Fungal Spore DNA Can Be Detected In Tissue And Body Fluids Of Patients With History Of Exposure To Toxin Producing Molds

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ABSTRACT (REVISED)

Background: The goal of these studies was to determine if DNA from 23 species of toxin producing mold could be identified in human tissue and body fluids from patients exposed to these organisms from their environment (i.e., home, work). The presence of various types of fungi in nasal mucus, skin, and many tissues has been established in patients who are immunocompromised (i.e., malignancies, HIV). A SmartCycler® II real-time PCR (RT-PCR) platform for the qualitative detection of toxin producing molds in human tissue and fluids was employed. **Methods:** Eighteen human upper respiratory (UR) specimens, and 34 tissues from biopsies, and/or autopsy specimens were examined from patients with histories of exposure to toxic molds. Twelve negative (UR) specimens and 15 autopsy/surgical specimens from patients with no known history to toxin producing molds were used as negative controls. Positive and negative controls for each target of interest (Primer/Probe sets) and an internal Geometrica sp. control were processed with each clinical sample in each RT-PCR run. Any amplification crossing a baseline fluorescence of > 30 between cycles 1 and 40 was interpreted as positive. **Results**: Nucleic acid from 10 different fungal spores was detected in 35% of UR specimens and in 42% of human tissue from exposed patients tested. All control specimens were negative. Conventional cultures and special stains on all specimens were negative. *Aspergillus niger* was detected in UR, lung, liver, brain, and skin specimens. *Aspergillus species* (*flavus*, *sydowii*, *ustus*), *Penicillium simplisticum* and *Eurotium amsteledami* were also detected in various tissues and UR specimens. The internal control and negative control were correct in all samples. **Conclusions**: DNA from toxin producing molds can be detected in human tissue and body fluids after mold exposure.

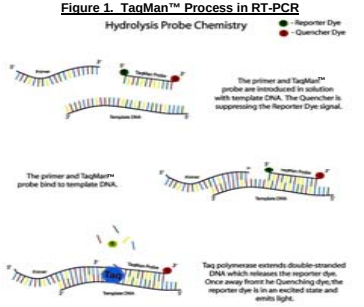
INTRODUCTION

The Real-time PCR (RT-PCR) method used in these assays utilize the hybridization of a species-specific probe to a complimentary DNA strand to amplify and detect fungal DNA. RT-PCR allows for the instant detection of infectious molds (*i.e.*, fungi) by simultaneously combining probe hybridization and amplification to achieve sensitive and specific detection. Several real-time platforms are available and for these assays, the Cepheid SmartCycler® System was used which is capable of multiplexing utilizing a four-color detection system. The integrated software system allows for the performance of Cepheid kits or user developed home-brew assays. This study was conducted in a CLIA-certified clinical lab and was properly validated. The 23 species detection assays utilize specific target probes and primers to demonstrate that real-time PCR can generate a qualitative determination of the presence or absence of fungal spores in clinical fluid and tissue.

Over the past 15-20 years, toxic mold exposure has become more hazardous and frequent. Climate changes, improper building environmental controls, poor building maintenance and energy efficient building practices have created opportunities for people to have a greater exposure to black toxic molds. Scientific and medical literature has demonstrated more awareness of black molds as possible pathogens in human disease. After hurricane Katrina, CDC issued new revised warnings as to health effects of toxic mold exposure. At present, there is no testing readily available to clinicians for helping establish the diagnosis of infections or illness due to such exposure. Currently, tests such as an ELISA test for antibodies to Aspergillus and the use of polymerase chain reaction (PCR) for the detection of mold DNA in human specimens have been limited to clinicians investigating mold infections in immunocompromised (cancer, HIV, etc)patients.

This study demonstrates that RT-PCR can be used to evaluate tissues and fluids from patients who have been exposed to toxigenic mold in the environment. The 23 species detection assays utilize specific target probes and primers to generate a qualitative determination of the presence or absence of fungal spores in clinical fluid and tissue. There is a need to not only know if black molds are in the patient's environment, but if these molds and their toxins are present in the patient and are causing their medical problem.

Figure 1. TaqMan™ Process in RT-PCR



MATERIALS AND METHODS

SAMPLES: Nasal secretions were obtained from hospital patients or out-patients. Fixed autopsy and surgical biopsy specimens from private autopsy specimens, pathology department surgical specimens, or coroner specimens were obtained from patients with a history of exposure to mycotoxins or fungi. Negative control groups were the same type of specimens taken from patients with no known exposure to mycotoxins or molds. All specimens were placed into two groups. Group 1 comprised samples from individuals with no reported symptoms or known fungi or mycotoxin exposure.. Group 2 comprised samples from individuals with reported exposure to non-identified fungi or chemicals.. Common symptoms of patients corresponding to group 2 samples included blurred vision, memory loss, fatigue, headache, nausea, loss of balance, cognitive deficits, rhinitis, sinusitis, rashes, and allergies. A detailed history and symptoms were provided corresponding to each patient sample.

