Original Article

Occupational Exposure to β-D-Glucans, Mould Allergens, Endotoxins and Cultivable Fungi in Pig Farms

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Submitted 14 April 2022; revised 21 July 2022; editorial decision 22 July 2022; revised version accepted 28 July 2022.

Abstract

Airborne concentrations of organic dust on animal farms are known to be very high. This dust is partly composed of microorganisms such as bacteria, fungi and their components [endotoxins, (1→3)-β-D-glucans, mould allergens, mycotoxins], recognised as being responsible for numerous health effects. Several cross-sectional studies have measured levels of airborne bacteria, fungi and endotoxins on pig farms. However, the temporal dynamics of organic dust’s components throughout the year have rarely been assessed, and airborne concentrations of (1→3)-β-D-glucans and mould allergens remain poorly understood in these work environments. This longitudinal, four-season study measured cultivable fungi, endotoxins, (1→3)-β-D-glucans, Aspergillus versicolor (AveX), Aspergillus fumigatus (Asp f1) and Alternaria sp (Alt a1) allergens on 31 pig farms in Switzerland. Results showed that exposure to AveX occurred in all four seasons. Total mean airborne concentration of endotoxins were between 3 and 4 times higher than the Swiss recommended limit value of 1000 EU m⁻³ and mean airborne concentrations of fungi were between 30 and 50 times higher than the Swiss recommended limit value of 1000 cfu m⁻³. Finally, accumulations of faecal matter on floors, humidity and dusty pathways were associated with increased concentrations of (1→3)-β-D-glucans. In conclusion, pig farmers require better information about biological occupational risks, and measures to improve air quality should be implemented, especially in winter.

Keywords: Alt a1; Asp f1; AveX; bioaerosol; fungal exposure; occupational health; swine confinement building
Introduction

Numerous studies have observed that animal-farm workers face daily exposure to very high levels of bioaerosols (Thorne et al., 2009; O’Shaughnessy et al., 2012; Basinas et al., 2013; Masclaux et al., 2013). Consequently, they are more at risk of developing respiratory health problems. Chronic exposure to airborne organic dust is associated with a variety of symptoms among pig-farm workers, including chronic cough, rhinitis, irritation, lung inflammation and decline in lung function (Vogelzang et al., 1998; Cole et al., 2000; Heederik et al., 2007; Senthilselvan et al., 2007). The organic dust present on pig farms contains different biological compounds emitted by pig dander, faecal matter and foraging activities. These components become aerosolised in densely populated, enclosed farm buildings. Reduced winter ventilation rates, often observed to avoid heat loss, have been demonstrated to increase concentrations of organic dust, fungi, microorganisms and endotoxins (Chang et al., 2001; Masclaux et al., 2013). Although a precise characterisation of airborne dust is essential to better understanding associations between dust exposure and health effects, some airborne biological components have never or rarely been measured in such environments. For example, several studies have reported the presence of the fungal species of Aspergillus versicolor, A. fumigatus and Alternaria alternata on pig farms (Viegas et al., 2013; Gladding et al., 2020; White et al., 2021), but levels of mould allergens and (1→3)-β-D-glucans (hereon referred to as β-glucans) remain unknown, despite their significant adverse health effects. Occupational risk assessments linked to the presence of these moulds usually consist of quantifying the numbers of cultivable or total spores of these fungal species. However, many hyphae fragments—that are neither cultivable nor countable—may be present in the air, and these potentially allergenic or pro-inflammatory particles are not considered when assessing the health risks associated with dust particles. Completing investigations into cultivable/uncultivable fungal contamination with a measurement of the specific airborne allergenic proteins derived from these fungal species would thus be very useful. β-Glucans molecules belong to a family of polysaccharides produced by eukaryotes and prokaryotes, and they compose parts of the cell walls of most microscopic fungi, upper fungi, yeasts, algae and certain bacteria. As with endotoxins, these components can cause inflammatory lung and airway reactions, activate the immune system when inhaled or be responsible for organic dust toxic syndrome (Madsen et al., 2012). They are rarely measured on pig farms (Sauve et al., 2020).

