

Development of a Dot-Blot Assay for the Detection of Mould-Specific IgE in the Belgian Population

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Abstract

Background Data on mould sensitization in the general population are scarce and mostly on *Aspergillus fumigatus*, *Alternaria alternata* and *Cladosporium herbarum*.

Objectives To validate a dot-blot assay for the detection of specific IgE and evaluate the prevalence of mould sensitization in a healthy population.

Methods The dot-blot assay was validated against the CAP test. Sensitization rate to ten common indoor and outdoor mould species in 344 serum samples was calculated. For each serum with more than one reactivity, the “major sensitization” defined as the

strongest response against a single mould species was calculated.

Results Intra- and inter-assay variations were both below 20%, and the positivity threshold of the test was of 0.418 kU/L for *A. fumigatus*. Correlation with CAP results was strong. The overall prevalence of sensitization was 32.8%, and the commonest sensitizations were against *A. alternaria*, *A. flavus* and *A. niger* (around 15%). The most frequent “major reactivities” were against *A. niger* and *A. alternata* (20–30%). In 25.1% of the samples, “major reactivities” were directed against a group of moulds commonly found indoor (*Penicillium* spp., *Aspergillus versicolor*, *Cladosporium sphaerospermum* and *Cladosporium cladosporioides*).

Conclusions The dot-blot assay was validated for the detection of mould-specific IgE. In the general population, sensitization to indoor species was common

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and accounted for 25% of overall mould sensitizations.

Keywords IgE detection · Dot-blot · Mould sensitization · Indoor mould species

Abbreviation

SPT skin prick test

Introduction

Fungi are eukaryotic, filamentous spore bearing organisms that can live as saprophytes or as parasites of humans, animals and plants. Since they are ubiquitous, humans are constantly, and in large concentrations, exposed to fungi. Spores vary in size (between 2 and 250 µm), but the majority are smaller than 10 µm, are inhalable and can reach the upper and/or lower airways. In most people, spores are recognized by innate defence mechanisms and eliminated. However, in a small proportion of individuals, exposure causes a sensitization, triggers an allergic response or leads to a colonization of the lung and the respiratory tract [1].

It is estimated that there exist between 1 and 1.5 million fungal species worldwide. Among them, 112 genera are potentially allergenic, 111 allergens have been described from 29 species and the International Allergen Nomenclature Sub-committee (WHO-IUIS) regularly updates its database (www.allergen.org). Among mould genera, four of them, *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*, are often related to a risk of development of clinical allergy [2]. The exact prevalence of sensitization against moulds is unknown but is estimated to vary between 3 and 10% in the general population [2]. Mould sensitization is commonly observed in asthma patients, and moreover, many studies have reported on the association between mould sensitization and asthma severity. *Alternaria alternata*, *Cladosporium herbarum* and *Aspergillus fumigatus* are the most frequently incriminated species for their association with asthma severity [3–7].

Over the years, the improvement of insulation techniques led to an increase in relative humidity in housings, thereby accelerating the development of moulds and the colonization of dwellings (ranging

from 5–10 to 10–30% depending on the climates) [8]. While *A. alternata* and *C. herbarum* are generally considered as outdoor moulds, *A. fumigatus* and *A. niger* are equally common outdoors and indoors. *Penicillium* spp, *A. versicolor* and *A. flavus* or *C. cladosporioides* and *C. sphaerospermum* are predominantly considered as indoor moulds [9–13]. To our knowledge, no data are available regarding the frequency of sensitization against indoor moulds species in the population.

Our objective was to develop a dot-blot assay for the detection of specific IgE and to document the prevalence of sensitization to a panel of moulds commonly isolated indoors (air, dust or visible marks) in Western Europe [14, 15] in a healthy adult Belgian population with the aim to determine whether indoor species could represent an issue for public health. In addition to the frequently studied *A. alternata*, *C. herbarum* and *A. fumigatus*, species like *A. niger*, *A. flavus*, *A. versicolor*, *P. chrysogenum*, *P. brevicompactum*, *C. cladosporioides* and *C. sphaerospermum*, with a more pronounced indoor tropism and frequently isolated in Belgium, were included in our panel [14, 15]. The dot-blot assay, with its ability to easily detect antibodies against a larger panel of moulds extracts than what is routinely used, could represent an interesting tool to combine with SPT and/or CAP when exploring mould sensitization in large populations or as tailor-made solution based on clinical needs.

Material and Methods

Moulds Extracts

Extracts from *A. alternata*, *P. chrysogenum*, *A. fumigatus*, *A. niger*, *A. flavus*, *C. herbarum* and *C. sphaerospermum* were purchased from Greer laboratories (Lenoir-NC, USA). They were supplied as lyophilized cakes meant to be suspended in aqueous solution (µg/mL).

Strains of *A. versicolor* (1129 and 18884), *P. brevicompactum* (5522) and *C. cladosporioides* (6032) were obtained from BCCM/IHEM (Belgian Coordinated Collection of Microorganisms/Biomedical Fungi and Yeasts; WIV-ISP-Brussels, Belgium). Extracts were prepared from mycelial mats cultured during 3 weeks in liquid Czapek media. Mats were

grinded in liquid nitrogen and proteins extracted as described by Achatz et al. [16]. Protein concentration was measured according to the Bradford method and extracts were stored at -20°C .

Study Population

The study population consisted of 344 serum samples from Belgian subjects part of an anonymized serum bank. Samples were collected across the country in adult donors aged 20–39 years as left-over diagnostic samples of clinical chemistry laboratory of six hospitals (CE2011/71) [17]. No clinical data are available for these samples, except that serum samples collected for investigations of respiratory-related diseases were excluded. There is no particular reason to consider that allergic sensitization was under- or over-estimated in this adult population.

