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Journal of Microbiological Methods 70 (2007) 86–95
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Evaluation of fungal aerosols using Temporal Temperature Gradient Electrophoresis (TTGE) and comparison with culture

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Received 4 December 2006; received in revised form 23 March 2007; accepted 28 March 2007
Available online 12 April 2007

Abstract

Information obtained from fungal air samples can assist in the assessment of health hazards and can be useful in proactive indoor air quality monitoring. The objective of the present study was to evaluate the PCR–TTGE technique for the analysis of fungal diversity in the air. Eleven air samples were collected in five different sites using the bioimpactor CIP 10-M (Arelco). After a 2 hours sampling period, the collection liquid was recovered for subsequent cultivation and PCR–TTGE. A set of three fungi-specific primers (Fungcont 1, Fungcont 2+GC and Fungcont 3) was designed for the partial amplification of the 18S rRNA gene. The amplification was obtained in a single reaction tube by a semi-nested PCR. For identification, the TTGE bands were extracted and sequenced. PCR–TTGE allowed the clear separation of amplicons corresponding to distinct fungal species (both Ascomycota and Basidiomycota) that may be encountered in air. The number of fungal taxa detected after culture was systematically higher than the number of taxa found using PCR–TTGE. However, few fungal species were detected by PCR–TTGE and not by cultivation, suggesting that the combination of these approaches may provide a better analysis of fungal diversity in air samples than either method alone.

Keywords: Fungal aerosols; Indoor; Air samples; PCR–TTGE

1. Introduction

Fungal spores represent a significant part of the biological contaminants that could be detected in air. Among the large number of aerosolized fungal species, some are being proposed as a cause of adverse health effects (Cooley et al., 1998; Fung and Hughson, 2003; Johanning et al., 1999; Kauffman et al., 1995; Piecková and Jesenská, 1999; Samson et al., 1994). Exposure to fungal spores or hyphal fragments has been associated with several types of human or animal health problems including irritations, infections, allergies, and toxic effects. Spores of *Aspergillus fumigatus* pose special risks to neutropenic humans. Spores of *Aspergillus, Cladosporium* and *Penicillium* generated in damp buildings can cause bouts of asthma and/or rhinitis among atopic occupants (Kauffman et al., 1995; Crameri et al., 2006). Furthermore, it has been suggested that toxigenic fungi are the cause of additional adverse health effects (Dewey et al., 1995; Hintikka, 1978; Johanning et al., 1993; Kuhn and Ghannoum, 2003; Piecková and Jesenská, 1999). *Stac hybotrys chartarum, Fusarium* spp. or *Aspergillus versicolor* produce toxins, which may be baneful on respiratory system (Etzel et al., 1998; Hodgson et al., 1998; Johanning et al., 1996). In animal facilities, environmental conditions are usually favorable for the development of molds. These environmental conditions may cause adverse health effects in workers (Chang et al., 2001; Lee et al., 2006) and in animals, especially avian species (Tell, 2005; Lair-Fulleringer et al., 2006).

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doi:10.1016/j.mimet.2007.03.021

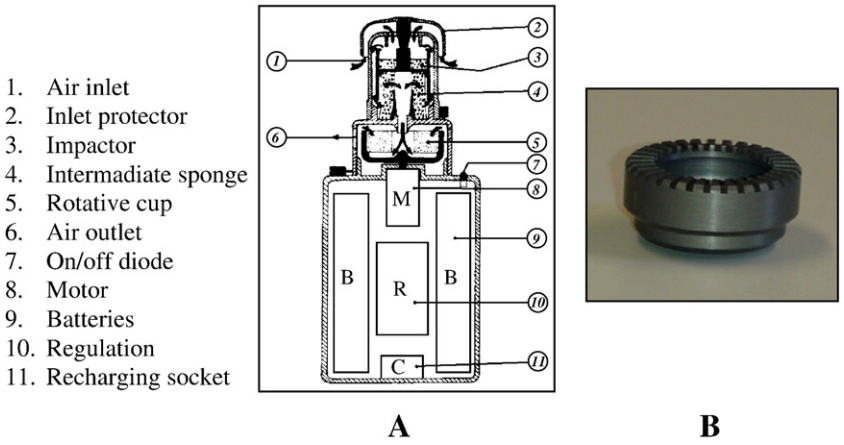

Fig. 1. Diagram of the rotating cup apparatus CIP 10-M (Arelo Company) (A); closer view of the rotative cup (B).

