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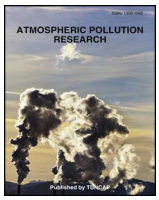

Interest of the qPCR method calibrated with flow cytometry to quantify *Aspergillus versicolor* in mold-damaged homes and comparison with the cultural approach

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ABSTRACT

The contamination of indoor air by molds constitutes an economic and health concern in many countries. Among fungi found in bioaerosols, some species like *Aspergillus versicolor* are known to be recurrent and toxinogenic. Several methods can be used to quantify this mold in bioaerosols, however, the lack of a standardized and rapid method to assess the human exposure to *A. versicolor* in indoor environments is a problem. In this study, the flow cytometry and qPCR techniques were used to quantitate *A. versicolor* in bioaerosols collected in 38 mold-damaged homes. A statistical analysis showed a correlation between qPCR and cultural approaches ($r = 0.704$, $p < 0.001$), and no significant difference ($p = 0.153$) between quantification with cultural and molecular approaches. Therefore a qPCR calibrated with flow cytometry could be routinely used to monitor *A. versicolor* in mold-damaged homes.

1. Introduction

Exposure to molds in houses is common in industrialized countries and constitutes a serious problem, especially because people spend 90% of their time indoors. In European countries, 10–50% of houses are known to have moisture problems, which can cause the deterioration of materials as well as fungal proliferation (WHO, 2009). Dampness and mold growth can be caused by climatic factors (such as heavy rains or floodings) or building defects, and is sometimes increased by the habits of the inhabitants (Andersen et al., 2011).

There is a lack of studies concerning the biological characterization of air in dwellings. Airborne molds represent a significant part of these biological contaminants in bioaerosols, and exposure to fungal particles may cause a variety of health effects including chronic respiratory affections and asthmatic symptoms (Douwes et al., 2003). Living in mold-damaged houses has been associated with adverse health effects, mainly respiratory symptoms, aggravation of asthma or infections in immunodeficient patients (Fisk et al., 2007; Platt et al., 1989; Stark et al., 2005). While some work may be required to better understand the microbial communities of bioaerosols and their impact on human health, some molds have been demonstrated to be characteristic of damp houses, such as *Aspergillus versicolor* and *Penicillium chrysogenum* (Beguin and Nolard, 1994).

A. versicolor represents the most reported species among *Aspergillus* section Versicolores which contains 14 different species (Jurjevic et al., 2012). *A. versicolor* caused aspergillosis in animals and humans (Baddley et al., 2009; Zhang et al., 2012) and is known to produce sterigmatocystin, a precursor of aflatoxins that has cytotoxic and mutagenic properties (Jussila et al., 2002). This mold has been found in many studies to be a recurrent fungal species as well as an indicator species of water-damaged homes (Andersen et al., 2011; Pottier et al., 2014; Tuomi et al., 2000).

Cultural methods are mainly used to evaluate the fungal contamination of bioaerosols allowing both the identification and quantification of viable fungal species, but these methods require special expertise (Gutarowska and Piotrowska, 2007). In addition, cultural methods are also time-consuming, and can be limited by the choice of culture medium, the growth of proliferant species and the presence of non-viable fungal particles. Over the last few years, real-time PCR has been increasingly employed in environmental microbiology to assess

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the levels of contamination by molds (Haugland et al., 2004; Méheust et al., 2012) or to quantify the presence of some fungal species (Morrison et al., 2004; Russell and Paterson, 2006).

Currently, most of the qPCR assays are based on ERMI developed by the US Environmental Protection Agency (EPA). Theses assays are based on TaqMan® chemistry, and the probe recognizes the internal transcribed spacers (ITS) (Hauglant et al., 2004). This region is located between the genes of the large subunit and the small subunit of rRNA genes, and have been extensively used in fungi characterization (Hinrinkson et al., 2005).

SYBR® Green technology can also be used in qPCR to quantify DNA because of its capacity to generate melting curves that could be useful to check competition with foreign DNA when we work on heavily contaminated samples such as bioaerosols collected in mold-damaged homes.

Usual method for spore counting needed to build calibration curve is hemocytometer which is a method that is both time consuming and subject to low repeatability (Smith et al., 1988). Flow cytometry could be an alternative method characterized by its fastness and reliability to count particles, and has previously shown its capacity to count fungal spores (Prigione et al., 2004).

In this study, we coupled cytometry to qPCR method in order to quantify *A. versicolor* in 38 bioaerosols collected from mold-damaged homes. Cytometry was used to calibrate spore suspension and allowed to build calibration curves for the qPCR approach previously developed by Libert et al. (2015). The results of this approach were compared with traditional cultural method.

2. Material and methods

2.1. Selection of homes and bioaerosol collection

The bioaerosols were collected in 38 mold-damaged houses located in Normandy. These houses were selected by local partners (health advisors, hygiene inspectors and tenant associations), the criteria being the presence of visible mold growth in home.

The air samples were taken using a liquid cyclone air sampler (Coriolis, Bertin Technologies, France), with a flow rate of 300 l/min during 10 min. The aerial particles were collected in sterile water containing 0.02% of Tween 80. For each home studied, four samples were taken in the main room at a height of 1 m. After collection, the samples were immediately tested for their fungal content using the cultural method, and an aliquot was also conserved at −20 °C until a DNA extraction and a qPCR quantification.