Specific IgE Detection

A dot blot was developed for the detection of specific IgE against mould extracts. Extracts, diluted in borate buffer (pH 10) (5 and 50 $\mu\text{g}/\text{ml}$ for commercial and in-house extracts, respectively), were coated on a nitrocellulose membrane (Amersham Protran 0.45 NC-GE Lifesciences) using a 96-well dot-blot system (Mini-fold® I Dot-Blot System; Whatman). The membrane was removed from the device and blocked in 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at RT in TBS. The membrane was then incubated with human's sera (dilution 1/5 in TBS–Tween 20 0.1%–BSA 0.1%, 1 h 30, RT). For inhibition experiments, patients' sera (diluted 1/2.5) were pre-incubated for 30 min with a mould extract chosen as inhibitor diluted at a concentration of 100 $\mu\text{g}/\text{mL}$ (final concentration 50 $\mu\text{g}/\text{mL}$) (ratio 1:1) except for the inhibition experiment performed with *P. brevicompactum* as inhibitor. In this experiment, the final concentration of *P. brevicompactum* was 500 $\mu\text{g}/\text{mL}$. Extracts chosen as inhibitors were mould extracts against which samples previously reacted positively. Finally, the membrane was incubated for 1h30 at RT with rat monoclonal anti-human IgE coupled to HRP (LO-HE 17-HRP and LO-HE 10-HRP—Institute of Experimental and Clinical Research, Université Catholique de Louvain) diluted 1/5000 in TBS–Tween 20 0.1%–BSA 0.1%. Between each incubation step, excess antibodies were removed by washing 3 times with

TBS for 3 min, then 3 times with TBS–Tween 20 0.05% for 5 min and then again 3 times with TBS for 3 min. The membrane was developed by addition of Amersham ECL Prime reagent (GE Lifesciences), and readings were performed on a Bioanalyzer CCD-Camera (LAS 4000Mini; GE Lifesciences). Results given as pixel counts were analysed with the ImageQuant TL software (GE Lifesciences). Two positive controls were used: the Non-WHO Human anti-*A. fumigatus* IgE Reference Serum (Product number 78/545 National Institute for Biological Standards and Controls—NIBSC—Hertfordshire, UK) (concentration 25.6 kU/L measured by ImmunoCAP (Phadia 1000 FEIA analyser—Thermo Scientific)) and the serum of a volunteer with established sensitizations to several mould species (CAP test *P. chrysogenum*: 0.5 kU/L; *C. herbarum*: 0.625 kU/L; *A. fumigatus*: 0.43 kU/L and *A. alternata*: 0.875 kU/L). Two negative controls, which had neither shown reactivity against any mould of the panel, were used. The background was estimated based on the signal from an uncoated membrane (sample dilution buffer). Results, obtained as pixel counts, were then converted into indexes to standardize their expression with the following formula:

$$\text{Index} = \frac{(\text{Pixel count for the tested serum} - \text{background})}{(\text{Pixel count for the first negative control} - \text{background})}$$

For each mould extract, positivity thresholds were established from 43 serial analyses of the second negative control serum. Positivity thresholds were expressed as the mean index response obtained for each extract + 3 standard deviations.

Samples Used for the Assay Validation

A pool of serum samples from allergic subjects (25 mould-positive and 29 mould-negative sera) and previously analysed in CHU Brugmann with ImmunoCAP (CAP) for the detection of IgE antibodies against *A. fumigatus*, *A. alternata*, *P. chrysogenum* and *C. herbarum* was used for the validation of the dot-blot assay. These samples were analysed with the dot-blot assay for the same four mould species and the results compared with CAP results. Mould-positive samples for which enough volume was available were later used for inhibition experiments. According to the manufacturer, CAP test has a limit of detection of 0.1 kU/L and a positivity threshold of 0.35 kU/L.

Statistical Analysis

Spearman's correlation coefficient test was used (Graph Pad Prism 6 for Windows, Version 6.01) to determine correlations between CAP and dot-blot results but also to study correlations between IgE levels for each mould. A p value <0.05 was considered significant.

Results

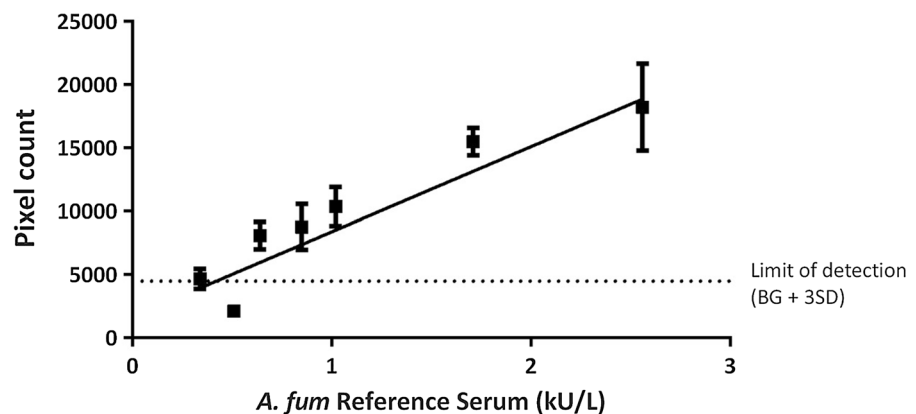
To assess the performance of the dot-blot assay, a dilution curve was performed with decreasing concentrations of the Non-WHO Human Reference Serum anti-*A. fumigatus* (each dilution was performed in ten replicates), on a *A. fumigatus* extract coated membrane. The limit of detection was set at mean pixel count + 3SD of the background and the calculated positivity threshold of the assay was 0.418 kU/L for *A. fumigatus* (Fig. 1). Intra-assay (i.e. repeatability) and inter-assay (i.e. reproducibility) variations were also evaluated using the Reference Serum. To estimate the repeatability, the dot blot was performed on a membrane with the extract of *A. fumigatus* coated in 11 replicates. The coefficient of variation was found to be of 18.9% (Supplementary data Table S1A). To assess its reproducibility, this experiment was performed 3 days in a row and the coefficient of variation was of 17.8% (Supplementary data Table S1B). To further validate the assay, a group of serum samples received from CHU Brugmann and tested by CAP tests for *A. alternata*, *C. herbarum*, *P. chrysogenum* and *A. fumigatus* was analysed by dot-blot assay with the same species. The concordance between CAP and

