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Utilizing Pyrosequencing and Quantitative PCR to Characterize Fungal Populations among House Dust Samples

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Abstract

Molecular techniques are replacing culturing and counting methods in quantifying indoor fungal contamination. Pyrosequencing offers the possibility of identifying unexpected indoor fungi. In this study, 50 house dust samples were collected from homes in the Yakima Valley, WA. Each sample was analyzed by quantitative PCR (QPCR) for 36 common fungi and by fungal tag-encoded flexible (FLX) amplicon pyrosequencing (fTEFAP) for these and additional fungi. Only 24 of the samples yielded amplified results using fTEFAP but QPCR successfully amplified all 50 samples. Over 450 fungal species were detected by fTEFAP but most were rare. Twenty-two fungi were found by fTEFAP to occur with at least an average of 0.5% relative occurrence. Many of these fungi seem to be associated with plants, soil or human skin. Combining fTEFAP and QPCR can enhance studies of fungal contamination in homes.

For many years culture-based techniques dominated the field of fungal identification and quantification in environmental samples.¹ However, these methods have many limitations, including only the viable fungi will grow; the media utilized will select for certain fungi; sampling time is often limited to reduce over-crowding of the culture plates; significant mycological experience is required to identify the many different fungi. More recently, DNA based analyses, like quantitative PCR (QPCR), have been developed which utilize

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evolutionarily stable genes to identify fungi.^{2,3} However, these methods are limited to the fungi for which there are validated assays. Therefore, some fungi in the samples may not be identified and quantified because there are no validated QPCR assays for them. The method of pyrosequencing (PS) offers the possibility of discovering fungal species in environmental samples that have not been previously identified.⁴

The fungal tag-encoded flexible (FLX) amplicon pyrosequencing (fTEFAP) method was developed to classify fungi in complex environments.^{5,6} The DNA in the extracted sample is sequenced using universal primers to target specific 18S ribosomal DNA sequences. The fTEFAP methodology results in the characterization of the relative percentage fungi in the sample, often at the species level. However, fTEFAP does not provide an absolute quantification, only the relative proportion of fungi in the sample. Therefore, the purpose of this study was to perform a comparison of QPCR and fTEFAP using aliquots of the same house dust samples.

MATERIALS AND METHODS

Sampling

The dust samples were collected under a Children's Health Center study (CHC) in the Yakima Valley, an agricultural valley region of Washington State and a part of the shrub steppe ecosystem.^{7,8} The Yakima Valley has a steppe climate with Mediterranean precipitation pattern that supports the growth of apples, pears, peaches, cherries, grapes, and hops. For this analysis, a set of 50 rural home dust samples were obtained in either 2005 or 2011.

Dust was collected by Nilfisk vacuums or Metropolitan VM500 vacuums. The size of the area vacuumed depended on the floor type, and ranged from a 1 m² area for plush carpets to a 4 m² area for hard or smooth floors. All dust samples were initially stored at -10°C in the field office laboratory.^{9,10} Samples were subsequently transferred to the University of Washington Children's Health Risks Research Biorepository for further storage at -10°C. Dust samples were transferred from the vacuum cleaner bags to 150 µm pore size metal sieves (VWR, West Chester, PA) and shook for 10 min (Shaker Model RX-24; WS Tyler Inc, Mentor, OH). All sieved dust samples were stored at -20°C until analyzed. Dust aliquots were shipped from the repository to either the US EPA laboratory (US Environmental Protection Agency, Cincinnati, OH) for QPCR analysis or to the PS Laboratory (Research and Testing Laboratory, Lubbock, TX) for pyrosequencing.

DNA Extraction

For QPCR analysis, each 5.0 mg dust sample was spiked with 1×10^6 conidia of *Geotrichum candidum* at the time of extraction as an external reference (Haugland et al 2002). Each extraction tube was shaken in the bead beater (Biospec Products, Bartlesville, OK) for one min and the DNA purified using the DNA-EZ extraction kit (GeneRite, Cherry Hill, NJ).