To evaluate the relationship between fungal aerosols and potential for adverse health effects in humans and animals, the fungal species and their relative frequencies in air need to be known. Traditional methods of fungal identification include culture and microscopic analysis. However, these methods are laborious, time-consuming and require a competent mycological expertise. In addition, some fungal species are non-culturable or are unable to produce classical structures under laboratory conditions that are necessary for identification. To circumvent the cultivation limits, several molecular techniques have been proposed: specific assays using single-step PCR, nested PCR and PCR followed by Southern blotting and probing (Williams et al., 2001), ribosomal DNA (rRNA gene) sequencing, rRNA gene restriction analysis (Wu et al., 2003). Techniques based on mass spectrometry (Szponar and Larson, 2001) and flow cytometry (Prigione et al., 2004) have also been

Table 1
List of primers sets evaluated in the present study

Primer name (and sequence 5′ to 3′)	Amplified region	Fragment size (bp)	PCR type	Reference or source
Yuniv1+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G AGC CTG AGA AAC GGC TAC CAC)	18S rDNA	244	Simple	Hernán-Gómez et al. (2000)
Yuniv3 (TTC AAC TAC GAG CTT TTT AA)				
NL1+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GCA TAT CAA TAA GCG GAG GAA AAG)	28S rDNA	640	Simple	Marshall et al. (2003)
NL4 (GGT CCG TGT TTC AAG ACG G)				
EF4 (GGA AGG GRT GTA TTT ATT AG)	18S rDNA	250	Semi-nested – PCR1: EF4/EF3 and EF4/FUNG5 – PCR2: EF4/NS3+GC	Smit et al. (1999)
EF3 (TCC TCT AAA TGA CCA AGT TTG)				
FUNG5 (GTA AAA GTC CTG GTT CCC C)				
NS3+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GCA AGT CTG GTG CCA GCA GCC)				
NS1 (GTA GTC ATA TGC TTG TCT C)	18S rDNA	545	Nested – PCR1: NS1/NS8 – PCR2: NS2+10/NS1+GC	Theelen et al. (2001)
NS8 (TCC GCA GGT TCA CCT ACG GA)				
NS2+10 (GAA TTA CCG CGG CTG CTG GC)				
NS1+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GTA GTC ATA TGC TTG TCT C)				
FF1+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GTT AAA AAG CTC GTA GTT GAA C)	18S rDNA	425	Semi-nested – PCR1: FF1+GC/FR1 – PCR2: FF2/FR1	Zhou et al. (2000)
FF2 (GGT TCT ATT TTG TTG GTT TCT A)				
FR1 (CTC TCA ATC TGT CAA TCC TTA TT)				
NL1+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GCA TAT CAA TAA GCG GAG GAA AAG)	28S rDNA	244	Simple	Marshall et al. (2003)
NL1-A (CCC AAA CAA CTC GAC TC)				
Fungcont 1 (CTC GCA TCG ATG AAG AAC)	18S rDNA	350	Semi-nested in one single reaction tube	This work
Fungcont 2+GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G GGG TAA TCC CAC CTG ATT)				
Fungcont 3 (CCG CTT ATT GAT ATG CTT)				

Original primers Fungcont 1–3 were finally selected for the PCR–TTGE technique.

Table 2
List of fungal isolates used for the selection of PCR primer sets and the adjustment of DNA extraction conditions (see Fig. 2)

Isolate	TTGE fragments no.	Closest sequence relative (species)	Genbank accession no.	% Identity
A	1 and 2	*Aspergillus versicolor*	AY373882.1	94
B	3 and 4	*Penicillium thiersii*	DQ532125.1	100
C	5	*Chrysosporium keratinophilum*	AJ131681.1	98
D	6 and 7	*Scopulariopsis brevicaulis*	AY625065.1	98
E	8	*Aspergillus flavus*	AY939785.1	99
F	9 and 10	*Fusarium culmorum*	AY147334.2	99
G	11	*Penicillium commune*	AY373905.1	99
H	12 and 13	*Absidia glauca*	AY944881.1	92
I	14 and 15	*Acremonium crotocinigenum*	AJ621773.1	94
J	16 and 17	*Fusarium culmorum*	AY147334.2	99
K	18	*A. flavus*	AY939785.1	99
L	19 and 20	*Scopulariopsis brevicaulis*	AY625065.1	98
M	21 and 22	*Alternaria gaisen*	AY762944.1	99
N	23	*C. keratinophilum*	AJ131681.1	98
O	24 and 25	*Aspergillus fumigatus*	AY373851.1	100
P	26	*Paecilomyces variotii*	AF033395.1	100
Q	27, 28 and 29	*Fusarium* sp.	AY633561.1	99
R	30 and 31	*Penicillium solitum*	AY373932.1	99

described. All these studies suggested that our current understanding of fungal diversity may be inadequate.

