AMPLIFICATION OF STACHYBOTRYS GENE USING PCR

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Abstract

Many fungi species are known to produce mycotoxins and or trichothecene that can be toxic to human. Fungi of the genus Stachybotrys isolated from water-damaged buildings are suspected to be responsible for Sick Building Syndrome (SBS) particularly S. chartarum which belongs to the trichothecenes producing family. The IT51/IT41 primers were used in this study initially described by Haugland and Heckman. The primer set could only amplify the ssrDNA in 7 of the 10 isolates due to the possibility of contamination in one of the isolates. Some regions show a high degree of conservation, while some others are very variable; IT51/IT41 regions were identical for isolate 00873 and 01297; Isolates 00011, 00453 and 00220 belong to a separate cluster from 00012, 00873, 00639 and 01297. Isolates 00011, 00453 and 00220 have nucleotide sequences similar to Penicillium species in the databases; Isolates 00012, 00873, 00639 and 01297 have sequences similar to Stachybotrys chartarum The method was tested by analyzing commercial and environmental samples. In all cases, the species with the presence of Trichodiene Synthase 5 (Tri5) gene sequence gave the highest signal intensity at an acceptable background level. This rapid and simple multiplex technique could be very important in gaining a better understanding of the physiological behavior of moulds exposed to radiation, and the role of fungi in the internal and external environment.

Key words: PCR Amplification, Water-damaged building, Sick Building Syndrome, Fungi Stachybotrys, Trichothecene, Trichodiene Synthase 5 (Tri5), IT51/IT41 Primers.

Introduction

Microbial infections are primarily suspected to be the major cause of severe health problem associated with water damaged buildings especially moulds**.** They are commonly found on the surface of damp inorganic matter such as glass and bare concrete covered by an invisible *biofilm.* Moulds tend to be inherently resilient and adaptable in varied habitations [40]. Floods, leaking pipes, leaking windows, and leaking roofs are all potential sources of moisture that can lead to mould infestation. Increased ambient humidity as a result of inadequate ventilation or improper drying of flooded areas can also lead to mould growth [11]. Moulds that are members of mycotoxin and macrocyclic trichothecene producing strains including Cladosporium, sphaerospermum, Penicillium chrysogenum, A. versicolor, Alternaria alternate and S. *chartarum* are thought to be the major cause of health problems [12, 22, 36, 59]. According to the Center for Disease Control (CDC) [11], some of the common health concerns linked with exposure to molds include non-specified disorders of the lower airways, eyes and skin, and hay fever-like allergy. The presence of *S. chartarum*-specific IgA or IgG antibodies in patients exposed to water-damaged building has been proposed to be evidence of recent or chronic exposure to the fungus respectively. Generally, the IgA antibodies are relatively short-lived, and the kinetics of IgA synthesis and breakdown show large variation among individuals. This phenomenom depends on the extent of exposure to related or cross-reacting antigens from *Aspergillus fumigatus* and *Alternaria alternate* [14, 33]. In addition, IBT Laboratory has also indicated that kinetics of decline for IgG antibodies are not predictable and cannot be used to establish the date of last exposure to *S. chartarum* or other fungal cross-reacting antigens. However, it was suggested that a patient with symptoms of S. *Chartarum* infection may have been exposed to an entirely different fungus that shares certain immunologic characteristics with *S. chartarum*. Thus, a positive *S. chartarum* test result does not necessarily mean the patient has developed antibodies to infection of *S. chartarum* [14].

Conventional laboratory procedure uses a Tease, or Scotch Tape Lactophenol Mount techniques to detect and identify fungi often requires culture isolation from environmental samples and/ or direct microscopic examination for morphological characterization such as viability on natural media, conidial color, septation, appearance of spores and mycelia [5]. The method requires high technical and analytical skills to perform; and it is prone to basic human error.

