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Verruculogen production in airborne and clinical isolates of *Aspergillus fumigatus* Fres.*

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Among airborne aspergilli sampled in outdoor air of the Zagreb area (2002/2003), *Aspergillus niger* (v. Teigh.) and *A. fumigatus* (Fres.) were the most abundant species (20–30%), with low mean annual concentrations (0.21–1.04 CFU m^{-3}). Higher concentrations of *A. fumigatus* were observed in autumn and winter (0.5–1.05 CFU m^{-3}) than in spring and summer (0–0.4 CFU m^{-3}). On the other hand, *A. fumigatus* was found to be the most frequent isolate from upper and/or lower respiratory tracts of imunocompromised patients in many studies. This species produces several mycotoxins, including the tremorgenic mycotoxin verruculogen that can be found in spores and during myceliar growth. Verruculogen production ability was tested on 30 airborne and 33 clinical isolates of *A. fumigatus*. In both groups, high percentage of verruculogen-producing strains was noticed (84% of airborne and 91% of clinical isolates). Verruculogen production was not significantly different in the groups of airborne isolates (0.34 ± 0.16 mg mL^{-1}), and clinical isolates (0.26 ± 0.19 mg mL^{-1}).

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The qualitative and quantitative composition of outdoor and indoor fungal aerosol depends on numerous factors, *e.g.* suitable growth substrate, biorhythms of the species, biotic and abiotic factors that influence the release, dispersion, and deposition of fungal propagules (1). Members of *Cladosporium*, *Alternaria*, *Penicillium* and *Aspergillus* genera were permanently present in many aeromycological surveys of outdoor and indoor environments. In general, in temperate climate, including the Zagreb area, *Cladosporium* (up to 80%) and *Alternaria* (up to 60%) are the most abundant fungal entities with peaks in late spring and summer, while *Penicillium* (up to 70%) and *Aspergillus* (up to 40%) are more frequent in autumn and winter (1–3). These fungi are sometimes associated with several respiratory diseases such as chronic bronchitis, asthma, hypersensitive pneumo-

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nitis and aspergillosis (4). Apart from the allergological potential, fungi are able to produce mycotoxins that can be found in both living and dead spores or airborne mycelial fragments (5). Among airborne aspergilli, *A. fumigatus* (Fres.) belongs to the most frequent airborne species (up to 30%). This species is often responsible for allergic bronchopulmonary aspergillosis (ABPA) in atopic patients, and invasive aspergillosis in immunocompromised patients (4, 6). Incidence of nosocomial aspergillosis in Croatia is 0–37% with mortality rate 40–95%. The rate of microbial isolates with *Aspergillus* (mostly *A. fumigatus*) was found to be 0.1/1000 hospital days in 1993 and increased to 5.31 in 2001 (7). Besides, *A. fumigatus* is capable of producing several mycotoxins including gliotoxin, fumigatin, fumigallin, trypacidin, monomethylsulochrin, triptoquivalins, triptoquivalon, fumitremorgens, fumigaclavines, pseurotin, anthraquinone, TR-2, verruculogen, and others (8).

Verruculogen is a low-molecular-weight secondary metabolite of various mould species of genera *Penicillium* and *Aspergillus*. It is a member of indole alkaloids group of mycotoxins that induce tremor in mice, rats and farm animals due to neurotoxic properties; these mycotoxins are called tremorgens (9). Several mechanisms of tremorgenic action have been proposed, such as reduction of the concentration of gamma-aminobutyric acid, an inhibitory neurotransmitter in the brain of experimental dogs (10). In the study of Peterson *et al.* (11), verruculogen-induced tremor in rats (treated intraperitoneally) showed an increased level of excitatory neurotransmitters, glutamate and aspartate in the lateral ventricle, suggesting subcortical action of verruculogen-induced tremorgenic activity. Verruculogen has an intraperitoneal ED_{50} of 390 μg kg^{-1} in mice (12).

Verruculogen is produced during myceliar growth of fungi, but it is also present in the spores of *A. fumigatus* strains. The effect of this mycotoxin upon health, especially of immunocompromised patients, is still unknown, as well as its influence on healthy population. Potential impact of inhalation of *A. fumigatus* spores with verruculogen and other toxins, and their role in the development of mycoses are not known.