dot-blot results was then analysed using the Spearman's correlation coefficient test. The concordance between positive results (data not shown) and the overall correlation (positive and negative samples) between the two tests were strong and significant ($\rho = 0.623$; $p < 0.0001$) (Fig. 2).

The dot-blot assay was then used in 344 samples from the Belgian adults aged 20–39 years for the study of mould sensitization rates. The overall prevalence of sensitization, i.e. the positive reaction against at least one mould extract, was 32.8% (113 out of 344 sera) with significant differences between mould species. The prevalence of sensitization against *A. alternata* and *A. flavus* reached 15%. Sensitization rates against *A. niger* were of 12.8%, *P. chrysogenum* 10.3%, *C. herbarum* 9.0% and *P. brevicompactum* 8.5%. *A. versicolor*, *A. fumigatus*, *C. cladosporioides*, *C. sphaerospermum* were recognized by less than 5% of the sera (Fig. 3a, b). 48.7% of the positive sera showed reactivity against only one species and among them, sensitization against *A. niger* was the most frequent monosensitization (32.7%).

Correlations between IgE responses were evaluated in positive samples (113/344). We found that IgE responses were positively correlated for 15 out of 45 pairs of extracts ($p < 0.05$) (Fig. 4). After analysis, three clusters of cross-reactive species were identified. Responses against *A. fumigatus* were highly significantly correlated ($\rho = 0.529$; $p < 0.0001$) with those against *C. herbarum*, and less significantly ($\rho = 0.191$ – 0.395 ; $p < 0.05$) with the responses against six other moulds. The responses against three out of these six moulds (*A. niger*, *C. cladosporioides*, *P. chrysogenum*) were also moderately correlated ($r = 0.399$ – 0.258) with the response against *C.*

Fig. 1 Determination of the dot-blot sensitivity. A dilution curve is performed with the anti-*A. fumigatus* IgE Reference Serum on a membrane coated with an *A. fumigatus* extract (dilution range 2.56–0.34 kU/L; 10 replicates per dilution). The limit of detection is set at mean pixel count + 3SD of the background and the calculated positivity threshold is 0.418 kU/L



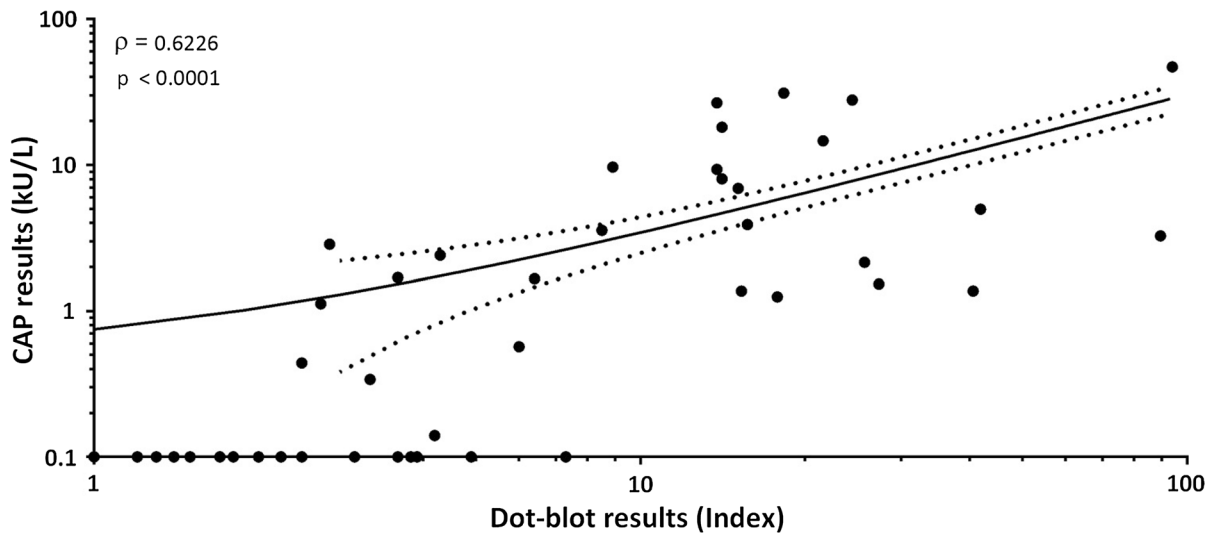


Fig. 2 Concordance between CAP and dot-blot results. Serum samples tested with CAP for *A. fumigatus*, *A. alternata*, *C. herbarum* and *P. chrysogenum* are also analysed with the dot-blot assay for the same mould species (n sera = 54; n assay = 123). Each spot represents CAP and dot results for

the same mould extract. Sixty-eight spots are outside the axis limits and do not appear on the graph. The overall concordance between CAP and dot-blot results for the four species is calculated with the Spearman's correlation coefficient test ($\rho = 0.6226$; $p < 0.0001$)

