

Levels of the fungal allergen Asp f 1 in dust from two sawmills in Croatia: a pilot study

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Abstract Asp f 1 (ribotoxin) is the main allergen of *Aspergillus fumigatus* and a critical factor in provoking allergic responses and bronchopulmonary aspergillosis. This study investigated the prevalence of allergen Asp f 1 in dust samples collected at two Croatian sawmills from different working sites (sawmilling, parquetry and sorting). A total of thirty-five floor dust samples were collected, extracted, and the mass fraction of Asp f 1 was determined using a commercially available enzyme-linked immunosorbent assay. More than 91 % of the collected dust samples had detectable levels of Asp f 1 (limit detection 3.6 ng g^{-1}). The median Asp f 1 mass fractions in Sawmill 1 and Sawmill 2 were 49.4 ng g^{-1} (range $<3.6\text{--}120 \text{ ng g}^{-1}$) and 35.5 ng g^{-1} (range $15.1\text{--}77.2 \text{ ng g}^{-1}$), respectively. At both sawmills, higher median Asp f 1 values were found in the dust from sawmilling than from parquetry sites. These preliminary findings suggest the abundance of allergen Asp f 1 in reservoir dust in the woodworking environments of both sawmills. Assessment of Asp f 1 in future studies will provide further insight into the role of this allergen in eliciting respiratory morbidity.

Keywords Aeroallergen · Mould · Sawmill · Industrial hygiene · Occupational exposure · Wood dust

1 Introduction

Approximately 3.6 million EU workers are employed in the wood industry (Kauppinen et al. 2006) and over 40,000 in Croatia (Puntarić et al. 2005). The wood industry is an environment with a high production of bioaerosol, which may present a potential risk of occupational respiratory diseases (Halpin et al. 1994a; Rusca et al. 2008; Park et al. 2010). Stored timber, spruce log, sand dust and defoliated barks are periodically colonized with moulds. Thus, the environments of woodworking sites are usually loaded with moulds at much higher levels than other indoor environments (Eduard 2009). Exposure to fungal contaminants in wood-related facilities may vary geographically. The most dominant mould genera in wood industry worldwide are *Aspergillus* (Dutkiewicz et al. 2001; Dillon et al. 2007), *Penicillium* (Halpin et al. 1994b; Opplinger et al. 2005; Verma et al. 2010) and *Cladosporium* (Verma et al. 2010; Nayar and Jothish 2013), all of which have been associated with respiratory morbidity. Also several studies identified *Rhizopus* and *Paecilomyces* as important aeroallergen and occupational hazard in sawmill operations, particularly in Norway (Eduard et al. 1992, 1993). These

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fungus contaminants have also been detected in air samples collected from Croatian sawmills (Šegvić Klarić et al. 2012). Several studies have reported that *A. fumigatus* was an important factor in occupational morbidity such as hypersensitivity pneumonitis (Singh and Singh 1999; Veillette et al. 2006). IUIS allergen nomenclature committee has approved 23 major and minor allergens from *A. fumigatus* as being responsible for allergy symptoms (<http://www.allergome.com>). Among them, Asp f 1 (18-kDa protein, ribotoxin) was the major virulence factor selectively expressed in *A. fumigatus* (Arruda et al. 1992; Kurup and Banerjee 2000; Rementeria et al. 2005; Bowyer et al. 2006; Abad et al. 2010). The germinating *A. fumigatus* conidia increase the expression and production of the Asp f 1 allergen in both spores and hyphae, suggesting that viable particles are more allergenic than non-viable ones (Sporik et al. 1993; Green et al. 2006; Cabral 2010). Therefore, the colonization of *A. fumigatus* on wood products may also affect environmental Asp f 1 levels and present an occupational inhalation biohazard to susceptible workers. The growth of fungal communities on specific surfaces depends on the available moisture, temperature, substrate and other environmental factors (Górny 2004; Piecková 2012). The conidia of *A. fumigatus* are of small dimensions (typically from 2 to 3 µm, aerodynamic diameter), which is why they remain airborne and respirable for extended periods of time (from several days to few weeks) and are potent sensitizers (Kildesø et al. 2003; Cho et al. 2005; Kanaani et al. 2009). Spores and fragments can be transported to the lower airways and lungs and trigger allergic reactions (Górny 2004).

