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Caractérisation de la flore (fongique, bactérienne, acariens) des logements par QPCR et impact sanitaire

Emeline Scherer

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Université de Bourgogne - Franche-Comté

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Ecole doctorale « Environnement, Santé »
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THESE

Pour obtenir le grade de
Docteur de l'Université de Bourgogne - Franche-Comté

Présentée par
Emeline SCHERER

Caractérisation de la microflore (fongique, bactérienne, acariens) des logements par QPCR et impact sanitaire

Soutenue le **01 décembre 2015** devant le jury composé de

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DEFINITIONS ET LISTE DES ABREVIATIONS

AI : Aspergillose Invasive.

ATOPIE : Aptitude à présenter, isolées ou associées, un certain nombre de manifestations cliniques « immédiates » (rhinite allergique, asthme bronchique, urticaire, eczéma constitutionnel, allergies alimentaires) et biologique (augmentation des IgE totales ou spécifiques), au contact d'allergènes banals, inoffensifs pour les sujets normaux.

Cq : Cycle seuil, nombre de cycles nécessaires pour détecter la fluorescence émise lors d'une analyse par QPCR. Il est inversement proportionnel à la quantité d'ADN présente au départ dans l'échantillon.

EBRA-ELFE : Environnement microBiotique et Risque Allergique sous cohorte nichée de la cohorte de l'Etude Française Longitudinale depuis l'Enfance.

EDC : *Electrostatic Dust Collector*, capteur électrostatique à poussière. Dispositif constitué d'une lingette électrostatique permettant le recueil de la poussière.

EPA : US Environmental Protection Agency, agence américaine pour la protection de l'environnement et de la santé publique. (<http://www.epa.org>)

ERMI : *Environmental Relative Moldiness Index*, correspond à un score calculé à partir de la quantification en QPCR de 36 moisissures (26 reconnues comme indicatrice de logements moisissés et 10 communes de l'environnement). L'ERMI pourrait permettre d'évaluer si un logement est à risque pour un enfant de développer de l'asthme ou non.

LPS : Lipopolysaccharides, composant essentiel de la membrane des bactéries à gram négatif. C'est une endotoxine pyrogène.

MALDI-TOF : instrument, spectromètre de masse couplant une source d'ionisation laser assistée par une matrice (MALDI, *Matrix-Assisted Laser Desorption/Ionisation*) et un analyseur à temps de vol (TOF, *time-of-flight mass spectrometry*). Le laser frappe la souche à identifier, associée à une matrice spécifique, les protéines libérées sont ensuite identifiées en fonction de leur masse et leur charge et le spectre obtenu comparé à une base de données pour fournir une identification en quelques minutes.

QPCR : *quantitative polymerase chain reaction*, ou PCR en temps réel, est une méthode permettant l'amplification exponentielle de 2 brins d'un segment d'ADN, au cours d'un cycle de température permettant alternativement la dénaturation de l'ADN, l'hybridation des amorces spécifiques de l'espèce étudiée, et l'élongation par une enzyme : l'ADN polymérase. Les produits d'amplification, peuvent être détectés au fur et à mesure qu'ils s'accumulent, en utilisant l'augmentation de l'émission de fluorescence produite au cours de l'amplification.

Résumé

Objectifs

L'objectif de la thèse est de caractériser par PCR quantitative en temps réel (QPCR) l'environnement fongique, bactérien ainsi que l'exposition à d'autres organismes allergisants (acariens et animaux de compagnie). Cette caractérisation des logements a pour but de mieux comprendre les interactions entre les différents micro-organismes de l'environnement intérieur et leurs relations aux maladies allergiques, dans le cadre d'une étude de grande cohorte.

L'objectif secondaire est la mise en place de nouvelles QPCR dans d'autres circonstances sanitaires (infections, PHS) afin de mieux caractériser l'impact des microorganismes de l'environnement.

L' étude EBRA-ELFE

L'étude **ELFE** incluant 18 319 enfants est la première cohorte française qui s'intéresse au développement des enfants de la naissance à leur majorité dans une approche multi-disciplinaire. Une étude nichée EBRA (Environnement microBiotique et Risque Allergique) est centrée sur l'étude microbiologique des poussières dans les chambres d'enfant.

Le mode d'échantillonnage (par capteur électrostatique à poussière, EDC) et d'analyse (QPCR) ont été validés et optimisés pour garantir l'acceptabilité du dispositif et la qualité des quantifications. Un panel de 10 cibles (moisissures, acariens, bactéries) sélectionnées pour leur caractère allergisant, toxique, infectieux ou potentiellement protecteur vis-à-vis des maladies allergiques a été initialement utilisé.

Ainsi lors de la première collecte à la naissance des enfants en 2011, 3217 CEP ont été quantifiés par QPCR. Les concentrations de six moisissures (*Aspergillus fumigatus*, *Aspergillus versicolor*, *Alternaria alternata*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Stachybotrys chartarum*), trois groupes de bactéries (Enterobacteriaceae, *Mycobacteria* et *Streptomyces*) et un acarien (*Dermatophagoïdes pteronissynus*) de l'environnement immédiat du nouveau-né ont été obtenues. Ces données ont permis d'identifier six profils de logements différents avec un gradient géographique E-O recouvrant la distribution des sifflements de l'asthme en France.

Un deuxième panel de 10 cibles a été choisi et documenté. Il inclut entre autres des cibles « chien » et « chat » pour proposer un test complet comprenant les allergènes principaux des logements. La collaboration avec le groupe ELFE continue et un projet pour une deuxième campagne d'échantillonnage à 5 ans se met en place.

L'utilisation de la QPCR pour caractériser d'autres situations à risque

La QPCR est un outil de quantification standardisé et reproductible et ses applications sont multiples. Au cours de ce travail de thèse, elle a été utilisée pour caractériser d'autres situations à risque : quantifier la présence de *Thermoactinomyces vulgaris* dans les aérosols des stations de compostages, détecter la présence d'*Aspergillus fumigatus* dans des environnements pouvant recevoir des patients immunodéprimés, mettre en évidence la présence d'ADN d' *Aspergillus fumigatus* ou de mucorales dans le sérum de patients immunodéprimés.

Conclusion

Les outils développés et les résultats obtenus pendant la thèse représentent une avancée pour une meilleure connaissance du monde microbien qui nous entoure et contribueront à mieux comprendre les mécanismes de développement des maladies allergiques.

Mots clés : PCR quantitative en temps réel, flore intérieure, monde microbien, profil de logements, cohorte, asthme et maladie allergique, développement de l'enfant.

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I. INTRODUCTION : BESOIN DE MESURER L'INVISIBLE, DE L'ENVIRONNEMENT A L'INDIVIDU

1. Caractériser l'environnement

1.1. Impact des micro-organismes

Depuis la crise pétrolière de 1973, les pays industrialisés se sont tournés vers une politique d'économie d'énergie. Un phénomène d'accroissement d'humidité au sein des logements, favorable au développement des micro-organismes, a ainsi été observé suite à la réduction des températures dans les habitats et aux problèmes d'isolation et de ventilation mal maîtrisée. En France, et dans d'autres pays « riches », différentes études montrent que 20 à 50% des logements présenteraient des problèmes d'humidité et de contaminations aux moisissures (Boutin-Forzano et al. 2006, Host et al. 2010). De par la petite taille de leurs spores, les moisissures peuvent atteindre les voies respiratoires et être responsables de maladies infectieuses, allergiques ou toxiques (Kuhn and Ghannoum 2003).

a. Allergique

Emergence des maladies allergiques et rôle des micro-organismes

L'allergie est une réponse inflammatoire à l'exposition à un ou plusieurs agents de l'environnement extérieur (pollens, moisissures) ou intérieur (acariens, moisissures) qui entraînent une réponse immunitaire exagérée (notamment en Immunoglobulines E) par rapport aux agents en cause (Denning et al. 2014). L'allergie se manifeste par différents symptômes cliniques tels que la rhinite, la conjonctivite ou encore l'asthme. Le coût de l'asthme a été estimé à 3 milliards d'euros / an en Europe en 2005 (European Environment Agency) incluant des milliers de décès et des jours d'absentéisme scolaire et professionnel. En France, l'asthme aurait concerné 6,7 % de la population en 2006, selon l'institut de recherche et de documentation en économie de la santé, avec une prévalence cumulée approchant 10% (Delmas et al. 2009). Au niveau mondial, l'asthme a été classé comme la 28ème cause de "disability-adjusted life years", ou DALYs, c'est-à-dire de nombre de perte des années de vie par diminution de l'espérance de vie et d'années vécues avec une invalidité liée à la maladie (Murray et al. 2012).

Parmi les facteurs impliqués dans l'asthme, des expositions actuelles plus longues dans l'environnement intérieur ont été mises en cause (Kasznia-Kocot et al. 2010). Vingt et un pourcent des cas d'asthme ont été attribués à l'exposition aux moisissures (Mudarri and Fisk 2007).

Une récente méta-analyse australienne a montré que les 4 genres de moisissures régulièrement retrouvées dans les logements étaient impliqués dans les phénomènes allergiques. Malgré les modes

d'échantillonnage et d'analyse différents, les auteurs concluent que *Cladosporium*, *Alternaria*, *Aspergillus* et *Penicillium* sont présents à des concentrations supérieures chez les personnes asthmatiques, et que leur présence augmente le risque d'exacerbation de l'asthme (Sharpe et al. 2015).

Allergies de l'enfant, asthme

L'asthme est la maladie chronique la plus fréquente chez l'enfant, sa prévalence est d'environ 10% en France (Saadeh et al. 2015) et reste cependant une pathologie difficile à diagnostiquer.

Avant 3 ans, on parle d'asthme du nourrisson : tout épisode dyspnéique avec sibilants qui se reproduit au moins trois fois avant l'âge de 2 ans. Après 3 ans, l'asthme est défini comme une maladie inflammatoire chronique des voies aériennes. Cette inflammation provoque des symptômes récidivants : sifflements, essoufflement, toux, particulièrement la nuit et au petit matin, associés à une obstruction diffuse des voies aériennes. En cours de crise, l'auscultation est caractéristique, avec de nombreux râles ronflants bronchiques et des sibilants (râle sonore aigu) (Marchac 2007).

L'asthme est une pathologie inflammatoire, allergique causée par plusieurs facteurs. La notion d'antécédents familiaux (allergiques, atopiques, chez les parents, d'eczéma atopique chez l'enfant concerné) est primordiale dans le diagnostic. Les conditions de survenue des crises peuvent aussi orienter sur les allergènes en cause. Les plus fréquemment rencontrés sont les acariens, les phanères d'animaux (chat), les pollens, les blattes et les moisissures.

Bien que la relation dose/effet entre quantité inhalée et apparition des symptômes allergiques reste mal connue, de nombreuses études rapportent le fait que des enfants vivant dans des logements humides et contaminés par les moisissures présentent un risque accru de développer des pathologies respiratoires (Kasznia-Kocot et al. 2010).

Cependant, d'autres auteurs sont partisans d'une théorie inverse (dite « théorie hygiéniste») : certains micro-organismes de l'environnement intérieur pourraient avoir des effets bénéfiques. Dans les années 80, Strachan a été le premier à proposer cette théorie, en se basant sur une observation : les phénomènes allergiques liés aux pollens et les dermatites atopiques sont moins fréquents chez les enfants vivants dans une fratrie multiple, probablement parce qu'ils sont exposés à plus de micro-organismes que les enfants uniques (Strachan 1989).

Plusieurs études en Europe, dont certaines menées en collaboration avec le laboratoire Chrono-Environnement (Riedler et al. 2001, Sudre et al. 2009, Vacheyrou et al. 2011, von Mutius 2007), ont montré que des enfants ayant grandi en côtoyant des fermes, des animaux, du lait non pasteurisé et

des boissons fermentées développaient moins d'asthme et de sensibilisations allergiques que des enfants n'ayant pas fréquenté ces environnements. Dans une étude plus récente (Lynch et al. 2014), l'exposition des enfants durant la première année de leur vie à certaines bactéries de la poussière domestique a été évoquée comme ayant un rôle protecteur vis-à-vis des sifflements respiratoires à 3 ans. Cette étude est particulièrement intéressante car elle évalue l'exposition sur la durée. Elle exprime l'exposition à différents allergènes domestiques à un instant donné mais également en valeurs cumulatives. Les auteurs montrent que l'exposition cumulée aux allergènes de blattes, souris et acariens sur les premières années de la vie est significativement associée à une augmentation de la sensibilisation, elle-même associée aux sifflements. L'exposition aux allergènes de chat, blattes, souris dans la première année de vie uniquement pourrait avoir un rôle protecteur vis-à-vis des sifflements à 3 ans.

Les allergies alimentaires sont rarement des causes isolées d'asthme et ne seront pas abordées à ce niveau du travail. Toutefois, on commence à percevoir le rôle du microbiote intestinal sur le développement de ce type d'allergie et le développement plus large de l'enfant. Hors le microbiote intestinal des enfants est probablement influencé par les micro-organismes rencontrés dans l'environnement au cours de la petite enfance (Konya et al. 2014).

Allergie professionnelle, pneumopathies d'hypersensibilité

La pneumopathie d'hypersensibilité (PHS) est une pneumopathie de mécanisme immuno-allergique complexe dû à l'inhalation de substances antigéniques le plus souvent organiques auxquelles le sujet a été préalablement sensibilisé. Elle requiert l'inhalation chronique, mais parfois intermittente, de concentrations élevées de microorganismes bactériens ou fongiques ou de substances aviaires. Ces conditions d'exposition sont observées le plus souvent en milieu professionnel, comme pour la maladie du poumon de fermier par exemple, qui est la forme la plus courante des pneumopathies d'hypersensibilité (Dalphin 2012).

Le micro-organisme initialement mis en cause dans la maladie du poumon de fermier est un actinomycète thermophile, *Saccharopolyspora rectivirgula* (*Micropolyspora faeni*) (PEPYS et al. 1963). Depuis, de nombreuses études ont complété la liste des micro-organismes pouvant être incriminés et également certaines situations, professionnelles ou non, où cette exposition répétée à des micro-organismes pouvait provoquer une PHS. La PHS domestique est au quatrième rang de fréquence après les PHS d'origines aviaires, celles liées aux jacuzzis ou spa et le poumon de fermier. De nombreuses autres expositions sont décrites, notamment aux mycobactéries présentes dans les huiles de coupe dans le domaine industriel (Murat et al. 2012), aux moisissures intervenant dans les

étapes de fabrication du saucisson, aux moisissures et acariens intervenant dans celle des fromages... (Thaon et al. 2006). Là où il y a prolifération de micro-organismes, avec une aérosolisation liée à l'activité et une exposition répétée, il y a un risque de PHS. Les domaines où cette pathologie survient et les agents impliqués sont encore aujourd'hui probablement sous-estimés. De nouvelles pratiques, comme le compostage, peuvent représenter des expositions professionnelles potentiellement à risque de par la prolifération de micro-organismes.

b. Infectieux

Les infections superficielles sont les atteintes fongiques les plus fréquentes dans le monde. Les mycoses cutanées touchent 20 à 25 % de la population générale à travers le monde. (Havlickova, Czaika, and Friedrich 2008). Elles sont le plus souvent liées à une infection par des dermatophytes, qui selon leur espèce, peuvent avoir comme réservoir l'homme, l'animal ou l'environnement (dont l'environnement intérieur, potentiellement riche en kératine) mais également par des levures, majoritairement représentées par le genre *Candida*. Ces infections sont généralement bénignes chez l'immunocompétent.

D'autres champignons environnementaux (*Aspergillus*, particulièrement *Aspergillus fumigatus* mais également *A. flavus*, *Cryptococcus*, particulièrement *C. neoformans*) et également les levures du genre *Candida*, sont de plus en plus impliqués dans des infections invasives, gravissimes chez les patients immunodéprimés. L'absence de réponse immunitaire à l'infection provoque chez les patients neutropéniques, transplantés d'organes solides ou sous thérapie immunosuppressive 200 000 aspergilloses invasives par an, dont le taux de mortalité est de 50 % sous traitement et de 100 % en l'absence de traitement (Brown et al. 2012).

Les mucorales sont également capables de provoquer des infections invasives. Plusieurs espèces sont impliquées, comme *Rhizopus*, *Lichtheimia corymbifera*, *Rhizomucor*. Les mucorales sont composées de filaments larges, non septés, et si leur implication en pathologie est moins fréquente, elle est en nette augmentation. En France, de 1996 à 2007, chez les patients atteints de pathologie hématologiques malignes, l'incidence des mucormycoses a augmenté de 24 % par an (Bitar et al. 2009).

Aujourd'hui, les personnes atteintes du VIH ne représentent plus la seule population à risque d'infections opportunistes : les thérapeutiques utilisées en oncologie, hématologie (dont les greffes de moelle osseuse), dans le cadre des traitements anti-rejet des greffes d'organes solides, sont autant de situations altérant le système immunitaire et pouvant laisser une place de pathogène à des

micro-organismes « colonisants », bien contrôlés chez l'immunocompétent, et dont la présence est fréquente en milieu hospitalier mais également au domicile.

c. Toxique

Les champignons ont également un rôle toxique en pathologie humaine. Les produits intracellulaires comme les β -glucanes ou l'ergostérol qui se trouvent dans les spores ou éléments mycéliens et les produits extracellulaires tels que les mycotoxines ou les composés organiques volatils (COV), sécrétés par les moisissures, ont un effet toxique (Kuhn and Ghannoum 2003).

Stachybotrys chartarum est une moisissure bien connue pour sa production de mycotoxines (dont la safra toxine et la Roridine E) et serait impliqué dans des phénomènes inflammatoires. De nombreuses autres moisissures (dont *Aspergillus* et *Penicillium*) sont capables de produire des mycotoxines (dont la gliotoxine et la patuline). Ces mycotoxines pourraient, par l'inflammation qu'elles provoquent au niveau des voies respiratoires, orienter la réponse immunitaire vers un profil Th2 (Schütze et al. 2010). Or l'asthme se caractérise par une hyperréactivité des voies aériennes liée à une réponse immunitaire orientée en profil Th2 en réponse à des allergènes respiratoires.

Les composants de la paroi des champignons (le Beta glucane, composé majoritaire de la paroi des cellules fongiques, les polysaccharides extracellulaires, émis dans l'environnement par le champignon lors de sa croissance) et les endotoxines (composants de la paroi des bactéries) sont des composés très présents dans l'environnement dont l'impact sur la santé humaine reste à préciser (Tischer et al. 2011).

d. Emergence de liens entre micro-organismes et pathologies autres que l'allergie

L'impact de l'environnement sur les pathologies allergiques est largement étudié, mais cette stimulation du système immunitaire par les micro-organismes pourrait également conduire à d'autres pathologies.

Ainsi, Jedrychowski, en 2011, a montré pour la première fois que l'exposition post natale, prolongée, aux moisissures (exposition évaluée par questionnaire répété tous les 3 mois) avait un rôle péjoratif sur le développement cognitif des enfants à 6 ans (en utilisant le WISC, mesure de QI par Wechsler Intelligence Scale for Children) (Jedrychowski et al. 2011).

Une étude allemande, menée sur 4860 enfants a mis en évidence une augmentation des difficultés de comportements et de symptômes émotionnels chez des enfants âgés de 10 ans qui avaient été

exposés à des moisissures visibles dans leur environnement intérieur au cours de ces 10 années (Casas et al. 2013), difficultés probablement médiées par une neurotoxicité et une activation des voies de l'inflammation.

La même équipe a travaillé sur une cohorte espagnole de 482 enfants, dont 424 ont bénéficié de tests psychométriques à l'âge de 4 ans. L'humidité persistante dans le logement ou dans la chambre de l'enfant dans les deux premières années de vie est associée à une diminution des scores obtenus aux tests psychométriques (MSCA, McCarthy Scales of Children's Abilities et CPSCS California Preshool Social Competence Scale respectivement)(Casas et al. 2013) . La présence de chien et de chat, si elle n'est pas associée à une différence de résultats aux tests, est associée à une augmentation des endotoxines dans le logement.

Il a également été montré chez l'adulte un lien entre moisissures, humidité et dépression, sans que de réelles mesures n'aient pu être réalisées (Shenassa et al. 2007).

Certaines expositions précoces à des facteurs environnementaux (comme le contact avec chien et chat) ont été mis en lien avec le développement d'autres maladies comme le diabète de type I, désordre immunitaire touchant la fonction pancréatique (Virtanen et al. 2014)

L'environnement et la poussière en particulier, sont des réservoirs de micro-organismes qui modifient la composition en bactéries (ou microbiote) de l'intestin de l'enfant (Konya et al. 2014). Les connaissances concernant l'étendue de l'impact des micro-organismes sur l'homme et particulièrement sur le développement de l'enfant sont en constante évolution. Cet impact pourrait être beaucoup plus large que le champ de l'allergie, et le nombre de domaines où les micro-organismes sont impliqués augmente.

De par le manque de mesures, de visibilité de cette flore qui nous entoure, le rôle de chacun reste encore mal connu.

1.2. Micro-organismes : une flore mal connue, caractéristique d'un environnement

a. Moisissures

Les micromycètes, également appelés moisissures, sont des organismes eucaryotes, sans chlorophylle, ce qui les distingue des végétaux. L'organisation n'est pas tissulaire, mais en hyphes, ou thalle, uni ou pluri cellulaire. Un ensemble d'hyphes forme le mycélium. Il s'agit d'organismes immobiles, dont la paroi est riche en sucre et en chitine qui confèrent à la membrane une certaine

résistance. Le nombre d'espèces et la diversité sont considérables. Des centaines d'espèces sont découvertes chaque année.

Il s'agit d'organismes hétérotrophes vis-à-vis du carbone, ils n'incorporent pas directement le carbone minéral mais dépendent de matières organiques préformées, présentes et produites par d'autres êtres vivants.

Les moisissures ont une grande capacité d'adaptation aux différents substrats. Leur présence dans de nombreux environnements et leurs effets visibles peuvent être délétères (destruction de matières organiques, dégradation des habitations) ou bénéfiques (industrie fromagère, production des antibiotiques). La température et l'humidité (ainsi que le milieu de culture) optimales de croissance sont propres à chaque espèce.

Leurs effets invisibles, sur la modification de la flore intérieure, la contamination de personnes susceptibles, l'inflammation des voies respiratoires est plus difficile à mettre en évidence et à évaluer. Une récente étude menée en France, au cours de laquelle 150 logements ont bénéficié d'échantillonnage d'air et de poussière a montré que des espèces comme *Cladosporium* et *Penicillium* étaient présentes dans 90 % des prélèvements d'air et présente dans 92 et 80 % des prélèvements de poussières (Dallongeville et al. 2015).

b. Bactéries

Les bactéries sont des organismes unicellulaires se multipliant par scissiparité. On les sépare classiquement en bactéries à gram positif (parmi lesquels on retrouve les actinomycètes, comme *Streptomyces*) et bactéries à gram négatif (parmi lesquelles toutes les entérobactéries, dont les parois contiennent des lipopolysaccharides, ou LPS, qui est une endotoxine pyrogène, très répandue dans l'environnement) selon leur résistance à la décoloration acido-alcoolique. Bien connues en pathologies humaines, elles sont impliquées dans de nombreuses infections qu'il serait impossible de lister.

Elles sont également très présentes dans la flore digestive et respiratoire de l'homme. L'intestin humain contient environ 10^{14} bactéries, représentées au moins par 400 à 500 espèces différentes. Ces bactéries commensales créent un environnement qui permettrait de protéger l'homme de pathogènes bactériens et de contribuer à l'activité métabolique de l'intestin. Cette flore intestinale, chez l'enfant, pourrait être influencée par la composition en bactéries dans la poussière domestique, celle-là même étant influencée par la présence d'un chien au domicile (Konya et al. 2014). Cette interaction entre bactéries environnementales, microbiote, développement de maladies est de plus en plus étudiée. Ainsi, le microbiote respiratoire de 60 enfants a été étudié par séquençage haut-débit. Le suivi de ces enfants a permis de montrer qu'il existait des profils de colonisation et que

certaines étaient associées à un plus petit nombre d'épisodes infectieux dans l'enfance (Biesbroek et al. 2014).

Au niveau des logements, les bactéries, comme les moisissures, peuvent croître sur différents milieux, dont les matériaux de construction et peuvent, au cours de cette croissance produire des métabolites toxiques ou inflammatoires. Si les bactéries ont toujours été présentes dans l'environnement, les nouveaux matériaux de construction et l'évolution des modes de vie leur fournissent de nouvelles niches écologiques. Cela nous conduit à chercher à les identifier et les quantifier (Nevalainen and Seuri 2005). Au vu de la grande diversité des bactéries existantes et des difficultés d'identification (les techniques d'identification usuelles ont prioritairement été mises au point pour les bactéries d'intérêt médical), les équipes ont cherché à évaluer leur présence par leur « charge », leur présence globale. Il a ainsi été mis au point des QPCR ciblant les bactéries gram positives et gram négatives (Kärkkäinen et al. 2010). Cette évaluation générale de « toutes les bactéries » gram négatives a pu être mise en corrélation avec la présence d'endotoxines dans les logements (Adhikari et al. 2014). Cette même étude, caractérisant les logements par QPCR bactérienne et par 36 QPCR fongiques (ou index ERMI) a permis de montrer que les logements fortement contaminés en moisissures avaient une concentration en bactéries gram négatives dans l'air supérieure à celle des logements moins contaminés.

Cette présence simultanée des bactéries et des moisissures, décrite également pour les bactéries du type mycobactéries (Torvinen et al. 2006) n'est pas anodine. Certaines (comme *Streptomyces californicus*, *Mycobacterium terrae*) sont connues pour leur effet d'induction de l'inflammation et leur effet cytotoxique sur les macrophages de la souris, effet plus important que pour les champignons (Hirvonen, Huttunen, and Roponen 2005). Or par exemple, *Stachybotrys chartarum* potentialise l'effet inflammatoire de *Streptomyces californicus* (Huttunen et al. 2004). L'ensemble des interactions possibles à l'échelle d'un environnement n'a pas pu être étudié mais les micro-organismes interagissent et c'est le résultat de ces interactions et non seulement leur présence, qui impacte notre santé.

c. Acariens

Les acariens sont des micro-organismes invisibles à l'œil nu, appartenant à la classe des arachnides, et à la sous classe des acariens. Il existe plusieurs milliers d'espèces identifiées, dont certaines ont un rôle de parasite de l'homme (c'est le cas de *Sarcoptes scabiei*, agent de la gale) et d'autres de contaminant de logements, on les appelle alors les acariens domestiques. Ces acariens domestiques sont séparés en deux groupes : les acariens de la poussière, essentiellement, en Europe,

Dermatophagoïdes pteronyssinus, *Dermatophagoïdes farinae*, et *Euroglyphus maynei*, alors que les acariens dits « de stockage », sont plutôt retrouvés dans les aliments et sont essentiellement représentés par *Acarus siro*, *Tyrophagus putrescentiae* et *Glycyphagus domesticus*.

Les *feces* des acariens de poussières sont connus pour être des allergènes majeurs dans le développement de l'asthme allergique (Celedón et al. 2007). Ces allergènes majeurs sont régulièrement quantifiés dans les études de cohortes portant sur l'allergie et leur présence est très répandue (Dallongeville et al. 2015, Weiler et al. 2015).

Au-delà de son rôle allergique propre, ce micro-organisme de taille beaucoup plus élevée que les autres composants de la flore domestique, pourrait interagir avec eux, soit en transportant et en assurant la dissémination des spores dans le logement, soit en modifiant la composition en spores par leur consommation préférentielle de certaines moisissures. A l'inverse, la présence de certaines moisissures, comme *Aspergillus penicilloïdes* (montré en culture) pourrait réduire la population d'acariens (Van Asselt 1999).

d. Blattes

Les blattes en général, et particulièrement la blatte germanique en France et en Allemagne (petite espèce de cafard mesurant 10 à 16 mm de long retrouvée en Europe), peuvent occuper des endroits chauds, sombres et humides des logements. Une étude menée à New York a montré que la présence de blattes pouvait indiquer un problème de moisissures dans le logement (Crawford et al. 2015). L'exposition aux blattes augmente le risque de sifflement chez les enfants (Gaffin, Kanchongkittiphon, and Phipatanakul 2014) mais jusqu'à aujourd'hui la présence de blatte n'a pu être évaluée que par questionnaire ou par mesure d'allergènes dans la poussière aspirée.

e. Autres... (*pneumocystis, virus*)

Considéré jusqu'en 1988 comme un parasite, *Pneumocystis carinii*, est un champignon à part. Il ne peut pas se cultiver sur milieux usuels, il ne produit pas de mycélium, il n'est pas sensible aux thérapeutiques habituellement utilisées contre les champignons. Il a comme point commun avec *Aspergillus fumigatus* par exemple d'être présent dans l'environnement, dans l'air, et de ne devenir pathogène pour l'homme que dans un contexte d'immunosuppression, particulièrement chez les patients atteints de VIH. Difficilement accessible par technique de culture, on commence à mieux connaître *Pneumocystis* depuis l'essor de la biologie moléculaire. Il a été montré, par sérologie, que de nombreux enfants étaient en contact avec ce champignon dans la petite enfance : 85 % feraient une séroconversion avant l'âge de 20 mois. Les jeunes enfants pourraient être un réservoir de

Pneumocystis (Vargas et al. 2001). La transmission se fait *via* les aérosols. (Brown et al. 2012). L'impact sur le développement de maladies respiratoires n'a pas du tout été étudié. Ce champignon peut être recherché dans l'air (Choukri et al. 2010). Toutefois, ce pathogène ne sera pas abordé dans ce travail de thèse, car, de structure différente des autres moisissures, il s'est avéré que le mode d'extraction initialement choisi dans nos études environnementales ne permettait pas d'extraire l'ADN de *Pneumocystis*. Cela reste une question en suspens, un projet à mettre en place au vu des données à venir.

Concernant les virus, ils font partie intégrante du développement de l'enfant et interviennent dans la mise en place de l'immunité et de l'allergie (Gern et al. 2009). Ils sont alors recherchés dans le tractus respiratoire des enfants et non dans l'environnement et ne feront donc pas non plus partie de ce travail.

1.3. Méthodes d'échantillonnage et représentativité de l'environnement

En reprenant plusieurs études, B. Flannigan montre la difficulté du prélèvement d'air (Flannigan 1997). La libération des spores provenant des tapis, murs et autres surfaces introduit une variabilité importante dans la mesure des concentrations en spores de l'air. Elle dépend du type et du degré d'activité des occupants.

Par exemple, la marche sur un tapis augmente de façon significative le nombre de spores fongiques retrouvées dans l'air.

Les différences de taille, de structure et donc de vitesse de sédimentation des spores affectent également la composition en moisissures que l'on peut retrouver dans l'air. Hunter et al. ont montré que les grandes spores d'*Ulocladium* provenant des murs sédimentent rapidement, et ne peuvent donc être retrouvées dans l'air que si le prélèvement a lieu à courte distance de la perturbation ayant provoqué la mise en suspension (Hunter et al. 1988).

Cependant, les prélèvements d'air sont largement répandus pour évaluer la concentration en spores fongiques dans les logements. Il existe plusieurs modes de prélèvement de l'air et des bioaérosols (c'est-à-dire les particules biologiques retrouvées dans l'air). Le choix du mode de prélèvement doit être fait en fonction de l'intensité de contamination du milieu et de la technique d'analyse prévue. Le temps d'échantillonnage et le nombre de prélèvements nécessaires pour permettre une représentativité du milieu, doivent également être discutés. Il n'existe pas de méthode unique appropriée à toutes les études (Sergey et al. 2007).

Les différentes méthodes couramment utilisées sont les suivantes :

La sédimentation

C'est une méthode de faible coût, qui expose de façon passive un support nutritif à la chute naturelle des spores sous l'effet de la gravitation. Le risque de dessiccation du milieu et l'absence d'évaluation du volume d'air prélevé ont entraîné son abandon.

L'impaction

La méthode d'impaction sépare les particules du flux d'air en utilisant leurs propriétés d'inertie. Les particules sont impactées directement sur les milieux de culture. Elle ne permet donc pas la mise en évidence des spores non vivantes, des spores non adaptées au milieu de culture choisi, et le «stress» provoqué par le prélèvement peut rendre certaines spores non cultivables. L'analyse microbienne peut ensuite se faire par culture, et même plus récemment par QPCR (Bellanger et al. 2010) en utilisant des milieux gélosés spécifiques.

La filtration

Les particules sont recueillies à travers un filtre, de nature variable (polyvinyle, fibre de verre, cellulose, téflon), disposé dans un porte-filtre, associé à une pompe aspirante de faible débit (3 à 5 L/min). Le filtre peut ensuite être examiné au microscope, mis directement en culture ou être rincé avec un liquide stérile, qui sera alors utilisé comme échantillon. Les inconvénients majeurs sont la perte de viabilité, essentiellement par dessiccation, et l'efficacité imparfaite du rinçage. De plus, la dilution induit une sélection des espèces les plus présentes au détriment des espèces moins représentées dans l'échantillon.

Le biocollecteur en milieu liquide

Il s'agit d'une nouvelle génération d'échantillonneur d'air, basée sur la technologie cyclonique. Son principe de fonctionnement est d'aspirer à un fort débit l'air et les particules aéroportées. L'air est entraîné dans un mouvement tourbillonnant à l'intérieur d'un cône. Ce mouvement engendre la centrifugation des particules sur les parois du cône et la formation d'un vortex de liquide qui balaie

ces parois. Cette technique permet donc la récupération des bio-aérosols en milieu liquide. Le liquide peut ensuite être utilisé pour la culture ou l'analyse en biologie moléculaire. Grâce à un débit d'air élevé, cette technique permet une durée de prélèvement moindre, et pourrait être utilisée pour les contrôles de la qualité de l'air de milieux faiblement contaminés (Vu-Thien et al. 2008).

Le capteur individuel de poussière ou CIP

C'est un appareil autonome destiné aux prélèvements de poussières en vue d'en évaluer les teneurs susceptibles d'être inhalées par l'homme. L'échantillonnage de l'air est assuré par la rotation d'une mousse dont les alvéoles engendrent un débit d'aspiration de 10 L/min, similaire au débit respiratoire de l'être humain et une filtration de l'air par un filtre rotatif en mousse poreuse. Ces prélèvements d'air sont réalisés sur des temps très courts (de 10 minutes à 1 heure le plus souvent, jusqu'à huit heures) et peuvent donc ne pas être représentatifs de l'exposition chronique. Pour permettre une évaluation de la contamination d'un logement, plusieurs prélèvements répétés sont souhaitables. De plus, ces prélèvements d'air soumettent les organismes à un stress pouvant conduire à une diminution de viabilité.

Autre appareil individuel capable de capter la fraction inhalée : l'échantillonneur IOM, est utilisé dans le monde entier. Les particules sont aspirées dans le dispositif à travers un orifice d'entrée circulaire de 15 mm sous un débit d'aspiration de 2 L par minute. Le capteur contient une cassette en plastique avec un filtre de 25 mm.

Prélèvements de surface

D'autres types de prélèvements sont utilisés, comme les prélèvements de surface (effectués à l'aide d'adhésifs, d'écouvillons, de boîtes « contact »), mais ils ne prennent pas en compte la facilité du champignon à se remettre en suspension. Ils ne sont donc pas révélateurs des quantités de spores inhalées et dépendent de la zone échantillonnée (McGinnis 2007).

Prélèvements de poussières

Les prélèvements de poussières sont également couramment utilisés (Lignell et al. 2008, Chew et al. 2003, Meyer et al. 2005, Pitkaranta et al. 2008). Les poussières peuvent être collectées à l'aide d'un aspirateur puissant. L'aspiration est standardisée en temps et en surface. Cependant, ils ne

permettent pas d'évaluer l'exposition au long terme, dans la mesure où la poussière déposée au sol est nettoyée, plus ou moins fréquemment, par les habitants. De plus, ils ne représentent pas la fraction de moisissures « respirées » car la composition en moisissures de la poussière déposée peut être très différente de celle de l'air (Flannigan 1997).

Plus récemment, certains auteurs ont utilisé des dispositifs pour recueillir de la poussière de manière passive dans les logements :

Capteur Electrostatique de Poussière, EDC « Electrostatic Dust Collector »

Würtz et al. utilisent un dispositif constitué d'un support couvert d'une feuille d'aluminium et protégé par une grille. Laisse en place durant 140 jours, il permet de recueillir la poussière, qui peut alors être pesée et exploitée pour la mesure des moisissures cultivables de l'environnement et les (1-3)- β -D-glucanes (constituant des spores fongiques). L'étude montre que si le dispositif n'est pas adapté à l'estimation de la concentration en glucanes, il peut être utilisé pour l'évaluation de la concentration en spores fongiques cultivables (Wurtz et al. 2005).

Thorne et al. ont testé l'utilisation de lingettes pour la mesure des endotoxines. Ils proposent un essuyage actif par les participants des surfaces à l'aide de lingettes commerciales et d'un protocole établi (Thorne et al. 2005). Ce mode de recueil a pour but de simplifier le prélèvement, et donc de diminuer les coûts, facilitant ainsi les études de cohortes. L'étude des moisissures ou des particules viables n'a pas été réalisée.

Enfin, en 2008, Noss et al. mettent au point un collecteur électrostatique de poussière de faible coût, recueillant de façon standardisée la poussière tombante. Le dispositif est constitué de quatre lingettes, d'une surface totale de 42 par 29,6 cm, disposées dans une pochette en polypropylène. Il est laissé en place durant 14 jours et peut être retourné par la poste au laboratoire. Les prélèvements ont été réalisés sur deux périodes différentes et les résultats ont montré une bonne corrélation sur ces deux périodes. Cela tend à prouver la possibilité d'une évaluation de l'exposition chronique par ce système. L'étude conclut à la possible alternative (ou à un complément) aux échantillonnages de poussière par aspiration pour l'étude des endotoxines. Il n'a pas été étudié pour les moisissures ou les particules viables.

Ces différentes techniques d'échantillonnage ont pu être comparées. Filtration, impaction en milieu liquide, EDC, collecteur, et poussières aspirées ont été comparés pour une analyse par culture. Il en

ressort que la corrélation entre prélèvement d'air et EDC est correcte pour les moisissures, sans avoir pu être démontrée pour les bactéries et les endotoxines (Frankel et al. 2012). Une récente revue de la littérature sur les modes d'échantillonnage et d'analyse de la flore des environnements intérieurs montre la difficulté de comparer les résultats issus d'échantillonnage (et d'analyse) différents et rappelle que le choix dépend de la stratégie de l'étude (Méheust et al. 2014).

1.4. Méthodes d'analyses et quantification de l'environnement

Culture

La culture est utilisée pour l'analyse des moisissures, mais également des bactéries, des levures et des actinomycètes. Elle permet une détermination sans *a priori* de la contamination du logement en micro-organismes mais les compétitions entre espèces peuvent conduire à des résultats qui ne reflètent pas la composition réelle en micro-organismes du logement. De plus, la culture ne met en évidence que les éléments viables et cultivables. Or, les spores mortes mais également les fragments de filaments mycéliens peuvent provoquer des effets sur la santé (Gorny 2004). De plus, il n'existe pas de milieu idéal pour toutes les espèces. On sait par exemple que pour *Stachybotrys chartarum*, même ensemencée sur le milieu adéquat, une suspension de spores de concentration connue ne donnera en culture qu'1 % de la quantité attendue (Dillon et al. 1999). L'expertise microscopique pour l'identification peut aujourd'hui être complétée par une identification par séquençage ou spectrométrie de masse (ou Maldi-tof).

PCR

Le concept de PCR (*polymerase chain reaction*), découvert en 1983 par Kary Mullis (prix Nobel de chimie en 1993), est une méthode sensible et spécifique permettant l'amplification exponentielle de 2 brins d'un segment d'ADN, au cours d'un cycle de température permettant alternativement la dénaturation de l'ADN, l'hybridation des amorces spécifiques de l'espèce étudiée, et l'élongation par une enzyme : l'ADN polymérase.

Les produits d'amplification, lorsqu'ils sont en quantité suffisante, peuvent être mis en évidence par électrophorèse. La présence et la taille des amplicons peuvent ainsi être testées.

Russell Higuchi fut l'un des premiers à faire l'analyse des cinétiques de la PCR en détectant le produit de PCR au fur et à mesure qu'il s'accumulait, en utilisant une mesure à chaque cycle de la

fluorescence produite au cours de l'amplification (Higuchi et al. 1992) : par PCR quantitative en temps réel ou QPCR.

En utilisant une sonde d'hydrolyse, à chaque cycle d'amplification du brin d'ADN, la sonde, spécifique de la partie à amplifier, est détachée de sa cible par la Taq polymérase et libère ainsi sa fluorescence. Le cycle seuil, ou Cq, est le nombre de cycles d'amplification qui a été nécessaire pour que la fluorescence libérée par la sonde soit supérieure au bruit de fond. Ainsi, plus la quantité d'ADN présente dans l'échantillon est importante, plus le nombre de cycles nécessaires pour détecter le signal Cq est faible. En rapportant le Cq à ceux obtenus par une gamme d'étalonnage de concentration en ADN connue, il est possible de quantifier l'ADN présent dans les échantillons.

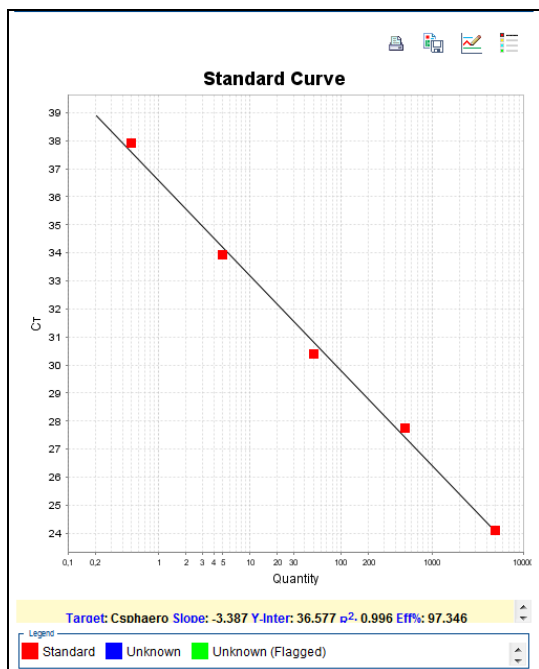


Image 1 : Courbe d'étalonnage, Cq en fonction de la quantité d'ADN, concentration des points de la gamme calibrée et connue

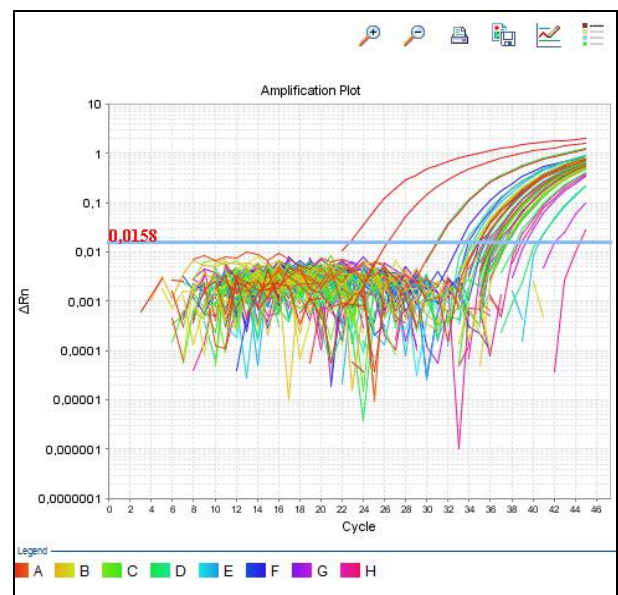


Image 2 : Courbes d'amplification, le Cq de l'échantillon est déterminé à l'intersection avec la ligne de seuil

L'EPA , Agence Américaine pour la Protection de l'Environnement a mis à disposition une liste de systèmes d'amorces et sondes spécifiques pour la quantification de 74 moisissures environnementales (voir Annexe 1, liste au 24 09 2015) (en cache dans Google : (<http://www.epa.gov/nerlcwww/moldtech.htm%23primers>)).

Des cibles spécifiques des moisissures, mais également des bactéries, acariens ont été dessinées permettant la quantification de plusieurs cibles et leur analyse éventuelle par score comme l'ERMI (Méheust et al. 2012, Reponen et al. 2011, Vesper et al. 2007).

Mesure des composants des microorganismes : allergènes, endotoxines, mycotoxines, glucanes

Les acariens sont les plus « grands » émetteurs d'allergènes dans les logements. Ces allergènes sont décrits par les trois premières lettres du genre, la première lettre de l'espèce et un chiffre (par ordre de découverte). Les allergènes les plus fréquemment dosés dans les logements sont ceux de *Dermatophagoïdes ptenonyssinus* et *D. farinae*, respectivement Der p 1 et Der f1.

Après les acariens, les blattes sont les allergènes les plus importants de la poussière de maison. Les allergènes majeurs de *Blatella germanica* sont Bla g1 et Bla g2.

L'allergène principal du chat, nommé Fel d1 est présent sur les poils, la peau, les glandes sébacées et sudoripares de l'animal. Il semble que les chats mâles soient plus allergisants que les femelles ou les chats castrés (Verloet et al. 2003). Ces allergènes peuvent persister plusieurs mois après le départ définitif de l'animal. L'allergène principal du chien est nommé Can f1. Il est également très présent dans la poussière de maison.

Les allergènes, protéines préalablement identifiées pour induire une réponse allergique, sont mesurables dans la poussière par ELISA, Enzym- Linke- ImmunoSorbent Assay (Dallongeville et al. 2015). Certaines études l'utilisent pour évaluer la présence des allergènes sur d'autres supports que la poussière de maison, comme sur les jouets (Wu et al. 2014).

Une technique avec Luminex, commercialisée par Indoor Biotechnology consistant à mélanger l'échantillon de poussière avec des billes marquées par des composés fluorescents et liées à des anticorps spécifiques des 9 antigènes domestiques proposés, est également utilisée (Weiler et al. 2015). Les billes sont ensuite lues par un appareil équipé d'un laser capable de mesurer l'intensité de fluorescence liée à la quantité d'antigène fixé. Cette fluorescence est ensuite rapportée à une gamme de calibration permettant de quantifier l'antigène.

Les autres composants sont également classiquement dosés par Elisa avec des étapes de préparation de l'échantillon qui leur sont propres. Une revue de la littérature a montré que l'influence de ces composants sur le développement de maladies allergiques restait à préciser, malgré les études

existantes. Cette difficulté à conclure est là encore en lien avec une évaluation de l'exposition à des temps et selon des modalités qui ne sont guère comparables d'une étude à l'autre (Heinrich 2011).

Séquençage et analyse métagénomique

Pitkäranta et al. ont utilisé le séquençage large échelle des sous-unités ribosomales de l'ADN pour caractériser la flore fongique de la poussière intérieure après clonage. Ils ont ainsi pu mettre en évidence des espèces n'ayant jamais été retrouvées par les autres techniques, mais cette technique peut s'avérer de manipulation et d'interprétation délicates, lié, entre autres, à des lacunes des bases de données (Pitkaranta et al. 2007). Les auteurs concluaient alors que séquençage, culture et QPCR étaient complémentaires et permettaient de mieux appréhender la flore globale

La métagénomique est une approche qui permet l'analyse de la composition des communautés microbiennes grâce au séquençage haut-débit de régions conservées du génome microbien (généralement, pour les micromycètes, les gènes codant pour les régions « ITS » (Internal transcribed spacer) (Schoch et al. 2012).

L'intérêt majeur est d'amplifier les gènes de l'ensemble des espèces fongiques présentes, y compris les espèces non cultivables. L'ADN provenant d'éléments fongiques non viables est cependant également détecté. Cette technique permet une approche unique de la diversité présente dans le logement. Toutefois, toutes les espèces ne sont pas détectées avec la même facilité (Yamamoto et al. 2014) et l'analyse du nombre considérable de données reste une difficulté (Dannemiller, Reeves, et al. 2014). Son utilisation dans des cohortes d'enfants a permis d'identifier de nouveaux micro-organismes associés à un risque accru de développement de l'asthme, comme le genre *Cryptococcus* (Dannemiller, Mendell, et al. 2014).

2. Etudier un grand nombre d'échantillons pour comprendre les impacts (cohortes)

L'asthme est une maladie plurifactorielle, les techniques d'échantillonnage environnemental et celles d'analyses sont multiples également, et parfois d'interprétation délicate. Il existe un vrai besoin d'un indicateur reproductible, capable de caractériser les logements des patients asthmatiques et non asthmatiques afin de mieux comprendre les facteurs influençant cette maladie. Cet indicateur doit permettre d'assurer un suivi tout au long des 20 années de l'étude, pour cela, il doit s'avérer dynamique. Cet indicateur pourrait avoir des valeurs seuils capables d'évaluer le risque de développement de la maladie allergique.

Des études de cohortes ont été menées dans plusieurs pays. Cependant, peu d'études de cohorte s'intéressent au cumul de l'exposition en reproduisant les analyses à des temps différents (Reponen et al. 2011, Pongracic et al. 2010). L'équipe de Reponen a montré que l'exposition précoce (1 an) aux moisissures et non celle à 7 ans augmentait le risque d'asthme à 7 ans mais aucune mesure n'avait été faite entre 1 et 7 ans, ni aucun suivi de enfants après 7 ans.

L'étude Longitudinale Française depuis l'Enfance (ELFE) est la première cohorte française pluridisciplinaire consacrée au suivi du développement des enfants en France métropolitaine, de la naissance à l'âge adulte. L'étude ELFE aborde les multiples aspects de la vie de l'enfant sous l'angle des sciences sociales, de la santé et de l'environnement, pour mieux comprendre leurs interactions. Des sujets aussi variés que les problèmes de santé, le recours aux soins, les vaccinations, les conditions sociales et économiques, les évolutions de la structure familiale, les contextes éducatifs, l'alimentation, les expositions à des polluants chimiques et l'environnement physique sont explorés au fil des différentes étapes de recueil au cours du suivi de l'enfant et de sa famille.

Le consortium ELFE est un GIS (Groupement d'Intérêt Scientifique) regroupant, entre autre, l'Institut national d'études démographiques (Ined), l'Institut national de la santé et de la recherche médicale (Inserm), l'Etablissement français du sang (EFS), l'Institut de veille sanitaire (InVS), l'Institut national statistiques et études économiques (Insee).

La cohorte ELFE inclue 18 329 enfants français nés en 2011 : 1 bébé sur 50 nés en 2011 est donc un bébé Elfe. Le recrutement des enfants s'est fait à leur naissance en 2011, dans 344 maternités françaises, selon quatre périodes de l'année, sélectionnées pour représenter chaque saison. Cette cohorte sera suivie pendant 18 ans (Vandentorren et al. 2009).

II. OBJECTIFS DE LA THESE :

1. Mettre au point un système de caractérisation de l'environnement par QPCR, adapté et pertinent dans le cadre d'une étude de cohorte et d'un suivi longitudinal

L'objectif principal de la thèse est de valider un système de mesure applicable à une cohorte de grande ampleur, d'étudier les limites du système, d'intégrer ces données à la réalisation des analyses de la cohorte, de coordonner la réalisation des mesures et leur analyse statistique avec les données de santé issues des questionnaires.

Le travail a donc fait l'objet de développement de nouvelles cibles de qPCR différentes de celles proposées par l'EPA vis-à-vis d'espèces jugées pertinentes (comme par exemple le chat ou la blatte).

L'une des exigences du projet était que le système développé soit pertinent et adapté pour une participation aux campagnes ultérieures au cours du suivi longitudinal et ainsi obtenir des données d'exposition cumulatives dans le temps.

2. Adapter l'outil pour caractériser d'autres situations à risque

Le deuxième objectif est d'utiliser ces connaissances concernant les avantages et les limites de la qPCR en situation environnementale pour caractériser d'autres échantillons environnementaux ou diagnostic.

3. Elargir les connaissances sur les micro-organismes autres que microbiens, notamment les acariens, dans le cadre de l'étude de l'environnement intérieur

Le troisième objectif est, en envisageant la flore intérieure comme un ensemble interactif de micro-organismes, d'améliorer les connaissances sur le rôle des acariens dans ce milieu.

III. CHAPITRE 1 : ETUDE LONGITUDINALE FRANÇAISE DEPUIS L'ENFANCE : MISE EN PLACE ET PREMIERS RESULTATS DE LA COHORTE

1. Echantillonneur et logistique : Etude pilote

Titre de l'article : *qPCR standard operating procedure for measuring microorganisms in dust from dwellings in large cohort studies*

Auteurs : Emeline Scherer, Steffi Rocchi, Gabriel Reboux, Stéphanie Vandentorren, Sandrine Roussel, Mallory Vacheyrou, Chantal Raheison, Laurence Millon

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A. Contexte

Une étude de cohorte d'une telle ampleur met plusieurs années à se mettre en place et mobilise de nombreuses instances nationales (Ined, Inserm, Insee, Caf, ministère du développement durable...) et de nombreuses équipes de recherche (selon trois axes principaux : sciences sociales, santé, santé-environnement). C'est un projet qui s'inscrit dans le long terme. Le groupe d'organisation d'ELFE a dans un premier temps fait un appel à projets pour évaluer les équipes susceptibles de participer à aux différents groupes de travail. Parmi les disciplines abordées, le groupe de recherche « Maladies respiratoires » s'intéresse particulièrement à l'asthme et prévoit une sous cohorte nichée (EBRA-ELFE) pour étudier spécifiquement les relations entre environnement biologique et risque allergique. Cette cohorte est prévue sur 18 ans. Le caractère principal des mesures à conduire devait être la continuité : la reproductibilité technique, mais également une excellente acceptabilité des participants, permettant ainsi leur adhésion à l'ensemble du projet et la possibilité de campagnes ultérieures.

La mesure de moisissures par qPCR à partir d'un échantillonneur silencieux, discret, ne nécessitant pas de déplacement au domicile a d'emblée été vue comme un atout pour le projet, en particulier au sein du groupe respiratoire. Plusieurs réunions ont été conduites avec les responsables du groupe pour ajuster les besoins puis une étude pilote a été lancée. Une partie du projet pilote a été organisée par le consortium ELFE, elle concernait 50 logements. Nous avons recruté 25 logements supplémentaires pour augmenter la quantité de résultats et de recul sur le dispositif.

Dans le cadre de ce pilote, 75 capteurs, constitués de lingettes électrostatiques collées dans une pochette à rabats en plastique au format A4 ont été distribués. La pochette devait être ouverte sur un meuble pendant une durée de 10 semaines. A la fin de la période de prélèvement, la lingette est retournée par la Poste. Elle est ensuite rincée et le liquide de rinçage est utilisé pour l'analyse par culture et par QPCR spécifiques ciblant 10 micro-organismes : 6 champignons filamenteux (*Aspergillus fumigatus*, *Aspergillus versicolor*, *Penicillium chrysogenum*, *Cladosporium sphaerospermum*, *Stachybotrys chartarum*, *Alternaria alternata*, choisis pour leur présence connue

dans les logements et leurs effets allergisants, toxiques ou infectieux), 3 bactéries (*Streptomyces* sp., *Mycobacteria* sp, entérobactéries, ciblant *Serratia*, *Enterobacter*, *Klebsiella*) et un acarien domestique (*Dermatophagoïdes pteronyssinus*).

Cette démarche simple, qui n'avait jamais été réalisée auparavant pour une analyse par qPCR a soulevé plusieurs questions d'ordre technique et nécessité des tests pour connaître au mieux les limites du dispositif. Certains de ces tests ont été présentés dans l'article suivant. D'autres ont fait parti de la mise en place de la méthode standardisée et de l'amélioration de notre expérience et n'ont pas été publiés. Toutefois, les étapes principales des différents choix techniques ont été reportées dans l'article, à savoir :

- Choix de la matière première de lingette la plus à même de restituer les spores au moment du rinçage, en comparant 6 marques commercialisées.
- Choix des cibles (forcément limité par le coût et par le temps d'analyse) les plus pertinentes selon les connaissances actuelles.
- Evaluation des performances analytiques : limites de détection, recherche d'inhibiteurs en utilisant l'ajout d'une quantité connue de levure, *geotrichum candidum*, recherche de détections croisées entre les espèces.
- Evaluation de la capacité à caractériser un logement, c'est-à-dire à mettre en évidence des différences.

B. Principaux résultats

Acceptabilité : Condition *sine qua non* pour poursuivre le projet, nous avons pu montrer que le dispositif était bien accepté, correctement utilisé et retourné au laboratoire dans de bonnes conditions dans 57 % des cas. Les participants ont fait la remarque que le dispositif était grand (2*A4 une fois ouvert) et difficile à placer sur un meuble. Cette remarque a été intégrée dans la fabrication du nouveau dispositif pour la cohorte : un boîtier DVD transparent contenant une seule lingette.



Photo 1 : Capteur utilisé pour le pilote, trop encombrant



Photo 2 : Capteur moins encombrant utilisé pour la cohorte

Maitrise des limites techniques de la méthode : Les séries sur des gammes d'ADN ont permis d'identifier des seuils au-delà desquels la méthode n'est plus reproductible et le résultat non quantifiable. Le seuil est différent selon la cible et il est donc indispensable de réaliser ces tests pour chaque cible.

La recherche systématique d'inhibiteurs sur l'ensemble des échantillons du pilote n'a pas mis en évidence d'échantillon « inhibé », phénomène probablement dû à la dilution de l'échantillon lors des phases de rinçage. Nous avons, pour le pilote, ajouté une quantité connue de *geotrichum candidum* dans chaque puits réactionnel et comparé le résultat en qPCR avec le résultat attendu. La recherche d'inhibiteurs est indispensable en qPCR car de nombreux agents peuvent empêcher l'activité de la Taq Polymérase, mais l'ajout d'ADN supplémentaire représente un coût et un risque de contamination non négligeable. Ce résultat a donc été primordial pour l'optimisation de notre système en nous permettant de nous affranchir de la recherche systématique d'inhibiteurs pour les analyses des échantillons de la cohorte.

