored in an Applied Biosystems Prism model 7700 sequence detection instrument. Thermal cycling conditions consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument.

Amplification factors and extrapolated sensitivities of QPCR assays

DNA extracts from 10 ml aliquots of calibrator stocks of each of the species were prepared by bead milling (as described above) but using 200 ml of AE buffer in place of the lysis and binding buffers and without use of DNeasy purification. Ten-fold serial dilutions of these DNA extracts were analyzed by QPCR using the appropriate assay. The undiluted extracts were also analyzed using the *G. candidum* reference assay. The δC_T values were calculated by subtracting the reference assay C_T result for each extract from the target assay results for each dilution of that extract. The relationship between δC_T and the number of target cell equivalents (N) in undiluted and diluted sample extracts is expressed by $N = ME^{-\delta C_T}$, where M represents standard cell equivalents and E is the amplification factor (ideally $E = 2$). This implies a linear relationship between δC_T and $\log_{10}(N)$:

$$\delta C_T = C_{T,\text{target}} - C_{T,\text{reference}} = a + b \log_{10}(N) \quad (1)$$

where $a = \log_{10}(M)/\log_{10}(E)$ and $b = -1/\log_{10}(E)$. For the purpose of estimating theoretical cell detection limits, the mean reference C_T value of 18 for all strains was used for $C_{T,\text{reference}}$ in Equation (1). The detection limit then was taken to be that value of N for which $C_{T,\text{target}}$ would be expected to reach 40 by Equation (1). Calculations of amplification factors (E) and extrapolated cell detection limits were performed as previously described (Haugland et al. 2004).

QPCR enumeration of target cells

Determinations of the numbers of cell equivalents in the samples were made using a standard calibration curve that was developed for each assay. These were the same curves that were used to determine amplification efficiency, as described above. The numbers of cells were estimated using the inverse of Equation (1) as

$$\log_{10}(N) = (\delta C_T - a)/b \quad (2)$$

in which δC_T was the difference in observed C_T between the target and reference organisms for the respective sample.

RESULTS

Table 1 shows the results of the analysis of the occurrence of *Aspergillus* opportunistic pathogens in home tap water samples. Only 10 samples out of 60 were positive for *Aspergillus* DNA of the four target species. (One sample contained both *A. terreus* and *A. fumigatus* DNA.) *Aspergillus terreus* DNA was detected in 16.7% of the samples and *A. fumigatus* DNA in 1.7%. No *A. niger* or *A. flavus* DNA was detected in the water samples from these homes. Concentrations of *A. terreus* ranged from 3 to 125 cell equivalents per 500 ml of water. None of these four fungal species were detected in any of the hospital water samples.

DISCUSSION

Earlier studies have shown that many yeasts and filamentous fungi can survive in drinking water (Burman 1965;

Table 1 | Occurrence of *Aspergillus* species in tap water as measured by quantitative PCR (QPCR). CE = cell equivalents

Positive sample	Pathogen sample from state	QPCR CE/500 ml	Standard deviation
A	<i>Aspergillus terreus</i>	125	27
B	<i>A. terreus</i>	28	17
	<i>Apergillus fumigatus</i>	5	4
C	<i>A. terreus</i>	16	14
D	<i>A. terreus</i>	27	28
E	<i>A. terreus</i>	20	4
F	<i>A. terreus</i>	9	6
G	<i>A. terreus</i>	8	7
H	<i>A. terreus</i>	3	4
I	<i>A. terreus</i>	4	9
J	<i>A. terreus</i>	3	4

Bays *et al.* 1970). Rosenzweig *et al.* (1986) showed that some fungal conidia were highly resistant to chlorine inactivation and survived conventional water treatment.

In studies of four US water distribution systems, more than 50% of the samples, taken from various locations in the distribution system, contained fungi (Rosenzweig *et al.* 1986). Doggett (2000) isolated 39 different species of fungi from a Springfield, MO water distribution system. The filamentous fungal densities ranged from 4.0 to 25.2 CFU/ml. In Californian drinking water, filamentous fungi were found at concentrations of 15 to 40 CFU per 100 ml drinking water (Nagy & Olson 1982). Similar studies have been carried out in France (Hinzelin & Block 1985), Finland (Niemi *et al.* 1982), Bratislava (Frankova & Horecka 1995) and Greece (Arvanitidou *et al.* 1999). In all cases, fungi were found in the potable water.

The homes of patients diagnosed with legionellosis were selected for this study in order to increase the chances of finding *Aspergillus* in the water supply, because growth of the thermo-tolerant *Aspergillus* species would also be expected to be promoted under the same conditions as

Legionella (Anaissie *et al.* 2002c). Although none of the patients in this study were diagnosed with aspergillosis, one water sample had measurable *A. fumigatus* DNA. However, *A. terreus* was fairly commonly detected.

A. terreus is increasingly reported as a cause of pneumonia and disseminated infections (Tritz & Woods 1993; Iwen *et al.* 1998; Baddley *et al.* 2003; Walsh *et al.* 2003). In one Alabama hospital, in a period from 1996 to 2001, *A. terreus* infections increased significantly compared to infections caused by other *Aspergillus* species (Baddley *et al.* 2003). In part this may be due to the relative resistance of *A. terreus* to amphotericin B. The environmental source of *A. terreus* was not considered in this Alabama study.

In the most comprehensive study of *Aspergillus* in a hospital water system, *A. fumigatus* and *A. terreus* were found in water samples from an Arkansas hospital by culture methods at a rate of 11 and 9%, respectively (Anaissie *et al.* 2003). We did not detect any *Aspergillus* DNA in any of the water samples from the hospital in Cincinnati in our limited survey. The major difference between the Cincinnati hospital and the Little Rock hospital is that the latter uses water storage tanks in the handling of the water and the Burn's Hospital does not. Anaissie *et al.* (2003) suggested that the water storage tanks may be the source of fungal amplification.

QPCR offers a more rapid and sensitive method than culture-based techniques for the screening of water or air samples (Neely *et al.* 2004). If opportunistic pathogens are detected, then steps to reduce or eliminate these organisms may be possible (Anaissie *et al.* 2002b). Such recommendations have been made for hospitals to control waterborne nosocomial infections (Anaissie *et al.* 2002a). Similar steps may prove useful in preventing waterborne infections from being acquired at home.

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