The purpose of our study was to determine the frequency and seasonal variations of the concentration of airborne *A. fumigatus* at different locations in Zagreb, the capital of Croatia, and to test and compare verruculogen production ability in airborne and clinical isolates of *A. fumigatus*.

EXPERIMENTAL

Reagents and solvents

Verruculogen standard, streptomycin-sulphate, penicillin and chloramphenicol were purchased from Sigma (USA), and chloroform, ethanol (96% *V/V*), ethyl acetate, toluene, formic acid, Na_2SO_4, $AgNO_3$, MgSO4 x 7 H_2O, $NaNO_3$, KH_2PO_4, KCl and $FeSO_4$, were purchased from Kemika (Croatia). Sabouraud-glucose agar and yeast extract were purchased from Fluka (Germany). Thin-layer chromatography plates (silica gel with fluorescence indicator UV_{254}, dimension 20 x 20 cm, thickness 0.25 mm) were purchased from Macherey-Nagel (Germany).

All reagents were of analytical grade.

Airborne Aspergillus spp. *sampling*

Sampling of airspora was conducted at three locations in the Zagreb area: centre of the city (C), Pharmaceutical Botanical Garden »Fran Kušan« (BG) and mountain Medvednica (M). The first sampling site C is characterized by traffic and streetcars as public transportation. The second location BG is a residential area with less traffic. The Pharmaceutical Botanical Garden (2.4 ha) contains continental and Mediterranean medicinal plant species. The third station M is a picnic area with the mountain house »Puntijarka« at the altitude of 957 meters. Samples were collected during autumn, winter, spring and summer between October 2002 and September 2003. Seventy-two samples per location were obtained for each season (total of 288 samples per location). Fungi were sampled using an Air-sampler Mas 100 Eco (Merck, Germany) (hole-to-agar impactor), with 400 holes, and Sabouraud-glucose agar plates (9 cm diameter) with antibiotics streptomycin/penicillin (6:4). Airflow rate was 100 L min^{-1}. The sampling height was 0.7–1.2 m and the sampling time was 2 min. After field sampling, the plates were incubated at 25 ± 2 ºC for 5 days, and the fungal colony forming units per cubic meter (CFU m^{-3}) were counted. Fellers correction was not applied. *Aspergillus* species were identified on the basis of their macro- and microscopic characteristics after subculturing on Czapek agar (composition: $MgSO_4$ x 7 H_2O 0.5 g, $FeSO_4$ 0.01 g, KCl 0.5 g, KH_2PO_4 1 g, $NaNO_3$ 3 g, sucrose 30 g, agar 15 g, pH 7.3 ± 0.2) according to a key (13).

Clinical isolates of Aspergillus fumigatus

Clinical isolates of *A. fumigatus* were isolated with swabs from the sputum, nose, aspirate and trachea of immunosuppressive patients (with probable or proved aspergillosis) from the haematological unit (University Hospital Zagreb) with underlying myeloic or lymphatic leukaemia, Hodgkin's and non-Hodgkin's diseases. Swabs were cultivated on Sabouraud-glucose agar with chloramphenicol (50 mg L^{-1}) for five days at 37 °C. *A. fumigatus* was identified on the basis of macro- and microscopic characteristics after subculture on Czapek agar according to a key (13). The strains of *A. fumigatus* used were randomly collected during a one-year period.

Biosynthesis, extraction and detection of verruculogen

Verruculogen production capacity of airborne (n = 30) and clinical (n = 33) isolates of *A. fumigatus* was examined *in vitro* in yeast-extract sucrose broth (YES) (composition: yeast extract 3%, sucrose 4%, distilled water 1000 g, pH 5.8 ± 0.2). The one milliliter suspensions of conidia (10^6 mL^{-1}) of *A. fumigatus* strains were inoculated into 50 mL of YES and incubated at 30 ± 2 °C for 6 days with daily shaking. After 6 days, the biomass was homogenized with 25 mL of chloroform in an electric homogenizer (3500 rpm). Filtrate was extracted with 25 mL chloroform and filtrated through anhydrous Na_2SO_4. Extracts were evaporated to dryness under vacuum at 60 °C. Dried extracts were dissolved in 0.5 mL chloroform prior to TLC analysis.

