

Original article

Identification of spore allergens from the indoor mould *Aspergillus versicolor*

Background: Indoor mould growth and dampness are associated with respiratory health effects and allergies and several studies demonstrated that mainly *Aspergillus versicolor* and *Penicillium expansum* are responsible for indoor mould exposure. In contrast, commercialized test systems to diagnose allergic reactions to this mould species are not available. In this study, allergenic proteins from spores of the indoor relevant species *A. versicolor* and *P. expansum* should get detected and identified.

Methods: We used two-dimensional (2D)-gel electrophoresis of spore proteins and immunoblotting with sera from patients participating in an epidemiologic study about indoor exposure of moulds and their influence on the development of allergies (ESTERSPEGA). Sera were screened for IgE antibodies specific for proteins from *A. versicolor*, *A. fumigatus* and *P. expansum* in one-dimensional blots and in 2D immunoblots. From the 2D gels, the corresponding spots were picked and identified by mass spectrometry.

Results: More than 20 allergens from *A. versicolor* were identified; in particular, seven major allergens were selected, which were detected by more than 90% of the positive sera. The most abundant allergen was glyceraldehyde-3-phosphate dehydrogenase, followed by an unnamed protein, which displays a high homology to sobitol/xylose reductase. The other allergens were identified as catalase A, hypothetical protein AN6918.2, enolase, hypothetical protein AN0297.2 and a protein with homology to a fungal malate dehydrogenase.

Conclusions: The results indicate an important role of spore proteins from *A. versicolor* for sensitization against indoor moulds and identification of the major allergens might enable species-specific diagnosis of allergic reactions.

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Human health is increasingly affected by allergies caused by naturally occurring compounds as well as by synthetically produced compounds. The identification of the allergenic compounds is essential for successful diagnosis and therapy of patients with allergic symptoms. Apart from food, inhalation is the most important route of exposure. From the natural airborne allergens, pollen of grass and trees are the most important outdoor allergenic sources. Other important allergenic sources are airborne mould. There is a high prevalence of sensitization to *Alternaria* and *Cladosporium* (up to 20%) and a significant association with asthma (1). However, several studies could demonstrate a spatial and seasonal distribution of airborne mould. Whereas *Alternaria* and *Cladosporium* occur typically outdoors with a maximum of spore concentration in summer and autumn, species of

Aspergillus and *Penicillium*, especially *A. versicolor*, *P. expansum* and *P. chrysogenum*, dominate indoors in winter in humid and cold climates (2–5). Particularly, *A. versicolor* was isolated and identified often from indoor air of moisture-damaged buildings (6, 7) and can be associated with negative health effects in humans (8).

To diagnose a sensitization to mould allergens, a set of diagnostic tools is available. Well analysed mould allergens are mainly the outdoor relevant species like: *Alternaria alternata*, *Cladosporium herbarum*, *C. cladosporoides*, *Botrytis cinerea*, *Fusarium solanii*, *Rhizopus stolonifer* and *Candida albicans* (9–11). Allergens from *Aspergillus* and *Penicillium* species are investigated from *A. fumigatus*, *A. niger* as well as from *P. chrysogenum* (10). *Aspergillus fumigatus* is known as a disease causing agent for allergic bronchopulmonary aspergillosis (12, 13). The allergen Asp f 1, the major fungal allergen produced by the mycelia of *A. fumigatus*, is widely used for diagnosis of a sensitization to this species. Surveys of the Asp f 1 allergens as well as of *A. fumigatus* spores demonstrated a very low level in indoor environments (14, 15). However,

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

allergens from the major indoor relevant *Aspergillus* species – *A. versicolor* – are until now unknown. The intention of this work was to detect new main allergens from indoor relevant moulds. Because of the dominance of the inhalation pathway of exposure, the investigation was focussed on spore proteins, because the number and mass of spores incorporated by inhalation are much higher than those of proteins from the mycelium.

