A novel qPCR based-method for detection and quantification of three recurrent species of Penicillium isolated from bioaerosols in mold-damaged homes

Antoine Géry, A. Delanoë, N. Heutte, E. Chosson, J. Bonhomme, David Garon

A novel qPCR based-method for detection and quantification of three recurrent species of *Penicillium* isolated from bioaerosols in mold-damaged homes.

Géry A.[1], Delanoë A.[1], Heutte N.[2], Chosson E.[1], Bonhomme J.[3], Garon D.[1]

[1] Normandie Univ, Unicaen & Unirouen, ToxEMAC-ABTE, Centre F. Baclesse, 14000 Caen, France

[2] Normandie Univ, Unirouen, CETAPS, 76000 Rouen, France

[3] Service de Microbiologie, CHU de Caen, 14000 Caen, France

Corresponding author : David Garon, Centre F. Baclesse, ABTE EA4651-ToxEMAC, avenue Général Harris, BP5026, 14076 CAEN Cedex05, France – david.garon@unicaen.fr

Highlights

- Indoor air quality is a major concern in industrialized and developing countries.
- *Penicillium* species present in bioaerosols can be involved in respiratory diseases.
- *P. brevicompactum, P. chrysogenum* and *P. crustosum* are among the most recurrent.
- A qPCR method was developed to detect and quantify these three *Penicillium* species.
- This new method opens up new prospects in bioaerosols study and monitoring.

Abstract

Fungal contamination of indoor environments can cause respiratory diseases and induce damages to building materials. Among the fungal species found in mold-damaged homes, *Penicillium brevicompactum*, *P. chrysogenum* and *P. crustosum* can be considered as recurrent strains. In this study, we therefore propose a rapid and novel qPCR-based method in order to allow the monitoring of these three fungal species. The method developed allows the quantification of the target DNA of these three *Penicillium* species with a limit of quantification of 0.01 ng/µL without significant difference with spectrophotometry quantification assay for DNA concentrations between 5 and 100 ng/µL. This technique also enables the rapid detection of these three species in complex mixtures of DNA extracted from 15 bioaerosols collected in mold-damaged homes and previously cultured on agar plate. This new *Penicillium* qPCR, sensitive and specific technique can thus be easily integrated into bioaerosol studies.

Keywords: *Penicillium*, qPCR, mold-damaged homes, indoor air

1. Introduction

In many industrialized countries, indoor air quality has become a major concern. Indeed, in these countries, it is estimated that people spend up to 90% of their time inside buildings (WHO, 1983) which can be exacerbated by confinement. Indoor air quality can be altered by humidity problems and the development of biological pollutants like molds (Andersen et al., 2011). This deterioration of indoor air quality in the habitats has impacted on the quality of life, and can lead to economic consequences in terms of health expenditure and renovation costs.

According to the WHO (2015), 10-50% of buildings in Europe have moisture problems that facilitate the growth of molds and the formation of bioaerosols. Some of these molds can cause respiratory diseases or aggravation of asthma and allergies, and sometimes infections in immunocompromised people (Douwes et al., 2003; Fisk et al., 2007, 2010; Platt et al., 1989; Stark et al., 2005; Eduard, 2009). Some micromycetes are also known for their ability to produce mycotoxins that may possess cytotoxic (Smith et al., 2018; Zhang et al., 2015) and/or genotoxic (Domijan et al., 2015; Liu et al., 2003) properties.

It is estimated that molds of the genus *Penicillium* are present in at least 90% of degraded habitats (Dallongeville et al., 2015). In this study, we are particularly interested in three species of *Penicillium* frequently isolated from indoor air samples collected in mold-damaged homes (Delanoë et al., 2018): *Penicillium chrysogenum*, *Penicillium crustosum* and *Penicillium brevicompactum*, which have been respectively found in 75%, 67%, and 63% of bioaerosols collected in our previous study (Delanoë et al., 2020).

These three *Penicillium* species are opportunistic pathogens that can cause allergies or lung damage (D'Antonio et al., 1997; De la Cámara et al., 1996; Geltner et al., 2013; Lyratzopoulos et al., 2002) and endophthalmitis (Eschete et al., 1981) in

immunocompromised patients. *P. brevicompactum* and *P. crustosum* are also considered as toxigenic species as *P. crustosum* synthesizes tremorigenic mycotoxins like penitrems (El-Banna and Leistner, 1988; Moldes-Anaya et al., 2012) and *P. brevicompactum* produces mycophenolic acid (Patel et al., 2018, 2016).