For pyrosequencing analysis, a 5.0 mg dust sample was resuspended in 200 µl of molecular grade water. This suspension was then added to the PowerBead Tubes from a MoBio

PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA). To each sample 60 μ l of kit Solution C1 was added to each sample. Each PowerBead Tube was then shaken on a Qiagen TissueLyser (Qiagen, Valencia, CA), run at 15Hz for 10 min. The tubes were centrifuged at $10,000 \times g$ for 30 sec and the supernatant was transferred to a new tube containing 250 μ l of kit Solution C2 and incubated at 4°C for 5 min. Tubes were centrifuged for 1 min at $10,000 \times g$ and 600 μ l of the supernatant was transferred to a new tube containing 200 μ l of kit Solution C3 and incubated at 4°C for 5 min. Tubes were centrifuged at $10,000 \times g$ and up to 750 μ l of supernatant was transferred to a new tube containing 1200 μ l of kit Solution C4. A 650 μ l aliquot of this mixture was added to a spin filter and spun at $10,000 \times g$ for 1 min and the flow-through discarded. This step was repeated 3 times until all of the supernatant had been added. The spin filter was then washed with 500 μ l of kit Solution C5 and spun for 30 sec at $10,000 \times g$ the flow-through was discarded and the filter spun an additional 1 min at $10,000 \times g$. Finally, the filter was added to a new tube, and 50 μ l of kit Solution C5 was added to the filter. The filter was spun at $10,000 \times g$ for 1 min and discarded and the flow-through was used in the analysis of each sample.

Fungal Quantification using QPCR

Methods and assays have been reported for performing QPCR analyses.^{2,3} Briefly, the standard reaction assays contained 12.5 μ l of “Universal Master Mix” (Applied Biosystems Inc., Foster City, CA), 1 μ l of a mixture of forward and reverse primers at 25 μ M each, 2.5 μ l of a 400 nM TaqMan probe (Applied Biosystems Inc.), 2.5 μ l of 2 mg/ml fraction V bovine serum albumin (Sigma Chemical, St. Louis, MO) and 2.5 μ l of DNA free water (Cepheid, Sunnyvale, CA). To this mix was added 5 μ l of the DNA extract from the sample. All primer and probe sequences used in the assays as well as known species comprising the assay groups are at the website: <http://www.epa.gov/nerlcwww/moldtech.htm>. Primers and probes were synthesized commercially (Applied Biosystems Inc., Foster City, CA).

Reactions were performed with thermal cycling conditions consisting of 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C for template denaturation and 1 minute at 60°C for probe and primer annealing and primer extension. The Cycle threshold determinations were automatically performed by the instrument using default parameters. Assays for each target species and the internal reference (*Geotrichum candidum*) were performed in separate tubes of the 96-well plate format.

The analysis of the 36 fungal species produced an index value called the Environmental Relative Moldiness Index (ERMI) that describes the fungal burden in each home based on a random national sampling of homes.¹¹ Of the 36 fungi, there are 26 Group 1 fungi that indicate water-damage and 10 Group 2 species that are often found in homes, even without water-damage, which primarily come from outdoors.¹²

Pyrosequencing of Fungi in Dust Samples: Massively Parallel fTEFAP Titanium

Fungal tag-encoded FLX amplicon pyrosequencing (fTEFAP) were performed as previously described.¹³ Following sequencing, any non-fungal ribosomal DNA sequences were removed. All tags, low quality sequence ends, and failed sequence reads and chimeras were also removed using custom software and the Black Box Chimera Check software B2C2 both

of which have been used previously to classify ribosomal DNA from fungi.^{6,13,14} Sequences less than 250 base pairs were also removed.

Fungal identification in pyrosequenced samples

To determine the identity of fungi, the sequences were sorted such that the FASTA formatted file contained reads from longest to shortest. These sequences were then clustered into operational taxonomic unit (OTU) clusters with 96.5% identity (3.5% divergence) using USEARCH.¹⁵ For each cluster the seed sequence was placed in a FASTA formatted sequence file. This file was then queried against a database of high quality sequences derived from NCBI using a distributed .NET algorithm that utilizes BLASTn+ (KrakenBLAST www.krakenblast.com). The BLASTn+ outputs were compiled using a .NET and C# analysis pipeline. The data reduction analysis performed as previously described.¹⁵⁻¹⁸

Based upon the above BLASTn+ derived sequence identity (percent of total length query sequence which aligns with a given database sequence) and validation using taxonomic distance methods, the fungi were classified at the genus and species taxonomic levels. Approximately, 82,584 sequences representing 14,438 fungal species were present in the database used for classification.¹⁹ Sequences with identity scores were compared to known or well characterized ribosomal DNA sequences. Sequence identities greater than 97% (<3% divergence) were resolved at the species level and between 95 and 97% at the genus level. Any match below this percent identity was discarded. In addition, the High Score Pair was at least 75% of the query sequence or it was discarded, regardless of identity.

