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Ochratoxin A in airborne dust and fungal conidia *

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Abstract

Farm workers are often exposed to high concentrations of airborne organic dust and fungal conidia, especially when working with plant materials. The purpose of this investigation was to study the possibility of exposure to the mycotoxin ochratoxin A (OTA) through inhalation of organic dust and conidia. Dust and aerosol samples were collected from three local cowsheds. Aerosol samples for determination of total conidia and dust concentrations were collected by stationary sampling on polycarbonate filters. Total dust was analysed by gravimetry, and conidia were counted using scanning electron microscopy. A method was developed for extraction and determination of OTA in small samples of settled dust. OTA was extracted with a mixture of methanol, chloroform, HCl, and water, purified on immunoaffinity column, and analysed by ion-pair HPLC with fluorescence detection. Recovery of OTA from spiked dust samples (0.9–1.0 μg/kg) was 74% (quantitation limit 0.150 μg/kg). OTA was found in 6 out of 14 settled dust samples (0.2–70 μg/kg). The total concentration of airborne conidia ranged from $< 1.1 \times 10^4$ to 3.9×15^5 per m^3, and the airborne dust concentration ranged from 0.08 to 0.21 mg/m^3. Conidia collected from cultures of *Penicillium verrucosum* and *Aspergillus ochraceus* contained 0.4–0.7 and 0.02–0.06 pg OTA per conidium, respectively. Testing of conidial extracts from these fungi in a *Bacillus subtilis* bioassay indicated the presence of toxic compounds in addition to OTA. The results show that airborne dust and fungal conidia can be sources of OTA. Peak exposures to airborne OTA may be significant, e.g., in agricultural environments.

Key words: agriculture, dust, health hazard, inhalation, mycotoxins, ochratoxin

Introduction

Mycotoxins are secondary metabolites produced by a number of mould species. Exposure to mycotoxins can occur by ingestion of contaminated food and feed. In addition, exposure to mycotoxins from inhalation has received increased attention in recent years. In environments highly contaminated with airborne fungi, e.g., in water-damaged buildings and in agriculture, inhalatory exposure to mycotoxins may constitute a potential health hazard [1–4].

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic, teratogenic, and immunosuppressive mycotoxin [5] frequently found as a contaminant in human blood and milk samples [6–12]. OTA can contaminate feedstuffs and a number of food commodities [13–17]. Cereals and cereal products are generally considered the most important dietary sources of OTA [18, 19].

OTA is produced by several *Penicillium* and *Aspergillus* species [20–22]. In the colder climatic zones, such as Scandinavia, *P. verrucosum* is the main OTA producer [20, 21, 23, 24]. *P. verrucosum* is frequently found on stored cereals [25–27] and in forages and grain feeds [24, 28]. Conidia of *P. verrucosum* can also be present in indoor domestic atmosphere [29] and in air samples from dairy farms [30]. Although many potential toxigenic mould species can be found in air, presence of mycotoxins in air has been reported in only a few studies [1, 29, 31].

The present investigation was initiated to study the possibility of OTA exposure through inhalation of airborne organic dust and conidia in an agricul-

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tural environment. Farmers often have a high exposure to airborne dust and fungi, especially when handling mouldy feeding and bedding materials [32]. Airborne dust in cowsheds is a complex mixture of organic components, consisting of plant fragments from feed and bedding materials, conidia, skin scales, bacteria, mite fragments, and faeces [33, 34]. Current methods for extraction and determination of OTA, in e.g., cereals, are applicable to large sample size (e.g., 50 grams). To detect possible OTA in airborne dust, a method had to be developed which was suitable for extraction of OTA from small amounts (50–200 mg) of heterogeneous, fine dust samples. Conidia collected from cultures of *Aspergillus ochraceus* and *Penicillium verrucosum* were tested for the presence of OTA, and for toxic properties in a bacterial bioassay.