The development of DNA sequencing in the 1990s allowed sequence-based identification. Indeed, the internal transcribed spacer rDNA area (ITS) is now considered as the official barcode for fungi (Schoch et al., 2012) and is widely used as a sequenced marker to allow species identification of fungi. However, the ITS does not show enough variations to distinguish closely related species of fungi such as those belonging to the *Penicillium* genus. Therefore, the use of beta-tubulin gene (*benA*) associated with ITS as a secondary barcode is recommended to ensure the identification of isolates from *Penicillium* genus (Visagie et al., 2014).

Moreover, real-time PCR allows for the quantification of specific fungal DNAs and, thus, a better appreciation of bioaerosols content (Wu et al., 2002; Zeng et al., 2006). Although it is time-consuming to develop such a standardized method to be used, this one then considerably reduces the analysis time and allows to detect and quantify target DNA of scarce fungal species, non-viable fungal elements, or non-culturable molds which is not achieved by cultural methods.

The assessment of human exposure to fungal bioaerosols remains difficult due to methodological difficulties (collection and analysis of bioaerosols) and complexity of bioaerosols (fungal diversity) (Nevalainen et al., 2015). This study presents an original and rapid qPCR analysis method with the aim to detect and quantify DNA of three *Penicillium* species (*P. brevicompactum* (Pbrev), *P. chrysogenum* (Pchry), and *P crustosum* (Pcrus)) frequently isolated in mold-damaged homes.

2. Materials and methods

2.1. Fungal strains

Different fungal strains were used in this study (Table 1). Reference strains of *P. brevicompactum* (CBS 257.29), *P. chrysogenum* (CBS 306.48) and *P. crustosum* (CBS 115503) were purchased from Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). For each mold-damaged home, an aliquot of 1 mL of collection liquid and dilutions (10^{-1}; 10^{-2}; 10^{-3}) were deposited into Petri plates, and malt extract agar (MEA) (Merck, Darmstadt, Germany) supplemented with chloramphenicol (0.02 g/L) (Cooper, USA) was poured. Plates were incubated at 25 °C for 7 days, checked daily, and the number of colonies was counted and reported as Colony Forming Units per m^3 of air (CFU/m^3). Each different fungal colony was isolated and purified in MEA medium (Delanoë et al., 2020). In total, 25 fungal strains were also isolated from indoor air and were preserved in our laboratory's fungal collection. These isolates which belonging to *Aspergillus* and *Penicillium* genera were previously purified and identified using macroscopic, microscopic and molecular features (Choi et al., 2019; Domsch et al., 1980; Houbraken et al., 2020; Klich, 2002; Pitt, 2000, 1979; Samson et al., 2002; Samson and Frisvad, 2004; Visagie et al., 2014; Von Arx, 1981).

Table 1. Fungal strains used in this study.

Fungal species	Isolate number
Penicillium brevicompactum	CBS 257.29, 122_HAB10, 389_HAB24, 846_HAB53, 857_HAB54, 862_HAB55
Penicillium chrysogenum	CBS 306.48, 18_HAB04, 634_HAB39, 708_HAB44, 803_HAB50, 864_HAB55

Penicillium crustosum	CBS 115503, 103_HAB09, 801_HAB50, 839_HAB53, 843_HAB54, HOSP10
Penicillium glabrum	386_HAB24, 451_HAB28
Penicillium granulatum	345_HAB22, 819_HAB51
Penicillium olsonii	118_HAB10, 603_HAB38
Aspergillus sydowii	530_HAB34, 963_HAB62
Aspergillus versicolor	275_HAB18, 458_HAB28

2.2. DNA extraction

DNA extraction was performed using a modified protocol of the Nucleospin™ Plant II kit (Macherey-Nagel, Duren, Germany). Fungal colony was introduced in a 2 mL microtube with glass beads. The microtube was then placed into a Qiagen TissueLyser with 400 µL of lysis buffer PL1 (Macherey-Nagel, Duren, Germany) for 15 min at 20 Hz, and then incubated with 10 µL of RNAse and 20 µL of proteinase K at 10 mg/mL (Sigma-Aldricht, St. Louis, MO, USA) (Macherey-Nagel, Duren, Germany) at 65 °C for 15 min. After that, 400 µL of chloroform (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 extraction from the bioaerosol collection liquid was performed from 5 mL centrifuged at 4500 g. The supernatant was removed and DNA extraction was performed on the pellet as described above.