The most straightforward approach to find new allergens is to use sera of sensitized patients to detect allergens in protein extracts from the allergenic source. In this experimental strategy, the separation of proteins can be achieved in an efficient way by two-dimensional (2D)-gel electrophoresis (16). In addition, to ensure to find patients who are reacting by the type I of sensitization, it is advisable to focus on IgE-specific detection of allergens (17). Allergens found in such an approach can be used first for species-specific diagnosis of sensitization and once such an analysis is established, epidemiological studies can be set up to clarify the real incidence of sensitization to indoor moulds. Finally, some main allergens might even be proven to be suitable for hyposensitization.

Material and methods

Sera collection

Within an epidemiological study (ESTERSPEGA) on effects of indoor mould exposure on health, respiratory diseases and allergies in Erfurt, Germany, data collection through questionnaires on living conditions and health effects, analyses of contamination with mould spores and clinical investigations were carried out. Sera samples from 97 participating patients were collected to investigate the specific IgE against various mould species. Sera were obtained by taking of blood samples with special sera monovettes (Fa. Sarstedt). Samples were centrifuged for 10 min with 2500 *g*, aliquoted and until further examinations stored at –20°C. For the characterization of allergic sensitizations of the patients, IgG, IgM, IgA and specific IgE for *P. chrysogenum*, *C. herbarum*, *A. fumigatus* and *Alternaria tenuis* (Pharmacia) were determined.

Culturing of moulds and preparation of spore extracts

Aspergillus versicolor, *A. fumigatus* and *P. expansum* were cultivated for 21 days on dicloran–glycerol–agar with chloramphenicol (DG 18; Oxoid), a selective medium for xerophilic moulds (18). The spores were collected by rinsing the cultures with a washing solution (Tris 50 mM Tris–HCl; pH 7.5; 0.1 g/100 ml chloramphenicol; 1 mM PMSF) and enriched by centrifugation for 30 min at 4°C and 15 000 *g* and lysed by rotation in a bead mill (Retsch GmbH, Haan, Germany) after addition of 1–1.5 mg beads ($d_m = 0.75$ –1 mm) per 1 ml for 1.5 h at 4°C. The suspensions were centrifuged twice for 20 min at 4°C and 16 000 *g* and the supernatants were kept frozen at –20°C until further usage. The protein concentration was determined as described by Lowry (1951) (19).

SDS-PAGE, 2D-gel electrophoresis and Western blotting

For one-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) 50 µg protein was precipitated

with a final concentration of 10% TCA and washed twice with acetone (21). The final pellets were resolubilized in 10 µl sample buffer, heated for 5 min at 60°C and applied to the gel. For 2D analysis, 500 µg of protein was purified by phenol extraction with subsequent solvent precipitation and washing steps. The resulting protein pellets were air dried and dissolved in 135 µl DeStreak rehydration solution with 0.5% immobilized pH gradient (IPG) pH 3–10 nonlinear (NL) buffer (v/v) (GE Healthcare, Uppsala, Sweden). To remove precipitates, the solution was centrifuged for 15 min at 62 000 *g* and loaded on 7 cm Immobiline DryStrips pH 3–10 NL (GE Healthcare). After rehydration, proteins were focussed using IPG Phor electrophoresis unit overnight (GE Healthcare) for 14000 Vh. The same procedure was used with varied dimensions for preparation of a large master gel as described previously (22). Gels were stained by Coomassie Brilliant Blue G-250 staining (Roth, Kassel, Germany) (23). Alternatively, the proteins were transferred by electroblotting to a nitrocellulose membrane (0.2 µm; Schleicher & Schuell, Dassel, Germany) using a Western blot chamber (XCell II Blot Module; Invitrogen, Karlsruhe, Germany) and 10 mM CAPS buffer pH 11 (24). A current of 1.5 mA/cm² was applied for 90 min at ambient temperature. The blotting efficiency was validated by reversible PonceauS stain (0.2% PonceauS in 3% TCA). Afterwards, the membranes were washed with water and blocked with 3% BSA in TBS (20 mM Tris, 500 mM NaCl, pH 7.5), overnight. For immunological detection, the membranes were incubated with or without the patients' sera diluted to 1 : 40 in antibody buffer (TBS with 0.05% Tween20 and 1% BSA) for 120 min at room temperature. Following washing twice with TTBS (0.05% Tween20 in TBS), the membranes were incubated with goat anti-human IgE secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, Geisenhofen, Germany) in a dilution of 1 : 4000. After incubation, the membranes were washed twice with TTBS and once with TBS. The binding of antibodies was detected by staining with the AP Conjugate Kit (Bio-Rad, Geisenhofen, Germany).

