Environmental contamination by *Aspergillus* spp. in laying hen farms and associated health risks for farm workers

Claudia Cafarchia,1 Antonio Camarda,1 Roberta Iatta,1 Patrizia Danesi,2 Vincenza Favuzzi,1 Giancarlo Di Paola,1 Nicola Pugliese,1 Anna Caroli,1 Maria Teresa Montagna3 and Domenico Otranto1

Correspondence
Claudia Cafarchia
claudia.cafarchia@uniba.it

1Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Str. prov. le per Casamassima Km 3, 70010 Valenzano, Bari, Italy
2Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, 1035020 Legnaro (PD), Italy
3Dipartimento di Scienze Biomediche ed Oncologia Umana, Università degli Studi di Bari, Piazza G. Cesare 11, 70124 Bari, Italy

Data on the occurrence and epidemiology of *Aspergillus* spp. in laying hens farms are scant. With the aims of determining levels of airborne contamination in laying hen farms and evaluating the potential risk of infection for workers and animals, 57 air samples from 19 sheds (Group I), 69 from faeces (Group II), 19 from poultry feedstuffs (Group III) and 60 from three anatomical sites (i.e. nostrils, pharynx, ears) of 20 farm workers (Group IV) were cultured. The *Aspergillus* spp. prevalence in samples ranged from 31.6 % (Group III) to 55.5 % (Group IV), whereas the highest conidia concentration was retrieved in Group II (1.2×10^4 c.f.u. g^-1) and in Group III (1.9×10^3 c.f.u. g^-1). The mean concentration of airborne *Aspergillus* spp. conidia was 70 c.f.u. m^-3 with *Aspergillus fumigatus* (27.3%) being the most frequently detected species, followed by *Aspergillus flavus* (6.3%). These *Aspergillus* spp. were also isolated from human nostrils (40 %) and ears (35 %) (P<0.05) (Group IV). No clinical aspergillosis was diagnosed in hens.

The results demonstrate a relationship between the environmental contamination in hen farms and presence of *Aspergillus* spp. on animals and humans. Even if the concentration of airborne *Aspergillus* spp. conidia (i.e. 70 c.f.u. m^-3) herein detected does not trigger clinical disease in hens, it causes human colonization. Correct management of hen farms is necessary to control environmental contamination by *Aspergillus* spp., and could lead to a significant reduction of animal and human colonization.

INTRODUCTION

*Aspergillus* spp. are saprophytic fungi, commonly found in soil, decaying vegetation, seeds and grains (Hajjeh & Warnock, 2001). Only a few species are recognized pathogens for humans and animals, such as *Aspergillus fumigatus*, which may invade the lungs of many species, including birds and humans (Leenders et al., 1999; Tell, 2005; Ozhak-Baysan et al., 2010; Arné et al., 2011; Binder & Lass-Flörl, 2011). In addition, *Aspergillus terreus*, *A. niger*, *A. flavus*, and the *A. glaucus* group have been occasionally reported as pathogens (Barton et al., 1992; Tell, 2005; Arné et al., 2011; Binder & Lass-Flörl, 2011). Generally, infections in susceptible hosts occur as a consequence of a high concentration of conidia and long-term exposure (Tell, 2005; Arné et al., 2011; Binder & Lass-Flörl, 2011). Spores are usually 2 to 10 µm in size and they easily penetrate the lower airways, causing allergic or invasive infections in humans (Chapman, 2006; Fairs et al., 2010; Chotirmall et al., 2013). The invasive infections mostly occur in severely immunocompromised patients whereas the allergic responses occur in immunocompetent hosts as a result of close contact with high levels of airborne propagules such as in the case of *A. fumigatus*, which may be a causative agent of ‘farmer’s lung’ (Gregory & Lacey, 1963). Similarly, *Aspergillus clavatus* may induce

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the molecularly confirmed species of *Aspergillus* in this work are KF651175 (*A. fumigatus*), KF651176 (*A. flavus*), KF651177 (*A. niger*), KF651178 (*A. ochraceus*) and KF651179 (*A. candidus*).
Aspergillus spp. have also been described as opportunistic pathogens of domestic animals (e.g. dogs, horses and cattle), and as the primary cause of a severe respiratory condition in birds (Tell, 2005; Arné et al., 2011). Bird aspergillosis has been described either as an acute infection leading to severe outbreaks in young birds, or as a chronic condition responsible for low productivity and economic losses in adult birds (Knudtson & Meincke, 1972; Morris & Fletcher, 1988; Ghazikhianan, 1989; Tell, 2005), for instance via contamination of egg albumin and yolk with A. fumigatus conidia (Kunkle, 2003). The epidemiology of both forms of aspergillosis in poultry remains poorly understood; however, it is known that exposure to large numbers of Aspergillus conidia in the breeding environment or in poultry litter represents a major risk for public health (Dyar et al., 1984; Richard et al., 1984; Fulleringer et al., 2006; Fairs et al., 2010; Arné et al., 2011).

