Potential poultry and meat products contamination by aflatoxin B1 due to fungal presence in Portuguese poultry units

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Abstract

The impact of mycotoxins on human and animal health is well recognized. Aflatoxin B_1 (AFB_1) is by far the most prevalent and the most potent natural carcinogen and is usually the major aflatoxin produced by toxigenic fungal strains. Data available, points to an increasing frequency of poultry feed contamination by aflatoxins. Since aflatoxin residues may accumulate in body tissues, this represents a high risk to human health. Samples from commercial poultry birds have already presented detectable levels of aflatoxin in liver. A descriptive study was developed in order to assess fungal contamination by species from *Aspergillus flavus* complex in seven Portuguese poultry units. Air fungal contamination was studied by conventional and molecular methods. Air, litter and surfaces samples were collected. To apply molecular methods, air samples of 300L were collected using the Coriolis μ air sampler (Bertin Technologies), at 300 L/min airflow rate. For conventional methodologies, all the collected samples were incubated at 27ºC for five to seven days. Through conventional methods, *Aspergillus flavus* was the third fungal species (7%) most frequently found in 27 indoor air samples analysed and the most commonly isolated species (75%) in air samples containing only the *Aspergillus* genus.

Moreover, *A. flavus* presented the highest level of airborne spores (>2000 CFU/m^3). Using Real-Time PCR, aflatoxigenic strains of *A. flavus* complex were obtained in three indoor samples from two different poultry units. Concerning new litter *A. flavus* was isolated in 9.9% of the samples and was not found in the used litter. Regarding surfaces *A. flavus* was found in 24% of the samples. It seems relevant to suggest that regular survey of aflatoxins should be done to evaluate food chain contamination aiming to create data to permit a detailed health risk assessment to poultry meat consumers.

Key words: poultry, aflatoxins, aflatoxin B1, meat contamination.

1   Introduction

In recent decades, the question of mould toxicity has attracted attention, especially in the fields of agriculture and food industry. Microscopic filamentous fungi often contaminate vegetal and animal products, and are commonly a source of diseases in man and animals [1]. The reason for an increasing interest in this field is their ability to produce secondary metabolites – mycotoxins – that can cause adverse effects, such as carcinogenesis and mutagenicity [2]. The presence of mycotoxins in food and feed stuff is result of a complex series of interaction among the causative fungi, the contaminated products, the various environmental factors and the intoxicated host [2].

Conditions that favor contamination by mycotoxins include excessive moisture both in field and post harvest storage, high humidity, extreme temperature, drought stress, and insect damage to crops [3].

Humans are exposed directly to mycotoxins through consumption of contaminated foods. Handling contaminated feed can also result in exposure to mycotoxins through the skin and by inhalation [4].

The impact of mycotoxins on human and animal health is well recognized. However, establishing a causal relationship between mycotoxins exposure and human disease can be difficult [5].

Aflatoxins, toxic metabolites mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are naturally occurring contaminants of food. Although aflatoxins have been a problem throughout history, they have been recognized as significant contaminants within agriculture only since the 1960s. *Aspergillus* growth is influenced mainly by temperature, moisture content and storage time. As aflatoxins often accumulate during food storage, post-harvest control at the farms aims to minimize fungal growth and, subsequently, aflatoxin production [6].

The four major types of the aflatoxins are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green, respectively). Aflatoxin B_1 (AFB_1) is by far the most prevalent and the most potent natural carcinogen and is usually the major aflatoxin produced by toxigenic strains [7–9].

Indirect exposure of humans to aflatoxins occurs through foods (primarily milk, liver, and eggs) derived from animals that consume contaminated feeds [10]. Aflatoxins are deleterious to poultry and their contamination in feed is practically unavoidable [11, 12].

Crops contaminated with aflatoxins are a worldwide problem and approximately 25% of world's food supply is contaminated with mycotoxins annually [13, 14]. Conditions that favor contamination by mycotoxins include excessive moisture both in field and post harvest storage, high humidity, temperature extremes, drought stress, and insect damage to crops [3].