Materials and methods

Farms

Aerosol and dust samples were collected from three dairy farms located in Hedmark County, Norway. The number of milking cows in the cowshed varied from 24 to 75. One of the farms had loose-housed cows in deep litter bedding, and the other two had tied cows. Cattle were fed dried hay, round bale silage, and standard concentrate. In addition, oats and pea-flour were also used at one of the farms. Dried straw, wood shavings, and sawdust were used as bedding materials.

Aerosol sampling

Three aerosol samples were collected from each cowshed, using stationary samplers located in the centre of the cowshed, at a height of 150 cm. Two samples were collected during one hour of animal tending, e.g., feeding, milking, and distribution of bedding materials, and the third sample was collected over a period of 7 h. The samples were collected on polycarbonate filters with pore size 0.4 μm in closed-face 25 mm aerosol monitors made of graphite-filled polypropylene, using portable battery powered pumps (AFC 123, Casella Ltd., London, UK). The flow rate was 2 l/min, which was measured with a calibrated rotameter at the start and the end of the sampling.

Total airborne dust was analysed by gravimetry with a detection limit of 3 μg (Satorius ultramicrobalance model S4, Goettingen, Germany). Airborne conidia were counted by scanning electron microscopy with a detection limit of 1–8 $\times$ 10^4 conidia, depending on the sampled volume of air [35]. Micrographs [36] were used for classification of conidia from *Penicillium verrucosum*.

Extraction of OTA from settled dust

Fourteen samples of settled dust were collected from horizontal surfaces 1.5–2 m above the floor, at different places in the cowsheds. The samples were stored in sterile plastic tubes at −20 °C until extraction and analysis. All glassware and centrifugation tubes used were silanized (Sigmacote, SL-2, Sigma Chemical CO., St. Louis, MO, USA) to avoid loss of OTA from adsorption. A 100 mg subsample of settled dust was placed in a 50 ml conical centrifugation tube and mixed with 2 ml methanol for 2 min (vortex mixer), before adding 6 ml chloroform, and 0.6 ml 1 M HCI. The extraction mixture was automatically shaken on a laboratory shaker for 60 min. A volume of 2.8 ml water was added, and the solution was shaken for another 15 min. The chloroform phase was separated by centrifugation at 10,000 $\times$ g for 15 min. A volume of 4 ml of the chloroform phase was drawn from the bottom of the tube, and evaporated to dryness by nitrogen gas. The residue was dissolved in 44 ml phosphate buffered saline pH 7.4 (PBS) with 5% acetonitrile by ultrasonication for 15 min. The extract was filtered through a 0.80 μm (Millipore AA) filter, and purified on an immunoaffinity column (Ochraprep®, Rhône Diagnostics). The immunoaffinity column was washed with 20 ml water (5 ml/min), and OTA was eluated from the column using 1.5 ml acetic acid/methanol (2:98 v/v) followed by 1.5 ml water. The eluate was evaporated to dryness by nitrogen gas, and the residue was immediately dissolved in 300 μl of the HPLC mobile phase by ultrasonication.

Determination of OTA by HPLC

The HPLC system consisted of a Merck Hitachi L-6200 A Intelligent Pump, fluorescence detector (Merck Hitachi F-1080), interface (Merck Hitachi D-6000), a Rheodyne manual injector, and chromatography software (model Hitachi D-6000 HPLC Manager). Separations were carried out on a Spherisorb S3ODS2 (C-18) column, 4.6 $\times$ 150 mm, with 3 μm particles. Crystalline OTA (benzene free, from *Aspergillus ochraceus*), was purchased from Sigma Chemical CO. (St Louis, MO, USA).