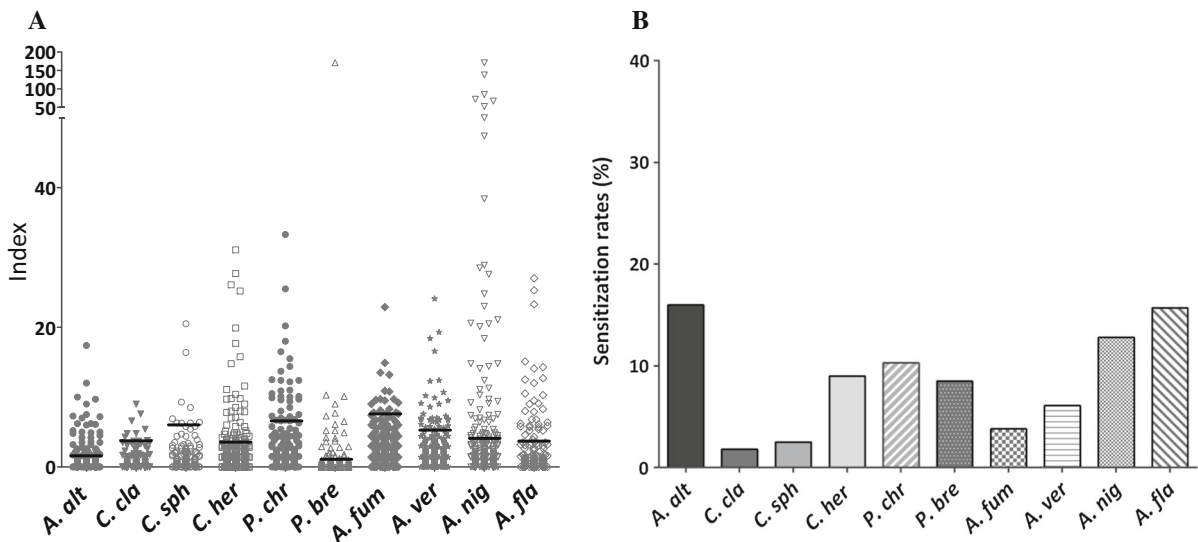


Fig. 3 Sensitization frequencies against a panel of indoor and outdoor moulds in serum samples from Belgian adults. The presence of specific IgE against 10 different mould species is investigated in a total of 344 sera collected across the country in adults aged 20–39 years and part of an anonymous serum bank. **a** Each dot represents the response of a single serum against each

extract. Horizontal bars represent, for each mould, the positivity threshold. This threshold corresponds to the mean response + 3 standard deviations (43 determinations) of a negative serum, on each mould extract. **b** Sensitization frequencies (%) for each mould of the panel ($n = 344$)

herbarum, defining a first group of five cross-reactive moulds. Responses against *P. chrysogenum* were highly significantly associated ($\rho = 0.56$ $p < 0.0001$) with those against *A. flavus*, creating therefore another cluster

of three cross-reactive moulds (*P. chrysogenum*, *A. flavus* and *A. fumigatus*). Responses against *P. brevicompactum* and against *C. cladosporioides* ($\rho = 0.421$; $p < 0.0001$) were also correlated. Of note, we did not

A. alt										
P. chr	0.402 p < 0.0001									
P. bre	0.136 p = 0.191	0.006 p = 0.951								
C. her	0.121 p = 0.244	0.300 p = 0.003	0.191 p = 0.062							
C. cla	0.005 p = 0.960	0.004 p = 0.964	0.421 p < 0.0001	0.258 p = 0.007						
C. sph	-0.159 p = 0.515	-0.514 p = 0.072	0.321 p = 0.284	-0.052 p = 0.842	0.296 p = 0.248					
A. fum	0.225 p = 0.029	0.395 p < 0.0001	0.304 p = 0.003	0.529 p < 0.0001	0.263 p = 0.006	0.145 p = 0.444				
A. nig	0.132 p = 0.205	0.330 p = 0.001	-0.094 p = 0.364	0.275 p = 0.003	-0.057 p = 0.558	-0.060 p = 0.755	0.191 p = 0.043			
A. fla	0.152 p = 0.153	0.560 p < 0.0001	0.220 p = 0.036	0.138 p = 0.189	-0.048 p = 0.656	-0.406 p = 0.044	0.326 p = 0.001	-0.070 p = 0.505		
A. ver	-0.236 p = 0.022	-0.073 p = 0.474	-0.260 p = 0.011	-0.070 p = 0.458	0.005 p = 0.955	0.133 p = 0.485	0.055 p = 0.564	0.019 p = 0.841	-0.076 p = 0.469	
	A. alt	P. chr	P. bre	C. her	C. cla	C. sph	A. fum	A. nig	A. fla	A. ver

Fig. 4 Correlations between the responses against moulds extracts. The Spearman's correlation coefficient test is used to estimate correlations between antigenic responses in sera showing at least one positive reaction against moulds of the panel ($n = 113$). Spearman correlation coefficient (ρ) and the

find any significant correlation of responses against *A. versicolor* and *C. sphaerospermum* with other species.

Multiple sensitizations, detected in 51.3% of the samples, were likely the expression of cross-reactions rather than “true” multiple sensitizations. Therefore, we aimed to determine the “major sensitization” for each serum. The “major sensitization” was defined as the response against a single extract showing the highest difference as compared to the variation of response of the negative control. It was calculated with the formula:

$$\Delta \text{ for each extract} = \frac{(\text{experimental serum index} - \text{index of the second negative control serum})}{\text{standard deviation of the second negative control serum}}.$$

This formula was used because it considers not only the gap between the response of the serum and the negative control for a given extract but also the variation of the response. In mould-sensitized individuals from our Belgian cohort, the commonest

p values are mentioned for each couple. The statistically significant values ($p < 0.05$) are indicated in **bold**. Boxes with p values < 0.05 are shaded in *light grey*, with p values < 0.01 in *intermediate grey*, with p values 0.001 in *dark grey* and with p values < 0.0001 in *darkest grey*

“major sensitizers” were *A. niger* and *A. alternata* (31.9 and 22.3% of the positive sera, respectively). Four other species (*A. flavus*, *C. herbarum*, *P. brevicompactum* and *A. versicolor*) were “major sensitizers” for 10–15% of the positive sera. No “major sensitizations” were found against *A. fumigatus* and *C. sphaerospermum* (Fig. 5).

