Aflatoxin production by *Aspergillus flavus* isolates from green– tiger shrimps (*Penaeus semisulcatus*)

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ABSTRACT

**Backgrounds and Objectives:** to obtain information about aflatoxigenicity of isolated *Aspergillus flavus* strains from shrimps.

**Material and Methods:** Forty – three isolates of *Aspergillus flavus* from cultured green tiger shrimps of Persian Gulf were examined for their ability to produce aflatoxins. Initially two media; Aflatoxin Producing Ability medium and Coconut Agar medium were used to detect fluorescence under UV light, later the presence of aflatoxin in culture extract was confirmed and quantified by high pressure liquid chromatography.

**Results:** Only 2 (4.6%) isolates fluoresced on Aflatoxin Producing Ability medium and Coconut Agar medium under UV light. In sum, 9 (20.93%) isolates (including the 2 above mentioned isolates) were confirmed to be aflatoxigenic by High Performance Liquid Chromatography. Eight (18.7%) of isolates produced aflatoxin B₁ ranging from 0.32 to 12.18 ppb, while 1 (2.3%) of isolates produced 18.88 ppb and 0.36 ppb of aflatoxin B₁ and aflatoxin B₂ respectively. *Aspergillus oryzae* did not produce any detectable aflatoxins. Although highest level of aflatoxin B₁ (18.88 ppb) was detected in an isolate from a hepato-pancreatic sample, no histopathological change was observed in that tissue.

**Conclusion:** Some *Aspergillus flavus* strains which were isolated from shrimps showed aflatoxin producing ability without any histopathological changes in tissues of contaminated shrimps.

**Keyword:** Green Tiger shrimp, aflatoxin, HPLC, Iran.

INTRODUCTION

The aflatoxins are a group of mycotoxins produced by certain Aspergillus species, in particular *A. parasiticus, A. flavus, A. nomius,* and *A. pseudotamarii* (1). *Aspergillus flavus* is an important *Aspergillus* species with aflatoxin (AF) producing capability. Aflatoxins (AFs) B₁, B₂, G₁, G₂ (AFB₁, AFB₂, AFG₁ and AFG₂) are a group of toxic, mutagenic, carcinogenic and teratogenic polypeptide secondary metabolites with health hazards to humans and animals and can adversely affect agricultural productivity (2,3). AFB₁ was evaluated as a class 1 human carcinogen (1). The incidence of AF in food and feed is relatively high in tropical and subtropical regions where the warm and humid climate provides optimal conditions for the growth of moulds (4). Because moulds are present in soil and plant debris and are spread by wind currents, insects, and rain, they are frequently found in/on foods together with their associated mycotoxins (1). On the other hand, the culture of various species of shrimp has an economical importance in many parts of the world as well as in Iran. One of the problems facing the culture of shrimp farming is widespread disease which lead to heavy losses to industry. Additionally, disease caused by other factors, such as culture environments and feed, also influence the success of shrimp culture (5). One such factor with a global food safety concern is AF, a contaminant produced by *Aspergillus* during the processing and storage of feed. The green tiger shrimp (*Penaeus semisulcatus*) is the endemic species of Persian Gulf and is cultured in Helleh shrimp farm complex, located in the humid tropical environment of Bushehr in south of Iran. Therefore, aflatoxin producing potency of the *A. flavus* isolates from above mentioned shrimps and water of their culture ponds were examined.
MATERIALS AND METHODS

Fungal isolates. A total of 43 A. flavus strains that were isolated as mycoflora from cultured green tiger shrimps and water of their ponds were included in the present study. These strains were identified based on morphological characteristics during our previous study (6) and their aflatoxin producing ability was examined in the current study. Two additional toxigenic A. parasiticus (NRRL 2999) with capability to produce both aflatoxin B (B₁ & B₂) and G (G₁ & G₂) and also non-toxigenic A. oryzae (IMI 126842) were also included as positive and negative quality controls. These isolates were stored in distilled water and kept at room temperature until required for use.

Cultivation and observation of fluorescence. Stock cultures were maintained on plates containing potato dextrose agar (PDA) (E.Merck, Germany). Inoculations were done by conidial transfer to center of plates containing Aflatoxin Producing Ability (APA) medium supplemented with 0.3% β-cyclodextrin and Coconut agar medium (CAM) as described by Hara et al., Davis et al. and Gupa et al. respectively (7-9). The APA and CAM plates were then incubated at 28°C in the dark for 2-5 days and 7 to 10 days respectively. The reverse side of colonies were periodically observed under long – wave (365 nm) UV light for blue fluorescence.

