Implementation of MALDI-TOF mass spectrometry to identify moulds from the indoor environment as an added value to the classical microscopic identification tool

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ABSTRACT

During the last decades, the presence of moulds in the indoor environment has raised concern regarding their potential adverse health effects. The genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Alternaria* and yeasts, the most common fungi found indoors, include species with a high allergenic and toxic potential. Identification of these moulds is generally performed by microscopy. However, this method rarely enables identification to the species level. In order to increase the accuracy of identification, MALDI-TOF MS analysis can be performed. In this study, 104 fungal air and dust isolates from 27 dwellings in Brussels were identified by both microscopy and MALDI-TOF MS analysis. A comparison of results obtained with both methods indicates an increased precision in identifications with MALDI-TOF MS analysis, emphasizing its highly added value to the standard microscopic analysis in routine practice.

INTRODUCTION

The quality of indoor air has become a subject of great interest as people are spending most of their time indoors (Cincinelli & Martellini, 2017). Biological pollutants such as moulds however can affect this air quality and can cause adverse health effects as fungal particles such as spores but also smaller fragments such as secondary metabolites (mycotoxins, allergens, fungal volatile organic compounds, …) can induce allergic, toxic or infectious effects (Fréalle et al., 2017). The genera *Aspergillus*, *Cladosporium*, *Penicillium, Alternaria* and yeasts are the most common fungi found indoors (Moldoveanu, 2015). A selected number of species within these genera have a high allergenic potential and can produce a significant amount of mycotoxins, capable to provoke adverse health effects in humans like allergic sinusitis, allergic rhinitis, allergic asthma, atopic dermatitis, mycotoxicose or hypersensitivity pneumonitis. In addition, some species within these genera are also capable of causing invasive infections such as candidiasis or invasive aspergillosis (Fromme et al., 2016; Fukutomi & Taniguchi, 2015; Mousavi et al., 2016; Simon-Nobbe et al., 2008). In the case of immunocompromised patients, the presence of these species indoors can represent an enormous health threat. Besides the specificity of action and pathologies related to some specific moulds, the patient's health background has to be taken into account as well while investigating adverse health effects that can be caused by mould problems indoors (Janbon et al., 2019)

As health effects caused by moulds can be dose-response related (Mendell & Kumagai, 2016), the quantification of the loads of the different mould species indoors is important. The latter also allows to compare the mould species diversity and their quantities indoors with the situation outdoors, being required in order to asses a potential indoor air mould contamination. Therefore, a reference sample of the outdoor environment, defining the mould species diversity and their quantities outdoors is always necessary (ANSES, 2016).

Regarding this and the ability of certain mould species to cause specific symptoms and illnesses, it is necessary to seek for fast and accurate tools, enabling an identification to the species level in order to guide general practitioners in their search for the underlying cause of a health problem.

Identification of moulds found in the indoor environment is generally performed by microscopy. This standard method has, however, some limitations as it needs mycologists with high expertise while identification is often limited to the genus level (Fréalle et al., 2017; Vesper, 2010). Molecular methods can offer relief (Fréalle et al., 2017). However, DNA based analysis techniques such as polymerase chain reactions are limited in the panel of species they can identify and multi-locus gene sequencing (the "gold standard" for the identification of filamentous fungi) is very expensive, time consuming and prone to environmental contamination. An alternative method is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), offering the advantage of being fast, easy to perform and cost-effective (Becker et al., 2014; Hendrickx, 2017). This analysis method has been used since the beginning of this century for the identification of bacteria, yeasts and moulds (Hendrickx, 2017). The principle of MALDI-TOF MS is based on the ionisation of fungal proteins by a laser, followed by the creation of a

spectrum, representing a species specific protein fingerprint. This spectrum is compared to a database of reference spectra and the similarity between the spectra is expressed in the form of a reliability index ("logscore") (Alanio et al., 2011; Cassagne et al., 2011; Hendrickx, 2017).

In order to increase the accuracy of identification, MALDI-TOF MS can be an added value to microscopy. The results of both microscopic and MALDI-TOF MS analyses will be compared to highlight the added value of MALDI-TOF MS to the identification of moulds and to validate the accuracy of the microscopic identifications.

