Molecular Identification of Airborne Fungal Spores from Caguas and San Juan, Puerto Rico

Elizabeth Quintero
Sistema Universitario Ana G. Méndez, Puerto Rico, gquintero2@suagm.edu

Benjamín Bolaños
Sistema Universitario Ana G. Méndez, Puerto Rico, bbolanos@rcmupr.edu

Sharon Cantrell
Sistema Universitario Ana G. Méndez, Puerto Rico, scantrell@suagm.edu

ABSTRACT

Aerobiology can be defined as the study of airborne particles of biological origin including sources, liberation, dispersal, deposition and impact on other living organisms. Fungal spores are universal atmospheric components that are found indoors and outdoors and are recognized as important causes of respiratory diseases. Conventional methods for detecting airborne microorganisms rely on either optical identification or culturing and are very time consuming. Also, their accuracy depends on the researcher's experience. Recent developments have tended to reduce emphasis on visual identification in favor of more automated methods of detection. Sequences were compiled using Auto Assembler (ABI) software. Sequence similarities were obtained using the BLAST tool from NCBI. A total of 65 clones were analyzed, 37 for Caguas and 28 for San Juan. The majority of clones in Caguas (20 clones) and San Juan (14 clones) were basidiomycetes. In general this study showed that PCR-based assays used in conjunction with conventional visual identification have potential as a method for detecting airborne fungal spores, essential procedure for aerobiological studies more efficient.

Keywords: DNA extraction, Fungal spores, PCR, Cloning, Sequencing.

RESUMEN

Aerobiología se puede definir como el estudio de las partículas en el aire de origen biológico, incluyendo fuentes, la liberación, dispersión, deposición e impacto en otros organismos vivos. Las esporas de hongos son universales componentes atmosféricos que se encuentran en interiores como en exteriores y son reconocidos como causas importantes de enfermedades respiratorias. Los métodos convencionales para la detección de microorganismos en el aire o bien se basan en la identificación óptica o el cultivo y se consume mucho tiempo. Además, su precisión depende de la experiencia del investigador. Los acontecimientos recientes han tendido a reducir el énfasis en la identificación visual en favor de métodos más automatizados de detección. El muestreador de aire Allergenco (MK3) trampa de esporas se utilizó para capturar el aire esporas de hongos, una muestra para el análisis microscópico y el otro para la identificación molecular. El ADN se extrajo directamente de portaobjetos de vidrio con el suelo Power ® DNA Isolation Kit - Mobio y fúngica / bacteriana Investigación ADN Kit-Zymo. La concentración y pureza del ADN extraído se midió con un Biophotometer 22331 Hamburgo (Eppendorf AG). Todas las reacciones de PCR se realizaron en ABI 2720 Thermal Cycler. Para la evaluación de la comunidad fúngica, la región ITS (espaciador transcrito interno Región) fue amplificado utilizando los cebadores ITS1F y ITS4A y ITS4B. Los amplicones fueron clonados con el sistema pGEM ®-T Vector de clonación y secuenciado en ambas direcciones se realizó con Big Dye Terminator en un analizador ABI 3130genetic. Las secuencias fueron compiladas utilizando Auto Assembler (ABI) de software. Similitudes de secuencia se obtuvieron usando la herramienta de BLAST del NCBI. Un total de 65 clones fueron analizados, 37 y 28 de Caguas a San Juan. La

mayoría de los clones en Caguas (20 clones) y San Juan (14 clones) fueron basidiomicetos. En general, este estudio demostró que los ensayos basados en PCR utilizados en conjunción con la identificación visual convencional tienen potencial como un método para la detección de esporas de hongos en el aire, procedimiento esencial para estudios aerobiológicos más eficientes.

Palabras claves: Extracción DNA, esporas fúngicas, PCR, clonación, secuenciación

1. INTRODUCTION

It has been estimated that 34% of Puerto Rican population are afflicted with asthma, with or without allergic rhinitis (Seguinot-Medina and Rivera-Rentas 2006; Quintero et al. 2010). In a large number of these cases, inhalant allergens, particularly molds, are involved and are one of most common taggers of human atopic diseases (Agarwal and Gupta 2010).