2.3. DNA purification and quantification

Extracted DNA was purified using NucleoSpin gDNA Clean-up kit (Macherey-Nagel, Duren, Germany) following the instructions of the manufacturer. For each isolate, purified DNA extracted was quantified using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA,

USA). To assess DNA quality, optical density values (A260/A280 and A260/A230) were compared to the reference values (1.8–2.0 and 2.0-2.2) using NanoDrop (Desjardins and Conklin, 2010). DNA extracted from reference strains CBS 257.29 (Pbrev), CBS 306.48 (Pchry) and CBS 115503 (Pcrus) was then diluted with sterile ddH_2O to a final concentration of 50 ng/µL.

2.4. Primers design

Primers were designed based on *benA* sequences downloaded on GenBank®/NCBI and aligned on ClustalW2® software (Conway Institute UCD, Dublin, Ireland) (Rodríguez et al., 2015). In order to ensure the specificity of primers (Table 2), the expected PCR products were compared using BLAST (Basic Local Alignment Search Tool, NCBI) to the sequences of the CoreNucleotide, dbEST and dbGSS databases. The expected PCR products all had a query coverage and percent identity of 100% for the corresponding species and no other species were identified in the request.

Table 2. List of accession numbers and sequences of specific primers used for qPCR assays.

Species	Strains	Accession Number	Primer sequences (5'-3')	Annealing temperature	Expected product length
Pbrev	CBS 110067 CBS 110069 CBS 257.29	AY674438 AY674435 KF499573	Forward (Bt_Pbrev_F3) : AGTAAGTGGACGACTGTG Reverse (Bt_Pbrev_R4) : CTCCAAGTCGACGAGGA	61 °C	207 bp
Pchry	CBS 282.97 CBS 306.48 CBS 109613	JX996925 JF909955 KJ866978	Forward (Bt_Pchry_F2) : AACAGTGATGGGGATTCTGG Reverse (Bt_Pchry_R5) : CGCCCGATCAGATGATGCA	64 °C	128 bp
Pcrus	CBS 47184	AY674352	Forward (Bt_Pcrus_F2) :	65 °C	100 bp

	CBS 101025 CBS 115503	AY674351 MN969379	TAGGGGTTTCCGGTGGATTA Reverse (Bt_Pcrus_R3) : TTCGGTTTCCTGTCGTTGGA		

130

131 Primers were synthesized by Eurogentec (Liège, Belgium), diluted with sterile ddH_2O to a
132 final concentration of 10 pmol/µL and stored at −20 °C before use.

133 ### 2.5. qPCR amplification

134 The qPCR assays were performed on the QuantStudio™ 5 System (Thermo Fisher Scientific,
135 Waltham, MA, USA) using SYBR™ Select Master Mix 2X (Thermo Fisher Scientific,
136 Waltham, MA, USA) containing SYBR® GreenER™ dye, ROX, AmpliTaq® DNA
137 Polymerase and oligonucleotides. The data collected were processed with QuantStudio™
138 Design & Analysis Software (Thermo Fisher Scientific, Waltham, MA, USA).

139 The program was composed of three stages: hold stage, PCR stage and melt curve stage. The
140 hold stage consisted in two steps: temperature with a ramping rate of 1.6 °C/s up to 50 °C
141 held 2 min (step 1); temperature with a ramping rate of 1.6 °C/s up to 95 °C held 10 min (step
142 2). The PCR stage consisted also in two steps: 15 sec at 95 °C (step 1); temperature decreased
143 down by 1.6 °C/s to the annealing temperature (Table 2) held 1 min before rising up anew by
144 1.6 °C/s to 95 °C (step 2) for 40 cycles. The melt curve stage consisted in 3 steps :
145 temperature rose by 1.6 °C/s up to 95 °C held 15 s (step 1); temperature decreased by 1.6 °C/s
146 down to 60 °C (step 2); temperature rose anew by 0.15 °C/s up to 95 °C (step 3) before the
147 end of the program.