After resolution based upon these parameters, the percentage of each fungal identity was individually analyzed for the sample by providing relative abundance information based upon relative numbers of 18s DNA sequences within a given sample. The primary taxonomic identification of the sample sequences was resolved to its closest relative or species level, when possible.

The intersection of the pyrosequencing analysis and the QPCR data for the 36 ERMI fungi were shown visually using a “Double Dendrogram” or “heat map”. The highest agreements in occurrence were shown as the “hottest” color (red) and progressively lower agreement to “cooler” colors of the spectrum (toward blue).

RESULTS

About half of the samples were not able to be amplified sufficiently by fTEFAP. However, all of the samples were adequately analyzed by MSQPCR with no evidence of inhibition. The average ERMI was 3.8 for the 50 samples and 2.3 for the 25 samples that could be pyrosequenced (Table 1). Pyrosequencing detected about 450 fungal species in one or more of the samples. However, most of these fungal species were not present in all of the samples (data not shown). The fungal species with an average of 0.5% relative occurrence in the 24 pyrosequenced samples are shown in Table 2. The fungus *Chalara longipes* (9% relative occurrence) was in the highest relative percentage occurrence among these samples;

followed by *Aureobasidium pullulans* (3%), *Malassezia globosa* (2.7%), and *Cladosporium cladosporioides* (2.4%).

Of the 36 fungi tested for with QPCR, *Cladosporium herbarum* was not detected in the PS samples; however an “unknown species” of *Cladosporium* was detected with an average relative percent occurrence of 0.2%. Similarly, *Alternaria alternata* was not detected by PS but an “unknown species” of *Alternaria* was detected at a rate of 1.25%. For these two species, the value for the relative percent occurrence of the “unknown species” was also listed in Table 1 (column 3) and used in the comparison of relative concentrations (by QPCR) or occurrence (by PS).

The 18 fungi detected by both QPCR and PS were ranked from highest to lowest by either cells/mg of dust or relative abundance (Table 3). *Aureobasidium pullulans* occurred at the highest concentration (cells/mg of dust) by QPCR and the highest relative percentage by PS. The next five species measured by QPCR (*C. herbarum* to *Penicillium brevicompactum*) were also included within the next seven fungi in occurrence as estimated by PS. The *Mucor* group and *A. niger* were detected at higher concentrations by QPCR (ranking 5 and 7, respectively) than their relative ranking based-on PS (Ranking 11 and 14, respectively) (Table 3). A few species have higher apparent relative occurrence rates based-on PS compared to QPCR. For example, *Chaetomium globosum* and *Aspergillus fumigatus* had higher ranking positions (6 and 8, respectively) based-on PS than the ranking based-on QPCR (17 and 15, respectively). The other fungi detected had fairly close rankings by both QPCR and PS (Table 3).

DISCUSSION

In this study we have combined the “wide-net” of PS with the “quantitativeness” of QPCR to evaluate the populations of fungi in a set of dust samples from rural homes in Washington State. The 36 fungi that make-up the ERMI occurred commonly in homes across the US¹¹ and these species were also common in the Yakima Valley homes. However, these 36 fungi likely represent only a fraction of the species present in home dust.

The intersection of the pyrosequencing data and the ERMI data can be visualized using a “Double Dendrogram” or “heat-map” (Figure 1). Fungi like *Aureobasidium pullulans*, *Cladosporium herbarum*, *C. cladosporioides* and *Alternaria alternata* were abundant based-on either QPCR or PS, as shown by the dark red areas (Figure 1). The rarer of the 36 molds are shown in progressively “cooler” colors (towards blue) and some were not detected by PS. However, PS detected many additional species at high relative occurrence proportions (Table 2).

The fungus *Chalara longipes* was three times more common in the PS analyzed samples than even *A. pullulans*. The presence of this fungal species is associated with the degradation of pine needles²⁰ and may reflect the forested environment of Washington State as these homes were located 10 to 40 miles from heavily forested regions. The Yakima Valley is one of the largest fruit tree growing areas in the US which may explain the occurrence of *Monilinia laxa*, a pathogen of peach trees²¹ or *Oidium aloysiae*, from the

powdery mildew family of fungal pathogens.²² The somewhat surprising finding of relative high concentrations of *Juncigena adarca*, typically associated with salt marsh plants, may be explained by the Westerly's and the winds that predominate during the winter months and which are known to bring a maritime presence to the valley from the coastal area.²³

Many of the fungi detected by PS, e.g. *Fusarium oxysporum*²⁴ and *F. equiseti*²⁵ are soil inhabitants that might have been carried indoors by the wind or foot traffic. Surprisingly, a soil inhabitant, *Dactylella candida*, best known for its nematode-lassoing ability, was found in high relative concentrations.²⁶

On the other hand, *Malassezia* species may be widespread because of their growth on human skin and scalp.²⁷ Many *Candida* species were also detected by PS and these too may reflect the shedding from the homes' inhabitants.