Les lingettes électrostatiques n'ont pas toutes les mêmes structures, elles n'ont pas toutes la même capacité à capter les spores ni à les restituer lors d'une étape de lavage. Leur capacité « électrostatique » n'a pas pu être testée, mais leur comportement lors du rinçage a montré des différences : les modèles avec les mailles les moins tissées relarguent plus de spores dans le liquide de rinçage. La perte de matériel lors de l'étape de rinçage est importante (rinçage avec un volume de 20 mL) mais l'ensemble des échantillons étant traité de la même manière, en choisissant un modèle de lingette ayant un comportement reproductible, les résultats seront comparables. Le pourcentage de spores retrouvé dans le liquide analysé s'échelonne de 1 à 5,9 % selon la marque. Ces tests ont permis de choisir le fournisseur pour la réalisation des 7000 capteurs de la première campagne d'EBRA-ELFE.

Résultats sur les échantillons issus de logements :

Sur les 53 logements analysés, un seul était négatif pour l'ensemble des espèces. En présentant les résultats sous forme de quartile par espèce (tableau 1) et par analyse statistique par Analyse en Composante Principale, nous avons pu montrer que les espèces n'étaient pas corrélées entre elles et que les logements avaient des profils propres, différents les uns des autres. Le système a donc été validé comme discriminant de la contamination intérieure des logements (tableaux 4 et 5 de l'article).

Dans les résultats qui n'ont pas été décrits dans l'article, il y a par exemple :

- la comparaison entre l'EDC et des prélèvements d'air répétés pendant la période sur 6 logements,
- Le test de plusieurs méthodes d'essorage après rinçage pour garantir un essorage reproductible : des lavages successifs de la lingette ont été réalisés, suivis d'une analyse par culture pour estimer la perte liée aux spores prisonnières de la lingette lors du premier essorage,
- le choix d'un mix réactionnel garantissant un équilibre satisfaisant entre spécificité et sensibilité mais également d'un automate de PCR haut débit répondant à la problématique d'échantillons environnementaux complexes et nombreux.

Cette étape de pilote n'a évidemment pas permis de mettre en évidence de différences significatives en fonction des données de santé, mais elle garanti une bonne maitrise de l'outil, sa reproductibilité, son optimisation et a remporté l'adhésion du groupe respiratoire ELFE pour participer à la cohorte et rendu possible son utilisation dans la sous-cohorte EBRA-ELFE.



qPCR standard operating procedure for measuring microorganisms in dust from dwellings in large cohort studies



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HIGHLIGHTS

- Pilot study of the qPCR analysis of dust collected by an Electrostatic Collector
- Standard Operational Procedure fulfills feasibility, cost and efficacy criteria.
- Chosen targets were 6 fungi, *Enterobacteria*, *Mycobacteria*, *Streptomyces* and mites.
- With this SOP, dwellings can be classified according to microbial DNA quantity.

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ABSTRACT

The aim of the present study was to assess performance, feasibility and relevance of a Standard Operational Procedure (SOP) for large-scale use in the microbial analysis of children's indoor environments.

We analyzed dust settled on Electrostatic Dust Fall Collectors (EDCs) by using qPCR which targeted 6 molds, 3 bacteria and 1 mite, chosen for their involvement in allergic or inflammatory processes. Six types of commercialized electrostatic wipes were tested for their releasing capacity of fungal DNA from fungal spores captured by the wipes. Specificity, repeatability and detection limits of the qPCR procedure were tested using calibrated microbial suspensions. The feasibility and relevance of this sampling and analysis method were assessed in a 75-home pilot study.

Our result showed that one specific make of wipe was more effective than the others in releasing fungal DNA. qPCR procedure showed good repeatability. The quantification limit was about 5 fg DNA/μL for all species except *Penicillium chrysogenum* (0.5 fg DNA/μL) and *Dermatophagoïdes pteronyssinus* (10 fg DNA/μL). No cross-reactivity was observed.

DNA concentrations in the 53/75 homes participating in the pilot study were between 0 and 24 625, 0 and 69 738 equivalent cells per cm² for the fungi and bacteria, and between 0 and 1 equivalent mites per cm² for *D. pteronyssinus*. Using the SOP described, we were able to classify the 53 dwellings from the least to the most contaminated according to the quantity of DNA measured for each species.

Our SOP measured fungi, bacteria and mites using a cost-efficient, discreet and well-accepted sampling method with just one qPCR tool. The whole procedure can be used for microbial analysis in large cohort studies such as the ELFE study ("Etude Longitudinale Française depuis l'Enfance") and could help improve our understanding of the interactions between the environment, allergic diseases and child development.

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1. Introduction

Asthma and related atopic diseases are now among the most common childhood diseases in industrialized countries (Asher et al., 2006; Eder et al., 2006). The combined effect of environmental exposure to indoor chemical pollutants and microbial contaminants, and genetic

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predisposition is unknown (Braun-Fährlander et al., 1999; von Mutius, 2007b). A significant difference in mold exposure between non-asthmatic and severely asthmatic populations has also been demonstrated (Reboux et al., 2009; Reponen et al., 2011; Vesper et al., 2008). Some studies have shown that exposure to higher levels of endotoxin protects against allergy (Ownby et al., 2010). Despite extensive research, doubt remains that exposure to indoor allergens causes asthma and allergy, because various studies have presented conflicting evidence, as reported by Arshad after a review of the literature. He concludes that the greatest effect of exposure to indoor allergens (molds, mites, pets, etc.) probably occurs during the postnatal period (Arshad, 2010).

The purpose of the ELFE “Etude Longitudinale Française depuis l’Enfance” project is to establish a nationally representative cohort of 20 000 children to be followed from birth to adulthood using a multidisciplinary approach to characterize the effects of environmental exposure, disease and the socio-economic context on health (Vandentorren et al., 2009). Many factors are known to influence child development and children’s health and different working groups have been convened, including a specific group focusing on asthma and allergic respiratory diseases.

To study environmental exposure we suggested using Electrostatic Dust Fall Collector (EDC) (Normand et al., 2009; Noss et al., 2008; Frankel et al., 2012) and analyzing settled dust from the EDCs using qPCR, targeting 6 molds (*Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Stachybotrys chartarum*), 3 bacteria (*Enterobacteriaceae*, *Mycobacteria*, *Streptomyces*) and 1 mite (*Dermatophagoides pteronissynus*). Literature concerning microbial species involved in allergic diseases is abundant and controversial. The 10 target microorganisms were chosen because they belong to classes or genera that are phylogenetically very distinct, and because they have been implicated in human diseases due to their allergic, toxic or infectious effects.

EDC is a newly developed assessment method that provides an alternative to surface or air sampling in epidemiological studies (Frankel et al., 2012; Tischer and Heinrich, 2013). Previous studies have shown that qPCR quantification of targeted species is an easy and reliable tool for characterizing the homes of allergic patients, and have demonstrated its advantages for studies in homes (Kaarakainen et al., 2009; Reponen et al., 2011; Rintala et al., 2004; Vesper, 2011; Vesper et al., 2007).

In the present study, we assessed the optimization, feasibility and relevance of this sampling and analysis method in a 75-home pilot study with the aim of determining a Standard Operational Procedure (SOP) that can be used in large-scale microbial and allergenic analyses of children’s indoor environments; we had the ELFE study specifically in mind.

2. Materials and methods

2.1. Dust collection

2.1.1. Sampling device (EDC)

The EDC consists of commercially available electrostatic wipes described by Noss et al. (2008) set in a plastic case, which the participant opens. The collection involves capturing settled airborne dust via the electrostatic properties of the wipe. The EDC may be considered as a surrogate for the assessment of indoor airborne microbial exposure. The EDC is also easy to use, as it can be sent by mail (Frankel et al., 2012). Wipes were first sterilized in an autoclave (124 °C for 30 min) and stuck on a disinfected (Surfanios™, Anios®, Lille-Hellemmes, France) polypropylene case (resembling a DVD case) with white glue (UHU®, Bühl, Germany).

2.1.2. Wipe material selection

Six different types of commercialized electrostatic wipe cloths, each with different textures, were tested for their capacity to release fungal

DNA from the fungal spores captured on them (Table 1). Three wipes of each type were impregnated with two 1 mL suspensions, one of *P. chrysogenum* and one of *S. chartarum* in known concentrations (7×10^5 and 4×10^5 spores/mL respectively) and dried overnight. Each wipe was put in a plastic bag with a washing solution of 20 mL of 0.1% Tween 80 solution (Merck®, Darmstadt, Germany) and shaken for ten minutes in a Stomacher™ (AES®, Combourg, France). Ten (± 0.5) mL of washing solution was then collected.

The qPCR protocol as described below was then applied to the DNA extract from the 2 initial fungal spore suspensions and from the washing solution from the wipe. The capacity of the wipe to release microbial DNA was deduced by comparing the two qPCR results taking into consideration the dilution by the washing liquid volume (20 mL). Tests were also performed using volumes of washing liquid of 40 mL.

2.2. qPCR protocol

2.2.1. DNA extraction

Rapid DNA extraction, chosen for its simplicity, cost and efficiency (Haugland et al., 2002), was performed as previously described by Keswani et al. with the following changes (Keswani et al., 2005).

Extraction was carried out using an initial sample volume of 200 μ L of fungal spore suspension or 200 μ L of washing solution from the wipe. The samples were placed in 2 mL conical-bottom screw-cap tubes (MagNA Lyser Green Beads, Roche Applied Science®, Mannheim, Germany) containing 1.4 mm diameter ceramic beads and 200 μ L of brain-heart infusion (BBL™, Becton Dickinson®, Sparks, NJ, USA). The tubes were shaken in a MagNA Lyser Instrument (Roche Applied Science®, Mannheim, Germany) three times for ten seconds at the maximum speed with one minute in the cooling block (4 °C). The tubes were then heated in a boiling water bath for ten minutes. The samples were placed on ice for ten minutes, followed by centrifugation for two minutes at 8000 rpm at room temperature. The supernatant above the beads was removed and stored at 4 °C for subsequent qPCR analysis.

A sterile distilled water sample was used as a negative control in each extraction series.

Table 1

Different makes of wipes and their capacity to release microbial DNA. Wipes were impregnated with suspension of *P. chrysogenum* and *S. chartarum*, then washed as described in “Materials and methods”. The qPCR protocol was applied to the DNA extract from the initial suspension and from the washing solution. Each experiment was done in triplicate. Quantitative results were expressed by determining the detection threshold, or quantification cycle (Cq), that marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the Cq, the smaller the amount of DNA in the sample.

	Make of wipes	<i>Penicillium chrysogenum</i> (Pc)	<i>Stachybotrys chartarum</i> (Sc)	Recovery efficiency (%)
Fungal suspension (sp/mL)		7×10^5	4×10^5	(Pc) (Sc)
Cq (cycles)		32.3	23.8	
Cq from the EDC washing liquid (mean Cq/SD)	Wipes 1 (Apta top budget)	36.8/1.1	28.5/0.8	5.9% 5%
	Wipes 2 (Dia)	4.1×10^4 sp/mL	2×10^4 sp/mL	5.6% 2.5%
	Wipes 3 (Casino)	36.9/0.9	29.1/0.6	
	Wipes 4 (Casino-First)	3.9×10^4 sp/mL	1×10^4 sp/mL	5.6% 1.3%
	Wipes 5 (Wiffer)	36.9/1.7	30.5/1.4	
	Wipes 6 (Super U)	3.9×10^4 sp/mL	5×10^3 sp/mL	4.9% 1%
		37.1/1.1	30.8/1.4	
		3.4×10^4 sp/mL	4×10^3 sp/mL	3.1% 2.5%
		37.8/1.2	29.1/0.9	
		2.2×10^4 sp/mL	1×10^4 sp/mL	1.9% 1.8%
		38.6/0.7	30.1/0.9	
		1.3×10^4 sp/mL	7×10^3 sp/mL	

sp/mL: spores per milliliters; Cq: quantification cycle; SD: standard deviation.

2.2.2. Selected target species

A limited number of targets were chosen for their involvement in allergic, infectious or inflammatory processes and their presence in indoor air.

- ✓ Six fungi targeted species: *A. alternata*, *A. fumigatus*, *A. versicolor*, *C. sphaerospermum*, *P. chrysogenum*, *S. chartarum*.
- ✓ Three families of bacteria or genus: *Enterobacteriaceae*, *Mycobacterium* spp., *Streptomyces* spp.
- ✓ One house dust mite: *Dermatophagoïdes pteronyssinus*.

2.2.3. DNA amplification

DNA from *A. alternata*, *A. fumigatus*, *A. versicolor*, *C. sphaerospermum*, *P. chrysogenum* and *S. chartarum* was detected by qPCR using primers and TaqMan™ probes previously described by Haugland et al. (2004) (<http://www.epa.gov/nerlcwww/moldtech.htm>). DNA from *Streptomyces* spp. and *Mycobacterium* spp. was detected by qPCR using the primers described by Rintala and Nevalainen (2006) and Torvinen et al. (2010). *D. pteronyssinus* was detected using primers and probes designed and developed in our laboratory (Roussel et al., 2013). Enterobacteria DNA was detected using a unique system of primers and probes targeting three different bacterial species: *Serratia* sp., *Enterobacter* sp. and *Klebsiella* sp. (Sen and Asher, 2001). See Table 2 for the sequences of primers and probes that were used.

The PCR mix was prepared identically for each of the ten targets in a 20-µL final volume using the Brilliant III Ultra-fast qPCR Master Mix (Agilent technologies®, Massy, France), with ROX as a passive reference, including 5 µL DNA, 1000 nM primers, 200 nM probe and DNA-free water (except primer AversF2 which was used at a concentration of 5000 nM). Reactions were prepared in a 96 well optical reaction plate (Life Technologies, Carlsbad, CA, USA). PCR setup was automated (Qiagility UV/HEPA™, Qiagen®, Hilden, Germany). The amplification protocol was as follows: three min at 95 °C, followed by 45 cycles for 15 s at 95 °C for template denaturation and one min at 60 °C for primer and probe annealing and extension. PCR's reactions and absolute

quantification were run and monitored using Applied Biosystems 7500 Fast System (Life Technologies, Carlsbad, CA, USA). Sterile distilled water samples were used as negative controls in each series of amplification.

2.2.4. Calibration scale

Absolute quantification was applied, using calibrated suspensions of environmental reference strains prepared as follows: *A. alternata* (BCCM/IHEM 2669), *A. fumigatus* (BCCM/IHEM 22670), *A. versicolor* (BCCM/IHEM 22671), *C. sphaerospermum* (BCCM/IHEM 18883), *P. chrysogenum* (BCCM/IHEM 22667), *S. chartarum* (BCCM/IHEM 22672), *Klebsiella oxytoca* (laboratory strain), *Mycobacterium terrae* (DSMZ 43277), and *Streptomyces coelicolor* (DSMZ 40233) were prepared from a culture plate and *D. pteronyssinus* (identification confirmed by sequencing) from laboratory farming. DNA was extracted as previously described followed by a purification step, using the NucleoSpin Plant II kit™ (MachereyNagel®, Düren, Germany). DNA was then quantified using a Nanodrop® (Thermo Fisher Scientific®, Waltham, MA, USA). The first calibration point was adjusted to 100 pg/µL DNA for *D. pteronyssinus* and to 5000 fg/µL for other species, and then 10-fold diluted.

2.2.5. Detection limits and repeatability

For each of the targeted species, dilutions of the calibration DNA (from 100 to 0.01 pg/µL for *D. pteronyssinus* and from 5000 to 0.5 fg/µL for the other species) were amplified in qPCR for seven times. Limit quantification is defined as the smallest amount of DNA at which seven successful detections can be observed out of seven trials (Hospodsky et al., 2010).

2.3. Application of the process in a pilot study

2.3.1. Sampling

To test the SOP before applying this process to the ELFE study (5000 dwellings), a pilot study was conducted in 75 households.

Table 2
Sequences and sources of the primers and probes used.

Name of the targeted species	Sequences (primers and probe)	Source
<i>Alternaria alternata</i>	Forward primer AaltrF1: 5'-GGCGGGCTGGAACCTC-3' Reverse primer AltrR1-1: 5'-GCAATTACAAAAGGTTTATGTTTGTCTGA-3' Probe AaltrP1: 5'-FAM-TTACAGCCTTGCTGAATTATCCACCTTGTCTTT-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Aspergillus fumigatus</i>	Forward primer AfumiF1: 5'-GCCCGCCGTTTCGAC-3' Reverse primer AfumiR1: 5'-CCGTTGTTGAAAGTTTAACTGATTAC-3' Probe AfumiP1: 5'-FAM-CCC GCCGAAGCCCCAACATG-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Aspergillus versicolor</i>	Forward primer AversF2 (5×): 5'-CGCGGGGAGCCCT-3' Reverse primer AversR1-1: 5'-CCATTGTTGAAAGTTTGAAGTCTGATcTTA-3' Probe versP1: 5'-FAM-AGACTGCATCACTCTCAGGCATGAAGTTAG-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Cladosporium sphaerospermum</i>	Forward primer CsphaF1: 5'-ACCGGCTGGTCTTTCCG-3' Reverse primer CsphaR1: 5'-GGGGTTGTTTACGGCGTG-3' Probe CsphaP1: 5'-FAM-CCCGCGGCACCCCTTACGCA-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Dermatophagoïdes pteronyssinus</i>	Primer Dpter F1: 5'-TGT TGT GGT TAA AAA GCT CGT AGT TG-3' Primer DpterR1: 5'-ATG CGA TAA TCT GCT CAG TAT GAC A-3' Probe DpterP1: 5'-FAM-CAG CTC ATG TAT GGC GGT CCA CCT G-TAMRA-3'	Roussel et al. (2013)
<i>Enterobacteriaceae</i> (<i>Serratia</i> sp., <i>Enterobacter</i> sp. and <i>Klebsiella</i> sp.)	Forward primer UF2: 5'-GGCGGCAGGCTAAC-3' Reverse primer UR2: 5'-CAGGCAGTTTCCAGACATTACT-3' Probe: 5'-FAM-AGCAAGCTCTCTGCTACCCGCTCGA-TAMRA-3'	Sen and Asher (2001)
<i>Mycobacteria</i>	Primer: Mf939: 5'-GAT GCA ACC CGA AGA ACC TT-3' Primer Mr1011: 5'-TGC ACC ACC TGCACA CAG G-3' Probe Mp960: 5'-FAM-CCT GGG TTTGAC ATG CAC AGG ACG-TAMRA-3'	Torvinen et al. (2006)
<i>Penicillium chrysogenum</i>	Forward primer PchryF4-1: 5'-GTTTGCCACTCAGAGAATACTGAAA-3' Reverse primer PchryR8: 5'-CCCCGGGATCGGAG-3' Probe PenP6: 5'-FAM-CCAACACACAAGCCGTGCTGAGG-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Stachybotrys chartarum</i>	Forward primer StacF4: 5'-TCCCAAACCTTATGTGAACC-3' Reverse primer StacR5: 5'-GTTTGCCACTCAGAGAATACTGAAA-3' Probe StacP2: 5'-FAM-CTGCGCCCGGATCCAGGC-TAMRA-3'	http://www.epa.gov/nerlcwww/moldtech.htm
<i>Streptomyces</i>	Primer SMfw8: 5'-GCC GAT TGT GGT GAA GTG GA-3' Primer Mrev9: 5'-GTA CGG GCC GCC ATG AAA-3' Probe: SMp6 5'-FAM-ATC CTA TGC TGT CGA GAA AAG CCT CTA GCG-TAMRA-3'	Rintala and Nevalainen (2006)

Seventy-five households were recruited in the Picardy and Burgundy regions and in the Territoire de Belfort area (France) by the INED (French National Institute of Demographic Studies) and by the DDASS (Local Division of Health and Social Affairs). Participants were volunteer mothers who had given birth less than six months previously. The households were visited by an investigator who set up an EDC.

No particular criterion for selecting the type of dwellings was used.

The EDC was exposed to air on a horizontal surface between 1.60 m and 1.80 m above the floor in the child's room, a height that was inaccessible for children and pets. Dust settling was allowed to occur over a ten week period. A previous assay showed that this duration was enough to detect an increased biodiversity and to generate a slightly greater qPCR signal compared to the results obtained with a collection phase lasting one month. The EDC was folded up by the participants and sent to the laboratory by mail in a pre-addressed envelope. The EDC was stored at -20°C before being analyzed. Microbial DNA was extracted from the EDC and the ten selected species were quantified as described above.

2.3.2. Search of inhibitors

Ten suspensions of 200 μL of DNA-free water with 2×10^2 conidia of *Geotrichum candidum* (BCCM/IHEM 5560) were prepared. DNA extraction was performed on the ten suspensions with the same rapid technique as for environmental samples (see above), and *G. candidum* DNA was quantified in the ten extracts (three qPCR replicates for each extract) using specific primers and probes (<http://www.epa.gov/nerlcwww/moldtech.htm>). A Reference Quantification Cycle ($C_{q\text{ref}}$) value was defined as the mean of the 30 measured values.

To assess the presence of inhibitors in the pilot ELFE study samples, 2×10^2 conidia of *G. candidum* were added as internal controls to the washing liquid of each wipe (Roe et al., 2001), and *G. candidum* DNA was quantified in separate tubes (Haugland et al., 1999). The C_q of *G. candidum* in samples was then compared to the $C_{q\text{ref}}$.

2.3.3. Expression of results

Each C_q below the quantification limit was expressed as a DNA concentration using absolute quantification with the Thermocycler software (7500 Software, 2.0.5, Life Technologies, Carlsbad, CA, USA). Samples with higher C_q were considered as negative. DNA concentration in the sample given by absolute quantification is converted to equivalent cells using the following equation: "DNA mass in one cell [pg] = (genome size [bp]) / $(0.978 \times 10^9 \text{ [bp/pg]})$ ". Equivalent cells are then related to the surface of the wipes (360 cm^2). The mean genome size of filamentous fungi has been estimated at 30 Mbp, and at 5 Mbp for bacteria and *Streptomyces* (GenBank: <http://www.ncbi.nlm.nih.gov/genbank/>) (Gibbons and Rokas, 2013; Yamamoto et al., 2011). As far as the expression of the mite results is concerned, the quantity of DNA in one specimen of *D. pteronyssinus* has been tested during qPCR development and is estimated at 400 pg of DNA per mite (data not shown).

The comparison between dwellings was expressed as follows: for each species, concentration levels were defined by the percentage of the maximum concentration found for each species in this series.

2.3.4. Statistical analysis

Independence between species was tested using two by two correlations with Spearman's rank correlation rho. A first analysis by PCA (Principal Component Analysis), using the DNA logarithmic concentration as a result, allowed us to determine species found simultaneously in dwellings with varying concentrations. Statistical analyses were performed using R 2.12.1 software (R Development Core Team, 2012).

3. Results

3.1. Selection of cloth texture and supplier

Tests performed with suspensions of *P. chrysogenum* and *S. chartarum* in known concentrations showed that the highest remaining quantities

of fungal DNA was obtained for the two species with the same make of wipe (Apta Top Budget™ Eurowipes®, Nogent le Rotrou, France) (see composition at (www.eurowipes.com/uk/lawful-information.php)). The difference between the C_q of the initial suspension and the C_q of the wipe washing fluid was 4.5 and 4.7 for *P. chrysogenum* and *S. chartarum* respectively (Table 1). The C_q was higher (+0.6) when wipes were washed using 40 mL of washing liquid, than with 20 mL. Washing with 20 mL was thus considered effective.

3.2. Detection limits and repeatability

Repeatability is expressed by the C_q standard deviation (Bustin et al., 2009). For each species targeted, mean C_q , C_q Standard Deviation (SD C_q), and the number of successful trials are reported in Table 3. The lowest concentration, with seven successful trials and SD $C_q < 1$ was considered as the limit of quantification. The correlation coefficient (r^2) of each calibration scale is also reported.

The quantification limit was about 5 fg DNA/ μL for all species except *P. chrysogenum* (0.5 fg DNA/ μL) and *D. pteronyssinus* (10 fg DNA/ μL).

3.3. Pilot study

3.3.1. Acceptability to households

Fifty-three EDCs were returned to our laboratory: 43 (57%) were spontaneously returned and ten others were returned after an additional phone call or mail request. Seventy-one percent of the installed devices were studied. Of the participants in the remaining households (29% of the total) who did not return the device, some had either moved or had had extensive renovation work carried out in their home.

3.3.2. Search for inhibitors

The mean $C_{q\text{ref}}$ of the ten assays, replicated three times, for *G. candidum* in water alone was 28.7 (SD = 0.12). When performed on environmental samples, the C_q for the *G. candidum* qPCR assay was < 29.5 cycles. We thus assume that the presence of inhibitors was very low in our series.

3.3.3. Detection of microbial DNA from EDC using qPCR

No positive results were observed for the negative control of amplification and extraction.

Cross-reactivity was checked by testing every primer/probe system with suspensions of the ten species targeted and *G. candidum*. No cross-reactivity was observed.

Quantities per cm^2 of equivalent cells or mites are presented in Table 4. DNA concentrations in the 53 dwellings were between 0 and 24 625 equivalent cells per cm^2 for fungi, between 0 and 69 738 equivalent cells per cm^2 for bacteria, and between 0 and 1 equivalent mites per cm^2 for *D. pteronyssinus*.

Percentages of the maximum concentration for each species and all dwellings are presented in Table 5. Five dwellings had more than two species higher than 75% of the maximum concentration found for these species in this series.

Only one dwelling (L16) had a negative signal for all ten targets. The species with the highest concentrations were *Enterobacteriaceae* with 94% of positive samples (a mean of 12 119 cells/ cm^2). *D. pteronyssinus* and *A. alternata* were detected in 55% (a mean of 0.04 cells/ cm^2) and 53% (a mean of 33.9 cells/ cm^2) of samples, respectively. *Mycobacteria* and *A. versicolor* were detected in 47% of cases (with a mean of 1020 and 884 cells/ cm^2 , respectively). Frequency of *P. chrysogenum*, *Streptomyces* spp. and *C. sphaerospermum* was 38%, 30% and 25% (with mean concentrations of 0.3, 74 and 9.4 cells/ cm^2 , respectively). Species with the lowest concentrations were *A. fumigatus* and *S. chartarum* with 7% and 9% of positive samples (mean concentrations of 3 and 2 cells/ cm^2).

Table 3
Detection limits and repeatability of the ten targets used in qPCR; correlation coefficient of the scale (r^2), mean of the Quantification Cycle obtained (Cq mean), Standard Deviation of these Cq (SD Cq), number of successful trials (n = 7) for each species. In bold are the concentrations chosen for quantification limit (7 successes and SD Cq < 1).

Concentration (fg/ μ L)	Cq mean	SD Cq	Number of successful trials	Concentration (fg/ μ L)	Cq mean	SD Cq	Number of successful trials
Concentration (fg/ μ L) <i>Alternaria alternata</i> ($r^2 = 0.97$)				Concentration (fg/ μ L) <i>Aspergillus versicolor</i> ($r^2 = 0.93$)			
5000	26.4	0.4	7	5000	24.7	0.1	7
500	29.8	0.1	7	500	30.5	0.3	7
50	33.2	0.8	7	50	32.0	0.2	7
5	35.8	0.2	7	5	35.1	0.8	7
0.5	38.0	1.0	7	0.5	38.6	2.1	6
Concentration (fg/ μ L) <i>Aspergillus fumigatus</i> ($r^2 = 0.96$)				Concentration (fg/ μ L) <i>Cladosporium sphaerospermum</i> ($r^2 = 0.98$)			
5000	27.0	0.3	7	5000	22.4	0.1	7
500	30.2	0.8	7	500	25.7	0.3	7
50	34.6	0.3	7	50	28.9	0.1	7
5	38.5	0.7	7	5	32.5	0.4	7
0.5	39.4	0.4	4	0.5	36.9	1.7	6
Concentration (pg/ μ L) <i>Dermatophagoïdes pteronyssinus</i> ($r^2 = 0.98$)				Concentration (fg/ μ L) Enterobacteria ($r^2 = 0.93$)			
100	28.4	0.1	7	5000	30.9	0.4	7
10	31.6	0.1	7	500	34.3	0.5	7
1	34.7	0.2	7	50	36.6	0.4	7
0.1	38.4	0.6	7	5	39.1	0.3	7
0.01	40.3	0.6	7	0.5	39.8	1.0	7
Concentration (fg/ μ L) <i>Mycobacteria</i> ($r^2 = 0.97$)				Concentration (fg/ μ L) <i>Penicillium chrysogenum</i> ($r^2 = 0.99$)			
5000	24.2	0.1	7	5000	24.6	0.1	7
500	27.6	0.2	7	500	28.5	0.3	7
50	30.6	0.1	7	50	31.0	0.1	7
5	33.9	0.4	7	5	34.7	0.3	7
0.5	36.9	2.1	6	0.5	37.6	1.00	7
Concentration (fg/ μ L) <i>Stachybotrys chartarum</i> ($r^2 = 0.98$)				Concentration (fg/ μ L) <i>Streptomyces</i> spp. ($r^2 = 0.95$)			
5000	27.9	0.0	7	5000	29.7	0.1	7
500	31.5	0.1	7	500	33.1	0.3	7
50	34.0	0.2	7	50	36.1	0.3	7
5	38.3	1.1	7	5	39.1	0.6	7
0.5	40.5	0.6	2	0.5	38.6		1

3.3.4. Correlation between species

No correlation between species was observed ($Rho < 0.5$ using the Spearman's test) except for *A. versicolor* and *Mycobacteria* ($Rho = 0.54$ using the Spearman's test).

The presence of two species could be associated using PCA analysis (Fig. 1): *A. versicolor* and *Mycobacteria*. *D. pteronyssinus* had completely distinct behavior. No link was found between species whose points on Fig. 1 are dispersed.

4. Discussion

In this paper, we report the analysis method chosen to respond to the requirements of the ELFE team which had asked that we offer an environmental sensor that is acceptable to participants, inexpensive and provides results that are representative of chronic exposure.

This environmental sensor will be placed in the homes of the 5000 children in the ELFE sub-cohort to evaluate indoor exposure.

Because of changes over time in bioaerosol concentrations and the small volume size of the air sample, the assessment of airborne microorganisms in an environment of interest often requires several air samples (Sergey et al., 2007). Dust sampling using a vacuum is generally indiscreet, whereas participants seemed to accept the presence of our device in their home. The percentage of responses to surveys without sampling usually varies from 40% to 67% (data from the French National Institute of Demographic Studies—INED). The number of EDC returns (71%) in our study, which included both a survey and sampling is satisfying, and suggests that the EDC could be used for large cohort studies.

Since this sampling method has been relatively underused up to now, there is no comparable study. To our knowledge, only one study

has reported qPCR quantification of microorganisms using wipe sampling (Yamamoto et al., 2011). However, in this study, cloths were used to actively wipe the floor, and they were not left exposed to the air for extended periods to collect dust.

In our study, fungi, mites, actinomycetes, mycobacteria and enterobacteria from standardized dust from the rooms of newborn babies sampled by EDC were analyzed by qPCR. The targets, which were limited in number by time and cost, were chosen for their involvement in allergic or inflammatory processes.

The genus *Cladosporium*, *Penicillium*, *Aspergillus* and *Alternaria* are the most commonly detected molds in indoor air (Chew et al., 2003; Kaarakainen et al., 2009; Meklin et al., 2004; Vandentorren et al., 2009; Zeng et al., 2006). *S. chartarum*, with its inflammatory potential (Huttunen et al., 2004) and complex role in toxicological pathology, must be quantified and analyzed (Kuhn and Ghannoum, 2003).

Mycobacteria and *Streptomyces* are difficult to cultivate, but several studies have found evidence of their presence in the indoor environment (Dawson, 1971; Kaarakainen et al., 2009; Lignell et al., 2008; Obihara et al., 2007; Rintala et al., 2004) and of their involvement in allergic phenomena. *Mycobacteria* and *Streptomyces* induce inflammatory and cytotoxic effects (Hirvonen et al., 2005) and could be associated with an increased concentration of fungi and thus with adverse health effects (Torvinen et al., 2006). Furthermore, co-cultivation with some fungi increases the production of certain mycotoxins (Hirvonen et al., 2005). Endotoxins are major components of the outer membrane of negative bacteria, and could have a protective effect on asthma in some circumstances (Gehring et al., 2007; von Mutius, 2007a). A previous study showed that most of the gram negative bacteria in indoor environments was *Enterobacteriaceae* (Bouillard et al., 2005). Therefore,

Table 4
Concentration of the ten microorganisms in 53 dwellings expressed in equivalent cells or mites per cm².

Housing	Concentration estimated in equivalent cells (or mite) per cm ²									
	A.alt	A.fumi	A.versi	C.spha	D.ptero	EnteroB	Mycob	P.chry	S.char	Strepto
L1	41	–	128	–	–	7261	2345	0.3	–	–
L2	41	–	–	–	–	3329	–	–	–	–
L3	–	–	–	45	0.12	19,023	–	–	–	–
L4	31	–	–	–	0.01	2620	1125	0.3	–	–
L5	–	–	–	–	0.03	–	1017	–	–	–
L6	50	–	173	–	–	14,599	–	–	–	135
L7	40	–	359	–	0.01	4440	1798	–	–	–
L8	–	–	–	36	–	5267	–	–	–	–
L9	–	–	–	–	–	1451	2325	0.5	27	–
L10	–	–	–	–	0.09	5329	–	–	–	–
L11	64	–	146	–	0.01	1763	1387	1.3	18	–
L12	–	–	389	–	0.01	26,305	5700	–	–	–
L13	–	–	–	–	0.03	2494	–	–	–	192
L14	121	44	32	–	–	13,621	–	1.1	–	–
L15	–	22	40	–	–	1557	1080	–	–	–
L16	–	–	–	–	–	–	–	–	–	–
L17	173	–	–	29	–	916	–	–	19	–
L18	61	–	19	–	–	4221	–	–	–	–
L19	63	33	–	–	–	3822	–	1.6	–	–
L20	193	60	967	–	–	2217	1142	0.4	–	–
L21	20	–	–	–	0.01	1558	1267	2.1	–	–
L22	115	–	45	21	0.01	10,294	–	–	–	293
L23	19	–	151	–	0.05	23,255	1870	0.8	–	–
L24	42	–	–	–	0.06	69,738	973	–	–	167
L25	–	–	113	–	0.03	21,680	–	0.4	–	–
L26	–	–	–	–	0.02	13,196	–	–	–	–
L27	34	–	–	20	0.03	6706	1096	–	–	355
L28	–	–	–	–	–	10,545	–	–	–	–
L29	55	–	–	29	0.03	13,781	948	–	24	259
L30	164	–	34	–	–	30,058	1269	–	–	376
L31	–	–	21	73	0.07	37,502	3635	–	–	140
L32	–	–	–	46	0.03	–	–	0.3	–	–
L33	–	–	24	–	–	6915	–	0.2	–	–
L34	–	–	–	–	0.01	1224	–	0.5	–	–
L35	48	–	–	–	0.02	9338	–	0.3	–	–
L36	–	–	24,625	27	0.07	22,944	5286	1.4	–	222
L37	21	–	39	–	–	988	–	–	–	–
L38	–	–	–	–	–	9519	1041	–	–	–
L39	37	–	–	–	–	1976	1038	–	–	–
L40	21	–	138	–	–	24,556	4050	2.1	–	545
L41	41	–	–	–	–	7020	–	–	–	–
L42	–	–	25	–	0.01	36,461	1006	–	–	260
L43	54	–	–	–	–	951	–	–	–	–
L44	90	–	–	–	–	8627	–	–	–	–
L45	43	–	54	–	0.03	13,757	1816	0.4	–	160
L46	–	–	–	–	0.02	37,403	–	–	–	243
L47	43	–	24	–	–	3063	–	0.8	–	194
L48	–	–	–	–	0.97	534	–	–	–	–
L49	–	–	19,113	93	0.05	29,139	5306	1.2	–	–
L50	–	–	–	–	–	903	–	–	–	–
L51	72	–	147	27	0.02	4108	1397	–	–	–
L52	–	–	29	31	0.02	62,902	4183	0.3	18	224
L53	–	–	21	24	0.32	1465	–	–	–	185

The ten targets were: *Alternaria alternata* (A.alt), *Aspergillus fumigatus* (A.fumi), *Aspergillus versicolor* (A.versi), *Cladosporium sphaerospermum* (C.spha), *Dermatophagoides pteronyssinus* (D.pter), *Enterobacteriaceae* (EnteroB), *Mycobacteria* sp. (Mycob), *Penicillium chrysogenum* (P.chry), *Stachybotrys chartarum* (S.char), *Streptomyces* spp. (Strepto). Negative or lower than the quantification limit results are indicated by "–".

we chose a primer/probe system that targets *Enterobacteriaceae* (Sen and Asher, 2001) to quantify bacteria that release endotoxins into the indoor environment.

House dust mites are a common allergen, and sensitization is frequently observed despite control measures (Vallance et al., 2006). Also, complex interactions between domestic mites and fungi have been observed (transportation and consumption of fungal spores) (Van Asselt, 1999). Extended exposure to fungi and mites enhances asthma symptoms (Harrison, 1999) and interactions could be further specified if these microorganisms were measured simultaneously using the same tool.

However, the target group could be extended if other species are shown to be involved by other studies (rinse suspension is maintained

at –80 °C). Quantifying these microorganisms with the same procedure and the same tool is a new and original approach. qPCR was the most appropriate tool for this purpose and gives quantitative results for a microbial database to be established for future comparisons with environmental and clinical data in the ELFE longitudinal study.

Although the DNA extraction method was run without the use of a commercial kit, it gave reliable results. There was a compromise between the sensitivity, the quality and the available quantity of the DNA extract. Extraction time and the cost of 20 samples were evaluated at 45 min and 60 Euros with our method, while they require 150 min and about 100 Euros using a commercial kit. Repeatability (seven times) enabled us to determine the limit of quantification for each species. All signals under this limit must be considered as a negative result.

Table 5
Relative concentrations of ten microorganisms in 53 dwellings expressed in percentages of the maximum concentration obtained in each species.

	Percentage of the maximum concentration for each species									
	A.alt	A.fumi	A.versi	C.spha	D.ptero	EnteroB	Mycob	P.chry	S.char	Strepto
L16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L50	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0
L37	10.8	0.0	0.2	0.0	0.0	1.4	0.0	0.0	0.0	0.0
L28	0.0	0.0	0.0	0.0	0.0	15.1	0.0	0.0	0.0	0.0
L10	0.0	0.0	0.0	0.0	8.8	7.6	0.0	0.0	0.0	0.0
L33	0.0	0.0	0.1	0.0	0.0	9.9	0.0	10.1	0.0	0.0
L5	0.0	0.0	0.0	0.0	2.7	0.0	17.9	0.0	0.0	0.0
L26	0.0	0.0	0.0	0.0	1.6	18.9	0.0	0.0	0.0	0.0
L34	0.0	0.0	0.0	0.0	0.6	1.8	0.0	23.7	0.0	0.0
L2	21.4	0.0	0.0	0.0	0.0	4.8	0.0	0.0	0.0	0.0
L43	27.9	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0
L41	21.1	0.0	0.0	0.0	0.0	10.1	0.0	0.0	0.0	0.0
L38	0.0	0.0	0.0	0.0	0.0	13.7	18.3	0.0	0.0	0.0
L18	31.7	0.0	0.1	0.0	0.0	6.1	0.0	0.0	0.0	0.0
L39	19.2	0.0	0.0	0.0	0.0	2.8	18.2	0.0	0.0	0.0
L13	0.0	0.0	0.0	0.0	2.9	3.6	0.0	0.0	0.0	35.2
L8	0.0	0.0	0.0	38.6	0.0	7.6	0.0	0.0	0.0	0.0
L25	0.0	0.0	0.5	0.0	2.9	31.1	0.0	18.0	0.0	0.0
L4	16.1	0.0	0.0	0.0	0.5	3.8	19.7	15.4	0.0	0.0
L35	25.1	0.0	0.0	0.0	2.3	13.4	0.0	15.4	0.0	0.0
L15	0.0	36.5	0.2	0.0	0.0	2.2	19.0	0.0	0.0	0.0
L44	46.7	0.0	0.0	0.0	0.0	12.4	0.0	0.0	0.0	0.0
L7	20.8	0.0	1.5	0.0	0.6	6.4	31.6	0.0	0.0	0.0
L32	0.0	0.0	0.0	50.1	3.4	0.0	0.0	14.7	0.0	0.0
L6	25.8	0.0	0.7	0.0	0.0	20.9	0.0	0.0	0.0	24.8
L1	21.3	0.0	0.5	0.0	0.0	10.4	41.1	13.8	0.0	0.0
L3	0.0	0.0	0.0	49.0	12.5	27.3	0.0	0.0	0.0	0.0
L53	0.0	0.0	0.1	25.8	32.7	2.1	0.0	0.0	0.0	34.0
L51	37.4	0.0	0.6	28.7	2.5	5.9	24.5	0.0	0.0	0.0
L46	0.0	0.0	0.0	0.0	2.2	53.6	0.0	0.0	0.0	44.5
L48	0.0	0.0	0.0	0.0	100.0	0.8	0.0	0.0	0.0	0.0
L47	22.5	0.0	0.1	0.0	0.0	4.4	0.0	39.9	0.0	35.7
L42	0.0	0.0	0.1	0.0	0.5	52.3	17.6	0.0	0.0	47.7
L23	9.6	0.0	0.6	0.0	5.5	33.4	32.8	40.0	0.0	0.0
L11	33.0	0.0	0.6	0.0	0.4	2.5	24.3	61.3	0.0	0.0
L17	89.8	0.0	0.0	31.3	0.0	1.3	0.0	0.0	0.0	0.0
L45	22.0	0.0	0.2	0.0	3.4	19.7	31.9	20.7	0.0	29.4
L21	10.2	0.0	0.0	0.0	1.3	2.2	22.2	100.0	0.0	0.0
L27	17.6	0.0	0.0	21.5	2.9	9.6	19.2	0.0	0.0	65.2
L12	0.0	0.0	1.6	0.0	1.4	37.7	100.0	0.0	0.0	0.0
L22	59.6	0.0	0.2	22.2	1.1	14.8	0.0	0.0	0.0	53.8
L19	32.6	54.9	0.0	0.0	0.0	5.5	0.0	74.7	0.0	0.0
L9	0.0	0.0	0.0	0.0	0.0	2.1	40.8	25.8	100.0	0.0
L24	21.6	0.0	0.0	0.0	5.8	100.0	17.1	0.0	0.0	30.7
L14	62.6	73.1	0.1	0.0	0.0	19.5	0.0	51.3	0.0	0.0
L30	85.0	0.0	0.1	0.0	0.0	43.1	22.3	0.0	0.0	69.0
L31	0.0	0.0	0.1	78.9	6.8	53.8	63.8	0.0	0.0	25.8
L29	28.4	0.0	0.0	31.3	3.3	19.8	16.6	0.0	88.6	47.5
L20	100.0	100.0	3.9	0.0	0.0	3.2	20.0	17.4	0.0	0.0
L40	10.7	0.0	0.6	0.0	0.0	35.2	71.1	97.0	0.0	100.0
L52	0.0	0.0	0.1	33.3	1.8	90.2	75.4	15.2	66.9	41.2
L36	0.0	0.0	100.0	28.6	7.6	32.9	92.7	67.3	0.0	40.7
L49	0.0	0.0	77.6	100.0	4.7	41.8	93.1	57.5	0.0	0.0

Squares with 0 to 25%, 25 to 50%, 50 to 75%, and >75% results are white, light gray, dark gray or black, respectively.

The ten targets were: *Alternaria alternata* (A.alt), *Aspergillus fumigatus* (A.fumi), *Aspergillus versicolor* (A.versi), *Cladosporium sphaerospermum* (C.spha), *Dermatophagoïdes pteronyssinus* (D.pter), *Enterobacteriaceae* (EnteroB), *Mycobacteria* sp. (Mycob), *Penicillium chrysogenum* (P.chry), *Stachybotrys chartarum* (S.char), *Streptomyces* spp. (Strepto).

These results also demonstrate the efficiency of the setup PCR machine. Because of the presence of brain–heart infusion proteins and sample dilution by rinsing, no qPCR inhibitors were found. Thus, the protocol can be simplified for the 5000 expected samples for the ELFE study, with no search for inhibitors.

The results of our study indicated the best choice of make of wipe (Eurowipes®, Nogent le Rotrou, France) for the EDCs for the ELFE

study because of its practicality and capacity to release spores. Recovery efficiency was 5.9% and 5% for *P. chrysogenum* and *S. chartarum*, respectively, which is lower than to those reported by Yamamoto et al. (10, 19 and 25% for *C. cladosporioides*, *A. fumigatus* and *P. chrysogenum*, respectively) (Yamamoto et al., 2011). However, the sensitivity of the technique is limited by a pre-analytical step that requires a large extraction liquid volume (20 mL). We tested rinsing wipes with a smaller

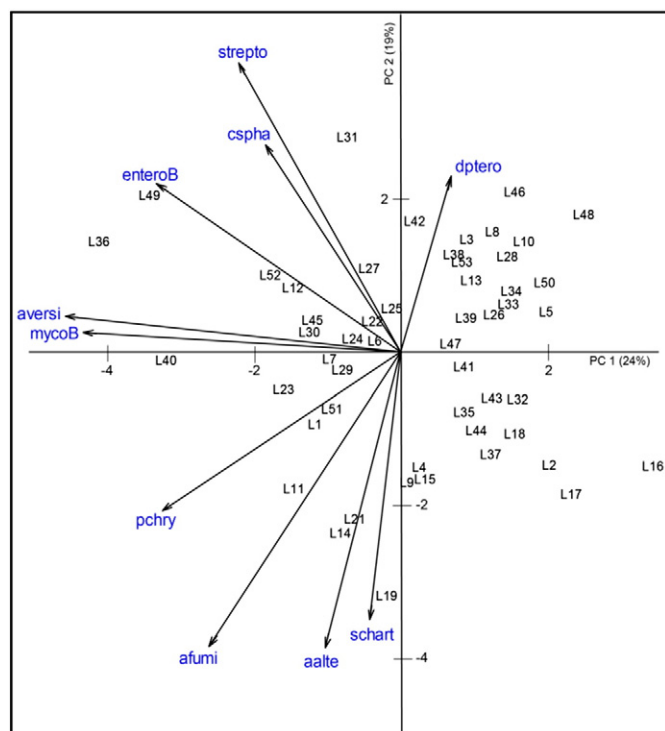


Fig. 1. Principal Component Analysis with 53 dwellings, using data from logarithmic concentrations (dwellings named L1 to L53). Axes are principal components, PC 1 and PC 2, with loading values. PCA Graphic analysis enables us to outline groups of microorganisms which could be correlated: the more the arrows are long and close to the axes, the more information they give on the data set. The ten targets are named as follows: *Alternaria alternata* (aalte), *Aspergillus fumigatus* (afumi), *Aspergillus versicolor* (avers), *Cladosporium sphaerospermum* (cspha), *Dermatophagoides pteronyssinus* (dptero), *Enterobacteriaceae* (EnteroB), *Mycobacteria* sp. (mycoB), *Penicillium chrysogenum* (pchry), *Stachybotrys chartarum* (schar), *Streptomyces* spp. (Strepto).

volume of liquid using the filtration step as described by Gibbons and Rokas (2013) and Yamamoto et al. (2011) but extraction efficiency proved inadequate and the results were less reproducible (data not shown). The results obtained for mite quantification are lower than one organism per cm^2 ; qPCR can detect all *D. pteronyssinus* cells left by the mite. Quantities detected for *P. chrysogenum* are low compared to other species. However, our aim was to identify differences that could have an impact on health and this method was able to differentiate between contaminated households and less contaminated ones, and to highlight the composition of species in each household.

Because no evidence of a correlation was found between species, we conclude that the presence of each one was entirely independent of other species. The analysis by PCA showed a possible link between *A. versicolor* and *Mycobacteria*, but this result is greatly influenced by just two dwellings: L36 and L49. We can thus assume that all species are independent and that it is appropriate to maintain the ten targets. Species were individually quantified and classified within their own usual ranges.

5. Conclusion

The requirements for ELFE study were as follows: one tool to evaluate allergenic components (fungi, bacteria and mites), a cost-efficient, discreet and well-accepted sampling method that can be easily carried out by participants, and a method that can provide qualitative and quantitative discrimination of microbiological data from children's dwellings. These preliminary tests have shown that the SOP we used meets these requirements. The overall procedure is a compromise between sensitivity and efficacy on the one hand, and economic feasibility

on the other. EDC sampling associated with qPCR provides quantitative data on several indoor microorganisms that should help improve our understanding of the interactions between the environment, allergic diseases and child development.

Conflict of interest

The authors have no competing interests.

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2. Première campagne : résultat de QPCR des 3193 logements analysés et établissement de profils

Titre de l'article : *Microbiological characterization of 3193 French dwellings of Elfe cohort children*

Auteurs : Steffi Rocchi, Gabriel Reboux, Victor Frossard, Emeline Scherer, Benoit Valot, Audrey Laboissière, Cécile Zaros, Mallory Vacheyrou, François Gillet, Sandrine Roussel, Chantal Raherison, Laurence Millon, *the Elfe team*

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A. Contexte

L'étude EBRA-ELFE est une étude nichée concernant certaines familles volontaires qui ont accepté d'installer un capteur électrostatique de poussières (EDC, *Electrostatic Dust Collector*) dans la chambre de l'enfant. La première phase de l'étude EBRA-ELFE (EBRA-ELFE (EST-10-40)) a bénéficié d'un financement ANSES (156 000 €- 2011-2014).

Au vu des résultats satisfaisants du pilote, la première campagne a débuté avec la cohorte ELFE en 2011. Les enfants ont été recrutés sur des jours de naissance définis, représentant les quatre saisons, à savoir ; du 1^{er} au 4 avril, du 27 juin au 4 juillet, du 27 septembre au 4 octobre et du 28 novembre au 5 décembre. Les EDC avaient préalablement été fabriqués au laboratoire (selon un format plus discret que celui du pilote) et ont été distribués aux participants à la maternité. Les familles ouvraient le capteur au retour à domicile et le laissaient en place pendant 10 semaines. L'EDC devait ensuite être renvoyé par la poste. Dès leur retour, le capteur était enregistré informatiquement, sur une base de données avec saisie sécurisée, puis congelé en attendant l'analyse.

Ainsi, 3193 EDC conformes nous ont été renvoyés par la poste. Ils ont été analysés par qPCR avec 10 microorganismes ciblés (6 moisissures, 3 groupes de bactéries et 1 acarien) :

- les moisissures : *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Stachybotrys chartarum*,
- les 3 groupes de bactéries : Enterobacteries, Mycobacteries, *Streptomyces* spp,
- l'acarien : *Dermatophagoïdes pteronyssinus*.

Trois questionnaires : un questionnaire médical, un questionnaire à la maternité, et un à l'âge de deux mois des nouveau-nés de retour dans le domicile, ont été remplis par famille. Plus de 200 variables ont ensuite été fournies par le consortium Elfe par l'intermédiaire d'une plateforme dédiée sécurisée.

B. Principaux résultats

Cette première approche statistique, utilisant des analyses multivariées (de type analyses en composantes principales), a permis de catégoriser les 3193 logements vis-à-vis des 10 cibles de qPCR. Les populations des différents micro-organismes présents dans l'environnement intérieur des enfants ont été analysées simultanément, et non deux à deux. Ainsi, six profils de logements différents, caractérisés par leur contamination en micro-organismes ont été définis :

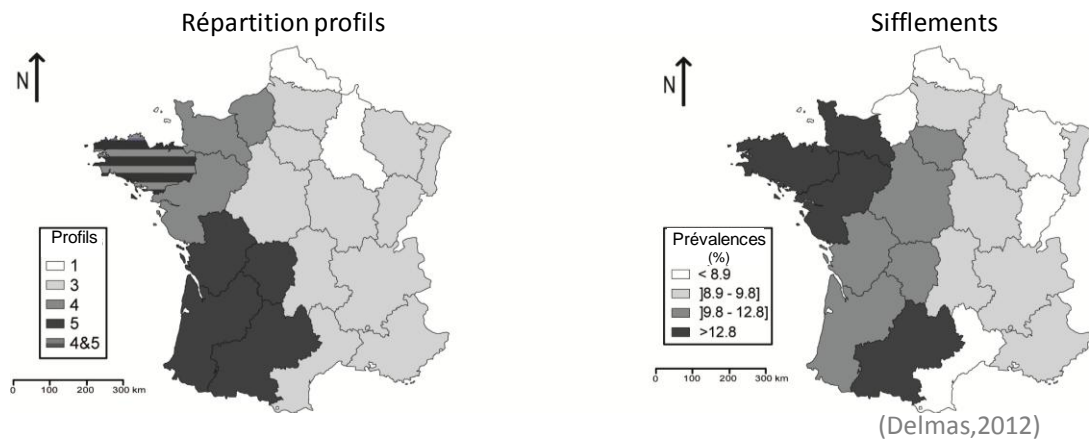
- (1) Un profil très peu contaminé,
- (2) Un profil peu contaminé par les bactéries,
- (3) Un profil dominé par les 3 bactéries et *Alternaria*,
- (4) Un profil dominé par les 3 bactéries et l'acarien,
- (5) Un profil dominé les 3 bactéries, *Alternaria* et l'acarien,
- (6) Un profil dominé par les 3 bactéries et *Alternaria*, toutes à fortes concentrations.

Ces six profils sont différemment représentés dans les différentes régions de France (la carte correspond à la figure 6 de l'article).

Le Nord de la France est dominé par le profil très peu contaminé (1), la Bretagne est dominée par les profils (4 & 5), bactéries/acarien (4) & bactéries/*Alternaria*/acarien (5), le Sud-Ouest est dominé par le profil (5) et le reste de la France par un profil bactéries/*Alternaria* (3).

Une approche statistique par analyse de redondance a pu mettre en évidence un lien entre ratio d'occupation (nombre d'habitant / unité de surface) au sein des logements et la contamination en micro-organismes observée (augmentation des concentrations de bactéries avec la promiscuité). De la même façon, en utilisant comme variable le type d'habitat (appartement ou maison), il a été mis en évidence une concentration en moisissures supérieure dans les maisons et en *Aspergillus fumigatus* supérieure dans les appartements. Enfin la présence d'animaux domestiques a pu être reliée à des concentrations plus importantes de moisissures et de *Streptomyces*.

Cette carte des profils de logements a été mise en parallèle avec celle établie lors de l'étude menée par Delmas en 2012. Elle concerne le suivi de 20 000 enfants de maternelle, en France, et présente plus particulièrement une carte relevant les sifflements, symptôme assez commun de l'asthme. La fréquence des enfants présentant des sifflements s'accroissaient d'Est en Ouest. La zone comportant la proportion la plus importante d'enfants « siffleurs » (à l'Ouest) coïncide à 85 % avec notre zone des profils 4 et 5.



(Delmas,2012)

Figure présentant la répartition des profils des 3193 logements (étude EBRA Elfe) et la répartition des sifflements chez 20 000 enfants (Delmas et al. 2012).

Ainsi, nos travaux sur la thématique allergique essayent de palier à la plupart des problèmes soulevés précédemment par les techniques d'observation directe ou par culture (Reboux and Nolard 2007) en utilisant des méthodes de prélèvement (EDC) couvrant une longue période de sédimentation des poussières et d'analyse (qPCR) standardisées pouvant mesurer indépendamment plusieurs micro-organismes avec la même efficacité.



Microbiological characterization of 3193 French dwellings of Elfe cohort children



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HIGHLIGHTS

- Electrostatic dust collector and qPCR analysis in 3193 Elfe children dwellings
- qPCR targets were 6 fungal species, 1 family and 2 genera of bacteria, 1 dust mite.
- 6 dwelling profiles across France have different geographical repartitions.
- Occupational ratio, pets, dwelling types influence microorganism concentration.
- High microorganism levels in West France in coherence with high wheezing area

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ABSTRACT

Although exposure to indoor microorganisms in early life has already been associated with respiratory illness or allergy protection, only a few studies have performed standardized samplings and specific microbial analysis. Moreover, most do not target the different groups of microorganisms involved in respiratory diseases (fungi, bacteria, dust mites).

In our study, ten specific qPCR targets (6 fungal species, 1 family and 2 genera of bacteria, 1 house dust mite) were used to analyze the microorganism composition of electrostatic dust fall collector (EDC) from 3193 dwellings of the Elfe French cohort study.

Multivariate analyses allowed us to show that the microbial composition of dwellings, assessed with simultaneous analysis of 10 microorganisms, can be characterized by four entities: three bacteria, house dust mite *Dermatophagoides pteronyssinus*, fungi *Alternaria alternata*, and five other molds.

Some dwellings' intrinsic characteristics (occupational ratio, type of dwelling and presence of pets) clearly influence microorganism distribution, and six different profiles of dwellings, characterized by their composition in microorganisms, have been described across France.

The use of these clusters seems promising in the evaluation of allergic risk. Allergic respiratory diseases will develop in the near future in some children of the Elfe cohort and will indicate to what extent our approach can be predictive of respiratory disease.

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1. Introduction

Several studies deal with microorganisms and their implication in allergic respiratory illness or symptoms such as wheezing, allergic rhinitis and asthma. Dust mite allergy is the most common, and some

molds like *Aspergillus* spp. (Agarwal and Gupta, 2010), *Alternaria* spp. (Salo et al., 2006), *Penicillium* spp. (Bundy et al., 2009), *Cladosporium* spp. (Garrett et al., 1998) are also involved in respiratory disorders. However, in their review, Bornehag et al. (2004) showed that only four publications out of the 547 studies on this subject (1998–2000) presented fungal concentration measures in dwellings. Tischer et al. (2011) found 1398 scientific publications with at least one keyword related to respiratory disorders, but only 36 publications included measurements of exposure and health outcomes validated by medical advice.

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Over the past 30 years, more than 100 cohorts of children have been created around the world (Bousquet et al., 2013) to study asthma and allergy (Keil et al., 2006).