METHODS

In collaboration with Brussels Environment RCIB/CRIPI (Regional Intervention Cell for Indoor Pollution), Sciensano's (Brussels, Belgium) Indoor Mycology unit performs measurements of fungal contaminations in Brussels dwellings in order to assess a link between a potential indoor air pollution and people's health problems. RCIB/CRIPI processes surveys in dwellings, sampling indoor air, dust, settled dust on furniture and visible contaminated surfaces in various rooms, especially those where the patient spends a noticeable amount of time. In addition, the outdoor air is always sampled as a reference.

Air and dust samples used for this research were collected by the RCIB/CRIPI team between 28 May 2019 and 01 December 2020 during investigations in 27 dwellings in Brussels. The air was sampled by a RCS+ impactor (volume of 80L). Air samples were incubated for 5 days at 25°C and grown on HS culture media (Rose Bengal agar + chloramphenicol). The dust was sampled by a vacuum cleaner containing a filter (filter « 3M filtrete » MC/US/diam 57 mm/PB). Dust samples were resuspended into a solution of Tween 80% and then poured in two culture media: MC (Malt extract agar + chloramphenicol) to search for hydrophilic moulds and M40Y+NaCl (Malt extrac agar + chloramphenicol + sodium salt) to search for xerophilic moulds. They were then incubated for 7 days at 25°C. After incubation and counting of the colonies for each sample, morphological identifications were performed for all the colonies grown on the culture media. The identifications were performed by observation of the macroscopic and microscopic characteristics of the colonies grown on the media.

For the colonies that could not be identified microscopically or in the cases where identification to the genus level or species complex level was not sufficient, a complementary MALDI-TOF MS analysis was performed.

In order to perform MALDI-TOF MS analysis, subcultures of 104 colonies were grown on SC culture medium (sabouraud chloramphenicol agar) by incubation during 3 to 4 days at 25°C. Samples were prepared and analysed following the method described for MALDI-TOF MS analysis in Becker et al. (2015), except from the identification with DNA analysis that was not performed in our study.

The obtained spectra were compared with the in-house created reference database containing over 1700 strains of the BCCM/IHEM fungal collection (https://msi.happy-dev.fr/login/). Logscore values were provided to express the similarity between the obtained spectra and the reference spectra from the database. Logscores are used to indicate that according to the manufacturer, a logscore <1.7 corresponds to an unreliable identification, a logscore between 1.7 and 1.99 indicates acceptable genus identification while a logscore equal to or above 2.0 indicates acceptable species level identification (Becker, 2014).

RESULTS

A total of 104 isolates including 18 yeasts and 86 filamentous fungi were analysed.

Microscopic analysis indicated 18 isolates as yeast species and allowed identification to the genus level for 47 of all the isolates analysed. Only 34 isolates could be identified to the species complex level. Four moulds could not be identified by microscopy and one was indicated as sterile mycelia. No isolate was identified to species level.

Analysis by MALDI-TOF MS identified 100 of the 104 isolates with a logscore > 1,7. Out of the 100 isolates, 94 were identified to the species level with a logscore > 2,0. For 6 isolates, identification was limited to the genus/section level with a logscore between 1,7 and 2,0. Only 4 isolates could not be identified by MALDI-TOF MS analysis.

The results of the identifications by both methods can be found in *Table 1*.

Compared to microscopic analysis, MALDI-TOF MS resulted in a more precise identification for 100 isolates (96%). Of the 65 isolates identified to the genus level or as belonging to yeasts and of the 34 isolates identified to the species complex level with microscopy, 58 and 29 respectively were confirmed as correctly identified when compared to the results obtained by MALDI-TOF MS analysis. The 29 correct microscopic identifications to the species complex level included 15 *Cladosporium* isolates (10 *Cladosporium herbarum* gr., 4 *Cladosporium cladosporioides* gr. and 1 *Cladosporium sphaerospermum* gr.) and 14 *Aspergillus* isolates (5 *Aspergillus versicolor* gr., 3 *Aspergillus fumigatus* gr., 3 *Aspergillus flavus* gr., 1 *Aspergillus glaucus* gr., 1 *Aspergillus niger* gr., 1 *Aspergillus restrictus* gr.) Microscopic identification of the 32 *Penicillium* isolates was limited to the genus level whereas yeasts were only referred to as "yeast species". In contrast, MALDI-TOF MS indicated a diversity of 10 different *Penicillium* species and 6 different yeasts species. Two isolates identified microscopically as yeasts could not be identified by MALDI-TOF MS (Table 1).