Detection and semiquantification of verruculogen was performed using the method of Land *et al.* (14) on TLC plates in the mobile phase toluene/ethyl acetate/formic acid (5:4:1, *V/V/V*). Verruculogen detection was performed under UV light (366 nm), after

spraying the developed plates with freshly prepared $AlCl_3$ (20% *w/V* in 50% *V/V* ethanol) and heating them for 10 min at 110 °C. Verruculogen appeared as a bluish green spot at R_f 0.5 (Fig. 1). Concentration of verruculogen from biosynthesis was determined by comparing the fluorescence intensity of the verruculogen standard solution spot (1 mg mL^{-1} in chloroform) and chloroformic extracts.

Statistics

The data obtained as CFU m^{-3} were statistically analyzed by a one-way analysis of variance (ANOVA), followed by a multiple comparison procedure (Bonferroni test). Verruculogen production capacity (mg mL^{-1}) of airborne and clinical isolates of *A. fumigatus* was tested by the Wilcoxon matched pairs test. The level of $p < 0.05$ was considered statistically significant for all the tests performed.

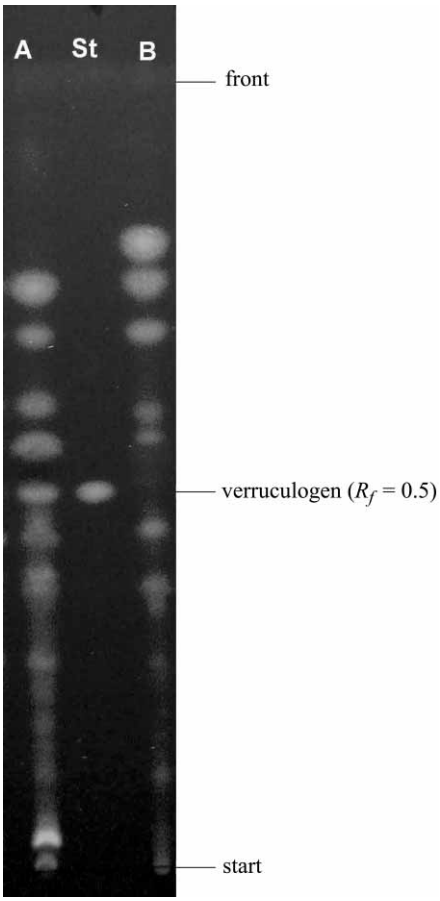

Fig. 1. TLC plate of *A. fumigataus* extracts under UV 366 nm (A – verruculogen-producing strain, B – non-producing strain, St – standard solution of verruculogen).

RESULTS AND DISCUSSION

Airborne aspergilli are listed in Table I in the descending order based on their mean annual concentrations. The frequency of *Aspergillus* species is calculated as the percentage of occurrence in the total of aspergilli positive samples. Seven *Aspergillus* species were permanently present in samples with low mean annual concentrations (0.16–1.04 CFU m^{-3}). Among airborne aspergilli, *A. niger* and *A. fumigatus* were the most abundant (20–30%), which is in agreement with previous reports from Italy (1, 2). Other aspergilli, including *A. flavus*, *A. glaucus*, *A. versicolor*, *A. ochraceus* and *A. nidulans*, have lower frequencies (1–13%) and mean annual concentrations (0.16–0.40 CFU m^{-3}). Cvetnić and Pepeljnjak (15) also observed a similar range of incidence (3–15%) of the mentioned airborne aspergilla from continental parts of Croatia. Higher concentrations of *A. fumigatus* were observed in autumn and winter (0.50–1.05 CFU m^{-3}) than in spring and summer (0–0.40 CFU m^{-3}) at all sampling locations and they were without significant differences (Fig. 2). In the Medvednica region, *A. fumigatus* was not found during spring and summer, which was probably influenced by meteorological factors (higher temperature and lower relative humidity) and prevalence of other fungal species. Even the *Aspergillus* was the permanently present entity; airsporal concentrations were extremely low compared to other constant airborne fungi, including species of *Cladosporium*, *Penicillium* and *Alternaria* (up to 300 CFU m^{-3}) (3). Also, in our previous reports on indoor airspora sampling (apartments, houses, air-conditioned buildings) low concentrations of *A. fumigatus* were observed (0.9–2.8 CFU m^{-3}) compared to other dominant fungal entities such as *Cladosporium*, *Alternaria* and *Penicillium* (up to 213 CFU m^{-3}) (16).