Children's exposure to high levels of fungi at 8 months of age has been linked to rhinitis and wheezing at 3 years and asthma at 7 (Reponen et al., 2012; Reponen et al., 2011). Conversely, proponents of the hygiene hypothesis (von Mutius, 2007), suggested that early-life exposure to microbial agents such as molds and bacteria may offer protection against atopic wheezing and asthma. In the ALEX study (Riedler et al., 2001), children younger than 1 year exposed to stables developed asthma less frequently (1% vs 11%) than older children. In the PARSIFAL study (Ege et al., 2011; Schram-Bijkerk et al., 2005), dust endotoxins, *Aspergillus* and *Penicillium* extracellular polysaccharides and β -1,3-glucan levels were higher in control children's dwellings than in wheezers' homes. In the PASTURE study (Karvonen et al., 2014), children exposed to molds, bacteria and/or endotoxins developed fewer allergic diseases than children less exposed.

Overall, many child cohort studies have been based on questioning parents to assess microorganism effects on respiratory health. In the International Study of Asthma and Allergies in Childhood (ISAAC) studies (phases I, II and III), parents answered questionnaires and the presence of dampness was linked to respiratory disorders. However, the use of metrology is now gaining in importance especially when studies such as those of Roussel et al. (2008) showed that 18% of sampled rooms had no damp smell nor visible molds on walls, though the air was, in fact, contaminated (>470 fungi colony forming unit/m³). Thus, some studies have included microbial measures such as biomarkers. The birth cohort Epidemiology of Home Allergens and Asthma Study (EHAAS) analyzed fungal and bacterial biomarkers such as β -d glucans, endotoxins, muramic acid and ergosterol in family room dust samples (Sordillo et al., 2011). Finally, only a few studies have done specific microbial measurement with standardized tools like quantitative Polymerase Chain Reaction (qPCR) (Karvonen et al., 2014; Reponen et al., 2012; Reponen et al., 2011).

In our study, we used the electrostatic dust fall collector (EDC) (Frankel et al., 2012; Noss et al., 2008) and qPCR, with the Standard Operational Procedure (SOP) previously described (Scherer, 2014), to assess the microbiology composition of a large number of dwellings. We analyzed a sample of children's dwellings included in the Elfe (Etude Longitudinale Française depuis l'Enfance) project (Vandentorren et al., 2009). Elfe was the first multidisciplinary French cohort devoted to monitoring children's development in France from birth to adulthood. This Elfe subsampling, called EBRA (Environnement Biologique et Risque Allergique) study, included 6390 children's dwellings. We targeted ten microorganisms by qPCR: 6 molds, 3 bacteria and 1 mite (Scherer et al., 2014) using a multi-group approach to study microorganisms involved in respiratory diseases.

The six fungal species were chosen because they are commonly found in homes and are known for their allergic and/or toxic effects (Chew et al., 2003; Huttunen et al., 2004; Kaarakainen et al., 2009; Kuhn and Ghannoum, 2003; Meklin et al., 2004). Mycobacteria and *Streptomyces* were selected for their allergic and inflammatory strength (Huttunen et al., 2004; Obihara et al., 2007). Enterobacteriaceae are used here as markers of endotoxins that may have a potential protective effect for asthma (Gehring et al., 2001; von Mutius, 2007) or play a major role in the respiratory morbidity of livestock farmers (Reynolds et al., 2013). Mites *Dermatophagoides pteronyssinus* are also sought because they are common allergens involved in asthma (Vallance et al., 2006).

The aim of our study was to describe the microbial composition of French dwellings included in the EBRA-Elfe study with a multivariate analysis to analyze the main factors involved: geographic distribution, meteorology, socioeconomic levels, parents' health and the intrinsic characteristics of dwellings. Finally, we sought to find a new proxy that could summarize the microbial composition of French housing. We do not yet know which children will develop asthma, but we

performed environmental measures of 10 microorganisms, linked to allergic diseases, in dwellings in which children spend the first months of their lives.

2. Material and methods

2.1. Recruitment of children and questionnaires

349 maternity hospitals were randomly selected among the 542 in France and 320 participated in the study. Children were recruited depending on their birth date and their birth region. From April 2011, all babies born in the included maternity wards on given recruitment days could be included with their parents' consent. Inclusion criteria were as follows: children born after 33 weeks of gestation, of mothers aged 18 years or older. Foreign families could also participate in the study, if mothers were able to read French, Arabic, Turkish or English. Recruitments were made over several four-day periods the same year: from April 1 to April 4, from June 27 to July 4, September 27 to October 4 and finally November 28 to December 5. EDCs were distributed in the 320 participating maternities based on the annual number of deliveries. Families agreeing to participate in the study installed an EDC for two months in the room where their child was sleeping. Thus, 6390 EDCs were distributed at the children's births. 3217 well-identified EDCs were returned to the laboratory in time and 3193 of the 3217 families who sent the EDCs responded to the questionnaire. In fact, mothers were interviewed at the maternity ward for medical information about their pregnancy and the newborn baby, their exposure to pollutants and their eating habits. Two months after the birth, telephone interviews with mothers and fathers took place and included different types of questions. The lists of variables were as follows:

- Demographic and socioeconomic variables including country of birth, educational level, profession, monthly income, number of family members.
- Dwelling characteristics such as type, surface area, number of rooms.
- Health variables of both children and parents: parents' asthma and eczema, mother's psychological difficulties, children's birth weight and height. Clinical and medical data related to pregnancy were also accessible and used as variables.

Families signed a consent form presenting the general aim of the study and all data were anonymous. For privacy concerns, the geographical location of the dwellings could only be indicated at the regional level.

2.2. Sample analysis

EDCs were washed and rapid DNA extraction was performed from the washing liquid using mechanical and thermal lysis (runs in MagNA Lyser Instrument (Roche Applied Science®, Mannheim, Germany), boiling water bath and ice) as previously described (Scherer et al., 2014). In each extraction series, a sterile distilled water sample was included as a negative control to be sure that manipulation was not contaminated.

Primers and hydrolyze probes were used in the qPCR analyses. Ten specific qPCR targets were used as previously described and distributed as follows:

- 6 fungal species: *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum* and *Stachybotrys chartarum* described by Haugland et al. (2004) (and available at <http://www.epa.gov/nerlcwww/moldtech.htm>);
- 1 family and 2 genera of bacteria: Enterobacteriaceae (Sen and Asher, 2001), Mycobacteria (Torvinen et al., 2006) and *Streptomyces* spp. (Rintala and Nevalainen, 2006);

- 1 house dust mite: *Dermatophagoïdes pteronyssinus* using primers and probes developed in our laboratory (Roussel et al., 2013). Negative and positive controls were added in each qPCR reaction. Specificity, repeatability and detection limits of the qPCR procedure were tested using calibrated microbial suspensions of reference strains.

2.3. Statistical analysis

Data analysis was done through multivariate analysis as principal component analysis (PCA) and redundancy analysis (RDA). PCA is a multivariate ordination analysis that simplifies a complex data set to a reduced number of dimensions or axes, called principal components (PC). The first axes are projected on one or two ordination plots in which samples (dwellings) and variables (qPCR targets) are represented by points and arrows, respectively (Legendre and Legendre, 2012). RDA is an extension of PCA that models response variables as a function of explanatory (environmental) variables. Rules of interpretation identify two close variables as positively correlated, two opposing variables as negatively correlated and two orthogonal variables as uncorrelated.

A single redundancy analysis (RDA) was performed to assess the structuring effects of region coordinates (latitude and longitude) and month of sampling on microorganism composition. A partial principal component analysis (pPCA) was performed to explore the relationships between the different qPCR targets in removing the effect of the temporal structure contained in the dataset by including sampling month as a conditional variable constraining the PCA. This analysis highlights the main underlying gradients of microorganism composition among the 3193 dwellings. The significance of the PCA axes was assessed graphically using the broken-stick model (Legendre and Legendre, 2012).

A cluster analysis was then performed using sample scores of pPCA axes supporting a significant amount of variance according to the broken-stick model (here, the first two axes). Ward's minimum variance clustering method, where the within-group sum of squares is minimized (Borcard et al., 2011) was applied to the matrix of Euclidean distances between sample scores. The gap statistic (Tibshirani et al.,

2001) was used to define the number of significant clusters that could be retained from the dendrogram.

The influence of environmental dwelling variables on community composition was assessed through partial redundancy analysis (pRDA). Here, explanatory variables constrain the first ordination axes in order to explain the species matrix. In pRDA, dwelling region and month of sampling were considered as covariables so that their influence was removed prior to assessing the effect of dwelling variables. This procedure allowed examination of the determinants of community variability at a dwelling level once regional and sampling month variability was accounted for. Thirteen explanatory variables related to dwelling characteristics (housework frequency, number of floors, number of windows, use of pesticides, plants, pets, dwelling type, occupation ratio, floor covering in bathroom, living room, kitchen, renovation and if parents were owners of the dwelling) were considered necessary to explain the structure of the microorganism community. Forward selection was used to select explanatory variables. Other pRDAs were performed to assess the influence of socioeconomic variables (mother's country of birth, father's country of birth, educational level, family month income) and parent's health variables (psychological difficulties, parent's asthma and eczema) on the microorganism community. Other variables such as ventilation and type of heating cannot be used in analyses.

DNA concentrations of qPCR targets were log-transformed prior to the statistical analyses. Statistical analyses and graphical displays were performed using R 3.0.2 (R Development Core Team, 2013) and the 'vegan' (Oksanen et al., 2012), 'mgcv' (Wood, 2006), 'mapdata' and 'maps' (Becker and Wilks, 2013) packages.

3. Results

3.1. Regional child recruitment

The 3193 children included in the study were recruited during four periods in the different French regions as presented in Table 1. EBRA-Elfe dwellings were located in 21 French regions and distributed in the same proportion as the Elfe total cohort (χ^2 test: p-value = 0.24, data not shown). Dwellings in Corsica were removed from our analysis because of their underrepresentation (only 10 dwellings).

Table 1
Number of dwellings included in the study by region and recruitment period.

Regions	Inhabitant numbers in 2011	Area (km ²)	Maternity unit numbers	Number of sampling dwellings by inclusion period				Total	
				April 1 April 4	June 27 July 4	September 27 October 4	November 28 December 5		
1	Alsace	1,852,325	8280	12	21	31	44	27	123
2	Aquitaine	3,254,233	41,308	15	40	48	29	31	148
3	Auvergne	1,350,682	26,013	3	10	17	16	17	60
4	Basse-Normandie	1,475,684	17,589	8	19	19	23	20	81
5	Bourgogne	1,642,734	31,582	10	25	22	24	28	99
6	Bretagne	3,217,767	27,208	19	42	51	58	47	198
7	Centre	2,556,835	39,151	11	30	31	32	28	121
8	Champagne-Ardenne	1,336,053	25,606	7	15	15	21	15	66
9	Franche-Comte	1,173,440	16,202	6	15	15	19	13	62
10	Haute-Normandie	1,839,393	12,317	8	24	33	30	20	107
11	Ile-de-France	11,852,851	12,012	62	139	151	151	129	570
12	Languedoc-Roussillon	2,670,046	27,376	16	32	32	37	38	139
13	Limousin	741,072	16,942	2	7	9	4	4	24
14	Lorraine	2,350,657	23,547	11	24	18	31	21	94
15	Midi-pyrenees	2,903,420	45,348	12	26	28	16	37	107
16	Nord-pas-de-Calais	4,042,015	12,414	23	50	74	63	62	249
17	PACA	4,916,069	31,400	28	49	65	58	50	222
18	Pays de Loire	3,601,113	32,082	15	50	60	60	66	236
19	Picardie	1,918,155	19,399	11	21	30	26	16	93
20	Poitou Charente	1,777,773	25,809	10	24	24	26	15	89
21	Rhone-Alpes	6,283,541	43,698	31	75	86	73	71	305
Total		62,755,858	535,283	320	738	859	841	755	3193

Table 2
Dwelling characteristics.

Qualitative variables		Quantitative variables	
Dwelling type		Housework daily time	
Appartment	1151	None	26
Home	1973	<0.5 h	414
NA	69	0.5–1 h	962
Family situation		1–2 h	1091
Owner	1895	2–3 h	412
Tenant	1018	>3 h	131
Free accomodation	102	NA	157
NA	178	Window number	
Pets (cats, dogs, birds, rodents)		Median	4
Yes	1406	Max	21
No	1489	NA	184
NA	298	Floor number	
Dwelling renovation		Median	1
Yes	1469	Max	20
No	1660	NA	73
NA	64	Occupation ratio (inhabitant/m²)	
Pesticide use (insects)		Median	0.04
Yes	769	Max	0.2
No	2252	NA	199
NA	172	Floor covering	
Potted plants		Bathroom	
Yes	2186	Tile	2300
No	833	Linoleum	406
NA	174	Parquet	218
NA = not available data.		Other	85
		NA	184
		Kitchen	
		Tile	2437
		Linoleum	337
		Parquet	167
		Other	76
		NA	176
		Living room	
		Tile	1778
		Linoleum	830
		Parquet	279
		Other	130
		NA	176

NA = not available data.

3.2. Family characteristics

EBRA-Elfe mothers were 33 and fathers 35 years old on average. Most parents (90% of fathers and 91.5% of mothers) were born in France. Fathers not born in France were born in North Africa, other countries in Africa, Eastern Europe, Western Europe, America and Asia in 2.4%, 2%, 1.1%, 0.8%, 0.3% and 0.2% of cases respectively. Mothers not born in France were born in Africa, North Africa, Western Europe, Eastern Europe, America and Asia in 1.9%, 1.6%, 1.5%, 0.9%, 0.5% and 0.5% of cases respectively. Mothers went to university in 74% of cases, and 93% of fathers had a job. The monthly family income was 3500€ on average. Three percent of fathers and 4% of mothers had unemployment benefits.

One-third of mothers smoked, but 60% of them quit during their pregnancy. 11% of mothers and 13% of fathers had asthma. 16% of mothers and 10% of fathers had eczema. 371 mothers had psychological difficulties during pregnancy. Gestational age at birth was between 33 and 42 weeks and 47% of children were girls. Dwellings characteristics are presented in Table 2.

3.3. Microorganism community features

Molds were detected by qPCR from 27% (*S. chartarum*) to 88% (*A. alternata*) of dwellings. Bacteria were detected from 92 to 98% and the mite in 48% of dwellings. The amount of DNA found in dwellings varied among targets (Fig. 1). The abundance of targets mainly varied between 0 and 1000 fg/μL except for *Enterobacteriaceae* and

D. pteronyssinus that exhibited the highest abundances, ten and hundred fold respectively. None of the 10 targets was detected in 22 dwellings (0.7%) and 6–9 targets out of 10 were detected in 76% of dwellings.

3.4. Spatio-temporal data set structure

According to the RDA (Fig 2), the microorganism distribution differed significantly both among region coordinates (p-value < 0.01) and months of sampling (p-value < 0.01). These two variables explain 13% of microorganism variability.

3.5. Microorganism ordination and classification

The initial dataset was significantly structured by the seasonal effect, the month of sampling explaining 9.7% of the variance. Using pPCA, the variance in community composition among dwellings was efficiently supported by the first two pPCA axes. Axis 1 (PC1) and axis 2 (PC2) accounted respectively for 41.2% and 20.7% of the variance of the dataset (61.9% of the total variance). According to the broken stick model, only these two axes supported a significant amount of variance. *A. alternata* and the dust mite *D. pteronyssinus* are the two most structuring targets that differentiated samples (longer arrow lengths, Fig. 3a). Their distributions are poorly related as indicated by their orthogonal position in the correlation plot. Quite differently, molds and bacteria had related distributions although molds were the less discriminant group of targets. All targets were positively correlated with PC1 indicating that the main gradient obtained by pPCA was a quantitative gradient of contamination. Thus, the higher PC1 score a dwelling has, the more contaminated it is. Complementarily, PC2 allowed discrimination especially among the *A. alternata* dominated community (high PC2 scores) and *D. pteronyssinus*-dominated community (low PC2 scores).

Following the gap statistic, three groups of dwellings could be defined according to their microorganism composition (Fig. 3b). Nonetheless, because of the large number of dwellings, each group was subdivided into two groups (according to hierarchical clustering) so that dwelling typology could be described in more detail (Fig. 3d). Thus, 6 different profiles can be identified in France.

Dwellings in clusters 1 and 2 have the lowest levels of microorganisms (negative coordinates on PC1 in Fig. 3c). Clusters 1, 2, 3 and 6 which follow the *A. alternata* and bacteria gradients (Fig. 3a, 3c) contained increasing amounts of microorganisms (cluster 1 < cluster 2 < cluster 3 < cluster 6). Clusters 4 and 5 contained molds, bacteria and *D. pteronyssinus* with a stronger mixed microbial composition in cluster 5. Mean abundance diagrams supporting interpretations of pPCA allow a thorough description of the microorganism community among the clusters (Fig. 4).

Thus, six different profiles of dwellings, characterized by their composition in microorganisms have been identified (Fig. 4):

- Clean profile (cluster 1);
- bacteria only profile (cluster 2);
- bacteria and *A. alternata* profile (cluster 3);
- bacteria and mite profile (cluster 4) – High level of all microorganism profile (cluster 5);
- high bacteria and *A. alternata* level profile (cluster 6).

3.6. Spatial clustering

Although all clusters were present in each French region, their occurrence differed greatly (Fig. 5). A dominant cluster was identified in each region. The regional dominant cluster represented from 22% to 47% of the total dwellings (Fig. 5). For one region (Bretagne), two clusters co-dominated. Mild bacteria and *A. alternata* level profile (cluster 3) was the most dominant cluster in France and dominated more than half of the regions (12/21). Bacteria and mite profile (cluster 4) and

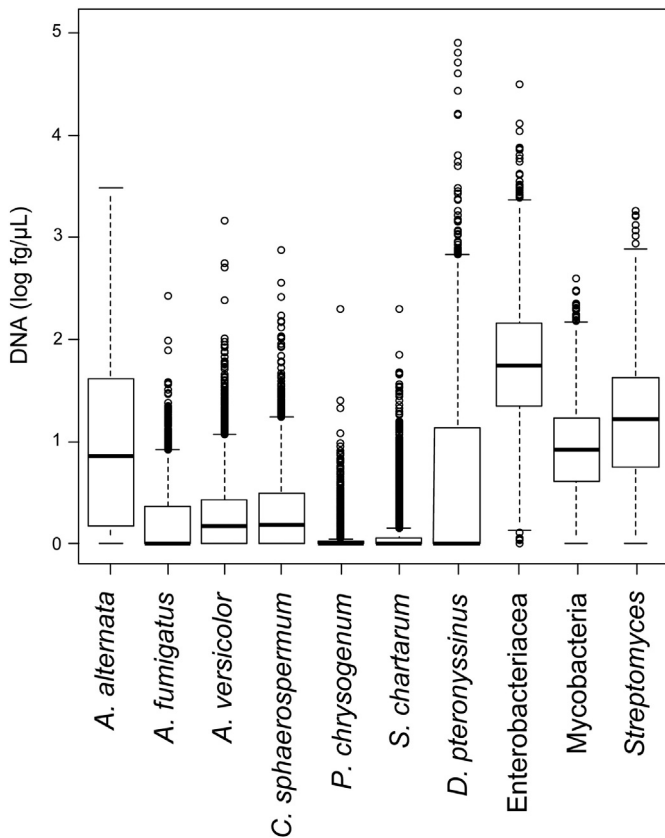


Fig. 1. Box plots for qPCR target concentrations (in log fg/μL) in the 3193 dwellings.

mixed strong profile (cluster 5), both with a strong mite component, were dominant in only four and six regions respectively. The few microorganism profile (cluster 1) was found dominant only in Champagne-Ardenne and Nord-Pas-de-Calais. Bacteria profile (cluster 2), strong bacteria and *A. alternata* level profile (cluster 6) were never dominant in the French regions. Once plotted on the national territory, the regional distribution of clusters appeared interestingly coherent.

Mild bacteria and *A. alternata* level profile (cluster 3) was found in central and eastern regions (Fig. 6a). Profiles with a mite component were found in western regions with cluster 5 dominant in south-western regions and cluster 4 in the north-western regions. The least contaminated profile (cluster 1) occurred in northern regions. This map (Fig. 6a) was compared to the distribution of wheezing in nursery school children (Delmas et al., 2012) (Fig. 6b), a single study conducted throughout France and dealing with a large number of children (>20,000 children). The recovery rate between the two most contaminated clusters (clusters 4 and 5) on our map and the highest wheezing prevalence on Delmas' map (>9.8%) is 85% of the undivided regional area. In the same way, the recovery rate between the two less contaminated clusters (clusters 1 and 3) on our map and the lowest wheezing prevalence on Delmas' map ($\leq 9.8\%$) is 88% of the undivided regional area.

3.7. In-dwelling determinant of microorganism community

Following the spatial analysis, a pRDA was conducted to search for variables within a dwelling that could shape the microorganism community. Considered together, months of sampling and regional coordinates accounted for 15% of the variance of the dataset. Once these effects were removed, the socioeconomic characteristics and presence of asthmatic parents, who could change the microorganism flora by implementing preventive measures (anti dust mite bedding for

example), failed to explain the remaining variability in microorganism community between dwellings ($p > 0.1$).

However, among the 13 dwelling characteristics, three variables, occupation ratio, dwelling type and presence/absence of pets, were significant ($p < 0.01$) in explaining a part of the microorganism distribution in dwellings; but they only explain a small fraction of the variance (3%). Species association observed in the correlation plot from pRDA differed from that of the pPCA for two reasons: (i) in pPCA, only the month of sampling effect was removed whereas in pRDA, the region of sampling effect was also removed; (ii) moreover, in a pRDA, observed graphical structure is constrained by the explanatory variables, i.e. occupation ratio, dwelling type and presence/absence of pets in the present analysis. *D. pteronyssinus* and Enterobacteriaceae and to a lesser extent *C. sphaerospermum* were mainly associated with occupation ratio (Fig. 7), whereas *Streptomyces* and secondarily Mycobacteria, *A. alternata* and *A. versicolor* were primarily related to both houses and the presence of pets. Alternatively, apartments with a low occupation ratio and no pets exhibited a low level of microorganisms.

4. Discussion

Our results showed that the microbial composition of dwellings, assessed using a combination of qPCR assays targeting 10 microorganisms, can be characterized by four entities: three bacteria, *D. pteronyssinus*, *A. alternata*, and five other molds. These community distributions made it possible to divide dwellings into 6 clusters with different profiles and regional distributions. Some dwellings' intrinsic characteristics clearly influence microorganism distribution as well.

The aim of the Elfe project was to follow a 20,000 child cohort representative of French births and thus include one child out of 50 born in France. With 18,300 children, the Elfe study has nearly reached its objectives. As recruitments in the EBRA-Elfe sub cohort resulted in a regional distribution with the same proportions as the Elfe cohort with regards to overall births in France. The average monthly family income is 3500€ in our study whereas the French National Institute of Statistics and Economic Studies (INSEE) estimated the average monthly income at 2998€ in 2011. From a sociological standpoint, our recruitment seems to be slightly different from the overall French population

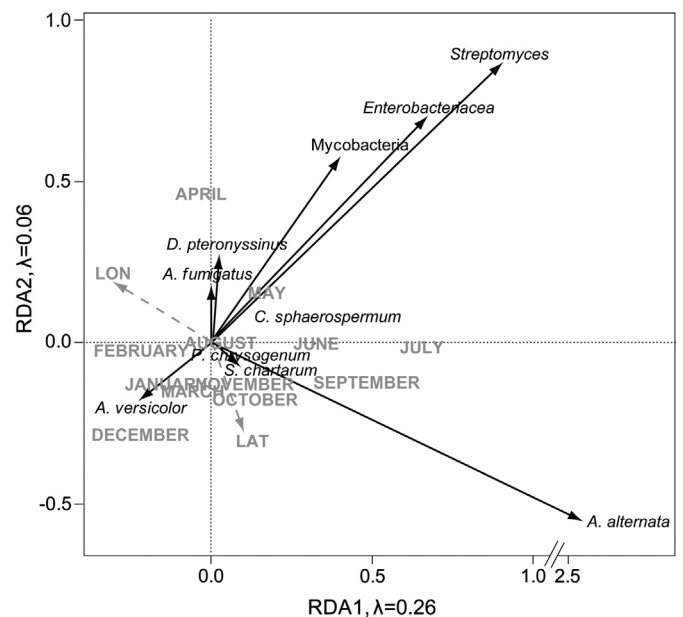


Fig. 2. Redundancy analysis (RDA) plot showing target microorganisms and spatio-temporal (regions coordinates and months of samplings). Solid arrows indicate microorganisms while dotted arrows indicate quantitative variables (LON: longitude and LAT: latitude). Months of samplings are qualitative variables and are represented without arrows.

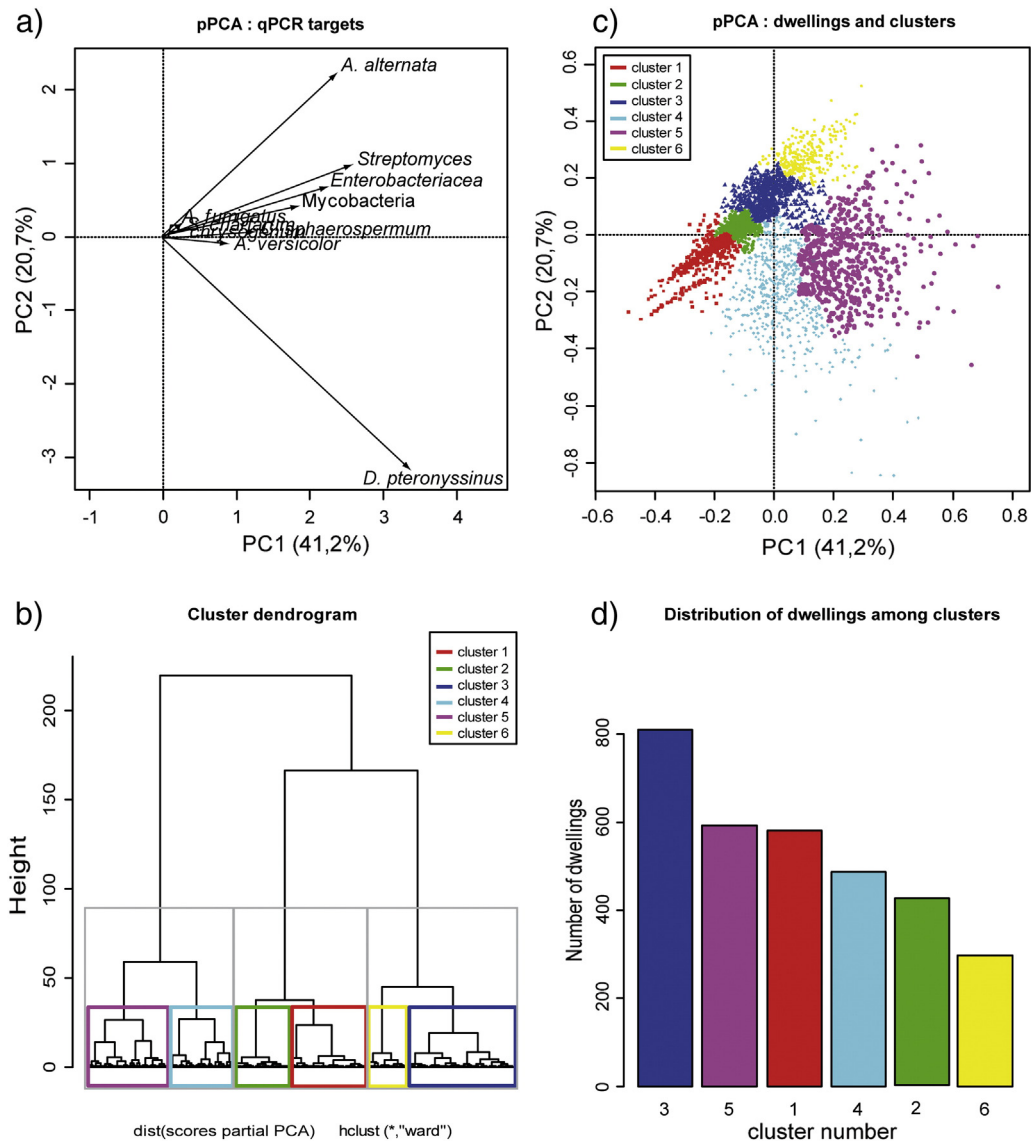


Fig. 3. Partial PCA (pPCA) and clustering analysis. a) Taxa correlation plot of the pPCA analysis. b) Hierarchical clustering constructed from PC1 and PC2 scores of the pPCA plots. c) Correlation plot showing dwelling distribution according to cluster obtained from hierarchical clustering. d) Distribution of dwellings among clusters.

based on a French reference study (DREES, 2010). In the EBRA-Elfe cohort, mothers pursued higher education in 74% of cases whereas the estimated proportion was 52.33%. Moreover, in our study, only 3% of fathers and 4% of mothers received unemployment benefits while the estimated unemployment rate was 12.64% for men and 12.1% for women (DREES, 2010).

The methodology used in this study allows measures of fungi, bacteria and mites using a single cost efficient sampling method (EDC) with qPCR, known to be a standardized quantitative method. This methodology (EDC + qPCR) has already been used in another recent important study which measures children's exposure at school (Jacobs et al., 2014). The EBRA-Elfe study targeted a small number of microorganisms focusing only on ten. However, when characterizing our dwellings, we used 5 very phylogenetically distinct families of microorganisms (fungi, mites, mycobacteria, Enterobacteriaceae and Actinomycetes) involved in human respiratory diseases. Moreover, multivariate analysis used in this study allowed us to obtain an overall view of the dwellings' microbial composition, and to observe how targets were distributed with respect to each other. The 10 targets were simultaneously analyzed and provided essential information about the microbiology of different dwellings. It is for this reason that our study is original with respect to

those that target only fungi communities (Reponen et al., 2013; Reponen et al., 2011; Vesper et al., 2007b; Vesper et al., 2007c) or studies which characterized cumulative microorganism exposure (Ege et al., 2011; Karvonen et al., 2014; Roussel et al., 2011).

Multivariate analysis was done in three steps. First, we showed a spatiotemporal effect on microorganism distribution. A seasonal effect has previously been observed in different studies in both indoor and outdoor environments (Madsen et al., 2012). Second, using pPCA allowed us to summarize in two significantly representative dimensions the complex distribution of ten microorganisms taking into account the month of sampling effect. Finally, we removed the spatio-temporal effects to keep only the variability explained by the characteristics inside the homes.

Bacteria, mites and molds are already known to be involved in respiratory disease, for their negative or positive effects. These three important groups have allowed us to clearly differentiate between different types of housing. In fact, *A. alternata*, bacteria and *D. pteronyssinus* are the targets with the heaviest weight in our analysis. *A. alternata* and *D. pteronyssinus* are the two most important microorganisms structuring our dataset, and the presence of one appeared to be independent of the other (orthogonality in Fig. 3a). *A. alternata* and *D. pteronyssinus*

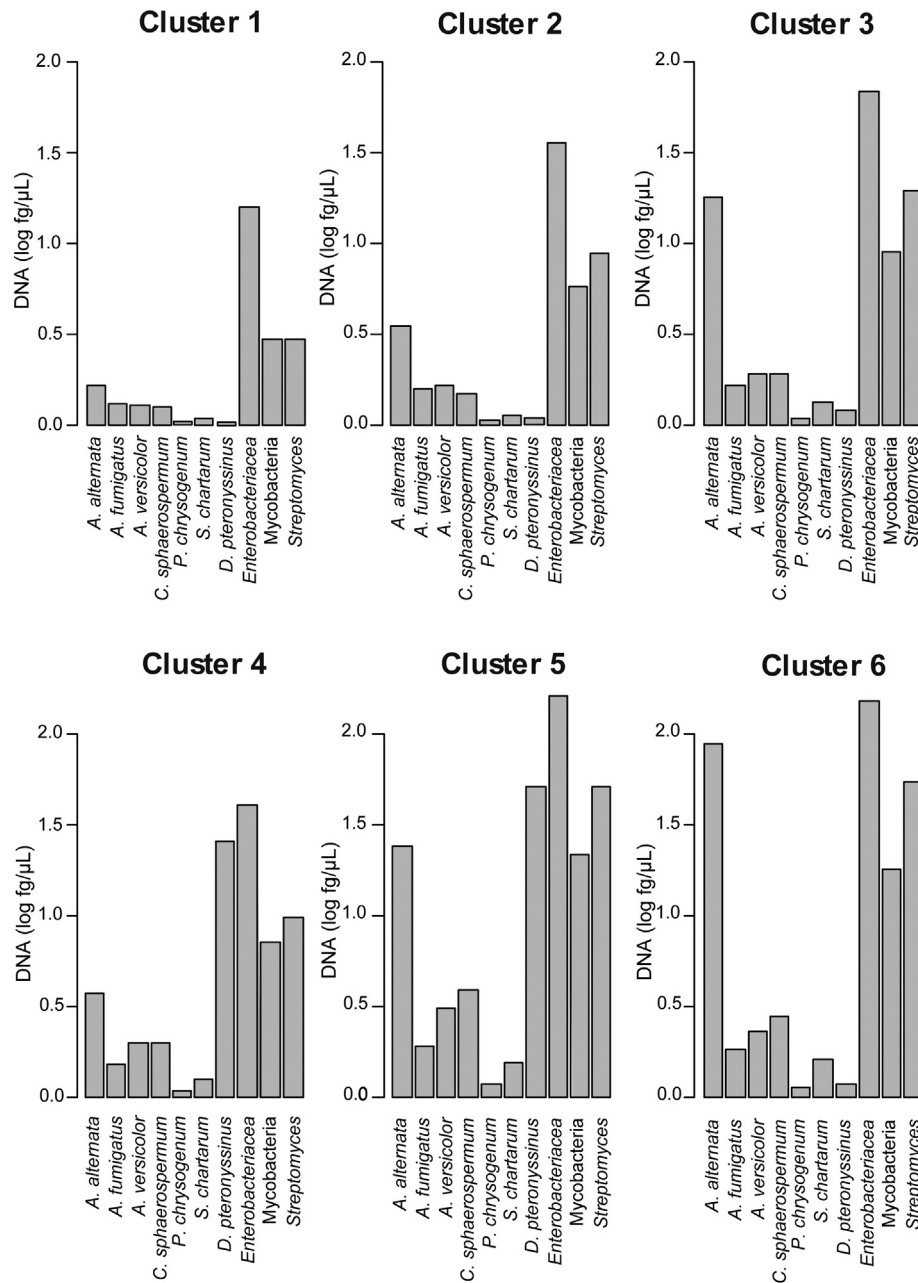


Fig. 4. Mean DNA concentration (log fg/μL) of qPCR targets by cluster.

are both part of the most described allergens (with grass, cats and dogs) tested with a prick test (Arshad et al., 2001; Bousquet et al., 2007). Both have been significantly associated with the severity of asthma (Zureik et al., 2002).

Contrary to other studies (Reponen et al., 2013; Reponen et al., 2011), the origin of the parents and their incomes do not influence the observed microbial composition in homes. We had previously supposed that asthmatic parents would take precautions to reduce the observed microorganism levels in their homes and thus influence the presence or absence of certain fungal species (Reboux et al., 2009). However, no link was found between asthmatic parents and dwelling microbial composition in the present study.

Different communities have been studied based on occupation ratio, dwelling type (house/apartment) and presence/absence of pets. The presence of occupants has already been shown to significantly influence the measured exposure to bioaerosol components (Madsen et al., 2012). Small but densely occupied apartments have been associated with

increased mold levels in Eastern France (Roussel et al., 2008). *Cladosporium cladosporioides* and *A. fumigatus* are the only two molds which seem to be influenced by the occupation ratio. Pets can influence the presence of home bacteria and molds (Sordillo et al., 2011). In our analysis, pets were associated with *A. alternata*, *A. versicolor*, Mycobacteria, *Streptomyces*, and also with the type of dwelling.

Region and sampling month variables accounted for 15% of the variability in the dataset whereas intrinsic characteristics accounted for 3%. It is very important to identify the indoor characteristics of dwellings taking into account the Geographical location.

The involvement of genetic factors in asthma is recognized but these factors alone cannot explain the geographic disparity of the disease's incidence already mentioned above. Vesper et al. (2007b) has quantified 36 molds in house dust using qPCR and established an Environmental Relative Moldiness Index (ERMI) to predict asthma in children. Overall, a higher index is found in houses of children with respiratory illness

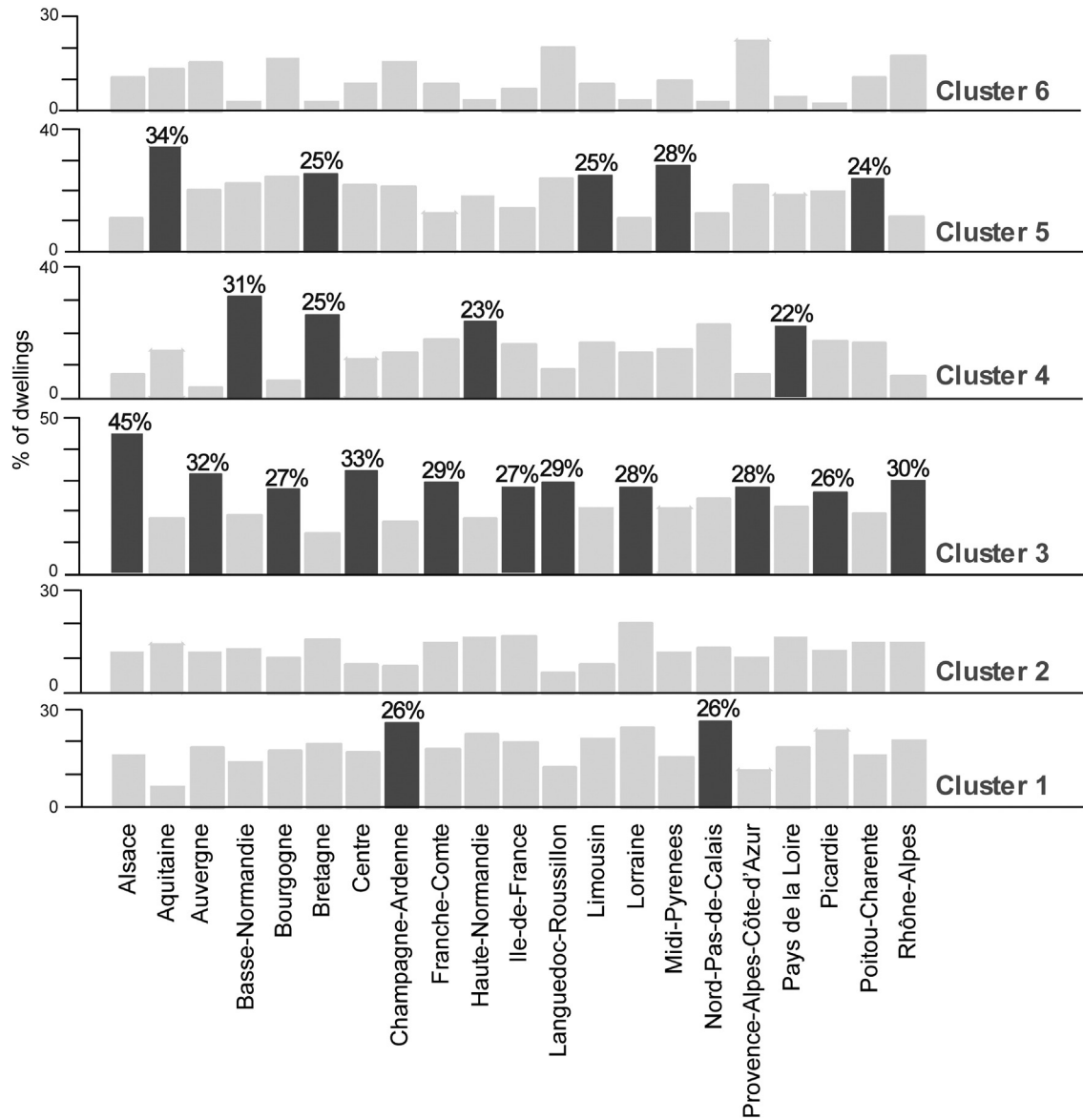


Fig. 5. Dwelling distribution by region and cluster. Dwellings of a dominant cluster in a given region are brightly colored. Others are paler. (Chi² test on regional cluster distribution yields p-value < 0.0001.)

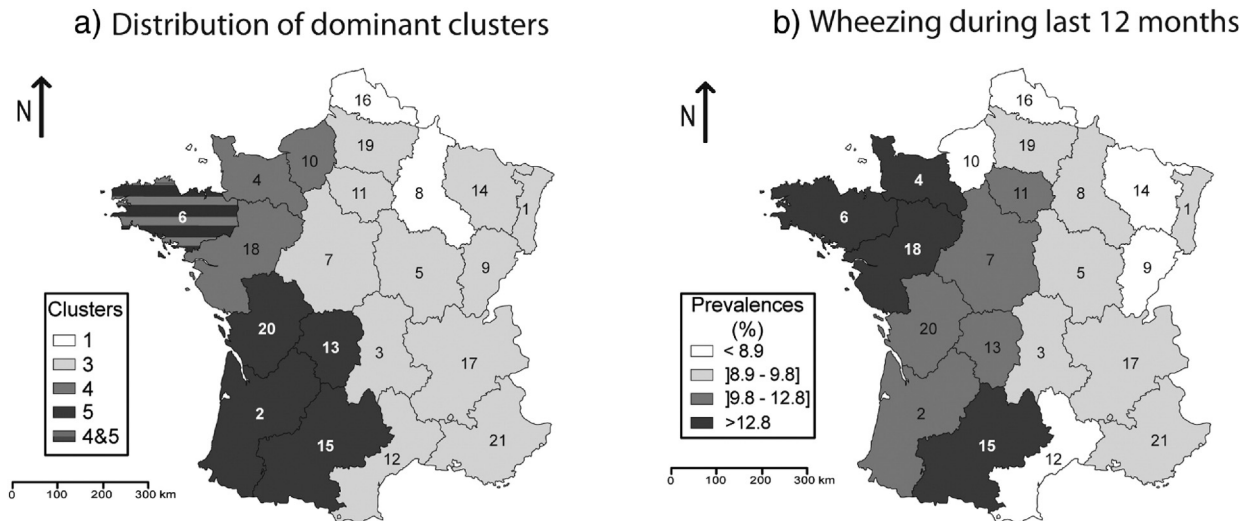


Fig. 6. Dominant cluster distribution (from our analysis) and wheezing distribution over the last 12 months in a study of 21,140 school children (Delmas et al., 2012).

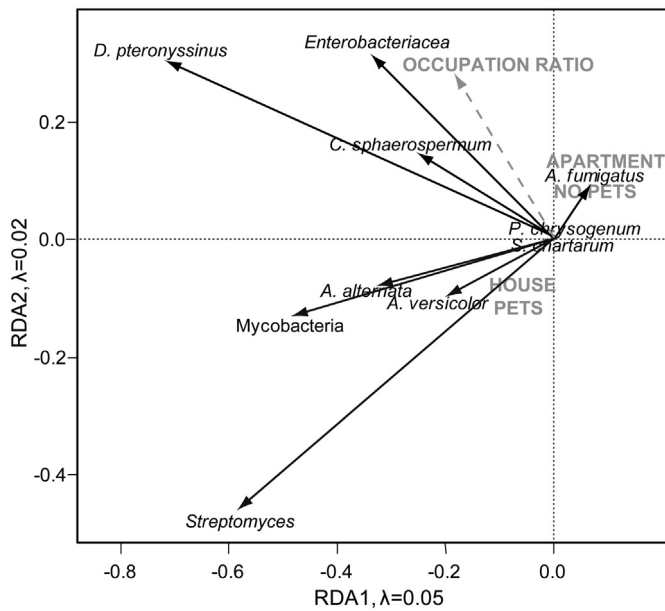


Fig. 7. Partial redundancy analysis (pRDA) plot showing target microorganisms and environmentally significant variables in dwellings ($p < 0.01$). Solid arrows indicate microorganisms while the dotted arrow indicates quantitative environmental variable. Other environmental variables without arrows are qualitative variables.

and has been linked to asthma when these children are 7 years old (Reponen et al., 2011). Yet, different targets have been related to asthma in children depending on the study location: *Scopulariopsis brevicaulis*, *Trichoderma viride* in Ohio (Vesper et al., 2006), *Chaetomium globosum*, *A. fumigatus*, *Eurotium* group in North California (Vesper et al., 2007a), *Aspergillus niger* and *Aspergillus unguis* in Michigan (Vesper et al., 2008).

In the present study, six clusters, built from pPCA scores, were defined to summarize a “microorganism cocktail” in the dwellings. These groups of dwellings, with different microbiological profiles were located throughout France. However, a mapping of France was carried out taking into account the dominant cluster represented in each region, showing a spatial effect on the microbial composition of dwellings.

As described by Kinney (2008), climate can influence the indoor distribution of microorganisms. Meteorological data (temperature, rainfall, humidity obtained from Météo France) do not allow us to explain the microorganism distribution on the pPCA (data not shown) but observed patterns of regional dominant cluster lead us to believe that there is indeed an influence on the local level Regional flora, linked to climate, soils, and agriculture may influence indoor microorganisms' flora. Other characteristics such as the architecture of buildings (data not available in our study) or type of heating (not exploitable data because of the low response rate to this question) could explain the observed differences.

This new proxy synthesizing microorganism level and diversity and reflecting the complexity and interaction of a panel of microorganisms in dwellings, could allow researchers to determine the precise link between microbiological environment and allergic diseases (probably when children are about six years old). In fact, our cluster mapping could be compared to asthma and the wheezing prick test or the prevalence of specific IgE results.

A study of about 21,140 children in nursery schools throughout France showed different prevalences of asthma and wheezing and notably a difference between the Nord, Western and Southwestern regions and those of the East and Centre (Delmas et al., 2012).

In our study, we found that clusters 4 (Bacteria and mite profile) and 5 (highest level of all the microorganism profiles) are dominant in western France. Dust mites (clusters 4 and 5) and *A. alternata* (cluster 5) characterized these two profiles and are known to be the allergens most closely related to asthma. Therefore, the allergy prevalence in

these regions will probably be greater. This hypothesis is consistent with the Delmas map which showed a wheezing gradient between the eastern and western parts of France. On the two maps (clusters and wheezing), both the Bretagne and southwest regions are very different from the others.

Our new proxy, profiles with a cluster approach and integrating a regional effect, seems, therefore, appropriate and promising. The results obtained through the simultaneous analysis of 10 microorganisms from 3 different types (bacteria, mold and mites) make it possible to analyze the indoor air biological pollutants of homes and provide a picture of the complex microbial composition in a large number of French dwellings throughout the country. In the future it will be easier to include new results from new samplings in this pPCA (without modifying the initial data set) to characterize new dwellings in the same way, with 1 of the 6 profiles.

Available samples have been kept and could be used for other qPCR target analyses in the near future to further develop and enhance our conclusions. For example, the *Dermatophagoides farinae* target, created in our laboratory (Roussel et al., 2013) could also be used. New EDC will be made and distributed to families to sample dwellings again when children are 3 1/2 years old and will enable us to monitor the children over time. Thus, we hope to obtain the most continuous and detailed approach possible of analyzing biological composition which will then help us to explain the occurrence of respiratory diseases in children. These diseases will develop in the near future in some children of the Elfe cohort and they will show us to what extent our approach can be predictive of respiratory disease.

Conflict of interest

The authors have no competing interests.

Acknowledgments

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3. Augmentation du nombre de cibles du panel, pour une analyse rétrospective sur les échantillons de la première campagne

Titre de l'article : *Quantification of pets and cockroaches in homes using qPCR: a new tool to evaluate exposure and risk of allergies*

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A. Contexte

Les résultats précédemment décrits sont très encourageants sur la capacité du dispositif à décrire l'environnement intérieur et à être utilisé comme marqueur d'une flore à mettre en lien avec des données de santé. Au vu de la complexité des pathologies étudiées (notamment l'asthme), des problématiques abordées par les différents groupes de travail (en particulier sur le développement cognitif de l'enfant) et de l'évolution des connaissances (par l'utilisation de la métagénomique entre autres), les 10 cibles initialement testées devaient être complétées par un nouveau panel de cibles.

Les liquides de rinçage de la première campagne ont été conservés à -80°C depuis la réception des échantillons. Pour tenter de répondre de façon plus complète à ces associations entre environnement et impact sur la santé humaine, une nouvelle série de 10 cibles vient d'être validée sur une étude pilote. Les dix nouvelles cibles choisies doivent répondre plus largement aux interrogations sur l'exposition intérieures

(1) Elargir la détection des moisissures (selon sonde et amorces décrites par EPA) :

- *Penicillium* « groupe 2 » : regroupant *Penicillium crustosum* /*camembertii* /*commune* /*echinulatum* /*solitum*.

Penicillium est fréquent dans les logements. La première campagne a montré des résultats quantitativement bas pour la cible de *penicillium* choisie, contrairement aux résultats attendus. Fréquent dans logement et récemment impliqué dans la littérature comme facteur de risque d'asthme, il convenait de compléter cette donnée.

- *Trichoderma viride* : producteur de mycotoxines (Bean, Jarvis, and Aboul-Nasr 1992).
- *Wallemia sebi* : difficilement retrouvé en culture mais pouvant avoir des interactions avec les acariens (Naegele et al. 2013).
- *Chaetomium globosum* : impliqué dans les phénomènes allergiques mais peu étudié car difficile à mettre en évidence en culture (Shi et al. 2014).

(2) Compléter le panel « champignon » par une levure :

- Genre *cryptococcus* (*hors neoformans*), récemment décrit comme protecteur (Dannemiller, Mendell, et al. 2014). Sonde et amorces ont été dessinées et validées au laboratoire.

(3) Elargir la détection des acariens (de poussière de logement et de stockage) :

- Dermatophagoïdes farinae.
- *Acarus siro* : Ces acariens sont également fréquents dans les logements et leurs communautés pourraient interagir. Ainsi, *Acarus siro* pourrait être plus présent dans les logements où les acariens de poussières sont plus rares. Sa présence dans le logement est étendue à la chambre et non pas limitée aux zones de stockage.

(4) Utiliser cet outil d'analyse commun pour quantifier d'autres allergènes :

- Chien,
- Chat,
- Blattes germaniques.

L'exposition aux chats, chiens, et blattes n'est quantifiée que par questionnaires ou dosage des allergènes. L'objectif de les quantifier par qPCR à partir des EDC est de compléter l'information apportée par les questionnaires (qui est limitée et ne prend pas en compte la persistance des allergènes, l'influence des habitudes de vies, les contacts indirects) et de fournir un résultat quantitatif selon un mode d'expression unique.

L'idée de ce panel complémentaire est de proposer, pour la première campagne, mais également pour les campagnes ultérieures, une analyse élargie en termes de diversité des agents mesurés, sans distinction de classe, dont les résultats pourront être mis en lien les uns avec les autres. L'analyse reste « restreinte » puisque le choix de 20 cibles ne peut décrire l'intégralité des espèces présentes dans un logement. Mais leur sélection pertinente selon les éléments de la littérature et leur analyse simultanée pourraient représenter un proxy du risque « microbiotique » représenté par le logement ou de son caractère protecteur.

Ces cibles sont en cours d'analyse sur la première campagne d'EBRA-ELFE. Elles ont été adaptées et validées sur des échantillons tests pour être passées en duplex (*A. siro*/*T. viride* ; Chat/*peni2* ; *W.*

sebi/Chien ; *Cryptococcus/C.glabosum* ; *D.farinae*/Blatte), afin d'optimiser les coûts et feront l'objet d'une publication ultérieure.

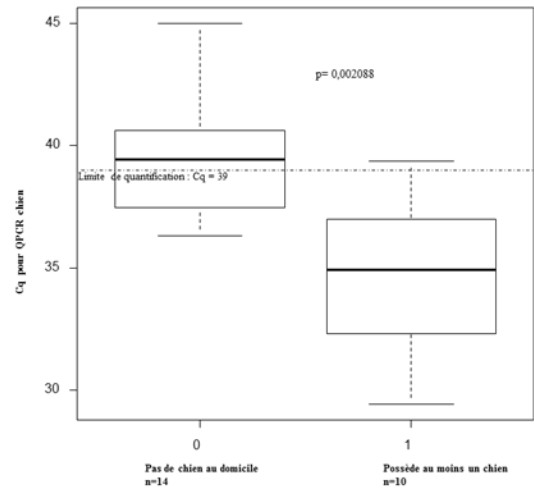
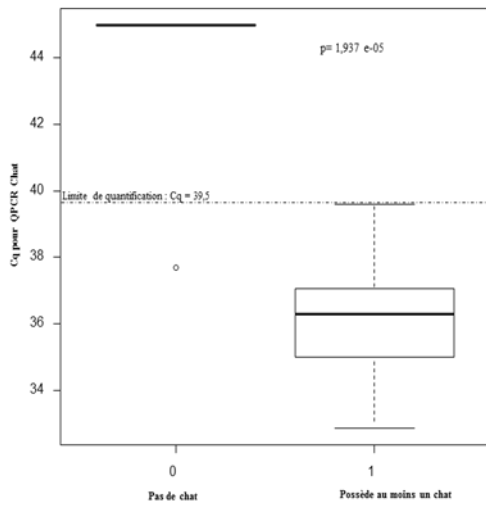
B. Principaux résultats de l'étude pilote visant à valider les qPCR chat, chien, blatte dans les logements

Les cibles chien et chat ont été dessinées au laboratoire pour l'identification des *feces* animales dans le cadre de recherche sur l'échinococcose. Avant de lancer les analyses sur les 3193 logements, nous avons validé la capacité de la qPCR à détecter la présence de chat et de chien dans un domicile mais aussi sa capacité à quantifier cette présence et à identifier d'éventuels contacts indirects.

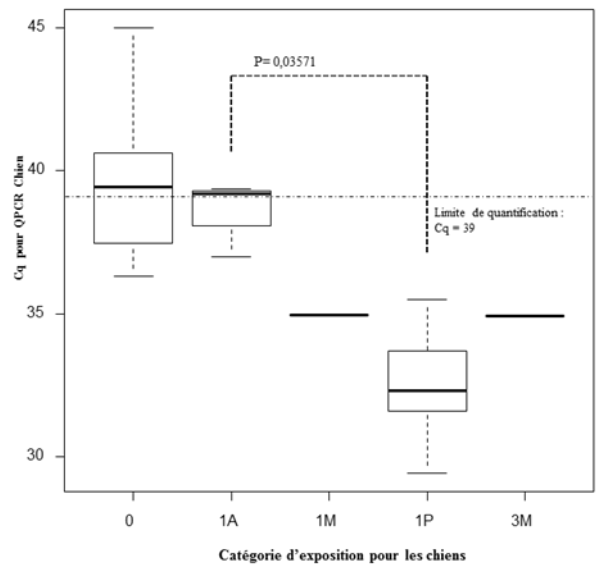
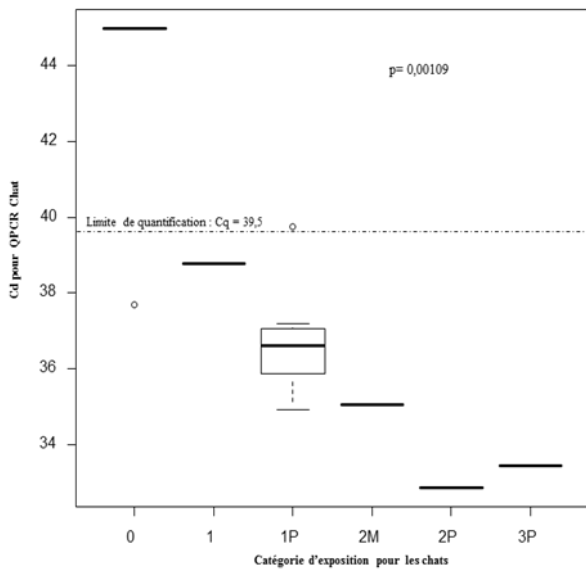
Vingt-quatre logements ont participé à l'étude. Un EDC a été déposé pendant 6 semaines et les étapes de rinçage et d'analyse ont été réalisées comme précédemment décrit. Le questionnaire rempli par les participants contenait entre autre les points suivants: nombre d'animaux, présence de l'animal dans la maison et la pièce échantillonnée, contact évident avec d'autres animaux à l'extérieur du logement.

Le premier résultat était que le signal de qPCR était significativement plus important dans les logements possédant au moins un animal (chat ou chien vs qPCR chat ou chien) (graphiques 1).

Dans un deuxième temps, l'exposition réelle aux animaux a été évaluée de la façon suivante : nombre d'animaux, associé à un groupe « A » si l'animal est systématiquement absent de la pièce, « M » s'il rentre parfois dans la maison, jamais dans la pièce échantillonnée et « P » s'il est souvent présent dans la pièce. Ainsi, pour les logements possédant un seul chien (catégorie la plus représentée dans notre petit échantillonnage) il y avait une différence significative entre les EDC posés dans une pièce où le chien allait régulièrement et ceux posés dans une pièce où le chien ne rentrait jamais (voir graphiques 2).



Graphiques 1 : Résultats des QPCR spécifiques en Cq Chat et Chien en fonction de la présence ou non d’animaux au domicile échantillonné



Graphiques 2 : Résultats des QPCR spécifiques en Cq Chat et chien en fonction de la catégorie d’exposition attribuée

De nombreux résultats étaient positifs en qPCR chien pour des logements dont les participants ne possédaient pas de chien. Pour tous les logements où le signal était inférieur à un Cq de 39, une exposition indirecte a été rapportée : garde exceptionnelle de chien au domicile, enfants gardés chez une assistante maternelle possédant des chiens ou famille régulièrement visitée possédant des chiens.

Cette étude pilote a permis de valider une cible mise au point pour une utilisation très différente en déterminant sa capacité à quantifier la présence d'animaux au domicile.