2.2. Quantification of molds by cultural method

For each sample, one ml aliquots (of collection liquid) and dilutions up to 10^{-3} were deposited into triplicates plates, and malt extract agar (MEA, Merck, Germany) supplemented with chloramphenicol (0.02 g/l) was poured. The plates were incubated at 25 °C for 7 days, and the fungal colonies were isolated, purified and identified during this period. The resulting colonies were counted and reported as Colony Forming Units (CFU) per m^3. The identification of fungal species was performed using macroscopic and microscopic features following the previous studies (Booth, 1966; Domsch et al., 1980; Klich, 2002; Pitt, 1979, 2000; Pottier et al., 2014; Samson et al., 2002; Samson and Frisvad, 2004; Von Arx, 1981).

2.3. DNA extraction

The DNA extractions were performed on aliquots of 2 ml of collection liquid using a modified protocol of the Nucleospin Plant II kit (Macherey-Nagel, Duren, Germany). The samples were centrifuged at 5000 g for 15 min and the supernatant was evacuated to leave only 100 µl containing the pellet. The pellet was then placed into a Qiagen TissueLyser with glass beads and 400 µl of lysis buffer for 15 min at 20 Hz, and then incubated at 65 °C for 15 min. After that, 400 µL of chloroform (Sigma-Aldricht, St. Louis, MO, USA) and 20 µL of Proteinase K at 10 mg/ml (Sigma-Aldricht, St. Louis, MO, USA) were added to the mixture. The aqueous phase was recovered and extracted using the kit precipitation and washing buffer according to the protocol described by the supplier.

DNA samples were stored at −20 °C and qPCR was performed within 3 days after the DNA extraction.

2.4. Flow cytometry for count of A. versicolor spores

A flow cytometry method was developed to count and calibrate spore concentrations of *A. versicolor* before their use in qPCR.

The spore suspensions (7 replicates) were prepared by harvesting the spores in sterile water additioned with 0.05% Tween 80 from a 10 days-culture of *A. versicolor* on Malt Extract Agar (MEA). The suspension was filtered on sterile cotton wool to remove big hyphal fragments, and then on a 5 µm pore cellulose filter (Millipore Corporation, Massachusetts, USA) to remove spore aggregates.

A flow Cytometric analysis was performed using a Gallios flow cytometer (Beckman Coulter). The data was acquired using the Gallios software (Beckman Coulter) and analyzed with the Kaluza analysis software (Beckman Coulter). Each cell was characterized by two diffraction parameters, namely, Forward Scatter (FS – Cell Size) and Side Scatter (SS - Granulosity). Samples were analyzed during 200 s and microspheres of 2 µm were used to determine the area of interest (Fluoresbrite YG Microspheres 2 µm, Polysciences). Fluorescent microspheres (Sphero Accucount Fluorescent Particules 5 µm, Interchim) were used to estimate the spore concentration in each cytometry run with the addition of 50 µl of these beads at a known concentration to 1 ml of each spore suspension.

2.5. qPCR for quantification of A. versicolor in bioaerosols

The qPCR was performed using a CFX Connect™ Real-Time PCR Detection System and the CFX manager 2.0 software (Bio-rad, Temse, Belgium) at the technical platform PROTEOGEN (UNICAEN). The SYBR green amplifications were done using the Takara master mix (Takara Bio, Japan) according to the producer recommendations, with the Aversi r/f primers (5′-AGTTCGCTGCGTTCTTCATC-3′/5′-CTGAGAGTG ATGCAGTCTGAGTCTG-3′) developed by Libert et al. (2015).

The specificity of primers was evaluated against several strains being recurrent in bioaerosols from mold-damaged homes: *Aspergillus fumigatus* (2 isolates), *Aspergillus flavus* (1 isolate), *Penicillium chrysogenum (2 isolates)*, *Penicillium crustosum* (1 isolate) and *Penicillium olsonii* (1 isolate).

The quantification limit and repeatability were evaluated by amplifying ten times dilutions of calibration of *A. versicolor* DNA (from 1 ng to 1 fg of DNA). The limit of quantification was defined as the lowest concentration of DNA when all ten amplifications were successful. Detection limit was evaluated according to determination of the LOD_6 described in the guidelines established by Broeders et al. (2014). We performed 3 separates runs of 6 replicates for 7 dilutions of gDNA corresponding to 10, 5, 2, 1, 0.5, 0.2 and 0.1 estimated copies of gDNA of *A. versicolor*. Repeatability was evaluated using the repeatability limit (r) and the relative standard deviation of the repeatability (RSDr) at the LOD_6 as described by Barbau-Piednoir et al. (2013).

For each run, a standard curve was realized using 1000 pg, 100 pg, 10 pg, 1 pg and 0.1 pg DNA as well as one negative consisting of pure water. The results obtained for the samples were reported to this standard curve.

It has been previously demonstrated that in some fungal species, the ITS region could be duplicated a random number of times between isolates belonging to the same species (Black et al., 2013). Although these repetitions allow an easier detection by PCR and qPCR as it