Conventional methods for identifying and enumerating airborne fungi and other microorganisms rely on microscopic or cultural techniques and, as a consequence, are time-consuming and laborious. Additionally, microscopy is unreliable for detection of the small, nondescript spores produced by many fungi, while cultural techniques are unsuitable for detection of spores that are slow growing or uncultivable in vitro and the choice of medium may influence which species can grow. These difficulties have restricted the use of routine air sampling in the study of environmental fungi (Dean et al. 2004; Karakousisa et al. 2006). Recently, molecular methods have been used in the development of diagnostic tests for a variety of fungi involved in diseases (Dean et al. 2004). While the potential of these techniques for detection of airborne spores has been recognized for some time (Peccia and Hernández 2006), there have been few reports in this area. DNA-based detection methods offer greater potential for sensitive and specific detection, and some progress has been made in the detection of airborne fungal spore using these techniques (Ward 2009).

PCR is a rapid and sensitive technique for specific amplification of a particular segment of DNA (Zhuo et al. 2000). Using carefully designed DNA primers, a genetic sequence representing a specific microorganism, a group of microorganisms, or a microbial function can be targeted, amplified, quantified, and sequenced. The aims of our work were to develop and optimize DNA extraction techniques and PCR-based methods suitable for the detection, cloning and sequencing of airborne fungal spores.

2. MATERIALS AND METHODS

Capture and preparation of samples- Four air samples were collected in Caguas and San Juan, with a Burkhard air sampler. Two were used for DNA extraction and the other two to count the number of spores present per usual visual methods. The total dust collected during a day was considered as integral sample.

DNA extraction from air samples- Two different methods were tested for the extraction of fungal DNA from air samples of different age and quality: (A) DNA was extracted directly from glass slide using the Power Soil DNA Kit (Mo BIO Laboratory). The protocol of the manufacturer was slightly modified. The sampler was removed with the use of a toothpick; the tips were placed in a tube and mixed with appropriate buffer for 5 min. The buffer with the suspension was centrifuged at 13 000 rpm for 1 min. The pellets were transfered to the extraction tube of kit. DNA was washed with appropriate solution and eluted from the binding matrix with 50 µL of solution S5 fron the kit. (B) DNA extraction was done following the protocol of kit Fungal/Bacterial DNA Kit™ (Zymo Research). The samples were processed in the same manner as for the previous kit. This kit presented one advanced strategy for identifying fungal communities. Briefly, this method combines chemical and mechanical steps which are important because the destroyed cells walls of fungi are very resistant. DNA crude extracts were used directly for Polymerase Chain Reaction (PCR) amplification analysis.

Concentration and purity of DNA- Using a Biophotometer 22331 Hamburg, (Eppendorf AG), the DNA concentration was measured at 260 nm and 280 nm absorbance.

PCR amplification of extracted DNA- All PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, CA). PCR was performed using: 12.5 µL Jump Start Red Taq Ready Mix (Sigma Aldrich, MO), 2 µL extracted DNA, 9.25 µL of deionized water, and 0.625 µL of each primer (20 µM stock) for a total reaction of 25

μL. For the assessment of the fungal community the ITS region (Internal Transcribed Spacer Region) was amplified using forward primers ITS1F and reverse primer ITS4A (for ascomycetes) and ITS4B (for basidiomycetes). The thermocycling program was as follows: 5 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 1 min extension at 72 °C. Ten minutes at 72 °C were used as a final extension step. PCR products were analyzed by electrophoresis in a 1% (w/v) agarose gel.
Cloning of amplicons and analysis of sequences- Amplicons were cloned with the pGEM® Vector Systems (Invitron, Carlsbad, CA). Sequencing in both directions was performed on an ABI 3130 genetic analyzer (ABI, Foster city, CA). Sequences were compiled using Auto Assembler (ABI) software. Sequence similarities were obtained using the BLAST tool from NCBI.