To have a better understanding of the composition and temporal dynamics of airborne fungal contamination on pig farms, this study measured airborne concentrations of β-glucans, endotoxins, cultivable fungi and major allergenic glycoproteins from A. versicolor, A. fumigatus and A. alternata (AveX, Asp f1 and Alt a1, respectively) in spring, summer, autumn and winter. Correlations between these different compounds were tested, as were the influences of different farm characteristics.

Methods

Study sites

We investigated 31 pig farms spread across western Switzerland four times between October 2014 and September 2015. Two or three pig farms were visited per day, and all consisted of either farrowing units alone or mixed farrowing and fattening units. Pigs per farm varied from 80 to 2700, with a mean of 483 pigs. Weaners were kept in boxes (12–20 pigs per box; 2–12 boxes per room) on wooden gratings and/or concrete with or without straw. Sows were kept with their suckling piglets in individual boxes (4–10 boxes per room) directly on concrete. Most buildings had a main pas sageway with animal boxes on one or both sides. All units were totally enclosed, with animals having no possibility of going outside. During each visit, the investigator used a checklist to evaluate the barn’s characteristics (floor type: concrete, wood, straw; feed type: liquid, solid; dung accumulation level: low, high; level of pathway dustiness: low, high). Temperature and relative humidity were also measured using an Ecolog device (Ecolog Th1, Elpro-Buchs, Switzerland).
Airborne cultivable fungi
At each pig-farm visit, 50 l of air were collected onto DG18 nutrient agar plates in triplicate (Thermo Fisher, Basel, Switzerland) using an impactor (MAS-100 Eco, MBV; Vevey, Switzerland) set to a flow rate of 100 l min⁻¹. Plates were incubated at 25°C for 7 days, with all plates checked daily. Results were expressed in colony-forming units (CFU) per cubic meter of air, based on the mean of the results from the three samples. After having performed a previous pilot study in these farms, we observed that A. versicolor were frequently dominant onto our nutrient plates. Therefore we decided to identify and count separately this fungi Aspergillus versicolor (Aspergillus sect Nidulantes) in order to attempt to see at a correlation between Avex allergens and A. versicolor. Identification was made according to the macroscopic and microscopic morphology of the colonies, hyphae and spores However, we are aware that A. versicolor can morphologically looks like other Aspergillus spp. and that only a molecular analysis could have confirmed the identification of such isolates.

Airborne dust collection for endotoxins, (1→3)-β-d-glucans and mould allergens
At each farm visit, airborne particles were sampled using a Coriolis μ air sampler (Bertin Technologies, Montigny-le-Bretonneux, France). The sampler was placed approximately 1.4 m above the ground, in the middle of the pig barn, and the airborne particles from 3 m³ air (0.3 m³ min⁻¹ for 10 min) were collected through a sterile cone containing 15 ml 0.005% Triton X-100 solution. All samples were immediately transported to the laboratory in a cold box (4°C). Aliquots (2 ml) were stored at −20°C for subsequent analyses.

Endotoxin and (1→3)-β-d-glucan analyses
For endotoxin measurements, 100 µl of liquid from the Coriolis samples were diluted (10×) and shaken at room temperature for 1 h in 10 ml of pyrogen-free water in a 50 ml conical polypropylene tube. These solutions were then analysed using a quantitative kinetic chromogenic LAL (limulus amoebocyte lysate) assay (Lanza, Switzerland) at 37°C. The Escherichia coli O55:B5 endotoxin was used as a calibration standard. Results were expressed in endotoxin units (EU) per cubic meter of air. (1→3)-β-d-glucans were analysed using the kinetic Glucatell® kit (Associates of Cape Cod Inc., Liverpool, UK), which uses the LAL factor G pathway. Briefly, 100 µl of liquid from the Coriolis samples were diluted (10×) and shaken on ice for 25 min in 10 ml of pyrogen-free water. These solutions were then analysed as per the manufacturer’s instructions. Results were obtained by comparing samples against the standard curve and are expressed in ng of (1→3)-β-d-glucans per cubic meter of air.