Recently, mould allergens and antigens have been analysed in various indoor environments (Prester 2011; Zahradnik et al. 2013). However, where the detection of specific mould allergens is concerned, sensitive enzyme immunoassays (ELISA) that use monoclonal or polyclonal antibodies are available for only a limited number of moulds, such as *A. fumigatus* (Asp f 1) and *A. versicolor* (Asp v 13) (Sporik et al. 1993; Shi et al. 2011; Zahradnik et al. 2013). This is due to the serious cross-reactivity with related and non-related moulds that limits the use of immunoassays in the detection of specific mould antigens in indoor environments (Schmechel et al. 2006; Bowyer et al. 2006; Cramer et al. 2008; Sander et al. 2012). Recent data have demonstrated that Alt a 1 a major

allergen of *Alternaria alternata* shows a high level of cross-reactivity with the Pleosporaceae family (*Ulocladium botrytis*, *Curvularia lunata* and *Stemphylium botryosum*) (Sáenz de Santamaría et al. 2006; Postigo et al. 2011). The detection of specific mould allergens, particularly in environments that generate high bio-aerosol emission, could give further information about the colonization of particular moulds in indoor environments and indicate the causative agent for certain symptoms and diseases (Feo Brito et al. 2012).

Only several studies have measured mould allergens in occupational settings. Most of the published studies focused on traditional methods for the assessment of exposure to moulds. However, the allergen Asp f 1 has been suggested as the most suitable marker for assessing the *A. fumigatus* exposure in wood facilities (Dillon et al. 2007). To our knowledge, there is no information available on Asp f 1 levels in reservoir dust from woodworking facilities. The aim of the present study was to determine the dust-borne Asp f 1 mass fraction at different woodworking sites (sawmilling, parquetry and sorting) at two Croatian sawmills. This investigation represents the continuation of our previous study aimed at determining the health effects of biopollutant exposure on the health of sawmill workers from two Croatian sawmills.

2 Materials and methods

2.1 Sampling

Two sawmills at geographically separate wood-processing plants located in the rural area of the Zagreb County (Croatia) were chosen for this study. Sawmill 1 primarily processed beech hardwood (*Fagus sylvatica* L.), oak (*Quercus Robur* L.) and fir softwood (*Abies Alba*), whereas Sawmill 2 mainly processed beech hardwood, oak and ash (*Fraxinus angustifolia* Vahl.). The mean summer temperature at both sawmills during the sampling was 27.8 °C, while the mean relative humidity was 51.2 %. At the work posts examined, no clearly visible signs of mould contamination were found.

2.2 Dust collection and extraction

Thirty-five bulk dust samples were collected from two sawmills after typical everyday settings during the

summer of 2007 (Sawmill 1) and the summer of 2008 (Sawmill 2). All of the dust samples were collected with a broom, placed in sterile plastic bags and stored at -20°C (within 8 h after collection) until extraction. At Sawmill 1, floor dust samples ($n = 15$) were collected from the floor during different operations (sawmilling, parquetry and sorting of logs). At Sawmill 2, dust samples ($n = 20$) were collected during sawmilling and parquet manufacturing. Before extraction, each sample was manually sieved (particle diameter $<300\text{-}\mu\text{m}$) and weighted. Aliquots (100 mg) of the fine dust were extracted with 2 mL of phosphate-buffer saline containing 0.05 % Tween-20 (PBS-T), as described in a previous paper (Prester et al. 2010). Dust extracts were stored at -20°C until analysis for allergen content.