En y associant les autres cibles citées, nous serons en mesure pour la première fois de caractériser avec la même démarche 5 grandes familles d'organismes, allergènes ou protecteurs, d'un logement et ainsi de décrire et classer, probablement sous forme de profil, les logements de la cohorte ELFE, de suivre leur évolution tout au long de la cohorte et de confronter ces données quantitatives aux évènements de santé à venir.

Quantification of pets and cockroaches in homes using qPCR: a new tool to evaluate exposure and risk of allergies

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Running title: QPCR for pets and cockroaches quantification

Highlights: QPCR is a widely-used tool that can be used to detect various organisms. Measuring cat, dog and cockroach DNA by QPCR is a new approach to characterize indoor contamination. QPCR for pets and cockroaches would be obtained when fungi and bacteria are measured. QPCR measurements should enable us to identify new home profiles which may be related to the development of asthma in children.

Abstract:

Contradictory results are found in the literature concerning fungi, bacteria, pet and cockroach exposure and the risk of developing asthma. All these allergens have been thoroughly studied separately in cohort studies, and a variety of sampling and analytical methods are used. It is already possible to characterize fungi, mites and bacteria by QPCR. The aim of our study is to evaluate QPCR systems to quantify the presence of cats, dogs and cockroaches in homes.

Twenty-four houses were sampled with an Electrostatic Dust Collector which was analyzed by QPCR. Questionnaires were completed on the presence of pets or cockroaches in homes.

The results from QPCR were correlated for real presence of cats and dogs, and highlighted indirect exposure.

No participants reported the presence of cockroaches in their homes and this was confirmed by the results of cockroach QPCR.

This study validated a screening tool that will be used in future in the ELFE cohort study.

Key words: QPCR, housing, cat, dog, cockroach, antigens

Introduction

Asthma has become the most common chronic childhood disease in the industrialized world (Eder et al. 2006). Asthma is described as an environmental disease resulting from the interaction between genetic predisposition and environmental exposure to pets, house dust mites (HDM) and molds, particularly indoor pollution, during the early years of life.

Many studies have attempted to explain environmental involvement in the development of allergic diseases. Real-time quantitative PCR (QPCR) technologies have highlighted a number of fungi involved in allergy or sensitization (Vesper et al. 2008) but their precise involvement remains inconclusive. Although some teams have identified some fungi species that are correlated with a high risk of developing asthma (Reponen et al. 2012, Sharpe et al. 2015), the role of the different agents and mechanisms is unknown. Cohort studies have reached different conclusions. The difficulty in reaching conclusions is related to the multifactorial characteristics of asthma, and to the heterogeneity of the methods used for indoor characterization. Questionnaires may be used instead of sampling; samples may include active dust, passive dust or air. These samples may be quantified in culture or with PCR, or through antigen measurements. It is also difficult to time these measurements accurately, as samples may vary considerably at different stages of a child's life, and may be taken once or repeatedly. Therefore, the available data cannot be compared and the conclusions of the different studies are often incomplete because they are so diverse.

QPCR is a useful technique for environmental micro-organism characterization and quantification and has recently been used in numerous cohort studies. The main advantages are accurate quantification, efficacy, sensitivity and reproducibility. QPCR has been used by several teams (Kaarakainen et al. 2009; Kaarakainen et al. 2011; Yamamoto et al. 2011; Reponen et al. 2012; Méheust et al. 2012; Vesper et al. 2013) but in all these studies, QPCR was restricted to detection and quantification of molds and bacteria.

The ELFE study (Etude Longitudinale Française depuis l'Enfance), which started in 2011, consists of a 20 000 birth cohort study conducted on a multidisciplinary basis. Several working groups have been established, one of them focusing on asthma and allergic respiratory diseases. This particular group required a tailored system for taking measurements.

In the first step of this study, ten specific QPCR targets (six fungal species, one family and two genera of bacteria, one house dust mite) were used to analyze the microorganism composition of an electrostatic dust fall collector (EDC) from 3193 dwellings in the Elfe cohort study. Multivariate analyses showed that the microbial composition of dwellings could be expressed in clusters, which seems promising for the evaluation of allergy risk (Rocchi et al. 2015). These profiles, which synthesize microorganism diversity (regardless of which type of living organism) and reflect the complexity and interaction of a panel of microorganisms in dwellings, could enhance our knowledge of the relationship between the microbiological environment and allergic diseases.

Because our results so far are so encouraging, it would be useful to improve these profiles by adding other allergenic agents which do not come from micro-organisms, i.e. pets and cockroaches.

The relationship between exposure to dogs and cats in early childhood and the incidence of allergies is still controversial (Chen et al. 2010; Lodge et al. 2012). It has been suggested that contact with pets increases the risk of allergic diseases and sensitization (Pyrhönen et al. 2015), or protects against them (Gaffin et al. 2012). Allergen measurements correlated with the presence of animals at home (Almqvist et al. 2003), and questionnaires are the current methods used to determine pet exposure in cohort studies (Crawford et al. 2015). Cockroaches, particularly *Blattella germanica*, can be found in inner cities and their presence may indicate chronic damp in homes (Crawford et al. 2015). Exposure has been shown to increase the risk of wheezing in children of atopic and non-atopic parents (Gaffin et al. 2014) but this exposure has only been quantified by measuring antigens on vacuum dust.

The aim of our study was to assess QPCR techniques that were previously developed to detect cat and dog DNA in samples of feces containing parasitological diseases (Knapp et al. 2015). We intended to measure exposure to pets in dwellings by obtaining quantitative results that can be integrated in the profile model previously used to measure environmental conditions in dwellings from the ELFE cohort. The second objective was to design a new QPCR system to quantify cockroaches, to complete the panel of potential allergens to be quantified in dwellings.

The final step would be to establish a unique result in the form of a profile synthesizing the allergen mixture of each dwelling. This will include micro-organisms, mites, pets and cockroaches, all of which could be related to health data of children from the ELFE Cohort.

Materials and methods

qPCR development

QPCR system for pets

The system was developed to identify carnivore feces using the high sensitivity of the real-time PCR technique. Probes and primers were previously designed for dogs and cats using the Primer Express 3.0 for the host fecal test (Knapp et al. 2015), shown in Table 1.

QPCR system for cockroaches

Probes and primers were designed for these studies on the 18s DNA, using the Primer Express 3.0. VIC TAMRA labeling was used because duplex QPCR quantification was required for the ELFE study (Table 1).

Efficacy, limit of detection and specificity

DNA from the epipydim and ear tip was extracted using the High Pure PCR Template Preparation kit (Roche Diagnostic, Penzberg, Germany), as recommended by the manufacturer and was used to define the calibration scale of dogs and cats respectively. The concentration of the first calibration point was 50 pg/ μ L.

DNA from *Blattella germanica* was obtained from specimens provided by the "Centre des sciences du gout et de l'alimentation" (faculty of Sciences, Dijon, France). Extraction was performed using mechanical lysis (runs in MagNA Lyser Instrument) (Roche Applied Science[®], Mannheim, Germany), and then using the same kit, as recommended by the manufacturer. The concentration of the first calibration point was 50 pg/ μ L.

For each of these targeted species, DNA extract was diluted from 50 000 to 0.5 fg/ μ L. Each calibrating point was run seven times. Limit quantification is defined as the smallest amount of DNA in which seven successful detections are observed in seven QPCR trials (Hospodsky et al. 2010).

Specificity was tested using DNA extract from 52 indoor micro-organisms currently found in houses and belonging to groups of mold (n=38), actinomycetes (n=4), yeasts (n=4), mycobacteria (n=2), enterobacteria (n=1) and dust mite (n= 3). DNA concentration in the sample tested was 500fg/ μ L, except for dust mites (10fg/ μ L). Cultivable micro-organisms were cultivated on adapted media at the correct temperature and extraction was performed as previously described (Scherer et al. 2014).

QPCR analysis

QPCR reaction was performed in a final volume of 20 μ L, containing 10 μ L of Hydrolysis Gene Expression Master mix 2X (Applied Biosystem, Foster City, CA), 1000nM primers et 200nM probe and 5 μ L of DNA extract. The amplification protocol

was as follows: two minutes at 50°C, ten min at 95 °C for the activation, followed by 45 cycles for 15 s at 95 °C for template denaturation and one min at 60 °C for primer and probe annealing and extension. PCR reactions and absolute quantification were run and monitored using Applied Biosystems[®] 7500 Fast System (Life Technologies, Carlsbad, CA, USA). Sterile distilled water samples were used as negative controls in each series of amplification. The results are expressed with the Quantification Cycle, Cq. Negative results are expressed as Cq=45.

Application of QPCR on dwellings with and without pet occupation

Sampling and QPCR Analysis of dwellings

Twenty-four houses were recruited in eastern France (Franche-Comté region). No environmental or medical criteria for selecting the type of dwellings were used. An Electrostatic Dust Collector (EDC) was exposed to the air on a horizontal surface between 1.60 m and 1.80 m above the floor in an occupied room. Dust was allowed to settle over a six week period in winter. A questionnaire on the type of housing, pets and the presence of cockroaches was given to the participants. Participants returned the questionnaires and the EDC at the end of the sampling period.

DNA extraction from the EDC was performed as previously described (Scherer et al. 2014). DNA from cats, dogs and cockroaches was amplified using the QPCR assays described above.

Estimation of real exposure to pets in homes

Participants had to respond not only to questions about the presence of cats or dogs in their homes, but also about the number of pets and the frequency of the pets' presence in the house and in the room tested (Direct Exposure). Using all of this information, three categories of exposure were determined. The cat(s) or dog(s) was identified with the letter P if the animal was often in the tested room, and with the letter A if the pet was never in the tested room. In cases where animals were often in the house but rarely in the tested room, the letter M was used.

Participants also had to give information about regular contact with relatives who had a cat or dog at home (considered as Indirect Exposure (Almqvist et al. 2003)).

Statistical analysis

Non-parametric tests (Wilcoxon rank sum test and Kruskal-Wallis test) were used to investigate possible differences in Cq results, the presence or absence of pets at home and type of exposure, and the category of exposure. Statistical analyses were

performed using R 2.13.1 (R Development Core Team, 2012).

Results

QPCR efficacy

Repeatability is expressed by the C_q standard deviation (Bustin et al. 2009). For each species targeted, mean C_q, C_q Standard Deviation (SD C_q), and the number of successful trials are reported (Table 2). The lowest concentration, with seven successful trials and SD C_q<1 was considered as the limit of quantification. The quantification limit was about 5fg DNA/μL for *Blatella germanica* and dogs, corresponding to C_q =39.8 and 39.0, respectively. For cats, we considered C_q=39.5 as the limit of detection because the sample with 5fg/μL was detected in the 5/7 run, and the sample with 0.5 fg was not detected at all.

QPCR specificity

The results of QPCR analyses using the primers and probes designed for cat, dog and cockroach detection were negative (C_q > 40 cycles) when applied to DNA extract from the 52 micro-organisms analyzed in specificity tests.

Dwelling recruitment

Eighteen houses were in urban areas, and six in rural areas. The mean time of exposure was 45 days (minimum 24 days, maximum 60 days).

Of the participants, 46% declared having at least one cat, 42% at least one dog, 21% a cat and a dog. The number of dogs is divided into five categories, and the number of cats into six categories (Table 3).

None of the participants lived in inner city areas, and none reported seeing cockroaches in their homes.

qPCR on dwelling samples

Correlation between QPCR results and presence of pets in homes

The number of animals, exposure category and C_q results are shown for each sample in Table 4.

Cat QPCR was positive in 50% of houses, and dog QPCR was positive in 87.5% of houses. All houses with cats were cat QPCR-positive (C_q ≤39,5 cycles).

A higher DNA concentration (i.e. lower C_q in QPCR) was measured in houses with the highest number of cats. Cat QPCR C_q was highly correlated with the presence or absence of cats (p-value=<0.0001) (Figure 1), and with the category of exposure (p-value< 0.005) (Figure 3). The QPCR signal was significantly higher (p-value < 0.01) when more than one cat was present (Figure 2).

Only one house without a cat presented a positive qPCR signal (H 17; C_q = 37.7), but indirect exposure was reported by frequent visits to and from family with cats and dogs.

All houses with dogs were dog-QPCR positive. The Dog-C_q is highly correlated with the presence or absence of dogs (p-value <0.005) (Figure 4). Kruskal–Wallis was used to test the difference between categories of exposure in dog owners. The difference was statistically significant between Dog-C_q when dogs were present in the room (1P) compared to when they were never in the room (1A) (Figure 5).

In 11 cases, a positive qPCR signal was detected when no dogs were reported in the home. In six cases, the C_q was lower than 39 (our previously determined limit of quantification) but indirect exposure was reported. In the five other cases, C_q was higher than 39.2. The difference between dog QPCR signal when indirect exposure or no exposure was reported was statistically significant (p-value<0.01) (Figure 6).

Cockroach results

Cockroach QPCR was negative in all the houses and no participants reported seeing cockroaches in their homes.

Discussion

When used together with EDC sampling, QPCR is a simple and accurate technique that can be applied to large numbers of samples. In this study, we confirmed the usefulness of QPCR techniques to quantify exposure to dogs and cats in houses, and we developed a cockroach QPCR assay. If we associate these new assays with fungi, bacteria and mite QPCR as previously described (Scherer et al. 2014), we will provide a new tool to quantify the overall allergenic risk in housing with the same sampling and analytical methods. The results will also be expressed in the same way.

The primers and probes system performed well, as shown by the analytical results. The quantification limit is set by seven out of seven successful detections, so it is strict in relation to the complex environmental situation. If we apply this limit (C_q = 36.5; DNA concentration = 50fg/μL) to cats, many of the results from houses would have been interpreted as unquantifiable, even though it corresponded to real exposure. The analytical limits of the method must be understood, but it is important to adapt these to the environmental situation. When results are between 36.5 and 39.5 cycles, in 5/7 cases, the real amount of cat DNA is successfully quantified. We therefore accept a limit of quantification C_q = 39.5 (DNA concentration = 5fg/μL),

knowing that the results from some houses may be false negatives.

The limit of quantification is 5 fg/ μ L ($C_q=39$) for dog QPCR reaction, which seemed to correspond to the limit in terms of contact with dogs (direct and indirect) and a background signal, which could be an unquantifiable but detectable real signal.

The QPCR signal is correlated with the presence of animals at home, and with gradation linked to frequency of presence in the tested room and with the number of animals in the home. The correlation between the QPCR cat signal and category of exposure, the significant difference between having a dog indoors or outdoors and the ability to detect indirect dog exposure are convincing results. They show that this tool is effective for quantification and provides more detailed information than a questionnaire alone.

There seems to be a background dog QPCR signal. This background also exists when allergens are measured (Almqvist et al. 2003). In the Arbes study, 100% and 99.9% of houses tested positive for dog and cat allergen respectively (Arbes et al. 2004). He suggests that the social environment is a major source of dog and cat allergen due to the transport of allergens. Indeed, this is what we observe with a positive QPCR signal when indirect pet contact is reported. When the limit of quantification was higher, the signal usually highlighted a low presence of dog particles, lower than with known indirect exposure. This should be interpreted as a positive result with no quantification. This is why it is essential to take measurements; questionnaires alone cannot accurately evaluate pet exposure.

It is unsurprising that we did not detect *blatella*. Participants were recruited from all over Besançon and the city center, but not in inner city areas. Selection criteria were not used. *Blatella* was never observed by the volunteer participants, the houses were modern and we did not expect to find a signal.

We have developed a new tool which allows us to quantitatively measure pets and cockroach exposure with a unique sampling and analytical technique. Associated with fungi, bacteria and mite QPCR as previously described, it provides a multi-parameter profile of the houses. This overall evaluation is needed to better understand the implications of chronic exposure, and to analyze the synergistic role of different micro-organisms and other allergens in the development of children's allergies. Another advantage of the proposed tool is that sampling can be done using EDC, which is very cheap

and easy to use and does not require investigator intervention. Therefore, sampling can be repeated to better analyze early childhood and later on in childhood in longitudinal studies (Gaffin et al. 2014; Pyrhönen et al. 2015).

Despite the small number of houses, these results are encouraging. The quantification of animals was well correlated with real exposure (unquantifiable by the questionnaire) and the analysis of these data, associated with other data on fungi, bacteria and house dust mites could explain the interaction between the different organisms in the environment and help to understand the relationship between complex exposure and allergic diseases. The ELFE cohort study is collecting measurements from the dwellings of many children. The EDC has already been used once when the children were newborns and these children will be followed until they are aged 18. Another round of EDC sampling will be performed when the children are aged five. These new targets described in this article will be tested in the first frozen samples. Further investigations will be made to collect homogenous and quantitative data on allergenic exposure in dwellings.

Tables

Table 1. Primers and hydrolysis probes designed to identify cats, dogs and cockroaches by QPCR.

Target	primer & probe	Sequence (5' - 3')	Reference
Cat (<i>Felis catus</i>)	forward primer	CCCTTCTAGGAGTCTGCCTAATCTT	Knapp et al., sub. paper
	reverse primer	CGGTATATGTGCTGATGTGTAGTGT	
	probe	(FAM)AAATCCTCACCGGCCTCTTTTTGGC(TAMRA)	
Dog (<i>Canis lupus familiaris</i>)	forward primer	CCACCCACTAGCCAAAATTGTT	Knapp et al., sub. paper
	reverse primer	AAGTTCCATCAAGCAGAGATGTTAGA	
	probe	(VIC)ATAACTCATTGACCTCCCAGCGCC(TAMRA)	
cockroach	forward primer	TTCGTGTGGACGTCCTGTTG	this paper
	reverse primer	GGCAGACGGAATCCAGACA	
	probe	(VIC)TGGCCTTTTCCGAGCTGTGTCTTC(TAMRA)	

Table 2. Detection limits and repeatability of the three targets used in qPCR; correlation coefficient of the scale (r^2), mean of the Quantification Cycle obtained (Cq mean), Standard Deviation of these Cq (SD Cq), number of successful trials (n=7) for each species. In bold are the concentrations chosen for limit of quantification. (nd = absence of signal)

Conc°(fg/ μ L)	Cq mean	SD Cq	Number of success	Cq mean	SD Cq	Number of success	Cq mean	SD Cq	Number of success
	Dog ($r^2=0.992$)			Cat ($r^2 = 0,997$)			<i>Blatella germanica</i> ($r^2= 0.942$)		
50000	25.2	0.02	7	26.3	0.023	7	26.0	0.094	7
5000	28.7	0.085	7	29.6	0.049	7	29.2	0.121	7
500	32.2	0.072	7	33.0	0.206	7	35.7	0.214	7
50	35.5	0.30	7	36.5	0.391	7	36.8	0.273	7
5	39.0	0.98	7	39.5	0.466	5	39.8	0,82	7
0,5	39.8	1.93	2	nd	nd	0	40,5	0,01	2

Table 3. Distribution of housing according to categories of exposure: number of each pet, completed by A=pet never in tested room, M= presence at home but rarely in tested room, P= frequent presence in tested room

Exposition category	0	1A	1M	1P	2M	2P	3M	3P
Cat	13	0	1	7	1	1	0	1
Dog	14	3	1	5	0	0	1	0

Table 4. Number of animals, Exposure category and Cq results for each of the 24 samples. Grey cases correspond to sample with positive signal without the pet concern contact, "*" identify the sample where indirect exposure has been reported

Name of sample	Declared Number of cats	Exposure category of cat	Mean Cq for cat QPCR	Declared number of dogs	Exposure category of dog	Mean Cq for dog QPCR	Mean Cq for <i>blattella</i> QPCR
H1	0	0	45.0	0	0	45.0	45.0
H2	0	0	45.0	0	0	*36.7	45.0
H3	0	0	45.0	1	1P	35.5	45.0
H4	1	1P	36.3	1	1M	34.9	45.0
H5	1	1P	39.5	1	1A	39.4	45.0
H6	2	2M	35.1	3	3M	34.9	45.0
H7	0	0	45.0	0	0	40.6	45.0
H8	0	0	45.0	1	1P	29.4	45.0
H9	1	1M	38.8	0	0	40.6	45.0
H10	1	1P	36.9	0	0	45.0	45.0
H11	1	1P	36.6	0	0	*38.1	45.0
H12	1	1P	34.9	0	0	39.2	45.0
H13	0	0	45.0	0	0	40.6	45.0
H14	0	0	45.0	1	1P	33.7	45.0
H15	0	0	45.0	0	0	*36.3	45.0
H16	2	2P	32.8	0	0	39.7	45.0
H17	0	0	*37.7	0	0	*37.5	45.0
H18	3	3P	33.4	1	1P	32.3	45.0
H19	1	1P	37.2	1	1P	31.6	45.0
H20	1	1P	35.4	0	0	*39.2	45.0
H21	0	0	45.0	0	0	45.0	45.0
H22	0	0	45.0	0	0	*36.9	45.0
H23	0	0	45.0	1	1A	37.0	45.0
H24	0	0	45.0	1	1A	39.2	45.0

Figures

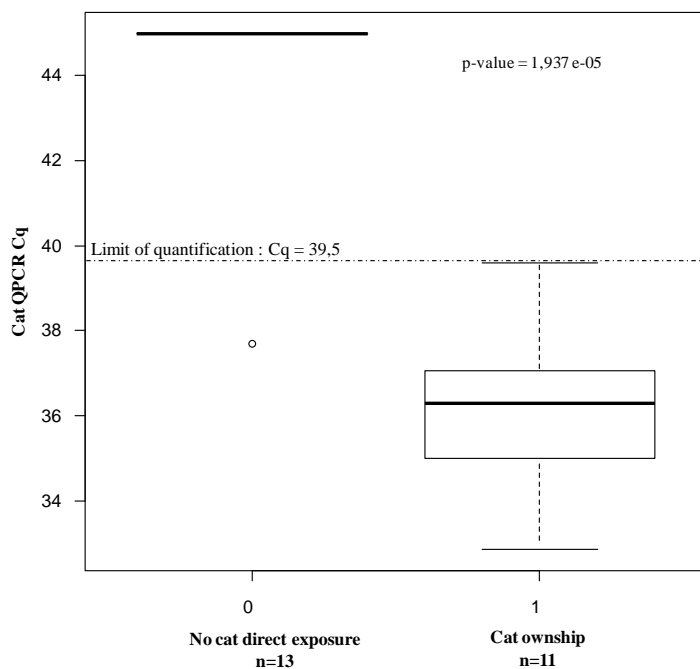


Figure 1. Boxplot with Cat QPCR results according to presence (1) or absence (0) of cat in the dwelling

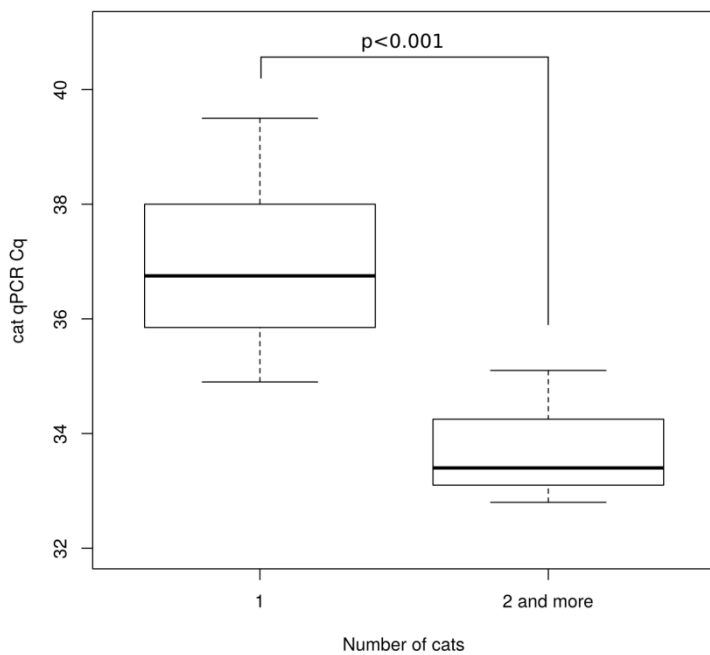


Figure 2. Boxplot with Cat QPCR results according to number of cats

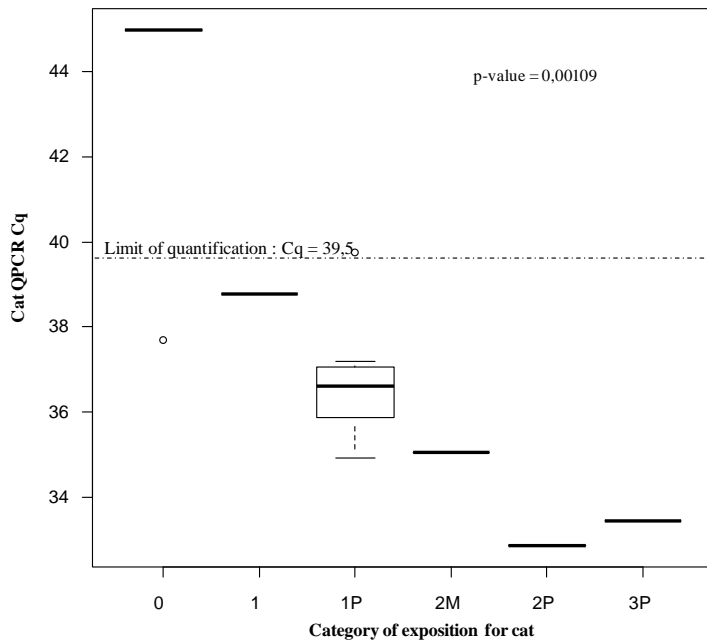


Figure 3. Boxplot with Cat QPCR results (in Cq – Inversly proportional to the concentration) according to the category of exposure (M=rarely in the room; P= often present in the room)

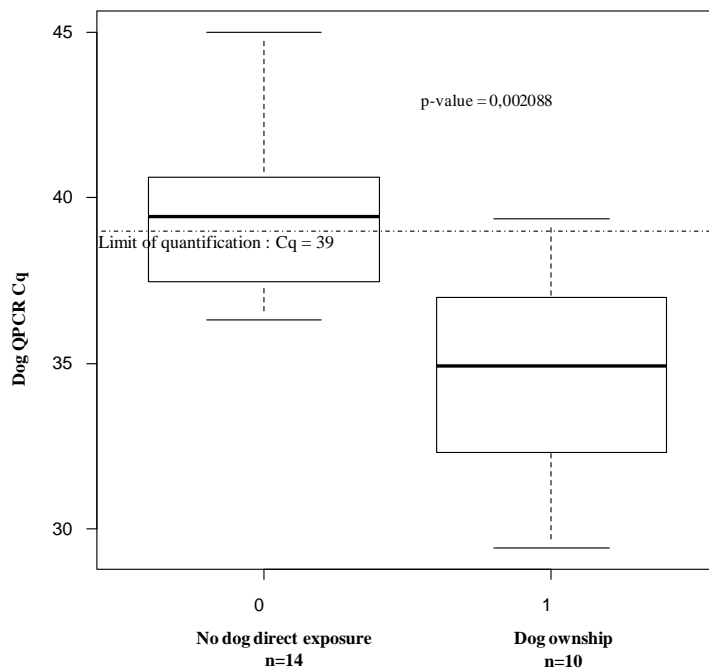


Figure 4. Boxplot with Dog QPCR results according to presence (1) or absence (0) of dogs in the dwelling

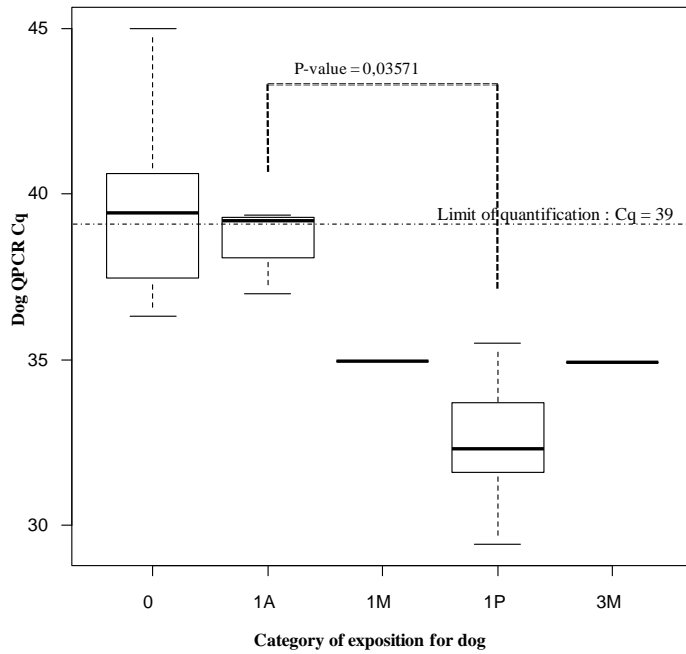


Figure 5. Boxplot with Dog QPCR results (in Cq – Inversely proportional to the concentration) according the category of exposure (A=never in the room; M=rarely in the room; P= often present in the room)

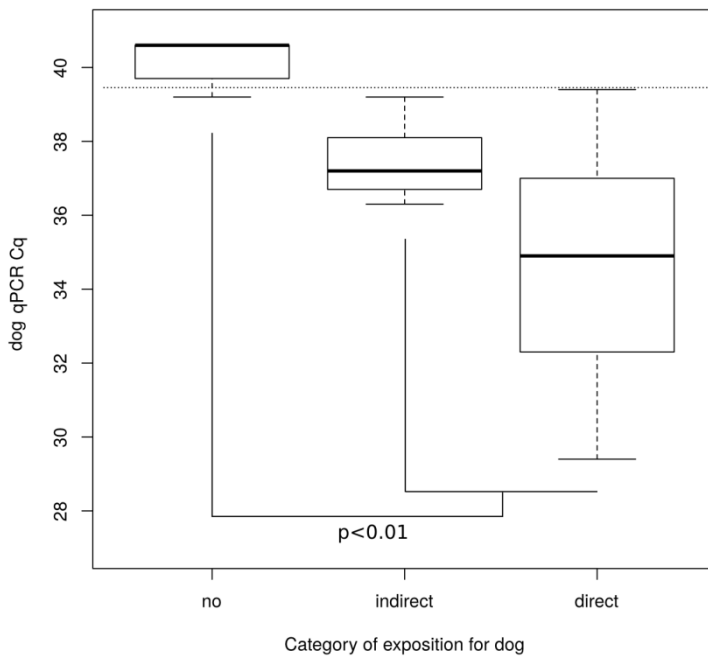


Figure 6. Boxplot with positive Dog QPCR results (in Cq – Inversely proportional to the concentration) according the type of exposure: no, indirect (outside contact but no dog residing permanently in the dwelling) direct (dog residing permanently in the dwelling)

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4. Suivi de la cohorte pour une campagne à 5 ans : le Projet Embrase

Titre du projet : EMBRASE, Environnement MicroBiotique et Risque Allergique, Suivi des Enfants à 5 ans

Collaborateurs: UMR CNRS 6249, Inserm Bordeaux, Ined

Demande financement : Demande ANR, dépôt d'une lettre d'intention, octobre 2015

Le projet EMBRASE prévoit qu'en janvier 2016, lorsque les enfants ELFE auront entre 4 et 5 ans, de nouveaux capteurs soient distribués dans les logements des familles qui avaient accepté de placer un capteur à la naissance de l'enfant (n=3193). L'objectif est de vérifier la constance (ou la variation) des profils de logements et d'évaluer le cumul de l'exposition au cours de la petite enfance. L'exposition mesurée, quel que soit le type d'échantillonneur utilisé, est partielle. Les individus ne vivent pas cloisonnés dans leurs logements et sont au contact d'autres environnements. Certaines équipes ont choisi d'étudier l'exposition dans les écoles (Jacobs et al. 2014). Toutefois, le temps passé à l'intérieur du domicile semble le plus représentatif, car il reste le plus long, notamment pour un enfant.

Il est attendu environ 80 % de participation par rapport à la première collecte de capteurs (soit un retour d'environ 2555 capteurs). Le projet EMBRASE prévoit l'analyse de ces 2555 capteurs déposés dans les logements des enfants à l'âge de 5 ans, par qPCR, sur les 20 cibles.

Dans cette même période (2015-2017), certains de ces enfants « Elfe » développeront de l'asthme et/ou d'autres symptômes allergiques. Les chiffres d'incidence de l'asthme en France étant d'environ 7 %, le chiffre attendu est d'environ 180 enfants atteints d'asthme dans la sous cohorte EBRA-Elfe.

A partir des capteurs déposés dans les logements de 120 enfants avec asthme, ainsi que dans 120 logements d'enfants sans maladie respiratoire, nous réaliserons une analyse métagénomique, permettant de dresser un bilan microbiologique complémentaire quasi exhaustif. Une liste de tous les champignons présents, y compris ceux ne cultivant pas, pourra être dressée dans les différents logements. Cet outil moléculaire, en caractérisant de façon exhaustive les agents biologiques présents dans les logements des deux groupes, permettra d'évaluer l'impact de la diversité sur les risques de développer de l'asthme et d'identifier les micro-organismes présentant un danger ou un risque particulier pour l'homme.

Une fois identifiées, les espèces (2 espèces envisagées dans un premier temps) les plus caractéristiques (par leur présence ou leur absence) des logements des enfants asthmatiques seront quantifiées par qPCR sur l'ensemble des logements de la cohorte EMBRASE (échantillonnage à la naissance et à 5 ans). Les résultats seront confrontés aux données des différents questionnaires, soit comme des données isolées, soit agrégées aux profils liés à l'asthme.

Le premier objectif est de caractériser l'environnement intérieur des jeunes enfants afin d'évaluer le cumul de l'exposition au cours de l'enfance pour les 20 cibles choisies puis d'évaluer l'impact de ce cumul d'exposition sur le développement des maladies allergiques, et notamment de l'asthme.

Le deuxième objectif est de décrire, par des analyses de séquençage haut débit, les principales espèces fongiques présentes dans les domiciles de 120 enfants présentant de l'asthme et de 120 « enfants témoins » sans asthme. Par comparaison, les micro-organismes présentant potentiellement un risque et au contraire, les micro-organismes « protecteurs », pourront être identifiés. Ces agents identifiés seront quantifiés par de nouvelles qPCR afin d'optimiser l'indicateur en le basant sur un nombre restreint de paramètres choisis parmi les 20 cibles testées *a priori* et celles testées après analyse métagénomique.

Le troisième objectif est d'identifier des relations possibles entre les micro-organismes présents dans le logement et les événements de croissance de l'enfant. En effet, parallèlement à ces objectifs concernant l'asthme, les données de croissance et de développement de l'enfant disponibles par l'intermédiaire des questionnaires ELFE (apparition de maladie autres qu'allergiques, développement cognitif de l'enfant, la dépression des parents) seront mises en lien avec les profils microbiologiques des logements.

IV. CHAPITRE 2 : CARACTERISATION D'AUTRES SITUATIONS A RISQUE, ADAPTATION DES OUTILS A LA QUESTION ENVIRONNEMENTALE POSEE

1. Composteurs industriel et risque de PHS

Titre de l'article : *Development and evaluation of a method for the quantification of airborne Thermoactinomyces vulgaris by real-time PCR*

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A. Contexte

Comme les nouveaux matériaux de construction peuvent modifier les flores de nos logements, les nouvelles activités peuvent influencer la composition de l'air en micro-organismes et avoir un impact sur la santé. Ainsi, les actinomycètes thermophiles peuvent induire des pneumopathies d'hypersensibilité en milieu professionnel. Si cette exposition (et ses conséquences) sont connues dans le cadre du poumon de fermier, elles n'ont pas été documentées pour les plateformes de compostage, où les végétaux en décomposition peuvent être une source d'actinomycètes thermophiles et leur aérosolisation un mode d'exposition pour les travailleurs.

B. Principaux résultats

Un système de sonde d'hydrolyse et d'amorces pour qPCR a été dessiné sur la zone GyrB de *Thermoactinomyces vulgaris* et validé sur 10 souches de *Thermoactinomyces vulgaris* et 82 souches d'actinomyces ou bactéries autres. Le système a montré une bonne spécificité (seules les souches de *Thermoactinomyces vulgaris* ont présenté un Cq < 25, les rares autres souches avec un signal positif (n=7) avaient un Cq >35), et une bonne sensibilité (la limite de quantification est estimée 20 copies du gene GyrB par réaction).

Quarante-quatre échantillons d'air ont été prélevés à différents points de 5 stations de compostage : 43 des 44 échantillons étaient positifs avec des valeurs comprises entre 3×10^2 et 3×10^6 copies de GyrB.m⁻³, décrivant ainsi pour la première fois l'exposition à *T. vulgaris* sur les stations de compostage.

L'article ci-dessus s'intéresse aux activités de compostage « industrielles » mais cette pratique est également de plus en plus fréquente chez les particuliers. La présence d'un bio-seau à l'intérieur du

logement soulève les mêmes interrogations. Une première étude a déjà été menée et montre que la flore ne semble pas affectée à une distance supérieure à 50 cm du seau (Naegele, Reboux, Vacheyrou, et al. 2015).



Development and evaluation of a method for the quantification of airborne *Thermoactinomyces vulgaris* by real-time PCR



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ABSTRACT

Actinomycetes are ubiquitous and some can be potentially pathogenic for humans when present in the air of some working areas. It's notably the case for *Thermoactinomyces vulgaris* in composting facilities where aerial concentrations can reach high values of more than 10^7 CFU·m⁻³. Workers exposure to these inhalable bioaerosols can be the source of various diseases. The literature reveals a lack of knowledge about risk assessment: there is neither dose-effects relationship for most agents, or threshold limit value. The objectives of this study were to develop and standardize a method to quantify workers exposure to bioaerosols. We have developed and evaluated a method to quantify airborne *T. vulgaris* based on DNA extraction of aerial microbial communities and qPCR. Four DNA extraction protocols were compared, and primers and a hydrolysis probe were designed for specific amplification of the target species (*gyrB* gene). This method was compared to traditional methods based on viable or cultivable counting by epifluorescence microscopy or plating on selective media. The method was applied on environmental bioaerosols sampled under real exposure conditions in composting plants. We demonstrate that the method to quantify *T. vulgaris* in bioaerosols is specific, sensitive and repeatable. We demonstrate the occurrence and quantified *T. vulgaris* in the atmosphere of composting facilities with concentrations ranging from 3×10^2 to 3×10^6 m⁻³.

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1. Introduction

Actinomycetes are Gram positive bacteria producing branched hyphae and spores. They are ubiquitous in the environment and are associated with organic matter and biodegradation process. Actinomycetes can be present at high concentration levels in the air of numerous working areas, particularly those associated with agricultural activities and waste composting (Miyashita et al., 1982; Strom, 1985; Lacey, 1997; Le Goff et al., 2010). Actinomycetes (i.e. *Streptomyces albus*, *Micromonospora halophytica*, *Saccharomonospora viridis*) are able to produce small spores with aerodynamic diameters ranging from 0.9 to 1.5 μm (Grinshpun et al., 1997; Reponen et al., 1998; Madelin and Johnson, 1992). They are able to enter the respiratory track with a high probability and may deposit in the deepest part of the lungs (ICRP, 1994; CEN, 1993). Worker exposure to bioaerosols containing thermophilic actinomycetes spores may induce numerous health disorders, hypersensitivity pneumonitis (HP), being the most severe (Kagen et al., 1981; Kleyn et al., 1981;

Pepys et al., 1990; Lacey and Dutkiewicz, 1994; Dalphin et al., 1998–1999; Jamey, 2008). Risks associated with occupational exposure to *Thermoactinomyces vulgaris*, i.e. dose-effects relationship and threshold limit value for occupational exposure, remain to be assessed.

Among thermophilic actinomycetes, *T. vulgaris* represents one of the main causing agents of HP, such as farmer's lung disease, mushroom-worker's lung disease and bagassosis, (Barboriak et al., 1972; Kleyn et al., 1981; Pepys et al., 1990; Reijula, 1993; Dalphin et al., 1998–1999). This species is widely present in high temperature biotopes like composts during the heating phase of the composting process (Millner et al., 1980; Lacey, 1997). It produces true endospores ranging in size from 0.5 to 1.5 μm diameter (Cross, 1970; Lacey, 1989; Reponen et al., 1998).

The most often used technique for analyses of microbiological aerosols containing actinomycetes is plate counts on agar media. Well-adapted for the investigation of new unknown environments, standard culture has however some drawbacks: the need for a huge diversity of culture media and incubation parameters and the underestimation of microbial concentrations in the samples. Plate counts only give access to cultivable microorganisms and colony masking may occur due to overgrowth of fast growing microorganisms on Petri dishes. Alternative culture-independent methods such

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as, epifluorescence microscopy (after DAPI or acridine orange staining), flow cytometry or fluorescence in situ hybridization (FISH), exist methods but have been poorly assessed for the enumeration of actinomycetes.

Previous studies reported fungi in bioaerosols such as *Penicillium* sp. (Lignell et al., 2008), *Aspergillus* sp. (Haugland et al., 2002; McDevitt et al., 2004; Lignell et al., 2008; Pietarinen et al., 2008), *Cladosporium* sp. (Zeng et al., 2006; Lignell et al., 2008; Pietarinen et al., 2008) or *Stachybotrys* sp. (Haugland et al., 2002; Lignell et al., 2008; Pietarinen et al., 2008), *Fusarium circinatum* (Schweigkofler et al., 2004), or yeast-type fungi like *Candida albicans* species (Haugland et al., 2002). Several pathogenic bacteria were also reported in bioaerosols such as *Mycobacterium tuberculosis* (Chen and Li, 2005), *Legionella pneumophila* (Sirigul et al., 2006), *Bacillus anthracis*, *Brucella* sp. or *Yersinia pestis* (Makino and Cheun, 2003; Fykse et al., 2008).

The use of quantitative real-time polymerase chain reaction (qPCR) based protocols represents a very attractive alternative for the detection and the quantification of microorganisms in bioaerosols (Bustin et al., 2009). This method is increasingly used for environmental detection and quantification of microorganisms, on substrates (dust) as well as in indoor air (house, water-damaged buildings, working areas...). To date, mold specific quantitative PCR (MSQPCR) was designed for environmental detection of more than 100 molds, and combined results of the 36 of them were used to design the Environmental Relative Moldiness Index (ERMI) for mold exposure assessment of different patient groups (Reponen et al., 2011; Vesper, 2011). As well, qPCR was used for global assessment of bacterial load in aerosols from contaminated environments (Oppliger et al., 2008; Blais Lecours et al., 2012), and environmental studies targeting mainly *Streptomyces* species, in dust or building materials (Rintala and Nevalainen, 2006; Pietarinen et al., 2008). To date, implementation of species-specific or genus-specific qPCR for airborne actinomycetes detection is scarce, and was only recently developed to quantify *Saccharopolyspora rectivirgula* and *Streptomyces* spp. in bioaerosols (Schafer et al., 2011; Kaarakainen et al., 2011; Blais Lecours et al., 2012).

The aim of this study was to develop a standardized method, based on qPCR, for the survey of worker exposure to bioaerosols contaminated with actinomycetes. *T. vulgaris* was used as a model microorganism because of its wide distribution in the environment and its potential health hazard. A qPCR based method was designed and then validated for the quantification of *T. vulgaris* in bioaerosols. This method has been compared with traditional plate count method and validated under laboratory conditions using pure cultures and experimental bioaerosols. Then, it is has been evaluated in real exposure conditions to monitor the occurrence of *T. vulgaris* in the atmosphere of five composting plants.

2. Materials and methods

2.1. Origin, maintenance and growth conditions of microbial strains

The strains used for sequence alignment and oligonucleotide specificity testing are listed in Table 1. They were used to design specific primers and a hydrolysis probe for the specific quantification of *T. vulgaris* by qPCR. Collection strains were from DSMZ (German Collection of Microorganisms and Cell Cultures), CIP or UMIP (Pasteur Institute Collection), IHEM (Biomedical Collection of the Scientific Institute of Public Health) and ATCC (American Type Culture Collection). Strains were cultured according to the protocols given by the supplier. Strains from the environment were isolated from hay at the University Hospital of Besançon (Jamey, 2008) and from bioaerosols sampled in composting plants by filtration (37 mm closed-face cassettes). Environmental strains were isolated and identified by 16S rDNA sequencing at the University Hospital of Besançon. The model microorganism chosen for the rest of the study is the type strain of *T. vulgaris* (ATCC 43649). Stock culture was prepared by inoculation of the strain in 750 mL cell culture flask containing 90 mL of BBL™ Trypticase™ Soy Agar TSA

Table 1

Type of wastes treated in the five composting plants where environmental bioaerosols were sampled. Green waste corresponds to plant wastes (garden trimmings, wood...). Biowaste corresponds to the biodegradable fraction of municipal solid waste (after selective sorting).

Composting facility designation	Type of waste treated			
	Green waste	Sewage sludge	Biowaste	Municipal solid waste
1	x	x	x	
2	x		x	x
3	x	x		
4	x			x
5	x		x	

(Becton Dickinson, USA) and incubation at 50 °C for 15 days, under high relative humidity.

2.2. Design of the primers and probe for qPCR

We targeted the gene *gyrB* which encodes the beta subunit of DNA gyrase (accession number AB242203) in *T. vulgaris*. *gyrB* is often used as a target gene for qPCR in bacteria (Kasai et al., 2000; Shen et al., 2005). *gyrB* gene is considered as a single copy gene present in all bacteria (Dauga, 2002). However, Rodrigues and Tiedje (2007) reported from the analysis of 194 genomes from the Joint Genome Institute (<http://genome.jgi.doe.gov/>) that 11.8% of the bacterial species harbored 2 to 3 copies of the *gyrB* gene. In the absence of complete genome data for *T. vulgaris* we cannot exclude the occurrence of multiple copies of this gene in *T. vulgaris*. *gyrB* sequences from several actinomycetes species (*Thermoactinomyces* sp., *Laceyella* sp., *Mechercharimyces* sp...) were retrieved from GenBank and aligned with ClustalW (through the bioinformatic platform Mobyle, Pasteur Institute). From the alignment, variable regions of *gyrB* were selected and used to design primers and a hydrolysis probe specific for *T. vulgaris* using the Primer Express software (Applied Biosystems, USA). The specificity of the primers and probe was tested "in silico" against the GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) on the National Center for Biotechnology Information (NCBI) website. Selected primers were: forward primer Tvu-F751 (5'-GCC GAT CAT TTC CAA ACC TAT TT-3'), reverse primer Tvu-R824 (5'-GCC ATC ACG GCT TTG TTA ATA ATC-3') and the hydrolysis probe was Tvu-S776 (5'-FAM-ACG AGC ATC CCG CCG AAG CG-TAMRA-3'). The corresponding PCR product has a 74 bp length. All purified oligonucleotides (Reverse-phase cartridge Purification for primers and High Performance Liquid Chromatography for probes) were obtained from Sigma-Aldrich (USA).

2.3. Genomic DNA extraction

Four protocols for genomic DNA extraction from bioaerosols were tested and compared. The first protocol is based on the FastDNA® SPIN kit for soil (MP Biomedicals, USA). The second protocol is based on the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, USA). The two last protocols tested are based on the DNeasy® Blood and Tissue Kit (Qiagen®, Netherlands); DNA was extracted following the supplier recommendations, using either the Gram negative bacteria specific protocol or Gram positive bacteria specific protocol. In preliminary experiments, we determined that the FastDNA® SPIN kit for soil associated to the FastPrep automated homogenizer (MP Biomedicals, USA) and following the manufacturer's protocols was the most efficient method to extract DNA from environmental bioaerosols (higher DNA yield). This DNA extraction method was chosen and used for both experimental and environmental bioaerosols as well as for DNA extraction from pure cultures of *T. vulgaris*.

2.4. qPCR assay

PCR reactions contained: 12.5 μL of iQTM supermix (Bio-Rad®, USA) at a final concentration of 1X, 1 μL of each primer (final concentration of 300 nM), 1 μL of the probe (final concentration of 200 nM), 7.5 μL of ultrapure water for molecular biology and 2 μL of DNA sample. PCR assays were done on a Bio-Rad® CFX96TM real-time PCR system with the following cycles: 95 °C for 10 min followed by 40 cycles 95 °C for 10 s and 60 °C for 1 min. Results were analyzed with the software Bio-Rad CFX Manager, version 1.0.1035.131 (Bio-Rad, USA). Absolute quantification was achieved using standard DNA dilutions. We used as a standard the recombinant plasmid containing one copy of the 74 bp *gyrB* fragment from *T. vulgaris* (ATCC 43649) cloned in the pCRII-TOPO vector (Invitrogen). A standard curve was obtained by amplifying serial dilutions of this plasmid from 1 to 10⁵ copies per reaction (Fig. 1). Standard curves were validated according to the PCR efficiency (in the range of 80% to 120%) and to the coefficient of determination r^2 (above 0.98). For bioaerosols, final results were expressed as *gyrB* copy number $\cdot\text{m}^{-3}$ of sampled air.

2.5. Quantification of *T. vulgaris* in liquid cultures

An experiment was carried out in laboratory conditions to compare qPCR, epifluorescence microscopy and culture for the enumeration of *T. vulgaris* in liquid culture. For the comparison, *T. vulgaris* strain (ATCC 43649) was grown in Tryptic Soy Broth TSB (AES Chemunex, France), at 50 °C with shaking at 80 rpm for 15 days. Then, the culture was centrifuged during 7 min at 7000 rpm and at 4 °C. The supernatant was eliminated and pellet was resuspended in ultrapure sterile water. Five serial 10 fold dilutions of the suspension were done in a solution of Tween® 80 at 0.05% and each sample was divided into three aliquots (replicates). Each aliquot was analyzed to quantify *T. vulgaris* DNA (qPCR following DNA extraction), total spores by epifluorescence and cultivable bacteria.

2.6. Quantification of *T. vulgaris* in experimental bioaerosols

An experiment was carried out in laboratory conditions to compare qPCR, epifluorescence microscopy and culture for the enumeration of *T. vulgaris* in experimental bioaerosols. Bioaerosols were produced according to the protocol we previously optimized and described (Simon et al., 2013). Six cell culture flasks containing freshly grown *T. vulgaris* strain were prepared as described above. Cells (spores and mycelium) were then washed from the agar surface by adding 15 mL of a solution of Tween 80 (Merck, Germany) at 0.05% and approx. 400 sterile glass beads (1.5 mm diameter) in the flasks.

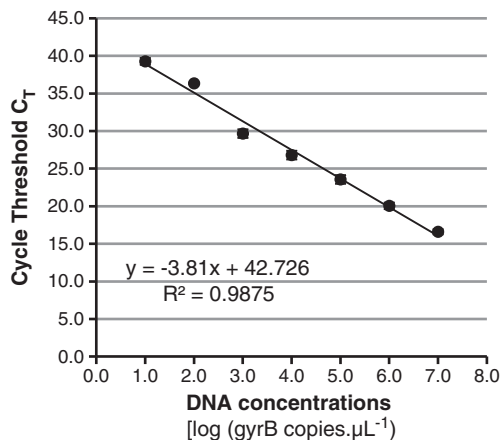


Fig. 1. Standard curve used for absolute quantification of *T. vulgaris* in samples by qPCR (mean values of 54 standard curves realized during the work). Error bars represent the 95% confidence interval associated to the values.

After a gentle agitation, the solutions recovered from the 6 flasks were mixed and filtered through a sintered glass filter (10 to 16 μm pore size). The obtained suspension was centrifuged 7 min at 7000 rpm, 4 °C and the pellet was re-suspended in 50 mL of ultrapure sterile water. The latter suspension was immediately used to produce an experimental bioaerosol using the bioaerosol generator device fully described by Simon et al. (2011). Briefly, the device includes a bubbling-type generator and a 12 L chamber for conditioning and sampling bioaerosols. Microorganisms are aerosolized by bubbling compressed air through a film of liquid culture. The sampling chamber is equipped with 6 sampling tubular probes allowing the simultaneous connection of 5 bioaerosol samplers and a particle counter. For the whole study, the generation parameters were those described previously (Simon et al., 2013): a complementary air flow rate Q_E of 20 $\text{L} \cdot \text{min}^{-1}$, a liquid film height H_{liq} of 8 mm and a relative humidity HR of 50 (± 2) %. Bioaerosols with different cell concentrations were produced by changing the bubbling flow rate Q_G from 0.4 to 4.7 $\text{L} \cdot \text{min}^{-1}$. The entire generation process has been repeated 12th in our study. Each generated bioaerosol was simultaneously sampled using five 37 mm closed-face cassettes (CFCs) connected to the chamber (see below). The duration of sampling was about 50 (± 5) min with stable bioaerosol. All CFCs were analyzed to quantify *T. vulgaris* DNA (qPCR following DNA extraction), total spores by epifluorescence and cultivable bacteria.

2.7. Detection and quantification of airborne *T. vulgaris* in composting facilities

Environmental bioaerosols were sampled from the atmosphere of composting plants. Sampling campaigns were organized, between April and June 2011 in 5 composting plants processing different types of waste (Table 1). Stationary samples were collected with 37 mm open-face cassettes (OFCs) held at 1.7 m above ground level. The duration of sampling was about 4 h. For each composting facility, samples were collected during the 3 main exposing tasks and for three consecutive days. Samples were then brought to the laboratory and analyzed within 24 h to quantify *T. vulgaris* by qPCR following DNA extraction and enumeration of thermophilic cultivable bacteria.

2.8. Bioaerosol sampling methods

Bioaerosol sampling was performed using the filtration method but the two versions of the method were used throughout the study depending on the aims of the measurements. For the first version, sampling was done by air filtration using 37 mm polystyrene 3-piece closed-face cassettes (CFCs) provided by Millipore. Each CFC contained a support pad (thick cellulose absorbent pad, Millipore®, USA) and a polycarbonate membrane (NucleporeTM membrane, 0.8 μm pore size, Whatman®, United Kingdom). It was connected to a personal sampling pump Gilian® GilAir-3 or GilAir-5 (Sensidyne®, Germany) at an average flow rate of 2 $\text{L} \cdot \text{min}^{-1}$ for about 50 (± 5) min when the bioaerosol was stabilized. For the second version, airborne microbiological particles were sampled by the filtration method using 37 mm polystyrene 3-piece open-face cassettes (OFCs) provided by Millipore. Samples were collected with OFCs mounted with a sterile polycarbonate filter (Nuclepore®, 0.8 mm pore size) and a backing cellulose pad (Millipore®, thick cellulose absorbent pad). Each OFC was connected to a pump operating at a 25 $\text{L} \cdot \text{min}^{-1}$ average flow rate (General Electrics, models 1531-176A-G445-X and -G629-X). CFCs were used for assays with experimental bioaerosols and for the isolation and identification of environmental actinomycetes strains (duration of sampling approx. 90 min.). OFCs were used for bioaerosol sampling on composting facilities (duration of sampling approx. 300 min.).

2.9. Pre-treatment of bioaerosol samples before analysis

Microbial particles collected on polycarbonate membranes were resuspended in a sterile solution before being analyzed. This was done during an elution step described previously (Duquenne et al., 2012). Briefly, 10 mL of a sterile solution containing $0.1 \text{ g}\cdot\text{L}^{-1}$ of Tween 80® (Merck, Germany) and $1 \text{ g}\cdot\text{L}^{-1}$ of peptone (Oxoid, United Kingdom) were introduced into the cassette. The cassettes were then shaken at 2000 rpm for 20 min (Multi reax, Heidolph®, UK) and the elution was recovered using a syringe and transferred to a sterile tube for further analysis.

2.10. Total spores counting by epifluorescence

Epifluorescence microscopy after DAPI (4',6-diamidino-2-phenylindole) staining allows us to count all the cells present in a sample (live and dead cells) (Kepner and Pratt, 1994). A minimal volume of 10 mL of sample was mixed with DAPI (Sigma-Aldrich®, USA) solution ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$). The suspension was then filtered onto a 25 mm polycarbonate membrane (Millipore®, Isopore™ black membrane, $0.22 \mu\text{m}$ pore size). The membrane was rinsed with sterile ultrapure water, mounted between slide and cover slip with paraffin oil immersion. Spores were observed using a fluorescence microscope (Leica DM2500) at $1000\times$ magnification. The microscope is connected to a video camera (Qicam Qimaging Fast1394) and spores were counted on 20 or 30 spaced fields using the software Histolab version 7.4.0 (Microvision Instruments). The concentrations were expressed in number of spores per milliliter of suspension analyzed (Mesa et al., 2003) or were given per cubic meter of sampled air ($\text{spores}\cdot\text{m}^{-3}$).

2.11. Cultivable thermophilic bacteria enumeration

Thermophilic bacteria were enumerated by the spread plate technique. This was done directly from liquid culture or bacterial suspension obtained from CFCs and OFCs after elution. Bacterial suspensions were serially diluted in tryptone-salt solution (AES Chemunex, France). Appropriate dilutions were spread plated on BBL™ Trypticase™ Soy Agar (Becton Dickinson, USA). Two Petri dishes were inoculated per dilution for three successive dilutions and incubated at $50 \text{ }^\circ\text{C}$ (for laboratory assays) or $56 \text{ }^\circ\text{C}$ (for real exposure condition assays) for 3 days. Colonies were counted daily on each dish. After incubation, only dishes containing fewer than 300 colonies were used to calculate the sample concentration of viable cultivable. The calculations are described in a food microbiology standard (AFNOR, 1996a). Cultivable thermophilic bacteria concentrations were expressed as Colony Forming Units per milliliter of suspension analyzed ($\text{CFU}\cdot\text{mL}^{-1}$) or per cubic meter ($\text{CFU}\cdot\text{m}^{-3}$).

2.12. Statistical analysis

All statistical analysis (arithmetic means, standard deviations, and confidence intervals) and graphics were computed with Microsoft® Office Excel® 2007 (Microsoft Corporation, USA). ANOVA tests (95% confidence) were done with log transformed values using the software Statgraphics Centurion XV, version 15.2.00 (StatPoint Technologies, USA).