Verruculogen toxigenic potential was observed in 84% of investigated airborne and 91% of clinical *A. fumigatus* strains (Table II), which was higher number of verruculogen-positive strains than in previously published studies (14). Verruculogen was detected in the spores of *A. fumigatus* isolated from biowaste-handling facilities (17), in pure culture of four from six strains, but not in dust (5). In the study of Land *et al.* (14), only 32% of 73 *A. fumigatus* strains isolated from sawmills produced tremorgenic mycotoxins fumitremorgen B and verruculogen *in vitro*. The amount of tremorgen mycotoxins detected in conidia *A. fumigatus* was between 0.6 and 8.0 μg 10^{-8} conidia, which was 0.18%

Table I. Airborne aspergilla detected in the centre of Zagreb (C), in the Pharmaceutical Botanical Garden (BG) and Medvednica mountain (M) with their mean annual concentrations (CFU m^{-3}) and frequencies (%)

Aspergillus species	C		BG		M	
	CFU m^{-3}	(%)	CFU m^{-3}	(%)	CFU m^{-3}	(%)
A. niger	1.04	(30.0)	0.70	(30.0)	0.23	(35.0)
A. fumigatus	0.50	(20.0)	0.50	(20.0)	0.21	(20.0)
A. *flavus*	0.22	(9.4)	0.28	(11.0)	0.08	(10.0)
A. glaucus	0.30	(9.4)	0.21	(8.5)	0.05	(8.0)
A. versicolor	0.40	(13.0)	0.34	(11.1)	0.05	(8.0)
A. ochraceus	0.22	(9.0)	0.12	(6.0)	0.05	(8.0)
A. nidulans	0.16	(5.0)	0.05	(1.3)	0.02	(1.0)
other aspergilli	0.16	(8.0)	0.21	(10.0)	0.02	(1.0)

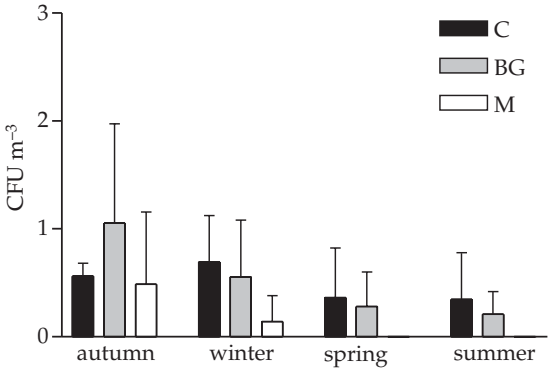

Fig. 2. Seasonal variations in concentrations of airborne *Aspergillus fumigatus* in the Zagreb area. Concentrations (CFU m^{-3}) of *Aspergillus fumigatus* in the city centre (C), in the Pharmaceutical Botanical Garden (BG) and Medvednica mountain (M) are presented as the mean and standard deviation.

of conidial weight (14). In another study, 40% (16 of 40 strains) of *A. fumigatus* isolates from saltern produced verruculogen, as well as other tremorgens fumitremorgens B and C and fumigaclavine A (18).

Verruculogen was produced in biosynthesis of airborne *A. fumigatus* strains in a concentration range from 0.1 to 0.6 mg mL^{-1} (average 0.34 mg mL^{-1}), and from 0.13 to 0.60 mg mL^{-1} (average 0.26 mg mL^{-1}) in the group of clinical isolates (Table II). Concentrations did not differ significantly (Table II). Our results of verruculogen-positive strains of *A. fumigatus* detected by thin-layer chromatography showed a high percentage of positive strains, which can be even higher because of the detection limit of verruculogen by TLC. It is expected that all *A. fumigatus* strains isolated from the air can produce verruculogen.