AF Production and Analysis. For assessment of the AFs, Koehler et al. method (10), with some modification, was used. All isolates were cultured in 250 ml Erlenmeyer flasks containing 50 ml yeast extract–sucrose (YES) broth medium (2% yeast extract, 20% sucrose) (E.Merck, Germany). The cultures were incubated at 30°C for 8 days in the dark. Afterwards, the culture was filtered through Whatman no.1 filter paper. AFs were extracted from culture filtrates by adding 50 ml of chloroform to each flask and shaking for 15 min in gyro rotary shaker. The chloroformic extracts were then concentrated by rotary evaporator (Eyela N-1000 Japan) near to dryness.

The residue of each sample was resolved in 10 ml of 40% methanol (HPLC grade) and diluted in 60 ml PBS (pH 7.8). Regarding the AOAC official methods 999.07 (11), aflatest immunoaffinity columns (IACs) (Vicam, Water Town, MA) were used for clean-up of samples. For this purpose, 10 ml PBS was passed through the IAC at a flow rate of 2-3 ml / min. Whole diluted extract was then applied to the column, at a steady flow rate of 3 ml / min. Thereafter, after washing the column with 15 ml of double-distilled water, air was drawn through the column until dry. AF was eluted from the column into a 3 ml flask in a two step procedure. Initially, 0.5 ml methanol was applied on the column and passed by gravity. After 1 min, the second portion of 0.75 ml methanol was applied and collected. Finally, eluate was diluted with water (final volume 3 ml) and analyzed by High – Pressure Liquid Chromatography (HPLC).

Analysis of AFs using HPLC. Reverse-phase HPLC was mainly applied to quantitate AFs along with fluorescence detector followed by post column derivatization (PCD) involving bromination using a water HPLC system (pump 1525; fluorescence detector 2475, analytical column Nova – pack – (18 250 × 406 mm : 4 µm)) (12). We used Kobra cell and added bromide to the mobile phase to achieve PCD. 100 µl of diluted AF eluate was then injected into HPLC. Mobile phase was water–methanol- acetonitrile mixture with the 600: 300: 200 (V/V/V) ratio in addition to 350 µl of nitric acid 4mol / l and 120 mg potassium bromide with a 1 ml / min flow rate. The fluorescence detector was operated at wavelengths of 365 nm and 435 nm as excitation and emission respectively. AF levels were calculated by measuring the areas under the chromatogram and standard curves with C-R3A chromatopac (Shimadzu).

Histopathological Examination. Tissue sections were obtained from the other half of the specimens of hepato-pancreas, gills and external shells of shrimps which were previously collected and kept in Davidson's fixative (13). At this stage, only tissue specimens were included in the study, from which the aflatoxigenic A. flavus strains were isolated and their aflatoxigenicities established and quantitated by HPLC. Tissue sections were stained with haematoxylin and eosin and examined microscopically for presence of histopathological alterations.

RESULTS

Of 43 isolates of A. flavus, 9 (20.93%) produced some AFs on YES medium with mean concentration of 4.38 ± 6.95 ppb when analyzed by HPLC. Whereas only 2 (4.6 %) isolates were determined to be aflatoxigenic by presence of blue fluorescence on the reverse side of colonies after exposure to UV light on APA and CAM. AF production of these two isolates was also confirmed by HPLC. The results revealed that only the A. flavus isolates which produced AFs at concentrations of 19.25 and 12.18 ppb fluoresced on APA and CAM media. From these AF producer strains, 8 (18.7%) isolates produced only AFB₁ and
one strain produced both AFB1 and AFB2. In no case did aflatoxin G1 or G2 contribute to the total toxin produced. Production patterns of AFs by aflatoxigenic A. flavus isolates are presented in Table 1. The results indicated that AFB1 was the most commonly detected AF from A. flavus isolates ranging from 0.32 to 19.25 ppb (mean ± StD = 4.36 ± 6.58). Although in the present study highest level of AFB1 (18.88 ppb) was detected in an isolate from hepato-pancreatic sample, no histopathological changes were observed in that tissue. Moreover, none of the other shrimp specimens from which toxigenic A. flavus strains (with AFB1 concentrations of 0.32-12.18 ppb) were isolated, showed any histopathological changes.