A study by Zhihong et Al. [62] illustrated a current attempt to use PCR for amplification of specific DNA fragment from fungi without any cross-amplification with DNA from bacteria cell. Normally, PCR is highly specific and sensitive in amplification of short fragments of nucleic acid for quantitative analysis. PCR amplifies species-specific gene sequence based on the small sequence ribosomal RNA (ssRNA) and TS regions of the Stachybotrys isolates. The gene amplification occurs as long as the desired segment is present in the sample regardless of the culture density or viability of the organisms on culture media. Toxicity test was used to detect production of trichothecene toxins by searching for the presence or absence of the Tri5 gene which acts on Trichodiene Synthase, an enzyme required in the first stages of Trichothecene metabolic pathway. To yield consistent result for determination of nucleotide polymorphism using PCR, a crucial step by step protocol involves a standardized nucleic acid sampling, extraction, purification, and amplification.

Experimental Design

Environmental isolates of various species of *Stachybotrys* were obtained from stock cultures kept in the Environmental Biotechnology Laboratory at the Lawrence Berkeley National Laboratory, Berkeley,

California [Table 2]. These isolates were a part of microbial cultures previously collected from the Chernobyl Exclusion Zone in Ukraine. They have been exposed to high levels of Cesium 137 radiation due to the explosion of reactor number 4 at the Chernobyl Nuclear Plant in April, 1986, and preserved under – 20°C. The conidia stock were grown and harvested on potato dextrose agar (PDA) at 23°C for 5 - 7 days. The fungal mycelia were transfered from PDA to culture tubes containing 5mL Yeast Peptone Dextrose (YPD) broth and incubated in an incubator shaker for 7 –10 days at 23°C and 150 rpm.

Genomic DNA Extraction using FastDNA Kit

The instrument is designed to purify a single or double stranded DNA fragments from PCR-ready Genomic DNA in a homogenized sample such as a plant and animal tissue, fungi, bacteria, or yeast cells etc., by centrifuging with a QIAquick spin columns micro centrifuge in less than 30 minutes. Fragments ranging from 100bp up to 10kb were obtained from analysis of the PCR product using the QIAquick Gel Extraction Kit. Exposure to shortwave ultraviolet light was minimized in order to avoid the formation of pyrimidine dimmers.

Polymerase Chain Reaction (PCR) Condition

A Master Mix containing 10X Buffer that was prepared with 500 mM KCl, 100mM Tris pH 8.3, 25mM $MgCl_2$, and 1mg/mL gelatin. The mixture was done in a PCR hood sterilized with UV light for 1 - 2 hours. A total number of 12 reaction with 10 samples, one negative control, and an extra reaction set for convenient pipetting were set up. PCR was performed under stringent thermo cycling condition with a bench top Stratagene RoboCycler® Gradient 96 Temperature Cycler, and Perkin Elmer Amplitaq polymerase.

Thermo Cycling Condition

Denaturing was carried out at 94°C for 2:00 minutes 1 Cycle

Step cycle complete mix 30 cycles at

- 94ºC for 30 seconds
- 54ºC for 1 minute
- 72°C for 1 minute 30 seconds
- After Final Cycle Incubate at 68ºC for 7 min

Sequencing of PCR Amplicons

Multiple sequence alignment programs - Genedoc and Chromas were used to examine, align, and edit DNA sequence files. The sequence data generated were analyzed for phylogenetic similarity based on number of base pair and consensus scores from pair wise alignment using CLUSTALW [Table 3]. The computer programs involved a 6-step protocol which includes:-

Read Forward and Reverse Sequencing in Genedoc >> Renaming Sequences and Changing Reverse Sequence to Inverse Complement >> Aligning of Sequences >> Removal Extra 5' and 3' sequences >> Rectifying of Sequences and Filling in the Gaps >> Copying the Consensus Sequence to a New File Containing All of the Sequences.

