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# Aflatoxin B1 Contamination Correlation with *Aspergillus flavus* in Animal Feed Raw Materials Imported through the Port of Tanjung Perak Surabaya, Indonesia

**Yunetta Putri Arios[1]*, Agustin Indrawati[2] and Joko Pamungkas[2]**

*[1]Indonesian Agricultural Quarantine Agency, Ministry of Agriculture, Stasiun Karantina Pertanian Kelas I Merauke, Indonesia*
*[2]Department of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural Universty, Bogor 16680, West Java, Indonesia*

***Corresponding author.***

## Abstract

This study aimed to measure the incidence rate of aflatoxin B1 contamination in feed raw materials (MBM and PPM) imported through Port of Tanjung Perak Surabaya, and its relation to the growth of the fungi that produce it. ELISA (Enzyme Linked Immunosorbent Assay) competitive was conducted to identify Aflatoxin B1 and followed by HPLC (High Performance Liquid Chromatography). Fungal identification was conducted as a macroscopic and microscopic observation. ELISA test showed 55 of 84 (55.95%) samples were positive for aflatoxin B1 contamination, while 3 of 7 (42.85%) samples tested by HPLC were positive. The result of fungal identification showed 1 of 12 samples positive *Aspergillus flavus*, and others types of fungi. AFB1 contamination in animal feed raw materials is likely to have occurred in the country of origin, whereas the growth of the fungus is due to the level of humidity and temperature during the transportation and storage process.

## Article Info

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## Keywords

Aflatoxin B1
*Aspergillus flavus*
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## Introduction

Human resources quality of a nation is influenced by many factors including the nutritional intake, especially the adequacy of animal protein. In this case, livestock has an important role to fulfill the adequacy of animal protein. The quality of fodder plays a very important role, because the meat that is expected to meet the adequacy of protein is a healthy meat from healthy livestock. This will be obtained if the animals consume healthy feed. Feed is a single or mixed food ingredient, either processed or unprocessed, which is given to animals for survival, production, and reproduction. As one of the important and strategic factors, feed must be maintained in quality and safety. Healthy feeds should contain a complete nutritional value, are also not contaminated physically, chemically, and biologically (harmful micro-organisms).

Feed ingredients or feed composers generally can not stored for long. Indonesia's tropical climate with high temperature and high humidity will accelerate the decreasing of raw material quality and growth of fungus during storage. Several other factors that accelerate the

damage of feed raw materials are postharvest handling, forgery and contamination on feed ingredients, as well as feed material production process. One of the microorganisms that often contaminates the feed is fungus or molds. Several types of molds that can be found in feed and ingredients are *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and *Mucor* spp. (Atanda et al., 2011). Mold contamination can cause health problems such as mycosis for animals. Disruption or disease is not only caused by mold, but also by the toxin produced by the mold. Some factors that support the occurrence of contamination of molds and toxins in the feed mainly are moisture and temperature. In Indonesia, *Aspergillus flavus* is the dominant mold contaminant on feed and ingredients. *Aspergillus* produces mycotoxins called Aflatoxin (B1, B2, G1 and G2), which can contaminate food during production, harvesting, storage, and food processing, which the most toxic is aflatoxin B1 (Williams et al., 2004).

Meat bone meal (MBM) and poultry by product meal (PPM) are animal feed raw materials containing proteins and amino acids which are essential for animal growth. Affordable prices and good nutritional content make materials like them used as raw material for animal feed (Gumus and Baki, 2013; King'ori, 2012). Indonesia still unable to produce these animal feed raw materials so as to fulfill the need for raw materials, the government imports from several countries such as Australia, Canada, New Zealand and United States. Supervision of food safety through both materials is necessary and need to be tested with aflatoxin contamination on raw material of animal feed imported to Indonesia because the potential of causing healthy problems especially for animal and human.