3. RESULTS

Table 1 shows the total fungal particulate counted microscopically in Caguas and San Juan in March 22, 2010. On this day 15, genera were identified. Ascospores and basidiospores categories had the highest density. These categories include a large number of unidentifiable species. Only 4% (Caguas) and 6% (San Juan) of fungal spores corresponding were identifiable.
In this research, two methods for extracting DNA from air spores were tested: Power Soil DNA Kit (Mo Bio Laboratory) and Fungal / Bacterial DNA Kit (Zymo Research). Concentration and purity of DNA was measure with a spectrophotometer. The results in Table 6.0.2 indicate that the best concentration was obtained with the Power Soil Kit. The DNA obtained with the Power Soil kit was amplified by polymerase chain reaction (PCR) using primers for ascospores (ITS1F/ITS4A) and basidiospores (ITS1F/ITS4B). The amplicons are observed in Figure 1.
PCR products were ligated to pGEM ® Vector Systems and inoculated into competent cells. Table 3 shows the efficiency of the cloning process. This percentage depends on the volume of cells analyzed. Results for Caguas showed higher efficiency with a volume of 50 μL of competent cells, than in San Juan that showed the best efficiency for 75 μL of sample.

Table 1. Microscopic analysis of fungal particulates collected in Caguas and San Juan in March 22, 2010.

Spore type	**Caguas**		**San Juan**	
	∑spores/day	**Total/m^3**	**∑spores/day**	**Total/m^3**
Agaricus/Coprinus	20	769	5	192
Ascospores	195	7500	148	5692
Basisiospores	352	13731	56	2154
Cercospore sp.	5	192	6	231
Cladosporium sp.	6	231	30	1154
Curvilaria sp.	1	38	0	0
Diatrypaceae	2	77	3	115
Ganoderma sp.	8	308	12	462
Leptosphaeria sp.	8	308	3	115
Others	28	1077	25	962
Penicillium/Aspergillus	17	654	56	2154
Periconia sp.	1	38	0	0
Pithomyces sp.	0	0	5	195
Pleurotus sp.	45	1731	37	1423

Rust	2	77	1	38
Tremetes sp.	14	538	9	346
Total Spores	**709**	**27269**	**395**	**22115**

Table 2. Concentration and purity of DNA extracted from fungal spores with two molecular kits. Relation $A_{260}/A_{280} > 2$, is indicative of high purity.

Molecular kit	**A_{260}**	**A_{280}**	**A_{280}/A_{260}**	**[DNA, $\mu g/\mu l$]**
Power Soil DNA	0.162	0.082	1.97	5.6
(Mo BIO Laboratory	0.154	0.076	20.02	5.4
Fungal/Bacterial DNA	0.142	0.082	1.73	4.9
(Zymo Research	0.135	0.064	2.11	4.7

Cell above = Data Caguas Cell below = Data San Juan

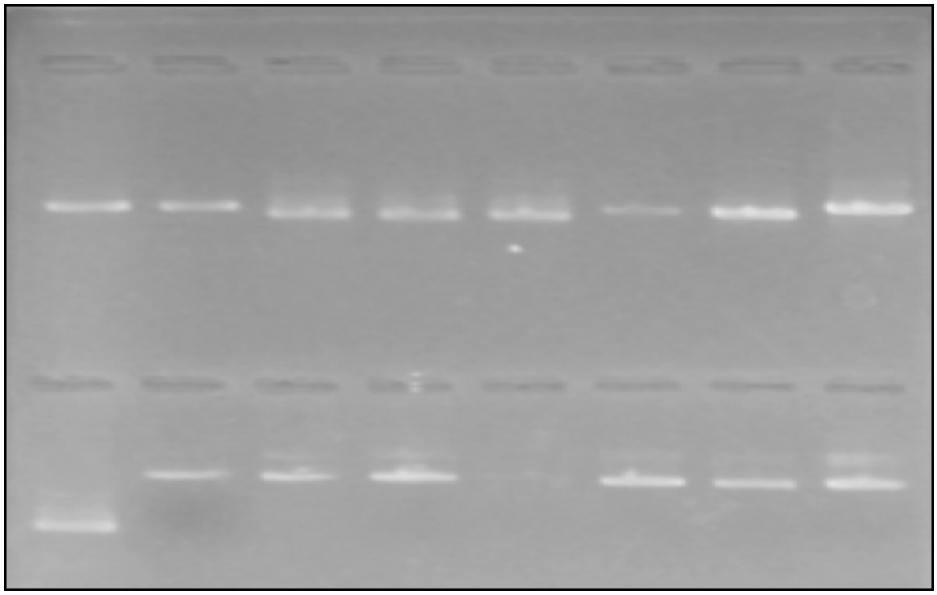

Figure 1. PCR products in 1% agarose gel. Top lines are sampler from Caguas and bottom lines are from San Juan.