By discovering some of these “unexpected” fungi in dust samples, future epidemiological studies could target these for analysis. However, all molecular approaches to fungal taxonomy are caught in the legacy of culture-based taxonomy. Therefore the fungal names provided through PS or QPCR are still dependent on molecular databases such as NCBI which are not complete, nor necessarily in agreement with classical taxonomy. A number of species that were in fairly high concentrations as measured by QPCR (e.g. *Wallemia sebi* and *Epicoccum nigrum*) were not detected by PS. This may be the result of the databases used to create the QPCR assays versus databases used for PS identification. Also, different primers are used for the PS and ERMI. PS primers are designed to be “universal” to amplify a larger number of targets. However, the design of the PS primers introduces a bias where some groups of fungi may not be amplified as efficiently as others and therefore may be underrepresented in the study sample. Lastly, reporting the prevalence of fungi in homes solely based on the relative abundance of fungi determined from PS is challenging as 18s targets maybe present at multiple locations in a given fungal genome. Therefore a positive bias may exist for some fungi identified. Furthermore, the depth of the sequencing in PS may not fully represent the diversity of fungi present in a complex environmental sample such as household dust.²⁸ However, even with these limitations, the combined use of multiple molecular techniques should provide an improvement in our understanding of the relationship between fungal exposures and health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

The intersection of the pyrosequencing analysis and the QPCR data for the 36 ERMI fungi were shown visually using a “Double Dendrogram” or “heat map”. The highest agreements in occurrence were shown as the “hottest” color (red) and progressively lower agreement to “cooler” colors of the spectrum (toward blue).

Table 1

Average concentration of fungi (cells per mg dust) as measured by quantitative PCR (QPCR) for all 50 samples. Average concentration of fungi (cells per mg dust) as measured by QPCR or as an average relative percentage occurrence based-on the 24 samples pyrosequenced (PS). (For purposes of calculating the average cells per mg dust, any non-detects were valued as 0.)

Group 1	Average (n=50) (cells/mg dust)	Average (n=24) (cells/mg dust)	Average (n=24) (%) Relative
<i>Aspergillus flavus</i>	4	1	0.01
<i>Aspergillus fumigatus</i>	8	2	0.08
<i>Aspergillus niger</i>	115	63	0.04
<i>Aspergillus ochraceus</i>	39	9	ND ^a
<i>Aspergillus penicillioides</i>	2	1	ND
<i>Aspergillus restrictus</i>	38	53	0.11
<i>Aspergillus sclerotiorum</i>	<1	1	ND
<i>Aspergillus sydowii</i>	9	1	ND
<i>Aspergillus unguis</i>	8	1	ND
<i>Aspergillus versicolor</i>	14	18	0.08
<i>Aureobasidium pullulans</i>	13,879	14,252	3.28
<i>Chaetomium globosum</i>	3	1	0.11
<i>Cladosporium sphaerospermum</i>	37	74	ND
<i>Eurotium</i> group	133	156	>0.01
<i>Paecilomyces variotii</i>	3	2	>0.01
<i>Penicillium brevicompactum</i>	138	100	0.47
<i>Penicillium corylophilum</i>	17	20	ND
<i>Penicillium</i> group/f or PS “ <i>P. crustosum</i> + <i>P. commune</i> ”	226	47	0.05
<i>Penicillium purpurogenum</i>	9	5	ND
<i>Penicillium spinulosum</i>	<1	<1	ND
<i>Penicillium variabile</i>	6	7	ND
<i>Scopulariopsis brevicaulis</i>	31	7	0.07
<i>Scopulariopsis chartarum</i>	50	55	ND
<i>Stachybotrys chartarum</i>	13	6	0.01
<i>Trichoderma viride</i>	54	104	ND
<i>Wallemia sebi</i>	553	393	ND
Sum of the Logs (Group 1):	20.57	18.10	NA
<i>Acremonium strictum</i>	5	5	ND
<i>Alternaria alternata</i> for PS “unknown <i>Alternaria</i> species”	513	356	0/1.25
<i>Aspergillus ustus</i>	9	2	ND
<i>Cladosporium cladosporioides</i> type 1	2,556	2,959	1.81
<i>Cladosporium cladosporioides</i> type 2	22	15	ND
<i>Cladosporium herbarum</i> / for PS “unknown <i>Cladosporium</i> species”	5,771	4,728	0/0.20
<i>Epicoccum nigrum</i>	272	236	ND
<i>Mucor</i> group	628	317	0.06