This study aimed to measure the level of aflatoxin B1 produced by *Aspergillus flavus* on raw animal feedstock (MBM and PPM) imported through Tanjung Perak Port of Surabaya, and to provide scientific data and information for the Indonesian Agricultural Quarantine Agency in order to establish aflatoxin B1 testing policy. The hypothesis of this research is the occurrence of aflatoxin B1 produced by *Aspergillus flavus* in animal feed raw materials (MBM and PPM) imported through Tanjung Perak Port of Surabaya.

## Materials and methods

Sampling was conducted at Tanjung Perak Port Surabaya. Sampling and testing were conducted from September 2016 to January 2017. Samples taken are MBM and PPM imported from Australia, USA and New Zealand. Testing of aflatoxin B1 contamination was carried out using enzyme linked immunosorbent assay (ELISA) method. Samples that showed positive results with ELISA test were confirmed by high performance liquid chromatography (HPLC) testing method and also cultured on the Sabouraud Dextrose Agar (SDA) medium to isolate fungus produce aflatoxin, *Aspergillus flavus*.

### Sample size

Samples tested were PPM originating from USA and MBM originating from USA, Australia and New Zealand. The sampling method follows The Grain Inspection Handbook (USDA, 2013). The sampling unit is selected on a container basis and uses a tool of shovels (long handles and short handles) and spears. Sample was taken (300 g), then inserted in a sterile plastic bag that had been code labeled, the date of collection and the country of origin of the sample.

The sample size is determined at 95% confidence level and calculated by equation of calculation formula of sample size according to Thrusfield (2006):

$$n = \frac{4PQ}{(L^2)}$$

Where, n = Sample size; P = Prevalence assumption (30%) (Bahri et al., 2005); Q = 1-P; L = Error desired (10%).

Based on the calculation, the sample size was 84 samples from 3 countries. The number of samples was taken proportionally based on the frequency and average time of PPM and MBM input during January-June 2016 (Table 1).

**Table 1.** The sizes per country based on import frequency of PPM and MBM in January-June 2016.

| Sl. No. | Country | Type of feed raw materials | Frequency Jan-Jun 2016 (x) | No. of samples |
|---|---|---|---|---|
| 1. | USA | PPM | 129 | 20 |
| 2. | USA | MBM | 287 | 34 |
| 3. | Australia | MBM | 89 | 12 |
| 4. | New Zealand | MBM | 138 | 18 |
| | **Total** | | | **84** |

### Sample MBM and PPM preparation

Sample was filtered with a 20 mesh filter, then weighed as much as ± 5 g and then put into the Erlenmeyer tube. Methanol 70% prepared by diluting absolute methanol with aquadest (comparison 7: 3). Preparation extracts of sample solution 70% methanol solution was added as much as 25 ml into an Erlenmeyer containing the sample. The samples were homogenized for 3 minutes (speed 200 rpm). The homogenized solution was filtered using Whatman paper no. 1. The filtrate solution is mixed with aquadest with a ratio of 1:1.

### ELISA aflatoxin test

Microplate that has been coated with captured antibodies directed against anti-aflatoxin antibodies prepared. Standard solution and filtrate solution were added as much as 50 µl per microplate well. A 50 µl conjugate enzyme was added at each well, after which 50 µl of anti-aflatoxin antibody solution was added to each well. The solution is mixed by rotating the plate slowly and incubated for 30 minutes at room temperature. The liquid in the well was removed by pressing the microplate on a clean filter paper to remove all liquids in the well. The washing solution was added as much as 250 µl at each well, then all the liquid was removed and repeated the leaching twice. After washing, a substrate solution of 100 µl was added to each well, mixing was done by rotating the plate slowly.

The plates were then incubated for 15 minutes at room temperature without light, then the stop solution was added as much as 100 µl to each well and homogenized slowly. Readings on ELISA reader can be done 15 minutes after giving stop solution. Data were obtained based on sample or standard absorbance readings on ELISA reader with wavelength 450 nm.