Results and Discussion

An earlier study by Zhihong et al. [62] on environmental monitoring of indoor air quality examined the selectivity of 53 sets of primers - Thirty-six fungal strains, representing 26 species from 14 genera of commonly occurring fungi, and 16 different bacterial strains, representing both Gram-negative and Gram-positive species, were included in the experiment. The research verified the specificity of 28 of the 53 sets of

primers, which were classified as universal fungal, universal bacterial, group or species specific. The PCR conditions required for optimal specificity were determined and the results served as a guide for the step-wise PCR-based detection and identification of airborne fungi commonly found in indoor environments. The results of this research reports on a quantitative PCR (QPCR) method for detection and quantification of S. *chartarum* demonstrated the presence of PCR inhibitors in some S. *chartarum* isolates [Fig. 3].

IT51/IT41 Primers for ssrDNA

The IT51/IT41 primers that were used in this study were initially described by Haugland and Heckman [16]. They observed that product bands of similar intensity were obtained from the templates of all species examined in their study when the universally conserved reverse primer IT41 was used in combination with IT51 forward primer [Table 3]. Table 2 shows the proposed identifications based on the comparison of the small ribosomal subunit sequences of the isolated with those in the database. The primer set could only amplify the ssrDNA in 7 of the 10 isolates due to the possibility of contamination in one of the isolates. Some regions show a high degree of conservation, while some others are very variable; IT51/IT41 regions were identical for isolates 00873 and 01297; Isolates 00011, 00453 and 00220 belong to a separate cluster from 00012, 00873, 00639 and 01297 [Figure 2, 3, and 4]. Isolates 00011, 00453 and 00220 have nucleotide sequences similar to *Penicillium species* in the databases; Isolates 00012, 00873, 00639 and 01297 have sequences similar to *Stachybotrys chartarum* [Table 2].

Trichodiene Synthase 5 Gene

Fungi produce low molecular weight secondary metabolites such as antibiotics and mycotoxins - trichothecene. Antibiotics cure diseases whereas mycotoxins cause diseases in plants, animals and human beings. Species such as *Aspergillus, Fusarium, Penicillium* and *Stachybotrys* are known to produce mycotoxins that accumulate in processed foods and feeds, although the incidence of infection occurs before processing during the growth cycle of the organism. The presence or absence of the trichodiene synthase gene in a mycotoxin –producing organism is an important step in determining toxicity. The presence of the gene shows a potential inherent ability in the organism in question to be toxic, and on the other hand, the absence of the gene means that the organism is not capable of producing the toxin. Trichodiene is produced in larger amounts by *Stachybotrys* isolates [Figure 4], which also produce satratoxins, but it will be difficult to utilize this metabolite to detect toxic isolates in buildings due to the relatively small amounts excreted. The relationship between the production of trichodiene and various non-volatile trichothecenes for several moulds was investigated. *Stachybotrys chartarum* isolates producing macrocyclic trichothecenes secreted significantly larger amounts of trichodiene and other sesquiterpenes than isolates which only produced simple trichothecenes. The amounts of secreted trichodiene were relatively small in all cases. With the exception of *Memnoniella* which excreted small amounts of sesquiterpenes; the other isolates produced varying amounts of sesquiterpenes, including trichodiene, and simple trichothecenes. However, the Memnoniella produced large amounts of griseofulvin derivatives. In *Stachybotrys* there is apparently a correlation between trichodiene and macrocyclic trichothecene production in contrast to the remaining isolates [60 and 61].

The results obtained from this study have shown that 4 out of the 10 isolates may be potentially toxic; however, the presence of *Stachybotrys chartarum* in an indoor environment does not necessarily pose a health risk as regards to the production of trichothecene family of toxins. The Tri5 gene sequences in these isolates generally show a high degree of sequence conservation. Isolates 00012 and 00638 are exactly the same, hence may be the same organism. There is a 99% similarity between the Tri5 genes the isolates (Table: 3).