Inhibition experiments were then finally conducted to validate the concept of “major sensitization”. Our assumption was that pre-incubation with an extract against which the serum showed reactivity but which

was not the “major sensitizer” should likely inhibit all responses except the response against the “major sensitization”. In a first experiment, a sample with *C. herbarum* as “major sensitizer” was pre-incubated with *A. alternata*. The dot blot was then performed as

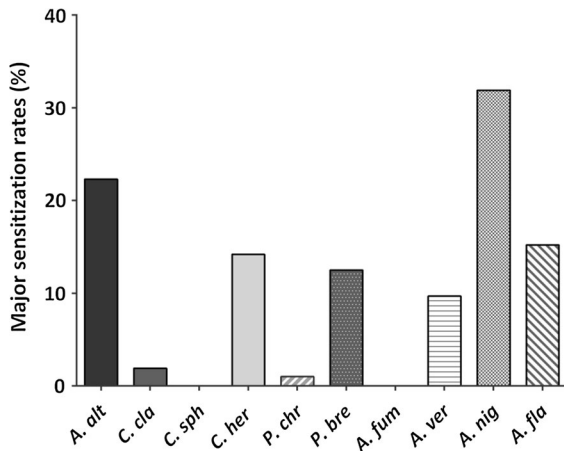


Fig. 5 “Major sensitizations” rates. “Major sensitization” frequencies (%) for each mould of the panel. For each positive serum ($n = 113$), the “major sensitization” is defined as the response against a single extract showing the highest difference as compared to the variation of response of the negative control serum (see “Material and Methods” section)

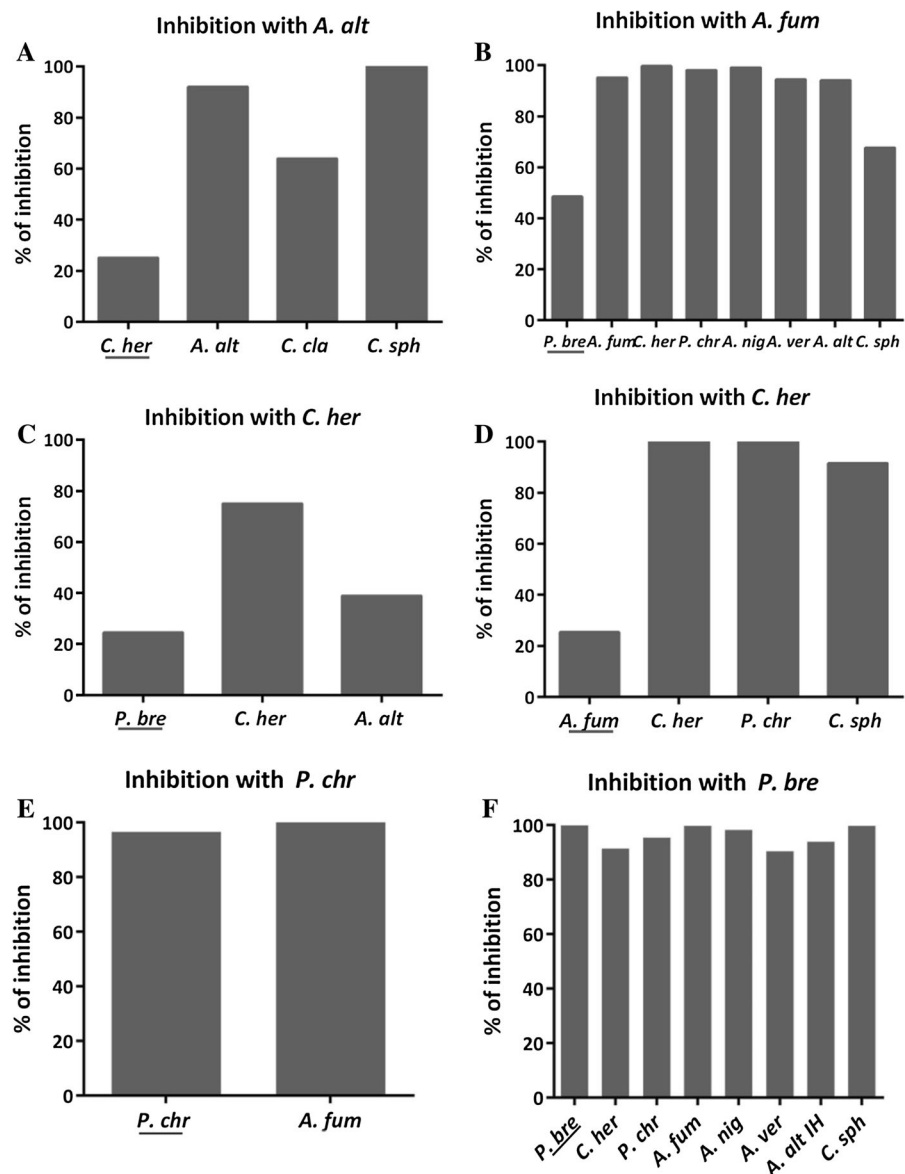
usual. This treatment only decreased partially the “major response” but strongly reduced the reactivity against *C. cladosporioides* and abolished the recognition of *A. alternata* and *C. sphaerospermum* (Fig. 6a). A second sample showing reactivity against multiple moulds was pre-incubated with *A. fumigatus* extract. The response against *P. brevicompactum*, the “major sensitizer”, was only inhibited by 50%. However, this pre-incubation step completely abrogated the response against *A. fumigatus* and five other species. The response against *C. sphaerospermum* was also partially inhibited (Fig. 6b). In another sample, pre-incubation with *C. herbarum* extract only slightly inhibited the response against the “major sensitizer” *P. brevicompactum* (22% inhibition) and decreased the response against *A. alternata* (40% inhibition) (Fig. 6c). In a fourth experiment, responses against *P. chrysogenum* and *C. sphaerospermum* were completely abrogated by pre-incubation with *C. herbarum*. The response against *A. fumigatus*, the “major sensitizer”, was again only slightly decreased (20% inhibition) (Fig. 6d). The last two experiments were conducted with the “major sensitizer” as inhibitor to confirm that after such a pre-incubation step, all responses were abolished. Results showed that a pre-incubation step with mould extracts defined as their “major sensitization completely abrogated all responses (Fig. 6e, f).