Molecular methods currently used to study microbial communities include broad-range PCR, using primers that target highly-conserved regions of genes allowing the simultaneous amplification of DNA from large groups of microorganisms present in one sample in a single-step. The different amplicons are then subsequently separated with sequence-specific separation tools such as SSCP (Single Strand Conformation Polymorphism), TGGE (Temperature Gradient Gel Electrophoresis), TTGE (Temporal Temperature Gradient Gel Electrophoresis) and DGGE (Denaturing Gradient Gel Electrophoresis). DGGE and TGGE have already been applied to elucidate fungal populations structures in different environments: the soil (Heuer and Smalla, 1997; Pennanen et al., 2001), wine (Cocolin et al., 2000; Hernán-Gómez et al., 2000), wood (Van Elsas et al., 2000; Vaino and Hantula, 2000), rhizospheres of marram grass (Kowalchuk et al., 1997), vanilla curing and corn silage (Röling et al., 2001; May et al., 2001), maize and wheat (Ben Omar and Ampe, 2000; Smit et al., 1999). DGGE, TGGE and TTGE are based on same principle. These techniques allow the separation of PCR products on the basis of the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels. For DGGE, the polyacrylamide gel is composed of a linear gradient of DNA denaturants (a mixture of urea and formamide) in a bath at a constant temperature. For TGGE and TTGE, the polyacrylamide gel includes a constant of DNA denaturant but PCR products are separated along a linear temperature gradient. Temperature gradient denaturing gradient gel electrophoreses (TGGE and TTGE) were suggested to remove the disadvantages of DGGE by avoiding both the draw backs of laborious optimization procedure and the need for casting a chemical denaturing gradient (Borrensen-Dale et al., 1997; Shina et al., 2001). TGGE replaces the spatial chemical denaturing gradient by using a spatial temperature gradient and TTGE replaces it by using a temporal temperature denaturing gradient. These two last methods have very similar characteristics and the differences are only related to the apparatus providing spatial or temporal temperature gradient.

The objective of the present study was to evaluate the PCR–TTGE method for the characterization of fungal diversity in air. Samples were collected in five sites, which have been selected because they were supposed to be contaminated by different

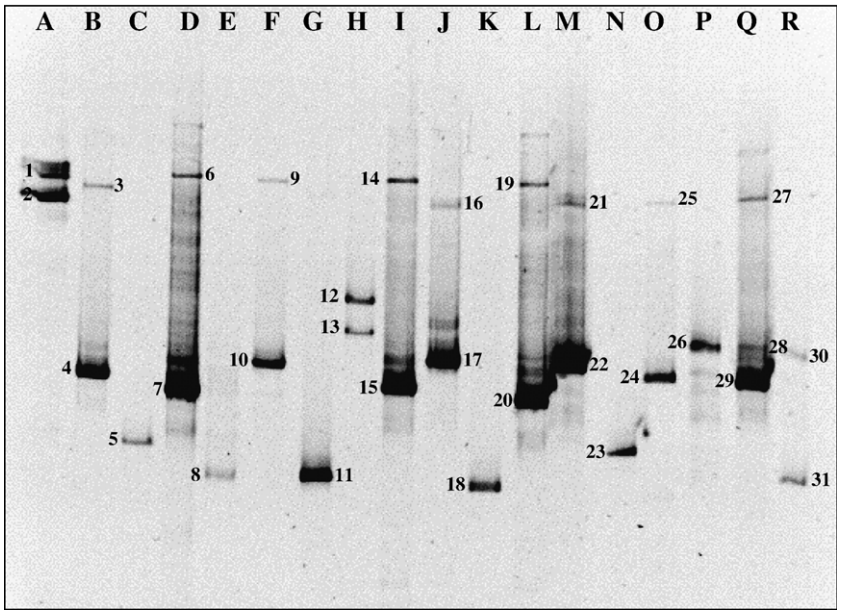

Fig. 2. TTGE of PCR amplicons of 18S rDNA fragments representing the fungal isolates. PCR Primers Fungcont 1–3 were used. The following species were tested: strain A *Aspergillus versicolor*; strain B *Penicillium thiersii*; strain C *Chrysosporium keratinophilum*; strain D *Scopulariopsis brevicaulis*; strain E *Aspergillus flavus*; strain F *Fusarium culmorum*; strain G *Penicillium commune*; strain H *Absidia glauca*; strain I *Acremonium crotocinigenum*; strain J *F. culmorum*; strain K *A. flavus*; strain L *S. brevicaulis*; strain M *Alternaria gaisen*; strain N *C. keratinophilum*; strain O *A. fumigatus*; strain P *Paecilomyces variotii*; strain Q *Fusarium* sp.; strain R *Penicillium solitum*.

types of aerosolized fungal organisms. At those sites, we used the air sampler CIP 10-M (*Capteur Individuel de Poussières Microbiologiques*). This device allows the collection of air particles in a liquid that could be used for subsequent cultivation and molecular analyses. For PCR–TTGE, broad-range primers for aerosolized fungal species were designed. The range of species that could be amplified by the primers was tested with a collection of different fungal isolates from all major groups (Ascomycota, Basidiomycota and Zygomycota). Primer sets were subsequently used to generate PCR products from air samples. TTGE results were complemented by sequencing of PCR products and compared to mycological culture results, which were used as control.

2. Materials and methods

2.1. Sampling site selection

Five sampling sites were selected because the air fungal diversity was expected to be high: a cowshed and a stable at the veterinary school of Alfort, a metropolitan station in Paris and two henhouses with static ventilation. In the cowshed and the stable, animals (sheep and horses, respectively) were housed and stocks of straw were maintained. Each henhouse held about 4300 chickens (4 week-old females or males, respectively).