Several studies [16, 21, 27, 28, 60 and 61] have alluded to the possibility that not all *Stachybotrys chartarum* species produce macrocyclic trichothecenes. The report supports the evidence obtained in this study regarding the observation that only five of the ten isolates were identified to have the gene, Trichodiene synthase 5 (Figure:4). The implication of this is that the mere fact that *Stachybotrys species* are found in an indoor environment does not necessarily mean that they are toxic or that they even have the potential to be toxic.

Wilkins and Kristan [61] reported that S. *chartarum* isolates producing macrocyclic trichothecenes secreted significantly larger amounts of trichodiene as observed in this study, and other sesquiterpenes than isolates which only produced simple trichothecenes. In Stachybotrys there is apparently a correlation between trichodiene and macrocyclic trichothecene production. The presence or absence of the trichodiene synthase gene in a mycotoxin – producing organism is an important step in determining toxicity. These reports support the evidence obtained in this study regarding the observation that only five of the ten isolates were identified to have the gene, Trichodiene synthase 5 Table: 3.

Conclusion

The role of Stachybotrys species in causing health risks as a result of exposure to water-damaged buildings is still unknown based on the scope of this study. In other words, to fully understand the impact of S. *chatarum* metabolites with respect to SBS, more information is required from in vivo interaction with other proteins. Although, the PCR amplification methodology described in this experiment does not require professionally trained scientist to perform; it is very credible and cost effective. Moreover, since it is an instrument-based method, it eliminates human errors, and standardizes quality assurance and quality control procedures that could be acceptable in case of litigation.

With the current trend of technology advancement, it is possible to develop Biosensors or Biochips consisting of specific nucleotide sequences of different fungi for identification of several species at the same time, and monitoring the presence of fungi over a given period of time. For example, a year round sampling can be done to study seasonal changes in occurrence. As a result, it could be possible to detect fungi where the periodicity of the toxins discharge may be an issue.

TABLE 1: Sequence of DNA fragments from the different extracts was amplified using two primer sets [13, 16].

	Trichodiene Synthase 5 (Tri5) gene Primer set	IT51 and IT41 Primers set
Forward Primer	5'-CATCAATCCAACAGTTTCAC-3'	5'-GAGGAAGTAAAAGTCGTAACAAGGT-3' – IT51
Reverse Primer	5'- GCAACCTTCAAAGACTATTG-3'	5'-GATATGCTTAAGTTCAGCGGGTA-3' – IT41

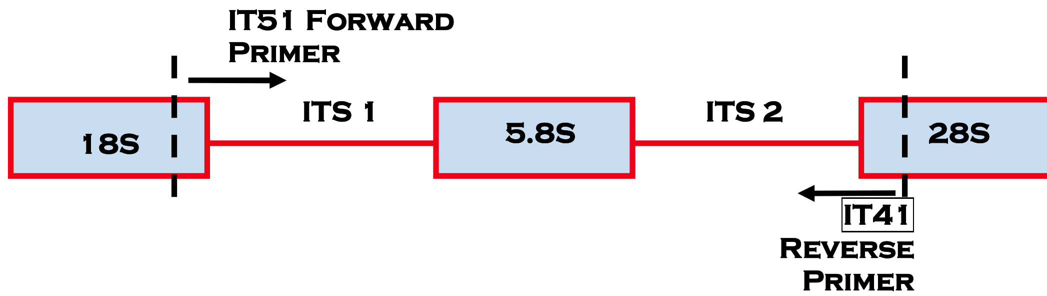

FIGURE 1: Map of the rDNA Regions. Regions sequenced in this study show target sites of the forward and reverse primers; by Invitrogen Life Technologies.

TABLE 2: Identification and comparison of the S. c*hartarum* based small Ribosomal subunit sequence of the isolates.