### Isolation and identification of *Aspergillus* sp.

Isolation was performed based on Dharmaputra et al. (2013). Isolation was done from the $10^{-1}$ to $10^{-5}$ series dilution stage, then each dilution was continued by the pour plate method. PPM/MBM weighed 25 g, then put into a 500 ml measuring cup and added sterile distilled water to a volume of 250 ml, the result of the dilution was $1:10^{-1}$ feed suspension. Then the suspension was transferred to a 500 ml erlenmeyer tube and homogenized using a shaker at a rate of 150 rpm for 1 minute. A total of 10 ml was transferred to 250 ml of an erlenmeyer tube and 90 ml of sterile dehydrated water was added, then homogenized.

The result of the obtained suspension was 1:100 or $10^{-2}$. The dilution series was made up to a $10^{-5}$ dilution suspension. Subsequently each dilution was transferred into 2 Petri dishes with 1 ml and added with 10 ml of liquid DG18 medium. The suspension and liquid in the Petri dish were then homogenized to form 8 to be well mixed and subsequently incubated at room temperature for 5-7 days.

### Fungal macroscopic observations

Each of the fungal colonies grown on Dichloran 18 Glycerol agar (DG18) medium were purified in Sabouraud Dextrose agar (SDA) medium, then incubated for 7 days and observed macroscopically. Observations including colony color, colony diameter, radial lines, and concentric circles. Fungal identification refers to the identification guidelines according to Larone (2002) and Campbell et al. (1996).

### Fungal microscopic observations

Identification of fungi microscopically is done by using slide culture Riddel method, i.e., glass object and glass cover is inserted into Petri dish which then sterilized by using autoclave, then sterile SDA media on Petri dish is cut in cube shape and the piece is placed by using ose needle on top Glass object. The spores that have been grown are inoculated on the sides of the cube-shaped SDA media pieces by means of an ose needle, then covered with a glass cover and incubated at 35°C for 48 hrs. After incubation, the closing glass was transferred to a sterile object glass that had been dyeed with lactophenol cotton blue, then performed under microscope.

### HPLC test method

### Sample extraction

The method of extraction was adopted from the method developed by Wijayanti (2010). A total of 50 g of sample was weighed and mixed with 5 g NaCl. Methanol and aquadest in the 80:20 ratio were added to the mixture of 100 ml. Once homogenized, the mixture was filtered with filter paper. A total of 10 ml of filtrate was mixed with 40 ml of distilled water.

The filtrate was filtered and accommodated in siring so that 10 ml of volume was obtained. The immunoafinity purification system begins by passing 10 ml of filtrate through the column at a rate of 1-2 drops/sec. Flushing was done with 10 ml water at a rate of 2 drops/sec. Affinity columns were then eluted with 1 ml methanol at the rate of 1-2 drops/sec. The resultant filtrate (1 ml ) was added to aquades test and injected into the HPLC system.

## Results analysis

The results analysis was conducted qualitatively by looking at peak and quantitative profiles by measuring concentrations based on standard curves. Setting the HPLC system with a flow rate of 1 ml/min, using methanol phase of motion : Aquadest (70:30), Shimpack ODS C18 diameter phase 5µm long 150 mm. Wave reading on Y 365 nm ultraviolet spectrophotometer detector at room temperature (25$^{o}$C).

## Data analysis

Data analysis used in this research is descriptive analysis by presenting data in the form of drawings and tables to describe the growing molds and aflatoxin B1 contamination.

## Results

The test using competitive ELISA showed 47 of 84 samples (55.95%) containing aflatoxin B1 contamination (Table 2). The highest results were PPM from USA as many as 15 of 20 samples (75%), followed by MBM from USA 21 of 34 samples (61.76%), MBM from New Zealand 9 of 18 samples (50.00%) and MBM from Australia as many 2 of 12 samples (16.67%) (Table 2).