2.2. Air sampling procedure

Air samples were obtained using the bioimpactor CIP 10-M (*Capteur Individuel de Poussières Microbiologiques*) (Courbon et al., 1988) (Arelco company, Fontenay-sous-Bois, France). This device, described in Fig. 1, was originally validated for the detection of the non-culturable fungi of the genus *Pneumocystis* (Guillot et al., 1999). CIP 10-M has been recently used for the characterization of exposure to fungi by measurement of ergosterol in air (Robine et al., 2005). The ambient air is aspirated at a sampling flow rate of 10 L/min via a selector. Particles are aspirated, then driven by centrifugal force toward a liquid collection layer, maintained inside the rotative cup. The air is then exhausted via a tangential outlet on the wall of the apparatus. The configuration of CIP 10-M causes minimal stress of mechanical impaction or pressure shock. The sampler exhibits a physical efficiency >50% for >1.8 μm in aerodynamic diameter, and >95% for particles >2.8 μm (Gorner et al., 2006). In the present study, the rotative cup was filled with 2 mL of sterile water. The CIP 10-M was placed at 1 m from the floor in a vertical position. The sampling duration was 120 min. After each sampling period, 1.5 mL of the collection liquid was recovered for subsequent cultivation and molecular analysis. For mycological culture, 0.5 mL of the liquid was immediately used. For molecular analysis, 1 mL was stored at −80 °C until use (a few days after the air sampling). After each sampling period, the rotative cup was first washed with alcohol and then with sterile water and dripped dry.

One air sampling (1200 L) was made in the cowshed and the metropolitan station. Three air samples were obtained at one month intervals in the stable. Six air samplings were performed in the henhouses (the same day).

Each of the 11 air sample was analyzed by PCR–TTGE and cultivation.

2.3. DNA extraction

Several extraction methods and commercial kits were tested (Fast DNA Spin kit for soil; T1, NucleoSpin®Tissue, Hoerdt, France). We finally selected a protocol, which included the use of a bead beater and the commercial kit NucleoSpin®Tissue. 1 mL of the collection liquid collected from the rotative cup was placed in a 2 mL sterile microtube containing 2 sterile microbeads (4 mm diameter) (Qiagen, Courtabeuf, France) and sand. Each tube was cooled at −80 °C for 1 h and crushed by shaking with a bead beater (mixer mill MM301, Qiagen, Courtabeuf, France) for 2 cycles of 15 min at a frequency of 20 Hz. DNA was further extracted using the NucleoSpin®Tissue kit (Macherey-Nagel, Hoerdt, France).

2.4. Polymerase chain reaction

To obtain fungi-specific amplicons suitable for TTGE, seven primer sets have been tested (Table 1). For the first six primer

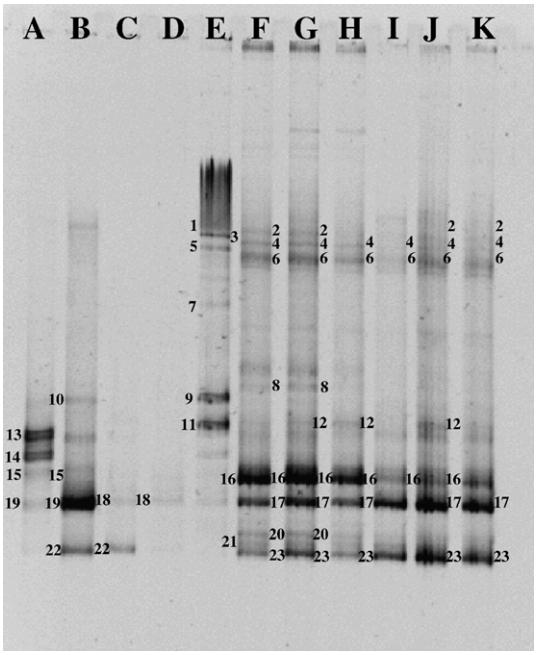

Fig. 3. TTGE of PCR amplicons of 18S rDNA fragments representing the fungal biodiversity in air samples. A, B, C: stable; D: cowshed; E: metropolitan station in Paris; F, G, H, I, J, K, I: henhouses. After purification and sequencing, the following species were identified: *Alternaria infectoria* (band 22), *Alternaria* sp. (band 23); *Alternaria triticina* (band 18); *Arthrinium* sp. (band 12); *Ascochyta* sp. (band 10); *Aspergillus caesiellus* (band 20); *Bulleromyces albus* (band 9); *Candida catenulate* (band 2), *Cladosporium cladosporioides* (band 15); *Cladosporium* sp. (bands 6 and 17); *Cochliobolus heterostrophus* (band 21); *Dioszegia hungarisca* (band 7); *Eurotium rubrum* (bands 8 and 16); *Fusarium arthrosporioides* (band 13); *Paecilomyces variotii* (band 11); *Penicillium* sp. (band 4); *Physcia aipolia* (band 19); *Sepedonium chalcipori* (band 14); *Sporobolomyces jilinensis* (band 3); *Sporobolomyces roseus* (bands 1 and 5).