3. Results

3.1. Validation of the qPCR detection system

The primer pair Tvu-F751/Tvu-R824 and the Taqman probe Tvu-S776, respectively corresponding to the nucleotides 751–773, 824–847 and 776–795 of the *gyrB* gene of *T. vulgaris* were found to be specific for this species. The BLAST analysis showed that the corresponding oligonucleotide sequences matched perfectly to *T. vulgaris* *gyrB* gene sequence (coverage of 100% associated with an e-value of

1.0×10^{-9}). Significantly similar sequences were found only in *Thermoactinomyces intermedius* (query coverage 64%, e-value of 2.0×10^{-8}). The qPCR detection system was then tested using genomic DNA extracted from 93 bacterial strains belonging to 19 genera. The results are summarized in Table 2. Ten of the eleven *T. vulgaris* tested strains were detected with cycle threshold (C_T) values below 25 cycles. One *T. vulgaris* strain was detected with a C_T value of 35 cycles. All the other tested strains (82 microorganisms belonging to 18 different genera) were not detected ($n=75$) or gave a C_T value above 35 cycles ($n=7$). The absolute quantification from standard curve appears to be highly reproducible (Fig. 1). The log (gene copy number) and the C_T over a wide range of concentrations (1 to 10^7 gene copies per μl of solution) were strongly correlated ($n=7$; $r=-0.9937$; $p=0.0000$). The detection limit of the qPCR detection system is low (20 copies of *gyrB* gene per PCR reaction) indicating a high sensitivity.

Pure cultures of *T. vulgaris* strain ATCC 43649 were analyzed by total spore counting (epifluorescence), enumeration of cultivable bacteria and qPCR. Results are given in Fig. 2. A linear regression between log (*gyrB* copy number) and log (cultivable bacteria concentration) variables is highly significant ($n=6$; $r=0.997$; $p=0.0000$). The adjusted model explained 99.31% of the log (*gyrB* copy number) variability and there was no serial correlation of the residuals ($p=0.4296$) which confirms model validation. Similar results were obtained between log (*gyrB* copy number) and log (total spore concentration) ($n=6$; $r=0.998$; $p=0.0000$). The model explains 99.52% of log (*gyrB* copy number) variability. As previously, this model is validated by the lack of a serial correlation of the residuals ($p=0.8947$).

3.2. Detection of *T. vulgaris* in experimental bioaerosols

We were able to detect *T. vulgaris* in experimental bioaerosols by qPCR targeting the *gyrB* gene. Results are summarized in Fig. 3. Linear regressions were computed between log (*gyrB* copy number) and log (concentration of cultivable *T. vulgaris*) or log (total spore concentration) for variable cell concentrations in bioaerosol. The linear regression between log (*gyrB* copy number) and log (cultivable bacteria concentration) variables was highly significant ($n=12$; $r=0.861$; $p=0.0003$) (Fig. 3). The adjusted model explains 74.09% of variability from the log (*gyrB* copy number) variable and there was no serial correlation of the residuals ($p=0.7388$) which confirms model validation. The linear regression between log (*gyrB* copy number) and log (total spore concentration) variables was also significant ($n=12$; $r=0.819$; $p=0.0011$). The model explains 67.11% of log (*gyrB* copy number) variability. This model was validated by the lack of a serial correlation of the residuals ($p=0.6714$).

Finally, DNA concentration is on average 16 times higher than cultivable concentration; the averaged difference between log (*gyrB* copy number) and log (cultivable bacteria concentration) is 1.11 ± 0.36 . qPCR overestimated *T. vulgaris* concentration in experimental bioaerosols by about one log (16 fold) compared to classical cultivable enumeration.

3.3. Detection and quantification of *T. vulgaris* in the atmosphere of composting plants

Environmental bioaerosol samples were collected in real exposure conditions in composting plants and analyzed by the culture method and by qPCR. Forty four samples were collected during the campaign and one was lost during the transport. Levels of cultivable thermophilic bacteria in the air depended either on the composting plant, the working activity and the period at which the samples were collected (Fig. 4). Two samples from the composting plant 1 lead to concentrations over $7.2\times 10^6 \text{ CFU}\cdot\text{m}^{-3}$ (overlapping colonies on Petri dishes). When exploiting the other samples, cultivable thermophilic bacteria numbers ranged from 10^3 to $10^7 \text{ CFU}\cdot\text{m}^{-3}$ of the air of the composting facilities that we investigated (Fig. 4). *T. vulgaris* was detected by qPCR in 43 samples among the 44 collected samples at concentrations between

Table 2

Organisms used for testing oligonucleotides specificity and associated results. DSMZ, CIP, UMIP, ATCC and IHEM are collection strains while WT strains environmental isolates identified by the CHU Besançon. Results are given with C_T (cycle threshold) and ND (not detected) means that no signal was observed after 40 PCR cycles.

SPECIES (samples with a DNA mean concentration of $6.76 \pm 2.17 \mu\text{g} \cdot \text{mL}^{-1}$)	C_T
<i>Thermoactinomyces vulgaris</i> ATCC 43649 and WT 01	<25
<i>Laceyella sacchari</i> WT 02	>35
<i>Bacillus pumilus</i> DSMZ 27	ND
<i>Escherichia coli</i> CIP 53.126	ND
<i>Laceyella putida</i> DSMZ 44608	ND
<i>L. sacchari</i> WT 06	ND
<i>Marinactinospora thermotolerans</i> WT 02	ND
<i>Micromonospora halophytica</i> ATCC 27596	ND
<i>Nocardioopsis alba</i> DSMZ 43377, DSMZ 44552 and WT 06	ND
<i>Nocardioopsis composita</i> DSMZ 44551	ND
<i>Pseudomonas fluorescens</i> CIP 69.13	ND
<i>Saccharomonospora azurea</i> DSMZ 44631	ND
<i>Saccharomonospora caesia</i> DSMZ 43068	ND
<i>Saccharomonospora cyanea</i> DSMZ 44106	ND
<i>Saccharomonospora glauca</i> DSMZ 43769, DSMZ 43770, WT 03 and 05	ND
<i>Saccharomonospora viridis</i> DSMZ 43017	ND
<i>Saccharopolyspora rectivirgula</i> DSMZ 43747	ND
<i>Streptomyces albus</i> subsp. <i>albus</i> DSMZ 40313 and ATCC 3004	ND
<i>Streptomyces californicus</i> ATCC 3312	ND
<i>Streptomyces coelicolor</i> DSMZ 40233	ND
<i>Streptomyces griseoflavus</i> IHEM 14577	ND
<i>Streptomyces</i> sp. WT 03 and 10	ND
<i>Thermoactinomyces intermedius</i> DSMZ 43846	ND
<i>Thermobifida alba</i> DSMZ 43795	ND
<i>Thermobifida fusca</i> DSMZ 43792	ND
SPECIES (samples with a DNA mean concentration of $12.74 \pm 1.96 \mu\text{g} \cdot \text{mL}^{-1}$)	C_T
<i>T. vulgaris</i> WT 02, 03, 04, 05, 07, 08, 09 and 11	<25
<i>Brevibacillus borstelensis</i> WT 01	>35
<i>L. sacchari</i> WT 07 and 09	>35
<i>S. azurea</i> WT 01	>35
<i>Streptomyces</i> sp. WT 05	>35
<i>T. vulgaris</i> WT 06	>35
<i>T. fusca</i> ATCC BAA-629	>35
<i>Aspergillus niger</i> UMIP 1431.83	ND
<i>Bacillus atropheus</i> CIP 107159	ND
<i>Enterococcus hirae</i> CIP 58.55	ND
<i>L. sacchari</i> WT 03, 04, 05 and 08	ND
<i>M. thermotolerans</i> WT 01, 03 and 04	ND
<i>Nocardia carnea</i> WT 01	ND
<i>N. alba</i> WT 01, 02, 03, 04, 05, 07 and 08	ND
<i>Pseudomonas aeruginosa</i> CIP 82.118	ND
<i>Saccharomonospora azurea</i> WT 02, 03 and 04	ND
<i>Saccharomonospora glauca</i> WT 01, 02 and 04	ND
<i>Saccharomonospora viridis</i> WT 01	ND
<i>Saccharopolyspora</i> sp. WT 01	ND
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> CIP 4.83	ND
<i>Streptomyces cellulosa</i> WT 01 and 02	ND
<i>Streptomyces</i> sp. WT 01, 02, 04, 06 to 09 and 11 to 15	ND
<i>Streptomyces thermoatroviridis</i> WT 01	ND
<i>T. alba</i> WT 01 and 02	ND

3×10^2 and 3×10^6 *gyrB* copies $\cdot \text{m}^{-3}$. The PCR negative sample was collected at the exit of a fermentation tunnel (5th sample from composting plant number 4). *T. vulgaris* was found in the two other samples collected at the same sampling point (1st and 6th sample from composting plant number 4). Concentrations of cultivable thermophilic bacteria were from 0.4 to 585 higher than those measured for *T. vulgaris* by qPCR, except for one sample from the composting plant number 3. A significant and moderately strong correlation was observed between cultivable bacteria and *T. vulgaris* by qPCR ($n=40$; $r=0.57$; $p=0.0001$).

4. Discussion

We have designed a new method based on real time PCR to detect and quantify *T. vulgaris* in bioaerosols. This method is specific for

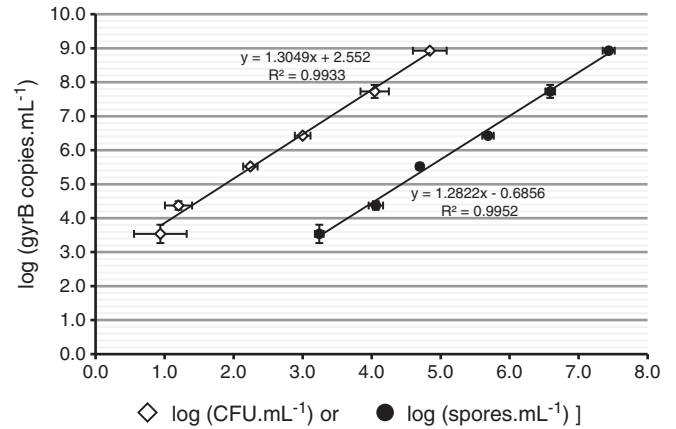


Fig. 2. Correlation between *gyrB* copy number detected by qPCR and microbial counts from pure cultures of *T. vulgaris*: cultivable bacteria concentration \diamond , total spores (\bullet). Error bars represent the 95% confidence interval of three repetitions.

T. vulgaris as demonstrated by the absence (or weak) PCR signals obtained with closely related actinomycetes species tested.

Laboratory experiments using pure liquid culture and experimental bioaerosols showed that *gyrB* copy numbers of *T. vulgaris* were directly correlated to the numbers of bacterial cells given by plate counts and epifluorescence microscopy. These results demonstrate the usefulness of the qPCR method we developed to quantitatively assess *T. vulgaris* in controlled conditions. They also provided data proving the robustness of the method.

Data from laboratory experiments revealed that total bacterial counts (epifluorescence and qPCR) are higher than cultivable ones. Indeed, assays with *T. vulgaris* liquid cultures revealed that counts of cultivable bacteria were from 280 to 1300 times lower than the total bacteria counted by epifluorescence and from 1370 to 20000 times lower than the total bacteria counted by qPCR. These results were expected since culture methods are known to underestimate the real number of naturally existing microorganisms (Amann et al., 1995). The underestimation is attributed to the selective culture conditions (culture medium, growth temperature...) as well as the physiological status of cultured microbial cells (viable but non-cultivable, dead, dormant...) that do not support the growth of the entire microbial population on Petri dishes. On the other hand, epifluorescence microscopy was used after DAPI staining on *T. vulgaris* cell cultures and experimental bioaerosols to monitor total spores. Thus, we did not take into account the vegetative cells (mycelium form) in our study. Furthermore, we didn't use this technique on environmental bioaerosols as several drawbacks were reported as in previous studies for its application in highly

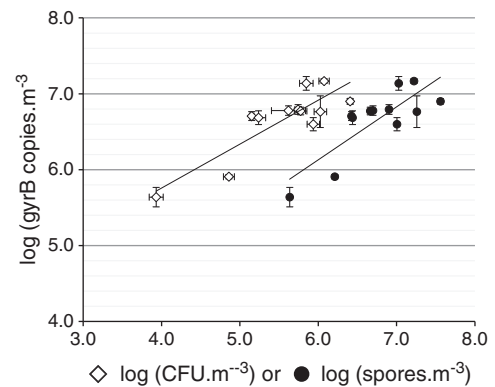


Fig. 3. Correlation between microbial counts and *gyrB* copy number detected by real time PCR from experimental bioaerosols of *T. vulgaris*: cultivable bacteria concentration \diamond and total spore concentration (\bullet). Error bars represent the 95% confidence interval of five repetitions. There is no repetition for the results of total spores.

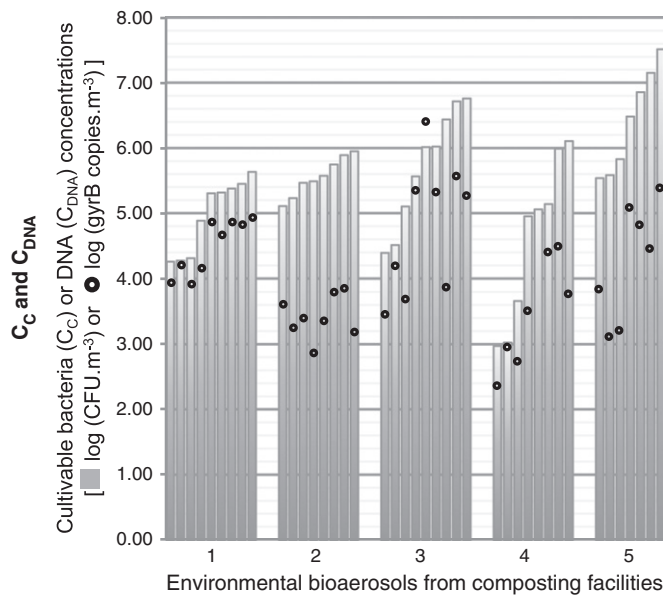


Fig. 4. DNA and cultivable thermophilic bacteria concentrations found in environmental bioaerosols sampled in five composting plants: viable cultivable concentrations (C_C) log (■) and DNA concentrations (C_{DNA}) log (●). Samplers were open-face cassettes; average flow rate: $25 \text{ L} \cdot \text{min}^{-1}$; sampling length: 167 to 375 min. No repetitions were made. 1 to 5: composting plant facilities described in Table 1.

contaminated environments such as composting facilities (Duquenne et al., 2012; Cartwright et al., 2009). The qPCR technique counts gene copies whatever their origins (mycelium, spores, free DNA...).

Results from *T. vulgaris* experimental bioaerosols revealed the same tendency but with lower ratio between cultivable and total counts. Indeed, counts of cultivable bacteria in experimental bioaerosols were from 2 to 10 lower than the total bacteria counted by epifluorescence and from 3 to 45 lower than the total bacteria counted by qPCR. The differences between liquid culture and experimental bioaerosols were assigned to the physiological status of *T. vulgaris* spores. On one hand, the cultural conditions for the enumeration of *T. vulgaris* were chosen among several conditions we have tested in a separated study (not detailed in the present manuscript). This was done by simultaneously comparing 3 growth temperatures ($45 \text{ }^\circ\text{C}$, $50 \text{ }^\circ\text{C}$ and $56 \text{ }^\circ\text{C}$) and 3 agar media (non selective agar TSA, Amner's R8 thermophilic actinomycetes (Amner et al., 1989) and DifcoTM actinomycete isolation agar supplemented with glycerol (Becton Dickinson, USA)). Results showed that growths of *T. vulgaris* on TSA agar at $50 \text{ }^\circ\text{C}$, were optimal conditions for enumeration of cultivable bacteria (data not shown) limiting the cause of non cultivability. On the other hand, the development cycle of *T. vulgaris* involves the formation of endogenous spores also called endospores. Most actinomycetes spores can only germinate upon favorable environmental conditions (nutrients, growth temperature...), but *Thermoactinomyces sp.* endospores are constitutively dormant and need to be activated to germinate (Ensign, 1978; Locci, 2006). Indeed, spore dormancy would lead to the lack of culture on agar without prior activation and considerably underestimates the number of viable and cultivable *T. vulgaris* measured by the plate count method in samples (Attwell and Colwell, 1982). This may partly explain the non cultivability observed in our experiments with liquid cultures. Previous studies have shown that *T. vulgaris* dormant spores could be thermally reactivated by both a brief cooling followed by a heat shock or a prolonged cooling without heat shock (Kalakoutsii and Agre, 1976; Ensign, 1978). Our results from experimental bioaerosols suggest that the analytical process we used (preparation of the bacterial culture, generation airborne spores, and method for bioaerosol sampling) have impacted the dormant state of spores, improving their germination and increasing cultivable counts.

Total bacteria counted by qPCR were from 7 to 36 higher than those counted by epifluorescence for the liquid culture assays and from 1 to 10 for the experimental bioaerosol assays. The difference between the two techniques may be due to the vegetative cells (mycelium form) that were not taken into account in the epifluorescence microscopy protocol we used whereas they are counted with qPCR. The ratio between total counts and DNA is not well documented in literature and depends on the copy number of the target gene in cells. The copy number of *gyrB* gene in *T. vulgaris* gene is believed to one but this can't be easily extrapolated from our data.

Results from real exposure conditions experiments show that the concentrations of cultivable thermophilic bacteria measured in the air of the composting facilities vary in a wide range and up to $10^7 \text{ CFU} \cdot \text{m}^{-3}$ (Fig. 4). These data are in agreement with the range of ambient concentrations (10^2 – $10^9 \text{ CFU} \cdot \text{m}^{-3}$) reported in other compost studies for thermophilic bacteria (Duquenne et al., 2012; Persoons et al., 2010). The concentrations measured inside composting facilities exceeded those reported in the literature, i.e. between 11 and $1.6 \times 10^3 \text{ CFU} \cdot \text{m}^{-3}$ in ambient outdoor concentrations without emission source (Jones and Cookson, 1983; Grigorevski-Limaa et al., 2006). Our study confirms that bioaerosols are emitted at high and variable levels during composting operations. The use of the developed qPCR method to assess specific components of bioaerosols allowed us to demonstrate the occurrence airborne *T. vulgaris* of in the investigated composting facilities. Indeed, *T. vulgaris* has been detected in 98% of the collected bioaerosol samples. Around 50% of the measured concentrations were below $10^4 \text{ gyrB copies} \cdot \text{m}^{-3}$ and 28% were between 10^4 and $10^5 \text{ gyrB copies} \cdot \text{m}^{-3}$. The sampling plan designed for our study did not allow us to demonstrate any significant effect of either the composting operations in progress close to the sampling points or the type of waste processed in the composting facilities, on the airborne concentration of *T. vulgaris* DNA or of cultivable bacteria. Concentrations of *T. vulgaris* DNA were generally lower than those measured for cultivable thermophilic bacteria (from 0.4 to 585 fold) (Fig. 5). This was particularly true for the composting plant 2. Cultivation of bacteria on TSA medium at $56 \text{ }^\circ\text{C}$ promote the growth of several thermophilic bacteria, belonging to actinomycetes genera and *Bacillus* species. Thus, *T. vulgaris* is not the only bacteria strain able to grow in the culture conditions we used. These results suggest that *T. vulgaris* may not be the major component of airborne microbial particles in bioaerosol produced during composting operations in our study. To our knowledge, this is the first reported data of airborne *T. vulgaris* using a qPCR based method in composting facilities.

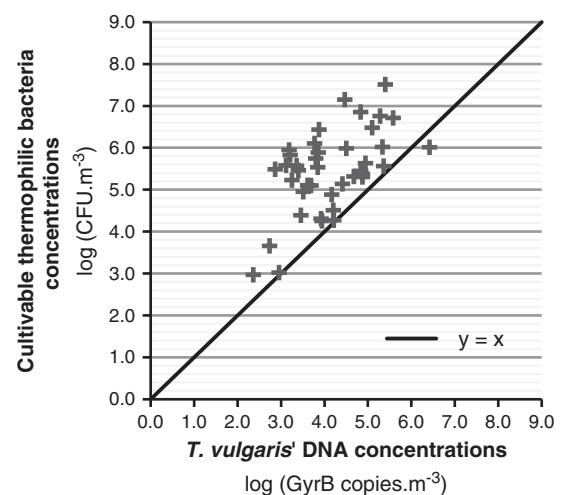


Fig. 5. Comparison of the concentrations of cultivable thermophilic bacteria and *T. vulgaris* DNA concentrations, from the 41 samples of environmental bioaerosols sampled in 5 composting plants. No repetitions were made.

Loss of DNA due to sample elution, DNA extraction or matrix interferences, was not taken into account in our study. Inhibition of PCR is reported in literature, often due to biological compounds found in the sample matrix such as bile salts in feces (Lantz et al., 1997), humic acid compounds from soil or plant (Watson and Blackwell, 2000), immunoglobulin in blood (Al-Soud et al., 2000). The materials and the reagents used throughout the measurement process may also constitute sources of inhibitors for PCR. Potential sources of inhibitors in our assays could be the sampling cassettes (polystyrene), the absorbent pads (cellulose fibers), the membrane for the filtration sampling (polycarbonate) or the extraction solution (containing Tween 80 and peptone). Bej et al. (1991) showed that the filters most widely used for bioaerosol sampling by filtration, including cellulose acetate filters or polycarbonate membranes were PCR inhibitors when DNA extraction is directly done on these filters. In our assays, DNA extraction was not performed directly on the polycarbonate membranes and we assumed that PCR inhibition due to polycarbonate was meaningless. The other compounds such as polystyrene from sampling cassettes or sodium chloride contained in peptone would have created interference as they are not known as inhibitors or as they are used in too small amount. As cellulose fibers and/or polycarbonate could be released in the elution solution during shaking at 2000 rpm for 20 min we suggest that interferences should be further investigated.

5. Conclusion

We have developed a method to quantify *T. vulgaris* in bioaerosol by qPCR. The quantification of *T. vulgaris* DNA is based on the specific enumeration of the *gyrB* gene copies. Since *T. vulgaris* genome is not fully sequenced, we cannot exclude the presence of multiple *gyrB* gene copies per genome, which might lead to a 2 fold overestimation of *T. vulgaris* numbers in samples. The method has been developed and improved by working both on cells culture samples and experimental bioaerosols, before its application on environmental bioaerosols in real exposure conditions. In laboratory experiments, qPCR counts were correlated to both epifluorescence and cultivable counts and gave a specific, sensitive and repeatable evaluation of *T. vulgaris* density in samples. It also overcame some drawbacks of the traditional counting methods (culture and epifluorescence). In real exposure conditions the method provided the first reported concentrations of airborne *T. vulgaris* in composting facilities.

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2. Détection des moisissures concernant le risque aspergillaire

Dans les deux études suivantes, *Aspergillus fumigatus* est recherché dans l'air intérieur, par qPCR et par culture, avec des modes d'échantillonnage différents. L'objectif est dans les deux cas d'évaluer l'exposition environnementale pour les patients à risque.

A. Contexte et résultats principaux de la première étude

Titre de l'article : *Contribution of Cyclonic- based Air Collector for detection of Aspergillus fumigatus in air samples*

Auteurs : Anne Pauline Bellanger, Gabriel Reboux, Emeline Scherer, Mallory Vacheyrou, Laurence Millon

Journal et date de publication : *Journal of Occupational and Environmental Hygiene*, 2012

La surveillance des couloirs généraux d'un d'hôpital et des couloirs spécifiques des services recevant des patients à haut risque d'infections fongiques invasives (les services d'hématologie, en particulier ceux réalisant des greffes de moelle ou de CSH) est une pratique recommandée pour veiller au risque aspergillaire (Reboux et al. 2014). Lorsqu'un « pic » de concentration en moisissures opportuniste est décelé, des consignes (concernant par exemple la ventilation, la protection de zones de travaux, la protection des patients lors des déplacements) peuvent être données pour limiter le risque d'exposition à ces moisissures. Au CHRU de Besançon, cette surveillance est réalisée de façon hebdomadaire depuis 2002. Les prélèvements d'air sont réalisés par impaction sur milieux adaptés. Les milieux sont ensuite incubés. Les colonies obtenues sont dénombrées et identifiées selon leurs caractères macroscopiques et microscopiques. Le délai entre le prélèvement et le rendu des résultats est donc de 5 à 7 jours. Les consignes peuvent alors être décalées de l'événement causal.

L'objectif de l'étude était de comparer ces prélèvements d'air par impaction à des prélèvements réalisés à l'aide d'un appareil récent au moment de l'étude : le Coriolis. Le prélèvement par le Coriolis se fait par un passage cyclonique de l'air dans 15 mL de liquide. Le liquide peut ensuite être utilisé pour une analyse par qPCR. Ainsi le délai de rendu peut être raccourci à 1 ou 2 jours.

Dans les couloirs généraux et dans les couloirs des services d'hématologie, trente-trois prélèvements d'air par impaction et par Coriolis ont été réalisés en double et analysés respectivement, par culture et par qPCR. Neuf prélèvements étaient positifs par culture et par qPCR, avec des Cq allant de 37,9 à 41,15. Un des prélèvements était positif uniquement par culture. Les 23 autres prélèvements étaient négatifs dans les deux techniques. Le Coriolis s'est avéré être moins pratique d'utilisation que le Mas 100® (impacteur utilisé au CHRU de Besançon) et la dilution des spores dans 15 mL de liquide un frein à la sensibilité de la technique.

B. Contexte et résultats principaux de la deuxième étude:

Titre de l'article : *Evaluation of invasive aspergillosis risk of immunocompromised patients alternatively hospitalized in hematology intensive care unit and at home*

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Journal et date de publication : *Indoor Air*, 2014

Si ce risque aspergillaire au sein de la structure hospitalière est correctement surveillé, il n'a jamais été évalué chez les patients à risque lors de leur retour à domicile.

L'objectif de cette étude était de caractériser l'environnement fongique du domicile des patients à risque d'infections fongiques invasives à leur sortie de l'hôpital. Cette caractérisation se faisait par culture avec prélèvement d'air par impaction et par un EDC placé 10 semaines au domicile, retourné par la Poste et traité comme précédemment décrit (avec une analyse par qPCR limitée aux 12 espèces suivantes: *Aspergillus fumigatus*, *A. versicolor*, *Alternaria alternata*, *Penicillium brevicompactum*, *P. chrysogenum*, *Cladosporium sphaerospermum*, *C. herbarum*, *C. cladosporioides*, *Eurotium* sp., *Lichtheimia* spp., *Mucor* spp. et *Rhizomucor* spp.).

Cinquante-trois patients à risque (c'est-à-dire des patients neutropéniques du service de soins intensifs d'hématologie) ont accepté de participer à l'étude et 26 % d'entre eux ont présenté une infection fongique invasive. L'analyse par EDC-QPCR n'a pas permis de discriminer les logements des patients ayant fait une aspergillose invasive (AI). Les résultats par impaction-culture ont montré un risque significativement augmenté de faire une AI quand le pourcentage d'*Aspergillus fumigatus* et d'*A. flavus*, comparé aux moisissures totales, était supérieur à 15 %. Le délai entre la contamination et l'infection ainsi que les sources de contamination restant mal connues, il est difficile de conclure formellement sur des seuils à mettre en place. Mais la culture, en permettant la mise en évidence de l'ensemble des espèces cultivables présentes, semble plus à même dans ce contexte, d'identifier des logements à risque.

C. Conclusion pour les deux études

La QPCR est un outil efficace pour caractériser l'environnement lorsque les espèces ciblées sont bien identifiées. Mais les étapes d'échantillonnage et d'extraction ont un impact sur la sensibilité et la reproductibilité de la méthode. Elles doivent être adaptées en fonction du projet pour répondre aux objectifs visés. Le risque aspergillaire est difficile à évaluer. Les spores d'*Aspergillus fumigatus* sont très fréquentes et d'autres moisissures peuvent avoir les mêmes effets infectieux. Il est indispensable

de poursuivre la surveillance dans le domaine hospitalier, milieu où sont amenés à circuler les patients dans leur phases de neutropénie profonde. Les aspergilloses invasives restent des pathologies rares et il faudrait un nombre plus important de cas pour pouvoir établir des seuils définissant les domiciles à risque. Mais dès maintenant, il semble pertinent de proposer ce « diagnostic » aspergillaire avant le retour au domicile et de conseiller au patient, en cas de contamination forte, un autre hébergement ou des limitations d'accès à certaines pièces d'habitation.

Analytical Performance Issues Contribution of a Cyclonic-Based Liquid Air Collector for Detecting *Aspergillus Fumigatus* by QPCR in Air Samples

5

INTRODUCTION

Fungal infections represent a constant threat in hospitals, especially for high-risk patients hospitalized in hematology and bone marrow transplant units. While routine air sampling to predict the fungal contamination risk is discussed by some authors,^(1,2) French hospital guidelines specify that each hospital is responsible for the air it provides to patients and that air control efficiency must be monitored by specific methodologies.⁽³⁾

Traditional monitoring involves impacted culture media that are incubated for several days so that the fungal species may grow and be identified on the basis of macroscopic and microscopic criteria.⁽⁴⁾ A variety of portable air impactors are commercially available and validated,^(5,6) but these systems are limited by their link with culture techniques, involving a time-lapse to results of around 7 days and by a maximal sampling rate of 100 L/min.

As optimal environmental monitoring requires large sample sizes and fast accurate detection of microorganisms contained within the samples,^(7,8) new techniques concurring with these characteristics are regularly evaluated. Quantitative polymerase chain reaction (QPCR) represents a very attractive alternative to culture techniques as it allows *Aspergillus fumigatus*, which is the preponderant etiologic agent of invasive aspergillosis, to be specifically detected in under 2 days. Reducing the time to results is key to permit intervention to prevent *Aspergillus* exposure in immunocompromised patients.

In this way, QPCR was previously used to amplify *Aspergillus fumigatus* DNA on various substrates, such as tap water,⁽⁹⁾ carpets,⁽⁷⁾ air filters,⁽¹⁰⁾ and impacted low-melt agar plates.⁽¹¹⁾ A new device, the Coriolis μ air sampler (Bertin Technologies, Montigny, France), based on a cyclonic system, was recently proposed for collecting large volumes of air on liquid medium quickly. This device was previously assessed for different areas other than fungal aero-contamination monitoring: analysis of pollen grain distribution^(12,13) and detection of *Pneumocystis jiroveci* DNA by QPCR.⁽¹⁴⁾

A preliminary study was carried out to test this cyclonic-based liquid device, combined with QPCR, and to assess its performance for detecting *A. fumigatus* DNA.

MATERIAL AND METHODS

Comparison of the Two Sampling Devices

The characteristics (weight, battery autonomy, sound level, and ease of use) of the two air samplers (the MAS 100 impactor and the Coriolis μ air sampler) were compared to assess their respective practicality. Both devices allow sampling of both

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40 conidia (under 5 μm in size) and hyphal fragments which vary markedly in size (5–100 μm).

Air Sampling

Air sampling was carried out in the corridors of the hematology units and in non-hematology areas during a period of 45 30 days with two devices:

1. 100 L of air was collected using a MAS 100 impactor (Merck, Darmstadt, Germany) on culture media, which is the reference technique used in standard routine environmental controls in our university hospital. With this system, the sampled air is blown through a perforated plate (400 holes of 0.7 mm in diameter) and is propelled on to a media plate with an impact speed of 10.8 m/s. Several other portable battery-powered air impactors are commercially available, and all present similar operational capability.⁽⁶⁾
2. 1.5 m³ of air was collected using a Coriolis μ air sampler in 15 mL of sterile phosphate buffered saline (PBS) + 0.002% Tween 80. With this device, the air is drawn into the liquid in a whirling motion, pulling the particles against the wall of the tube by centrifugal force and separated from air to be concentrated into the liquid. For now, there is no other commercially available device that provides the same operational capability.

30 Thirty-three samples were taken simultaneously with the two devices (same time/same place). Fourteen samples were taken in corridors of the hematology units, and 19 samples were taken in non-hematology hospital areas. In all cases, both devices were placed 1 m from the floor.

Detection of *A. Fumigatus* by Culture Technique

70 Impacted culture media were dichloran-18% glycerol (DG18) (Oxoid, Basingstoke, Hampshire, UK), incubated at 30°, and malt agar extract (3%) (Difco, Detroit, Mich.) incubated at 37°C. Cultures were checked after 3 and 7 days of incubation. Fungal species were identified by macroscopic and microscopic examinations. Results were expressed in colony forming unit (CFU) per 100 L of air.

Detection of *A. fumigatus* DNA by QPCR

DNA Extraction

80 Rapid DNA extraction was performed as described previously,⁽¹⁵⁾ with the following modifications: each liquid sample was centrifuged at 1300 g for 10 min, and the supernatant was carefully removed to leave a 1-mL pellet that was subsequently transferred into 2-mL screw-cap tubes prefilled with 1.4-mm ceramic beads. Samples were placed in a mini-bead beater (Magna Lyser; Roche Diagnostic, Meylan, France) three times for 10 sec, then heated in a boiling water bath for 10 min. Tubes were stored on ice for 10 min and then centrifuged in a micro-centrifuge (12000 g for 2 min). The supernatants above the beads were carefully removed and stored at –20°C before 90 QPCR analysis.

QPCR

DNA from *A. fumigatus* was detected using primers and Taqman probes as described previously by Haugland et al.^(16,17) QPCR was carried out in a 20 μL final volume using the LightCycler FastStart Master HybProbe (Roche): 2 μL of Master Mix 1a/1b, 2.2 μL of MgCl₂ (25 mM stock solution), 1.6 μL probe (AFUMI P1, 1 μM), 4 μL of each primer (A FUMI F1/R1, 5 μM), 0.2 μL of uracil DNA glycosylase (USB, Cleveland, Ohio), and 10 μL of sample extract. The thermal cycling conditions were: a denaturation step at 95°C for 10 min followed by amplification for 45 cycles at 60°C for 1 min.

Quantitative results were expressed as cycle quantification (Cq), which indicates the cycle during which fluorescence of the sample became exponential in comparison with the baseline signal. The Cq was inversely proportional to the amount of DNA in the sample: the higher the Cq, the less DNA in the sample. All samples were tested in duplicate and differences between two replicates never exceeded 1 Cq.

Standard Curve for *A. fumigatus*

A suspension containing 10⁶ *A. fumigatus* conidia/mL (BCCM/IHEM 23670) was prepared after counting in Malassez cells. Six ten-fold dilutions of the initial suspension were prepared, and the dilutions were counted again. This process was repeated three times. The standard curve was established using the mean Cq of the three extracts obtained for each conidia/mL concentration.

Specificity of the QPCR Assay

The specificity of the QPCR assay was determined using conidia suspensions of *Lichteimia corymbifera*, *Aspergillus versicolor*, *Penicillium chrysogenum*, *Alternaria alternata*, *Stachybotrys chartarum*, *Eurotium* sp., *Aspergillus sydowii*, *Aspergillus penicillioides*, *Walleimia sebi*, *Penicillium brevicompactum*, *Penicillium olsoni*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus flavus*, *Penicillium variotii*, *Penicillium citrinum*, and *Penicillium crustosum* (10⁴ conidia/mL for each species).

RESULTS

Comparison of the Two Sampling Devices

The different characteristics of the two disposals used are presented in Table I. The Coriolis μ air sampler was slightly less practical to use than the MAS 100: it was heavier, was not furnished with an adapted transport case, made more noise, and had shorter battery autonomy.

Standard curve for *A. fumigatus*

QPCR results obtained for the *A. fumigatus* conidia suspensions showed that *A. fumigatus* conidia were detected at a concentration of less than 10 conidia/mL with a Cq of approximately 40 (Figure 1).

TABLE I. Differential Characteristics of the Two Disposals Used for Air Sampling

	Mas 100 Impactor	Coriolis μ Air Sampler
Weight	≈ 2 kg	≈ 3 kg
Physical resistance	+++	++
Practicality	+++	++
Sound level	+	++
Maximal airflow rate	100 L/min	300 L/min
Battery autonomy	7 hr	2 hr
Easiness to use	+++	++

Specificity of QPCR

140 The QPCR was specific to *A. fumigatus*; no false-positive results were observed when using conidia suspensions from other fungal species.

Detection of *A. fumigatus* in air samples from hematology units

145 The 14 air samples from hematology units were negative for *A. fumigatus* with both techniques (culture and QPCR). *

Detection of *A. fumigatus* in Air Samples from Non-Hematology Hospital Areas

After Sampling with the MAS 100

150 Ten of the 19 air samples from non-hematology hospital areas were culture positive for *A. fumigatus* after impaction with the MAS 100 with a mean and standard deviation of 2.11 ± 3.13 CFU/100 L of air (Table II)

TABLE II. Detection of *A. fumigatus* in the Air Samples from Non-Hematology Hospital Areas

Air Samples	Culture after Impaction with MAS 100	QPCR after Sampling with Coriolis μ
1	1 CFU/100L	Cq >45
2	1 CFU/100L	Cq = 39.9
3	9 CFU/100L	Cq = 39.25
4	10 CFU/100L	Cq = 37.8
5	4 CFU/100L	Cq = 40.3
6	6 CFU/100L	Cq = 38.5
7	4 CFU/100L	Cq = 40.08
8	2 CFU/100L	Cq = 38.2
9	2 CFU/100L	Cq = 39.2
10	1 CFU/100L	Cq = 41.15

After Sampling with the Coriolis μ

Nine of the 19 air samples from non-hematology hospital areas were QPCR positive for *A. fumigatus* after sampling using the Coriolis μ with a mean and standard deviation of Cq of 39.38 ± 1.09 (Table II).

Comparison of the Results Obtained Using the MAS 100 and the Coriolis μ

160 Twenty-three of the 33 samples (70%) were negative for *A. fumigatus* with both techniques (culture and QPCR). Nine of the 33 samples (27%) were positive with both techniques (detailed results in Table II). One of the 19 samples was positive in culture after impaction (with one CFU/100 L of air), whereas it was negative after liquid-based collection and QPCR analysis. 165

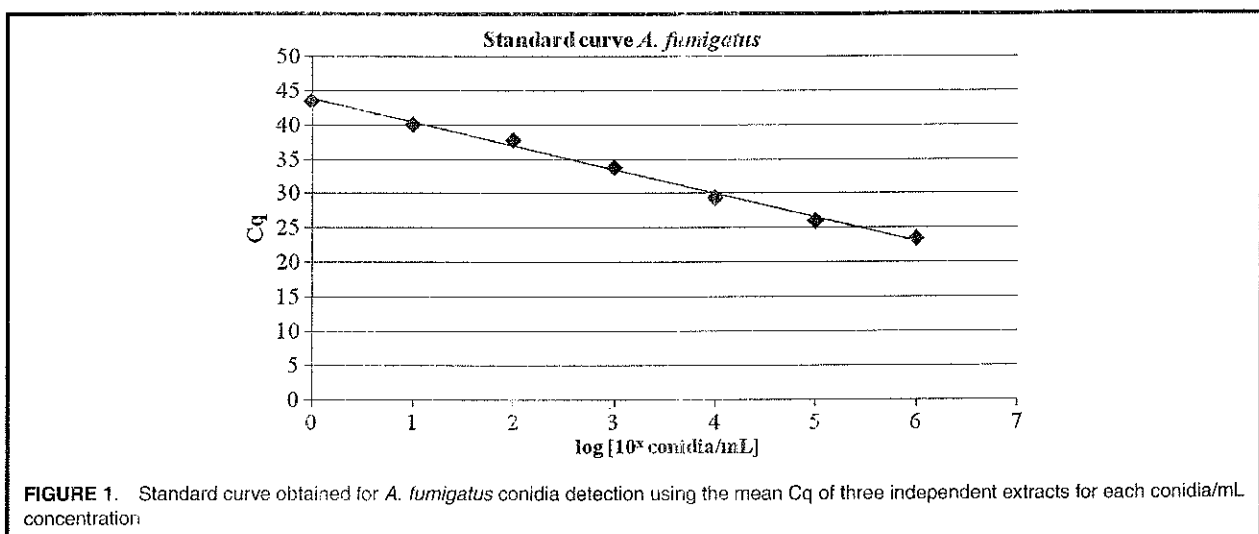


FIGURE 1. Standard curve obtained for *A. fumigatus* conidia detection using the mean Cq of three independent extracts for each conidia/mL concentration

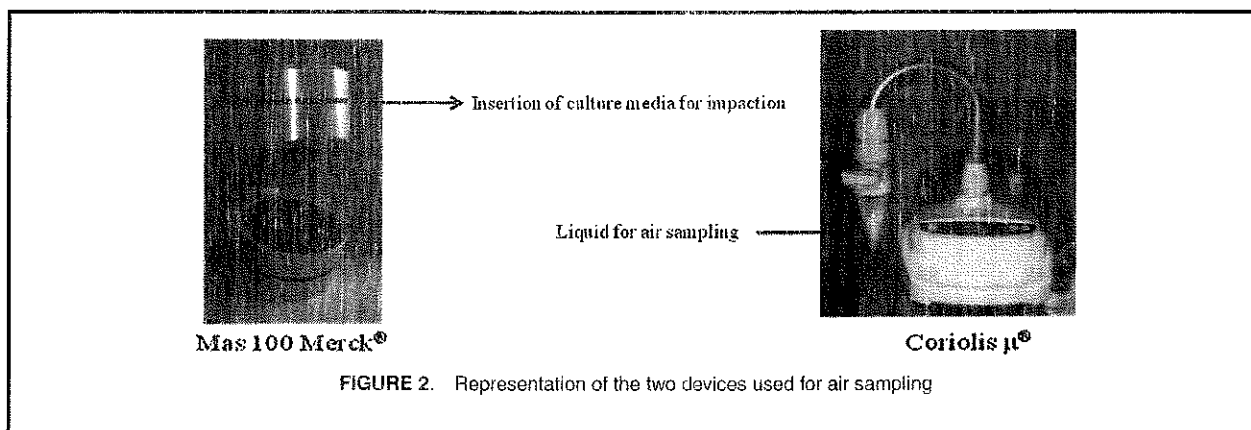


FIGURE 2. Representation of the two devices used for air sampling

DISCUSSION

The aim of this study was to evaluate the performance of a new air sampling device, the Coriolis μ , which is able to sample large volumes of air over a short period, uses a higher airflow rate than the MAS 100, and provides substrates for analysis techniques such as QPCR.

In this experiment, a good concordance was observed, in terms of positive and negative results for the detection of *A. fumigatus*, between the two devices. Although the number of samples of this preliminary assay was limited, the Coriolis μ seems promising and could be an alternative for routine monitoring of fungal aero-contamination. Using QPCR makes it possible to react more quickly in terms of prevention and remediation and is particularly useful in cases of hospital building works.⁽¹⁰⁾

It may be that by sampling larger air volumes (100 L with the MAS 100 compared with 1500 L with the Coriolis μ), more samples would have been positive with the Coriolis μ . This could be explained by the fact that 15 mL of liquid is necessary for sampling with the Coriolis μ to generate the cyclonic reaction. The liquid was centrifuged to concentrate the particles, but fungal spores are light and some may have remained in the supernatants. One solution for improving the quantification of *A. fumigatus* conidia in the air could be to reduce the volume of liquid used for sampling so that the totality of the air sampled would be extracted. However, such a solution is not yet possible because the cyclonic reaction cannot be created with less than 15 mL of liquid medium. Another solution to concentrate the sample could be either to filter the 15 mL sampled and to extract the filter or to use ultra-centrifugal filter units.

In conclusion, the Coriolis μ could be an interesting alternative to culture techniques and allow *A. fumigatus* DNA to be detected quickly in air sampled by QPCR.

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Evaluation of invasive aspergillosis risk of immunocompromised patients alternatively hospitalized in hematology intensive care unit and at home

Abstract Contrary to hospital exposure, little is known about the indoor fungal exposure of hematology patients at home. The aim of our study was to investigate the mold exposure of hematology patients both at home and at hospital to assess their invasive aspergillosis (IA) risk. Fungal exposure was assessed by quantifying opportunistic molds at hospital during hospitalization and in homes of 53 hematology patients. IA was diagnosed in 13 of 53 patients and invasive fungal infection (IFI) in one patient. In hospital, no opportunistic species, or low levels of opportunistic species, were found in 98% of weekly controls. Only 2% of hematology intensive care unit (ICU) controls showed a high level of *Aspergillus fumigatus* spores in corridor air. Five patients IA were hospitalized during these periods. Seven dwellings of 53 (5/14 dwellings of patients with IA/IFI and 2/39 dwellings of non-IA patients) had a percentage of *A. fumigatus* and *Aspergillus flavus* to total mold (significant predictor variable of IA/IFI in our study, general linear model, P -value = 0.02) as high as 15%. Maintaining a 'zero *Aspergillus*' goal at hospital is essential, and establishing specific and individually opportunistic mold monitoring at home could help to further reduce the IA risk through continuous surveillance.

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Practical Implications

This study emphasizes the fact that preventive measures should not be aimed only at the hospital setting: among patients diagnosed with invasive aspergillosis/invasive fungal infection (IA/IFI), 5 of 14 (36%) were exposed to opportunistic fungal species at home exclusively. Moreover, four of these five patients were living in homes having the highest percentage of *Aspergillus fumigatus* and *Aspergillus flavus* (>15%), one of which had 48% of *A. fumigatus*. Therefore, our work supports the need for a counselor to carry out an environmental survey in patients' homes.

Introduction

By improving the care of patients with hematologic malignancies, the number of hematopoietic stem cell transplantations (HSCT), and modern immunosuppressive therapies, the survival of patients can be extended, but there is still a major risk of invasive

fungal infections (IFI) in these patients. Despite advances in early diagnosis, IFI are related to mortality with rates between 30% and 70% (Marr et al., 2002; Panackal et al., 2010). Although *Aspergillus fumigatus* is the main etiologic agent of most mold infections, other opportunistic filamentous fungi may be involved such as *Mucor* spp., *Lichtheimia* spp.

(formerly *Absidia* spp.), *Rhizomucor* spp., *Rhizopus* spp., *Scedosporium* spp., or *Fusarium* spp. (Garcia-Vidal et al., 2008). For patients with hematologic malignancies, invasive aspergillosis (IA) incidence follows a two-peak pattern: one just after graft for inpatients and the second approximately 100 days later when patients are at home (Viscoli and Castagnola, 2010). The prognosis is severe with a 57.5% mortality rate at 12 weeks among allogenic HSCT patients (Baddley et al., 2010) and an 80% death rate in these patients the year following an IFI (Marr et al., 2002).

Many studies dealing with environmental fungal measures and IFI risk were conducted in hospitals (Bénet et al., 2007; Cornet et al., 2002; Fournieret-Vivier et al., 2006). The importance of preventive measures at hospital, especially during building construction or repairs, is well recognized (Bouza et al., 2002). Specific measures against contamination by fungal spores must be applied during construction work such as efficient and controlled ventilation, protective isolation of patients and bio-cleaning (Gangneux et al., 2012). However, the contribution of systematic environmental surveys is debatable (Falvey and Streifel, 2007; Rupp et al., 2008). No guidelines about monitoring in intensive care units (ICUs) exist in France, but the 2000 consensus conference recommended quarterly controls in hematology ICUs (rooms and corridors) to reach the 'zero *Aspergillus*' goal. Since 2002, in addition to quarterly controls, weekly environmental measures have been implemented in corridors of the hematology ICU at the Besançon University Hospital and served as indicators. These control checks were useful to anticipate increased prevention in high efficiency particulate air (HEPA) filtered rooms when high levels of opportunistic species were detected in ICU corridors, especially during building periods (Gangneux et al., 2012).

Unfortunately, despite chemoprophylaxis and all usual preventive and monitoring measures used in hospitals, IFI incidence remains high with IA incidence between 5% and 10% in allogenic HSCT patients (Panackal et al., 2010). These patients return home between two hospitalization periods with recurrent neutropenia episodes. Once at home, some of them still have impaired immune status, and most of them spend more than 90% of their time inside, in an environment where fungal contamination is unknown. Recommendations are given when patients leave the hospital (no pets, no carpets, no potted plants or flower arrangements at home...); but, unlike the rigorous hospital environmental surveillance, no environmental measures are taken at home while various domestic activities, such as sweeping for example, are known to increase airborne fungi (Lehtonen et al., 1993). The aim of our study was to investigate the fungal exposure of hematology patients both at home and at hospital and thus to assess their IA risk.

Patients and methods

Hematology ICU

The Hematology ICU of the University Hospital of Besançon hospitalizes around 275 patients per year, corresponding to approximately a cohort of 125 followed patients. Each year, around 40 chemotherapy with aplasia (induction or consolidation) and 50 allografts are performed in the hematology ICU.

Patients

From January 2010, all neutropenic patients from the hematology ICU were contacted (within 7–23 days after the return home) to obtain their agreement to participate in the study. Data from patients were collected from the date of malignancy diagnosis to May 2012 (end of the study). Clinical, biological, and radiological data useful for IA diagnosis or fungal risk assessment were collected from numeric medical records. Dates of hospitalization, chemotherapy, allograft, neutropenia episodes and recovery of polynuclear neutrophils, mycological culture (bronchoalveolar lavage and sputum), galactomannan antigen, histopathology, high-resolution computed tomography (CT) findings, and antifungal treatments were compiled.

Patients who developed IA were classified into three groups (possible, probable, and proven) according to the standard definitions of the European Organization for Research and Treatment of Cancer (EORTC; De Pauw et al., 2008). Proven and probable cases were then reported to the French network for surveillance of IFI.

Weekly environmental surveillance in hematology ICU

Environmental surveillance of hematology ICU corridors was performed weekly at the Besançon University Hospital. Five samples of air were taken in corridors by a MAS100™ impactor (Merck, Darmstadt, Germany) on DG18 medium for 2.5 min/sample (250 l of air). Media were incubated for 7 days at 30°C. Molds were identified according to microscopic and macroscopic characteristics, and results were expressed in colony-forming units per cubic meter of air (CFU/m³). ICU rooms of patients were sampled during quarterly controls (4 rooms/15 sampled during each quarterly control with three swabbing samples of 25 cm² and 2 air samples of 500 l).

Environmental surveys at home

When patients agreed to participate in the study, they received one home visit the following week. Home visiting in summer was avoided to exclude the variation

due to the opening of windows and doors (Roussel et al., 2008). The patients were asked to keep windows and doors closed for at least one hour before the visit to avoid interferences with outdoor spores. Air samples were carried out in different rooms (bedroom, bathroom, and the two other rooms where patients spent most of their time) as previously described for hematology ICU corridors. Air concentrations were expressed as averages per dwelling in CFU/m³. Each patient had only one visit at home over the 3 years of the study. Electrostatic dust fall collectors (EDC) were deposited in one room of each dwelling during a 10-week period and returned by post to the Mycology Department afterward by the patients. This sample device makes it possible to estimate the chronic fungal contamination of the environment (Scherer et al., 2014). Twelve real-time quantitative polymerase chain reactions (qPCR; targeted *A. fumigatus*, *A. versicolor*, *Alternaria alternata*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Cladosporium sphaerospermum*, *Cladosporium herbarum*, *Cladosporium cladosporoides*, *Eurotium* spp., *Lichteimia* spp., *Mucor* spp., and *Rhizomucor* spp.) were performed on the washing solution of the electrostatic wipe.

Evaluation of risk periods

Little is known about the incubation period, inasmuch as the patient colonization may be possible before hospitalization (Fairs et al., 2013). Thus, in addition to usual risk factors such as severe and prolonged neutropenia (polynuclear neutrophils <0.5 G cells/l for more than 15 days), severe neutropenia (<0.5 G cells/l for less than 15 days), immunosuppressive and corticoid therapy, and all situations of mold exposure in the 3 months prior to IA diagnosis were analyzed. Mold exposure in the 3 months prior to IA only involved potential fungal exposure during hospitalization periods.

As environmental samples at hospital were collected every week, if an air sample was positive (>1 CFU/m³) for the most frequent opportunistic species (*A. fumigatus* complex, *Aspergillus flavus*, *Mucor* spp., *Lichteimia* spp., *Rhizomucor* spp., *Rhizopus* spp., *Scedosporium* spp., and *F. solani*), an inpatient, hospitalized 7 days before or after environment sampling, was considered as potentially exposed. At home, mold exposure was considered possible if opportunistic species were found in air samples.

Statistical analyses

Nonparametric tests (Wilcoxon rank sum test and Kruskal–Wallis test) were used to investigate possible differences in total and opportunistic mold levels regarding the types of rooms and the characteristics of dwellings.

A generalized linear model with logit-link function for binomial distribution and odds ratio (OR) with confidence intervals were used to compare the development of IFI and potential predictor variables. Statistical analyses were performed using R 2.13.1 (R Development Core Team, 2012).

Results

Patients

Seventy-nine patients were contacted when they left the hospital: 53 agreed to participate in the study. During the study period, patients came to the hematology ICU three times on average (from 1 to 9 times) and were hospitalized for 27 days (on average). Severe and prolonged neutropenia episodes were observed for 42 patients of 53. At the end of the study, 41 patients had undergone allogeneic HSCT (four before January 2010 and 37 during the 3 years of the study).

Invasive aspergillosis and IFI were diagnosed for 14 of 53 patients (26%), from 31 to 56 years old.

During the present study, the rate of IA/IFI diagnosed was high compared with the frequency reported in the literature (Bitar et al., 2013). Three main reasons may explain this result: (i) the numerous building projects in the University Hospital over the past few years, (ii) the active screening strategy and recording of IFI conducted by our Mycology Department (using systematic galactomannan twice a week and *A. fumigatus* qPCR; Millon et al., 2011), and (iii) the large cohort of patients presenting IFI at risk factors as defined by the EORTC/mycoses study group (EORTC/MSG) of the hematology ICU.

Thirteen patients had acute myelogenous leukemia (AML) and one patient had a lymphoma. One patient had a proven IFI, nine had probable IA, and four had possible IA according to EORTC/MSG standardized definitions. Data for the 14 cases are presented in Table 1.

Possible IA cases were included in the analysis, as in other works (Chai et al., 2012; Ghosh et al., 2013), because imaging arguments (halo, nodule) were present for all of them and three improved under antifungal therapy. The fourth died the day of diagnosis, before mycological data could be obtained. For the case of proven IFI (P1), CT indicated a halo sign and biopsy showed mycelium, but galactomannan antigenemia and culture remained negative. The term IA/IFI will be used throughout the manuscript to speak about the case of IFI and the 13 cases of IA. During the 90 days before IA/IFI, one patient had immunosuppressive therapy and seven had corticoid therapy (two patients for a long period and five patients for a few days). Nine patients had antifungal prophylaxis with *Aspergillus*-active molecules before the first IA/IFI sign (four were treated with caspofungin, three with voriconazole, one with posaconazole, and one with amphotericin B).

Table 1 Clinical and biological data of patients with IA/IFI at the time of IA/IFI diagnosis

Patients	Underlying disease	Sex/Age	IFI	IFI diagnosis period	IFI localization	GM index detection ^a	CT imaging ^b	Mycological criteria	Curative Antifungal therapy	Outcomes (90 days)
P1	AML	M/32	Proven IFI	Induction	Pulmonary	0.44	Halo sign	Biopsy filaments	Voriconazole amphotericin B	Alive
P2	AML	F/36	Probable IA	Induction	Pulmonary	0.58	Cavitation	0	Posaconazole	Alive
P3	Lymphoma	M/34	Probable IA	Allograft	Pulmonary	0.99	Nodules	0	Amphotericin B	Alive
P4	AML	M/55	Probable IA	Ambulatory	Disseminated	4.87	Sinus	0	Posaconazole	Alive
P5	AML	M/55	Probable IA	Consolidation	Pulmonary	0.54	Nodules	0	Voriconazole	Alive
P6	AML	F/58	Probable IA	Consolidation	Pulmonary	0.88	Nodules	0	Voriconazole	Death (2 months)
P7	AML	F/65	Probable IA	Induction	Pulmonary	0.98	Nodules	0	Voriconazole	Alive
P8	AML	M/55	Probable IA	Consolidation	Pulmonary	1.19	2 infection foci	0	Voriconazole	Alive
P9	AML	F/66	Probable IA	Induction	Pulmonary	0.91	Nodules	Expectoration	Voriconazole	Alive
P10	AML	F/56	Probable IA	Induction	Pulmonary	0.52	Nodules	0	Voriconazole	Alive
P11	AML	M/34	Possible IA	Induction	Pulmonary	0	Nodules	0	Voriconazole	Alive
P12	AML	M/37	Possible AI	Consolidation	Pulmonary	0	Nodules	0	Voriconazole amphotericin B	Alive
P13	AML	F/31	Possible IA	Other hospitalization	Pulmonary	0	Halo sign nodules	0	Voriconazole	Death (17 days)
P14	AML	M/55	Possible IA	Hospitalization for fever	Pulmonary	0	Nodules	0	Voriconazole	Death (2 months)

AML, acute myeloblastic leukemia; M, male; F, female; IFI, invasive fungal infection; IA, invasive aspergillosis.

^aThe threshold of positivity for Galactomannan antigenemia index (GM) was 0.5.

^bHigh-resolution computed tomography (CT) findings.

Among the 39 patients without IA (Table 2), aged from 4 to 68, 20 patients had AML, 5 myeloma, 4 lymphoma, 4 acute lymphoid leukemia (ALL), 4 myelodysplasia, 1 chronic lymphoid leukemia (CLL), and 1 chronic myeloid leukemia (CML). At the time of recruitment, after hospitalization periods, 15 patients had antifungal prophylaxis with anti-*Aspergillus*-active molecules (3 with voriconazole and 12 with posaconazole).

Weekly environmental surveillance at hospital

In general, hematology ICU corridors were presented a low level of molds. During the study period (January 2010–May 2012), three different situations were observed: (i) no opportunistic species were found in 101 of the 126 total weekly controls (80%), (ii) low levels of opportunistic species (mean of 1 CFU/m³) were found in 22 of the 126 (18%) weekly controls, and (iii) high levels of *A. fumigatus* spores in hematology ICU corridors (9, 14, and 25 CFU/m³) were found in 3 of the 126 weekly controls (2%). However, no *A. fumigatus* were detected in rooms when hematology ICU corridors were contaminated.

Environmental surveys of patients' homes

Forty-two dwellings were rural and 11 urban. Mean indoor temperature of dwellings was 21.8°C (range 20.3–24.7°C) and mean relative humidity 38.5% (range 30–53%). Around 56% of patients (30/53) had more than five plants in their dwelling and 58.5% of patients (22/53) had pets.