2.3 Asp f 1 ELISA

The concentration of allergen Asp f 1 was determined using capture ELISA with a commercial kit (Indoor Biotechnologies Ltd, Cardiff, UK) (Prester et al. 2010). The purified mAb 4A6 (mouse anti-Asp f 1, IgG1) (Product code: MA-4A6; lot no. 2764) was used as the capture antibody ($1\text{ }\mu\text{g mL}^{-1}$ carbonate-bicarbonate buffer, pH 9.6). Rabbit polyclonal anti-Asp f 1 (IgG) raised against partially purified Asp f 1 ($1\text{ }\mu\text{g mL}^{-1}$ of PBS-T) (Product code: PA-AF1; lot no. 2765) was used as the secondary antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG ($1\text{ }\mu\text{g mL}^{-1}$ of PBS-T) (BioSource, Camarillo, CA, USA) was used for detection. As a reference standard, a purified natural Asp f 1 extract was used ($400\text{ ng Asp f 1 per 1 mL}$) (Product code: ST-AF1; lot no. 2521). Twofold serially diluted standards (concentrations ranged from 6.4 to 0.1 ng mL^{-1}) were used to construct a standard curve. All of the antibodies (except capture mAb), standards, dust extracts, positive and negative controls were diluted in PBS-T containing 1 % bovine serum albumin (BSA) (Sigma) (PBS-T-BSA). Assay background controls were processed in parallel, but they contained PBS-T instead of antibodies. All details on Asp f 1 ELISA analyses have been previously published (Prester et al. 2010). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with capture mAb ($100\text{ }\mu\text{L/well}$) overnight at room temperature. Following this and each subsequent incubation steps (at room temperature), the plates were aspirated and

washed (three times with PBS-T). The plates were blocked with PBS-T-BSA for 30 min. Diluted dust extracts (three to sixfold), serially diluted standards, controls and background controls ($100\text{ }\mu\text{L/well}$) were incubated for 1 h. The secondary polyclonal antibody ($100\text{ }\mu\text{L/well}$) was added to each well and incubated for 1 h. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG ($100\text{ }\mu\text{L/well}$) after 1 h of incubation. The colour was developed with tetramethylbenzidine (TMB substrate reagent set) by incubation for 10–15 min. The reaction was quenched by the addition of sulphuric acid, and the absorbance was read in an ELISA plate reader (Personal LAB, IASON, Graz, Austria) at 450 nm. The Asp f 1 concentrations were calculated using linear regression analysis and were expressed as the ng of Asp f 1 per g of sieved dust (ng g^{-1}). All of the samples were measured in triplicate. The limit of detection (LOD) for this assay was 3.6 ng g^{-1} (Prester et al. 2010).

2.4 Statistical analysis

Statistical analysis of data was performed using Stata/SE 11.2 for Windows (StataCorp LP, USA). The results are presented as median and range (minimum–maximum). Differences in Asp f 1 levels between the sawmills and between the woodworking locations (sawmilling and parquetry) at each sawmill were analysed by Mann–Whitney U test since the data were not normally distributed. The results were considered statistically significant at a value of $P < 0.05$.

3 Results and discussion

Table 1 shows Asp f 1 values in settled dust samples collected from two Croatian sawmills. In Sawmill 1 and Sawmill 2, Asp f 1 was detected in 80 and 100 % of dust samples, respectively. In Sawmill 1, which processes deciduous and coniferous wood, Asp f 1 values were higher in dust samples collected at sawmilling sites (median = 65.6 ng g^{-1} , range $<\text{LOD}$ – 120 ng g^{-1}) than at parquetry (median = 40.7 ng g^{-1} , range 21.1 – 118.5 ng g^{-1}) sites. Similarly, in Sawmill 2, which processed deciduous wood, Asp f 1 mass fraction in reservoir dust collected at sawmilling was higher than at parquetry (median = 39.2 ng g^{-1} , range 15.1 – 77.2 ng g^{-1} vs. median = 30.3 ng g^{-1} , range 15.1 – 44.8 ng g^{-1} , respectively). Average Asp f 1 mass

Table 1 Median and range of Asp f 1, the main allergen of *A. fumigatus*, in floor dust collected from different indoor locations at two Croatian sawmills