The concentration of OTA was determined by HPLC ion-pair technique, at an alkaline pH and with

fluorescence detection based on the method described by Breitholtz-Emanuelsson et al. [8]. The extract was filtered through a 0.45 μm filter (Cameo 3N Syringe Filter, 3 mm diameter), and a 50 μl aliquot of the sample extract was injected into the chromatograph. The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol-potassium phosphate buffer (pH 7.5 and ionic strength 0.1) mixture. The ratio of methanol to potassium phosphate buffer was 51:49. Flow rate was 0.8 ml/min. The determinations were performed at 380 nm (excitation wavelength) and 450 nm (emission wavelength). The detection limit (signal-to-noise ratio of 3) for standard solutions of OTA was 1.5 pg.

Analytical quality control

A solution of OTA (10 μg/ml in methanol) was calibrated spectrophotometrically at 333 nm, using the value of 6640 for the extinction coefficient [37]. The OTA solution was diluted to 10^{-8} M in methanol and stored at −20 °C. Working standard solutions for calibration were prepared every day by dilution of the 10^{-8} M OTA stock solution with HPLC mobile phase. For quantitation, peak heights were measured by a Merck Hitachi integrator. The calibration curves used for quantitation were calculated by the least-squares method. Positive control samples for recovery experiments and for calculation of the quantitation limit (signal-to-noise ratio of 10) were made by adding 2 ml of a diluted OTA/methanol solution to 100 mg of a negative dust sample, mixed by vortexing for 2 min, before adding acidic chloroform and water. A negative dust sample (100 mg) added 2 ml methanol was used as a negative control.

Qualitative confirmation of positive samples were performed in two ways: (1) All sample extracts shown to contain OTA during initial HPLC analysis were analysed in duplicate, with a direct spiking of the second aliquot. This was performed by adding an amount of OTA stock solution directly to the injected aliquot. The chromatograms of the unspiked and the spiked sample extract were then compared. (2) Derivatization of OTA through methylation of the extracts with subsequent HPLC analysis was also used for qualitative confirmation of positive samples [11]. A 200 μl aliquot of the purified sample extract was evaporated to dryness, and the residue was dissolved in 2.5 ml methanol and 0.1 ml conc. HCl. The mixture was kept overnight at room temperature. After evaporating the mixture to dryness, the residue was dissolved in 200 μl mobile phase and 50 μl of it was injected onto the HPLC column. Confirmation was based on disappearance of the OTA peak and appearance of a new peak with approximately double retention time, corresponding to OTA methyl ester.

Analyses of conidia

Isolates of *Aspergillus ochraceus* CBS 263.67 and *Penicillium verrucosum* IBC 5075 (generous gift from Dr. J.C. Frisvad, Dept. of Technol. Technical University of Denmark, Lyngby, Denmark) were grown on Sabouraud agar plates (135 mm in diameter), pH 5.9, and incubated at 28 °C in the dark for 2–3 weeks. The conidia were collected from the mycelial mat by washing with sterile saline, counted, and extracted with acidic chloroform as previously described [38]. The conidial extracts were evaporated and dissolved in methanol to give a concentration corresponding to 1×10^5 conidia per μl. The conidial extracts were used for OTA determination and toxicity testing. For OTA determination, the extract was evaporated to dryness, the residue was dissolved in HPLC mobile phase by ultrasonication, and injected onto the HPLC column without preceding purification. Toxic effects of conidial extracts were tested in a bacterial bioassay. *Bacillus sublilis* ATCC 6633 obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was grown on a minimal medium containing glucose as the carbon source [39]. The pH of the medium was adjusted to 6.0 with 1 M HCl. The medium was prepared in plates of 4 mm depth. Paper discs (6 mm in diameter, AB Biodisk, Solna, Sweden) were impregnated with extracts from 2×10^6 conidia, dried, and applied to the agar plates. Paper discs impregnated with OTA (10 μg), citrinin (2.5 μg and 6.3 μg), and methanol (negative control) were used for comparison. The bacteria were inoculated onto plates in a suspension of about 10^5 cells/ml to give a semiconfluent to confluent growth. The plates were incubated at 37 °C. Toxic effects of the conidial extracts were measured as growth inhibition zones (mm diameter) after 24 h of incubation.