Adverse weather conditions pre-harvest in many parts of the world arising from global climate change can conduct to an increasing frequency of mycotoxin contamination of poultry feeds. The complex nature of modern poultry rations including the increasing use of potentially contaminated by-products such distillers' dried grains adds the possibility of toxicological synergy between combinations of mycotoxins thereby increasing the severity of the response of poultry to contaminated feeds [15].

Acute or chronic aflatoxicosis in poultry birds results in decreased meat and egg production, immunosuppressant, and hepatotoxicosis [16–18]. Aflatoxin residues may also appear in body tissues and samples collected from the commercial poultry birds presented detectable levels of aflatoxin in liver [19–21].

As in human species, the liver is the most severely affected organ in poultry, primary consequences being hepatotoxicity and carcinogenicity [11, 22].

As far as public health problems are concerned, food contamination by aflatoxins poses a high risk to human health including acute aflatoxicosis, hepatocellular carcinoma, hepatitis B virus infection and growth impairment [23].

In humans, AFB_1 is activated by cytochromes P450 to AFB_1-8, 9-*exo*epoxide and AFB_1-8, 9-*endo*-epoxide, but it is the *exo*-epoxide which binds to DNA to form the predominant 8,9-dihydro-8- (N7-guanyl)-9-hydroxy-AFB_1 (AFB_1–N7-Gua) adduct [24]). AFB_1–N7-Gua confers the mutagenic properties of the compound [25, 26]). Other metabolites are formed from AFB_1, including AFQ1, AFM1 and AFP1. These metabolites and other naturally occurring aflatoxins (G1, B2 and G2) are poorer substrates for epoxidation and, consequently, are less mutagenic, carcinogenic and toxic than AFB_1 [27].

Besides being an important public health issue, aflatoxins result in economic losses to poultry industry due to reductions in growth rate, hatchability, feed efficiency and immunity towards diseases [3, 11, 28].

Keeping in mind the concerns above mentioned a descriptive study was developed in order to assess fungal contamination by species from *Aspergillus flavus* complex in seven Portuguese poultry units.

2   Materials and methods

This study was carried out in seven poultry farms located in the district of Lisbon, Portugal, between January and May 2011. All the units have more than one pavilion where birds are kept. The pavilions have natural and mechanical ventilation, but the latter is generally only activated in summer, when temperatures rise.

Air fungal contamination was studied by conventional and molecular methods. Twenty-seven air samples from seven poultries were collected at 140 L/minute, at one meter height, onto malt extract agar supplemented with chloramphenicol (MEA) for conventional methods. In order to apply molecular methods, air samples (300L) were collected using the Coriolis μ air sampler (Bertin Technologies), at 300 L/min airflow rate. Each air sample was collected into a conic sterile tube containing 10 ml sterile phosphate buffered saline and 0.05% Triton X-100. Five ml from the collection liquid was centrifuged at 2500g for 10 min and supernatant was removed to leave a 250 μl pellet that was subsequently used for DNA extraction. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's recommendations. Confirmation of fungal DNA extraction was obtained by amplifying a fragment of the 18SrDNA gene with universal fungal primers [29]. Aflatoxigenic fungi identification was achieved by using nor-1 gene specific primers and TaqMan probes in a Real Time PCR using an iQ Real Time PCR Detection System (Bio-Rad). Reactions included 1X iQ Supermix (Bio-Rad), 0.5 μM of each primer and 0.375 μM of TaqMan probe in a total volume of 20 μl. Amplification followed a three step PCR (polymerase chain reaction): 40 cycles with denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec.

Litter samples were collected and washed in 100 mL of sterilized distilled water, for 30 min at 100 rpm and 0.2 mL of this suspension was spread onto three malt extract agar plates (2%) with chloramphenicol (0.05 g/L). Surface samples of indoor spaces were collected by swabbing the surfaces using a 10 by 10 cm square stencil and then platted onto MEA. All the collected samples were incubated at 27ºC for five to seven days. After laboratory processing and incubation of the collected samples, quantitative (CFU/m^3, CFU/g and CFU/cm^2) and qualitative results were obtained, with identification of the isolated fungal species belonging to *Aspergillus flavus* complex.