Identification of proteins by mass spectrometry

Protein bands of interest were cut from polyacrylamide gels and digested overnight using trypsin (Sigma-Aldrich) as described elsewhere (25). The resulting peptides were eluted, concentrated by vacuum centrifugation and thereafter separated by RP nano-LC (LC1100 series, Agilent Technologies, Palo Alto, CA, USA; column: Zorbax 300SB-C18, 3.5 µm, 150 × 0.075 mm; eluate: 0.1% formic acid, 0–60% ACN). The peptides were identified by on-line MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies) (22). Thereafter, a database search was conducted using the MS/MS ion search (MASCOT, <http://www.matrixscience.com>) against all fungal entries of NCBI nr (GenBank) with subsequent parameters: trypsin digestion, up to one missed cleavage, fixed modifications: carbamidomethyl (C) and with the following variable modifications: oxidation (M), peptide tol.: ±1.2 Da, MS/MS tol.: ±0.6 Da and peptide charge: +1, +2 and +3.

Results

Screening of sera in 1D blots underlined the importance of allergens from *A. versicolor*

The collected 97 sera were tested for IgE specific immunoreactivity on 1D blots of *A. versicolor*, *A. fumigatus* and *P. expansum*. A representative collection is shown in Fig. 1. The serum from patient EF-011-01 is an example

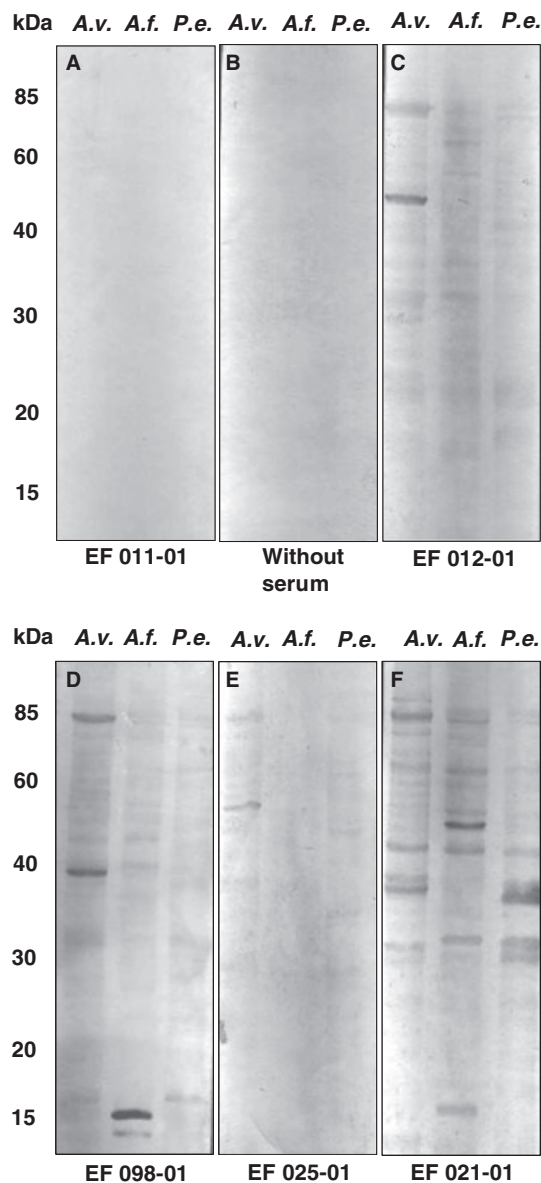


Figure 1. Screening of IgE specific immune reactivity of patients' sera towards proteins extracted from *A. versicolor* (*A.v.*), *A. fumigatus* (*A.f.*) and *P. expansum* (*P.e.*) in 1D blot analysis. In each lane 50 µg protein of spore extracts was applied and blotted on nitrocellulose membrane after 1D SDS-PAGE. The blots were developed by an anti-IgE antibody conjugated with alkaline phosphatase. In an immune negative serum (A) and in a control without primary antibodies (B), no signals were detected.