Table 3
List of fungal species identified by cultivation or PCR–TTGE from air samples

Sampling site	Fungal species identified from air samples[a]	
	by cultivation (CFU / m^3)[b]	by PCR-TTGE (fragment size, GenBank sequence identity)
Stable (samples A, B & C)		
	Acremonium strictum (–; 17; –)	
	Alternaria sp. (–; 29; 43)	*Alternaria infectoria* (347 bp, Y17066 99%) *Alternaria triticina* (334 bp, AY278834 99%)
	Arthonia sardoa (–; 6; –)	
		Ascochyta sp. (334 bp, AY305377 100%)
	Aspergillus fumigatus (17; 6; 29)	
	Aspergillus niger (–; 6; –)	
	Aspergillus sp. (35; 17; –)	
	Aspergillus versicolor (–; 11; –)	
	Bulleromyces albus (–; 6; 14)	
		Cladosporium cladosporioides (347 bp, AY361994 100%)
	Cladosporium herbarum (–; 11; –)	
		Fusarium arthrosporioides (334 bp, AF111065 97%)
	Fusarium proliferatum (26; 11; 29)	
	Glomerella cingulata (–; 6; –)	
	Mucor circinelloides (–; 11; –)	
	Mucor sp. (17; –; –)	
	Parmelia saxatilis (–; 6; –)	
	Peacilomyces variotii (–; 11; –)	
	Penicillium commune (26; 11; 43)	
	Penicillium griseofulvum (–; 11; –)	
		Physcia aipolia (321 bp, AY3031 35 100%)
	Protoblastenia calva (–; 6; –)	
	Scutellinia sp. (–; 6; –)	
		Sepedonium chalcipori (351 bp, AF054865 93%)
	Rhizopus sp. (35; –; –)	
	Trichoderma sp. (17; 11; 14)	
Cowshed (sample D)		
	Alternaria alternata (37)	
		A. triticina (334 bp, AY278834 99%)
	Aspergillus sp. (37)	
	F. proliferatum (25)	
	P. griseofulvum (25)	
	Penicillium sp. (37)	
	Trichoderma sp. (12)	
Metropolitan station (sample E)		
	Aspergillus flavus (25)	
	Aspergillus nidulans (37)	
	Aspergillus sp. (37)	
		B. albus (347 bp, AB093527 98%)
		Dioszegia hungarisca (347 bp, AB049614 99%)
		Paecilomyces variotii (334 bp, AY373941 100%)
	Penicillium mangini (25)	
	Penicillium sp. (50)	
		Sporobolomyces jilinensis (321 bp, AY364838 97%)
		Sporobolomyces roseus (351 bp, AB030351 100%)

When results were in accordance (at the fungal genus level), species have been framed.
[a] when several air samples were collected in the same site, cultivation and PCR–TTGE results were pooled (in the two corresponding columns).
[b] >200: uncountable colonies. –: below the detection threshold of the sampling techniques.

sets, PCR conditions were in accordance with previous studies (Hernán-Gómez et al., 2000; Marshall et al., 2003; Smit et al., 1999; Theelen et al., 2001; Zhou et al., 2000). For the present study, we decided to design a new set of primers (Fungcont1-3). For that purpose the following primer design procedure was adopted: a small 18S rRNA sequences database was constituted from GenBank. The database included 40 different fungal species (Ascomycota, Basidiomycota or Zygomycota), which are commonly detected in air. The sequences were aligned using Clustal X software (version 1.63b, December 1997) (Thompson et al., 1997) and the primers were selected in highly-conserved regions. The best combination of primers was determined using Oligo 4.0 software. The composition of the primers is the following: Fungcont 1 (5′-CTC GCA TCG ATG AAG AAC-3′); Fungcont 2 (5′-GGG TAA TCC CAC CTG ATT CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G-3′); Fungcont 3 (5′-CCG CTT ATT GAT ATG CTT-3′). These primers were used in a semi-nested PCR in a single reaction tube. They allowed the amplification of 350 bp amplicons from 18S rRNA fungal sequences. Primer Fungcont 2 contained a 5′-

Table 3 (*continued*)