Culture-based results of impactor samples. Mold concentrations in homes varied from 23 to 1263 CFU/m³ (Table S1). According to classification previously developed by our group (Reboux et al., 2009), most homes (35/53) had low level of molds (<170 CFU/m³), whereas two homes had very high levels of molds (>1000 CFU/m³). No difference in mold contamination was observed between the four rooms sampled in a given dwelling, which is consistent with our previous results on a larger cohort (Reboux et al., 2009). Three to 12 different molds were found in dwellings, with *Penicillium* spp. found in 100% of the studied homes, *Eurotium amstelodami* in 91%, *A. versicolor* in 81%, *Cladosporium* spp. in 53%, and *Alternaria* spp. in 19%. Opportunistic molds were identified in 68% of homes (36/53) by culture after impaction, and in 58% of cases (31/53), *A. fumigatus* and *A. flavus* were found (Figure 1). Other opportunistic molds such as mucorales and *Scedosporium* spp. were observed in 38% and 2% of homes, respectively.

Seven dwellings of 53 (5/14 dwellings of patients IA/IFI and 2/39 dwellings of non-IA patients) had a percentage of *A. fumigatus* and *A. flavus*, the two most common thermophilic *Aspergillus* involved in IA, to total molds as high as 15% (Table 3). The four dwellings (P14, P1, P3, and P7) with the highest percentage of *A. fumigatus* and *A. flavus* to total molds (48, 35, 27, and 22%) were homes of patients with IA/IFI (Figure 1).

No significant difference between total contamination or *A. fumigatus* and *A. flavus* percentage in samples and the environment type (rural, urban) or the type of dwelling (apartment, house) was found.

Table 2 Characteristics of patients without IA at the time of recruitment

Patients	Underlying disease	Sex/Age	Hospitalization before recruitment	GM index detection ^a	Antifungal therapy (outpatient)	Outcomes (end of the study) Death (after recruitment)
P15	Myeloma	F/47	Allograft	0	Posaconazole	Death (9 months)
P16	AML	M/29	Consolidation	0	0	Alive
P17	AML	M/53	Allograft	0	Voriconazole	Alive
P18	AML	M/14	Allograft	0	Posaconazole	Alive
P19	AML	M/60	Allograft	0	0	Alive
P20	CML	M/47	Induction	0	0	Alive
P21	AML	F/33	Allograft	0.68	0	Alive
P22	Lymphoma	F/40	Allograft	0	0	Alive
P23	AML	F/46	Induction	0	0	Alive
P24	ALL	M/68	Consolidation	0	0	Alive
P25	Myeloma	F/47	Allograft	0	0	Alive
P26	AML	M/18	Aplasia + fever	0	Voriconazole	Alive
P27	AML	F/54	Allograft	0	Posaconazole	Death (2 months)
P28	AML	F/38	Other hospitalization	0	0	Alive
P29	Myelodysplasia	M/67	Consolidation	0.67	0	Alive
P30	ALL	F/51	Consolidation	0	0	Alive
P31	CLL	M/59	Other hospitalization	0	Voriconazole	Alive
P32	AML	M/48	Induction	0	Posaconazole	Alive
P33	AML	M/48	Allograft	0	Posaconazole	Death (1 year)
P34	AML	M/60	Other hospitalization	0	0	Alive
P35	AML	F/36	Induction	2.05	0	Alive
P36	AML	F/22	Induction	0	0	Alive
P37	Lymphoma	M/57	Allograft	0	0	Alive
P38	ALL	M/24	Rescue	0.58	0	Alive
P39	Myelodysplasia	M/35	Allograft	0	0	Alive
P40	Anemia	F/41	Allograft	0	0	Death (8 months)
P41	AML	M/42	Consolidation	0	0	Alive
P42	AML	F/59	Allograft	0	Posaconazole	Alive
P43	Lymphoma	M/61	Allograft	0	Posaconazole	Alive
P44	Lymphoma	F/46	Allograft	0	0	Alive
P45	Myeloma	M/51	Allograft	0	Posaconazole	Alive
P46	ALL	F/4	Allograft	0	0	Alive
P47	AML	F/44	Allograft	0	0	Alive
P48	AML	F/49	Allograft	0	Posaconazole	Alive
P49	AML	M/25	Consolidation	0	0	Alive
P50	AML	F/22	Induction	0	0	Alive
P51	Myeloma	F/38	Allograft	0	Posaconazole	Alive
P52	Myeloma	M/56	Other hospitalization	0	Posaconazole	Death (1 year)
P53	Myelodysplasia	M/56	Allograft	0	Posaconazole	Alive

AML, acute myeloblastic leukemia; ALL, acute lymphoid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; M, male; F, female; IA, invasive aspergillosis.

^aThe threshold of positivity for Galactomannan antigenemia index (GM) was 0.5.

Variables such as ventilation, opening windows, the presence of visible mold, and the presence of plants and pets were taken into account for the analysis. No proxy could be established for housing contamination with opportunistic species and number of species.

qPCR results of EDC samples. QPCR targets were detected in 84–100% of samples. Infective and non-infective spores were concomitantly quantified; thus, no discrimination could indicate whether or not there was actually an IA risk (Table S2).

IFI and fungal exposure

The fact that recruitment was based on the presence of neutropenia (aplasia episode) aimed at eliminating

neutropenia as a confusing factor, we can better analyze the role of environmental exposure to opportunistic species, at home and/or at hospital (Table 1). The absence of a significant link between neutropenia and patients with IFI was checked by statistical analyses.

Patients with IA/IFI. As indicated in Tables 2 and 3, IA/IFI occurred during severe neutropenia for 10 patients (six patients during induction chemotherapy, two patients in consolidation therapy, one in allograft hospitalization, and one admitted to the hematology ICU for relapse). IA occurred during a non-neutropenic period for four patients (two patients in consolidation therapy, one with ambulatory treatment, and one hospitalized for fever). The hospitalization periods of five patients who developed IA (P2, P3, P5, P6, and

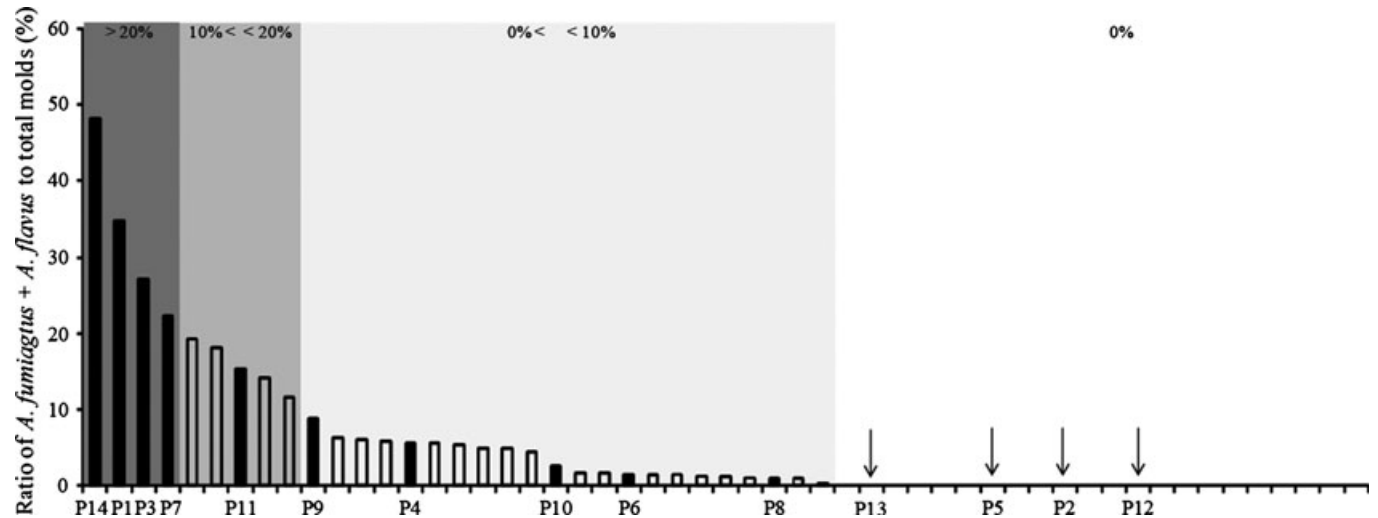


Fig. 1 Percentage of *Aspergillus fumigatus* and *Aspergillus flavus* to total molds in the 53 dwellings. Black bars represent dwellings of patients with invasive aspergillosis/invasive fungal infection (IA/IFI; P1–P14) and empty bars dwellings of patients without IA. Arrows represent dwellings of patients with IA without *A. fumigatus* and *A. flavus* (P5, P2 and P12) detected at home. The four dwellings with the highest percentages of *A. fumigatus* and *A. flavus* are those of patients with IA/IFI (P14, P1, P3 and P7). The percentage of *A. fumigatus* and *A. flavus* at home was a predictor variable for the development of IA/IFI (general linear model, P -value = 0.02)

P13) were concomitant with abnormally high levels of *A. fumigatus* (−75 to +3 days from the day of IA diagnosis) in hematology ICU corridors (14 and 25 CFU/m³). Four patients (P4, P8, P10, and P12) were hospitalized when fungal contamination was low (1 CFU/m³ on average), including two patients (P10 and P12) who were in severe neutropenia at the time. No fungal contamination was observed during the hospitalization periods of the five remaining patients (P1, P7, P9, P11, and P14) diagnosed with IA/IFI. However, *A. fumigatus* and *A. flavus* were found in the homes of these five patients with percentages of 35%, 22%, 9%, 15%, and 48%, respectively (Table 3).

Non-IA patients. If we consider neutropenia as the first risk factor for IA, the most critical periods for the 39 patients without IA were as follows: 25 allograft, 5 induction, 7 consolidation, and 2 other hospitalization. Twenty-three patients suffered from severe and prolonged neutropenia, 11 suffered from severe neutropenia, and 5 did not suffer from severe neutropenia. Two patients (P44 and P52) were not exposed at home or at hospital. Thirty-two patients were hospitalized during a contamination in hematology ICU corridors (11 hospitalizations with high levels and 21 hospitalizations with low levels). *A. fumigatus* and *A. flavus* were found in 21 dwellings of the 39 non-IA patients with 19 dwellings with a percentage of *A. fumigatus* and *A. flavus* to total mold below 14%. The two remaining patients (P15 and P39) had dwellings with a percentage of *A. fumigatus* and *A. flavus* to total mold as high as 19% and 18% and were also exposed in hospital during the period of severe neutropenia (Table 3).

General linear model ($P = 0.02$) and odds ratio (OR = 1.09 and 95% interval confidence between 1.02

and 1.2) both determined that the percentage of *A. fumigatus* and *A. flavus* at home was a predictor variable for the development of IA/IFI.

Discussion

This study investigated fungal risk exposure for at-risk hematological patients during their hospitalization in the hematology ICU but also when returning home. As it deals with IA risk, we focused on *A. fumigatus* and *A. flavus*, the two most common thermophilic *Aspergillus* involved in IA. The present study demonstrated that among patients diagnosed with IA/IFI, 6 of 14 patients (43%) were potentially exposed to opportunistic molds both at home and in hospital, 3 of 14 (21%) were potentially exposed only in hospital, and 5 of 14 (36%) were exposed at home exclusively. Moreover, four of these last five patients, exposed only at home, were living in homes having the highest percentage of *A. fumigatus* and *A. flavus* (>15%), one of which had 48% of *A. fumigatus*.

An environmental survey of hematology ICU is recommended, but no referent threshold has been proposed thus far, in France or in any other country. Alberti et al. (2001) demonstrated in a previous study that an increase of more than 2 CFU/m³ of *A. fumigatus* had a determining role in IA occurrence for hematological patients. According to Warris et al. (2001), the risk of IA can be avoided in inpatients if fungal contamination remains under 5 CFU/m³ in an isolation area and under 0.1 CFU/m³ in a HEPA-filtered area. In our study, the few positive mycological results from environmental surveillance in hematology ICU corridors were concomitant with the hospitalization of patients who developed IA (9/13 patients potentially

Table 3 Host factors and exposure to opportunistic molds at home and at hospital in patients with and without invasive aspergillosis/invasive fungal infection (IA/IFI)

Patients	Clinical data			Environmental exposure	
	IA/IFI	Hospitalization ^a	Neutropenia ^a	Level of <i>A. fumigatus</i> in hematology corridors ^b	<i>A. fumigatus</i> + <i>A. flavus</i> percentages at home (%) ^c
P1	Proven IFI	Induction	Severe prolonged	0	35
P2	Probable IA	Induction	Severe prolonged	High level	0
P3	Probable IA	Allograft	Severe prolonged	High level	27
P4	Probable IA	Ambulatory	No	Low level	6
P5	Probable IA	Consolidation	Severe	High level	0
P6	Probable IA	Consolidation	No	High level	1
P7	Probable IA	Induction	Severe prolonged	0	22
P8	Probable IA	Consolidation	No	Low level	1
P9	Probable IA	Induction	Severe prolonged	0	9
P10	Probable IA	Induction	Severe prolonged	Low level	3
P11	Possible IA	Induction	Severe prolonged	0	15
P12	Possible IA	Consolidation	Severe	Low level	0
P13	Possible IA	Other hospitalization	Severe prolonged	High level	0
P14	Possible IA	Hospitalization for fever	No	0	48
P15	No	Allograft	Severe prolonged	High level	19
P16	No	Consolidation	Severe	Low level	4
P17	No	Induction	Severe prolonged	Low level	1
P18	No	Allograft	Severe prolonged	Low level	1
P19	No	Allograft	Severe	0	5
P20	No	Allograft	Severe	0	1
P21	No	Allograft	Severe prolonged	High level	6
P22	No	Allograft	Severe	0	6
P23	No	Induction	Severe prolonged	Low level	0
P24	No	Consolidation	Severe	Low level	0
P25	No	Allograft	Severe	High level	6
P26	No	Consolidation	Severe	Low level	2
P27	No	Allograft	No	High level	0
P28	No	Other hospitalization	No	High level	0
P29	No	Consolidation	Severe prolonged	Low level	0
P30	No	Consolidation	Severe	Low level	0
P31	No	Allograft	Severe prolonged	0	1
P32	No	Induction	Severe prolonged	Low level	1
P33	No	Allograft	No	High level	0
P34	No	Allograft	Severe prolonged	Low level	0
P35	No	Induction	Severe prolonged	Low level	0
P36	No	Allograft	Severe prolonged	Low level	0
P37	No	Allograft	Severe	Low level	0
P38	No	Consolidation	Severe	High level	6
P39	No	Allograft	Severe prolonged	Low level	18
P40	No	Allograft	Severe prolonged	Low level	1
P41	No	Other hospitalization	Severe	High level	0
P42	No	Allograft	No	Low level	0
P43	No	Allograft	Severe prolonged	Low level	5
P44	No	Allograft	Severe prolonged	0	0
P45	No	Allograft	Severe prolonged	Low level	0
P46	No	Allograft	Severe prolonged	High level	12
P47	No	Allograft	No	Low level	0
P48	No	Allograft	Severe prolonged	0	2
P49	No	Induction	Severe prolonged	Low level	14
P50	No	Consolidation	Severe prolonged	High level	5
P51	No	Allograft	Severe prolonged	Low level	0
P52	No	Allograft	Severe prolonged	0	0
P53	No	Allograft	Severe prolonged	High level	0

^aFor patients with IA/IFI, the hospitalization period indicated corresponded to the period when infections were diagnosed. For patients without IA, the described period was the most critical period for the patient if we consider neutropenia as the first risk factor for IA and environmental mold contamination as the second factor. Severe neutropenia, <0.5 Giga of polynuclear neutrophils/L, prolonged, for more than 15 days.

^bLow levels of *Aspergillus fumigatus* = 1 CFU/m³ on average and high levels of *A. fumigatus* = 9, 14 and 25 CFU/m³.

^c*A. fumigatus* and *Aspergillus flavus* percentage relative to the total count of molds at home.

exposed at hospital). This observation corroborates the need of efficient systematic fungal environmental surveillance. Environmental studies about water contamination (Warris et al., 2010), food contamination (Marr et al., 2009), and the influence of weather conditions on fungal contamination (Boff et al., 2013; Panackal et al., 2010) have already been conducted.

There is a growing interest in the issue of outpatient safety, especially because several antifungal drugs are now available for prophylaxis (Kontoyiannis, 2013). However, antifungal prophylaxis has shown varying success rates in preventing fungal infections (Kontoyiannis, 2011; Kousha et al., 2011). Prolonged antifungal prophylaxis in hematological patients seems inadequate to eliminate fungal risk without careful infection control and educating patients to avoid fungal exposure (Partridge-Hinckley et al., 2009). However, although nurses and physicians explain preventive measures to patients when they leave the hospital (no pets, no carpet, no flower arrangements...), home visits often showed that these measures were not applied. This observation illustrates the need for an explanatory educational document indicating the measures to prevent fungal exposure at home. It could be given to patients to further explain and reinforce the information provided by hematologists. While environment risk factors are beginning to be recognized, the importance of a dwelling survey after hospitalization is rarely mentioned by authors in the literature (Kontoyiannis, 2013). However, our study illustrates that when significant exposure to *A. fumigatus* and *A. flavus* is detected in homes of at-risk hematological patients, the risk of developing IA/IFI exists.

Unlike the regular weekly hospital environment surveillance, there was only one home visit in our study. A previous study dealing with reproducibility showed that 92% of air fungal concentration in dwellings remained constant when sampling was performed again 3–7 months afterward (Reboux et al., 2009). Thus, we considered that a single visit was enough to assess the level of fungal contamination in homes and that this level was stable during the study period. In the present study, dwellings showed relatively low contamination and are comparable with the standard housing findings presented in the Reboux et al. (2009) study conducted in Eastern France.

While a relatively common distribution of usual species among dwellings was observed, homes with the highest count of total mold were not those with the highest number of *A. fumigatus* and *A. flavus*. Moreover, the five patients who were only exposed at home are among those who lived in dwellings with the highest percentage of *A. fumigatus* and *A. flavus*. Neither the total contamination nor the *A. fumigatus/A. flavus* count was significantly linked to the IA/IFI cases. Other explanatory variables such as the count or the percentage of other *Aspergillus* (such as *Eurotium*

amstelodami) were also not significantly linked to the fungal infections. The *A. fumigatus/A. flavus* percentage is the only predictive factor for IA/IFI highlighted by our statistical analysis.

However, two patients with the same immunocompromised status and dwellings with high *A. fumigatus* and *A. flavus* percentage did not develop IA. Thus, an approach using the *A. fumigatus* and *A. flavus* percentage relative to the total count of molds (especially >15%) could be an original and appropriate strategy to assess the IA risk in chronically immunocompromised patients' homes. However, environmental home mold contamination should be integrated into the series of host factors (genetic factor, underlying disease, immune system, and immunosuppressive therapy) in this approach (Chamilos et al., 2006; Kousha et al., 2011; Meersseman et al., 2007; Pagano et al., 2006). Moreover, as any new concept, this approach using *A. fumigatus* and *A. flavus* percentage must be tested on a different set of patients.

Investigating a patient's home exposure for longer sampling times would have been an improvement. Therefore, we tested EDC devices over a 10-week period followed by qPCR quantification of target species, which has been proven to be a reliable way to investigate allergic risk (Scherer et al., 2014). However, as qPCR quantified equally viable (infective) and non-viable (non-infective) spores, the results obtained were not useful in discriminating which patients were at risk of IA.

Neither the 'type of dwelling' variable, nor any of the habit or lifestyle variables tested (such as opening windows, the presence of plants, efficient ventilation, or the presence of pets) significantly influenced total fungal contamination. Nevertheless, the two homes most contaminated with fungi were uncommon: one attached to a farm and one above a restaurant. In addition to the type of work, already mentioned by Pagano et al. (2011), personal habits, hobbies, and lifestyles are known to expose inhabitants to pathogenic fungi (Sipsas and Kontoyiannis, 2008). Another example from our study showed that the highest quantities of *A. fumigatus* were found in a home (P14) decorated with plaited dried wheat in all rooms (considered as a lucky charm in rural culture).

To conclude, this study emphasizes the fact that preventive measures should not be aimed only at the hospital setting and, therefore, supports the need for a counselor to carry out an environmental survey in patients' homes. Future monitoring at home, focusing on *A. fumigatus* and *A. flavus* percentage, could help to detect IA risk and substantiate the usefulness of specific preventive measures. Advice may be given such as avoiding activity in highly contaminated rooms (cellar, attic, garage, etc.), cleaning contaminated rooms, installing air treatment devices, delaying hospital release, or introducing health monitoring before

patients return home if a high risk of fungal infection is suspected.

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Conflict of interest

The authors report no declarations of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Mold contamination of dwellings in colony-forming units per cubic meter of air (CFU/m³).

Table S2. Quantities of DNA (in fg/μl) obtained by electrostatic dust fall collectors (EDC) qPCR analyses for 12 different qPCR molds targets.

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3. Détection des mucorales et *Aspergillus* au niveau sanguin pour le diagnostic précoce des infections fongiques invasives

Certaines espèces d'*Aspergillus* thermophiles et de mucorales peuvent provoquer, chez les patients à risque, des infections fongiques invasives gravissimes. Nous venons de voir l'intérêt de les rechercher dans l'environnement, par qPCR et par culture, pour limiter au maximum l'exposition des personnes fragiles aux spores potentiellement infectieuses.

Ces techniques de culture et de qPCR peuvent également être utilisées pour réaliser le diagnostic de l'infection. Dans les deux études suivantes, les mêmes systèmes d'amorces et de sondes ont été utilisés pour apporter et améliorer le diagnostic en médecine humaine.

A. Contexte et résultats principaux de la première étude

Titre de l'article : *Ribosomal and Mitochondrial DNA Target for Real-Time PCR Diagnosis of Invasive Aspergillosis*

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Journal, année publication : *Journal of Microbiology*, 2012

La mortalité des aspergilloses invasives est supérieure à 50 %. Seule la précocité du diagnostic et la mise en place d'un traitement adapté permet de réduire cette mortalité. Les patients à risque sont régulièrement suivis par des dosages sanguins de galactomannane (GM). Le GM est un composant de la paroi du champignon. IL est utilisé comme marqueur de sa prolifération. Son dosage sanguin est outil de screening validé pour diagnostiquer les aspergilloses invasives. Cependant, le GM ne permet pas un diagnostic de certitude et la qPCR spécifique d'*Aspergillus fumigatus* est utilisée en complément pour argumenter le diagnostic biologique. Une cible mitochondriale était déjà mise en place au laboratoire du CHU de Besançon dès qu'un patient présentait un dosage de GM positif.

L'objectif de l'étude est de tester une deuxième qPCR spécifique d'*Aspergillus fumigatus* ciblant une zone ribosomale et de valider ses performances, associées à celle du test immuno-enzymatique GM et de la qPCR mitochondriale.

Les sérums de 26 patients, ayant présenté deux réactions positives successives au test immunoenzymatique et chez qui une AI prouvée ou probable (selon les critères EORTC-MSG : European Organization for Research and Treatment of Cancer, Mycose Study Group) a été diagnostiquée, ont été analysés.

Ces patients, ayant eu un antigène GM positif, avait déjà bénéficié d'une qPCR mitochondriale. La qPCR ribosomale a été réalisée rétrospectivement. En parallèle, les limites de linéarité, la spécificité vis-à-vis d'autres moisissures ont été testées pour les deux qPCR.

Les résultats montrent une plus grande spécificité de la cible ribosomale. Toutefois, les réactions croisées de la cible mitochondriale peuvent être un atout pour le diagnostic : elles ont été observées pour *Aspergillus flavus*, *A. nidulans*, *A. niger*, *A. terreus*. Or *A. flavus* est une moisissure dont l'implication dans les AI augmente. Dans deux des cas où la qPCRm était positive avec une qPCRr négative, la culture était positive pour *A. flavus*. Par contre, la limite de détection pour la qPCRr est plus faible et permet une meilleure sensibilité diagnostique. En combinant les deux qPCR, la sensibilité diagnostique est augmentée et atteint 65 %.

B. Contexte et principaux résultats de la deuxième étude

Titre de l'article : *Quantitative Polymerase Chain Reaction Detection of Circulating DNA in Serum for Early Diagnosis of Mucormycosis in Immunocompromised Patients*

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Journal, année publication : *Clinical Infectious Diseases*, 2012

Si la qPCR détectant *Aspergillus fumigatus* est testée par plusieurs équipes depuis plusieurs années, le diagnostic des mucormycoses reposait sur l'examen histologique des tissus et sur la culture.

Le but de l'étude est de tester rétrospectivement 3 cibles de qPCR spécifiques de mucorales sur des sérums de patients pour lesquels une mucormycose a été diagnostiquée.

Ainsi 10 patients, avec un diagnostic certain et des sérums disponibles à -20°C, ont été inclus dans l'étude. L'ensemble des sérums, avant et après le diagnostic histologique, ont été extraits et analysés par qPCR spécifique : *Absidia corymbifera* (actuellement nommée *Lichtheimia corymbifera*), *Mucor* (cible capable d'amplifier plusieurs espèces dont *Mucor racemosus*, *circinelloïdes* et *Rhizopus*) et *Rhizomucor* (plusieurs espèces également). Les sondes et amorces utilisées sont celles publiées sur le site de l'EPA (annexe). Elles sont identiques à celles utilisées pour la qPCR dans les logements.

Sur les 10 patients, la qPCR aurait permis de faire le diagnostic plus précocement dans 9 cas : de 68 à 3 jours avant le diagnostic histologique. Dans les 9 cas, la cible positive correspondait à l'espèce retrouvée en culture ou par séquençage de la pièce paraffinée.

C. Conclusion pour les deux études

L'excellente sensibilité diagnostique des qPCR « mucorales », associée à l'amélioration du diagnostic des AI par ajout d'une deuxième cible, permet un screening efficace chez les patients à risque, et ce, sur un seul prélèvement sanguin et une seule extraction d'ADN.

Dans le cadre de ce diagnostic médical, la sensibilité est primordiale, la spécificité doit être intégrée à l'interprétation. Les systèmes de sondes et amorces utilisés en environnement s'avèrent pertinents. La bonne connaissance des limites de l'analyse permet au biologiste d'aider le clinicien dans sa décision de mise en place d'une thérapeutique adaptée. De la même façon, « l'échantillonnage » doit être intégré à l'interprétation : la présence d'antifongique, la date de prélèvement par rapport au développement fongique et la qualité de la phase pré analytique sont des éléments influençant le résultat.

Ribosomal and Mitochondrial DNA Target for Real-Time PCR Diagnosis of Invasive Aspergillosis[∇]

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The aim of the present study was to assess the diagnostic efficacy of a combination of two quantitative *Aspergillus* PCR assays, targeting a mitochondrial and a ribosomal target, in patients with risk factors for invasive aspergillosis (IA) and positive galactomannan (GM) antigen. Forty-four patients with hematological malignancies and risk factors for IA according to revised European Organization for Research on Treatment of Cancer and the Mycoses Study Group criteria (EORTC/MSG) criteria and presenting at least two sequential GM-positive sera were included in the study. Mitochondrial PCR was carried out prospectively on all GM-positive serum samples. Ribosomal PCR was carried out retrospectively on frozen stored sera. The sensitivities of mitochondrial and ribosomal PCRs were 58% and 50%, respectively. The diagnostic test performance was improved by using a combination of both PCR assays and by considering a patient PCR positive when at least two positive results were obtained. The sensitivity, specificity, and positive and negative likelihood ratios were 65%, 94%, and 11.8 and 0.37, respectively. A significant association between fatal outcome at 90 days and positive results of ribosomal PCR assays was observed (adjusted hazard ratio = 8.2; 95% confidence interval [CI] = 1.0 to 65.8; $P = 0.048$). Our results showed that the combination of two PCR assays targeting mitochondrial and ribosomal *Aspergillus* DNA improves the sensitivity of PCR in the diagnosis of IA in hematological patients with risk factors and positive GM results. This study also confirms that a positive PCR result is associated with a poor prognosis in these patients and should lead to specific antifungal therapy being introduced immediately.

Invasive aspergillosis (IA) is a life-threatening infection whose incidence is still increasing in immunocompromised patients. There has been a significant decrease in mortality in patients diagnosed with IA following hematopoietic cell transplantation in recent years, but the average death rate continues to exceed 50% (26). Early diagnosis and treatment are essential to improve the prognosis. Serial testing for detection of galactomannan (GM) in serum using the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad Laboratories) has been proposed as a screening tool for diagnosis of IA, as a positive GM result has been reported to appear before clinical signs, especially when a cutoff value of 0.5 is used (19, 20). However, false-positive GM results frequently occur (21, 27). A combination of GM detection with other types of biomarker detection, including circulating DNA, could be a good way to improve early diagnosis of IA (12, 15, 29).

Although hundreds of publications have reported the use of PCR for diagnosing IA, PCR is still not included in the European Organization for Research on Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) criteria (10). The reason for not including PCR is mainly the absence of a com-

mercially available system and the lack of standardization. Up to now, only methodological recommendations concerning DNA extraction from whole-blood specimens have been determined by the European *Aspergillus* PCR Initiative (EAPCRI) working group (28). There is a major consensus in favor of real-time PCR technology due to the sensitivity and rapidity of the techniques, as well as the absence of contamination by PCR products. However, a range of chemistry types are currently used (SYBR green, hybridization probes, and hydrolysis probes), and another highly debated area is the choice of the target DNA. Several multicopy genes, such as ribosomal genes—5.8S (24), 28S (2, 5), and, most frequently, 18S (4, 15, 16, 18), and the mitochondrial gene (3, 7, 25)—have been investigated for the assessment of real-time PCR assay in the diagnosis of IA. However, the designs of these studies are very diverse, making it impossible to compare the diagnostic performance with each type of DNA target.

In a previous study, we showed that detecting circulating *Aspergillus* DNA on the first GM-positive serum sample could help diagnose IA and assist in the decision to start antifungal therapy (22). Consequently, a strategy was implemented in our hospital in 2005 that consists of carrying out the detection of *Aspergillus* DNA by a real-time PCR assay targeting mitochondrial DNA on each GM-positive sample. The aim of the present study was to evaluate retrospectively a real-time PCR assay targeting 18S ribosomal DNA on the same sample and to assess the diagnostic efficacy of a combination of the two PCR

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assays, focusing on the ability to manage the initiation of specific antifungal therapy in high-risk patients with an initial positive GM result.

MATERIALS AND METHODS

Strains. Fourteen strains from species most frequently isolated in human infection, either from an international collection (Belgian Coordinated Collections of Microorganisms/Institute of Hygiene and Epidemiology—Mycology section [BCCM/IHEM], Brussels, Belgium; Centraalbureau voor Schimmelcultures [CBS], Utrecht, Netherlands; Institut Pasteur [IP] collection, Paris, France) or from our own laboratory collection (Mycology laboratory—Univ-hospital Besançon [BES]), were used in this study: *Candida albicans* IHEM 9559, *Candida glabrata* CBS 138, *Candida tropicalis* CBS 192, *Saccharomyces cerevisiae* IHEM 6036, *Aspergillus fumigatus* IP 2279, *Aspergillus nidulans* BES 1022, *Aspergillus terreus* BES 1429, *Aspergillus niger* BES 1798, *Aspergillus flavus* BES 1561, *Lichtheimia corymbifera* BES 227, *Rhizopus oryzae* BES 1798, *Scedosporium proliferans* IHEM 23387, *Scedosporium apiospermum* BES 328, and *Fusarium solani* complex BES 308.

Fungal DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Meylan, France), according to the manufacturer's recommendations, after the mechanical disruption of mycelia in liquid nitrogen using a pestle for molds. *A. fumigatus* DNA extract was serially diluted from 3×10^5 fg/10 μ l to 0.03 fg/10 μ l for sensitivity testing. For all other species, DNA concentrations were adjusted to 3×10^3 fg/10 μ l for real-time PCR specificity testing.

Patients. Four hundred eighty-nine patients followed in the hematology unit in Besançon University Hospital were screened for GM antigenemia from September 2005 to January 2009. Forty-four of them presented at least two sequential GM-positive sera over a period of less than 30 days and a risk factor for IA according to EORTC/MSG criteria (10) and were included in this study. The mean (minimum-maximum) age of patients was 46 (12 to 74) years. Hematological malignancies were as follows: acute myeloblastic leukemia ($n = 21$), acute lymphoblastic leukemia ($n = 6$), chronic lymphocytic leukemia ($n = 5$), lymphoma ($n = 4$), chronic myelocytic leukemia ($n = 2$), multiple myeloma ($n = 1$), aplastic anemia ($n = 1$), sickle-cell anemia ($n = 1$), granulocytic sarcoma ($n = 1$), acute plasmablastic leukemia ($n = 1$), and myelodysplastic syndrome ($n = 1$). Sixteen of them received a hematopoietic stem cell transplant (HSCT).

Clinical, radiological, and biological data were prospectively recorded using the information sheet from the French network for surveillance of IA. Antibiotic and antifungal therapies were collected from the nominative prescription system of anti-infective agents used in our hospital.

Cases of IA were classified as proven, probable IA, or no IA according to EORTC/MSG criteria revised in 2008 (10) (Table 1). There were 4 proven cases, 22 probable cases, and 18 no-IA patients. The 18 patients classified as no IA had host criteria and positive GM, but no clinical criteria; 3/18 received piperacillin-tazobactam at the time of the positive GM test.

Patients with possible IA (i.e., radiological signs but no positive biomarker) were not included in this study because the strategy implemented in 2005 in our hospital was to perform mitochondrial PCR only when patients had positive GM results.

Patients with only one positive GM result were also excluded from the study, because from 2005 to 2008, according to previous recommendations for classification of IA (1), two sequential positive tests were necessary to consider GM a microbiological criterion for probable cases of IA, and thus, clinical data were not recorded prospectively and sera were not stored for all these patients.

GM testing. The GM test was performed as part of twice-a-week systematic surveillance of all the patients presenting with host factors of IA monitored in our hematology unit. GM antigenemia was determined using the Platelia *Aspergillus* sandwich enzyme-linked immunosorbent assay (Bio-Rad, Marnes la Coquette, France), using an index of 0.5 as a positive threshold.

Real-time PCR assays. Mitochondrial real-time PCR was carried out immediately on each GM-positive serum sample. Automatic DNA extraction was performed using the MagNa Pure Compact Nucleic Acid Isolation Kit I on a MagNa Pure Compact apparatus (Roche Diagnostics, Meylan, France). Two independent DNA extractions were performed for each serum. Detection of mitochondrial *Aspergillus* DNA was performed using 10 μ l of each of the two extracts and a dual hybridization probe and primers, as previously described (7). Quantitative results were expressed by determining the detection threshold, or quantification cycle (Cq), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the Cq, the smaller the amount of DNA in the sample.

All DNA extracts were stored at -20°C . Ribosomal real-time PCR was respectively performed on stored DNA extracts, using primers AfumiR1/AfumiF1 and a hydrolysis probe (AfumiP1) targeting the 18S gene, as described by Haugland et al. (13; <http://www.epa.gov/microbes/moldtech.htm>). The PCR mixture was prepared in a 20- μ l final volume using the LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Meylan, France): 2 μ l of Master mix 1a/1b, 2.4 μ l of MgCl_2 (25 mM, stock solution), 1.6 μ l of probe (P1; 1 pM), 4 μ l primers (F1/R1; 5 pM) (Pharmacia Amersham, Uppsala, Sweden) and 10 μ l of DNA. PCRs were run on a LightCycler 2.0 (Roche Diagnostics). The thermal-cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C .

For both PCR assays, samples were considered positive when at least one replicate had a Cq of ≤ 45 cycles. Results were expressed as the mean Cq of the two extracts or the Cq of the positive replicate if only one replicate was ≤ 45 cycles. Indeed, a very small amount of fungal DNA is found in blood specimens from patients suffering from invasive aspergillosis (7, 18). The DNA concentration calculated after the positive control never exceeded a few femtograms per microliter. According to Poisson's law, the PCR result cannot be consistently positive at these very low concentrations. However, for decision making concerning hematology patients, we previously suggested considering a sample to be positive even if only one replicate is positive (22).

DNA sequencing. Real-time PCR products were subsequently sequenced when discordant results between the 2 PCR assays were observed. Sequencing was performed using a BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA) with the same primers used in the PCR step on an ABI Prism 3130 DNA analyzer (Applied Biosystems). Sequences were compared to those available in the GenBank database using BLAST software (<http://www.ncbi.nlm.nih.gov/blast>).

Statistical analysis. Data were entered in an electronic Excel database. We evaluated the ribosomal and mitochondrial PCR assays for IA diagnosis by determining various indicators of diagnostic test performance, including sensitivity (Se), specificity (Sp), and positive and negative likelihood ratios (LR+ and LR-), with a 95% confidence interval (95% CI). LR+ and LR- describe the discriminatory abilities of positive and negative test results, respectively. An LR+ above 10 and an LR- below 0.1 are considered to provide strong evidence in favor of or against diagnoses, respectively, in most circumstances (9). A receiver operating characteristic (ROC) analysis was performed to establish a decision threshold in terms of the number of positive PCR results. Indeed, four positivity thresholds were assessed for the combination of both PCR tests. These threshold values were level 1 (≤ 1 positive PCR result), level 2 (a single positive result in each PCR), level 3 (≥ 2 positive results in the same PCR and none in the other), and level 4 (≥ 2 positive results in each PCR). We assessed the significance of differences between groups by carrying out the Wilcoxon signed-rank test for continuous paired data and the Pearson χ^2 test or Fisher's exact test for categorical variables, as appropriate.

Kaplan-Meier survival curves, the log rank test, and Cox proportional-hazards models were performed to compare survival patterns. All analyses were two tailed, and a P value of less than 0.05 was considered significant. The software package Stata, version 10.0 (Stata Corp., College Station, TX), was used for the analysis.

RESULTS

Cross-reactivity and detection limits of ribosomal and mitochondrial PCRs. The ribosomal PCR assay was specific for *A. fumigatus*. The mitochondrial PCR assay was less specific, as DNA extracts from 4 other species of *Aspergillus* (*A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*) and from one species of zygomycete (*L. corymbifera*) were also amplified. The Cq values were similar, about 30 cycles, for *A. flavus* and *A. niger* (100% identity between *A. fumigatus*, *A. flavus*, and *A. niger* mitochondrial sequences). The Cq was higher for *A. nidulans* (37 cycles), showing that amplification was possible, but with less efficacy due to primer and/or probe mismatches with the target sequence (5 mismatches in primer sequences for *A. nidulans* [GenBank J01390.1]). The Cq value was also higher with *A. terreus* (42 cycles) and *L. corymbifera* (36 cycles), probably also due to primer and/or probe mismatches, but the mitochondrial sequences of these species are unknown. No

TABLE 1. Radiological findings, results of GM and PCR assays, antifungal treatment, and outcomes for 26 patients with proven IA and probable IA

Patient no.	Primary disease(s) ^a	Sex ^b	Age (yr)	Radiologic sign	No. of samples tested/positive ^c			Mycological culture	I.v. antifungal(s) ^f	Outcome at day 90 after diagnosis
					GM (index minimum-maximum)	PCRm (Cq minimum-maximum)	PCRr (Cq minimum-maximum)			
Proven IA (n = 4)										
1	AML	F	13	Halo	42/28 (0.5–5.5)	28/2 (43–41)	28/2 (40–41)		VOR, CAS, AMB	Survival
2	AML, HSCT	F	22	Nodules	30/21 (0.5–15.3)	21/6 (34–40)	21/none	<i>A. flavus</i> (palate biopsy)	AMB	Survival
3	AML	M	38		26/7 (0.6–3.7)	7/3 (41–43)	7/none	<i>A. flavus</i> (palate biopsy)	AMB, CAS	Survival
4	Aplastic anemia	M	74	Not done	4/2 (0.4–0.5)	2/2 (35–37)	2/2 (34–34)	<i>A. fumigatus</i> , <i>F. oxysporum</i> (lung biopsy)	AMB, CAS	Death
Probable IA (n = 22)										
1	AML	M	50	Nodules	34/6 (0.5–0.8)	6/none	6/none		CAS, AMB	Survival
2	AML, HSCT	F	34	Nodules	7/4 (0.5–1)	6/none	6/none			Survival
3	CLL	M	72	Halo	10/7 ^d (1.1–6)	7/2 (40–40)	7/2 (38–38)		VOR, AMB	Survival
4	Lymphoma	M	46	Nodules, halo	3/3 ^d (1.6–4.4)	3/2 (40–41)	3/2 (38–38)		AMB, VOR	Death
5	AML	F	54	Nodules	9/3 ^d (1.6–5.4)	3/2 (42–46)	3/2 (40–42)		CAS, VOR	Survival
6	CLL	M	56	Cerebral lesion	3/3 (1.2–4.9)	3/3 (38–45)	3/3 (33–45)		CAS, AMB	Death
7	AML, HSCT	M	54	Nodules	19/4 (0.5–1.7)	4/none	4/none		VOR	Survival
8	AML	F	16	Nodules, halo	14/4 (0.5–0.6)	4/none	4/none		AMB	Survival
9	CLL	M	62	Nodules	8/3 (0.6–0.7)	3/none	3/none		VOR	Survival
10	Sickle-cell anemia, HSCT	M	16	Air crescent sign	16/5 ^d (0.6–0.8)	5/3 (41–45)	5/none		VOR, CAS	Survival
11	AML, HSCT	F	36	Nodules	17/6 (0.6–4.7)	6/2 (45–45)	6/6 (39–41)		AMB, VOR, CAS	Death
12	Lymphoma	M	47	Nodules	4/2 (0.9–1)	2/none	2/none			Survival
13	ALL, HSCT	M	22	Halo	4/2 ^d (0.6–1.3)	2/none	2/none		VOR	Death
14	AML, HSCT	F	28	Halo	11/5 ^d (0.5–3.2)	5/none	5/none		VOR, CAS, AMB	Death
15	CLL	F	52	Nodules	27/16 ^d (0.6–6.8)	16/3 (42–44)	16/3 (41–42)		AMB, CAS	Survival
16	AML	F	57	Nodules	27/9 ^d (0.5–1.3)	9/3 (39–44)	9/5 (40–45)		AMB, CAS	Survival
17	CLL	M	61	Halo	8/4 (0.5–1.5)	4/none	4/none		VOR	Survival
18	AML	F	59	Nodules, halo	8/6 ^d (0.6–2.6)	6/3 (39–43)	6/none		AMB, VOR, CAS	Survival
19	ALL	F	39	Halo	11/3 ^d (0.6–2)	3/2 (41–43)	2/2 (38–40)		VOR, AMB	Death
20	AML	F	28	Nodules	9/2 (0.5–3.4)	2/1 (41)	2/1 (40)		AMB	Survival
21	CML, HSCT	F	48	Nodules	13/2 (0.7–2.1)	2/none	2/2 (37–41)	<i>Scopulariopsis</i> sp. (BAL ^e)	VOR	Death
22	AML, HSCT	M	56	Cerebral lesion	6/6 ^d (0.8–4.9)	6/none	6/3 (38–41)	<i>Phanerochaete chrysosporium</i> (sputum)	CAS, VOR, AMB	Death

^a AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; HSCT, hematopoietic stem cell transplantation.

^b M, male; F, female.

^c AMB, liposomal amphotericin B; CAS, caspofungin; VOR, voriconazole. According to the guidelines for antifungal therapies used in our institution (which are quite similar to those of the Infectious Diseases Society of America [IDSA]), caspofungin or L-Amb was used as a first-line treatment for febrile neutropenia and L-Amb was used when physicians suspected mold infection. Voriconazole was the first-line treatment for invasive aspergillosis. A combination of antifungals was used for cerebral and/or sinus aspergillosis, invasive aspergillosis with extrapulmonary foci, and bilateral IA with multiple nodules in highly immunocompromised patients.

^d Patient receiving piperacillin-tazobactam.

^e BAL, bronchoalveolar lavage.

^f PCRm, mitochondrial PCR; PCRr, ribosomal PCR.

TABLE 2. PCR results of calibrated dilution of DNA extract from *A. fumigatus* (IP 2279)

Amt of <i>A. fumigatus</i> DNA in vol tested (10 µl)	Quantification cycle (Cq)			
	Mitochondrial PCR		Ribosomal PCR	
	Extract 1	Extract 2	Extract 1	Extract 2
300 pg	22.3	22.3	22.9	22.8
30 pg	25.0	26.0	25.9	25.8
3 pg	29.9	29.8	29.4	29.8
300 fg	33.7	33.9	33.4	32.9
30 fg	38.2	38.6	36.1	36.7
3 fg	41.8	>46	39.8	40.6
0.3 fg	>46	>46	41.8	>46
0.03 fg	>46	>46	>46	>46

amplification was obtained with DNA extracts from other species.

An experiment with a serial dilution of *A. fumigatus* extract showed that for a low concentration of DNA (≤ 300 fg/10 µl), the Cq was lower with ribosomal PCR than with the mitochondrial PCR assay: the differences in the mean Cq values were 0.7, 2, and 1.6 with concentrations of 300 fg/10 µl, 30 fg/10 µl, and 3 fg/10 µl, respectively (Table 2). When the concentration was ≤ 3 fg/10 µl, the ribosomal PCR assay was positive for 3/6 extracts while the mitochondrial PCR assay was positive for only 1 of the 6 extracts. When clinical samples were tested, 11 sera from 11 different patients were positive for both mitochondrial and ribosomal PCR assays. The Cq of the ribosomal PCR assay was significantly lower than the Cq of the mitochondrial PCR assay (mean difference \pm standard deviation, 1.77 cycles \pm 2.18; $P = 0.022$).

Ribosomal and mitochondrial PCR for IA diagnosis and prognosis. (i) **ROC analysis and indicators of diagnostic performance.** The ROC analysis revealed that the optimal threshold was level 3 (Se = 61.5%; Sp = 100%). However, a threshold of level 2 (Se = 65.4%; Sp = 94.4%) gave the same result as a threshold level of 3 in terms of patients correctly classified (77.3%). For the diagnosis of IA (highly lethal), high sensitivity is the most important element. Thus, the positivity threshold retained was level 2. The area under the curve was equal to 0.82 (95% CI = 0.67 to 0.92; $P < 0.001$).

The diagnostic efficacy of each PCR and the association of both PCR assays, when performed in patients with at least 2

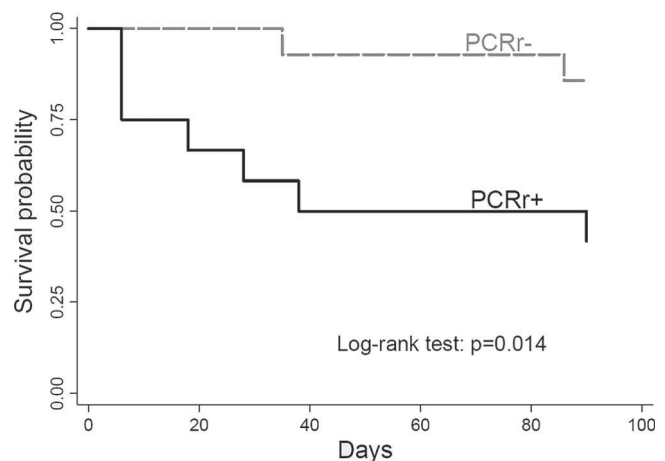


FIG. 1. Kaplan-Meier survival curves of patients with positive and negative ribosomal PCR results (PCRr+ and PCRr-) among 26 patients with proven or probable invasive aspergillosis.

positive GM results, were evaluated by comparing the results in probable and proven cases versus no-IA cases. The Se, Sp, LR+, and LR- of the PCR diagnosis were improved by using the association of both PCR assays and by considering a patient PCR positive when at least two positive results were obtained (i.e., at least one positive mitochondrial PCR test and one positive ribosomal PCR test, or at least two positive ribosomal PCR tests, or at least two positive mitochondrial PCR tests). Under these conditions, the Se, Sp, LR+, and LR- were 65%, 94%, 11.8, and 0.37, respectively (Table 3).

(ii) **Analysis of discordant PCR results.** For 2 probable cases (patients 21 and 22), mitochondrial PCR was negative while ribosomal PCR was positive. The ribosomal PCR product was sequenced, and in the two cases, the presence of *A. fumigatus* DNA was confirmed (100% identity with part of the *A. fumigatus* ribosomal DNA available in the GenBank database).

For 2 proven cases (patients 2 and 3) and 2 probable cases (patients 10 and 18), mitochondrial PCR was positive while ribosomal DNA was negative. In the 2 proven cases, *A. flavus* had been isolated by mycological culture of a palate biopsy specimen. We failed to sequence mitochondrial PCR products.

(iii) **PCR results and outcomes for the 26 patients with proven or probable IA.** Kaplan-Meier survival curves (Fig. 1)

TABLE 3. Indicators of diagnostic test performance

Test ^a	Result ^b					
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
≥ 1 positive result						
PCRm	57.7 (37.2–76)	94.4 (70.6–99.7)	93.8 (67.7–99.7)	60.7 (40.7–77.9)	10.4 (1.33–81.1)	0.45 (0.37–0.54)
PCRr	50 (30.4–69.6)	66.7 (41.2–85.6)	68.4 (43.5–86.4)	48 (28.3–68.2)	1.50 (0.93–2.42)	0.75 (0.59–0.95)
PCRm-PCRr	65.4 (44.4–82.1)	66.7 (41.2–85.6)	73.9 (51.3–88.9)	57.1 (34.4–77.4)	1.96 (1.33–2.89)	0.52 (0.38–0.70)
≥ 2 positive results						
PCRm	53.8 (33.7–72.9)	100 (78.1–100)	100 (73.2–100)	60 (40.7–76.8)		0.46 (0.39–0.54)
PCRr	46.2 (27.1–66.3)	100 (78.1–100)	100 (69.9–100)	56.3 (37.9–73.2)		0.54 (0.47–0.62)
PCRm-PCRr	65.4 (44.4–82.1)	94.4 (70.6–99.7)	94.4 (70.6–99.7)	65.4 (44.4–82.1)	11.8 (1.56–88.8)	0.37 (0.29–0.46)

^a PCRm, mitochondrial PCR; PCRr, ribosomal PCR; PCRm-PCRr, combination of both PCR assays.

^b PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio of a positive test; LR-, likelihood ratio of a negative test. The 95% CI is in parentheses.

revealed significantly shorter survival (outcome at 90 days) among patients with positive ribosomal PCR than those with negative ribosomal PCR (log rank test, $P = 0.014$) results. Cox proportional-hazards models showed a significant association between fatal outcome at 90 days and positive results of a ribosomal PCR assay (after adjusting for "primary disease," categorized as follows: 1, acute myeloblastic leukemia; 2, lymphoma and chronic lymphocytic leukemia; 3, hematopoietic stem cell transplantation; 4, other; adjusted hazard ratio = 8.2; 95% CI = 1.0 to 65.8; $P = 0.048$). No significant association was found between the mitochondrial PCR result or the combination of both ribosomal and mitochondrial PCR results and patient outcome. In addition, the Cox regression analysis did not show a significant difference in patient survival, according to the number of antifungal agents received (≤ 1 versus > 1).

(iv) PCR results and outcomes for the 18 patients classified as no IA. One of 18 patients had two positive PCR results (ribosomal PCR and mitochondrial PCR); he had no symptoms of respiratory tract infection. However, he received intravenous (i.v.) antifungal therapy and piperacillin-tazobactam at the time of the positive results and was alive at day 90.

Five of 18 patients had only one positive PCR result (ribosomal PCR only). Two of them died within 90 days: one patient had a *Rhizomucor pusillus* sinus infection and died despite antifungal treatment with liposomal amphotericin B and posaconazole; the other patient who died had symptoms of respiratory tract infection but nonspecific radiological signs and received i.v. voriconazole treatment. The other 3 patients with only one positive PCR result had nonspecific clinical or radiological signs: one patient had a *Streptococcus pneumoniae* infection and received adapted antibiotherapy, the second had oral voriconazole, and the last one had no antifungal treatment.

Twelve of 18 patients had negative PCR results; 2 patients had nonspecific clinical or radiological signs, received i.v. antifungal therapy, and were alive at day 90; the last 10 patients, who presented 2 to 8 sequential GM-positive sera, had no clinical signs of invasive fungal infection and were alive in the absence of antifungal treatment, reinforcing the idea that the GM test result was false positive. Of these 10 patients, only 1 received piperacillin-tazobactam; the causes of false positivity for the other 9 patients remain unknown.

DISCUSSION

Our results showed that the combination of two PCR assays targeting mitochondrial and ribosomal *Aspergillus* DNA improves the sensitivity of PCR in the diagnosis of IA in patients with risk factors and positive GM results. This study also showed that, in such patients, a positive ribosomal PCR result is associated with a poor prognosis.

Serum samples allow free-circulating *Aspergillus* DNA to be detected, while the use of EDTA-whole-blood samples allows conidia, hyphal fragments, and/or free-circulating DNA to be detected (17). We chose serum samples because serum has been shown to be an appropriate source of *Aspergillus* DNA amplification (7, 8), and also for several practical reasons: the use of the same sample as for GM detection and the fact that it is easy to store frozen samples and that standardized DNA

extraction is straightforward using an automated commercial kit.

The selection of an appropriate target for amplification is another key point for standardizing a PCR-based detection method, and multicopy genes, such as ribosomal or mitochondrial genes, are the best candidates. Our study demonstrates that the combination of the two targets increased the diagnostic efficacy. Two positive results of one or both PCR assays were strongly predictive of a diagnosis of IA, as the LR+ was higher than 10. On the other hand, negative results for both PCR assays had a moderate informative value for ruling out the diagnosis of IA, as the LR- was between 0.2 and 0.5. Unlike the predictive values, which are used more frequently, likelihood ratios are not dependent on the prevalence of disease in the study sample. Thus, findings can be generalized beyond the study and across different contexts.

The size of the *A. fumigatus* genome is about 29 Mb, and it is organized in 8 chromosomes with 9,631 (23) to 9,906 (11) genes. The ribosomal DNA gene (5.8S, 18S, and 28S) is located on chromosome 4, and the copy number was first reported to be about 35 copies (23). The copy numbers of the 18S RNA were recently determined for a variety of isolates and were found to vary with the strain from 38 to 91 copies per genome (14). Mitochondrial DNA is made up of 16 genes and is 32 kb in size (23). The copy number of mitochondrial genes used as targets in this study was estimated at between 9 and 10 mitochondrial copies per single-copy gene (8). Thus, ribosomal DNA, with a number of copies at least three times higher than that of mitochondrial DNA, can theoretically generate a higher signal when the same initial DNA concentration is amplified. We actually observed better detection of low concentrations of DNA by using ribosomal targets in experiments with calibrated DNA extracts and significantly lower Cq for ribosomal PCR when both PCRs were positive in clinical samples. We also observed 2 probable IA cases with positive ribosomal PCR and negative mitochondrial PCR results. In these 2 cases, it was demonstrated, using sequencing, that *A. fumigatus* DNA was present; the amounts of DNA in serum were probably very small and were detected using a ribosomal target only.

Experiments with calibrated DNA extracts showed that the ribosomal PCR assay was very specific for *A. fumigatus*, while other opportunistic *Aspergillus* species were detected using the mitochondrial DNA target, notably, *A. flavus*. This is particularly interesting because *A. flavus* has been increasingly recognized as an opportunistic pathogen in several cancer centers (6, 30). In the 2 proven cases with positive mitochondrial PCR and negative ribosomal PCR results, the implication of *A. flavus* was demonstrated by culture.

Thus, sensitivity is increased by using both PCRs, because of the decreasing detection limit using a ribosomal target and the enhancement of detected species using a mitochondrial target. This should be balanced by the fact that repetition of the same PCR assay could also lead to increased sensitivity. Indeed, in a sample with very small amounts of DNA, according to the Poisson law, the probability of having a copy of the target in the volume of sample tested, and thus of having a positive PCR result, would have been increased if triplicates or more had been tested, regardless of the mitochondrial or ribosomal probe.

The ribosomal PCR positive result, when obtained in pa-

tients at risk of IA with positive GM, was associated with a poor prognosis. Indeed, survival was significantly shorter among patients with positive ribosomal PCR results after adjusting for primary disease. The association between a positive mitochondrial PCR result and a fatal outcome was not found in the present study, while it was significant in our previous study (22). The small number of patients in each of the two studies probably explains this discordance. However, this second study confirms that a positive PCR result in patients at risk of IA with a positive GM could reinforce the decision to initiate specific anti-*Aspergillus* antifungal therapy, with the understanding that the therapy may be stopped if the diagnosis of IA is not confirmed.

In this retrospective study, we showed that the combination of mitochondrial and ribosomal targets increased the sensitivity of PCR for the diagnosis of IA and could help in decision making for initiating specific antifungal therapy, but this should be checked in a prospective multicenter study on a larger cohort. Determining the most efficient sequence of testing (including both types of PCR, and GM and beta-glucan testing) and the cost-effectiveness of diagnostic strategies using such a combination should also be included in further investigations.

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Quantitative Polymerase Chain Reaction Detection of Circulating DNA in Serum for Early Diagnosis of Mucormycosis in Immunocompromised Patients

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Background. The aim of our study was to assess the detection of circulating DNA from the most common species of Mucorales for early diagnosis of mucormycosis in at-risk patients.

Methods. We retrospectively evaluated a combination of 3 quantitative polymerase chain reaction (qPCR) assays using hydrolysis probes targeting *Mucor/Rhizopus*, *Lichtheimia* (formerly *Absidia*), and *Rhizomucor* for circulating Mucorales detection. Serial serum samples from 10 patients diagnosed with proven mucormycosis (2–9 samples per patient) were analyzed.