of a nonimmune reactive serum, although the patient suffered from indifferent allergic symptoms (Fig. 1A). As control, the secondary antibody was tested for unspecific background signals without any sera providing the primary antibody and this experiment yielded no signal at all (Fig. 1B). Therefore, the immune reactivity of the other sera were validated and exhibited variable, patient dependent patterns of immunoreactivity in 1D blots. The

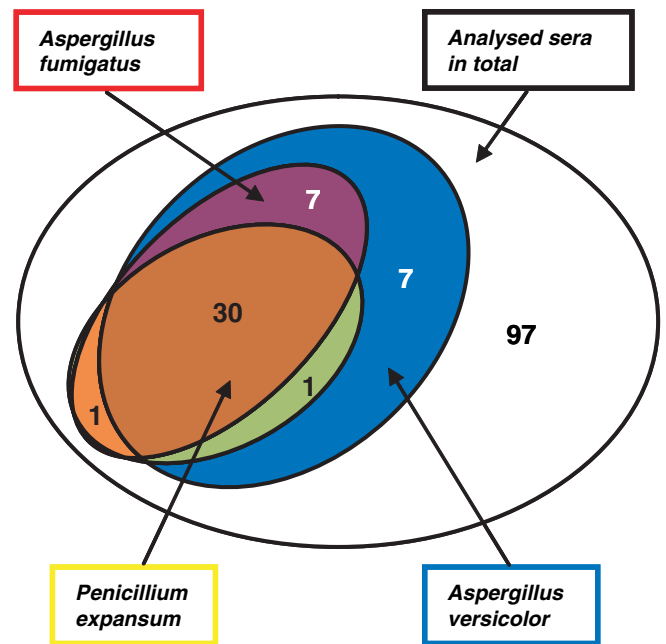


Figure 2. Immune reactivity towards the different fungi species. The outer circle represents the total amount of 97 sera; the 46 immune positive sera are displayed colour coded. A majority of 30 sera were tested positively for extracts from *A.f.*, *P.e.* and *A.f.* (30, orange), seven reacted only with *A.f.* and *A.v.* (purple), one with *P.e.* and seven exclusively with proteins from *A. versicolor* (blue).

serum of the patient EF-012-01 showed significant signals only for proteins from *A. versicolor* and here in particular for one protein at about 55 kDa as well as for one at about 85 kDa (Fig. 1C). The serum of patient EF-098-01 (Fig. 1D) yielded strong signals for proteins from *A. versicolor* (40 and 85 kDa) and for one protein from *A. fumigatus* of about 17 kDa. The other sera displayed either weak but species-specific signals (EF-025-01, Fig. 1E) or stronger specific signals although lacking species specificity (EF-021-01, Fig. 1F) based on the assumption that homologues proteins might display highly similar molecular weights. Interestingly, the minority of detected bands matched among the three species, leading to the assumption that different proteins were detected. The results of the 1D blot screening are summarized in Fig. 2. Of the 97 tested sera, 46 were found to react positively with at least one of the protein extracts from *A. versicolor*, *A. fumigatus* or *P. expansum*. The largest group, 30 patients, showed immune reactivity against extracts from all three fungi, although the reactivity could be directed towards different proteins. The extracts of *A. versicolor* were detected with the highest specificity. In detail, seven sera exclusively detected proteins from *A. versicolor*, whereas none of the sera detected exclusively *A. fumigatus* proteins. This fact points to an original sensitization rather by *A. versicolor* allergens than by proteins from *A. fumigatus*.