The aims of this study were (i) to determine the presence and the load of Aspergillus spp. in the air of laying hen farms and (ii) to identify the risks for farm workers and hens associated with high airborne fungal occurrence. Additionally, in order to assess the effects of exposure to Aspergillus spp. conidia, their occurrence in nostrils, pharynx and ears of farm workers was investigated.

**METHODS**

**Study population and farm management.** From May to November 2011, a large-scale study was carried out on laying hen farms and in farm workers in an area of the Apulia region, southern Italy (latitude 39°57′N to 41°47′N, longitude 18°17′E to 15°43′E). Overall, 12 commercial egg farms, housing approximately 500,000 laying hens in total, were examined. Each farm consisted of one to four sheds, each housing 10,000 to 33,000 birds. Each flock was reared in cages sized 40×55×40 cm, which were placed in three to five levels with four hens per cage, according to the appropriate legislation in force at the time of sampling.

A total of 19 sheds were analysed. For each farm and shed, information on capacity (i.e. number of hens housed in the shed at the time of sampling), altitude, relative humidity and daily temperatures registered throughout the month before sampling was recorded in individual files, along with the disinfection procedures and antifungal compounds used. Laying hen farms/sheds were located in rural or suburban areas at varying altitudes, and characterized by well-drained soil, and automatic feeding and watering systems. The buildings had no windows, however, they were equipped with forced longitudinal ventilation systems. In all farms, cages, feeders and watering equipment were sanitized once a week using commercially available solutions of quaternary salts; faeces were removed weekly.

A total of 20 farm workers from 12 laying hen farms were included in the study. All subjects were informed about the study and signed a consent form. Working hours were from Monday to Friday, 8 h per day, with an additional 5 h shift on Saturday. Each worker was asked to fill in a questionnaire, which included clinical information, such as episodes of past or current respiratory symptoms (i.e. dyspnoea, hypopnoea and apnoea, asthma, chronic bronchitis, allergic rhinitis), the occurrence of atopic dermatitis (i.e. pruritus and inflammatory localized or generalized dermatitis) and/or onychomycosis, the underlying occurrence of immunosuppressive disorders (i.e. diabetes, immune-mediated, haematological diseases), and therapies (i.e. with antibiotics, corticosteroids, immunosuppressive drugs).

**Sampling collection procedure.** Samples were collected from environmental air (Group I), litter (Group II) and feed (Group III) from each shed, and from different anatomical sites (i.e. nostrils, pharynx, ears) of each farm worker (Group IV).

Air samples (n=57; Group I) were collected using the SAS System (PBI International) and Petri dishes containing Sabouraud dextrose agar with chloramphenicol (0.5 %) (SDA; Liofilchem Diagnostici). Air sampling was performed for 40 s in three different areas of each shed (i.e. entrance, middle and end of the sheds), at the level of the second cage floor, in order to obtain a mean bioaerosol exposure in each laying hen shed. A total of 120 dm³ of air per sampling was collected.

Faeces collected from each level of cage were pooled (10 g per pool, n=69 samples; Group II): 1 g of each sample was suspended in 9 ml sterile saline solution (NaCl 0.9 %).

In addition, random samples of 500 g each (n=19 samples; Group III) were collected from different points in a batch of feed and mixed thoroughly to provide a composite sample of 100 g for subsequent analysis. These primary samples were homogenized and aliquots of 10 g each were suspended in 90 ml of 0.1 % peptone-water solution. Serial dilutions from 10⁻² to 10⁻⁶ were made from each sample from Groups II and III: 0.1 ml aliquots of these samples were used to inoculate SDA medium. Finally, samples were collected from the nostrils, pharynx and ears of each of the 20 farm workers using a sterile cotton swab (n=60 samples in total, Group IV).

**Mycological culture and identification procedures.** All samples were cultured on SDA and incubated at 25 °C for 15 days. Fungi population size was evaluated and expressed as c.f.u. m⁻³ for samples from Group I, c.f.u. g⁻¹ for samples from Groups II and III, and c.f.u. per swab for samples from Group IV, respectively. For each positive sample, four colonies were subcultured on SDA slants for species identification. Identification of isolates was based on their macro- and microscopic morphology, as described by de Hoog et al. (2011).