Of the 4 isolates that could not be identified by microscopy, MALDI-TOF MS analysis identified 3 isolates to the species level (*Hormographiella verticillata*, *Exophiala sideris* and *Plectosphaerella cucumerina*) and 1 to the genus level (*Parengyodontium* species) (Table 1). MALDI-TOF MS analysis indicated 5 microscopic misidentifications to the species complex level. Two isolates identified as *Cladosporium sphaerospermum* gr. by microscopy were identified by MALDI-TOF MS as *Cladosporium delicatulum* and *Cladosporium europaeum*, both members of the *Cladosporium cladosporioides* species complex. Another isolate microscopically identified as *Cladosporium herbarum* gr. was identified as *Cladosporium westerdijkiae* (*Cladosporium cladosporioides* gr.) with MALDI-TOF MS. Within the genus *Aspergillus*, 2 microscopic identifications proved wrong with MALDI-TOF MS: an *Aspergillus ochraceus* gr. isolate turned out to be *Aspergillus flavus* and an isolate of *Aspergillus flavus* gr. was identified as *Aspergillus persii* (*Aspergillus ochraceus* gr.) (Table 1).

In addition, 1 microscopically identified *Alternaria* isolate was identified as *Pseudopithomyces* species with MALDI-TOF MS analysis. One isolate identified as *Paecilomyces* species with the microscope was *Taloromyces wortmanii* according to MALDI-TOF MS and 2 *Acremonium* isolates were identified as *Acrodontium crateriforme* and *Calcarisporium* species with MALDI-TOF MS (Table 1).

DISCUSSION

A comparison between identifications performed by microscopic analysis and MALDI-TOF MS analysis highlights their difference in accuracy of identification. Microscopic analysis did not allow an identification to the genus level for none of the yeast isolates analysed and almost half of all microscopic identifications in this study were limited to an identification to the genus level. Less than one third of the isolates could be identified to the species complex level by microscopy. These findings highlight the difficulty of species differentiation within genera and within species complexes based on morphological characteristics. Indeed, species within most genera of moulds are often hard to differentiate, especially when it comes to specific structures such as spores (Vesper, 2010). Moreover, *Penicillium* isolates can hardly be identified to the species level and yeasts cannot be further identified by the standard microscopic method (Barton, 2010; Reboux et al., 2019), as indicated by the results in our study.

In contrast, MALDI-TOF MS analysis allowed for identification to the species level for most of the isolates (90%) analysed. The latter method enabled insight into the diversity of *Penicillium* found in the sampled dwellings. Over 65% of all *Penicillium* isolates identified by MALDI-TOF MS analysis appear to belong to *P. chrysogenum*, *P. brevicompactum*, *P. crustosum* and *P. rubens* with *P. chrysogenum* accounting for more than one third of all identifications, the latter being consistent with the study of Reboux et al. (2019). Indeed, *P. chrysogenum* is often put forward as the most common *Penicillium* species found in dwellings and is considered as an important cause of allergic reactions. In addition, together with *P. brevicompactum*, it is capable of producing numerous mycotoxins (Fromme et al., 2016; Reboux et al., 2019; Simon-Nobbe et al., 2008). In accordance with the results for *Penicillium* species, MALDI-TOF MS analysis also demonstrated a wide diversity of yeast species found in the indoor environment of Brussels dwellings analysed in this study. *Rhodotorula mucilaginosa* was the most present, followed by *Naganishia diffluens*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae,* the latter three being equally present. Of them, *Saccharomyces cerevisiae* can be implicated in allergic reactions such as atopic dermatitis and *Rhodotorula mucilaginosa* is also able to produce allergens (Simon-Nobbe et al., 2008). *Naganishia diffluens* can be an exacerbating factor in atopic dermatitis (Zhang et al., 2011) while *Debaryomyces hansenii* seems to be rarely associated with health problems in humans (Fitzpatrick & Butler, 2010).