Mould allergen analysis
Concentrations of extracted Asp f1 (A. fumigatus), Alt a1 (A. alternara) and AveX (A. versicolor) allergens were determined using a commercial ELISA kit (Indoor Biotechnologies Ltd, Cardiff, UK), as per the manufacturer’s instructions, and analysed using a Tecan microplate reader (Tecan Group, Switzerland). Purified natural Asp f1 (400 ng Asp f1 ml⁻¹), Alt a1 (1000 ng Alt a1 ml⁻¹) and AveX (10 000 U AveX ml⁻¹) extracts were used as reference standards. We formed standard curves by using serial twofold dilutions of these standards. Analyses were carried out in triplicate, and results were calculated from blank-corrected absorbance values using linear regression analysis. Results are expressed as ng of Asp f1 or Alt a1 per cubic meter of air and as units (U) of AveX m⁻³. Detection limits were 0.32 ng ml⁻¹ of Asp f1, 0.05 ng ml⁻¹ of Alt a1 and 16 U ml⁻¹ of AveX.

Statistical methods
All data were log-transformed before statistical analyses. The influences of different parameters and seasons on bioaerosols were tested using logistic regressions for binary data and a linear mixed model considering a random farm effect. Correlations between the different bioaerosols were tested using Pearson correlation tests. All statistics were calculated using Systat or STATA software (SYSTAT Software Inc. products Canada; StataCorp, Texas USA). Data are presented as arithmetic mean values and ranges (min–max). Geometric means are only presented in the table. For mould allergens, values below the limit of detection (LD) were considered to be a third of the LD.

Results
Airborne concentrations of endotoxins, fungi and their components
Seasonal bioaerosol levels are presented in Table 1. Mean airborne endotoxin concentrations in every season were three to four times higher than Switzerland’s recommended limit value of 1000 EU m⁻³. Mean airborne concentrations of fungi were 30–50 times higher than Switzerland’s recommended limit value of 1000 CFU m⁻³. Our measurements showed that 96% of the samples were above this threshold (119/124), and 8% had several
cultivable fungi present above the suggested lowest observed effect level of 10^5 spores m^-3 (Eduard, 2009), where lung function decline, respiratory symptoms and airway inflammation began to appear in a highly exposed working population. Concerning β-glucans, mean seasonal concentrations varied from about 20–37 ng m^-3.

Mean concentrations of *A. versicolor* allergens (AveX) were between 19 and 78 Units of AveX m^-3, and mean concentrations of cultivable *A. versicolor* were between 1300 and 4500 CFU m^-3. Only two samples (2/29) of *A. fumigatus* allergens (Asp f1) were found above the LD in autumn, with one sample each in winter and spring and no samples above the LD in summer. Most concentrations of *Alternaria* sp. allergens (Alt a1) were also below the LD. However, the number of samples above this limit was significantly higher in summer (13/31) and autumn (10/31) than in winter or spring (5/31 and 4/31) (Pearson chi-square = 9.3, P = 0.025). The mean number of *Alternaria* sp. CFU was also higher in summer and autumn (1056 and 1233 CFU m^-3) than in winter and spring (284 and 290 CFU m^-3).

**Effects of season and farm characteristics**

Mean seasonal outdoor/indoor temperatures were 9.5/17.8, 19.5/20.3, 25.4/25.4, 16.4/20.2°C, and mean outdoor/indoor humidities were 57/67, 42/53, 47/56, 51/65% in winter, spring, summer and autumn, respectively. A linear mixed model showed that the season had a significant influence on mean airborne concentrations of AveX and β-glucans, with higher levels of allergens in spring and higher levels of β-glucans in winter (Wald chi^2 = 10.14, P = 0.0174 and Wald chi^2 = 31.22, P < 0.001 respectively). Levels of all the other organic dust components measured were not influenced by season (P > 0.05, data not shown). Moreover, we observed a significant effect of dung accumulation (categorical data: high or low) on barn floors on concentrations of β-glucans (Wald chi^2 = 5.35, P = 0.02) as well as the effects of indoor humidity (Z = 2.84, P = 0.004) and pathway dust levels (categorical data: high or low) (Wald chi^2 = 4.68, P = 0.03) with high levels of dung accumulation, increasing humidity and high level of dust associated with an increase in β-glucans concentration.