148 For the standard range, the reaction mixture contained: 10 µL of SYBR™ Select Master Mix
149 2X, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of pure genomic DNA (gDNA) of
150 each *Penicillium* species reference (standard range : 0.01/0.1/1/10/100 ng of DNA) and

ultrapure water to 20 µL. 2.4 µL of $MgCl_2$ 25 mM was added for Pbrev to reach a sufficient efficiency of 90%.

2.6. Statistical analysis

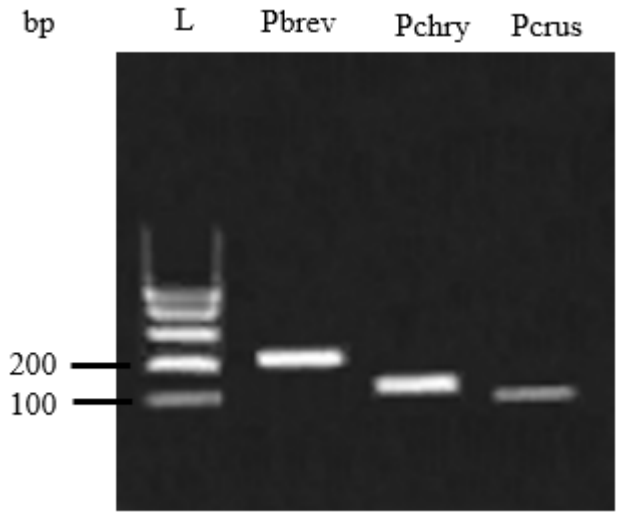
Descriptive statistics were calculated to provide information about the sample and paired t-test was also used to compare the two methods. Bland-Altman plots were used for descriptive evaluation of the concordance of the results from two methods. This was performed by plotting the difference for analysed variables between two methods against the mean results.

Only results at $p < 0.05$ were considered statistically significant. All statistical analyses were realized using SAS system version 9.4 (SAS Institute Inc. Cary, NC, USA).