Tables 4 and 5 the organisms identified molecularly are shown. In Caguas 17 clones of ascomycetes and 20 of basidiomycetes were identified. In San Juan, the clones were less: 14 ascomycetes and 14 basidiomycetes. In Caguas *Epicocum nigrun*, *Pleospora* sp.*, Curvularia geniculata*, *Trametes versicolor*, *Ganoderma japonicum* and *Exhidia globulosa* were identified in more than one occasion. In San Juan, several species of *Cladosporium*, *Curvularia clavata*, *Aspergillus fumigatus* and *Trametes versicolor* were identified several times from in genomic library.

Table 3. Cloning performance. Percentage indicates the number of white colonies with DNA insert.

Cloning and transformation efficiency						
Analyzed volume competent cells						
	50 µL			***75 µL***		
City	**CFU blue/ CFU white**	**%**	**Efficiency**	**CFU blue/ CFU white**	**%**	**Efficiency**
Caguas	36/95	71	$1.3x10^8$cfu/μg	84/132	61	$2x10^8$cfu/μg DNA
San Juan	45/62	58	$1.0x10^8$cfu/μg	59/143	71	$2x10^8$cfu/μg DNA
CFU White with product insert						

Table 4. Molecular identification of fungal spores captured in atmosphere of Caguas the day March 22, 2010.

Ascomycetes		***Basidiomycetes***	
Organism	**% of similarity**	**Organism**	**% of similarity**
* Epicocum nigrum (2)	91 - 87	Phlebiopsis flavidolba	90
* Pleospores sp.(2)	93 – 95	*Ganoderma lucidium	86
* Camillea obularia	94	*Trametes versicolor (3)	92 - 87 – 94
Xylaria sp.	85	Tetrapygros subcinerea	91
Camarosporum brabeji	86	*Pleurotus calyptratus	97 - 93
Annulohypoxilon stagium	98	Amylostereun chaittetii	92
* Periconia macrospinosa	88	*Inocybe obsolete	93
Curvularia geniculata (2)	90 – 92	Coryolopssis caperata	89
Aspergillus fumigatus	89	*Exidia globulosa (3)	94 – 87 – 91
Cladosporium cladosporioides	87	*Coprinus sp. (2)	87 – 92
Penicillium aethiopicum	91	Phellinus pini	95

Phomopsis sp.	88	Agaricus xantodermus	88
* Beauveria alba	90	*Ganoderma japonicum (2)	81 – 94

* Forward and reverse sequences available
The number into parentheses indicates number of times identified

Table 5. Molecular identification of fungal spores captured in atmosphere of San Juan the day March 22, 2010.

Ascomycetes		***Basidiomycetes***	
Organism	**% of similarity**	**Organism**	**% of similarity**
Epicocum nigrum	97	* Psilocybe fasciata	89
* Pleospores sp.	89	* Cortinarius sp.	91
Aspergillus fumigates (2)	91 – 89	Ganoderma applenatum	92
Cladosporium sphaerospermum (2)	89 – 92	* Annulohypoxylon stygium	89
Cladosporium cladosporioides (2)	98 – 95	Ganoderma japonicum	93
Curvularia clavata (2)	93 – 91	Eichleriella leveilleana	89
Penicillium aethiopicum	98	* Sporobolomyces sp.	91
* Eutypella sp.	89	* Agrocybe aegerita	96
* Malassezia restricta	92	* Trametes versicolor (3)	92 – 91 – 89
Pestalotiopsis mangiferae	93	* Aphyllophorales sp.	99
		Basidiomycetes nocultured	85
		*Auriculariales nocultured	89

* Forward and reverse sequences available
The number into parentheses indicates number of times identified

Figures 2 and 3 respectively showed examples of the spore molecularly identified in March 22 of 2011. Ascospores and basidiospores are shown.