**Discussion**

To the best of our knowledge, the present study was the first to investigate mould allergens and β-glucans in the air of many (31) pig farms.

**Airborne fungi concentrations**

Workers’ exposure to fungi was non-negligible since nearly every pig-farm had concentrations above Switzerland’s recommended occupational limits (1000 CFU m^-3). In particular, levels of *Aspergillus* sp. and *A. versicolor* were 1.5–4 times higher than Switzerland’s recommended values for total airborne cultivable fungi. By using only culture dependent-methods to quantify the airborne fungi load, we underestimated the real total concentration, which include both cultivable and non-cultivable strains. Indeed a study estimated that the values for cultivable airborne fungal fraction was 1–3 logarithms lower than the values obtained using molecular tools (Gauzere et al., 2013).

Our results confirmed what was previously shown in other studies concerning the high levels of exposure to cultivable fungi and more specifically *Aspergillus* spp.
pig farms (Jo and Kang, 2005; Letourneau et al., 2010; White et al., 2020).

Exposure to high levels of fungi can be problematic since many species are allergenic and some produce mycotoxins toxic to human and animal health (Viegas et al., 2019). A. versicolor, for instance, produces sterigmatocystin, a carcinogenic mycotoxin that can lead to liver and kidney necrosis. In Portuguese pig farms, A. versicolor presented with the highest level of airborne spores of Aspergillus sp. (Sabino et al., 2012). In contrast, in one Danish pig-farm, A. glaucus was the most frequent Aspergillus identified (White et al., 2020). The A. versicolor levels found in the present study were within the same range measured in Portuguese pig farm air (Viegas et al., 2013) and 38% (48/124) of our samples were above Switzerland’s 1000 CFU m\(^{-3}\) recommended limit value for airborne total cultivable fungi. This means that workers were at risk of developing allergic reactions or other symptoms, such as an aggravation of asthma or hypersensitivity reactions associated with high airborne concentrations of Aspergillus sp.

Mould allergens

Mould allergens have rarely been measured in occupational environments. A study from Poland measured Alt a1 in two libraries, a museum, an archive and a tannery (Skora et al., 2015), but these work environments cannot be compared to a farming environment. Among animal farms, only one study, carried out in Croatia, has reported on Alt a and Asp f concentrations in dust samples collected in poultry farms (Prester and Macan, 2010; Prester et al., 2010). The authors detected Alt a and Asp f allergens in 100% and 62% of 45 farms, respectively, but exposure levels were relatively low (median Alt a = 10.37 μg g\(^{-1}\), and median Asp f = 117.9 ng g\(^{-1}\)) and cannot be compared to the results of airborne measurements. However, it could be assumed that, with such a low level of allergens observed in dust, the probability of having a detectable concentration in the air is low. This agrees with our results since we detected Alt a1 in 13–42% (depending on the season) of our 31 farms but only detected Asp f1 in one.

Measurements of AveX have only previously been made in house dust samples (Benndorf et al., 2008; Shi et al., 2011), and we did not find it in our air samples. Thus, we could not compare our results with other work or domestic environments.

Even though we found no correlation between AveX and concentrations of cultivable A. versicolor, we noted that the mean A. versicolor level was well above Switzerland’s recommendation in every season.