Resolution of allergens in 2D blots refines analysis

From 97 sera tested in 1D blots, we selected 18 immune reactive and two immune-negative controls for IgE-based immune detection of protein extracts from *A. versicolor*, which were previously separated by 2D gel electrophoresis. The results from the identical sera, as in Fig. 1, are displayed in Fig. 3. The IgE antibodies in serum EF-025-01 detected five groups of spots (Fig. 3C). The strongest signal occurred at acidic pH and at a molecular weight of about 55 kDa, which fits to the measurement in 1D blots (Fig. 1C). In addition, the higher resolution of 2D electrophoresis unravels additional signals. The observed grouping effect leads to the assumption that these groups

are either formed by isoforms of proteins or by post-translational modifications. The serum from patient EF-012-01 (Fig. 3A) detected two groups of spots, which matched with the groups 1 and 2 detected by serum from patient EF-025-01. The serum EF-098-01 showed a strong reaction with a protein at about 90 kDa (group 3) and one at about 35 kDa at basic pH (group 2), which matches with group 2 detected by sera EF-012-01 and EF-025-01 respectively. The IgE antibodies in sera EF-021-01 (Fig. 3D) reacted with only one group of spots that was also detected by the other sera.

Identification of allergens

For identification of the allergens, we separated 2 mg of an extract of *A. versicolor* spores on a preparative large 2D gel (Fig. 4). From this master gel, the spots corresponding to the detected allergens were cut and identified by MS. Afterwards, the spots were ordered in groups according to the location in the 2D gels and to their identities. The allergens were numbered referring to the code of the allergome data base (26) with a three letter abbreviation of the genus, and a single letter for the

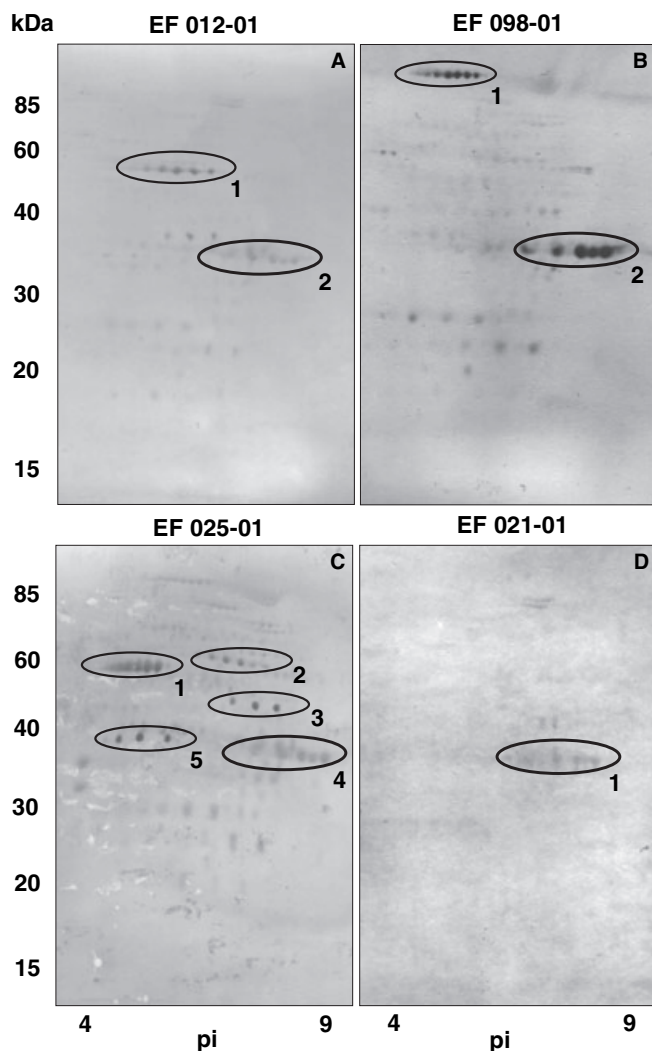


Figure 3. 2D blots with extracts from *Aspergillus versicolor* (*A.v.*), *A. fumigatus* (*A. f.*) and *P. expansum* (*P. e.*). 500 µg of protein was applied to 2D gel electrophoresis and was blotted onto nitrocellulose membranes. After incubation with sera from the patients, also shown in Fig. 1, the blots were developed with an anti-IgE antibody conjugated with alkaline phosphatase.