In aerobiological studies there are categories as ascospores, basidiospores and *Penicillium / Aspergillus* which are composed of several species and are located in the same group. These aspects underestimate the diversity and risk of atmospheric particulate; the molecular identification processes are more sensitive and accurate tool for identifying airborne spores (Williams et al. 2001).

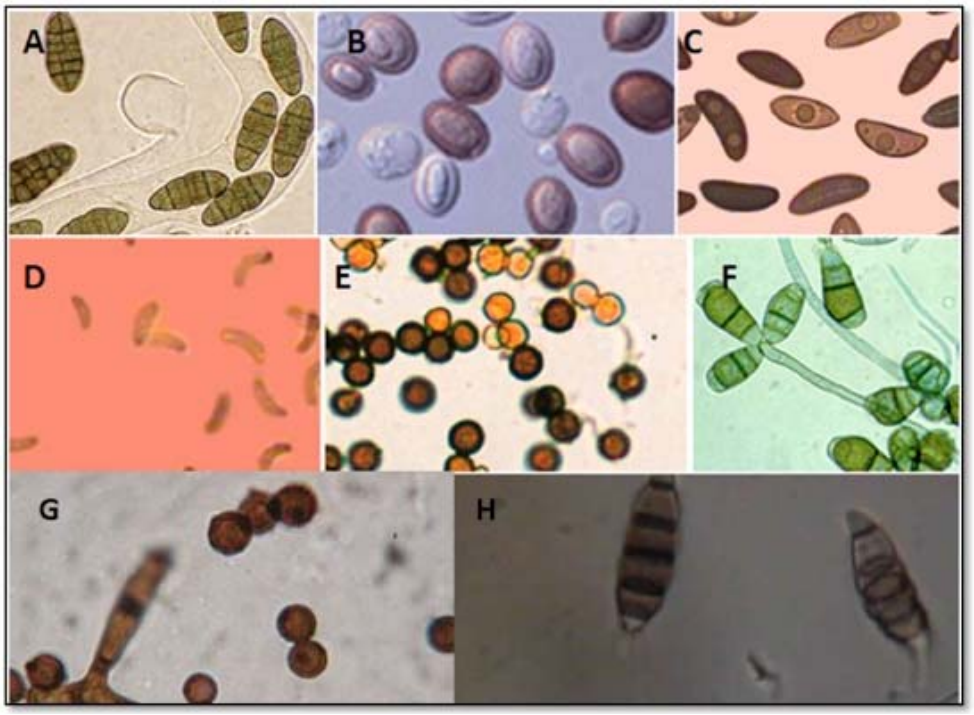

Figure 2. Asposcores identified for molecular analysis. (A) *Pleospora* sp., (B) *Annulohypoxilon stagium* (C) *Xylaria* sp., (D) *Eutypella* sp., (E) *Periconia macrospinosa*, (F) *Curvularia geniculata,* (G) *Cladosporium sphaerosporum,* (H) *Pestalotiopsis mangifera*.