β-Glucans

Occupational exposure to β-glucans is present in many different work environments (see examples in Cyprowski et al. (2011)). However, to date, not a single governmental organisation has set occupational exposure limits (OEL) or made recommendations for inhalation exposure to β-glucans. (Parker et al., 2021) recently derived an OEL of 150 ng m\(^{-3}\) for β-glucans based on the most relevant non-clinical study to date and inflammatory reactions. Compared to this proposed OEL, the mean concentrations measured in the present study were acceptable and in the same range as two studies carried out on pig farms in China and the USA (Yang et al., 2013; Sauve et al., 2020) and on other animal farms (Samadi et al., 2009; Lawniczek-Walczyk et al., 2013). We should nevertheless remember that farmers are always simultaneously exposed to non-negligible levels of endotoxins that induce direct inflammatory effects on the respiratory tract. Therefore, this cumulative, related health effect of exposure to both endotoxins and β-glucans should be considered when performing occupational risk assessments. Indeed, although each separate component may be below its recommended concentration, and therefore respects worker protection guidelines, it is likely that cumulative exposure to these two agents no longer protects them from adverse health effects. We should therefore base our assessments on values lower than those recommended.

Endotoxins

Within the 31 farms visited, levels of airborne endotoxins exceeded Switzerland’s recommended value of 1000 EU m\(^{-3}\) in 28, 22, 26 and 30 pig farms during the winter, spring, summer and autumn, respectively, and systematically exceeded the “no adverse health effects” level for chronic exposure fixed at 90 EU m\(^{-3}\) by the Dutch Expert Committee on Occupational Safety (DECOS, 2010). Chronic exposure to endotoxins has been shown to be a major determinant of lung function decline in pig farmers (Vogelzang et al., 1998). Most farms in Switzerland are medium- or small-sized; therefore, farmers are not frequently exposed to airborne endotoxins during 8 h day. However, the evolution of farmers’ lung function should be monitored regularly throughout their working lives by occupational or general practitioner physicians in order to prevent deleterious, irreversible impairments.

Seasonal influence on bioaerosol concentrations

Contrary to observations in China, where concentrated animal feeding operations showed no seasonal changes
observed in β-glucans concentrations (Yang et al., 2013), we found that the seasons had significant influences on the levels of airborne β-glucan and AveX, with their highest respective levels found in winter and spring. In Switzerland, for economic reasons, winter ventilation is usually kept to a minimum to avoid heat loss, while in summer, well-ventilated rooms are needed for animal well-being. Thus, the evacuation of bioaerosols seems to be more efficient in summer and occupational exposure lessened. These differences in climatic factors could explain the discrepancies found between Swiss and Chinese pig farms. However, it is surprising that differences in ventilation management between seasons in Switzerland have an influence only on β-glucan and AveX concentrations and not on the cultivable fungi and endotoxins levels as previously observed in Switzerland in 2012 (Masclaux et al., 2013). This highlights that the level of exposure to different bioaerosols in pig farms from the same geographical area can be season-dependent or not and is therefore unpredictable. In term of prevention, that means that we have always to consider that farmers face to the “worst situation”.

Conclusion

In conclusion, this was the first study to measure a variety of components for assessing fungal exposure on animal farms. We found that the allergenic AveX protein was present in 77% of the farms investigated. However, no correlation between this protein and the cultivable A. versicolor was observed. We also observed non-negligible concentrations of (1→3)-β-d-glucans that should be considered when assessing the risks associated with the exposure to endotoxins. Indeed, since both endotoxins and (1→3)-β-d-glucans are pro-inflammatory substances, it seems essential that their potential health effects should be considered together.

Pig-farm workers all year-round are exposed to high levels of fungal particles and their components, and we recommend that specific, detailed information about the biological risks linked to pig farming activities should be available to them.

Acknowledgements

The authors are grateful to all the pig farmers who participated in this study. We thank Pascal Wild for his help with statistical analyses and Eulallia Semaani for her advice and help recruiting farms. We are also grateful to Darren Hart from “admin@publish-or-perish.ch” for English editing.

Funding

This work was supported by Swiss National Science Foundation (SNF) grant 310030_152880 to MH and AO.

Conflict of Interests

The authors have no competing interests to declare.

Data Availability

The data underlying this article will be shared upon reasonable request made to the corresponding author.

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