Asp f 1	Sawmill 1			Sawmill 2	
	Sawmilling	Parquetry	Sorting	Sawmilling	Parquetry
Median (ng g ⁻¹)	65.6	40.7	–	39.2*	30.3
Range (min–max)	<LOD–120	21.1–118.5	<LOD–109.2	15.5–77.2	15.1–44.8
Samples (<i>n</i>)	6	6	3	12	8
<i>n</i> > LOD (%)	5	6	1	12	8

LOD limit of detection

* Significant difference between sawmilling in Sawmill 1 and Sawmill 2 (Mann–Whitney *U* test, *z* = 1.97, *P* = 0.049)

fraction in samples collected near the sawmilling sites at Sawmill 1 was about 40 % higher than at Sawmill 2 (*z* = 1.97, *P* = 0.049). In contrast, the average concentrations of Asp f 1 in dust extracts from both parquetrys were not statistically significant. However, due to the small number of samples and wide range of Asp f 1 values found among the manufacturing groups, statistical power could not be achieved. Thus, these preliminary data prevent any firm conclusion about the significance of Asp f 1 differences in reservoir dust collected from different locations at both sawmills and may suggest cross-contamination between manufacturing groups in sawmill.

The main finding of this pilot study was that detectable levels of Asp f 1 were found in 91.4 % (32/35) of the dust samples collected in two sawmills in Croatia. At Sawmill 1, the Asp f 1 from sawmilling dust showed a large variation (almost 20-fold), which is in line with reported differences in bioaerosol exposure among working sites at sawmills (Opplinger et al. 2005; Verma et al. 2010). The Asp f 1 levels reported in this study cannot be explained by the outdoor–indoor transport of fungal spores due to a very low outdoor *Aspergillus* spore concentration in the Zagreb area (Šegvić Klarić and Pepeljnjak 2006). Other studies have also reported that the contribution of *Aspergillus* contamination from outdoor areas to indoor areas was usually negligible in highly contaminated environments (Eduard 2009). An earlier study conducted in Croatian sawmills identified the airborne *A. fumigatus*, particularly in colder periods (Šegvić Klarić et al. 2012). It is well known that *A. fumigatus* grows well on indoor organic substrates such as wood dust (where organic materials are abundant), particularly in humid environments (Singh and Singh 1999). The results of this study, however, may suggest that

the contamination of sawmills with *A. fumigatus* has generated reservoirs of its allergens associated with hyphae, fragments and possible spores. Since Asp f 1 participates in dust emission, these results could partially explain the elevated emission of respirable and total wood dust observed in various Croatian wood-processing facilities (Kos et al. 2004; Puntarić et al. 2005).

To our knowledge, Asp f 1 levels have not yet been studied in the wood industry, except for the study carried out by Dillon et al. (2007). Through comparison with several markers of mould exposure (glucans, ergosterol and quantitative polymerase chain reaction), this study suggested Asp f 1 as the most suitable marker for assessing the *A. fumigatus* exposure in wood facilities. The Asp f 1 levels from the previous and present study cannot be compared due to different sampling strategies. In contrast to Asp f 1, the main allergen from *Alternaria alternata* (Alt a 1) was not detected in the settled dust collected from a hardwood-processing sawmill in continental Croatia, thus suggesting the low colonization of this mould (Prester and Macan 2010). Internationally accepted exposure thresholds or guidelines for any indoor mould allergen are yet to be established.

Besides the sawmills, Asp f 1 levels were also determined in other agricultural and non-agricultural settings. The background levels of Asp f 1 in the household dust of a general US, UK and Croatia population were very low and typically under the limit of detection of the method (Sporik et al. 1993; Prester 2011). Out of 296 dust samples, Asp f 1 was below the LOD in 95 % of dust extracts (Sporik et al. 1993). Similarly, background Asp f 1 levels in air and settled dust samples collected from certain US office environments were also detected as very low (Ryan et al. 2001).