Since the air is the most common source of fungal colonization of the upper respiratory tract of immunocompromised patients, mycotoxins in inhaled spores could influence easier colonization of the respiratory tract. A recent study by Khoufache *et al.* (19) showed that verruculogen is responsible for the electrophysiological modifications of human nasal epithelial cells, but there are no studies on other low-molecular-weight metabolites present in spores, such as fumitremorgens, trypacidin and tryptoquivaline. Endo- and exometabolites of *A. fumigatus* were found to have very potent ciliostatic activity in the

Table II. Verruculogen production in A. fumigatus *strains*

	Airborne isolates of *A. fumigatus* strains[a]	Clinical isolates of *A. fumigatus* strains[b]
Verruculogen-positive strains	84%	91%
Concentration of verruculogen (mg mL^{-1})	0.10–0.60	0.13–0.60
X ± SD	0.34 ± 0.16[c]	0.26 ± 0.19[c]

[a] $n = 30$
[b] $n = 33$
[c] Not statistically significant ($p > 0.05$).

experiment with chicken trachea after 24 hours of exposure to fungal extracts in a concentration of 20 μg mL^{-1} (20). These findings could be also related to the activity of verruculogen and/or other mentioned mycotoxins that can be produced by *A. fumigatus*.

Therefore, we will in future detect production of verruculogen and other secondary metabolites in tissues of immunocompomised patients infected with *A. fumigatus*.

CONCLUSIONS

According to the results of the one-year monitoring of airborne fungi, spores of *A. fumigatus* are permanently present in the outdoor air of the Zagreb area, showing a relatively high frequency and low seasonal concentrations. High percentage of verruculogen positive strains was found among airborne and clinical isolates of *A. fumigatus*, which produced relatively high amounts of this mycotoxin. Even *A. fumigatus* had a low airsporal concentration, high percentage of verruculogen positive strains and their toxigenic potential could have a possible role in the development of neurotoxicity, as well as colonization of the upper respiratory tract, particularly in immunocompromised patients since this mycotoxin can be found in spores.

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S A Ž E T A K

Tvorba verukulogena u kliničkih i iz zraka izoliranih sojeva vrste *Aspergillus fumigatus* Fres.

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Uzorkovanje spora plijesni na području grada Zagreba obavljeno je tijekom godine 2002./2003. Pri tome su *Aspergillus niger* (v. Teigh.) i *A. fumigatus* (Fres.) bile najučestalije (20–30%) među aerogenim *Aspergillus* vrstama. Srednje godišnje koncentracije njihovih spora bile su izrazito niske (0,21–1,04 CFU m^{-3}). Veće koncentracije spora *A. fumigatus* zabilježene su tijekom jeseni i zime (0,50–1,05 CFU m^{-3}) u odnosu na koncentracije izmjerene tijekom proljeća i ljeta (0–0,40 CFU m^{-3}). S druge strane, mnogobrojne studije ukazuju na visoku učestalost *A. fumigatus* izolata iz gornjih i donjih dišnih puteva imunokompromitiranih bolesnika. Ova plijesan tvori nekoliko različitih mikotoksina, uključujući i tremorgeni mikotoksin verukulogen koji se može naći u sporama i miceliju vrste *A. fumigatus*. Tvorba verukulogena ispitana je u 30 aerogenih i 33 klinička izolata *A. fumigatus* vrste. U obje skupine dokazana je visoka učestalost verukulogen-pozitivnih sojeva (84% aerogenih i 91% kliničkih izolata). Potencijal tvorbe verukulogena u aerogenih (0.34 ± 0.16 mg mL^{-1}) i kliničkih sojeva (0.26 ± 0.19 mg mL^{-1}) nije se značajnije razlikovao.

Ključne riječi: verukulogen, *Aspergillus fumigatus*, toksičnost, mikotoksin, ekstrakt kvasca

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