Results

A method was developed for extraction and analysis of OTA in small (50–200 mg) dust samples. The recovery of OTA from positive control samples (0.9–1.0 μg/kg) was 74.2 ± 20% (mean ± 1 SD, n = 12), and the

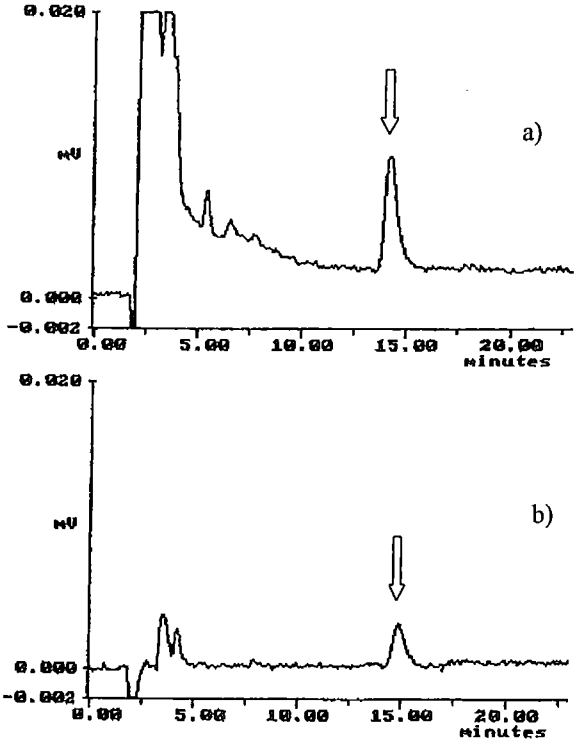

Figure 1. HPLC chromatograms of (a) naturally contaminated dust sample,(b) OTA standard. Sample extract is purified on immunoaffinity column.

quantitation limit was 0.150 μg/kg. OTA was found in 6 out of 14 settled dust samples collected from cowsheds (concentrations from 0.2–70 μg/kg, mean of positive samples: 27.5 μg/kg). HPLC chromatograms of a naturally contaminated dust sample, and of OTA standard are shown (Figure 1). The concentration of total airborne conidia in the cowsheds ranged from $<1.1 \times 10^4$ to 3.9×10^5 per m^3. Conidia morphologically similar to *P. verrucosum* were found in air samples from one of the cowsheds (deep litter bedding). The concentration of total airborne dust ranged from 0.08 to 0.21 mg/m^3.

Extracts of conidia collected from cultures of *P. verrucosum* (5 plates) and *A. ochraceus* (6 plates) contained from 0.4–0.7 and 0.02–0.06 pg OTA per conidium, respectively.

Conidial extracts from *P. verrucosum* and *A. ochraceus* possessed toxic effects to *B. subtilis*. The growth inhibition produced by conidial extracts of *P. verrucosum* and *A. ochraceus* were much stronger than the inhibition produced by 10 μg OTA, indicating that the conidia contain toxic substances in addition to OTA (Table 1).

Table 1. Bacillus subtilis bioassay: Extracts of 2×10^6 conidia collected from 3 plates cultured with *P. verrucosum*, and from 2 plates cultured with *A. ochraceus*. Toxic properties of extracts measured as growth inhibition zones (mm diameter with a complete inhibition).

Test extract	Amount of toxin (μg) in extract	Inhibition zone, mm
P. verrucosum: 2×10^6 conidia	0.8–1.4 OTA*	9
		11
		12
A. ochraceus: 2×10^6 conidia	0.04–0.12 OTA*	19
		20
OTA	10	0
Citrinin	2.5	0
	6.3	30
Methanol (negative control)		0

*Determined by HPLC analyses.