On the other hand, in poultry farms, Asp f 1 was detected in 62 % of dust samples (median 17.9 ng g⁻¹) (Prester et al. 2010). Based on these data, the concentrations of Asp f 1 in Croatian sawmills were about twice as high as in poultry farms and possibly much higher than in residential environments without water damage (Prester 2011). Furthermore, the high level of a mycotoxin-producing species such as *A. fumigatus*, which is commonly encountered in wood-related facilities, suggests that the mycotoxin concentration could be greater than the concentration of no toxicological concern (CoNTC) (Hardin et al. 2009).

The sampling and analytical methods employed to assess airborne moulds are varied and non-standardized (Martinez et al. 2004). Both settled dust and air sampling can be used to determine exposure to mould allergens in indoor environments. The reservoir dust sampled in the present study is a surrogate measure for bioaerosol exposure to moulds. Generally, floor dust sampling is used for assessing the cumulative and chronic exposure to moulds and may be particularly useful in epidemiological studies. In contrast, airborne fungal propagules have large temporal and spatial variability in indoor and outdoor environments and represent the actual mould load (Chew et al. 2003). Air sampling has previously been applied for the detection of Trip s 1, an obeche wood allergen (Kespohl et al. 2008), beta-1,3-glucans (Ronald et al. 2003; Seo et al. 2009) and mycotoxins (Brasel et al. 2005; Bloom et al. 2009) in mouldy indoor environments. Similarly as allergen Asp f 1, these compounds are associated with mould particles of various dimensions, including those that have long atmospheric residence times. At the present, the best measurement strategy for the detection of mould allergens may be a combination of both airborne and dustborne sampling methodologies, which may provide a wider range of data than a single sample strategy (Chew et al. 2003).

Typical wood-handling operations (sawing, shredding of waste wood, storing of chips, land-based log storage and clean-up jobs) generate elevated bioaerosol emission (Douwes et al. 2000; Ronald et al. 2003). Once inhaled, wood dust particles are deposited at different levels in the airways depending on their size (Reponen et al. 2001; Sercombe et al. 2006). Several studies have shown that mould particles with diameters below 2.4 µm (majority of *A. fumigatus* particles present in indoor environments), if inhaled, present the greatest danger to human health (Górny 2004). In

wood-processing environments, moulds are considered as a major harmful factor related to various symptoms (Mandryk et al. 1999; Rusca et al. 2008; Karvala et al. 2008; Jacobsen et al. 2009; Ljubičić Čalušić et al. 2012) and respiratory diseases such as extrinsic allergic alveolitis and occupational asthma (Halpin et al. 1994a; Veillette et al. 2006; Heikkilä et al. 2008). The inhalation of large amounts of moulds (*Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Rhizopus* sp. and *Alternaria* sp) has especially been associated with the appearance of the above-mentioned respiratory disorders among wood workers (Karvala et al. 2008). In addition, mould allergens have been suggested as the major factors contributing to the pro-inflammatory potency of microbial exposure rather than the fungal spores alone (Roponen et al. 2002; Kespohl et al. 2008). Furthermore, ambient air biocontaminants (glucans and endotoxins) and air pollutants (terpenes, tobacco smoking and anthropogenic gases, particularly CO₂) may interact with various aeroallergens and increase their allergenicity, which in turn will exacerbate symptoms in sensitized individuals (Wolf et al. 2010; Cakmak et al. 2012; Mazitova et al. 2012). Thus, the exposure–response relationships are complex depending on occupational allergens, co-exposure with other contaminants and genetic variations in occupants (Jones 2008; Ji and Khurana Hershey 2012).

To conclude, despite the small sample size of this study, these findings have shown that Asp f 1 allergen has been accumulated in the work environment during sawing and parquetry and that occupational exposure to this allergen calls for monitoring. The Asp f 1 ELISA may provide complementary information regarding the contamination and exposure to *A. fumigatus*. Further research should focus on determining mould allergens in both air and settled dust samples together with viable and non-viable analyses. The role of Asp f 1 in the aetiology of wood-related respiratory diseases should be evaluated in subsequent studies. The results of the present study stress the importance of monitoring of biopollutants, particularly allergenic moulds in wood industry in order to ensure a satisfactory level of industrial hygiene and safe environment for workers.

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