Isolate Code	Isolate Stock #	Labeled Identification	Proposed Identification
1	00011	*Stachybotrys chartarum*	*Penicillium species?*
2	00012	*Stachybotrys chartarum*	*Stachybotrys chartarum*
3	00220	*Stachybotrys elegans*	*Penicillium species?*
4	00453	*Stachybotrys bisbyi*	*Penicillium species?*
5	00638	*Stachybotrys chartarum*	*Stachybotrys chartarum*
6	00639	*Stachybotrys chartarum*	*Stachybotrys chartarum*
7	00640	*Stachybotrys chartarum*	*Unknown*
8	00873	*Stachybotrys albipes*	*Stachybotrys chartarum?*
9	01134	*Stachybotrys species*	*Unknown*
10	01297	*Stachybotrys chartarum*	*Stachybotrys chartarum*

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2	00012	*Stachybotrys chartarum*	*Stachybotrys chartarum*
3	00220	*Stachybotrys elegans*	*Penicillium species?*
4	00453	*Stachybotrys bisbyi*	*Penicillium species?*
5	00638	*Stachybotrys chartarum*	*Stachybotrys chartarum*
6	00639	*Stachybotrys chartarum*	*Stachybotrys chartarum*
7	00640	*Stachybotrys chartarum*	*Unknown*
8	00873	*Stachybotrys albipes*	*Stachybotrys chartarum?*

9	01134	*Stachybotrys species*	*Unknown*
10	01297	*Stachybotrys chartarum*	*Stachybotrys chartarum*

Table 3: CLUSTAL W Multiple Sequence Alignments (1.81) and (1.82) for IT51/IT41 Primers and Tri5 Gene Sequence respectively with Pearson Sequence format.

CLUSTALW Results for IT51/IT41 Primers	**CLUSTALW Result for the Tri5 Gene Sequences**
Sequence 1: 00011 522 bp Sequence 2: 00012 544 bp Sequence 3: 00220 548 bp Sequence 4: 00453 548 bp Sequence 5: 00639 533 bp Sequence 6: 00873 537 bp Sequence 7: 01297 537 bp Start of Pair wise alignments Sequences (1:2) Aligned. Score: 58 Sequences (1:3) Aligned. Score: 92 Sequences (1:4) Aligned. Score: 91 Sequences (1:5) Aligned. Score: 58 Sequences (1:6) Aligned. Score: 58 Sequences (1:7) Aligned. Score: 58 Sequences (2:3) Aligned. Score: 71 Sequences (2:4) Aligned. Score: 71 Sequences (2:5) Aligned. Score: 99 Sequences (2:6) Aligned. Score: 99 Sequences (2:7) Aligned. Score: 99 Sequences (3:4) Aligned. Score: 99 Sequences (3:5) Aligned. Score: 71 Sequences (3:6) Aligned. Score: 71 Sequences (3:7) Aligned. Score: 71 Sequences (4:5) Aligned. Score: 70 Sequences (4:6) Aligned. Score: 71 Sequences (4:7) Aligned. Score: 71 Sequences (5:6) Aligned. Score: 99 Sequences (5:7) Aligned. Score: 99 Sequences (6:7) Aligned. Score: 100 Start of Multiple Alignment Group 1: Sequences: 2 Score:10070 Group 2: Sequences: 3 Score:10127 Group 3: Sequences: 4 Score:10080 Group 4: Sequences: 2 Score:8995 Group 5: Sequences: 3 Score:9147 Group 6: Sequences: 7 Score:6443 Alignment Score 60551	Sequence type explicitly set to DNA Sequence 1: 00011Tri5 564 bp Sequence 2: 00012Tri5 561 bp Sequence 3: 00638Tri5 559 bp Sequence 4: 00873Tri5 560 bp Start of Pair wise alignments Sequences (1:2) Aligned. Score: 99 Sequences (2:3) Aligned. Score: 100 Sequences (1:3) Aligned. Score: 99 Sequences (2:4) Aligned. Score: 99 Sequences (3:4) Aligned. Score: 99 Sequences (1:4) Aligned. Score: 99 Start of Multiple Alignment There are 3 groups Group 1: Sequences: 2 Score:10602 Group 2: Sequences: 3 Score:10574 Group 3: Sequences: 4 Score:10535 Alignment Score 24610

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