3. Results

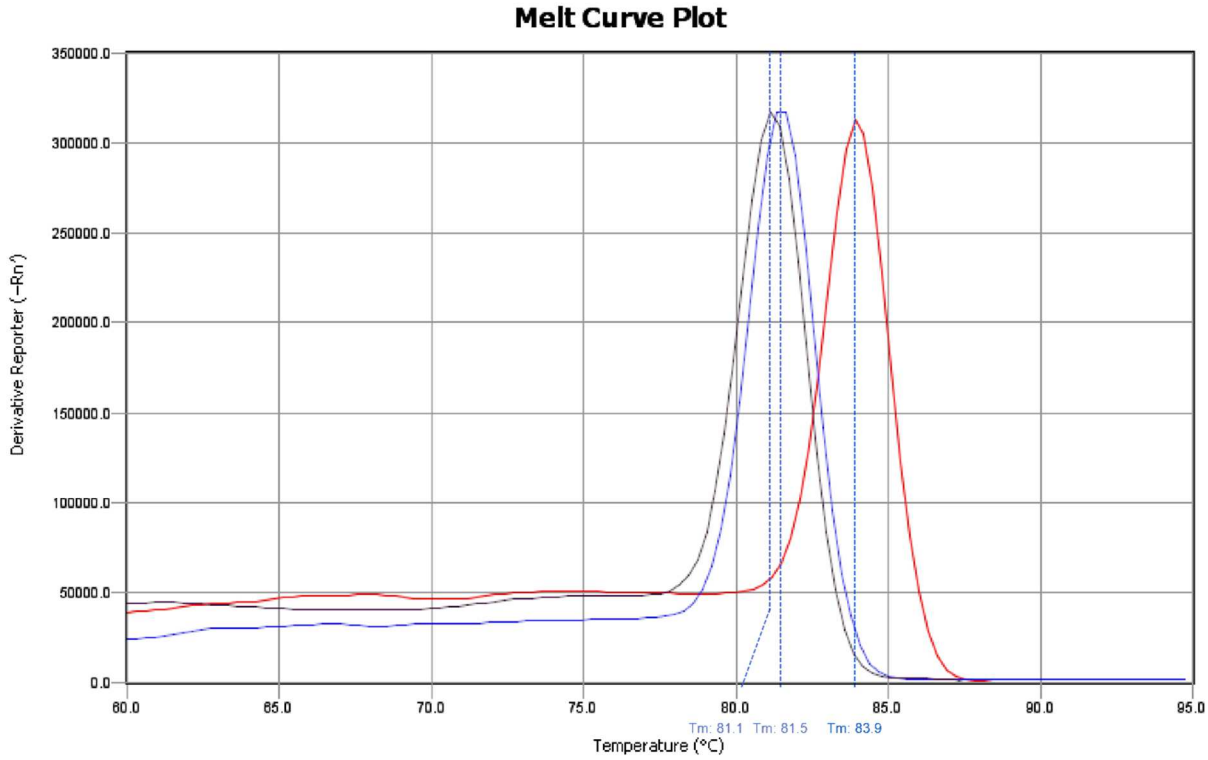
3.1. qPCR amplification

The amplified fragments from reference strains of Pbrev, Pchry and Pcrus showed bands around 200 bp, 125 bp and 100 bp respectively, without nonspecific bands (Figure 1), which was consistent with the length of expected fragments. The qPCR melting curves obtained for Pbrev, Pchry and Pcrus with this method showed three specific peaks with the following Tm values: 81.1 °C (Pcrus), 81.5 °C (Pchry) and 83.9 °C (Pbrev) (Figure 2).



167 Figure 1. PCR amplification of the *benA* fragment of three *Penicillium* (L: BenchTop 100 bp

168 DNA Ladder (Promega, Madison, WI, USA)).



169

170 Figure 2. Tm peaks obtained for Pchry— (81.1 °C), Pcrus— (81.5 °C) and Pbrev— (83.9

171 °C).

3.2. qPCR sensitivity

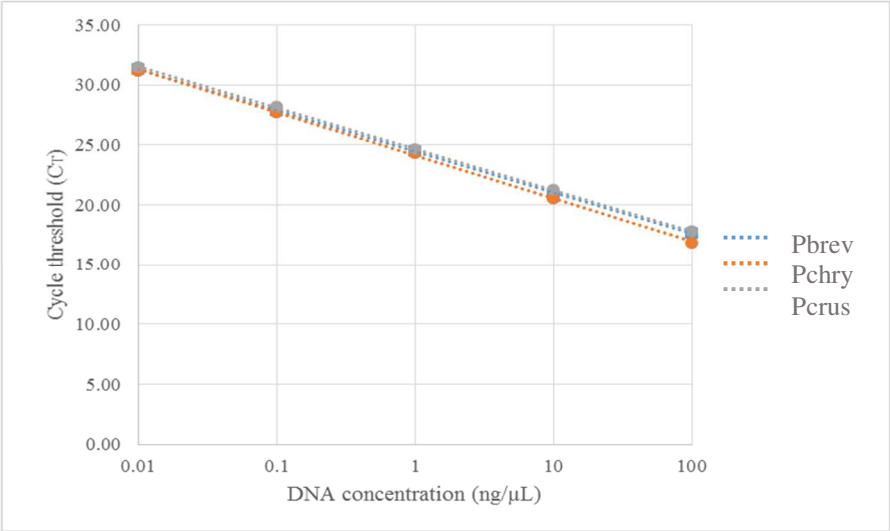


Figure 3. Standard curves of qPCR. Pbrev standard equation: $C_T = -3.4342x + 34.741$, $R^2 = 0.9998$, Efficiency = 95.52%; Pchry standard equation: $C_T = -3.5705x + 354.816$, $R^2 = 0.9994$, Efficiency = 90.58%; Pcrus standard equation: $C_T = -3.4357x + 34.978$, $R^2 = 0.9998$, Efficiency = 95.46%.

qPCR amplification using gDNA with different gradient concentrations demonstrated that there was a good linear correlation on standard curves between the cycle threshold and DNA concentration on the range from 0.01 ng/µL to 100 ng/µL (Figure 3). Detailed data are presented in supplementary material (Table S1). The reproducibility results showed that the coefficient of variation (CV) at C_T values for different concentrations of Pbrev, Pchry and Pcrus was less than one (Figure 2). Consequently, the intra-assay and inter-assay reproducibility of this method were good.