Discussion

There are few data on the prevalence of mould sensitization in the general population. In order to evaluate it, we developed a dot-blot assay for the detection of mould-specific IgE. The assay displayed a consistent performance with respect to its reproducibility and repeatability, although CVs are slightly higher than reported for CAP tests [18]. The dot-blot assay was validated by its strong correlation with CAP results for the four tested species (*A. alternata*, *A. fumigatus*, *C. herbarum* and *P. chrysogenum*) and its ability to screen multiple allergens simultaneously. The positivity threshold for *A. fumigatus*-specific IgE showed sensitivity (0.418 kU/L). Due to a lack of other fungal reference sera, we assumed that the sensitivity was comparable for the other species.

Results from environmental enquiries performed in Belgium (indoor air and settled dust samples) and in Eastern France (indoor air samples and visible mould marks analysis) showed that *Penicillium* spp, *A. versicolor*, *C. sphaerospermum* and *C. cladosporioides* were frequently isolated indoors [14, 15]. Therefore, based on these findings, ten mould species, mainly indoor species plus the classical *A. fumigatus*, *A. alternata* and *C. herbarum*, were selected for this study. The cohort consisted of more than 300 samples from adults donors aged 20–39 years. They were anonymously collected in clinical chemistry laboratories and even though no clinical information was available, we had no reason to suspect that allergic sensitizations in this group were not representative of the Belgian population of this age. We found an overall mould sensitization rate of 32.8% with responses mostly directed against *A. alternata*, *A. flavus* and *A. niger*. Reactivity against *A. alternata* and *C. herbarum* (16.0 and 9.0%, respectively) was higher than reported in previous studies. Indeed, in the European Community Health Survey conducted in 35 centres in 15 countries, a sensitization prevalence of 4.7% (range 0.2–14.4%) and 2.4% (range 0–11.9%) to *A. alternata* and *C. herbarum* was reported [19, 20]. Arshad et al. [21] obtained comparable results in a birth cohort study in the UK with sensitization prevalence of 4.8 and 2.5%, respectively. Another study analysing the sensitization to *A. alternata* alone in US citizens reported a prevalence of 12.9% [22]. Of note, all these studies were based on skin prick tests. In our study, we found a sensitization rate of 3.8% for *A.*

Fig. 6 Inhibition experiments. Inhibition experiments were performed according to the protocol defined in “Material and Methods” in selected sera provided by CHU Brugmann. Extracts used for these experiments (diluted at the concentration of 50 µg/mL) were chosen among the serum reactivities. “Major sensitizations” are underlined. Each bar represents the percentage of inhibition of the response against the indicated mould extract. A serum with *C. herbarum* defined as “major sensitizer” was inhibited with an *A. alternata* extract (a). Sera with *P. brevicompactum* defined as “major sensitizer” were inhibited with an extract of *A. fumigatus* (b) and an extract of *C. herbarum* (c). A serum with *A. fumigatus* defined as “major sensitization” was inhibited with an extract of *C. herbarum* (d). A serum with a “major sensitization” against *P. chrysogenum* was inhibited with an extract of the same species (e). A serum with a “major sensitization” against *P. brevicompactum* was inhibited with an extract of *P. brevicompactum* (f)



fumigatus and of 10.3% for *P. chrysogenum* which is higher than results from an older cohort study on 714 children where a prevalence of 2.4 and 2.2% was observed for *A. fumigatus* and *Penicillium* spp., respectively [23]. Monosensitization was observed in 48.7% of the tested samples, which is noticeably lower than what Mari et al. [24] reported previously in a large study in allergic patients (77%) with a panel of ten allergens. It is, however, higher than what Soeria-Atmadja et al. [18] found (almost 20%) in subjects with at least one allergic sensitization in a panel of 17 moulds. Additionally, we did not find any subject with

monosensitization to *A. fumigatus* or *P. chrysogenum* and only 1.4% of monosensitization to *C. herbarum* was recorded. These results are similar to the observation of Mari et al. [24] (<1% of monosensitization to these species).