3   Results

Through conventional methods, *Aspergillus flavus* was the third fungal species (7%) most frequently found in indoor air samples analysed. It was also the most commonly isolated species (75%) in air samples containing only the *Aspergillus* genus. Using Real-Time PCR, aflatoxigenic strains of *A. flavus* complex were obtained in three indoor samples from two different farms (Table 1). Moreover, *A. flavus* presented the highest level of airborne spores (>2000 CFU/m^3). Aflatoxigenic strains were not identified (by Real-Time PCR) in all samples where *A. flavus-complex* colonies were identified by conventional methods. On the other hand, we were able to detect the presence of aflatoxigenic strains in pavilions in which strains from *A. flavus-complex* did not grow in culture.

Concerning the new litter analysed, isolates from *A. flavus* complex was isolated in 9.9% of the samples but they were not found in the new litter. Regarding surfaces, isolates from *A. flavus* complex were found in 24% of the samples (Figure 1).

Table 1:   Distribution of aflatoxigenic strains of *A. flavus* complex.

Poultry farms	Air		Used litter	New litter	Surfaces
	Conventional	Molecular	Conventional	Conventional	Conventional
1	+	+	+	-	+
2	-	+	-	-	-
3	-	-	-	-	-
4	-	NA	-	-	+
5	-	-	-	-	-
6	+	-	-	-	-
7	-	NA	-	-	+

Note. For molecular biology, only air samples were tested. NA, not applicable.
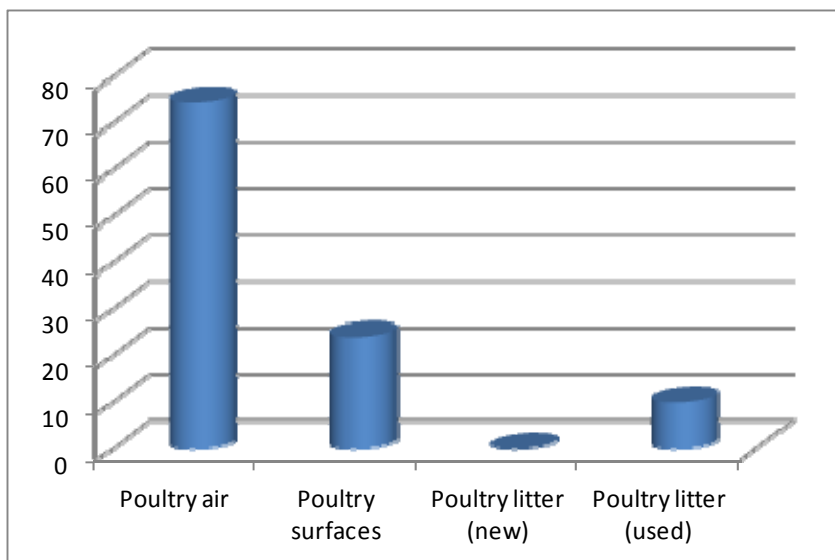

Figure 1:   *Aspergillus flavus* complex distribution among *Aspergillus* genus.

4   Discussion

Fungi are often used as indicators for mycotoxins presence in agricultural settings. However, in addition to quantification, fungi need to be identified at the species level in order to relate the identified species to a certain mycotoxin, and in case of aflatoxin production, at strain level because only some strains produce this mycotoxin while other strains are non-aflatoxigenic [30, 31].

Considering the results obtained for fungal contamination is possible to conclude that there is a high contamination by fungi responsible by aflatoxin production. Moreover, data obtained in this study allowed the discrimination of aflatoxigenic strains belonging to *A. flavus-* complex by the use of a specific and sensitive real time PCR to detect fungi with aflatoxin-producing potential. This methodology included the use of specific primers and probes for nor-1 gene,

which encodes a reductase enzyme (norsolorinic acid redutase) from the aflatoxin biosynthetic pathway. Using this approach, mycotoxin-producing strains were identified irrespective of fungal morphology and cultivability. Furthermore, PCR has the advantage of specific identification of fungal DNA, including all DNA-containing fungal structures, such as hyphae, which are important contributors to mycotoxin production and bioaerosol exposure [31]. The results showed the presence of fungal strains aflatoxin-producers in two out of five poultry units studied. From those, one had a "clean" outdoor environment for aflatoxigenic fungi. These results indicate that there are sources of toxigenic fungi inside the pavilions that may be either the cover materials or the animal feed [32–34]. In addition, it is important to consider that mycotoxins may be present in the environment long after death and disintegration of the producer [31, 35].