In addition to the few species specific microscopic identifications, several microscopic misidentifications occurred as well, i.e. between *A. flavus* and *A. ochraceus*, and between the species complexes of *C. herbarum*, *C cladosporioides* and *C. sphaerospermum*. Moreover, as for *Penicillium,* some *Aspergillus* and *Cladosporium* isolates could not be identified further than the genus level by microscopy.

Cladosporium herbarum gr. and *C. cladosporioides* gr. accounted for over 90% of all *Cladosporium* isolates analysed in this study. These results are in accordance with the findings of Segers et al. (2015), who identified *C. sphaerospermum* gr. as the less frequent *Cladosporium* species complex found in indoor air. Three isolates of the species complex *Cladosporium cladosporioides* were microscopically misidentified, one with the species complex *Cladosporium herbarum* and two with *Cladosporium sphaerospermum* gr. Although all three species complexes are known to produce allergenic proteins (Fromme et al., 2016; Fukutomi & Tanigushi, 2015), their relative presence is often not equal in indoor and outdoor environments, especially not in poorly ventilated houses (Segers et al., 2015). Therefore, an accurate identification of *Cladosporium* to the species level is very important in order to trace for a potential indoor contamination.

Considering *Aspergillus,* as both allergic and toxic reactions are mainly caused by *A. fumigatus, A. flavus, A. niger* and *A versicolor* (Fromme et al., 2016, Fukutomi & Tanigushi, 2015; Reboux et al., 2010; Simon-Nobbe et al., 2008), an accurate identification to the species level is of high importance for this genus as well.

Table 1. Identifications of the isolates by MALDI-TOF MS versus microscopy, including origin of isolates