Fungal cross-reactions are very well described and generally involve fungi that are phylogenetically related and share homology in the amino acid sequence, but cross-reactivity can also involve distant species such as *A. fumigatus* and *Candida boidiini* [25]. Cross-reactive carbohydrate determinants earlier considered as clinically relevant are now rather

questioned [26]. As expected, the Spearman's correlation coefficient test showed correlations between IgE responses for many mould extracts of the panel. Three clusters of cross-reactive moulds were identified, probably sharing cross-reactive epitopes. The first cluster involved *A. fumigatus*, *C. herbarum*, *A. niger*, *P. chrysogenum* and *C. cladosporioides*, the second one *P. chrysogenum*, *A. flavus* and *A. fumigatus* and the last one *P. brevicompactum* and *C. cladosporioides*. As expected, responses to phylogenetically close species were generally positively correlated. In a large study, Soeria-Atmadja et al. [18] investigated correlations between IgE responses (specific IgE detected by CAP test) against 17 allergenic fungal species, among them *A. alternata*, *P. chrysogenum*, *C. herbarum*, *A. fumigatus*, in a group of 668 individuals sensitized to at least one of the species. Our results are in accordance with their data as we observed correlations between the same species.

As discussed above, several samples showed reactivities against multiple mould extracts and we then aimed at distinguish the “major sensitization” from plausible cross-reactions. Even if multiple reactivities are likely the expression of cross-reactions, one should keep in mind that at the individual level, polysensitization can never be totally excluded. Inhibitions experiments confirmed the validity of our concept since after inhibition, the only response that was consistently and (almost) completely preserved was the response against the “major sensitizer”. In addition, when inhibitions were performed with the “major sensitizer”, all reactivities were completely abolished. These results were consistent with those obtained from the study of cross-reactions.

Aspergillus niger was found to be the most frequent “major sensitizer” (31.9%), followed by *A. alternata* (22.3%) and then by *A. flavus* and *C. herbarum* whose frequencies reached around 15%. While the noxious potential of *A. alternata* is well described [5, 27], the literature over *A. niger* is not so abundant but suggests that it can also trigger hypersensitivity reactions [28]. *A. flavus* is frequently cited for its implication on chronic fungal sinusitis and cutaneous infection [29–31]. A recent study in patients affected by allergic diseases reports of a frequency of sensitization against these species of 14.7 and 17%, respectively [32]. Quite strikingly, in this cohort from the general population, *A. fumigatus* was never identified as the “major sensitizer”, while it represents around 35% of “major

reactivities” in a cohort of mould-sensitized asthmatics (unpublished results) and was repeatedly associated with asthma severity [6, 7]. Noticeably, 25.1% of the sensitized subjects showed “major reactivities” against species frequently isolated in damp houses (*A. versicolor*, *C. cladosporioides*, *P. chrysogenum* and *P. brevicompactum*) as a consequence of water damage, dampness and lack of heating [10, 11, 14]. Many studies have already pointed at the adverse health effects of mould contamination but did not focus on particular species [33–35]. Our study, using a panel of ten moulds, shows that some species commonly found indoor can also elicit sensitizations.

Conclusions

The rate and pattern of sensitization to a large panel of moulds were analysed in Belgian adults using an in-house developed dot-blot assay. Given its performances and specifications, it could make a valuable tool that can be used for broad screening of mould sensitizations. Additionally, it can also be used to detect reactivities against less common mould species that do not exist as commercial allergen preparations for SPT or as CAP reagents. Based on the analysis of a large cohort of healthy subjects, we found that beyond classical outdoor species *A. alternata* and *C. herbarum* and pathological *A. fumigatus*, other mould species commonly found indoor are able to elicit true sensitizations as well. These findings suggest that these species should be included in future studies on mould exposure and adverse health effects.

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Authors' Contribution M.V, O.D and O.M designed the study protocol. M.V analysed and interpreted data and wrote the article. O.D reviewed data analysis and interpretation and revised the manuscript. O.M recruited the patients and provided critical review of the manuscript. F.C provided CAP results for allergic patients and made manuscript revision. M.R and K.H provided critical input in the manuscript framework and reviewed the paper. All authors approved the final version of the document.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