Sampling site	Fungal species identified from air samples[a]	
	by cultivation (CFU / m^3)[b]	by PCR-TTGE (fragment size, GenBank sequence identity)
Henhouses (samples F, G, H, I, J & K)		
	A. strictum (–; –; >200; –; –; 7)	
	Alternaria oregonensis (–; –; –; 10; 9; –) *Alternaria* sp. (–; 31; –; > 200; > 200; > 200)	*Alternaria* sp. (343 bp, AY305368 96%)
	Arthrinium sacchari (–; –; –; –; 9; 7)	*Arthrinium* sp. (346 bp, AY425967 95%)
		Aspergillus caesiellus (347 bp, AY373865 97%)
	A. flavus (17; –; 12; –; –; –) *A. fumigatus* (14; 17; 21; 23; 24; 26) *A. nidulans* (12; 15; 26; 32; >200) *Aspergillus* sp. (–; >200; –; –; –; 14) *A. versicolor* (–; 23; 24; –; –; 7)	
	B. albus (–; –; –; –; –;7)	
	Candida catenulata (>200 ; > 200; > 200; > 200; > 200; > 200)	*C. catenulata* (347 bp, AY493436 99%)
	Chrysonilia sp. (–; –; –; –; 9; –)	
	Cladosporium sp. (–; –; –; –; 9; 7)	*Cladosporium* sp. (334 bp, AY305374 98%)
		Cochliobolus heterostrophus (321 bp, BQ491489 90%)
	Cordyceps bassiana (–; –; –; –; 9; –)	
		Eurotium rubrum (351 bp, AY373891 98%)
	Fusarium oxysporum (–; –; –; –; –; 14) *Lophodermium niteus* (–; –; –; –; 7; –) *Mucor* sp. (17; –; –; –; –; –) *P. variotii* (17; 15; 24; 10; 9; –) *Penicillium roquefortii* (–; –; 12; –; –; –)	
	Penicillium sp. (17; –; –; 21; 17; 21) *Penicillium viridicatum* (–; –; 12; –; –; –)	*Penicillium* sp. (332 bp, AF177739 100%)
	Rhizopus oryzae (17; 23; 18; –; –; –) *Scopulariopsis* sp. (–; –; –; 10; 9; –) *Sporobolomyces* sp. (–; –; –; –; 7; –)	

GC clamp for direct melting during TTGE. Each reaction was carried out in 50 μL volume containing 2 pM of each primer, 2.5 mM of each dNTP, 0.9X PCR buffer, 1.5 U of *Taq* DNA polymerase (Takara, Saint Germain en Laye, France) and 5 μL of the DNA extract. To check for the presence of DNA inhibitors, DNA samples were used at the following dilutions: 1/1, 1/10, 1/100 and 1/1000. All PCR were performed in a Genamp thermocycler (Applied biosystem, Courtabeuf, France) with one cycle of denaturation (2 min, 95 °C), followed by 25 cycles of denaturation (30 s, 95 °C), annealing (30 s, 50 °C) and extension (1 min, 72 °C) and a final extension step (10 min, 72 °C). To avoid contamination, solutions were prepared with sterile and double distilled water and preparation of the mastermix, addition of template and gel electrophoresis of PCR products were carried out in separate rooms. DNA electrophoresis was carried out in 2% agarose gels containing ethidium bromide, and DNA fragments were visualized under ultraviolet light.

To adjust DNA extraction conditions and confirm the ability of Fungcont1-3 primers to amplify fungal DNA, we selected 18 isolates from the collection of the Mycology laboratory at the Veterinary College of Alfort (Table 2). These isolates represented some major fungal species that propagate via aerosolized conidia. Each isolate was maintained on Sabouraud dextrose agar (supplemented with 0.5 g of chloramphenicol) and subcultured at 27 °C for several days to two weeks, until sufficient hyphal growth and sporulation was observed. DNA from these fungal isolates was extracted as described above.

2.5. Temporal Temperature Gradient Electrophoresis

For sequence-specific separation of PCR products, the TTGE DCode System (Bio-Rad, Marnes-la-Coquette, France) was used. 8 μL of each PCR-amplified fragments were added to 8 μL of loading buffer (0.5 g L^{-1} bromophenol blue, 0.5 g L^{-1} xylene cyanol, 70% glycerol in dH_2O) and loaded onto the TTGE apparatus. Ten percent polyacrylamide gels (per 60 mL) were composed of 10% acrylamide–bisacrylamide (37.5:1), 1.25X Tris–acetate–EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8), 2.94 M urea, 55 μL of *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 9.2% ammonium persulfate. Electrophoresis was performed at a constant voltage of 80 V and with a temperature gradient of 55 °C to 63 °C and a temperature ramp rate of 1 °C/h were applied in 1.25X Tris–acetate–EDTA buffer. After electrophoresis, the gel was stained for 30 min in SYBR Gold nucleic acid gel stain (Amresco, Solon, USA). Gels were digitized using a gel Doc system (Bio-Rad, Marnes La Coquette, France).

2.6. DNA sequencing and data analysis

TTGE bands were excised and the DNA was eluted with 50 μL of elution buffer EB (Qiaquick PCR purification kit,

Qiagen, Courtabeuf, France) for 3 h at 58 °C before PCR amplification with the same primer set except that the primer Fungcont 2 was used without the GC clamp. The reaction conditions were similar to those described above. PCR products were purified and concentrated with a QIAquick spin PCR purification kit (Qiagen, Courtabeuf, France). PCR products were sequenced by Qiagen Genomic Services (Sequencing Services, Hilden, Germany).

18S rRNA sequences were compared with known sequences listed in the GenBank nucleotide sequence databases. The BLAST search option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) was used to search for close evolutionary relatives in the GenBank database (Altschul et al., 1990).