Results. No cross-reactivity was detected in the 3 qPCR assays using 19 reference strains of opportunistic fungi, and the limit of detection ranged from 3.7 to 15 femtograms/10 μ L, depending on the species. DNA from Mucorales was detected in the serum of 9 of 10 patients between 68 and 3 days before mucormycosis diagnosis was confirmed by histopathological examination and/or positive culture. All the qPCR results were concordant with culture and/or PCR-based identification of the causing agents in tissue (*Lichtheimia* species, *Rhizomucor* species, and *Mucor/Rhizopus* species in 4, 3, and 2 patients, respectively). Quantitative PCR was negative in only 1 patient with proven disseminated mucormycosis caused by *Lichtheimia* species.

Conclusion. Our study suggests that using specific qPCR targeting several species of Mucorales according to local ecology to screen at-risk patients could be useful in a clinical setting. The cost and efficacy of this strategy should be evaluated. However, given the human and economic cost of mucormycosis and the need for rapid diagnosis to initiate prompt directed antifungal therapy, this strategy could be highly attractive.

Keywords. quantitative PCR; mucormycosis; biomarker; circulating DNA.

Several recent studies report the increasing incidence of mucormycosis in hematopoietic stem cell transplant recipients, but also in other at-risk patients (ie, solid organ transplant, hematological malignancy, and

uncontrolled diabetes mellitus). Recent data from the United States shows that 63% (105/169) of non-*Aspergillus* mold infections in transplant recipients are Mucorales infections [1]. An increasing incidence of mucormycosis was reported in France between 1996 and 2007, particularly in patients with hematological malignancies (>24% per year) or bone marrow transplant (>15% per year) but also in the population of patients with diabetes (>9% per year) [2]. The increasing incidence of mucormycosis in Belgium between 2000 and 2009 was reported as probably related to the increase in the number of high-risk patients, particularly in those with an underlying hematological

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malignancy [3]. Mucormycosis is being diagnosed more and more frequently in developing countries, especially in India, China, and Latin America [4, 5]. Rhino-orbitocerebral presentation is the predominant form, possibly because of its association with diabetes. Renal mucormycosis has also emerged as a new clinical entity in India and China [6].

Rhizopus species are the most common genera in the United States and in France (55% and 49%, respectively). Distribution of other Mucorales species depends on geographical location. *Rhizopus* species were followed by *Mucor* species (19%), *Rhizomucor* species (7%), *Cunninghamella* species (9%), and *Lichtheimia* species (formerly *Absidia* species, 3%) in the United States, and by *Lichtheimia* species (29%), *Rhizomucor* species (7%), and *Mucor* species (3%) in France [7]. The prognosis of mucormycosis remains poor, with a reported mortality rate between 22% and 25% for cutaneous and rhinocerebral infections, to between 48% and 79% for pulmonary and disseminated forms [7].

Clinically and radiographically, mucormycosis is often indistinguishable from other invasive mold infections such as aspergillosis and remains difficult to diagnose. Coinfection with *Aspergillus* species may be frequent [3]. However, Mucorales species are not susceptible to voriconazole, which is often used as a first-line treatment in aspergillosis. Thus, early specific diagnosis and prompt therapeutic intervention with active antifungal treatment such as amphotericin B are essential for improving the outcome of mucormycosis.

Diagnosis is done by histopathological examination and positive culture of a Mucorales species. Mucorales polymerase chain reaction (PCR) performed on formalin-fixed, paraffin-embedded tissue samples followed by sequencing is useful for diagnosis confirmation in cases of culture-negative invasive mold infection [8, 9]. However, in a clinical setting, obtaining deep tissue samples or bronchoalveolar lavage fluids from a patient with suspected mucormycosis may be not feasible, especially in hematological patients. If done, histopathological identification of Mucorales in tissue specimens is difficult and requires significant expertise. Moreover, Mucorales species observed in tissue often fail to grow in fungal culture [8]. Up to now, none of the fungal biomarkers available (β -glucan, galactomannan) have been useful for mucormycosis diagnosis. Developing DNA or antigen-based detection for early diagnosis has been underlined as a key issue in mucormycosis research [10].

Circulating DNA from Mucorales has been detected in plasma samples obtained from an in vivo rabbit model of invasive pulmonary mucormycosis. Specific DNA was detected using quantitative PCR in plasma samples from rabbits infected with *Rhizopus*, *Mucor*, and *Cunninghamella* species, as early as day 1 after inoculation [11]. A molecular approach, detecting circulating DNA from Mucorales in at-risk patients,

may thus help to diagnose invasive mucormycosis quickly and to introduce directed therapy earlier.

The aim of our study was to assess the detection of circulating DNA from the most common species of Mucorales for early diagnosis of mucormycosis in at-risk patients. We retrospectively evaluated a combination of 3 quantitative PCR (qPCR) assays targeting *Mucor/Rhizopus*, *Lichtheimia*, and *Rhizomucor* species using serial serum samples from 10 patients diagnosed with proven mucormycosis between January 2004 and June 2012 in our hospital.

MATERIALS AND METHODS

Strains

Nineteen strains from the fungal species that are most frequently isolated in human infections, either from an international collection (Belgian Coordinated Collections of Microorganisms/Institute of Hygiene and Epidemiology–Mycology section [IHEM], Brussels, Belgium; Centraal bureau voor Schimmelcultures [CBS], Utrecht, Netherlands; Institut Pasteur [IP] collection, Paris, France) or from our own laboratory collection (Mycology laboratory—University Hospital Besançon [BES]), were used in this study, as follows: *Lichtheimia corymbifera* BES 227, *Lichtheimia ramosa* CBS582.65, *Lichtheimia ornata* CBS 291.66, *Rhizopus oryzae* BES 2224, *Rhizopus oryzae* BES 1683, *Mucor racemosus* BES1510, *Mucor ramosissimus* BES1439, *Rhizomucor pusillus* BES 2506, *Rhizomucor pusillus* BES 2495, *Aspergillus fumigatus* IP 2279, *Aspergillus nidulans* BES 1022, *Aspergillus terreus* BES 1429, *Scedosporium prolificans* IHEM 23387, *Scedosporium apiospermum* BES 328, *Fusarium solani* complex BES 308, *Candida albicans* IHEM 9559, *Candida glabrata* CBS 138, *Candida tropicalis* CBS 192, and *Saccharomyces cerevisiae* IHEM 6036.

Fungal DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Meylan, France), according to the manufacturer's recommendations, after the mechanical disruption of mycelia in liquid nitrogen using a pestle for molds. DNA extract from Mucorales species was serially diluted from 300 femtograms [fg]/10 μ L to 1 fg/10 μ L for qPCR sensitivity testing. For all other species, DNA concentrations were adjusted to 300 fg/10 μ L for qPCR specificity testing. For each qPCR assay, a positive control containing 30 fg/10 μ L of DNA from the main targeted species was prepared. These controls were run in 10 separate series to assess reproducibility.

Patients

Twenty-one patients were diagnosed with proven or probable mucormycosis at the University Hospital of Besançon between January 2004 and June 2012. Ten patients diagnosed with proven mucormycosis and with available sera at the time of

diagnosis were included in the study. The date of histopathological diagnosis was defined as day 0. Other patients presenting with probable mucormycosis, or without available sera between day -10 and day 10 were not included. Biological, radiological, and clinical data were collected at the time of diagnosis. Seven patients had hematological malignancy: acute myeloid leukemia (AML; n = 2), lymphoma (n = 2), acute lymphoid leukemia (n = 1), allogeneic hematopoietic stem cell transplant (allo-HSCT) for myelodysplasia (n = 1), severe aplastic anemia (n = 1); and 3 patients had other risk factors (renal transplantation, polytraumatism, alcoholism, and undernutrition).

The clinical presentations included pulmonary (n = 1), rhinocerebral (n = 2), cutaneous (n = 1), and disseminated (n = 6) localizations. All the cases were proven mucormycosis according to European Organization for Research on Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) consensual definitions (Table 1), with identification of causative Mucorales by culture and/or molecular techniques on biopsies. Positive culture was obtained in 7 patients: *Lichtheimia c.* (n = 3), *L. ramosa* (n = 1), *R. pusillus* (n = 2), and *R. oryzae* (n = 1). In 7 of 10 cases, fresh and/or paraffin-embedded tissues were available for investigation by PCR sequencing targeting 18S ribosomal DNA (18S rDNA), as described by Bialek et al [12], and/or internal transcribed spacer [13]. For the 3 patients without positive culture, the causative Mucorales species was identified using molecular techniques only: it was *Lichtheimia* species for patient 4, *R. oryzae* for patient 7, and *Rhizomucor* species for patient 10.

Negative Controls

Ten healthy patients without hematological malignancy and 10 patients with hematological malignancy but without invasive fungal infection described in a previous study [14] were included as negative controls. Sera from 17 patients with probable or proven invasive aspergillosis according to EORTC/MSG criteria [15] and sera from 14 patients with *Pneumocystis jirovecii* infection were tested using the 3 qPCR assays.

Quantitative PCR Assays

The following 3 qPCR assays, previously described by the US Environmental Protection Agency for environmental analysis, were adapted for detection of Mucorales DNA from serum: (1) *Absidia corymbifera* (assay name: Acory [qPCR assay was designed before the taxonomy was changed from *Absidia* to *Lichtheimia*]); (2) *Mucor amphibiorum/circinelloides/hiemalis/indicus/mucedo/racemosus/ramosissimus* and *Rhizopus azygosporus/homothalicus/microspor/oligosporus/oryzae* (assay name: Muc1); (3) *Rhizomucor meihei/pusillus/variabilis* (assay name: Rmuc).

Sequences from primers and probes can be found on the US Environmental Protection Agency website (<http://www.epa.gov/nerlcwww/moldtech.htm#primers>).

The PCR mix was prepared in a 20 μ L final volume using the LightCycler 480 Probes Master (Roche Diagnostics, Meylan, France): 11 μ L of Master Mix containing 0.4 μ L probe (P1, 4 μ M), 0.5 μ L primers (F1/R1, 40 μ M), and 9 μ L DNA. PCR reactions were run on a LightCycler 480 Instrument II (Roche Diagnostics). The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 minutes, followed by 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C. We did not use an internal control for inhibition because with the type of commercial kit we used, PCR inhibitors are rarely observed [16].

Detection of Circulating DNA in Serum

Detection of fungal DNA using qPCR was done retrospectively on 51 serum samples stored at -20°C. Sera sampled between day -75 and day 29 from the time of diagnosis of mucormycosis were tested (2-9 sera per patient).

Automatic DNA extraction was performed using 1 mL of serum with a Large Volume MagNa Pure Nucleic Acid Isolation Kit on a MagNa Pure Compact apparatus (Roche Diagnostics, Meylan, France). Elution was performed in a 50- μ L volume. Detection of fungal DNA was performed with 9 μ L of extract, using each of the 3 qPCR assays described above.

Quantitative results were expressed by determining the detection threshold, or quantification cycle (Cq), that marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the Cq, the smaller the amount of DNA in the sample.

RESULTS

Cross-Reactivity, Reproducibility, and Detection Limits of PCR

Amplification was obtained only with DNA extracts from the targeted species for each of the 3 qPCR assays: *L. corymbifera*, *L. ramosa*, *L. ornata* with the Acory assay; *R. oryzae*, *M. racemosus*, and *M. ramosissimus* with the Muc1 assay; and *R. pusillus* with the Rmuc assay. No cross-reactivity with other DNA fungal extracts was detected for any of the 3 qPCR assays. Serial dilution of DNA extracts showed that the limit of detection ranged from 3.7 to 15 fg/10 μ L depending on the species (Table 2). Reproducibility testing showed that the variation coefficient was <0.02 (mean Cq \pm 1) for each of the qPCR assays.

Detection of Circulating DNA in Serum

All of the 3 qPCR assays were negative in patients without hematological malignancy and in patients with hematological malignancy but without invasive fungal infection. All 3 qPCR

Table 1 Clinical, Histological, and Mycological Findings, Antifungal Treatment, Results of Quantitative Polymerase Chain Reaction Assays, and Outcomes for 10 Patients With Proven Mucormycosis

Patient No.	Sex ^a / Age	Underlying Disease	Localization of Infection, Date ^b of First Clinical Symptoms/First Radiological Signs	Date ^b of Fever Onset	Positive Histology (Day 0)/Molecular Diagnosis	Positive Mycological Cultures (Day)/Identification	Antifungal Therapy (Day of Initiation of L-AmB, POS)	Outcome at Day 90	qPCR Result Cq (Day)	Days From 1st Positive PCR to 1st Clinical Signs ^c	Days From 1st Positive PCR to Positive Histology ^d
1	F/65	Undernutrition, alcoholism	Disseminated D -1/not done	D -5	Skin/not done	Skin, urine (D0)/ <i>L. corymbifera</i>	FLU	Death (D1)	Acory Neg (D -8, D -1)	/	/
2	M/37	HL	Disseminated D -16/not done	D -28	Lung, liver/not done	Lung, liver (D0)/ <i>L. corymbifera</i>	L-AmB (D -1)	Death (D0)	Acory 28 (D -3), Neg (D -10)	+13	-3
3	M/51	ALL	Rhinocerebral D -1/D -1	D -9	Sinus/ <i>Lichtheimia corymbifera</i>	Sinus (D0)/ <i>L. corymbifera</i>	L-AmB (D0) POS (D0)	Death (D3)	Acory 36 (D -8), 32 (D -5), 30 (D -1), 25 (D0), 27 (D2), Neg (D -18, D -15, D -12)	-7	-8
4	F/48	Polytrauma	Cutaneous D -5/not done	D0	Skin/ <i>Lichtheimia</i> sp	Skin (D0)/negative	FLU, CAS	Death (D2)	Acory 27 (D0), Neg (D -2, D -9)	+5	0
5	M/59	NHL, diabetes mellitus	Disseminated D -5/D -22	D -14	Kidney/ <i>Lichtheimia</i> sp	Cerebrospinal fluid (D -10)/ <i>Lichtheimia ramosa</i>	FLU, CAS, L-AmB (D0)	Alive	Acory 32 (D -23) 38 (D -8), Neg (D -1, D5)	-18	-23
6	F/41	PNH, SAA	Disseminated D -30/D -4	D -17	Sinus/ <i>Rhizopus</i> sp	Sinus (D0)/ <i>R. oryzae</i>	CAS, POS (D -11) L-AmB (D0) deferasirox (D0)	Death (D15)	Muc1 36 (D -10), 35 (D -1), 38 (D2), 36 (D6), 38 (D9), 34 (D13)	+20	-10
7	F/13	AML	Pulmonary ^e D -62/D -61	D -65	Lung/ <i>Rhizopus oryzae</i>	Not done	VOR, L-AmB (D -60)	Alive	Muc1 35 (D -68), 40 (D -62), 36 (D -55), 38 (D -21), 36 (D10), Neg (D -75, D29)	-6	-68
8	M/57	MDS, allo-HSCT	Rhino-cerebral D -18/D -11	D0	Sinus/not done	Sinus (D0)/ <i>R. pusillus</i>	ITR (D -96), L-AmB (D0) POS (D0)	Alive	Rmuc 39 (D -18), 39 (D -1), Neg (D -11, D -8, D9, D13)	0	-18
9	F/55	Renal transplant	Disseminated D -24/D -11	D -30	Lung, heart/ <i>Rhizomucor pusillus</i>	Lung, heart (D0)/ <i>R. pusillus</i>	CAS	Death (D0)	Rmuc 29 (D -4), 22 (D -3), Neg (D -24, D -19)	+20	-4
10	M/60	AML	Disseminated D -50/D -47	D -50	Liver, spleen/ <i>Rhizomucor</i> sp	Liver, spleen (D0)/negative	VOR, L-AmB (D -44) POS (D -36)	Alive	Rmuc 41 (D -49), 28 (D -46), Neg (D -52, D -42, D -39, D -36, D -18, D -13, D -1)	+1	-49

Abbreviations: ALL, acute lymphoblastic leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplant; AML, acute myeloblastic leukemia; CAS, caspofungin; FLU, fluconazole; HL, Hodgkin lymphoma; ITR, itraconazole; L-AmB, liposomal amphotericin B; MDS, myelodysplasia; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction; PNH, paroxysmal nocturnal hemoglobinuria; POS, posaconazole; qPCR, quantitative polymerase chain reaction; SAA, severe aplastic anemia, VOR, voriconazole.

^a M, male; F, female.

^b D0 = date of positive histology.

^c A minus sign indicates that polymerase chain reaction was positive before the first clinical signs.

^d A minus sign indicates that polymerase chain reaction was positive before the positive histopathology.

^e *Aspergillus niger*-*Rhizopus oryzae* coinfection, based on molecular analysis of operative specimen (lobectomy).

Table 2. Quantitative Polymerase Chain Reaction Results for Calibrated DNA Extract From Several Mucorales Species Using the Acory, Muc1, and Rmuc Assays

Amount of Mucorales, DNA in Volume Tested (10 µL)	Quantification Cycle			
	Acory Assay	Muc1 Assay	Muc1 Assay	Rmuc Assay
	<i>Lichtheimia corymbifera</i> / <i>L. ramosa</i> / <i>L. ornata</i>	<i>Rhizopus</i> <i>oryzae</i>	<i>Mucor racemosus</i> / <i>M. ramosissimus</i>	<i>Rhizomucor</i> <i>pusillus</i>
300 fg	35/28/30	34	32/35	39
30 fg	37/32/33	36	35/37	41
15 fg	39/34/35	38	37/38	45
7.5 fg	42/39/41	43	>46/>46	>46
3.7 fg	>46/43/>46	>46	>46/>46	>46
1.9 fg	>46/>46/>46	>46	>46/>46	>46
0.9 fg	>46	>46	>46/>46	>46

assays were also negative in sera from patients with invasive aspergillosis and *Pneumocystis* infection. Quantitative PCR was negative in only 1 patient (patient 1) with proven disseminated mucormycosis, caused by *L. corymbifera*. The combination of 3 targeted qPCR assays was positive before diagnosis for the 9 other patients with proven mucormycosis. All the positive qPCR results were concordant with culture and/or PCR-based identification of the causing agents in tissue (*Lichtheimia*, *Rhizomucor*, and *Mucor/Rhizopus* qPCR assay positive in 4, 3, and 2 patients, respectively).

First serum with positive qPCR was sampled from 68 to 3 days before diagnosis, with Cq between 39 and 25 cycles (Table 1). In 5 of the 9 patients, circulating Mucorales DNA was detected 1–8 days before occurrence of the first clinical or radiological signs. Of note, 2 patients showed circulating DNA >6 weeks before diagnosis of proven mucormycosis (68 and 49 days, respectively).

Patient 7 presented with circulating *Rhizopus* DNA at day –68. She was originally diagnosed with probable pulmonary aspergillosis (nodules, halo sign, positive galactomannan in serum at day –62) during induction chemotherapy for AML. She received a combination of liposomal amphotericin B (L-AmB) 3 mg/kg/day and voriconazole for 14 days (day –61 to day –49), followed by combined voriconazole-caspofungin (day –48 to day –23), and later voriconazole–L-AmB (day –22 to day –5), voriconazole–caspofungin (day –4 to day 31), and voriconazole–L-AmB (day 32 to day 98, including allo-HSCT at day 50). Lobectomy was planned before allo-HSCT. Diagnosis of proven aspergillosis was performed by examination of lung operative specimen (day 0: positive histology, negative mycological culture). The diagnosis of *Aspergillus*–Mucorales coinfection was performed retrospectively 3 years later by another expert pathologist examination (2 distinct types of hyphae, including characteristic features of Mucorales hyphae) and molecular analysis of day 0 operative specimens.

Rhizomucor DNA was detectable at day –49 in patient 10, who presented with febrile neutropenia associated with pulmonary nodules during induction chemotherapy of AML. Initial antifungal therapy based on intravenous voriconazole was switched to L-AmB (day –44 to day –35), because voriconazole caused neurological adverse events to occur rapidly. The patient was discharged from hospital at day –35, with posaconazole therapy. Compliance to posaconazole therapy remained poor, however. Radiological evidence of liver and spleen abscesses (day –7) led to surgical resection and then diagnosis by direct examination of operative specimens (liver and spleen abscesses, day 0), confirmed by histology and molecular analysis performed on these specimens.

Only 4 patients underwent surgery (pulmonary lobectomy in patient 7, abdominal surgery in 2 patients (patients 5 and 10), and sinus debridement without enucleation in patient 8). All 4 of these patients were alive at day 90 after diagnosis, while the 6 patients who were not treated with surgery died before day 15 (5/6 patients died before day 3).

DISCUSSION

Detection of specific circulating DNA appears to be a noninvasive, sensitive, and specific technique that may be a useful tool for early diagnosis of infection involving Mucorales, especially in patients who cannot undergo a biopsy or a bronchoalveolar lavage due to thrombocytopenia, coagulopathy, or any other reason. We were able to detect Mucorales DNA in serum samples from 9 of 10 patients up to 68 days before mucormycosis diagnosis was confirmed by histopathological examination and/or positive culture. The combination of 3 targeted qPCR assays allowed us to diagnose the genera implicated accurately, and in particular could identify the *L. corymbifera* species, which is the most frequent cause of mucormycosis in our hospital (8/21 cases of proven or

probable mucormycosis diagnosed between January 2004 and June 2012).

Specificity of the 3 qPCR assays was checked: no cross-amplification with yeast species or other molds commonly causing invasive fungal infection (*Fusarium*, *Scedosporium*, and *Aspergillus* species) was observed. This very good specificity is the main advantage of the technique, especially in cases of double infections that are difficult to diagnose by histopathological examination. Indeed, infections with a concomitant fungal pathogen were observed in one-third of patients (8/24) with mucormycosis in one well-described series, including *Aspergillus*, *Fusarium*, or *Candida* coinfection [17]. Occurrence of such coinfections is probably underreported [9]. However, detection of double infections involving a Mucorales species is crucial for identifying an appropriate treatment. Detecting Mucorales circulating DNA, in association with the detection of other biomarkers (β -glucan, galactomannan, and *Aspergillus* circulating DNA), could be very helpful when making the decision to initiate directed therapy early in patients showing radiological signs of fungal infection. Patient 7 is a good example of this kind of coinfection, as she showed numerous positive galactomannanemia and positive *Aspergillus* qPCR, concomitantly with positive Mucorales DNA detection in sera.

We used primers and probes that targeted the 18S rDNA gene to detect circulating DNA from the most common species causing mucormycosis in Europe (*Mucor/Rhizopus* species, *Lichtheimia* species, and *Rhizomucor* species); targeting this multicopy gene allows us to detect a very small amount of DNA. Indeed, in our preliminary experiment, as few as 3.7–15 fg DNA could be detected, depending on the species and target studied. These values were quite similar to those we obtained previously (3 fg) with *Aspergillus* qPCR [14]. The copy numbers of the 18S rDNA varied between fungal species [18], and also between different strains of the same species, that is, *Aspergillus fumigatus* with 38–91 copies per genome [19]. Quantification of rDNA provided by qPCR and qPCR assay limit of detection depend on infecting species or strains, as the 18S rDNA copy number cannot be assumed based on another value previously determined from an unrelated strain.

The ability to detect a very small amount of DNA is essential for early diagnosis using qPCR techniques. There are numerous benefits of early mucormycosis diagnosis: prevention of progression to dissemination, reduced need for surgery or less surgical resection, less disfigurement and suffering, and improvement of outcome and survival [20]. Delayed therapy was shown to result in a 2-fold increase in mortality at 12 weeks [21]. In our series, 6 of the 10 patients died before day 90, including 3 patients without any Mucorales-active antifungal treatment, and 3 patients who died 1–15 days after implementation of L-AmB therapy. For all patients except 1 with a negative PCR result, appropriate antifungal treatment would have been given

between 1 and 23 days earlier and/or not temporarily discontinued (as in patient 7) if the PCR result had been known.

Taxonomy of the *L. corymbifera* complex was recently reevaluated and 3 distinct species are now recognized: *L. corymbifera*, *L. ramosa*, and *L. ornata* [22, 23]. Interestingly, the qPCR Acory assay is able to detect all 3 species. The *L. corymbifera* “complex” is a rare cause of mucormycosis in the United States [1] but has been reported as one of the main species identified in France (29%) [7], and accounted for 19% of species in a recent European series [24]. *Lichtheimia* species were identified in 38% of cases of probable and proven invasive mucormycosis since 2004 in our hospital, and were observed in 50% of the patients in this study. Environmental differences probably explain these contrasting data. Besançon is located in a rural area in eastern France, where the *L. corymbifera* complex has been shown to be one of the most frequent molds in farming environments and an etiological agent of farmer’s lung disease [25]. Four of the 5 patients with mucormycosis due to *Lichtheimia* species in our series had been living in a rural district (ie, villages with <2000 inhabitants).

Our strategy does not include a pan-Mucorales DNA assay or a specific qPCR assay targeting less frequent genera of Mucorales (ie, *Cunninghamella* species, *Saksenaia* species, *Apoophysomyces* species), contrary to previous qPCR studies [11, 26], but infections due to such fungi have never been diagnosed in our institution. Specific qPCR assays targeting these genera should be implemented to optimize the panel of species detected, if required by local ecology. Detection of additional Mucorales species from subtropical areas could be required in assays to be used in developing countries.

Our study suggests that using specific qPCR targeting several species of Mucorales according to local ecology for screening at-risk patients is feasible in a clinical setting. Indeed, the use of a high-throughput qPCR system reduces analysis time and cost. About a hundred samples can be analyzed with different targets in a single run of 2 hours, if harmonized amplification protocols are used. A key issue will be to identify the population of susceptible patients to increase the positive predictive value of the assay result [11]. First, the combination of the 3 qPCR assays targeting Mucorales species is now performed in our hospital in immunocompromised patients with clinical signs of fungal infection, when available biomarkers (galactomannan, β -glucan and qPCR targeting *Aspergillus* species) remain negative. Second, a strategy including a combination of the 3 qPCR assays targeting Mucorales species and 2 qPCR assays targeting *Aspergillus* species for twice-weekly screening of very high-risk patients (patients with acute leukemia or lymphoma receiving intensive chemotherapy, during the aplasia period, and allo-HSCT recipients, from day 0 to day 35 after the graft) is ongoing for prospective evaluation. The cost and efficacy of this strategy should be

evaluated. However, given the human and economic cost of mucormycosis, the importance of rapid diagnosis to initiate a prompt, efficient antifungal therapy, and the possible cost optimization of a high-throughput qPCR system, we believe this strategy could be highly attractive.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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V. CHAPITRE 3 : ELARGISSEMENT DE LA COMPREHENSION DE L'ENVIRONNEMENT INTERIEUR A L'ECHELLE MICROSCOPIQUE

A mesure du développement des technologies, la caractérisation du monde microbien va vers une meilleure reproductibilité et une représentativité élargie, mais également vers des proxy traduisant les micro-organismes et non plus les microorganismes eux même : de la culture, donnant des colonies identifiables, à la mesure d'allergènes ou de composant spécifiques, donnant un résultat en unité arbitraire, à la qPCR, exprimée dans un premier temps en équivalent spores (Bellanger et al. 2009, Scherer et al. 2014)) puis en concentration d'ADN (Rocchi et al. 2015, Millon et al. 2013) ; et enfin à la métagénomique, exprimant les résultats en nombre d'OTU (Operational Taxonomic Unit). Si ces améliorations sont un atout pour la recherche, il reste important de comprendre, par l'expérience, les différentes interactions pouvant exister à l'échelle microscopique de notre environnement intérieur.

1. Dessin de nouvelles cibles pour identifier spécifiquement ou par genre les acariens domestiques

Titre de l'article : *Detecting and quantifying mites in domestic dust: A novel application for real-time PCR*

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Contexte et principaux résultats de l'étude

La présence des acariens dans les poussières est mesurée le plus souvent par Elisa avec mesure des allergènes fréquents (comme Der p1 pour *Dermatophagoïdes pteronyssinus*) ou par test semi-quantitatif avec mesure de la guanine (type ACAREX®test).

L'objectif de l'étude était de dessiner plusieurs couples de sondes et amorces pour qPCR pour identifier spécifiquement les différentes espèces et groupes d'acariens présentes dans les logements. Les qPCR ont été testées sur des gammes d'ADN puis sur 19 poussières de matelas. Sur les 19 poussières de matelas ont également été réalisés des dosages d'allergènes et des ACAREX®tests.

Les qPCR ciblant *D. pteronyssinus* et *D. farinae* se sont montrées spécifiques, celle ciblant les deux espèces de *Dermatophagoïdes* permet de détecter celles-ci de façon équivalente et la qPCR ciblant « tout acariens » est positive vis-à-vis des deux *dermatophagoïdes*, de *Lepidoglyphus destructor*, *Glycophagus domesticus* et *acarus siro*. Les résultats de qPCR sur les poussières de matelas (en

utilisant la cible du genre *dermatophagoïdes*), exprimés en équivalents acariens par gramme de poussière, sont corrélés à ceux de l'Elisa mais pas à ceux de l'ACAREX®test.

La technique permet d'évaluer la quantité d'acariens par gramme de poussière sur le même extrait d'ADN que celui utilisé pour les moisissures. Le panel de cibles décrites permet de choisir la mesure la plus adaptée en fonction des objectifs des études à venir.

C'est à partir de cette étude que la première cible d'acarien a été intégrée au panel pour l'étude EBRA-ELFE. Puis l'utilisation de plusieurs de ces cibles dans des pièces différentes de logements nous a montré que les espèces d'acariens interagissaient entre elles (Naegele, Reboux, Valot, et al. 2015) et nous a conduits à augmenter le nombre de cible d'acariens dans le nouveau panel proposé.



Detecting and quantifying mites in domestic dust: A novel application for real-time PCR



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1. Introduction

Real-time quantitative PCR (qPCR) is a widely used method to quantify the abundance of organism or gene expression in the environment. qPCR based analyses combine classical DNA amplification by PCR with real time counting of amplicons during each cycle of PCR amplification. Target specificity of qPCR assays is determined by the design of the primers and in some cases internal probes. Indeed, qPCR analyses have been developed to quantify species belonging to fungi and protista kingdoms, and are currently used in human environment investigations of allergies to assess allergen exposure (Haugland et al., 2004; Kaarakainen et al., 2011; Vesper et al., 2007b). The advantage of the qPCR approach is its ability to quantify numerous individual species in a complex mixed community with a single tool. qPCR to detect fungi was used to investigate the relationship between mold exposure and asthma (Reponen et al., 2012; Vesper et al., 2007a) but has not yet been developed for house dust mite quantification, although there is good evidence suggesting a causal relationship

between mite exposure and allergic disorders such as asthma (Cole Johnson et al., 2004; Lodge et al., 2011; Platts-Mills et al., 1997). Mites found in house dust causing the development of IgE antibody responses are mainly house dust mites (from the Pyroglyphidae family, e.g. *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Dermatophagoides microceras* and *Euroglyphus maynei*), storage mites (e.g. *Lepidoglyphus destructor*, *Acarus siro* and *Tyrophagus putrescentiae*) and other species such as *Blomia tropicalis* (Colloff and Spieksma, 1992; Platts-Mills et al., 1992). The ELISA was the most commonly used method for investigating allergens from these mites in the environment (Dunn et al., 2008; Krizkova-Kudlikova et al., 2007). In our study, we propose to broaden the use of qPCR assays to quantify mites in the environment. Primers and probes were designed to detect and measure amounts of nucleic acids from *D. pteronyssinus*, *D. farinae*, *Dermatophagoides species* and, less specifically, from several mites present in domestic dust. Analytical characteristics (specificity, linear dynamic range, limit of detection and efficiency) were determined to validate each qPCR assay. qPCR was tested on 19 dust samples for mattresses and the results were compared to the results of Der p 1 quantification (ELISA) and to the results of guanine semi-quantification test (ACAREX® test).

2. Material and methods

2.1. Tested strains

D. pteronyssinus, *D. farinae*, *L. destructor*, *Glycyphagus domesticus* and *A. siro* (Mite collection, Laboratory of Parasitology, Faculty of Pharmacy, University of Basque Country, Vitoria, Spain) were used to test specificity. The strains were cultured on a 1:1 (wt:wt) autoclaved mixture of commercial mouse meal and dried yeast powder according to Cardona et al. (2004).

Individuals of *Ephestia kuehniella* were collected in a house using glue traps.

Fungal species used were those currently found in indoor dust samples. Stains are deposited in the fungal BCCM/IHEM collection (Scientific Institute of Public Health, Brussels, Belgium): *Aspergillus versicolor* (BCCM/IHEM 22975), *Cladosporium sphaerospermum* (BCCM/IHEM 18883), *Penicillium chrysogenum* (BCCM/IHEM 22667), *Aspergillus*

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fumigatus (BCCM/IHEM 22670), *Alternaria alternata* (BCCM/IHEM 22669), *Eurotium amstelodami* (BCCM/IHEM 16286) and *Wallemia sebi* (BCCM/IHEM 16284). Fungi were cultured onto Sabouraud or Malt Agar media (Beckton Dickinson, New Jersey, USA) at 20 or 30 °C for two weeks. Spores were removed using sterile distilled water and were recovered by centrifugation (10 min at 6000 ×g).

2.2. DNA isolation

Two hundred microliters of DNA-free water containing almost 400 mites, 3 individuals of *E. kuehniella* or 10 mm² fungal suspension was placed into 2.0 ml conical-bottom screw-cap tubes (MagNA Lyser Green Beads, RocheDiagnostics GmbH, Germany) containing 1.4 mm ceramic beads and 400 µl of lysis buffer from the NucleoSpin Plant II kit (Macherey-Nagel, Germany). The tubes were shaken in a MagNA Lyser Instrument (Roche Applied Science®, Germany) three times for ten seconds at the maximum speed, with one cooling minute in the cooling block. The tubes were then heated in a boiling water bath for 10 min. The samples were placed on ice for 10 min, followed by centrifugation for 2 min at 6000 ×g. DNA was then purified with NucleoSpin Plant II kit according to the manufacturer's instructions. The DNA concentration of each extract was determined by a dosage program (160 nm) with a spectrophotometer (ND-1000 Nanodrop, Thermo Fisher Scientific, USA).

2.3. QPCR

PCR reactions contain 10 µl two-fold concentrated TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), reverse and forward primers (final concentration 1 µM each) (Sigma-Aldrich, USA), a hydrolysis probe (final concentration 200 nM) (MWG Eurofins Operon, Germany), 5 µl of DNA sample and nuclease-free water to complete the 20 µl total volume of reaction mixture. Plates were centrifuged (one min at 1000 ×g) and PCR reactions were run and monitored using Applied Biosystems 7500 FAST Real-Time PCR System and 7500 Software v2.0.5. Sterile DNA-free water was extracted with samples and used as negative controls in each series of amplification. Thermal cycles consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.4. Design of probes and primers and analytical specificity

Eighteen rRNA sequences of the main mite species isolated indoors were retrieved from the GenBank database and were aligned using BioEdit Software (Tom Hall Ibis Biosciences, California) to choose the most appropriate areas for primer design. Aligned sequences were those of *D. pteronyssinus* (accession numbers: EU152579; DQ025512; DQ025511), *D. farinae* (GQ864309; EU152578; GQ864309), *Dermatophagoides evansi* (EU152577), *A. siro* (AF022023; EU152495), *L. destructor* (EF203771), and *T. putrescentiae* (DQ025510). Primers and probes were designed using Primer Express® 3.0 for Real-Time PCR software (Applied Biosystems). Parameters used to design primers and probes were chosen to ensure optimal conditions for qPCR. Indeed, the minimum and maximum melting temperatures (T_m) permitted for primers were 58 and 60 °C respectively. The minimum and maximum percentages of C and G contained by either primer were 30 and 80% respectively, with a maximum of two residues on the 3' end required to be a G or C. The optimal length allowed for each primer was 20 bases (9 bases minimum and 40 bases maximum). Minimum and maximum T_m allowed for probes were 68 and 80 °C respectively. The minimum and maximum percentages of G and C contained by the probes were 30 and 80% respectively for a length range of 13 to 30 bases. No more 3 G repeats were required in probe. The amplified region length permitted ranged from 50 to 150 bases.

Analytical specificity, which refers to the qPCR assay detecting the appropriate organism rather than the other, was tested against seven fungal and five mite DNA extracts at 1 pg/µl (Sections 2.1 and 2.2).

2.5. QPCR linear dynamic range, limit of detection and efficiency

To generate calibration curves, DNA extracts were adjusted at 100 pg/µl in the NucleoSpin Plant II kit elution buffer, and were then serially (1:10) diluted, resulting in a six point calibration curve range of 100 to 0.001 pg/µl. QPCR analysis was repeated three times for each concentration. For each qPCR assay, the calibration curve allowed us to calculate the linear dynamic range, the limit of detection (LOD) and PCR amplification efficiency. The linear dynamic range is the concentration range over which the quantification cycle (C_q) and concentration remain directly proportional to each other. Range interval and correlation coefficient (r^2 value) were then reported. The LOD was the lowest concentration at which DNA was detected in all three samples. PCR amplification efficiency was established as the slope of the log-linear portion of the calibration curve: PCR efficiency = $10^{-1/\text{slope}} - 1$.

2.6. Applying qPCR assays on dust from mattresses

Dust from 19 mattresses belonging to people with allergic rhinitis or asthma was collected using nylon sampling socks that were designed to fit on a vacuum cleaner. The whole area of the mattress from which the sample was taken was cleaned for 15 min. The socks were then removed from the vacuum cleaner and closed.

The ACAREX® test (Dyn'R, France) was used according to the manufacturer's instructions directly after dust collection. A colorimetric scale can determine the amount of guanine in dust according to four levels: none, low, medium and high.

In the laboratory, dust samples were filtered (hole of 1 mm in diameter) and stored at -20 °C. To prepare the DNA extracts as described in Section 2.2, 15 mg of dust was mixed with 200 µl of DNA-free water. QPCR assays were performed for each target three times on different days and by different technicians, with the aim of calculating reproducibility expressed by the coefficient of variation from the three points after conversion in pg/µl using calibration curves.

Concentrations of Der p I in dust were determined by ELISA assays (Der p I Assay, Citeq Biologics, The Netherlands) according to the manufacturer's instructions. Results were grouped into three categories according to calibration points: less than 0.05 µg Der p I per gram of dust, between 0.05 and 0.4 µg/g and more than 0.4 µg/g.

Table 1
Primers and probes^a list.

<i>Dermatophagoides pteronyssinus</i>			
Fwd	Dpter F1	TGTTGTGGTTAAAAAGCTCGTAGTTG	
Rev	DpterR1	ATGCGATAATCTGCTCAGTATGACA	
Probe	DpterP1	CAGCTCATGTATGGCGGTCCACCTG	
<i>Dermatophagoides farinae</i>			
Fwd	DfariF1	TGTTGTGGTTAAAAAGCTCGTAGTTG	
Rev	DfariR1	ATGCGATATTCTGCTCAGCATGACA	
Probe	DfariP1	CAGCTCATGTATGGTGGTCCACTTG	
<i>Dermatophagoides spp.</i>			
Fwd	DermaF1	CCTCGTGGTGAGTGCATT	
Rev	DermaR1	TCAGCTGCAGTATCCAGAGTCA	
Probe	DermaP1	ATTAGACCAAGACCAAAGGTGGCAACACC	
<i>Mites</i>			
Fwd	MisppF1	GAGGTTCGAAGGCGCATGACA	
Rev	MisppR1	TTGCTGGTTGGCATCGTTTA	
Probe	MisppP1	ACCCGCTAGTTCTA	

Fwd: forward primer; Rev: Reverse primer.

^a Labeled with FAM reporter and TAMRA quencher.

Table 2
Mean of threshold cycles (standard deviation) for the specificity study of qPCR.

Species extracts	DpterF1/ R1/P1	DfariF1/ R1/P1	DermaF1/ R1/P1	MisppF1/ R1/P1
<i>Dermatophagoides pteronyssinus</i>	35.10 (0.53)	Und.	36.84 (0.85)	37.59 (0.36)
<i>Dermatophagoides farinae</i>	Und.	35.35 (0.42)	36.15 (0.81)	37.31 (0.55)
<i>Lepidoglyphus destructor</i>	Und.	Und.	Und.	36.79 (0.55)
<i>Glycyphagus domesticus</i>	Und.	Und.	Und.	36.28 (0.98)
<i>Acarus siro</i>	Und.	Und.	Und.	39.99 (1.64)

Und. (undetermined): no amplification curve was obtained after 45 cycles of PCR.

3. Results

3.1. Design of primers/probes and analytical specificity

The alignment of mite sequences enabled us to choose areas for primer and probe designs. Primer and probe sequences are listed in Table 1. DpterF1/R1/P1 was designed specifically to amplify *D. pteronyssinus*; DfariF1/R1/P1 specifically to amplify *D. farinae*; and DermaF1/R1/P1 to amplify *D. pteronyssinus* and *D. farinae*. MisppF1/R1/P1 was designed in a conserved area in sequences, so as to detect several genera of mites.

Analytical specificity was first validated using BLAST searches, and then by qPCR assays against mites, *E. kuehniella* and fungal extracts. No fungal or insects DNA extracts were detected in qPCR assays using the four primers/probes designed. Mite extracts were amplified as reported in Table 2, showing a good analytical specificity: only the target species were detected. For MisppF1/R1/P1, we found 3.7 cycles of difference between *G. domesticus* and *A. siro* amplification, all tested at 1 pgDNA/μl. This suggests that there were differences in PCR efficiency or that target repetition was not the same for each of the two species.

3.2. Analytical characteristics of qPCR assays

Linear dynamic ranges and LOD of qPCRs were calculated using calibration curves of targeted organisms (Table 3). Linear dynamic ranges were between 100 pg/μl and 1 or 0.1 pg/μl depending on qPCR assays. Correlation coefficients were up to 0.99 for DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1, showing a good linearity between logarithms for DNA concentration and Cq. A weaker correlation coefficient was obtained with the MisppF1/R1/P1 assay performed with the *D. pteronyssinus* calibration curve. The limit of detection ranged from 10⁻³ to 10⁻⁵ equivalent mites per microliter of extract.

Coefficients of variation for reproducibility, established on dust sample extracts, were higher for MisppF1/R1/P1 than for the three other qPCR assays. Coefficients of variation higher than 0.8 are not

acceptable in qPCR because they represent a very high dispersion of Cq values for the same DNA extract.

3.3. Applying qPCR to mattress dust

qPCR assays on the 19 dust extracts were performed using DermaF1/R1/P1 primers and probes. Quantities of *Dermatophagoides* ranged from 1 to 532 mites per gram of dust, with a mean of 78 mites per gram (standard deviation (SD) = 136).

Acarex® test results showed that the amount of contamination caused by mites was low in five mattresses, medium in ten mattresses and high in four mattresses. There was no correlation between the qPCR and Acarex® test results in mattress dust (Fig. 1).

ELISA Der p I results were correlated with the quantities of *Dermatophagoides* determined by qPCR assays ($r^2 = 0.55$) (Fig. 2). A mean quantity of four *Dermatophagoides*/g of dust (SD = 2) were determined by qPCR when Der p I was found to be less than 0.05 μg/g of dust, 13 *Dermatophagoides*/g (SD = 8) when Der p I in dust was 0.05 to 0.4 μg/g and 140 *Dermatophagoides*/g (SD = 168) when Der p I was more than 0.4 μg/g.

4. Discussion

We proposed and validated a method to quantify house dust mites using qPCR. Four primers/probes were designated to target *D. pteronyssinus* (DpterF1/R1/P1), *D. farinae* (DfariF1/R1/P1), *Dermatophagoides* species (DermaF1/R1/P1) and the main mites present in domestic dust (MisppF1/R1/P1). The qPCR experiments were validated following the MIQE guidelines (Bustin et al., 2009). The specificity of targets was validated by tools for sequence aligning and by direct experimental assays. The linear dynamic ranges covered three or four orders of magnitude and was range to 3 or 2 log₁₀. Ideally, the linear dynamic range should be 5 log₁₀ but our assays were restricted by the limit of detection. We should thus have tested more concentrate DNA extracts to reach 5 log₁₀. The limit of detection for the qPCR experiments mainly ranged from 1 to 0.1 pg/μl, allowing us to detect 13 to 1 mites per gram of dust. This limit of detection is equivalent to those of the ELISA Der p I method (Lind, 1986) and is higher to those of the ELISA allowing detection of *A. siro* in grain (Krizkova-Kudlikova et al., 2007).

For the last twenty years, three methods have been used in environmental studies to estimate exposure to mites: mite counts, assays for specific mite allergens by ELISA, and measurements of guanine, the major nitrogenous excretory product of arachnids (Platts-Mills et al., 1992). These three methods are correlated with each other (Hallas et al., 1993; Hoyet et al., 1991; Lind, 1986; Van Bronswijk et al., 1989). Mite counts by microscopic analysis allow to specify the founding species and demonstrate a good correlation with group I allergen quantification (Platts-Mills et al., 1992). However, determining species requires expert knowledge and this method

Table 3
Performance of qPCR assays to detect specific mite strains extracted.

Targets		Linear dynamic range		Limit of detection		PCR efficiency in %	CV _{rep}
Primers/probes	DNA extract tested	Interval	r ²	In pg/μl	In mites/μl		
DpterF1/R1/P1	<i>D. pteronyssinus</i>	100 to 0.1 pg/μl	0.998	1	10 ⁻³	74	0.52
Dfari F1/R1/P1	<i>D. farinae</i>	100 to 1 pg/μl	0.990	0.1	10 ⁻⁴	73	0.41
DermaF1/R1/P1	<i>D. pteronyssinus</i>	100 to 1 pg/μl	0.995	1	10 ⁻³	70	0.43
DermaF1/R1/P1	<i>D. farinae</i>	100 to 1 pg/μl	0.996	0.1	10 ⁻⁴	79	0.40
MisppF1/R1/P1	<i>D. pteronyssinus</i>	100 to 1 pg/μl	0.974	0.1	10 ⁻⁴	63	0.59
MisppF1/R1/P1	<i>D. farinae</i>	100 to 1 pg/μl	0.997	0.1	10 ⁻⁴	78	0.77
MisppF1/R1/P1	<i>A. siro</i>	100 to 0.1 pg/μl	0.981	1	10 ⁻³	75	0.82
MisppF1/R1/P1	<i>L. destructor</i>	100 to 0.1 pg/μl	0.997	1	10 ⁻³	85	0.86
MisppF1/R1/P1	<i>G. domesticus</i>	100 to 1 pg/μl	0.999	0.01	10 ⁻⁵	81	0.90

CV_{rep}: coefficient of variation for reproducibility.

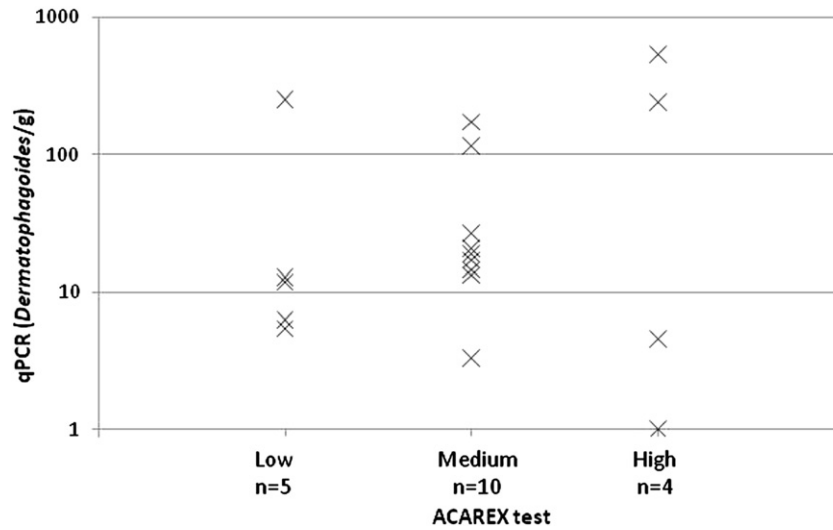


Fig. 1. Correlation between qPCR assays (DermaF1/R1/P1) and ACAREX® tests in mattress dust (n = 19).

takes too long to be applicable to a large number of samples. Guanine production of mites is also correlated with allergen Der p I and with mite counts (Koren et al., 2000). Quantification is limited by the amounts of guanine not originate to house dust mites (Hallas et al., 1993). The ELISA was the most commonly used method for investigating the human environment with species-specific monoclonal antibodies to bind group I (Der p I and Der f I) allergens. Measurements were usually performed on dust from floor and mattresses (Brunekreef et al., 2002; Gent et al., 2012). The generalized use of molecular biology has made it possible to propose new methods for quantifying mites in the environment.

The results of detection and quantification of *Dermatophagoides* species in dust by qPCR correlated with ELISA assays for Der p I quantification, as there is a correlation between mite counts and Der p I quantification (Lind, 1986). Quantities of dust necessary for assays are different for qPCR and ELISA tests: 100 mg of dust was needed to perform the ELISA whereas qPCR experiments were performed with 15 mg of dust. In qPCR experiments, a single dust extract allows us to quantify fungi and bacteria as well as house dust mites. The human environment is a complex biota in which these organisms interact (Naegel et al., 2012). The study of these interactions may be facilitated by a single method to quantify all organisms.

We did not show a clear relationship between the qPCR assay and ACAREX® test results. However, studies comparing ACAREX® and ELISA tests showed that ACAREX® was especially accurate in detecting negative results (level 0: none) and highly positive results (levels 2 and 3: medium and high) (Haouichat et al., 2000; Ridout et al., 1993). The ACAREX® test is simple to use in the domestic environment. It is used to identify sources of mites in houses. This colorimetric test has educational properties that can help involve the patient in finding remedies, but is not used to quantify allergen exposure.

Variation coefficients for reproducibility were better for assays specific to one or two species (DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1) than those detecting a large range of species (MisppF1/R1/P1). Furthermore, the correlation coefficient of calibration curves and PCR efficiency were also lower for MisppF1/R1/P1. We thus recommend using the DpterF1/R1/P1, DfariF1/R1/P1 and DermaF1/R1/P1 specific targets for assessing environmental exposure.

5. Conclusions

New developments in molecular biology have enabled us to quantify organisms in the human environment quickly. QPCR is thus used in epidemiological studies on asthma and allergy to quantify fungi.

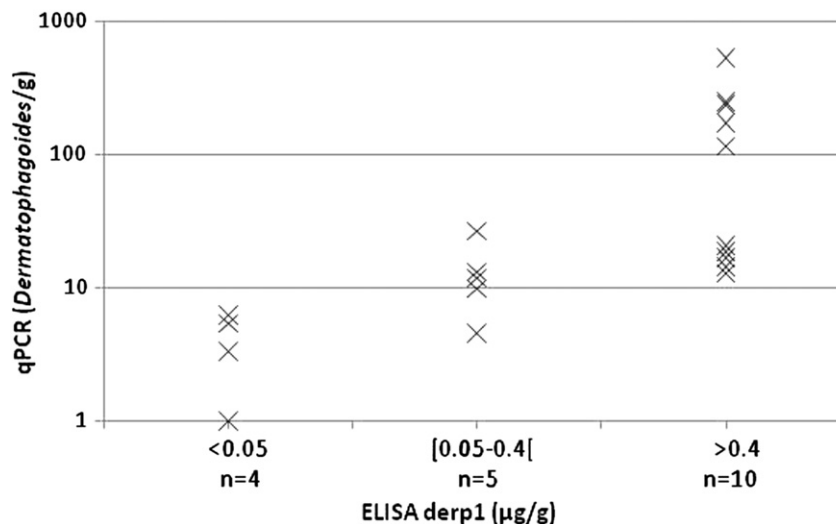


Fig. 2. Correlation between qPCR (DermaF1/R1/P1) and ELISA Der p 1 assays in mattress dust (n = 19).

We validated new qPCR assays to quantify house dust mites that have an important role in asthma and in the development of other allergic diseases. It would be useful to measure levels of exposure in epidemiological studies on human health.

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2. Place des acariens dans le monde invisible : quelles interactions avec les moisissures ?

Titre de l'article : *Fungal food choices of Dermatophagoides farinae affect indoor fungi selection and dispersal*

Auteurs : Alexandre Naegele, Gabriel Reboux, Emeline Scherer, Sandrine Roussel, Laurence Millon

Journal, année de publication : *International Journal of Environmental Health Research*, 2012

Contexte et résultats principaux de l'étude

Acariens et moisissures sont très présents dans les logements et impliqués dans les phénomènes d'allergie. L'objectif de l'étude était d'observer le comportement d'une espèce d'acariens (*Dermatophagoides farinae*) vis-à-vis de 6 espèces courantes de moisissures des logements.

Pour cela, un dispositif particulier a été utilisé : une boîte de Pétri remplie avec un milieu gélosé neutre, dans lequel le centre et des couloirs ont été évidés. Au bout de chaque couloir est déposé un dôme de milieu gélosé sur lequel est ensemencée (puis incubée) une suspension calibrée en spores de moisissure. Au centre du dispositif sont déposés 10 acariens. Leur position dans le dispositif est observée après 180 minutes.

En réalisant cette expérience avec 6 , puis 2, puis 1 seule moisissure(s), il a été montré qu'il existait des moisissures attractives pour cette espèce d'acarien : *Cladosporium sphaerospermum*, *Alternaria alternata*, *Wallemia sebi* et des espèces répulsives, que les acariens ne choisissent pas, même lorsqu'il n'y a qu'une seule espèce dans le dispositif : *Penicillium chrysogenum*, *Aspergillus versicolor*, *Stachybotrys chartarum*.

Au cours de l'étude, les acariens observés « après leur repas » étaient couverts de spores. Par leur consommation de certaines spores plus de d'autres, par le transport des spores sur leur corps ou par leur *feces*, les acariens, en grand nombre dans la poussière de logement, pourraient influencer les équilibres entre les différentes espèces de moisissures.

Fungal food choices of *Dermatophagoïdes farinae* affect indoor fungi selection and dispersal

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House dust mite (HDM) feces and molds are the main allergens involved in allergic asthma. Differences exist between the housing fungal biome of allergic patients and standard or unhealthy housing. House dust mite (HDM) feed off spores and transport them on their bodies, but do they have fungal food preferences? We observed *Dermatophagoïdes farinae* *in vitro* with 16 mold menus and repeated the experiment 10 times. This observation led us to define *Alternaria alternata*, *Cladosporium sphaerospermum*, and *Wallemia sebi* as “tasty” molds and *Penicillium chrysogenum*, *Aspergillus versicolor*, and *Stachybotrys chartarum* as “repulsive” molds. The food preferences of *D. farinae* may play a role in the following two phenomena: a decrease in spore numbers due to HDM consumption and a scattering of spores that stick to the bodies of HDMs. The extent of these two phenomena should be estimated in future studies for other common domestic HDM species.

Keywords: *Dermatophagoïdes farinae*; fungi; spores; selection; dispersal

Introduction

House dust mite (HDM) feces and molds are the main allergens involved in IgE-mediated allergic asthma (Vandentorren et al. 2003; Fisk et al. 2007). *Dermatophagoïdes pteronyssinus* (Trouessart) and *Dermatophagoïdes farinae* (Hughes) are present in dwellings in variable proportions. *Dermatophagoïdes farinae* is found in mattresses, carpets, and soft furnishings, whereas *D. pteronyssinus* is found most frequently in mattresses. The concomitant presence of HDMs and molds increases the frequency and severity of asthma attacks (Prescott 2003). The level of total IgE is significantly higher in children exposed to both fungi and HDMs (Su et al. 2005). House dust mites (HDMs) contribute to mold transportation (McGinnis 2004). Differences exist between the housing fungal biome of allergic patients and unhealthy housing (Vesper et al. 2006; Bellanger et al. 2009; Reboux et al. 2009). House dust mites (HDMs) may modify patient exposure to allergens in dwellings by selecting and transporting some molds in the same way as astigmatic mites do in stored grain silo (Van Asselt 1999).

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This study aimed to determine the food preferences of *D. farinae* between six fungal species commonly found in housing and responsible for allergic diseases.

Materials and methods

HDMs

Dermatophagoides farinae were identified according to morphological and genotypical characteristics and by sequencing of internal transcribed spacer (ITS) regions. House dust mites (HDMs) were bred at 21°C and relative humidity at 45%. House dust mites (HDMs) were fed *Saccharomyces boulardii*, fish food, and beard hair (Unpublished data supplied by Christian Bories (Assistant Professor, University of Paris 11, Chatenay Malabry, France)).

Molds

Six common indoor molds were used as food for HDMs: *Alternaria alternata*, *Wallemia sebi*, *Cladosporium sphaerospermum*, *Stachybotrys chartarum*, *Penicillium chrysogenum*, and *Aspergillus versicolor*, respectively, registered in the BBCM-IHEM collection (22669, 16284, 18883, 22672, 22667, 22671).

Device and protocols

In a Petri dish, six “corridors” (35 mm × 10 mm) connected with a central sector (diameter: 15 mm), were cut from agar (agar 2%) (thickness: 2 mm) (Figure 1). At the corridor extremity, 500 µl of agar was arranged to obtain six lenticular domes

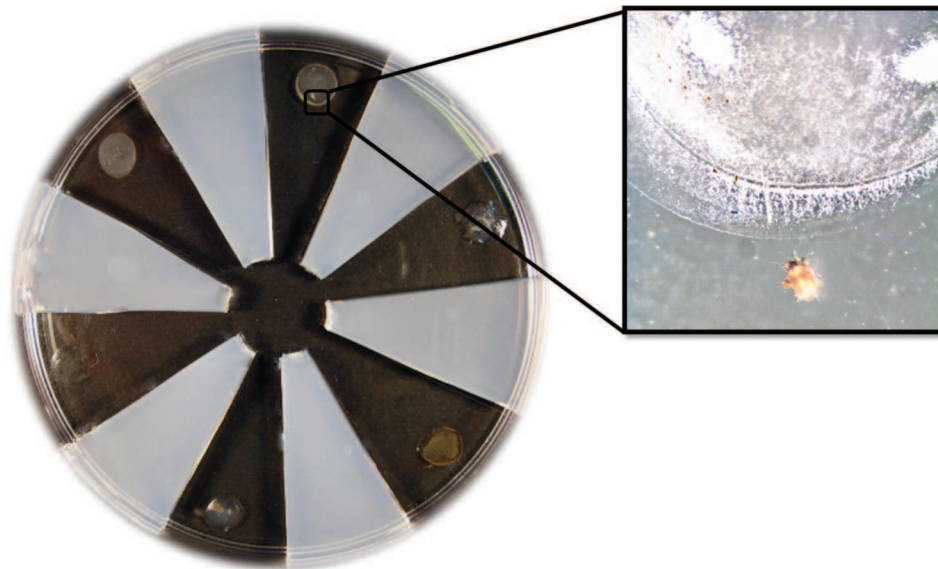


Figure 1. Six mold devices and HDMs eating *Wallemia sebi* spores. This device consists of six corridors cut from a gelose (Agar 2%) with six molds scattered on six lenticular domes and a central section for HDMs.