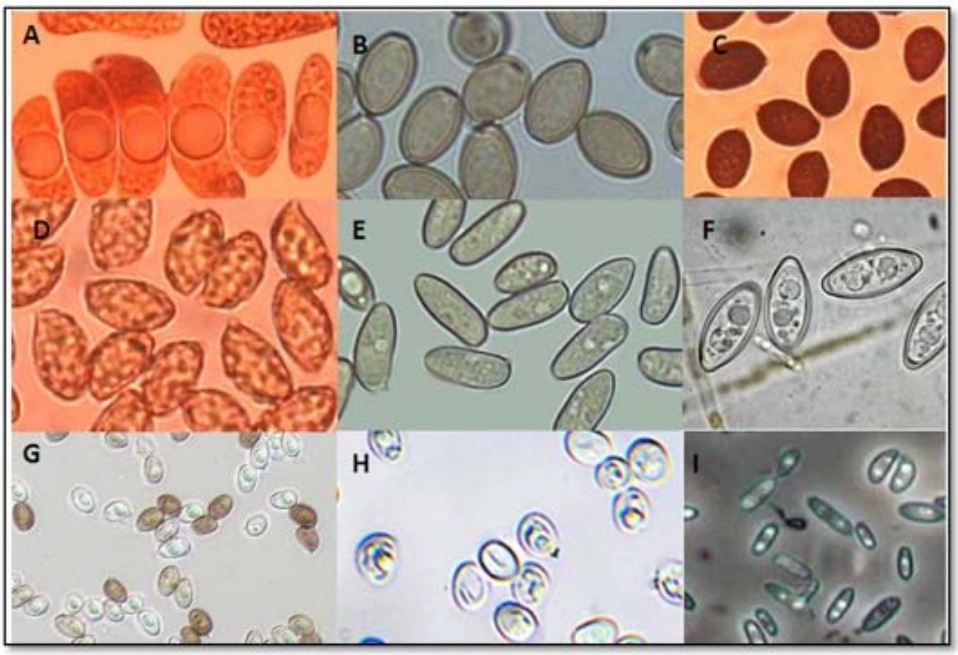

Figure 3. Basidiospores identified from molecular analysis. (A) *Auricularia* sp., (B) *Agrocye aegerita,* (C)*Coprinus* sp., (D) *Cortinarius* sp., (E) *Inocybe obsoleta*, (F) *Coryolopssis caperata,* (G) *Phellinus pini,* (H) *Sporobolomyces* sp.

4. DISCUSSION

Traditional methods for identification of fungal spores can be time consuming and laborious (Michaelsena et al. 2006). DNA based methods for fungal detection can be used to detect the spores allergenic for human (Angenent et al. 2005). Microscopically, a large amount of particulate was observed in the results, but not identified by lack of experience or due to the fungal diversity present in the atmosphere.

PCR analysis provides a sensitive and specific means to detect and monitor microorganisms in complex environmental samples (Miller et al. 1999). Successful detection and characterization of microbial DNA in the environment require efficient extraction of the DNA from environmental samples and adequate purification from the coextracted contaminants that inhibit PCR (Roose- Ansaleg et al. 2001; Williams et al. 2001).
Air in atmosphere varies greatly in chemical and organic composition. They also contain contaminant that is inhibitory to Taq DNA polymerase and other enzymes (Frohlich-Nowoiskya et al. 2009). Air is therefore one of the most challenging environmental matrices from which to obtain microbial DNA that will support PCR (Despres et al. 2007).
One problem of molecular work with air spores is the difficulty to concentrate the sample and obtaining sufficient material for the extraction of DNA, there is not commercial kit for this purpose and the option is to use the kit for plants or soil samples (Peccia and Hernández 2006).
A common practice in molecular biology is to perform a quick assessment of the purity of nucleic acid samples spectrophotometrically using the absorbance (A) at the wavelengths of 260 nm and 280 nm respectively. A_{260} is frequently used to measure DNA/RNA concentration and A_{280} is used to measure protein concentration. The ratio of A_{260}/A_{280} higher than 1.8 suggest little protein contamination in a DNA/RNA sample. In this study two types of kit were evaluated: Power Soil DNA Kit and Zymo Fungal / Bacterial DNA Kit. The results showed a high performance for the first kit considering the concentration and purity of DNA obtained. Similar results were obtained by Cotarlet et al. (2010) using pure isolates of *Streptomyces*. Many DNA sequence manipulations are made easier by cloning a PCR product into an appropriate vector. The pGEM Vector System is a linearized vector with a single 3′-terminal thymidine at both ends compatible with overhang of PCR products. Although, the efficiency of insertion of the vector is stipulated by the manufacturer as 2×10^8 cfu/μg DNA, it is necessary to know the efficiency obtained in each particular experiment, since this depends on factors such as quality and size of PCR product used, efficiency of competent cells and skills of investigator. The PCR amplicon interrupts the gen for β-galactosidase and recombinant clones can be identified by color screening on indicator plates. Usually, clones containing PCR products produce white Colónies.
In this research using 75 μL of competent cells the same cloning efficiency determined by the manufacturer was achieved. These results are similar to those reported in studies that have estimated the efficiency of three types of vectors. These studies considered pGEN® - T Vector the second best after TOPO TA Cloning® Vector (Litterer 2009).
Molecular techniques, based on DNA analysis, have been recognized as potential alternative methods for detecting airborne fungal spores (MacNeil et al.1995; McCartney et al.1997). But, little progress had been made in their use. Polymerase chain reaction (PCR) techniques have been used to detect a few species of fungi in air samples (Williams et al. 2001; Calderón et al. 2002; Kruger and Gargas 2006; Manter et al. 2007; Prewitt et al. 2008). Results in Caguas and San Juan demonstrated that using molecular techniques can be fungal air spores identified with high precision. However, molecular work involves a high economic cost that sometimes investigators cannot subsidize.
In general, this study showed that PCR-based assays used in conjunction with conventional visual identification have potential as a method for detecting airborne fungal spores.