All the Aspergillus spp. isolated from the environment were also identified molecularly at species complex level to confirm the morphological identification. Specifically, 32 Aspergillus isolates from Group I, morphologically identified as A. fumigatus (n=17), A. candidus (n=6), A. niger (n=4), A. flavus (n=3) and A. ochraceus (n=2), were subjected to molecular analysis; this was carried out by specific amplification and sequencing of target internal transcribed spacers (ITS) of the nuclear rDNA (White et al., 1990; Gadés & Bruns, 1993). The Aspergillus spp. isolates were cultured in SDA for 4 days at 25 °C and extraction of genomic DNA was performed from the cultured isolates using TRI Reagent (Sigma Aldrich), according to the manufacturer’s instructions. Briefly, the ITS region was amplified by using primer pairs ITS1/ITS4 (White et al., 1990) and ITS1-F/ITS4-B (Gardes & Bruns, 1993). PCRs were performed in 50 ml 0.2 mM each dNTP, 0.5 mM each primer, 1.2 U Taq (EuroClone) and 4 ml purified DNA. The

http://jmm.sgmjournals.org
Aspergillus spp. conidia were uniformly distributed in the environment without any statistically significant difference (P>0.05) among sampling areas (i.e. entrance, middle and end of the sheds; data not shown).

The occurrence of Aspergillus spp. in air samples, according to environmental and geographical data, is reported in Table 2. The airborne occurrence of Aspergillus spp. was significantly higher in sheds located at <90 m above sea level, housing >9000 hens, with temperature and humidity higher than 29 °C and 50 %, respectively. A high occurrence of airborne Aspergillus spp. conidia was observed in sheds where the concentration of Aspergillus spp. in foodstuffs was higher than 10^2 c.f.u. g^-1 (Table 2). An inverse correlation between the occurrence of airborne Aspergillus and conidia concentration in faeces was observed (P<0.05).

![Table 2. Occurrence of Aspergillus spp. in air samples according to environmental and geographical conditions in buildings housing laying hens](image)

Statistical differences are marked with the same superscript letter (lower case, P<0.05).
Table 3. Number and percentage (in brackets) of samples from air (Group I), pooled faeces (Group II), poultry feedstuffs (Group III) and farm workers (Group IV) positive for *Aspergillus* spp., divided according to the fungal species.

Number of isolates (I) for each species is also reported. Statistical differences among groups for each species are marked with the same superscript letter (lower case, P<0.05; upper case, P<0.01).

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Group I (n=57)</th>
<th>Group II (n=69)</th>
<th>Group III (n=19)</th>
<th>Group IV (n=60)</th>
<th>Total (n=205)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Positive/total (%)</td>
<td>I</td>
<td>Positive (%)</td>
<td>Positive/total (%)</td>
</tr>
<tr>
<td><em>Aspergillus candidus</em></td>
<td>6 (10.5)^A</td>
<td>0 (0.0)^A</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>3 (5.3)</td>
<td>7 (10.1)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>17 (29.8)^c</td>
<td>30 (43.5)^d</td>
<td>43</td>
<td>3 (15.8)^d</td>
<td>12</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>4 (7.0)^f</td>
<td>2 (2.9)^f</td>
<td>5</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td><em>Aspergillus ochraceus</em></td>
<td>2 (3.5)</td>
<td>1 (1.4)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>30* (52.6)^H</td>
<td>38* (55.1)^I</td>
<td>155 (52.4)</td>
<td>6* (31.6)</td>
<td>28 (9.4)</td>
</tr>
</tbody>
</table>

*Two samples were positive for two *Aspergillus* spp.

†One sample was positive for two *Aspergillus* spp.
spread via feedstuffs and faeces (Arnow et al., 1991; Humphreys et al., 1991; Groll et al., 1998; Alberti et al., 2001; Tell, 2005; Arné et al., 2011), whereas the latter may be introduced into the sheds from the outdoors. Indeed, up to 26% of outdoor airborne fungal spores can infiltrate indoors, carried by the wind or by workers (Fairs et al., 2010). Conversely, the presence of *A. flavus* in the environment and in the faeces, but not in the feedstuffs, might indicate that feedstuff is not the source of air contamination.

Although *A. flavus* was previously isolated from feedstuffs in other settings (Fulleringer et al., 2006), its absence in the feedstuffs herein tested indicates that they were screened for the presence of *A. flavus* and its toxin (e.g. aflatoxins) (Rodrigues et al., 2011).