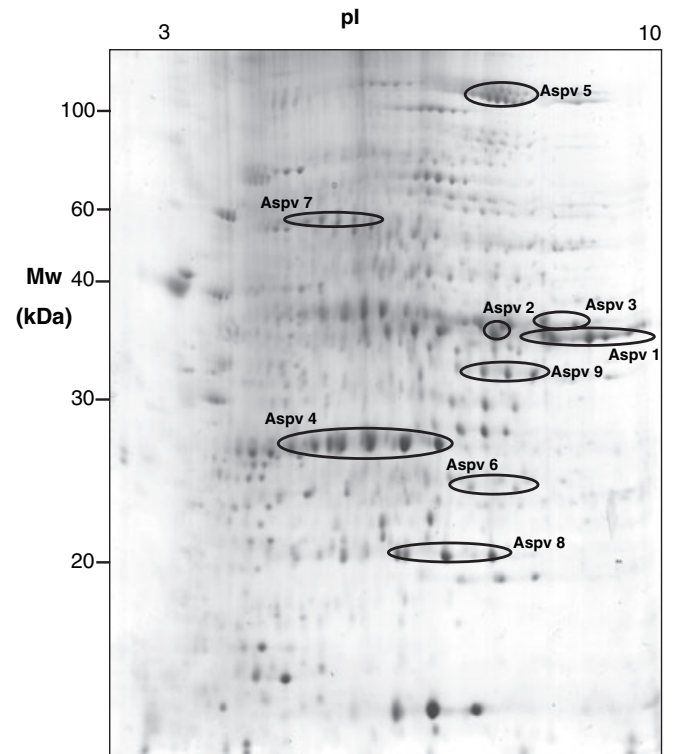


Figure 4. Preparative gel of spore extract from *Aspergillus versicolor*. 2 mg of protein from *A. versicolor* spore extract were applied to a large (18 cm) strip for the first dimension and a large gel for SDS-PAGE in the second dimension and the total protein was stained by Coomassie. All identified allergens are encircled, as we found protein identities most often in groups. The seven major allergens are encircled with bold lines.

Table 1. Identification of allergens from *Aspergillus versicolor*. The identified proteins are listed with the spot number taken from Fig. 4, the cross-species identified proteins, NCBI accession number, the MS/MS – Mowse Score and the number of peptides used in the Mowse search

Spot	Protein	Accession number	MS/MS-Mowse Score	Number of peptides
Asp v 1	Glyceraldehyde-3-phosphate dehydrogenase (<i>Aspergillus nidulans</i> FGSC A4)	gil120681	226	5
Asp v 2	Glyceraldehyde-3-phosphate dehydrogenase (<i>Aspergillus nidulans</i> FGSC A4)	gil120681	108	3
Asp v 3	Glyceraldehyde-3-phosphate dehydrogenase (<i>Aspergillus nidulans</i> FGSC A4)	gil120681	59	2
Asp v 4	Unnamed protein product (<i>Aspergillus oryzae</i>) 93% homology to sorbitol/xylulose reductase, putative (<i>Aspergillus clavatus</i> NPJIL 1)	gil83770356 gil121716507	250	6
Asp v 5	Catalase A (<i>Aspergillus nidulans</i> FGSC A4)	gil67903300	141	5
Asp v 6	Hypothetical protein AN69 18.2 (<i>Aspergillus nidulans</i> FGSC A4)	gil67541496	84	2
Asp v 7	Enolase (<i>Aspergillus nidulans</i> FGSC A4)	gil67539152	367	5
Asp v 8	Hypothetical protein AN0297.2 (<i>Aspergillus nidulans</i> FGSC A4)	gil67516031	114	2
Asp v 9	Hypothetical protein AN67 17.2 (<i>Aspergillus nidulans</i> FGSC A4) 91% homology to malate dehydrogenase, mitochondrial precursor (<i>Aspergillus terreus</i> NIH2624)	gil67541094 gil119141202	184	4