**DISCUSSION**

The AFs are extremely potent mutagens and suspected human carcinogens. They can adversely affect animal health and agricultural productivity. In aquatic animals, AF can cause abnormalities such as poor growth, physiological disorders and histological changes that decrease production. Problems can be caused by many factors such as low quality of feed ingredient and inappropriate methods of feed storage, which lead to contamination of food and feed with toxigenic fungi. Additionally, feed contamination indirectly causes a risk to human health through the ingestion of AF residue in animal products. Due to the importance of this group of mycotoxins to human and animal health, screening of food and feed for presence of the AFs particularly AFB1, the most potent member of the group is routinely considered by many countries (14). Production of AFs is mainly reported to occur by some strains belonging to the three major species; A. flavus, A. parasiticus and A. nomius (3) and less frequently by some other Aspergillus species including A. bombycis, A. pseudotamarii and A. ochraceus as well as two Emericella species (1). Aflatoxigenic A. flavus strains have been reported from various crops, agricultural commodities, and soils (15, 16) and also from clinical specimens, but little is known about shrimp isolates (17, 18). In the present study, AF producing ability of A. flavus isolates from green tiger shrimps was investigated.

HPLC analysis indicated that 18.7% of all aflatoxigenic A. flavus isolates were able to produce only AFB1. However, only 1 (2.3%) was capable

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Fungal isolates</th>
<th>Fluorescence (UV light)</th>
<th>Concentration of AFs (ppb) on YES medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA</td>
<td>APA</td>
<td>CAM</td>
<td>AFs</td>
</tr>
<tr>
<td>16</td>
<td>A. flavus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>A. flavus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
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</tr>
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<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43</td>
<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>126842</td>
<td>A. oryzae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2999 (+control)</td>
<td>A. parasiticus NRRL</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>A. flavus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>126842 (-control)</td>
<td>A. oryzae IMI</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

of producing both AFB₁ and AFB₂. Vaamonde et al. reported that A. flavus strains from various crops may be different in their AF producing ability (15). They showed that 29% of the total A. flavus isolates were able to produce aflatoxins. In the same manner, Wei and Jong (20) and Koehler et al. (10) reported 34.91 and 54% of A. flavus strains as aflatoxin producer respectively.

On the other hand it has been established that cultured shrimps generally suffer from toxic effects of dietary AFs (4, 21–25) but no information on the contamination of shrimp’s tissue with toxigenic A. flavus strains was found. Although in the current study HPLC analysis revealed that 9 (20.93%) of A. flavus isolates are aflatoxin producers, no abnormalities were detected in gills, shells and hepatopancreatic tissue samples of shrimps examined. These findings suggest that the possibility of aflatoxicosis occurring in shrimp via toxigenic A. flavus strains is limited. However, the definite role of colonization with such fungi in shrimp must be investigated to achieve a better conclusion in the future.

Histopathological studies indicated that no alterations have been observed in the hepatopancreatic tissue of shrimps. These results are in agreement with findings of Bintvihok et al. (4), who demonstrated that higher degrees of hepatopancreatic damage occurred by 20 and 10 ppb of the AFB₁ in diet. In contrast to Bintvihok et al. (4), Boomyaratpalin et al. (24) showed that AFB₁ at 0.17 and 37 ppb caused no histological changes in hepatopancreatic tissues, while alterations of hepatopancreatic tissue occurred at concentrations of 74, 126 and 220 ppb. Ostrowski – Missner et al. (22) reported abnormal hepatopancreatic and antennal gland tissues by AFB₁, at 50 ppb. Wiseman et al. (25) as well as Ostrowski (22) showed histologic change in shrimps fed 50 to 300 mg of AFB₁. Histopathological changes were also observed by Boutista et al. (20) in tissue of shrimps given diets with 26.5 µg / kg AFB₁.

Although the APA and CAM were reported as special aflatoxin detecting media (7- 9), the present study showed that these two media are only capable of detecting AF concentrations above the 12 ppb. In conclusion, although aflatoxin–producing A. flavus strains have been reported to contaminate shrimps, no pathological changes were observed due to those fungi.

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REFERENCES