2.7. Culture and mycological identification

For fungal cultures, we used Petri dishes filled with malt extract agar (20 g of malt extract [Fluka, Biochemika]; 1 g of peptone [Difco]; 20 g of glucose; 15 g of agar–agar [Labosi] and 0.5 g of chloramphenicol). The collection liquid recovered from the CIP10-M cup was poured onto the surface of the medium and incubated at 27 °C for 10 days. Molds were subcultured and identified by their macroscopic and microscopic appearance after lactophenol cotton blue staining (de Hoog et al., 2000). Yeasts were subcultured and identified with the 32C API system (BioMerieux, Marcy l'Etoile, France). Specific identifications were systematically confirmed by partial sequencing of 18S fungal rRNA gene. For each isolate, DNA was extracted and the PCR amplification was performed with the same procedures as that developed for air samples (except that the PCR primers were used without a GC clamp).

3. Results

3.1. Primer set selection

Seven primer sets (Table 1) have been tested using DNA from air samples and DNA from representative fungal species (Table 2). The primer sets already described in the literature did not provide satisfactory results. Some primers did not allow the amplification of all fungal species (especially Mucorales), When other primers (Yuniv1-3, for example) were used for PCR–TTGE from air samples, they amplified DNA from non fungal eukaryotes. Original primers Fungcont 1/Fungcont 2+GC/Fungcont 3 allowed the amplification of 350 bp amplicons in 18S rRNA fungal sequences from all representative fungal species (Ascomycota, Basidiomycota and Zygomycota) (Table 1). When they were tested with air samples, they yielded fungal products.

3.2. TTGE fingerprints

Using a semi-nested PCR approach with Fungcont 1/Fungcont 2+GC/Fungcont 3 primers, we were able to perform a TTGE analysis of 18S rRNA fragments from single fungal isolates (Fig. 2) and mixtures of fungal organisms from air samples (Fig. 3). Each fungal isolate was characterized by one to three bands in TTGE analysis. Sequencing confirmed that the band(s) obtained for each isolate corresponded to a unique sequence in accordance with the specific identification of the isolate (Table 1). Fingerprints from air samples collected in the stable, the cowshed, the metropolitan station or the henhouses were different. Each TTGE fingerprint (from a representative fungal isolate or from an air sample) was highly reproducible (data not shown). Fingerprints were similar when DNA samples were used at dilutions 1/1, 1/10, 1/100 and 1/1000, suggesting absence of PCR inhibitors in air. The identification results obtained by sequence analysis and subsequent nBLAST comparisons are reported in Tables 2 and 3. All TTGE sequenced bands corresponded to fungal DNA sequences. Bands from different lanes but with similar electrophoretic migration pattern corresponded to the same sequence (Fig. 3). The presence of several bands for the same 18S sequence was sometimes observed in TTGE fingerprints from air samples. A total number of 20 distinct fungal taxa were detected from air samples. These taxa represented 16 different fungal genera (13 Ascomycota and 3 Basidiomycota). No member of the Zygomycota was detected with TTGE analysis. *Alternaria* spp. (bands 18, 22 and 23) and *Cladosporium* spp. (bands 6, 15 and 17) were identified in fingerprints from different sampling sites. Other fungal taxa were detected only once. Henhouses were the sampling sites where the greatest diversity of fungal taxa could be detected (8 different taxa representing 8 distinct genera). On the opposite, the TTGE fingerprint from the cowshed was characterized by a single band (*Alternaria triticina*).

3.3. Cultivation results

Mycological cultures allowed the isolation of 21, 6, 5 and 24 fungal taxa from air samplings in the stable, the cowshed, the metropolitan station and the henhouses, respectively (Table 3). These isolates belonged to 23 different genera of the Ascomycota, one genera of the Basidiomycota and two genera of the Zygomycota. All these taxa represent common aerosolized organisms. Taxa of the genera *Aspergillus* and *Penicillium* were isolated from all air samples.

4. Discussion

Indoor air quality has become an area of major concern in recent years (Cooley et al., 1998; Fung and Hughson, 2003; Johanning et al., 1999; Kauffman et al., 1995; Piecková and Jesenská, 1999; Samson et al., 1994). Increased understanding of fungal populations in air should enable better interpretation of fungal exposure found in air quality investigations in building or animal facilities. In the present study, fungal populations were assessed by two different techniques: mycological culture which is still considered as the gold standard technique and an original molecular method based on the separation of amplicons in a polyacrylamide gel under a denaturing temperature gradient (TTGE).

4.1. Air sampling procedure

A large number of sampling devices have been used for the detection of fungi in air. The CIP 10-M was selected for the present study because it allows both culture and molecular analysis from the same air samples. This sampler is light and offers full work shift autonomy. Furthermore, CIP 10-M limits the mechanical stress on aerosolized microorganisms (Gorner et al., 2006). Its sampling flow rate of 10 L/min is much lower than that of other air samplers but with a sampling duration as long as 120 min, the CIP 10-M allowed the recovery of a large number of different fungal taxa from the stable, the cowshed, the henhouses and the metropolitan station.