Meanwhile, it is important to mention that the workers of these poultry farms were enrolled in a study aiming to assess occupational exposure to AFB_1. A biomarker of internal dose was used providing information regarding recent exposure to AFB_1 and its intensity. Eighteen from thirty one poultry workers displayed detectable levels of AFB_1, in contrast to all individuals used as controls (n=30) who showed no detectable values. These findings corroborate the hypothesis that occupational exposure to AFB_1 by inhalation was occurring [36]. Moreover they also corroborate the possibility of poultry birds being exposed to AFB_1. Therefore, the presence of AFB_1 in meat and eggs is a quite plausible scenario.

In some experimental studies, poultry birds where fed with aflatoxin contaminated rations and resulted in the presence of aflatoxin residues in their edible tissues like liver and muscles [37]. Residues of AFB_1 in liver of broiler and layer birds have been reported to vary from no detection to 3.0 ng/g by feeding 250–3310 ng/g AFB_1 for variable periods [19, 37–40].

The wide variations in the tissue of aflatoxin residue concentration suggested that these levels might be influenced by different factors including dietary aflatoxin levels, duration of administration, age, type of the birds, etc. However, the effect of such factors upon concentration of AFB_1 residues in poultry meat (liver and muscles), and clearance of AFB_1 from the body tissues after withdrawal of dietary aflatoxin, have not been adequately studied [19].

Considering possible sources of contamination of the poultry setting [32–34], the most mentioned in the literature is the feed and probably is the most responsible for bird's exposure by ingestion. Consequently, feed becomes an indoor source for fungal contamination and also their metabolites such as mycotoxins.

In 2004, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) from European Food Safety Authority (EFSA) concluded that the current maximum levels of AFB_1 in animal feed (0.020 mg AFB_1/kg, Directive 2002/32/EC) provided an adequate protection from adverse health effects in target animal species. Among its recommendations, and to avoid higher level of feed contamination, the Panel encouraged regular monitoring of the presence of AFB_1 in imported feedstuffs in all EU member states. In the case of Southern

Europe countries, monitoring programmes for feed materials, especially maize and products thereof should be extended to cover materials originating from those parts of Europe, where a subtropical climate and extensive agricultural practice favour fungal growth and subsequent formation of aflatoxins [41].

Regarding human food products, the European Community and many other countries have imposed 2ng/g AFB_1 as maximum tolerance level [42].

In the case of birds and following ingestion, aflatoxins are rapidly metabolized into nontoxic substances in the body [43, 44] and it may not represent a significant human health risk. However, in areas with no regulatory limits on AFB_1 levels of poultry feed for instance, the secondary exposure to aflatoxins through consumption of chicken liver and meat may pose a risk to consumer's health.

Following the ALARA principle ("as low as reasonably achievable") in terms of an intension to keep the exposure to carcinogenic substances at the lowest achievable level, the levels of aflatoxins and their metabolites in human foods, like poultry meat, should be kept as low as possible. In 2007, and taking into account this principle, the CONTAM Panel of EFSA concluded that exposure to aflatoxins from all sources should be as low as reasonably achievable, because aflatoxins are genotoxic and carcinogenic. The same work group stated that data available indicate that reduction of total dietary exposure to aflatoxins could be achieved by reducing the number of highly contaminated foods reaching the market through more effective enforcement and reducing exposure from food sources other than almonds, hazelnuts and pistachios.

5   Conclusions

It seems relevant to suggested that regular survey of aflatoxins should be done to evaluate food chain contamination aiming to create data to permit a detailed health risk assessment to poultry meat consumers. Additionally, in poultry industry all the potential indoor sources of fungal dissemination must be monitored, aiming to assess and prevent fungal and, consequently, mycotoxin contamination.

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