3.3. qPCR specificity

This qPCR method showed primers could specifically amplify *benA* from the 18 isolates of *Penicillium brevicompactum*, *Penicillium chrysogenum*, and *Penicillium crustosum*. No amplification was observed when the method was performed on gDNA of the other

environmental isolates of *Penicillium glabrum*, *P. granulatum*, *P. olsonii*, *Aspergillus versicolor* and *A. sydowii* frequently isolated from indoor bioaerosols.

3.4. Comparison between spectrophotometry and qPCR techniques

Because of its simplicity and rapidity, spectrophotometry is commonly used to quantify DNA. However, it indicates the concentration of total DNA in the DNA extract. To determine if our qPCR method can specifically quantify the DNA of these three *Penicillium* as finely as spectrophotometry, we quantified DNA extracts of each *Penicillium* with both methods and compared them.

For each *Penicillium* species, 30 DNA extracts obtained from our samples with concentrations between 5 and 100 ng/µL were performed in triplicates using Nanodrop and qPCR.

No significant difference could be found between these two methods (respectively *Penicillium brevicompactum*: $p = 0.940$; *Penicillium chrysogenum*: $p = 0.817$; *Penicillium crustosum*: $p = 0.106$). Bland-Altman plot analyses were also carried out to evaluate the bias between the mean differences of these two methods (Figure 4).