Discussion

The extraction and clean-up protocol for analysis of OTA in dust was developed in order to produce high extraction efficiency of OTA, and minimal coextracting interfering substances. Immunoaffinity column (IAC) provided clean extracts, as can be seen in Figure 1, and significantly improved the sensitivity of the HPLC determination as compared to clean-up using silica gel cartridge. The heterogeneous and complex nature of the sample matrix resulted in some variations in the recovery of OTA, ranging from 51 to 123% (mean 74%).

Settled dust samples from the cowsheds were contaminated with OTA, at levels from 0.2–70 μg/kg. OTA in the dust is likely to originate from contaminated feeding and bedding materials, and from airborne conidia. OTA levels from 10 to more than 30 000 μg/kg have been reported in feeds in Europe and Canada [5, 14, 40]. In addition, OTA may be produced by fungi growing in the settled dust. In a recent report, a very high level (1500 μg/kg) of OTA was found in dust collected from inside the ducts of the heating system in a household [41].

Conidia morphologically similar to *P. verrucosum* were found in air samples from one of the cowsheds. However, the morphology of these conidia was not specific as several other Penicillia produce conidia with similar shape and size.

Data on OTA levels in conidia are not available from the literature. To examine the occurrence of OTA in conidia, isolates of *P. verrucosum* and *A. ochraceus*

were grown on Sabouraud plates and conidial extracts were analysed by HPLC. The results indicate that OTA levels were on the order of 0.5 pg per conidium for *P. verrucosum*, and about ten times less for conidia from *A. ochraceus*. In addition to OTA, conidia may contain other toxic compounds. Several bacterial species, including *B. subtilis* have been used as tools for unspecific testing of mycotoxins [42]. The *B. subtilis* bioassay used in this study demonstrated that conidial extracts from *P. verrucosum* and *A. ochraceus* possessed toxic effects. The growth inhibition produced by the extracts was much stronger than would be expected from the amount of OTA in the conidia. This indicates the presence of additional toxic substances in the conidia, possibly citrinin. *P. verrucosum* contain citrinin in the range of 1.4–4.1 pg/conidium, located in the outer layer of the conidium [38]. These results emphasize the potential health risk of prolonged inhalation of conidia. Conidia from Penicillia are usually small, less than 5 μm in diameter, and are therefore easily inhaled into the lungs. Toxins associated with conidia may be absorbed via the respiratory epithelium and distributed to other sites, possibly causing systemic effects. A recent case report indicates that inhalation of OTA containing conidia has caused human illness. A female farm worker suffered from acute renal failure after working for 8 h in a granary which had been closed for several months. Inhalation of *A. ochraceus* conidia containing OTA was implicated [43].

With a minute ventilation of 6 l/min (moderate activity), a human inhales approximately 0.36 m^3 air per hour. If the concentration of airborne dust is 0.21 mg/m^3, the person will inhale 76 μg dust per hour. Suppose the concentration of OTA in the dust is 70 $\mu g/kg$, the inhaled amount of OTA from dust will be 5 pg OTA per hour. The levels of airborne dust and conidia measured in the present study were moderate to low compared to results from other studies [32, 44]. If the concentration of dust in inhaled air is 1.78 mg/m^3, as reported by Kullmann et al. [34], 45 pg of OTA will be inhaled per hour. In the work of Karlsson and Malmberg [30], air concentrations exceeding 10^{10} conidia per m^3 were measured (by personal sampling) on dairy farms where respiratory diseases had been reported. If 1% of the total airborne conidia were *P. verrucosum*, and they contained 0.5 pg OTA per conidium, 18 μg OTA could be inhaled per hour. Our results demonstrate that exposure to OTA from inhalation of dust and conidia is possible, and that peak exposures can be considerable. Even if the inhalatory intake of OTA may be low compared to dietary intake, inhalation may contribute significantly to the total intake of the toxin because the toxin is very efficiently absorbed from the lung [45].

Conclusion

Airborne dust and fungal conidia can be a source of OTA. Peak exposures to airborne OTA may be significant, e.g., in agricultural environments.

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