Genus / species complex	**MALDI-TOF MS identification** (number of isolates)	**Microscopic identification** (number of isolates)	**Isolate origin**
	Penicillium		
Penicillium species	*Penicillium chrysogenum* (10), *Penicillium brevicompactum* (4) *Penicillium crustosum* (4), *Penicillium rubens* (3), *Penicillium frequentans* (2), *Penicillium fellutanum* (2), *Penicillium bialowiezense* (2), *Penicillium griseofulvum* (1), *Penicillium olsonii* (1), *Penicillium* species (section *aspergilloides*) (1), *Penicillium* species (*brevicompactum* gr.?) (1)	*Penicillium* species (31)	Indoor air
	Penicillium polonicum (1)	*Penicillium* species (1)	Dust
	Cladosporium		
Cladosporium herbarum gr.	*Cladosporium allicinum* (8)	*Cladosporium herbarum* gr. (7), *Cladosporium* species (1)	Indoor air
	Cladosporium aggregatocicatricatum (2)	*Cladosporium herbarum* gr. (2)	Indoor air
	Cladosporium ramotenellum (2)	*Cladosporium herbarum* gr. (1), *Cladosporium* species (1)	Indoor air
Cladosporium cladosporioides gr.	*Cladosporium westerdijkiae* (1)	*Cladosporium herbarum* gr. (1)	Indoor air
	Cladosporium inversicolor (1)	*Cladosporium* species (1)	Indoor air
	Cladosporium delicatulum (3)	*Cladosporium cladosporioides* gr. (2), *Cladosporium sphaerospermum* gr. (1)	Indoor air
	Cladosporium cladosporioides (1)	*Cladosporium cladosporioides* gr. (1)	Indoor air
	Cladosporium pseudocladosporioides (1)	*Cladosporium cladosporioides* gr. (1)	Indoor air
	Cladosporium europaeum (1)	*Cladosporium sphaerospermum* gr. (1)	Indoor air
Cladosporium sphaerospermum gr.	*Cladosporium halotolerans* (1)	*Cladosporium* species (1)	Indoor air
	Cladosporium sphaerospermum (1)	*Cladosporium sphaerospermum* gr. (1)	Indoor air
	Aspergillus		
Aspergillus fumigatus gr.	*Aspergillus fumigatus* (3)	*Aspergillus fumigatus* gr. (3)	Indoor air
Aspergillus versicolor gr.	*Aspergillus creber* (3), *Aspergillus sidowii* (2)	*Aspergillus versicolor* gr. (5)	Indoor air
Aspergillus flavus gr.	*Aspergillus flavus* (4)	*Aspergillus flavus* gr. (3), *Aspergillus ochraceus* gr. (1)	Indoor air
Aspergillus niger gr.	*Aspergillus tubingensis* (3)	*Aspergillus niger* gr.(1), *Aspergillus* species (2)	Indoor air
Aspergillus glaucus gr.	*Aspergillus pseudoglaucus* (2)	*Aspergillus glaucus* gr. (1), *Aspergillus* species (1)	Indoor air
Aspergillus ochraceus gr.	*Aspergillus persii* (1)	*Aspergillus flavus* gr. (1)	Indoor air
Aspergillus nidulans gr.	*Aspergillus nidulans* (1)	*Aspergillus* species (1)	Indoor air
Aspergillus restrictus gr.	*Aspergillus restrictus* (1)	*Aspergillus restrictus* gr. (1)	Dust
	Aspergillus species (section *nidulantes*) (1)	*Aspergillus* species (1)	Indoor air
	Yeasts		
	Rhodotorula mucilaginosa (4), *Debaryomyces hansenii* (3), *Saccharomyces cerevisiae* (3), *Naganishia diffluens* (3), *Candida parapsilosis* (1), *Starmerella etchellsii* (1)	Yeast species (15)	Dust
	Rhodotorula mucilaginosa (1)	Yeast species (1)	Indoor air
	Other species		
	Pseudopithomyces species (1)	*Alternaria* species (1)	Indoor air
	Hormographiella verticillata (*Caprinellus domesticus*) (1)	Not identified (1)	Indoor air
	Exophiala sideris (1)	Not identified (1)	Dust
	Plectosphaerella cucumerina (1)	Not identified (1)	Indoor air
	Taloromyces wortmanii (1)	*Paecilomyces* species (1)	Indoor air
	Botrytis cinerea (1)	*Botrytis* species (1)	Indoor air
	Parengyodontium species (1)	Not identified (1)	Indoor air
	Acrodontium crateriforme (1)	*Acremonium* species (1)	Indoor air
	Calcarisporium species (1)	*Acremonium* species (1)	Indoor air
	Not identified (4)	Sterile mycelia (1), *Penicillium* species (1), yeast species (2)	Indoor air

Only a few environmental isolates (4%) could not be identified by MALDI-TOF MS analysis, which could be explained by the dominance of spectra from clinical isolates and the lack of environmental isolates in the current MALDI-TOF MS databases (including our in-house database). However, in order to strengthen this identification tool, our in-house database is currently being expanded with spectra of environmental isolates (using DNA extraction and sequencing).

The results in our study demonstrate the enormous capacity of MALDI-TOF MS in identifying fungal air and dust isolates to the species level. In contrast to morphological observations, MALDI-TOF MS is an automated tool, yielding results with a high objectivity level. In addition, the accuracy of MALDI-TOF MS is

increased here by applying our in-house created reference database, containing only highly controlled fungal strains from the BCCM/IHEM collection, both ISO9001 certified and ISO17025 accredited (Becker et al., 2014; 2015). These findings make MALDI-TOF MS a highly added value to microscopy in the identification of fungal isolates in routine analyses in our lab.

CONCLUSION

- The comparison of species diversity indoors versus outdoors can help to guide general practitioners in their search for the underlying cause of a health problem.
- A comparison between identifications of fungal isolates performed by microscopic analysis and MALDI-TOF MS analysis highlights the higher accuracy of the latter.
- The automated and objective MALDI-TOF MS tool can be a highly added value to the time-consuming standard microscopic analysis in routine practice aiming to identify moulds from dwellings.
- MALDI-TOF MS databases, being mostly built on spectra from clinical isolates, should be extended by including environmental isolates in order to strengthen the identification tool.

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