(diameter: 8 mm). Calibrated suspensions of 10^7 spores/mL were prepared for the six mold species. The domes were then seeded with 100 μ l of spore solution and incubated at 30°C for 7 days. Ten *D. farinae* individuals were then placed in the center of the device. House dust mites (HDMs) were observed in mold cultures through a binocular microscope with cold light (Leica S6D, Solms, Germany) after 180 min. This time span was determined by a preliminary test to establish HDM movement speed (7 mm/min.). Movement was preceded by an adaptation time due to the environmental change (breeding/Petri dish). Dust mites have to adapt to the space to turn to the tasty mold.

Each of the following tests was repeated 10 times (160 observations in all with 10 HDMs for each test). Only HDMs that were seen to consume mold were included in the analysis.

First, six molds were presented to HDMs in each device (Figure 1), in order to divide molds into two groups: “tasty fungi” and “repulsive fungi”. Molds chosen at least once were considered tasty and those not chosen were considered repulsive.

Second, each tasty fungus (*Alternaria/Cladosporium/Walleimia*) was placed alongside each repulsive fungus (*Penicillium/Stachybotrys/Aspergillus*) in a different dish (nine dish combinations in total).

Third, the movement of *D. farinae* was studied with each of the six isolated molds. *Dermatophagoides farinae* chose whether or not to eat the mold.

Results

The results are summarized in Table 1.

The first experiment (six molds) ($n = 10$) enabled us to divide molds into two groups: tasty: 20% of deposited HDMs chose *A. alternata*, 13% *C. sphaerospermum*, 8% *W. sebi* – and repulsive: 0% for the other three species (*P. chrysogenum*, *A. versicolor*, and *S. chartarum*).

The second experiment (with two molds) ($n = 30$) confirmed this classification: 48% of HDMs chose *A. alternata*, 35% *C. sphaerospermum*, 33% *W. sebi*, 9.3% *P. chrysogenum*, 12% *A. versicolor*, and 13% *S. chartarum*.

The third experiment ($n = 60$) showed that this choice was “deliberate”: 31% chose *A. alternata*, 36% *C. sphaerospermum*, 24% *W. sebi*, 7% *P. chrysogenum*, 3%

Table 1. Choice of molds consumed by *D. farinae*.

Mold species Experiments	<i>Alternaria alternata</i>	<i>Cladosporium sphaerospermum</i>	<i>Wallemia sebi</i>	<i>Penicillium chrysogenum</i>	<i>Aspergillus versicolor</i>	<i>Stachybotrys chartarum</i>
6 molds ($n = 10$)	2 (1.8)	1.3 (1.1)	0.8 (0.7)	0 (0)	0 (0)	0 (0)
		Tasty fungi			Repulsive fungi	
2 molds ($n = 90$)	4.8 (0.7)	3.5 (0.7)	3.3 (0.7)	0.9 (0.3)	1.2 (0.2)	1.3 (0.3)
1 mold ($n = 60$)	3.1 (0.7)	3.6 (0.4)	2.4 (0.8)	0.7 (0.4)	0.3 (0.4)	0.7 (0.4)

Notes: n : test for each device. In bold: mean number of *D. farinae* choosing the fungi species of 10 individuals. In brackets: standard deviation.

A. versicolor, and 7% *S. chartarum*. When offered these latter three species, more than 90% of the HDMs preferred to go without food.

Discussion

Our observation of *D. farinae* *in vitro* together with 16 menus consisting of six molds, repeated 10 times, led us to define *A. alternata*, *C. sphaerospermum*, and *W. sebi* as “tasty” and *P. chrysogenum*, *A. versicolor*, and *S. chartarum* as “repulsive” molds. Only certain mold species were consumed by *D. farinae*.

House dust mites (HDMs) moved towards “tasty” mold species, ate them and disseminated the spores attached to their body during movements (Figure 2) as has been shown in astigmatid mites (*Acarus siro*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*) (Hubert et al. 2004). In their study, the preferred mold species were *A. alternata*, *C. sphaerospermum*, and *W. sebi*. *Dermatophagoïdes farinae* were able to detect repulsive fungal species at a considerable distance: 45 mm (equivalent to 7 min of movement) and move further away. This suggests that *D. farinae* actively select molds.

A study on stored grain shows that tasty fungi as a food source for stored mites are more commonly dispersed than those not chosen. Fungal dispersions caused by mites depend on mite species (Hubert et al. 2003). This is also the case for plants (Gamliel-Atinsky et al. 2010), possibly through dispersal by an insect vector (Roets et al. 2009). However, the ability of mites to disperse specific fungal propagules remains questionable because it was not possible to prove selective eating and dissemination through the fecal pellets (Renker et al. 2005). The astigmatid mites degrade around 50% of spores. It is hard to determine if it is the eating or dispersal due to astigmatid mites which play the more important role (Nesvorna et al. 2012).

The large proportion of HDMs – more than 100 HDMs per grams of dust and more than 2 million per mattress (Korsgaard 1998) – suggests a potentially significant influence on fungal biome. The behavior of HDMs towards molds is modulated by the nature of fungi and the compounds they emit.

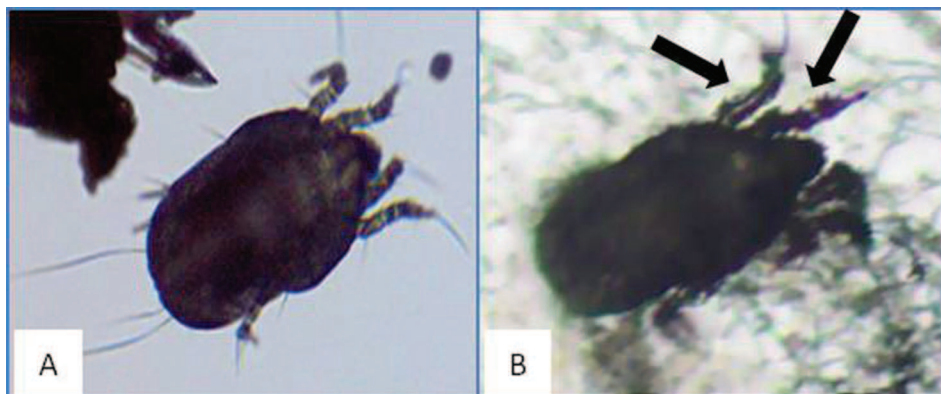


Figure 2. Observation of a *Dermatophagoïdes farinae* before (A) and after (B) consumption of *Wallemia sebi* spores and the girdle formed around the legs and body of the dust mites (Arrow) ($\times 40$, binocular microscope with cold light, Leica S6D[®], Solms, Germany).

An HDM prevention program (removing carpets, using anti-dust mite covers) modifies the indoor environment of allergic patients.

It is true that other factors are involved in the dispersal of fungi spores such as airflow, temperature, damp, and sometimes flooding. Research into mold exposure in asthma patients should examine the extent of the role of HDMs in destroying and dispersing mold colonies compared to the role of environmental factors.

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VI. DISCUSSION

Bilan des différentes études menées

Le temps passé à l'intérieur du logement augmente de par l'évolution de nos modes de vie. Ce temps peut encore être augmenté en période pré et post natale.

Le nombre de patient immunodéprimés, à risque de développer des infections graves à cause de micro-organismes opportunistes augmente également avec l'élargissement de l'utilisation de thérapeutiques immunosuppressives (par exemple dans le cadre de la prévention de rejet de greffe, de greffe de moelle osseuse, de pathologie auto-immune).

L'impact des micro-organismes sur notre santé est donc une préoccupation majeure et le niveau de compréhension du microbiote doit augmenter, grâce à l'amélioration des techniques d'étude.

Dans ce travail, la qPCR est la technique la plus utilisée, mais elle n'a pas pu être validée seule et sa mise en œuvre a évolué au cours des différents projets.

En effet, des étapes de culture et des validations sur des souches sont obligatoires. La qPCR est un outil, l'interprétation du signal et de sa significativité est le travail du scientifique. La comparaison à des méthodes pour lesquelles un plus grand recul existe est indispensable dans un premier temps.

Le matériel disponible a évolué au cours de ce travail. Les fournisseurs ont conscience des avantages de cette technologie et proposent des solutions réactionnelles pour tous les types d'échantillons (ou presque) sans que les compositions soient connues. Les efficacités des qPCR peuvent être considérablement modifiées et ces ajustements font partie des étapes de validation. Ces étapes de validation sont essentielles et font de la qPCR un outil fiable et reproductible pour les enquêtes environnementales comme en diagnostic médical.

Ce travail a débuté par le pilote de la cohorte ELFE et, depuis le pilote, la procédure s'est constamment améliorée. L'augmentation du panel de cibles fait partie de cette amélioration, tout comme le projet d'un échantillonnage à 5 ans. Au vu des données de santé des 18 années de suivi de l'enfant, d'autres problématiques pourront être soulevées et feront l'objet de nouveaux développements.

Cette procédure simple (EDC + qPCR) mise au point dans ce travail, adaptée et adaptable, d'un coût modéré pourrait devenir un test considéré comme un « diagnostic » de la contamination allergénique d'un logement et un élément de suivi de la qualité de l'air.

De la même façon, les qPCR mises au point dans le domaine du diagnostic sont aptes à être (et sont déjà) utilisées en routine pour le *screening* des patients à hauts risques de développer des infections fongiques invasives.

Dans les deux cas, environnemental et diagnostique, c'est l'application des méthodes sur un plus grand nombre d'échantillons qui permet d'évaluer leur pertinence et leurs limites biologiques.

Critiques des méthodes utilisées

Concernant la cohorte ELFE, le point principal des études menées est l'optimisation de la quantification (tant sur la technique que sur les cibles choisies) des allergènes ou agents potentiellement impliqués dans le risque allergique.

Le choix de l'échantillonnage reste discutable. L'EDC est pratique, bien accepté, utilisable en qPCR aussi bien qu'en culture, le volume restitué lors du rinçage du capteur permet un grand nombre d'analyses. Cependant, la dilution pendant le rinçage fait baisser la sensibilité de la qPCR. En contre partie, cette dilution dans une solution riche en protéines (bouillon cœur cerveau) garanti l'absence d'inhibiteurs de qPCR.

La plus grande inconnue vis-à-vis de ce dispositif est la reproductibilité dans un même logement. Elle a été testée sur deux lingettes différentes pour la recherche d'endotoxine. Elle n'a pas été testée pour l'analyse par qPCR. La poussière sédimentant pendant 10 semaines, il est probable que l'échantillon soit représentatif du niveau moyen de contamination, moins sensible aux variations ponctuelles que les prélèvements d'air, réalisés sur un temps beaucoup plus court. Un test portant sur 6 logements avait été réalisé en 2009 (Didier-Scherer 2009). Dans chaque logement deux EDC étaient déposés, l'un pendant 1 mois, l'autre pendant deux mois et demi. Des prélèvements d'air étaient également réalisés de façon bi-mensuelle.

Concernant la comparaison avec les prélèvements d'air, l'étude n'avait porté que sur 4 espèces de moisissures, mais pour *Cladosporium sphaerospermum*, plus le nombre de prélèvements d'air (n=7) positifs était important, plus le signal par qPCR sur la lingette était important. La lingette semble bien cumuler les spores circulant dans l'air des logements.

De la même façon, la durée d'exposition ne peut être strictement de 10 semaines pour chaque participant. Il est toléré quelques jours de décalage. Cette variabilité a été abordée dans l'étude préliminaire de 2009 citée plus haut, durant laquelle l'EDC était placé pendant deux durées différentes. Si les résultats ne montraient pas d'augmentation proportionnelle du signal entre un

mois et deux mois et demi, les EDC à deux mois et demi mettaient en évidence une plus grande diversité en micro-organismes en culture et une quantité moyenne plus importante par qPCR.

L'EDC est réellement un dispositif capable de mettre en évidence des concentrations différentes en moisissures et micro-organismes dans les logements. La durée de 10 semaines semble la plus propice à la mise en évidence de ces différences.

Les cibles de qPCR choisies sont également discutables. Le choix se fait selon les éléments disponibles dans la littérature et les possibilités techniques actuelles. Il s'agit cependant d'une avancée majeure pour l'analyse des logements. Les premières études montrant un lien entre moisissures et allergies étaient basées sur les constats d'humidité ou l'odeur de moisi. La culture a ensuite permis d'objectiver ce lien. A présent, il s'agit de mieux connaître et mieux comprendre les interactions et les effets de micro-organismes spécifiques, quantifiés avec précision et d'envisager cette démarche sur le long terme.

Certes, il s'agit d'un choix d'espèces *a priori*, mais il pourra être complété, notamment en fonction des résultats d'analyses métagénomiques. La validation des essais en duplex, c'est-à-dire en réalisant dans le même puits réactionnel l'amplification de deux cibles avec des sondes marquées par des fluorophores différents, diminue le prix (quantité de Mix commercial divisée par deux) et le nombre des réactions par deux sans altérer la qualité des résultats.

De plus, nous avons également pu montrer que la qPCR pouvait être utilisée comme marqueur d'une exposition visible et accessible par questionnaire (présence de chien et chat au domicile) et qu'elle apportait en plus une notion quantitative. Elle augmente ainsi les possibilités de comprendre les liens de cause à effet.

Le traitement statistique des premiers résultats par ACP a permis de mettre en évidence des profils de logements, avec une distribution géographique propre. Cette distribution géographique est comparable à celle des sifflements chez les enfants en France. Ces résultats très encourageants doivent être confirmés par les données de santé des enfants de la cohorte ELFE, mais ces données ne seront disponibles que dans les années à venir. Elles seront à ré évaluer tout au long de la croissance des enfants. Les profils seront complétés par de nouvelles cibles, mis en lien avec de nouvelles données de santé et l'ensemble fera l'objet d'une nouvelle analyse statistique.

VII. CONCLUSION ET PERSPECTIVES

Ce travail constitue la base solide du recueil de données environnementales pour ELFE. Il est la preuve de la pertinence de nos choix pour un travail ultérieur de plus grande ampleur. De plus, ce *screening* « complet » sur l'ensemble du territoire pourra également permettre de connaître la répartition privilégiée de certains micro-organismes en fonction du climat.

De la même façon, les autres utilisations de la qPCR présentées dans cette thèse constituent les bases techniques indispensables pour un usage à plus grande échelle. Elles montrent l'amélioration des connaissances et de l'expérience dans les différents domaines décrits. L'outil est convaincant et validé, il reste à être utilisé sur un grand nombre de sujets ou d'environnements (ou de séquences temporelles) pour établir des seuils utilisables en pratique.

Perspectives

La perspective concernant le projet ELFE est la réalisation du projet EMBRASE avec un nouvel échantillonnage à 5 ans. Cette donnée d'exposition cumulative est rarement disponible et pourtant probablement un élément clé de la compréhension de l'apparition et de l'exacerbation des symptômes de l'asthme. Elle permettra de différencier l'impact d'une exposition précoce à celle d'une exposition plus tardive.

En fonction des résultats des deux campagnes, des résultats de métagénomiques mais aussi des caractéristiques des événements de santé des enfants suivis, d'autres campagnes sont envisageables au cours des 14 années restantes. Si l'exposition aux micro-organismes semble plus impliquée dans la petite enfance vis-à-vis du développement de l'asthme, elle pourrait jouer un rôle tout autre dans le comportement ou la croissance lorsque l'enfant sera plus âgé. Ce suivi longitudinal est une occasion unique d'explorer conjointement des micro-organismes et des disciplines longtemps restées cloisonnées. C'est un des atouts majeurs de la technique choisie et un des enjeux de la cohorte ELFE : la multidisciplinarité.

Pneumocystis est une cible manquante dans le projet. La méthode d'extraction a été initialement choisie pour les moisissures. L'extraction de ce champignon atypique est inefficace avec cette extraction et la cible choisie de *Pneumocystis*. Il pourrait être intéressant de comprendre ce phénomène, de tenter d'y remédier et ainsi d'ajouter *Pneumocystis* aux prochaines cibles mesurées.

En effet, en situation médicale, la qPCR a considérablement augmenté la sensibilité des diagnostics de pneumocystose, cette amélioration pourrait être utilisée dans le domaine environnemental.

A l'inverse, pour *Aspergillus fumigatus* et les mucorales, la qPCR a amélioré la caractérisation environnementale de nombreuses espèces de moisissures et son adaptation en situation médicale a considérablement augmenté la sensibilité du diagnostic.

Environnement et santé sont des problématiques en constante interaction et l'amélioration de la connaissance de l'un va de pair avec l'amélioration de l'autre.

VALORISATION SCIENTIFIQUE

Communications orales et affichées

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- « *En direct* » journal de l'Université de Franche Comté, n° 245 nov-Déc 2012 p17.
- « La presse bisontine » Pour que bébé respire le bon air, n°143 Mai 2013.
- Reportage photographique illustrant les différentes étapes d'analyse des capteurs de l'Etude ELFE nous a été demandé par la personne chargée de communication de l'Etude ELFE. Ce reportage devrait paraître sur le site www.elfe-france.fr.
- Reportage sur l'Etude ELFE intitulé "Ce que bébé respire" a été réalisé par l'équipe de "mission culture scientifique" de l'université de Franche-comté. Ce reportage est visible à l'adresse suivante <http://sciences-en-culture.univ-fcomte.fr/pages/fr/menu232/activites/ce-que-bebe-respire...-7801-15786.html>

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ANNEXE : LISTES DES SONDÉS ET AMORCES DÉCRITES PAR EPA

Mise à jour du 24 septembre 2015

Ceci est le cache Google de <http://www.epa.gov/nerlcwww/moldtech.htm%23primers>. Il s'agit d'un instantané de la page telle qu'elle était affichée le 24 sept. 2015 19:12:20 GMT.

<http://webcache.googleusercontent.com/search?q=cache:SzbBcJVTmqQJ:www.epa.gov/nerlcwww/moldtech.htm%2523primers+&cd=1&hl=fr&ct=clnk&gl=us>

The presence of molds in homes, schools and business is of growing concern in the United States. To begin to understand the significance of exposure to these organisms, a standardized technology is needed for their identification and quantification. In May of 2002, the EPA was granted patent 6,387,652 which describes mold DNA sequences that makes their identification and quantification possible. This relatively inexpensive technology has been licensed by firms in the U.S., Germany and the United Kingdom.

The patented technology teaches the best available DNA sequences to use for over 130 of the major indoor air fungi. It is particularly useful, in that a licensed company can employ a technician or robotic system to analyze samples and obtain highly reproducible results in only a few hours -- eliminating the need for plating and culturing or identifying and counting. Other analytical techniques may take days or week to complete. Most typical sampling systems can be adapted for use with this technology.

One of the problems with mold exposure estimates has been the lack of a standardized method for describing the mold burden in a home (Vesper 2010, in press). United States Environmental Protection Agency (US EPA) and Department of Housing and Urban Development (HUD) researchers have developed a metric called the Environmental Relative Moldiness Index (ERMI) to objectively describe the home mold burden (Vesper et al. 2007). A DNA-based analysis called Mold Specific Quantitative PCR (MSQPCR) of 36 molds including, the 26 Group 1 species associated with homes with water damage and the 10 Group 2 species which are found in homes independent of water damage, forms the basis of the ERMI. (Figure 1, click once for values, click 2-5 times for quartiles) If the Sum of the Logs of the Group 2 (SLG2) molds is subtracted from the Sum of the Logs of the Group1 (SLG1) molds, a unit-less Environmental Relative Moldiness Index (ERMI) value is calculated which describes the mold burden in a home with a single numeric value relative to the National ERMI scale. The ERMI scale ranges from about -10 to 20 or even higher and is divided into quartiles. The homes in the lowest quartile have the lowest mold burden. Each higher quartile indicates more mold present in the home. The fourth quartile indicates homes with the highest mold burden (Vesper et al. 2007)

Literature references pertinent to this technology can be found at the end of this page.

Primers and Probes for Target Species

Absidia corymbifera (assay name: Acory)

Forward Primer NS92F: 5'-CACCGCCCGTCGCTAC
Reverse Primer AcoryR1: 5'-GCAAAGCGTTCCGAAGGACA
Probe AcoryP1: 5'-ATGGCACGAGCAAGCATTAGGGACG

Acremonium strictum (assay name: Astrc)

Forward Primer AstrcF1: 5'-CAACCCATTGTGAACCTACCAAAC
Reverse Primer AstrcR1: 5'-CGCCCTCAGAGAAATACGATT
Probe AstrcP1: 5'-TCAGCGCGCGGTGGCCTC

Alternaria alternata (assay name: Aaltr)

Forward Primer AaltrF1: 5'-GGCGGGCTGGAACCTC
Reverse Primer AltrR1-1: 5'-
GCAATTACAAAAGTTTATGTTTGTGCGTA
Probe AaltrP1: 5'-TTACAGCCTTGCTGAATTATCACCTTGCTCTT

Aspergillus auricomus (assay name: Aauri)

Forward Primer AcircF1: 5'-ATTACTGAGTGAGGGTCCCTCG
Reverse Primer AauriR1: 5'-GGCGCCGCGTAAAC
Probe AcircP1: 5'-CCCGCCAAGCAACAAGGTACG

Aspergillus caespitosus (assay name: Acaes)

Forward Primer AcaesF1: 5'-CTCCACCCGTGAATACCTT
Reverse Primer AcaesR1: 5'-GGCTCAGACGCAACTCTACAAT
Probe AcaesP1: 5'-CACTGTTGCTTCGGCGAGGAGCC

Aspergillus candidus (assay name: Acan3)

Forward Primer AcanF1: 5'-TTACCGAGTGAGGGTTTCTCTGA
Reverse Primer AcanR2: 5'-CCC GCCGAAGCAACAG
Probe* PenAspP1mgb: 5'-CCAACCTCCACCCGCTG

Aspergillus carbonarius (assay name: Acarb)

Forward Primer AcarbF1-1: 5'-CTTTGGGCCCAACCTCtAC
Reverse Primer AcarbR1: 5'-CCC GAGGGCAGAGATG
Probe AspP1: 5'-TTGCTTCGGCGGGCCC

Aspergillus cervinus (assay name: Acerv)

Forward Primer AcervF1: 5'-CCACCCGTGCTATTGTACCTT
Reverse Primer AcervR1-1: 5'-
CAACTCAGACTGCAATTCAGAActGT
Probe AspP1: 5'-TTGCTTCGGCGGGCCC

Aspergillus clavatus/giganteus (assay name: Aclav)

Forward Primer AclavF1: 5'-CCCGCGTCTTCGGA
Reverse Primer AclavR1: 5'-
CCGTTGTTGAAAGTTTTAACTGATTATG
Probe AfumiP1: 5'-CCCGCCAAGACCCCAACATG

Aspergillus flavus/oryzae (assay name: Aflav)

Forward Primer AflavF1: 5'-CGAGTGTAGGGTTCCTAGCGA
Reverse Primer AflavR1: 5'-CCGCGGCCATGAAT
Probe AflavP1: 5'-TCCCACCCGTGTTACTGTACCTAGTTGCT

Aspergillus fumigatus, Neosartorya fischeri (assay name: Afumi)
Forward Primer AfumiF1: 5'-GCCCGCCGTTTCGAC
Reverse Primer AfumiR1: 5'-CCGTTGTTGAAAGTTTAACTGATTAC
Probe AfumiP1: 5'-CCCGCCGAAGACCCCAACATG

Aspergillus flavipes (assay name: Aflvp2)
Forward Primer AflvpF1: 5'-CCACCGTGACTACTGTACCAC
Reverse Primer AflvpR2: 5'-AGGCTTTCAGAAACAGTGTTCG
Probe AspP1: 5'-TTGCTTCGGCGGGCCC

Aspergillus niger/awamori/foetidus/phoenicis (assay name: Anigr)
Forward Primer AnigrF1: 5'-GCCGGAGACCCCAACAC-3'
Reverse Primer AnigrR1: 5'-TGTTGAAAGTTTAACTGATTGCATT-3'
Probe AnigrP1: 5'-AATCAACTCAGACTGCACGCTTTCAGACAG

Aspergillus niveus (assay name: Anive)
Forward Primer AniveF1: 5'-ACCGTGCCTATTGTACCCT
Reverse Primer AniveR1: 5'-TGCAAAACATCACACTCAGACAC
Probe AspP1: 5'-TTGCTTCGGCGGGCCC

Aspergillus ochraceus/ostianus (assay name: Aochr1)
Forward Primer AochrF1: 5'-AACCTCCACCCGTTATACC-3'
Reverse Primer AochrR1: 5'-CCGCGGAGCGCTGTG-3'
Probe AochrP1: 5'-ACCTTGTGCTTCGGCGAGCCC

Aspergillus paradoxus (assay name: Apar)
Forward Primer AparF1: 5'-CGGGGGGCTTACGCT
Reverse Primer AparR1-1: 5'-GACTGCAACTTCATACAGATTGGT
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Aspergillus parasiticus/sojiae (assay name: Apara)
Forward Primer AflavF1: 5'-CGAGTGATAGGGTTCCTAGCGA-3'
Reverse Primer AparaR3: 5'-GCCCGGGGCTGACG
Probe AflavP1: 5'-TCCACCCGTTTACTGTACTCTAGTTGCT

Aspergillus penicillioides (assay name: Apeni2)
Forward Primer ApeniF2: 5'-CGCCGAGACCTCAACC
Reverse Primer ApeniR2: 5'-TCCGTTGTTGAAAGTTTAAACGA
Probe ApeniP2: 5'-TGAACACTGTCTGAAGGTTGCAGTCTGAGTATG

Aspergillus puniceus (assay name: Apuni)
Forward Primer ApuniF1: 5'-CATTACCGAGTGCAGGCCTC
Reverse Primer AustsR1: 5'-GCCGAAGCAACGTTGGTC
Probe ApuniP1: 5'-CCCCACAGGCAGGCCTAACCTC

Aspergillus restrictus/caesillus/conicus (assay name: Arest)
Forward Primer ArestF1: 5'-GGGCCCGCCTTCAT-3'
Reverse Primer ArestR1: 5'-GTTGTTGAAAGTTTAAACGATTTTTCT
Probe ArestP1: 5'-CCCGCCGAGACTCCAACATTG

Aspergillus sclerotiorum (assay name: Asclr)
Forward Primer AcircF1: 5'-ATTACTGAGTGAGGGTCCCTCG
Reverse Primer AsclrR1: 5'-CCTAGGGAGGGGGTTTGA
Probe AcircP1: 5'-CCCGCCGAAGCAACAAGGTACG

Aspergillus sydowii (assay name: Asydo3)
Forward Primer AsydoF1-1: 5'-CAACCTCCACCCGAGAA
Reverse Primer AversR1-1: 5'-CCATTGTTGAAAGTTTACTGATCTTA
Probe versP1: 5'-AGACTGCATCACTCTCAGGCATGAAGTTCAG

Aspergillus tamarii (assay name: Atama2)
Forward Primer AflavF1: 5'-CGAGTGATAGGGTTCCTAGCGA

Reverse Primer AtamaR2: 5'-CCCGCCGCTTAAAG
Probe AflavP1: 5'-TCCACCCGTTTACTGTACCTTAGTTGCT

Aspergillus terreus (assay name: Aterr2)
Forward Primer AterrF1-1: 5'-ATCATTACCGAGTGCCTGCTTTA
Reverse Primer AterrR2: 5'-CCCGCCGAAGCAACAAG
Probe AterrP2: 5'-CCCAACCTCCACCCGACTATTG

Aspergillus unguis (assay name: Aungu)
Forward Primer AunguF1: 5'-CAACCTCCACCTTGAATACT
Reverse Primer AunguR1: 5'-TCACTCTCAGGCATGAAGTTCAG
Probe AcaesP1: 5'-CACTGTTGCTTCGGCGAGGAGCC

Aspergillus ustus (assay name: Austs2)
Forward Primer AustsF2-1: 5'-AAGGATCATTACCGAGTGCATgT
Reverse Primer AustsR1: 5'-GCCGAAGCAACGTTGGTC
Probe AustsP1: 5'-CCCCGGGCGAGCCTAAC

Aspergillus versicolor (assay name: Avers2-2)
Forward Primer AversF2 (5x): 5'-CGGCGGGGAGCCCT
Reverse Primer AversR1-1: 5'-CCATTGTTGAAAGTTTACTGATCTTA
Probe versP1: 5'-AGACTGCATCACTCTCAGGCATGAAGTTCAG

Aspergillus wentii (assay name: Awent)
Forward Primer AwentF1: 5'-CATTACCGAGTGCAGCCTAAC
Reverse Primer AwentR1: 5'-CGGCGGCCACGAAT
Probe AcircP1: 5'-CCCGCCGAAGCAACAAGGTACG

Aureobasidium pullulans (assay name: Apull)
Forward Primer ApullF1: 5'-GATCATTAAAGATAAGGGTGCTCA
Reverse Primer ApullR1: 5'-GCTCGCTGGGACGAATC
Probe ApullP1: 5'-CGCCGACCTCCAACCTTTG

Candida albicans (assay name: Calbi)
Forward Primer CalbF1: 5'-CTTGGTATTTTGCATGTTGCTCTC
Reverse Primer CalbR1: 5'-GTCAGAGGCTATAACACACAGCAG
Probe CalbP1: 5'-TTTACCGGGCCAGCATCGGTTT

Candida dubliniensis (assay name: Cdub1)
Forward Primer CdubF1: 5'-AGATCAGACTTGGTATTTTGAAGTTA
Reverse Primer CdubR1: 5'-TAGGCTGGCAGTATCGTCAGA
Probe CdubP1: 5'-TTTACCGGGCCAGCATCGGTTT

Candida glabrata (assay name: Cglab)
Forward Primer Cglab F1: 5'-GCGCCCTTGCCTCTC
Reverse Primer Cglab R1: 5'-CCCAGGGCTATAACACTCTACACC
Probe Cglab P1: 5'-TGGGCTTGGACTCTCGCAGC

Candida (assay name: Pichia) *guilliermondii* (assay name: Cgui1)
Forward Primer CguiF1: 5'-CCTTCGTGGCGGGGTG
Reverse Primer CguiR1: 5'-GCAGGCAGCATCAACGC
Probe CguiP1: 5'-CCGCAGCTTATCGGGCCAGC

Candida haemulonii, svar. 1 (assay name: Chaem1)
Forward Primer ChaeF1: 5'-GGAGCGACAACGAGCAGTC
Reverse Primer ChaeR1: 5'-AGGAGCCGAAAGCAAGACG
Probe ChaeP1: 5'-ATGTAGTACAGCCCTCTGGGCTGTGCA

Candida haemulonii, svar. 2 (assay name: Chaem2)
Forward Primer Cha2F1: 5'-ATCGGGTGGAGCGGAAC
Reverse Primer Cha2R1: 5'-CGAAGCAGGAACCTCTGAGA
Probe Cha2P1: 5'-AAGTGGGAGCTGATGTAGCAACCC

Candida krusei (assay name: Ckrus)
Forward Primer CkruF1: 5'-CTCAGATTTGAAATCGTCTTTG

Reverse Primer CkruR1: 5'-GGGGCTCTCACCTCCTG
Probe CkruP1: 5'-CACGAGTTGTAGATTGCAGTTGGAGTCTG

Candida lipolytica (assay name: Clipo)
Forward Primer ClipF1: 5'-TAGCGAGACGAGGTTACAAATG
Reverse Primer ClipR1: 5'-CGTCGGTGGCAGTGTGGA
Probe ClipP1: 5'-CCTTCGGGCGTTCTCCCTAACCC

Candida lusitanae (assay name: Clusi)
Forward Primer Clus F2: 5'-GGGAATTGTAATTTGAAGTTTCGT-3'
Reverse Primer Clus R2: 5'-GTCGGCGTGCGCCATA-3'
Probe Clus P2: 5'-TCTGAGTCGGCCGCC-3'

Candida maltosa (assay name: Cmalt)
Forward Primer CmalF1: 5'-GGCCAGCATCAGTTTGAC
Reverse Primer CmalR1: 5'-TCTAGACTGGCAGTATCGACAGTG
Probe CmalP1: 5'-TAGGACAATTGCGGTGGAATGTGGC

Candida parapsilosis (assay name: Cpara)
Forward Primer CparF1: 5'-
GATCAGACTTGGTATTTGTATGTTACTCTC
Reverse Primer CparR1: 5'-CAGAGCCACATTTCTTGCAC
Probe CparP1: 5'-CCTCTACAGTTTACGGGCCAGCATCA

Candida sojae (assay name: Csoja)
Forward Primer CsojF1: 5'-CGGTTGTGTGTATAGCCTTCGTA
Reverse Primer CsojR1: 5'-ATCATTATGCCAACATCCTAGGTAAT
Probe CtropP2: 5'-CGCAGTCTCAGTCTAGGCTGGCAG

Candida tropicalis (assay name: Ctrop)
Forward Primer CtropF1: 5'-GCGGTAGGAGAATTGCGTT
Reverse Primer CtropR2: 5'-TCATTATGCCAACATCCTAGGTTTA
Probe CtropP2: 5'-CGCAGTCTCAGTCTAGGCTGGCAG

Candida viswanathii (assay name: Cvisw)
Forward Primer CvisF1: 5'-CGGCAGGACAATCGCGT
Reverse Primer CvisR1: 5'-TCTAGGCTGGCAGTATCCACG
Probe CvisP1: 5'-AATGTGGCACGCGCTCGGC

Candida zeylanoides (assay name: Czeyl)
Forward Primer Czey F1: 5'-
GTTGTAATTTGAAGAAGGTAACCTTTGATT
Reverse Primer Czey R1: 5'-GACTCTTCGAAAGCACTTTACATGG
Probe Czey P1: 5'-CCTTGGAACAGGACGTACAGAGGGT

Chaetomium globosum (assay name: Cglob)
Forward Primer CglobF1: 5'-CCGCAGGCCCTGAAAAG
Reverse Primer CglobR1: 5'-CGCGGCGCGACCA
Probe CglobP1: 5'-AGATGTATGCTACTACGCTCGGTGCGACAG

Cladosporium cladosporioides, svar. 1 (assay name: Cclad1)
Forward Primer Cclad1F1: 5'-CATTACAAGTGACCCCGGTCTAAC
Reverse Primer CcladR1: 5'-CCCCGGAGGCAACAGAG
Probe CcladP1: 5'-CCGGATGTTTCATAACCCCTTTGTTGTCC

Cladosporium cladosporioides, svar. 2 (assay name: Cclad2)
Forward Primer Cclad2F1: 5'-TACAAGTGACCCCGGTACG
Reverse Primer CcladR1: 5'-CCCCGGAGGCAACAGAG
Probe CcladP1: 5'-CCGGATGTTTCATAACCCCTTTGTTGTCC

Cladosporium herbarum (assay name: Cherb)
Forward Primer CherbF1: 5'-AAGAACGCCCGGGCTT
Reverse Primer CherbR1: 5'-CGCAAGAGTTTGAAGTCTCCAC
Probe CherbP1: 5'-CTGGTTATTCATAACCCCTTTGTTGTCCGACTCTG

Cladosporium sphaerospermum (assay name: Cspha)
Forward Primer CsphaF1: 5'-ACCGGCTGGGTCTTTCCG
Reverse Primer CsphaR1: 5'-GGGGTTGTTTTACGGCGTG
Probe CsphaP1: 5'-CCCGCGGCACCCCTTATGCCGA

Emericella (Aspergillus) nidulans/rugulosa/quadrilineata (assay name: Anidu2)
Forward Primer AversF1: 5'-CAACCTCCCACCCGTGAC
Reverse Primer AniduR1-1: 5'-
CCATTGTTGAAAGTTTGGACTGATaTGT
Probe versP1: 5'-AGACTGCATCACTCTCAGGCATGAAGTTCCAG

Emericella (Aspergillus) varicolor (assay name: Avari)
Forward Primer AversF2: 5'-CGCGGGGAGCCCT
Reverse Primer AvariR1-1: 5'-
CCATTGTTGAAAGTTTGGACTAAATTCTC
Probe AvariP1: 5'-CATCACTACAGGCATGAAGTTGCGGTGGT

Eurotium (Aspergillus) amstelodami/chevalieri/herbariorum/rubrum/repens (assay name: Eamst)
Forward Primer EamstF1: 5'-GTGGCGGCACCATGTCT
Reverse Primer EamstR1: 5'-CTGGTAAAAAGATTGGTTGCCA
Probe EamstP1: 5'-CAGCTGGACCTACGGGAGCGGG

Epicoccum nigrum (assay name: Enigr)
Forward Primer EnigrF1: 5'-TTGTAGACTTCGGTCTGCTACTCTCT
Reverse Primer EnigrR1: 5'-TGCAACTGCAAAGGGTTTGAAT
Probe EnigrP1: 5'-CATGTCTTTTGTAGTACCTTCGTTCTCGGC

Fusarium solani f.sp. *batatas* MP II (Fsola2)
Forward Primer FsolaF2: 5'-CGCCGAGCTTCCAAT
Reverse Primer FsolaR2: 5'-GATTCGAGGTCAACGTTCTAAAAGT
Probe* FsolaP1mgb: 5'-CGTAGTAGCTAACACCTC

Fusarium solani f.sp. *cucurbitae* MP I (Fsola1)
Forward Primer FsolaF1: 5'-CAGCCGAGCTTCCAGT
Reverse Primer FsolaR1-1: 5'-CGTGCCGAGCCTCTG
Probe* FsolaP1mgb: 5'-CGTAGTAGCTAACACCTC

Fusarium solani f.sp. *mari* MP III, *Fusarium solani* f.sp. *cucurbitae* MP V, *Fusarium solani* f.sp. *pisi* MP VI, *Fusarium solani* f.sp. *robiniae* MP VII (Fsola3)
Forward Primer FsolaF3: 5'-CGCCGAGCTTCCATC
Reverse Primer FsolaR3: 5'-GATTCGAGGTCAACTTCAGAAGAG
Probe* FsolaP1mgb: 5'-CGTAGTAGCTAACACCTC

Fusarium solani f.sp. *xanthoxyli* MP IV (Fsola4)
Forward Primer FsolaF4: 5'-CGCCGAGCTTCCAGT
Reverse Primer FsolaR4-1: 5'-GATTCGAGGTCAACTTCAGATgT
Probe* FsolaP1mgb: 5'-CGTAGTAGCTAACACCTC

Geotrichum candidum strain UAMH 7863 (assay name: Geo - reference strain only)
Forward Primer GeoF1: 5'-GATATTTCTTGTGAATTGCAGAAGTGA
Reverse Primer GeoR1: 5'-TTGATTCGAAATTTAGAAAGAGCAAA
Probe GeoP1: 5'-CAATTCGAAGAGAAACAACGCTCAAACAAG

Geotrichum candidum (assay name: Gcand2)
Forward Primer NS92F: 5'-CACCGCCGCTGCTAC
Reverse Primer GcandR1: 5'-AGAAAAGTTGCCCTCTCCAGT
Probe GeoP2: 5'-TCAATCCGGAAGCCTCACTAAGCCATT

Geotrichum klebahnii (assay name: Gkleb1)
Forward Primer GklebF1: 5'-GGGCGACTTTTCCGGC
Reverse Primer GklebR2: 5'-

TGGCACAAATTCTCCTCTAATTTATTTA
Probe GklebP1: 5'-
AAGCTAGTCAAACCTGGTCATTTAGAGGAAGTAAAAGTC

Memnoniella echinata (assay name: Mem)
Forward Primer StacF4 5'-TCCCAAACCTTATGTGAACC
Reverse Primer MemR1: 5'-
TGTTTATACCACTCAGACGATACTCAAGT
Probe MemP1: 5'-CTCGGGCCCGGAGTCAGGC

Mucor
amphibiorum/circinelloides/hiemalis/indicus/mucedo/racemosus/ramosissimus
and *Rhizopus*
azygosporus/homothalicus/microsporus/oligosporus/oryzae
(assay name: Muc1)
Forward Primer NS92F: 5'-CACCGCCCGTCGCTAC
Reverse Primer MucR1-1: 5'-CCTAGTTTGCCATAGTTCTCAGCAG
Probe MucP1: 5'-CCGATTGAATGGTTATAGTGAGCATATGGGATC

Myrothecium verrucaria (assay name: Mverr)
Forward Primer MyroF1: 5'-AGTTTACAACTCCCAAACCTTT
Reverse Primer MyroR1: 5'-GTGCTACTCAGAGGAGAAAACCA
Probe MyroP1: 5'-CGCCTGGTTCGGGGCCC

Paecilomyces lilacinus (assay name: Plila)
Forward Primer PlilaF1: 5'-CCCACTGTGAACCTTACCTCAG
Reverse Primer PlilaR1: 5'-GCTTGTGCAACTCAGAGAAGAAAT
Probe PlilaP1: 5'-CCGCCCGCTGGGCGTAATG

Paecilomyces variotii (assay name: Pvari2)
Forward Primer PvariF2: 5'-CGAAGACCCCTGGAACG
Reverse Primer PvariR1: 5'-
GTTGTTGAAAGTTTTAATTGATTGATTGT
Probe PvariP1: 5'-CTCAGACGGCAACCTTCCAGGCA

Penicillium aethiopicum (assay name: Paeth)
Forward Primer PaethF1-1: 5'-CGGGGGGCTCtCGCT
Reverse Primer PchryR1-1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGAgTA
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium atramentosum (assay name: Patra)
Forward Primer PgrisF1-1: 5'-ACCTGCGGAAGGATCATTtCT
Reverse Primer PatraR1: 5'-CCCCGGCGGCCATA
Probe PenP3: 5'-TCCAACCTCCCACCCGTGTTTATTT

Penicillium
aurantiogriseum/freii/polonicum/tricolor/viridicatum (assay
name: PenGrp1A)
Forward Primer PexpaF1-1: 5'-TTACCGAGTGAGGGCCgTT
Reverse Primer PauraR6: 5'-CCCGCGGCCAGTA
Probe PenP3: 5'-TCCAACCTCCCACCCGTGTTTATTT

Penicillium
aurantiogriseum/freii/hirsutum/polonicum/tricolor/viridicatum/
verrucosum, svar. 2 (assay name: PenGrp1)
Forward Primer PauraF1: 5'-CGGGCCCGCCTTAC
Reverse Primer PauraR1-1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGAGTT
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium brevicompactum/stoloniferum (assay name: Pbrev)
Forward Primer PbrevF3: 5'-GGCAGCCTGCCTTTTG
Reverse Primer PenR2: 5'-
GATCCGTTGTTGAAAGTTTTAAATAATTTATA
Probe PbrevP2: 5'-CTCGCCGAAGACACCTTAGAACTCTGTCTGA

Penicillium canescens (assay name: Pcan2)
Forward Primer PcanF1: 5'-TTACCGAGCGAGAATTCTCTGA
Reverse Primer PcanR2-1: 5'-CCGCCGAAGCAACAAGtTAC
Probe* PenAspP1mgb: 5'-CCAACCTCCCACCCGTG

Penicillium chrysogenum, svar. 2 (assay name: Pchry)
Forward Primer PchryF4-1: 5'-GCCTGTCCGAGCGTCACTT
Reverse Primer PchryR8: 5'-CCCCCGGGATCGGAG
Probe PenP6: 5'-CCAACACACAAGCCGTGCTTGAGG

Penicillium
chrysogenum/griseofulvum/glandicola/coprophilum/expansum
and *Eupenicillium crustaceum/egyptiacum* (assay name:
PenGrp3)
Forward Primer PchryF1: 5'-CGGGCCCGCCTTAAC
Reverse Primer PchryR1-1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGAGTA
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium citreonigrum (Pcteo2)
Forward Primer PcteoF2-1: 5'-GGGGGCATCTGCTCTCTG
Reverse Primer PcteoR2-1: 5'-
CCGTTGTTGAAAGTTTTAAGTTATCTAA
Probe* PenAspP1mgb: 5'-CCAACCTCCCACCCGTG

Penicillium citrinum/sartoryi/westlingi (Pcitr)
Forward Primer PcitrF1: 5'-CCGTGTTGCCCGAACCTA
Reverse Primer PcitrR1: 5'-
TTGTTGAAAGTTTTAACTAATTTCTGTTATAG
Probe PcitrP1: 5'-TGCAACTTCAGACAGCGTTTACGGGG

Penicillium coprophilum (assay name: Pcopr)
Forward Primer PcoprF1-1: 5'-GGGTCCAACCTCCCActCA
Reverse Primer PchryR1-1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGAgTA
Probe PenP1: 5'-CGCCTTAACTGGCCGCCGG

Penicillium corylophilum (assay name: Pcopy)
Forward Primer PcopyF1: 5'-GTCCAACCTCCCACCCA
Reverse Primer PcopyR3-1: 5'-GCTCAGACTGCAATCTTCACTGATG
Probe PcopyP1: 5'-CTGCCCTCTGCGCCCGG

Penicillium
crustosum/camembertii/commune/echinulatum/solitum (assay
name: PenGrp2)
Forward Primer PchryF1: 5'-CGGGCCCGCCTTAAC
Reverse Primer PauraR1-1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGAGTT
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium decumbens (assay name: Pdecu3)
Forward Primer PdecuF3: 5'-GGCCTCCGCTCCTCTTTG
Reverse Primer PdecuR4-1: 5'-GACCTACAGAGCGGGTATGA
Probe PenP7: 5'-CCGAAAGGCAGCGGGCGG

Penicillium digitatum (assay name: Pdigi)
Forward Primer PaethF1-1: 5'-CGGGGGGCTCtCGCT
Reverse Primer PdigiR1: 5'-
CGTTGTTGAAAGTTTTAAATAATTTCTG
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium expansum (assay name: Pexpa)
Forward Primer PexpaF1-1: 5'-TTACCGAGTGAGGGCCgTT
Reverse Primer PexpaR2-1: 5'-GCCCGCCGAAGCtACG
Probe PenP3: 5'-TCCAACCTCCCACCCGTGTTTATTT

Penicillium fellutanum/charlesii (assay name: Pfell2)
Forward Primer PfellF2: 5'-CTGAGTGC GGCCCTCT
Reverse Primer PfellR2: 5'-CGCCGAAGCAACTGTAAAG
Probe* PenAsp1mgb: 5'-CCAACCTCCCACCCGTG

Penicillium glandicola (assay name: Pglan)
Forward Primer PglanF1-1: 5'-CCGGGGGGCTTTCGT
Reverse Primer PchryR1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGACTA
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium griseofulvum (assay name: Pgris)
Forward Primer PgrisF1-1: 5'-ACCTGCGGAAGGATCATTtCT
Reverse Primer PchryR6: 5'-CCCGGCGGCCAGTT
Probe PenP3: 5'-TCCAACCTCCCACCCGTGTTTATTT

Penicillium implicatum (assay name: Pimpl)
Forward Primer PimplF1: 5'-GCCGAAGACCCCTGT
Reverse Primer PimplR1: 5'-CGTTGTTGAAAGTTTTGACTGATTGT
Probe PimplP1: 5'-AACGCTGTCTGAAGCTTGACGCTGAGC

Penicillium islandicum (assay name: PislA)
Forward Primer PislA1: 5'-CGAGTGC GGTTTCGACA
Reverse Primer PislAR1: 5'-GGCAACGCGGTAACGGTAG
Probe PislA1: 5'-AGCCCAACCTCCCACCCGTG

Penicillium italicum (assay name: Pital)
Forward Primer PitalF1-1: 5'-CTCCACCCGTGTTTATTTAtCA
Reverse Primer PexpaR1: 5'-TCACTCAGACGACAATCTTCAGG
Probe PenP1: 5'-CGCCTTAAGTGGCCGCGG

Penicillium melinii (assay name: Pmeli)
Forward Primer PmeliF1-1: 5'-CAGGCTGTGTGTTGGtCT
Reverse Primer PmeliR1: 5'-GGGCTACAAGAGCGGAA
Probe PenP7: 5'-CCGAAAGGCAGCGCGCGC

Penicillium miczynskii (assay name: Pmicz)
Forward Primer PmiczF1-1: 5'-GTGTTTAAACGAACTGTTGCaTT
Reverse Primer PmiczR1-1: 5'-CTCAGACTGCATACTCAGACaGA
Probe PsimpP1: 5'-CCGCTCACGCGCGCC

Penicillium olsonii (assay name: Polsn)
Forward Primer PolsnF1: 5'-GGCGAGCCTGCCTTCG
Reverse Primer PenR2: 5'-
GATCCGTTGTTGAAAGTTTTAAATAATTTATA
Probe PolsnP2: 5'-TCCGCTCGCCGAGAGC

Penicillium oxalicum (assay name: Poxal)
Forward Primer PoxalF1: 5'-GGGCCCCCTCACG
Reverse Primer PoxalR1: 5'-
GTTGTTGAAAGTTTTAACTGATTTAGTCAAGTA
Probe PoxalP1: 5'-ACAAGATTCGTTTGTGTGCTTCGCGC

Penicillium purpurogenum (assay name: Ppurp)
Forward Primer PpurpF1: 5'-AGGATCATTACTGAGTGC GGA
Reverse Primer PpurpR1: 5'-GCCAAGCAACAGGGTATTC
Probe PpurpP1: 5'-CCCTCGCGGGTCCAACCTCC

Penicillium raistrickii (assay name: Prais3)
Forward Primer PgrisF1-1: 5'-ACCTGCGGAAGGATCATTtCT
Reverse Primer PraisR2: 5'-CCGGGCGCCTGACG
Probe PenP3: 5'-TCCAACCTCCCACCCGTGTTTATTT

Penicillium restrictum (assay name: Prest2)
Forward Primer PrestF2-1: 5'-CACGGCTGTGTGTTGGtCT

Reverse Primer PrestR1-1: 5'-CGGCCGGGCCTaCAA
Probe PenP7: 5'-CCGAAAGGCAGCGCGCGC

Penicillium roquefortii (assay name: Proqu)
Forward Primer PchryF1: 5'-CGGGCCCCCTTAAC
Reverse Primer ProquR2: 5'-
TTAAATAATTTATATTTGTTCTCAGACTGCAT
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Penicillium sclerotiorum (assay name: Psclr)
Forward Primer PsclrF1: 5'-TTCCCCGGGAACAGG
Reverse Primer PsclrR1: 5'-GCCCATACGCTCGAGGAT
Probe PsclrP1: 5'-CCGAAAGGCAGTGGCGGCAC

Penicillium simplicissimum/ochrochloron (assay name: Psimp2)
Forward Primer PsimpF2-1: 5'-CGCCGAAGACACCATTGAtCT
Reverse Primer PsimpR4-1: 5'-CTGAATTCTGCAATTCACATaACG
Probe PsimpP2: 5'-TGCTGAAGATTGACGCTGAGCGATTAGC

Penicillium glabrum/lividum/pupurescens/spinulosum/thomii
(assay name: Pspin2)
Forward Primer PspinF2: 5'-CATTACTGAGTGAGGGCCCTCT
Reverse Primer PspinR2-1: 5'-CGTGAGGCGGGaGCA
Probe* PenAsp1mgb: 5'-CCAACCTCCCACCCGTG

Penicillium variabile (assay name: Pvarb2)
Forward Primer PvarbF2-1: 5'-TTACCGAGTGC GGTTCTaAA
Reverse Primer PvarbR2: 5'-CGAGGCAACGCGGTAAC
Probe* PenAsp1mgb: 5'-CCAACCTCCCACCCGTG

Penicillium verrucosum, svar. 1 (assay name: Pverr)
Forward Primer PverrF2: 5'-CGGGCCCCCTTTG
Reverse Primer PauraR1: 5'-
GAAAGTTTTAAATAATTTATATTTTCACTCAGACTT
Probe PenP2: 5'-CGCGCCCGCCGAAGACA

Rhizomucor meihei/pusillus/variabilis (assay name: Rmuc)
Forward Primer NS92F: 5'-CACCGCCCGTCGCTAC
Reverse Primer RmucR1: 5'-GTAGTTTGCCATAGTTCGGCTA
Probe RmucP1: 5'-TTGAATGGCTATAGTGAGCATATGGGAGGCT

Rhizopus stolonifer (assay name: Rstol)
Forward Primer NS92F: 5'-CACCGCCCGTCGCTAC
Reverse Primer RstolR1: 5'-GCTTAGTTTGCCATAGTTCCTAAACAA
Probe MucP1: 5'-CCGATTGAATGTTATAGTGAGCATATGGGATC

Scopulariopsis asperula (assay name: SCaspr)
Forward Primer SCbrvF1: 5'-CCCTGCGTAGTAGATCCTACAT
Reverse Primer SCasprR1: 5'-TCCGAGGTCAAACCATGAGTAA
Probe ScopP1: 5'-TCGCATCGGGTCCCGGCG

Scopulariopsis brevicaulis/fusca (assay name: SCbrv)
Forward Primer SCbrvF1: 5'-CCCTGCGTAGTAGATCCTACAT
Reverse Primer SCbrvR1: 5'-TCCGAGGTCAAACCATGAAATA
Probe ScopP1: 5'-TCGCATCGGGTCCCGGCG

Scopulariopsis brumptii (assay name: SCbrm)
Forward Primer SCbrmF1: 5'-CCCTGCGTAGTAGTAAACCA
Reverse Primer SCbrmR1: 5'-CCGAGGTCAAACATCTTTGG
Probe ScopP1: 5'-TCGCATCGGGTCCCGGCG

Scopulariopsis chartarum (assay name: SCchr)
Forward Primer SCchrF1: 5'-CCCTGCGTAGTAGTAAAGC
Reverse Primer SCchrR1: 5'-TCCGAGGTCAAACCATCAAG
Probe ScopP1: 5'-TCGCATCGGGTCCCGGCG

Scopulariopsis sphaerospora (assay name: SCsph)
Forward Primer SCsphF1: 5'-CCCCCTGCGTAGTAGTTTACAA
Reverse Primer SCsphR1: 5'-CCGAGGTCAAACCATCAAAAAG
Probe ScopP1: 5'-TCGCATCGGGTCCCGGCG

Stachybotrys chartarum (assay name: Stac)
Forward Primer StacF4: 5'-TCCCAAACCTTATGTGAACC
Reverse Primer StacR5: 5'-GTTTGCCACTCAGAGAATACTGAAA
Probe StacP2: 5'-CTGCGCCCGGATCCAGGC

Trichoderma asperellum/hamatum (assay name: Taspr1)
Forward Primer TasprF1: 5'-CCCAAACCAATGTGAACGT
Reverse Primer TasprR2-1: 5'-
GGACTACAGAAAGAGTTTGTTGCTT
Probe TridP1: 5'-CCAACTGTTGCCTCGGCGGG

Trichoderma harzianum (assay name: Tharz)
Forward Primer TharzF1: 5'-TTGCCTCGGCGGGAT
Reverse Primer TharzR1: 5'-ATTTTCGAAACGCCTACGAGA
Probe TharzP1: 5'-CTGCCCGGGTGCCTCG

Trichoderma longibrachiatum/citriniviride (assay name: Tlong)
Forward Primer TlongF1: 5'-TGCTCGGCGGGATTC
Reverse Primer TlongR1: 5'-CGAGAAAGGCTCAGAGCAAAAAT
Probe TlongP1: 5'-TCGCAGCCCGGATCCCA

Trichoderma viride/atroviride/koningii (assay name: Tviri)
Forward Primer TviriF1: 5'-CCCAAACCAATGTGAACCA
Reverse Primer TviriR1: 5'-TCCGCGAGGGGACTACAG
Probe TridP1: 5'-CCAACTGTTGCCTCGGCGGG

Ulocladium atrum (assay name: Uatrm)
Forward Primer UatrmF2: 5'-CGGGCTGGCATCCTTC
Reverse Primer UatrmR2: 5'-CTGATTGCAATTACAAAAGTTTATG
Probe UloP1: 5'-TGAATTATTACCCGTGTCTTTTGCCTACTTCT

Ulocladium botrytis (assay name: Ubotr)
Forward Primer UbotrF1: 5'-CCCCAGCAGTGCGTT
Reverse Primer UbotrR1: 5'-CTGATTGCAATTACAAAAGTTTATG
Probe UloP1: 5'-TGAATTATTACCCGTGTCTTTTGCCTACTTCT

Ulocladium chartarum (assay name: Uchar)
Forward Primer UcharF1-1: 5'-AGCGGGCTGGAATCCaTT
Reverse Primer UcharR1-1: 5'-
CTGATTGCAATTACAAAAGTTgAAT
Probe UloP1: 5'-TGAATTATTACCCGTGTCTTTTGCCTACTTCT

Wallemia sebi (assay name: Wsebi)
Forward Primer WsebiF1: 5'-GGCTTAGTGAATCCTTCGGAG
Reverse Primer WsebiR1: 5'-GTTTACCAACTTTGCAGTCCA
Probe WsebiP1: 5'-TGTGCCGTGCGCGCTCAAATAG

Universal *Penicillium*, *Aspergillus* and *Paecilomyces varioti*
(assay name: PenAsp1mgb)
Forward Primer PenAspF1: 5'-CGGAAGGATCATTACTGAGTG
Reverse Primer PenAspR1: 5'-GCCCGCCGAAGCAAC
Probe* PenAspP1mgb: 5'-CCAACCTCCACCCGTG

* Applied Biosystems MGB probe, labeled with 6FAM reporter and black hole quencher. Suggest labeling of all other probes with 6FAM reporter and TAMRA or black hole quencher.

Suggested probe and primer concentrations for all assays is 80 nM probe, 1000 nM primers. Primers with (5x) designations indicate suggested use of 5x higher concentration.

Lower case letters in primer sequences denote deliberate mismatches with target sequences.