References

- Kurup VP, Shen HD, Banerjee B. Respiratory fungal allergy. *Microbes Infect.* 2000;2:1101–10.
- Twaroch TE, Curin M, Valenta R, Swoboda I. Mold allergens in respiratory allergy: from structure to therapy. *Allergy Asthma Immunol Res.* 2015;7:205–20.
- Neukirch C, Henry C, Leynaert B, Liard R, Bousquet J, Neukirch F. Is sensitization to *Alternaria alternata* a risk factor for severe asthma? A population-based study. *J Allergy Clin Immunol.* 1999;103:709–11.
- Zureik M, Neukirch C, Leynaert B, Liard R, Bousquet J, Neukirch F. Sensitization to airborne moulds and severity of asthma: cross sectional study from European community respiratory health survey. *BMJ.* 2002;325:411–4.
- Black PN, Udy AA, Brodie SM. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy.* 2000;55:501–4.
- Fairs A, Agbetile J, Hargadon B, Bourne M, Monteiro WR, Brightling CE, Bradding P, Green RH, Motalithas K, Desai D, Pavord ID, Wardlaw AJ, Pashley CH. IgE sensitization to *Aspergillus fumigatus* is associated with reduced lung function in asthma. *Am J Respir Crit Care Med.* 2010;182:1362–8.
- Schwartz HJ, Citron KM, Chester EH, Kaimal J, Barlow PB, Baum GL, Schuyler MR. A comparison of the prevalence of sensitization to *Aspergillus* antigens among asthmatics in Cleveland and London. *J Allergy Clin Immunol.* 1978;62:9–14.
- Quansah R, Jaakkola MS, Hugg TT, Heikkinen SA, Jaakkola JJ. Residential dampness and molds and the risk of developing asthma: a systematic review and meta-analysis. *PLoS ONE.* 2012;7:e47526.
- Denning DW, Pashley C, Hartl D, Wardlaw A, Godet C, Del GS, Delhaes L, Sergejeva S. Fungal allergy in asthma—state of the art and research needs. *Clin Transl Allergy.* 2014;4:14.
- Chew GL, Rogers C, Burge HA, Muilenberg ML, Gold DR. Dustborne and airborne fungal propagules represent a different spectrum of fungi with differing relations to home characteristics. *Allergy.* 2003;58:13–20.
- Mahooti-Brooks N, Storey E, Yang C, Simcox NJ, Turner W, Hodgson M. Characterization of mold and moisture indicators in the home. *J Occup Environ Hyg.* 2004;1:826–39.
- Bellanger AP, Reboux G, Roussel S, Grenouillet F, Didier-Scherer E, Dalphin JC, Millon L. Indoor fungal contamination of moisture-damaged and allergic patient housing analysed using real-time PCR. *Lett Appl Microbiol.* 2009;49:260–6.
- Alshareef F, Robson GD. Prevalence, persistence, and phenotypic variation of *Aspergillus fumigatus* in the outdoor environment in Manchester, UK, over a 2-year period. *Med Mycol.* 2014;52:367–75.
- Beguín H. Mould biodiversity in homes I. Air and surfaces analysis of 130 dwellings. *Aerobiologia.* 1994;10:157–66.
- Reboux G, Bellanger AP, Roussel S, Grenouillet F, Sornin S, Piarroux R, Dalphin JC, Millon L. Indoor mold concentration in Eastern France. *Indoor Air.* 2009;19:446–53.
- Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, Ebner C, Prillinger H, Kraft D, Breitenbach M. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol Immunol.* 1995;32:213–27.
- Huygen K, Rodeghiero C, Govaerts D, Leroux-Roels I, Melin P, Reynders M, Van Der Meeren S, Van Den Wijngaert S, Pierard D. *Bordetella pertussis* seroprevalence in Belgian adults aged 20–39 years, 2012. *Epidemiol Infect.* 2014;142:724–8.
- Soeria-Atmadja D, Onell A, Borga A. IgE sensitization to fungi mirrors fungal phylogenetic systematics. *J Allergy Clin Immunol.* 2010;125:1379–86.
- Bousquet PJ, Chinn S, Janson C, Kogevinas M, Burney P, Jarvis D. Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European community respiratory health survey I. *Allergy.* 2007;62:301–9.
- Zock JP, Jarvis D, Luczynska C, Sunyer J, Burney P. Housing characteristics, reported mold exposure, and asthma in the European community respiratory health survey. *J Allergy Clin Immunol.* 2002;110:285–92.
- Arshad SH, Tariq SM, Matthews S, Hakim E. Sensitization to common allergens and its association with allergic disorders at age 4 years: a whole population birth cohort study. *Pediatrics.* 2001;108:E33.
- Arbes SJ Jr, Gergen PJ, Elliott L, Zeldin DC. Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. *J Allergy Clin Immunol.* 2005;116:377–83.
- Sears MR, Herbison GP, Holdaway MD, Hewitt CJ, Flannery EM, Silva PA. The relative risks of sensitivity to grass pollen, house dust mite and cat dander in the development of childhood asthma. *Clin Exp Allergy.* 1989;19:419–24.
- Mari A, Schneider P, Wally V, Breitenbach M, Simon-Nobbe B. Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts. *Clin Exp Allergy.* 2003;33:1429–38.
- Hemmann S, Blaser K, Cramer R. Allergens of *Aspergillus fumigatus* and *Candida boidinii* share IgE-binding epitopes. *Am J Respir Crit Care Med.* 1997;156:1956–62.
- Cramer R, Zeller S, Glaser AG, Vilhelmsson M, Rhyner C. Cross-reactivity among fungal allergens: a clinically relevant phenomenon? *Mycoses.* 2009;52:99–106.
- O'Hollaren MT, Yunginger JW, Offord KP, Somers MJ, O'Connell EJ, Ballard DJ, Sachs MI. Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. *N Engl J Med.* 1991;324:359–63.
- Baur X, Sander I, Posch A, Raulf-Heimsoth M. Baker's asthma due to the enzyme xylanase—a new occupational allergen. *Clin Exp Allergy.* 1998;28:1591–3.
- Wang DY, Hadj-Henni L, Thierry S, Arne P, Chermette R, Botterel F, Hadrich I, Makni F, Ayadi A, Ranque S, Huang WY, Guillot J. Simple and highly discriminatory VNTR-

- based multiplex PCR for tracing sources of *Aspergillus flavus* isolates. PLoS ONE. 2012;7:e44204.
30. Krishnan S, Manavathu EK, Chandrasekar PH. *Aspergillus flavus*: an emerging non-fumigatus *Aspergillus* species of significance. Mycoses. 2009;52:206–22.
 31. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology. 2007;153:1677–92.
 32. Vermani M, Vijayan VK, Agarwal MK. Identification of *Aspergillus* (*A. flavus* and *A. niger*) Allergens and Heterogeneity of Allergic Patients' IgE Response. Iran J Allergy Asthma Immunol. 2015;14:361–9.
 33. Karvonen AM, Hyvarinen A, Korppi M, Haverinen-Shaughnessy U, Renz H, Pfefferle PI, Remes S, Genuneit J, Pekkanen J. Moisture damage and asthma: a birth cohort study. Pediatrics. 2015;135:e598–606.
 34. Fisk WJ, Lei-Gomez Q, Mendell MJ. Meta-analyses of the associations of respiratory health effects with dampness and mold in homes. Indoor Air. 2007;17:284–96.
 35. Iossifova YY, Reponen T, Ryan PH, Levin L, Bernstein DI, Lockey JE, Hershey GK, Villareal M, LeMasters G. Mold exposure during infancy as a predictor of potential asthma development. Ann Allergy Asthma Immunol. 2009;102:131–7.