4.2. DNA extraction and PCR amplification

Conidia have strong cell walls, which are often resistant to traditional DNA extraction procedures (Van Burik et al., 1998; Cenis, 1992; Prosser, 2002). These difficulties have led to time-consuming (Blanchard and Nowotny, 1994) and expensive extraction methods involving ultracentrifugation (Specht et al., 1982) or column chromatography (Saunders et al., 1984). Many of these methods are not suited for routine analyses, where processing many samples simultaneously may be necessary. In the present study, we used the fine crushing with beads beater and DNA extraction with a commercial kit. The bead beater is considered as a convenient, rapid and very efficient device for disrupting fungal spores (Zhou et al., 2000; Kim et al., 1999; Haugland et al., 1999; Vaitilingom et al., 1998).

The other limitation in investigating fungal diversity in air samples is the suitability of available PCR primers. None of the already published primer sets were proved to be satisfactory for the present investigation. As a consequence, the challenge has been to design primers able to amplify as broad a taxonomic range of fungi as possible, but at the same time to prevent amplification of closely related eukaryotic DNA present in air samples. Primers Fungcont 1–3 allowed the amplification of 18S rRNA sequences from diverse fungal species, including members of the Ascomycota, Basidiomycota or Zygomycota. Using the three primers in a single reaction tube limited the risk of contamination.

4.3. TTGE fingerprints

DGGE, TGGE and TTGE have been the most widely adopted fingerprinting techniques in bacterial as well as fungal ecology in the last years (Anderson and Cairney, 2004). Most investigations concerned the analysis of soil microbial communities. The present study is the first one to use TTGE technique for the analysis of fungal diversity in the air. As already described, the technique benefits from the ability to analyze and compare several samples on a single gel and to investigate shifts or changes in community composition. The other advantage of TTGE technique is the ability to excise and sequence bands of interest. It has been shown that the intensity of bands in TTGE corresponded semiquantitatively with the abundance of species (Muyzer et al., 1993).

One of the main disadvantages of the TTGE technique is the formation of more than one band for a single rRNA sequence. We observed this phenomenon with reference isolates as well as some air samples. A similar situation has been reported in other investigations (Ferris and Ward, 1997; Fromin et al., 2002; Gadanho and Sampaio, 2004). This is probably caused by secondary structures formed in the DNA during electrophoresis (Anderson and Cairney, 2004; Doare-Lebrun et al., 2006).

4.4. Comparison of TTGE and cultivation results

The present investigation clearly demonstrated that the fungal diversity detected by TTGE was much lower than that revealed by culture. This result may be related to the inherent limitation of TTGE technique. First of all, preferential amplification of particular fungal groups from mixed community DNA samples could not be excluded. Although primer bias is an acknowledged problem (Smit et al., 1999) the potential bias associated with Fungcont 1–3 is difficult to estimate from our data set. Targeted investigations using defined template mixtures or synthetic fungal communities are still required. The second limitation associated with TTGE is the lack of sensitivity of conventional gel staining methods. It has been suggested that even the most sensitive staining methods may not be suitable to detect all the diversity present in a sample. Furthermore, single TTGE bands have been shown to comprise more than a single sequence (Anderson and Cairney, 2004).

The present investigation also demonstrated that fungal taxa detected by TTGE were different from pure cultures isolated on malt agar chloramphenicol in many instances. The same genera were detected by culture and TTGE in only 3 cases for air samples from the stable, in one case for the air sample from the cowshed and in six cases for air samples from the henhouses (Table 3). There was no similarity at all between TTGE and culture results for the air sample from the metropolitan station. This remains a surprising result even though the targets of the two detection methods are different. Cultivation allowed the detection of fungal taxa which were initially represented by viable conidia and which were able to grow on the selected mycological medium. We may imagine that DNA from non-viable conidia or fragments of hyphae could be amplified and identified by TTGE. This represents a major advantage for investigations about allergenic or toxigenic risks related to fungal aerocontamination. The fact that most of the cultured species (especially *Aspergillus*, *Penicillium* or Zygomycota) could not be detected by TTGE may be related to the different steps of the molecular technique. DNA extraction may not have the same efficiency according to the different types of conidia (size, shape, wall thickness, etc). The primer bias could not be excluded. Furthermore, the number of rRNA operon is known to vary according to the different fungal species (Hibbet, 1992). All these variations complicate the quantification of different fungal species in a mixed DNA pool. The presence of DNA inhibitors is unlikely as PCR–TTGE results were similar when DNA from air samples were diluted.

In conclusion, the present study demonstrated that there was no clear correlation between cultivation and PCR–TTGE results.

Cultivation was proved to give a much better overview of fungal diversity in air than did PCR–TTGE but the molecular detection may in some circumstances provide additional information by identifying non cultivable or non viable fungal elements. The combination of traditional methods and culture-independent techniques would certainly increase our understanding of fungal communities in air.

Acknowledgements

The authors are grateful to Christian Champion from Arelco Company for providing the CIP 10-M impactor. They also thank Valérie Bex from the Laboratoire d'Hygiène de Paris for giving the opportunity to examine one air sample from a metropolitan Station. This study was supported by a grant from the Association Nationale de la Recherche Technique (CIFRE convention 016/2005).

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