species followed by an increasing numbering. We performed a semi-quantitative scoring on the basis of both the frequency of detection of a certain group of spots and the intensity of the immune staining. The seven utmost highest ranked allergens (Asp v 1–9) are summarized in Table 1. The most important allergen, Asp v 1–3 was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and was found in 17 out of 18 immune-positive patients. As *A. versicolor* is not sequenced, all identifications were achieved by cross-species similarity, in this study, most often to *A. nidulans* sequences. The theoretical mass and pI matched well with the experimental data. The second most frequently found allergen (Asp v 4), was found to be an unnamed protein with homology to a sorbitol/xylose reductase. This allergen was found in 14 cases, accounting for 78%. The other allergens were identified as catalase A (Asp v 5), protein AN6918.2 (Asp v 6), enolase (Asp v 7), protein AN0297.2 (Asp v 8) and as malate dehydrogenase (Asp v 9) (Fig. 5).

Discussion

Correlations between medical status, mould concentrations and sensitization

In our cohort, some patients suffered from allergic reactions and several of them were tested positive to be sensitized against moulds. With currently available diagnostic tools, the specific IgEs to various moulds were detected. Positive reactions (RAST–CAP–class ≥1, IgE >0.35 kU/l, Pharmacia) were observed for eight *P. chrysogenum*, five *C. herbarum*, nine *A. fumigatus* and 10 *A. tenuis* patients.

In 1D immunoblots, sera from seven patients showed the presence of IgE antibodies recognizing exclusively spore extracts from *A. versicolor*. No significant correlations of these results with positive sensitizations to moulds, which were diagnosed within the clinical inves-

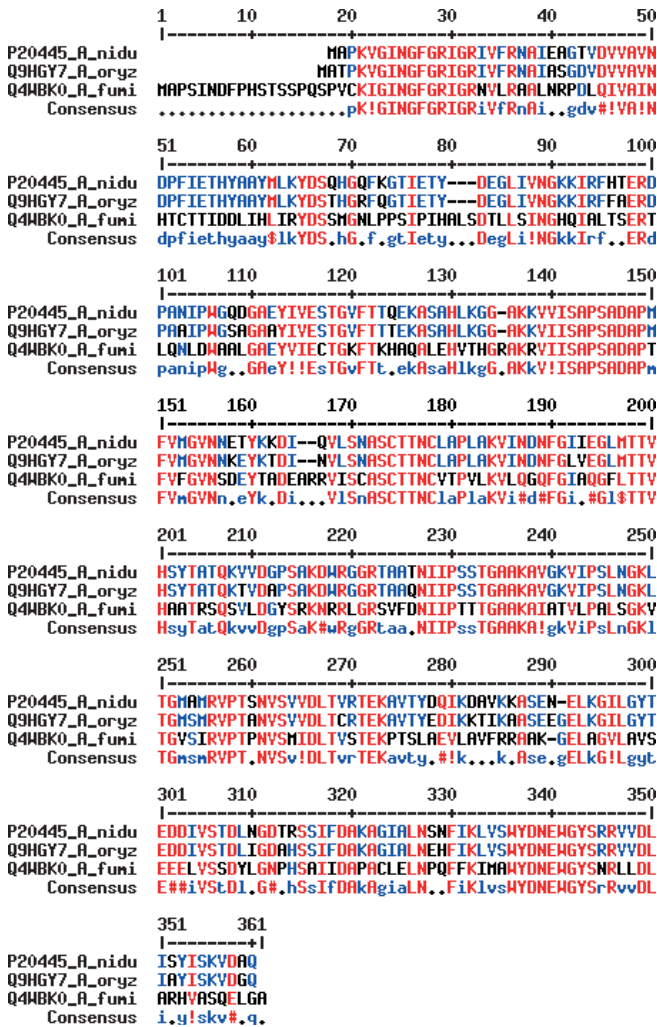


Figure 5. Alignment of GAPDH. The sequences of GAPDH from *Aspergillus fumigatus*, *A. nidulans* and *A. oryzae* were aligned by the software MULTALIGN, which highlights identical amino acids in red and partial matches in blue.