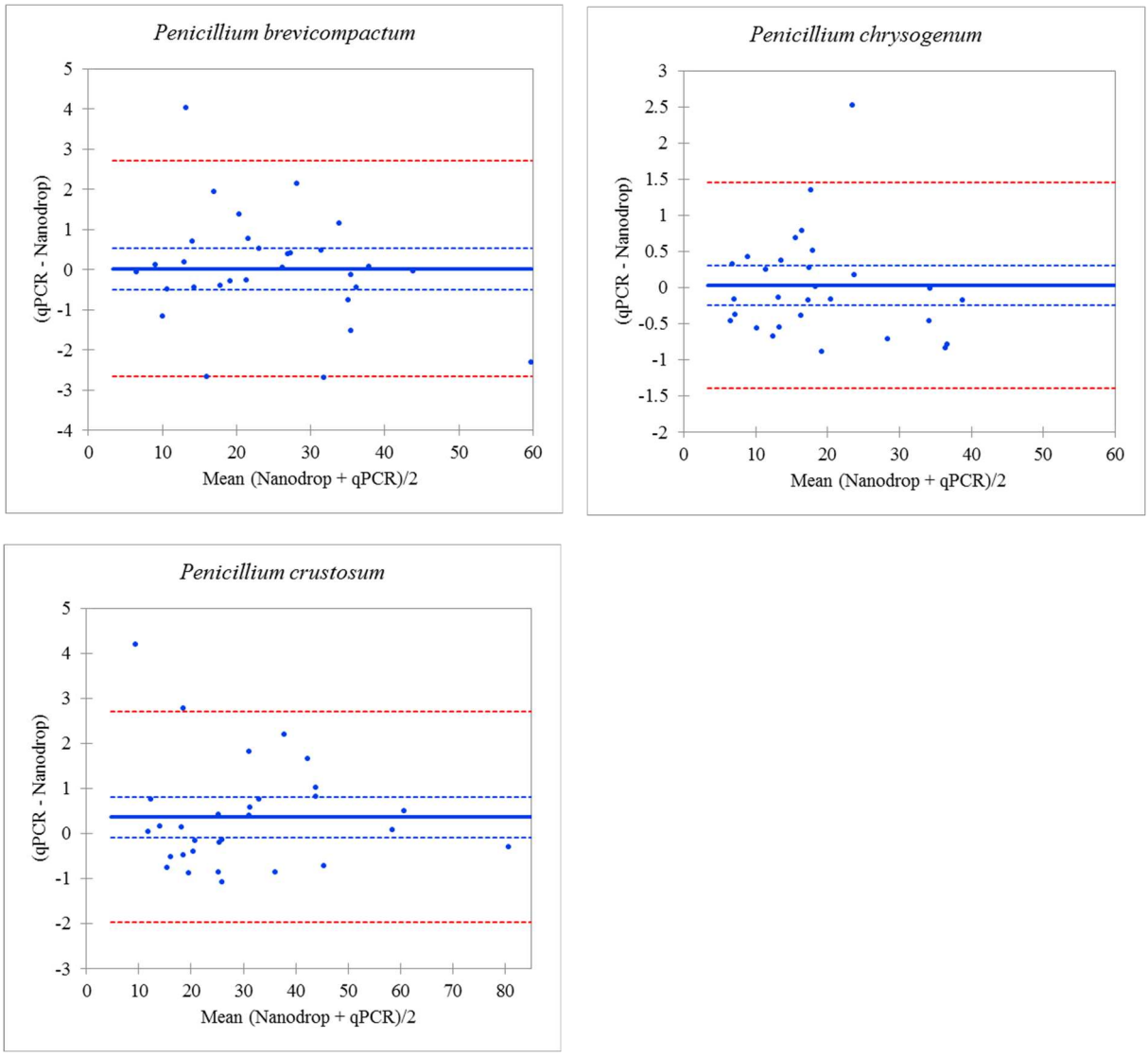


Figure 4. Bland-Altman plot analysis (: Bias; : CI Bias (95%); : CI (95%)).

3.5. Tests on environmental samples

DNA extractions were carried out from the agar plates obtained after culture of bioaerosols collected from 15 mold-damaged homes. The total DNA obtained was then tested to determine if Pbrev, Pchry and Pcrus could be detected using our technique. An example of culture plate before extraction was presented in supplementary material (Figure S1).

The DNA of these *Penicillium* species was found among the total extracted DNA. The macroscopic, microscopic and molecular study confirmed the presence of these *Penicillium* in the studied bioaerosols and highlighted other species as presented in Table 3.

231

232

233 Table 3. List of fungal species identified in 15 mold-damaged homes.

Sample ID	qPCR identification			Species isolated and identified using culture step
	Pbrev	Pchry	Pcrus	
HAB01	-	+	+	*Actinomucor elegans, Aspergillus sydowii, Aspergillus versicolor, Paecylomyces variotii,* ***Penicillium chrysogenum****,* ***Penicillium crustosum***
HAB02	-	+	+	*Aspergillus versicolor, Cladosporium cladosporioides,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium simplicissimum*
HAB03	+	+	+	*Aspergillus sydowii, Cladosporium cladosporioides, Paecylomyces variotii,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium griseofulvum, Penicillium simplicissimum*
HAB04	+	+	-	*Aphanocladium album, Aspergillus versicolor, Cladosporium sphaerospermum,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****, Penicillium expansum, Penicillium waksmanii*
HAB05	+	+	+	*Aspergillus flavus, Aspergillus versicolor,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium olsonii*
HAB06	+	+	+	*Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus sydowii, Aspergillus versicolor,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium olsonii, Penicillium purpurogenum, Penicillium verrucosum, Penicillium viridicatum*
HAB07	+	-	+	*Aspergillus europaeus, Aspergillus flavus, Aspergillus fumigatus, Botrytis cinerea, Cladosporium cladosporioides, Cladosporium herbarum, Penicillium aurantiogriseum,* ***Penicillium brevicompactum****,* ***Penicillium crustosum****, Penicillium glabrum, Penicillium simplicissimum, Ulocladium chartarum*
HAB08	+	-	+	*Alternaria alternata, Aspergillus versicolor, Cladosporium cladosporioides, Cladosporium sphaerospermum,* ***Penicillium brevicompactum****,* ***Penicillium crustosum****, Penicillium glabrum, Penicillium nalgiovense, Penicillium purpurogenum, Talaromyces flavus*
HAB09	-	-	-	*Actinomucor elegans, Aspergillus sydowii, Aspergillus ustus, Fusarium oxysporum, Penicillium glabrum, Penicillium purpurogenum, Penicillium variabile*
HAB10	-	+	+	*Arthrinium phaeospermum, Aspergillus melleus, Aspergillus versicolor, Cladosporium cladosporioides,* ***Penicillium chrysogenum****, Penicillium citrinum,* ***Penicillium crustosum***
HAB11	+	+	+	*Aspergillus versicolor,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium expansum, Penicillium glabrum, Penicillium olsonii*
HAB12	+	+	+	*Aspergillus fumigatus, Aspergillus ustus, Aspergillus versicolor,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****,* ***Penicillium crustosum****, Penicillium digitatum, Penicillium expansum, Penicillium variabile*
HAB13	+	+	-	*Aspergillus pseudoglaucus, Cladosporium cladosporioides,* ***Penicillium brevicompactum****,* ***Penicillium chrysogenum****, Penicillium glabrum, Penicillium viridicatum*