Group 1	Average (n=50) (cells/mg dust)	Average (n=24) (cells/mg dust)	Average (n=24) (% Relative)
<i>Penicillium chrysogenum</i> type 2	346	12	0.06
<i>Rhizopus stolonifer</i>	995	14	0.02
Sum of the Logs (Group 2):	16.76	15.77	NA ^b
ERMI	3.81	2.33	NA

^aND=no target DNA detected by pyrosequencing

^bNA= not applicable

Table 2

Fungal species with average relative occurrences of 0.5% as determined by pyrosequencing and possible sources.

Fungal Species	Average % Occurrence	Possible Source
<i>Chalara longipes</i>	9.04	Decomposer in pine forests
<i>Aureobasidium pullulans</i>	3.03	Phylosphere and broad ecology
<i>Malassezia globosa</i>	2.71	Skin
<i>Cladosporium cladosporioides</i>	2.44	Phylosphere
<i>Montagnula dura</i>	2.27	Plant associated
<i>Catenulostroma elginense</i>	2.12	Plant pathogen
<i>Dactylotia candida</i>	1.98	Nematode capturing fungus
<i>Juncigena adarca</i>	1.47	Salt marsh plants
<i>Malassezia restricta</i>	1.45	Skin
<i>Oidium aloysiae</i>	1.31	Powdery mildew fungus
<i>Alternaria alternata</i>	1.25	Phylosphere
<i>Dothidotthia symphoricarpi</i>	1.20	Cosmopolitan
<i>Leptosphaeria microscopic</i>	0.97	Phylosphere
<i>Fusarium oxysporum</i>	0.88	Plant Pathogen
<i>Ascobolus crenulatus</i>	0.83	Coprophilous/cellulose degrader
<i>Catenulostroma germanicum</i>	0.82	Environmental
<i>C. chromoblastomycosum</i>	0.78	Environmental
<i>Guignardia citricarpa</i>	0.75	Cosmopolitan/ endophyte of plants
<i>Mortierella alpina</i>	0.65	Cosmopolitan/soil inhabiting
<i>Fusarium equiseti</i>	0.65	Plant root colonizer
<i>Monilinia laxa</i>	0.54	Pathogen of peaches
<i>Teratosphaeria mexicana</i>	0.52	Phylosphere/possible plant pathogen

Table 3

Rank comparison for the 18 fungal species detected by both quantitative PCR (QPCR) and pyrosequencing (PS). The fungi were assembled from highest to lowest based on the average concentration (cells per mg dust) as measured by QPCR.

Fungi	QPCR Rank	Pyrosequenced Rank
<i>Aureobasidium pullulans</i>	1	1
<i>Cladosporium herbarum</i> / for PS “unknown <i>Cladosporium</i> species”	2	5
<i>Cladosporium cladosporioides</i> only type 1	3	2
<i>Alternaria alternata</i> / for PS “unknown <i>Alternaria</i> species”	4	3
<i>Mucor</i> group	5	11
<i>Penicillium brevicompactum</i>	6	4
<i>Aspergillus niger</i>	7	14
<i>Aspergillus restrictus</i>	8	6
<i>Penicillium</i> group/ for PS “ <i>P. crustosum</i> + <i>P. commune</i> ”	9	13
<i>Aspergillus versicolor</i>	10	8
<i>Rhizopus stolonifer</i>	11	15
<i>Penicillium chrysogenum</i> type 2	12	11
<i>Scopulariopsis brevicaulis</i>	13	10
<i>Stachybotrys chartarum</i>	14	16
<i>Aspergillus fumigatus</i>	15	8
<i>Paecilomyces variotii</i>	16	18
<i>Chaetomium globosum</i>	17	6
<i>Aspergillus flavus</i>	18	16