tigations, were observed. There was no correlation between recognition of the extracted allergens from spores of *A. fumigatus* and an increased level of its specific IgE. It can be assumed that in our assay other proteins from *A. fumigatus* were detected because we analysed spore proteins, whereas the major allergen Asp f 1 is found to be more abundant in hyphae than in spores (15) and therefore can be used as marker for aspergillosis.

Within this study, also the concentration of mould spores was determined. In 55% of the investigated households, exposure to mould was detected either as increased spore concentration in indoor air [which was assumed, if the difference between indoor and outdoor concentration (CFU/m³) exceeded a threshold of 100 CFU/m³ of each genera (2)], or as visible mould affection. More importantly, visible mould of *A. versicolor* as well as of *Penicillium* species was associated significantly with a positive reaction of sera to *A. versicolor* and *P. expansum*. Additionally, patients from households with water damages in the past have shown a significant association with a sensitization to *A. versicolor* ($p < 0.05$, Mann–Whitney *U*-test, STATISTICA 7.1). As water damage is very often associated with an exposure to *A. versicolor*, a significant risk to be sensitized to this mould was detected (OR: 4.08; CI: 1.17–14.2; $p = 0.025$). A significant correlation of an increased spore concentration with a sensitization to the investigated spore allergens was not observed. It seems that the actual burden of spores reflects more the actual state, but that for sensitization a prolonged or repeated exposure is necessary.

Finally, we could not find a correlation between the presence of allergens of *A. fumigatus*, neither with spore concentration, nor with water damages, or other living conditions. Spores from *A. fumigatus* occurred rather rarely, which is in concordance with other studies (14, 27).

Major allergens for use in diagnosis

Most known allergens are summarized in the allergome data base (26). This data base does not contain any entries

from *A. aspergillus* so far. Hence, we searched for homologues of the identified proteins in the AllFam database, in which the allergens are ordered by protein families. Within the large family of oxido-reductases, several allergens, also from fungi, are listed, but none of them showed a significant sequence homology with the dehydrogenases found in this study. In contrast, the enolases are highly conserved and the protein identified as allergen here has a significant homology to those 10 enolases, which are well known allergens. An intermediate homology was found for the malate dehydrogenase with an allergen (Mal f 4) found in *Malassezia furfur* (28; 63% identity and 74% homology). A similar degree of homology (49% identity and 64% homology) was found for the herein identified catalase with the reported allergen Pen c 30 from *Penicillium citrinum*. Taken together, especially the GAPDH from *A. versicolor* has the potential for species specific diagnosis, whereas possible cross reactions of IgE against other allergens have to be analysed with protein extracts from a greater variety of fungi.

Another common feature of the identified major allergens is their cytoplasmatic localization. None of them is known to be membrane-associated or even described to contain an extracellular domain, so the sensitization requires an uptake and degradation of the spore within the organism to get into contact with the intracellular allergens.

Major allergens are often high abundant proteins. This is also true of the most frequently detected allergen in *A. versicolor*, because GAPDH is one of the most abundant proteins in protein extracts from *A. versicolor*, as judged by the Coomassie stained gel (Asp 1–3, Fig. 4).

Furthermore, the appearance of nearly all allergens in groups in which the spots are strung like pearls points to modifications that affect the pI but not the mass of the proteins. Therefore, further studies evaluating the impact of modifications on allergenicity as well as confirming allergenicity of newly detected allergens of *A. versicolor* using highly purified or recombinant proteins are necessary.

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