HAB14	-	-	-	*Aspergillus versicolor, Botrytis cinerea, Cladosporium cladosporioides, Mucor plumbeus, Penicillium expansum, Penicillium purpurogenum*
HAB15	+	+	+	***Penicillium brevicompactum***, ***Penicillium chrysogenum***, *Penicillium citrinum,* ***Penicillium crustosum***, *Penicillium glabrum, Penicillium spinulosum, Penicillium waksmanii, Rhizopus oryzae*

234 In bold, *P. brevicompactum*, *P. chrysogenum* and *P. crustosum* isolated and identified by
235 culture method.

236 The DNA of these three species of *Penicillium* was also searched in the total DNA extracted
237 directly from the collection liquid (as decribed in 2.2) without any prior culture step. The
238 amount of DNA extracted this way was not sufficient to enable amplification to generate a
239 signal different from the negative control. Thus, for the same 15 mold-damaged homes, none
240 of these *Penicillium* species could be directly found in the bioaerosols collected.

241 ## 4. Discussion

242 The development of specific molecular techniques for the identification of molds marked a
243 turning point in the field of mycology. Although ITS and RPB fragments are widely used for
244 this purpose, *benA* allows a finer identification between phylogenetically related *Penicillium*
245 species (Visagie et al., 2014). Furthermore, the alignment of ITS and RPB fragments for these
246 three *Penicillium* species does not show sufficient genotypic heterogeneity to allow for
247 specific primer design.

248 In this study we developed an original qPCR based method for the identification of three
249 species of *Penicillium* frequently found in the air of degraded homes (Delanoë et al., 2020).
250 This identification is faster than the cultural approach and does not require the support of a
251 sequencing platform. In addition, specificity tests showed that the primers used did not cross-
252 react with the gDNA of other recurrent indoor mold species belonging to the genera
253 *Aspergillus* and *Penicillium*.

In addition, this technique can be standardized and therefore routinely used in the laboratory to allow the identification of a large number of isolates in a short time, and then to assess human exposure to *Penicillium* strains in indoor environment.

Our method did not show any significant difference with spectrophotometry in its ability to quantify the target DNA of *P. brevicompactum*, *P. chrysogenum* and *P. crustosum* while spectrophotometry only measures total DNA. It also has the major advantage of being able to specifically detect and quantify the target DNA of these three *Penicillium* species at concentrations ranging from 0.01 to 100 ng/µL, i.e. at concentrations up to 100 times lower than the spectrophometry detection limit (2.5 ng/µL for Nanodrop 2000^{TM}).

Although this method allows a rapid identification and quantification of the DNA of these three *Penicillium* by avoiding the many steps required for the isolation, purification and identification of each fungal colony, it requires a sampling technique that enables the collection of more fungal elements to be applicable to bioaerosols without a step of culture on agar plate.

Indeed, despite assays were lead on pre-amplification and nested-PCR methods our samples did not generate a signal different from the negative control. Next studies on bioaerosol analysis by qPCR would require a concentration of CFU/m^3 of air ten times higher than that observed in the mold-damaged houses included in our study where the maximum concentrations observed are respectively 38 CFU/m^3 for *Penicillium brevicompactum*; 2400 CFU/m^3 for *P. chrysogenum* and 38 CFU/m^3 for *P. crustosum* (Delanoë et al., 2020). Another approach would be to take air samples over much longer periods by equipping residents with portable pumps, for example.

5. Conclusion

This qPCR method allows to gain time throughout all the purification and identification steps and a specific quantification of DNA from *Penicillium brevicompactum*, *Penicillium chrysogenum* and *Penicillium crustosum*. In addition to its practicality, this method can easily be standardized due to its robustness and so open new prospects for the monitoring of specific indoor fungi, particularly in the field of the exposure to fungal bioaerosols.

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