Finally, the finding of all *Aspergillus* species in air samples (Group I), as well as in human samples (Group IV), suggests that laying hen farms might represent suitable vehicles of fungal infections for humans and/or birds, especially when suffering an underlying condition of immunosuppression and when coming in contact with a large number of airborne *Aspergillus* conidia (Morris et al., 2000; Alberti et al., 2001; Heinemann et al., 2004; Fulleringer et al., 2006; Prester, 2011; Sabino et al., 2012). However, the potential role of other environmental reservoirs of *Aspergillus* spp. propagules as a source of colonization/infection of farm workers cannot be ruled out. Moreover, it has been demonstrated that a concentration of *Aspergillus* spp. lower than 30 c.f.u. m\(^{-3}\) is not associated with clinical signs of aspergillosis in turkeys (Fulleringer et al., 2006), while 5 c.f.u. m\(^{-3}\) is the threshold concentration for human infections (Morris et al., 2000; Alberti et al., 2001). In the present investigation, the mean concentration of airborne *Aspergillus* conidia (70 c.f.u. m\(^{-3}\)) did not induce clinical disease in hens but it was enough to colonize farm workers. In fact, 40% of human nasal swabs and 35% of human ear swabs scored positive for *Aspergillus* spp. All these positive samples were collected from workers who declared they suffered respiratory disorders (i.e. asthma, dyspnoea, cough, rhinitis and/or bronchitis) or localized dermatitis. Although beyond the scope of the present study, the role of *Aspergillus* spp. as a potential aetiological agent of these clinical signs cannot be ruled out. Indeed, all *Aspergillus* spp. detected are known as major opportunistic pathogens for humans, being responsible for pathologies such as asthma, dyspnoea, headache, allergic and invasive infections (Jaakkola et al., 2002; Chapman, 2006; Sahakian et al., 2008; Fairs et al., 2010). Additionally, *A. fumigatus* has been proven to contaminate egg albumin and yolk, thus being responsible for significant economic losses (Kunkle, 2003).

Based on the results of this study, the risk factors associated with the occurrence of *Aspergillus* conidia in highly colonized environments should be accurately monitored (Arnow et al., 1991; Humphreys et al., 1991; Groll et al., 1998; Leenders et al., 1999; Alberti et al., 2001; Tell, 2005; Arné et al., 2011). Environmental and geographical conditions may influence the occurrence of *Aspergillus* conidia in the buildings. In particular, a temperature above 29 °C and a relative humidity higher than 50% were associated with contamination by *Aspergillus* spp. This is not surprising, considering that high temperature and humidity strongly affect the emission of the *Aspergillus* conidia from their sources (Fulleringer et al., 2006; Arné et al., 2011).

Finally, the inverse correlation between airborne *Aspergillus* occurrence and conidia concentration in faeces might be a consequence of animal movements under high stocking densities and of the ventilation system, which contributes to the dispersion of the conidia from the faeces to the air and, in turn, to the generation of a highly populated conidial aerosol.

In conclusion, the results of this study provide information on the distribution of *Aspergillus* airborne conidia in laying hen farms, and of the relationship between the occurrence of these fungi and human colonization. Even if the concentration of airborne *Aspergillus* conidia does not cause disease in birds, it can have an impact on

---

Table 4. Occurrence of *Aspergillus* spp. in farm workers, analysed according to answers to the questionnaire

<table>
<thead>
<tr>
<th>Symptoms/predisposing factors</th>
<th>Positive response (%)</th>
<th>Occurrence of <em>Aspergillus</em> spp. (%)</th>
<th>Negative response (%)</th>
<th>Occurrence of <em>Aspergillus</em> spp. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory</td>
<td>9 (45)</td>
<td>9/9 (100)(^A)</td>
<td>11 (55)</td>
<td>1/11 (9.0)(^A)</td>
</tr>
<tr>
<td>Dermatitis</td>
<td>6 (30)</td>
<td>6/6 (100)(^B)</td>
<td>14 (70)</td>
<td>0/14 (0)(^B)</td>
</tr>
<tr>
<td>Antibiotic therapies</td>
<td>2 (10)</td>
<td>1/2 (50)</td>
<td>18 (90)</td>
<td>0/18</td>
</tr>
<tr>
<td>Immunosuppressive disorders</td>
<td>0</td>
<td>0</td>
<td>20 (100)</td>
<td>0/20</td>
</tr>
<tr>
<td>Total</td>
<td>11 (55)</td>
<td>10/11 (90.9)(^C)</td>
<td>9 (45)</td>
<td>1/9 (11.1)(^C)</td>
</tr>
</tbody>
</table>

\(^*\)Six farm workers reported both dermatitis and respiratory symptoms.
human health. Hence, correct management of laying hen farms, including control of the microclimate (i.e. temperature and humidity) and appropriate cleaning procedures (e.g. removal of faeces several times a week, and/or use of proper disinfectants), is of importance to control the dispersion of airborne Aspergillus conidia, in order to reduce risk of animal and human infection, and to improve the hygienic quality and safety of table eggs.

ACKNOWLEDGEMENTS

We thank Dr Cánzia Cantacessi for revising the English text.

REFERENCES