EXTRACTION OF SAMPLES: Patient Samples and Negative Controls were extracted using a commercially available tissue extraction system that was optimized for the extraction of fungal genomic DNA. Small amounts of tissue (including paraffin embedded tissue) or other sample types containing spores were prepared for extraction by placing or pipetting the sample into an autoclaved screw top tube containing silica bead beating glass. A set volume of internal control spores was introduced to the sample and to the negative control. Samples and controls were pretreated with Proteinase K and a lysis solution optimized for the lysis of tissue and processed utilizing a bead-beater instrument. The tubes were removed from the bead-beater and incubated to complete the lysis of the samples. After incubation, the samples were further treated with a second lysis solution and incubated. Ethanol was added and the mixture pipetted onto a spin column containing a silica filter. DNA in the mixture was bound to the silica filter, washed several times to remove contaminants and eluted from the spin column filter utilizing a low salt solution.

Table 1.
Toxic Black Mold PCR information.

Target	Organism	Forward Primer Size (bp)	Reverse Primer Size (bp)	Probe Length (bp)	PCR Product Size
1	*S chartarum*	20	20	22	212
2	*A versicolor*	20	20	20	221
3	*A niger*	20	20	23	251
4	*P chrysogenum*	20	20	26	241
5	*P verrucosum*	20	20	19	241
6	*GEO*	21	22	25	218
7	*A flavus*	20	20	22	247
8	*A fumigatus*	20	20	26	250
9	*E nidulans*	20	20	20	220
10	*A ochraceus*	20	20	25	235
11	*A parasiticus*	20	20	20	245
12	*A sydowii*	20	20	20	221
13	*A ustus*	20	20	24	223
14	*F solani*	20	20	24	202
15	*P aurantiogriseum*	20	20	21	241
16	*P citrinum*	20	20	21	188
17	*P corylophilum*	20	20	25	239
18	*P crustosum*	20	18	20	258
19	*P expansium*	20	20	24	240
20	*P fellutanum*	20	20	19	236
21	*P roqueforti*	20	20	25	239
22	*P simplicissimum*	20	20	25	240
23	*S echinata*	20	20	22	211
24	*E amstelodami*	20	20	25	208

•"S= *Stachybotrys* ,*P = Penicillium*,*A = Aspergillus*

TARGET DESIGNS: Primers and probes specific for each of 23 fungal targets plus an internal control assay were designed by RealTime Labs, LLC and synthesized by Integrated DNA Technologies, Inc. All probes used for the assays are hydrolysis probes with the reporter FAM attached to the 5' end and the quencher BHQ attached to the 3' end. Primers and probes were received lyophilized and re-suspended and mixed into working stocks containing probe, primers and $MgCl_2$ at required concentrations. The enzyme for the reaction was purchased as a lyophilized bead from Cepheid manufactured specifically for the SmartCycler® system. Each 25.0 uL SmartCycler® reaction tube contained 0.5 bead, 16.5 uL of PCR grade water, 3.5 uL primer/probe working stock, and 5.0uL of extracted DNA. The internal control was run for each extracted sample and a negative control was added as an independent assay to insure a quality control step for the extraction. All 23 fungal assays and the internal control had a cycling profile containing an initial hot start followed by 45 denature and anneal cycles. All assays were designed and operated as qualitative detection assays. The capacity of a single SmartCycler® instrument varies from 16 to 96 reactions depending on the configuration. Multiple fungal assays can be processed on a single SmartCycler® run with each assay containing patient samples, a negative control and a positive control. Data generated from the assays was analyzed utilizing the SmartCycler® Software and positives were defined as any target that crosses the Cepheid recommended threshold of 30 on the fluorescence scale during processing on the instrument.

CONTROLS FOR RT-PCR TOXIGENIC MOLDS:

Internal Control – Each clinical sample processed was inoculated with spores from the internal control target Geometrica to show that a negative target result is a true negative result and not related to the extraction of the sample. The samples were processed through the extraction protocol and amplified and detected utilizing primer and probes specific for Geometrica.

Positive Control – Targets were spore extractions of the organisms listed in Table 1 which were obtained from ATCC. A positive control for each target of interest (Primer/Probe sets) was processed along with each clinical sample in each real-time PCR run. This positive control can be extracted from tissue or spore solutions but must be lot checked prior to use. The positive control shows that the primer/probe set for each target is not being inhibited and shows that a negative result is a true negative.

Negative Control – A negative control for each target of interest (Primer/Probe sets) was processed along with each clinical sample in each real-time PCR run. This negative control can be extracted from tissue or water but must be lot checked prior to use. The negative control shows that the primer/probe set, water and extraction reagents for each target is not contaminated with the target and shows that a positive result is a true positive.

Figure 2.
Example of aFAM Ct Graph showing six (6) positive ATCC fungal organisms .