REFERENCES

Agarwal R, Gupta D. 2010.Severe asthma and fungi: current evidence. Medical Mycology 1–8.

Angenent, L., Kelley, S., Amand, A.S., Pace, N.R., Hernandez, M., 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proceedings of the National Academy of Science 102, 4860–4865.

Calderón C, Ward E, Freeman J, McCartney HA. 2002. Detection of fungal spore sampled by rotating arm and Hirst type spore traps using polymerase chain reactions assays. Journal Aerosol Science 33:283-296.

Dean T, Betancourt D, Menetrez M. 2004. A rapid DNA extraction method for PCR identification of fungal indoor air contaminants. Journal of Microbiological Methods 56(3):431-434.

Després, V. R., Nowoisky, J. F., Klose, M., Conrad, R., Andreae, M. O. and co-authors. 2007. Characterization of primary biogenic aerosol particles in urban, rural, and high-alpine air by DNA sequence and restriction fragment analysis of ribosomal RNA genes. Biogeosciences, 4, 1127–1141.

Fröhlich-Nowoisky, J., Pickersgill, D. A., Després, V. R. and Pöschl, U. 2009. High diversity of fungi in air particulate matter. Proc. Natl. Acad. Sci. USA, 106, 12814–12819.

Karakousisa A, Tana L, Ellisb D, Alexioub H , Wormald PJ. 2006. An assessment of the efficiency of fungal DNA extraction methods for maximizing the detection of medically important fungi using PCR. Journal of Microbiological Methods 65(1):38-48.

Krüger D., Gargas A. 2006. New measures of topological stability in phylogenetic trees - taking taxon composition into account. Bioinformation 1, 327-330.

MacNeil, L., Kauri, T., Robertson, W., 1995. Molecular techniques and their potential application in monitoring the microbiological quality of indoor air. Canadian Journal of Microbiology 41, 657–665.

McCartney, H.A., Foster, S.J., Fraaije, B.A., Ward, E., 2003. Molecular diagnostics for fungal plant pathogen. Pest Management Science 59, 129–142.

Michaelsen, E., Soergel, U. and Thoennessen, U., 2006. Percep- tual Grouping in Automatic Detection of Man-Made Structure in High Resolution SAR Data. Pattern Recognition Letters 27(4), pp. 218–225.

Miller, D.N., Bryant, J.E., Madsen, E.L., Ghiorse, W.C., 1999. Evaluation and optimization of DNA extraction and pur- ification procedures for soil and sediment samples. Applied and Environmental Microbiology 65, 4715–4724.

Pecciaa J, Hernández M. 2006.Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. Atmospheric Environment 40:3941-3961.

Quintero E, Rivera-Merani F, Bolaños B. 2010. Analysis of environmental factors and their effects on fungal spores in the atmosphere of a tropical urban area (San Juan, Puerto Rico). Aerobiología 26(2):113-124.

Seguinot-Medina R, Rivera-Rentas A. 2006. Risk assessment and community participation model for environmental asthma management in an elementary public school: a case study in Puerto Rico. International Journal of Environmental Research Public Health 3(1):76-85.

Ward E. 2009. Use of molecular methods for the detection of fungal spores. Methods Molecular Biology 508:147-159.

Williams R, Ward E, Mccartney A.2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Applied and Environmental Microbiology 67(6):2453-2459.

Zhou G, Whong WZ, Ong T, Chen B. 2000. Development of a fungus specific PCR assay for detecting low-level fungi in an indoor environment. Molecular and Cellular Probes 14:339-348.

Authorization and Disclaimer

Authors authorize LACCEI to publish the paper in the conference proceedings. Neither LACCEI nor the editors are responsible either for the content or for the implications of what is expressed in the paper.