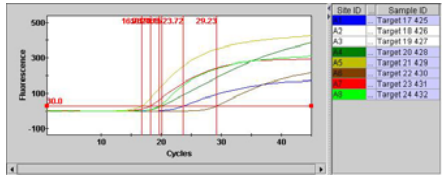


Table 2.
Isolates identified by RT-PCR by tissue

SPECIMEN TYPE	TOTAL	% POSITIVE	ORGANISM ISOLATED
Liver	6	50	*Penicillium chrysogenium* *Aspergillus niger* *Eurotium amstelodami*
Lung	7	57.2	*Penicillium chrysogenium* *Aspergillus niger* *Eurotium amstelodami* *Penicillium fellutanum* *Aspergillus fumigatus*
Skin	8	37.5	*Stachybotrys chartarum* *Aspergillus niger* *Penicillium chrysogenium* *Aspergillus ustus*
Brain	7	43.03	*Aspergillus niger* *Eurotium amstelodami* *Penicillim fellutanum*
Sputum/ U R *	18	33.3	*Aspergillus niger* *Stachybotrys chartarum* *Penicillium simplisticum*
Other	8	25	Aspergillus sydowii
TOTAL	55	40.0	

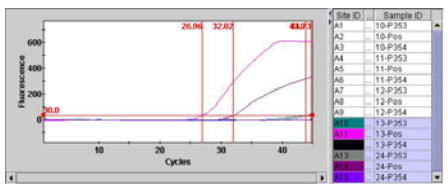
Validation: Each of the 23 fungal assays and the internal control assays were validated per CLIA requirements utilizing fungal spore solutions, spore spiked clinical tissues, and negative patient samples. Three serial dilutions were performed on each stock spore solution with each point run in triplicate. Each assay confirmed the precision and accuracy expected from observation during the development phase with each obtaining a positive qualitative determination at each point. In addition to the spore solutions, clinical samples were spiked with different combinations of spores and tested with each fungal assay. Each clinical sample containing spores for a particular target returned a positive qualitative result for the respective target. All clinical samples tested containing no spores for a particular assay obtained a negative qualitative result for the respective target. In summary, all positive and negative samples reproducibly presented positive and negative results, respectively.

RESULTS AND DISCUSSION

Case 1

40 y.o male with hx of chronic headaches X 2 years; with mold exposure in home.
CNS workup: negative
Pathology DX: Temporal dura and lobe: Perivascular inflammatory cell infiltrate. No neoplasm is identified. Hemosideridin filled macrophages. IPX stains show expression of GFAP by reactive astrocytes and CD66 by microglia. No Herpes simplex I or II, herpes type 8, CMV, or Toxoplasmosis. Negative for the JC Virus.
RT-PCR, Brain Bx.: Positive for ***Aspergillus ustus*** and ***Emericella amstelodami*** **(See Figure 3).**

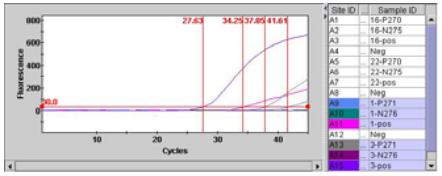
Figure 3. FAM-Ct Graph of Brain tissue



Case #2

56 y.o male, Las Vegas, NV, with multiple skin lesions, eczema, lentigo
Exposed to Stachybotrys chartarum, Asp. Niger, and Penicillium sp. (verified by Environmental studies).
Pathology Bx: Psoriasiform Spongiotic Dermatitis.
RT-PCR, Skin Biopsy: ***Stachybotyrous chartarum, Aspergillus niger*** **(See Figure 4).**

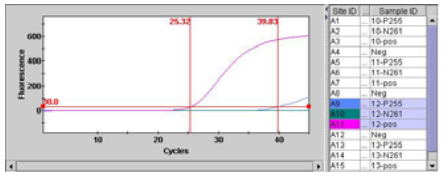
Figure 4. FAM Ct Graph of Skin



Case #3

84 y.o female with three mouth lesions; complaint: "cotton mouth" X 2 years.
History of mold exposure in home in Lousiana.
Pathology DX: Mouth: Parakeratosis, ulceration, no malignant cells, chronic inflammation, increased vascularity; consistent with a history of aphthous stomatitis
RT-PCR of oral mucosa: ***Aspergillus sydowii*** **(See Figure 5).**

Figure 5. FAM Ct. Graph of Oral Tissue



SUMMARY AND CONCLUSIONS

FUNGAL SPORE DNA CAN BE DETECTED IN HUMAN TISSUE AND BODY FLUIDS OF PATIENTS WHO HAVE BEEN EXPOSED TO TOXIN PRODUCING MOLDS IN THEIR ENVIRONMENT

Provisional Application Serial Nos. 60/787,754, filed on April 1, 2006, and 60/790,974, filed on April 11, 2006., Utility Patents filed on March 29, 2007.