**Table 2.** Results of aflatoxin B1 with competitive ELISA test.

| | MBM | | | | PPM | |
|---|---|---|---|---|---|---|
| | **USA** | **NZ** | **Australia** | **Amount** | **USA** | **Amount** |
| No. of Samples | 34 | 18 | 12 | 64 | 20 | 20 |
| Positive Samples | 21 | 9 | 2 | 32 | 15 | 15 |
| Positive (%) | 61.76 | 50 | 16.67 | - | 75 | - |
| Average (ppb) | 1.69 | 2.61 | 4.70 | - | 3.97 | - |
| Min (ppb) | 1.01 | 1.18 | 3.85 | - | 1.04 | - |
| Max (ppb) | 4.95 | 7.45 | 5.56 | - | 11.9 | - |

Samples tested by the HPLC (High Performance Liquid Chromatography) were taken from ELISA method results. The sample categorized into 3 groups: 1-4 ppb (3 samples), 5-8 ppb (3 samples), and 9-12 ppb (1 sample). There were 3 from 7 samples (42.85%) showed positive results (Table 3). Those are 2 PPM samples from USA (0.18 ppb, and 0.06 ppb) and 1 MBM sample from New Zealand (0.08 ppb). HPLC test also detects the presence of aflatoxin B2 (2.09 ppb and 0.02 ppb) in 2 PPM samples from USA and aflatoksin G1 (0.28 ppb) in sample PPM from USA (Table 3).

**Tabel 3**. Results of aflatoxin B1 with HPLC test.

| | MBM | | PPM | |
|---|---|---|---|---|
| | **New Zealand** | **Amount** | **USA** | **Amount** |
| No. of samples | 2 | 2 | 5 | 5 |
| Positive samples | 1 | 1 | 2 | 2 |
| Positive (%) | 50 | 50 | 40 | 40 |
| Average (ppb) | 0.08 | - | 0.10 | - |
| Min (ppb) | 0.08 | - | 0.03 | - |
| Max (ppb) | 0.08 | - | 0.18 | - |

The fungal identification showed varied results. Which 6 from 12 samples contain *Aspergillus niger, A. fumigatus* and *A. flavus*, which most of them was *Aspergillus fumigatus*. This can be seen from the preparation either macroscopically or microscopically. Each of the cultured samples appeared some other fungus (Table 4). The cultural and microscopic characteristics of fungal species

isolated from animal feed raw materials shown in Figs. 1-5.

Based on the culture of MBM and PPM conducted, it can be seen only 1 sample (sample number 7e, PPM from USA) which positively contains *Aspergillus flavus*. But there are several samples that also contain *Aspergillus* spp. such as *Aspergillus niger* and *Aspergillus fumigatus* (sample number 36, MBM from New Zealand; sample number 12, MBM from Australia; sample no. 3, MBM from New Zealand; sample no. 33, PPM from USA; sample number 5, MBM from Australia; and sample number 9e, PPM from USA) (Table 5).

**Tabel 4.** Results of culture of PPM and MBM on SDA medium.

| No. | No. of samples | Isolation (Total colonies) | No. of fungal colonies | Identification |
|---|---|---|---|---|
| 1. | 12 | 14 | 4 | *Penicillium* sp., *A. fumigatus*, *Cladosporium* sp. |
| 2. | 36 | 11 | 4 | *Cladosporium* sp., *A. fumigatus* |
| 3. | 54 | 3 | 3 | *Madurella* spp. |
| 4. | 3 | 4 | 2 | *Aspergillus fumigatus* |
| 5. | 9 | 4 | 3 | *Cladosporium* sp., *Synchepalastrum* sp., *Fusarium* sp. |
| 6. | 33 | 7 | 3 | *Cladosporium* sp., *Fusarium* sp., *Aspergillus* sp. |
| 7. | 5 | 9 | 6 | *Epidermophyton* sp., *Cunninghamella* sp., *Microsporum* sp., *A. niger*, *Trichophyton* sp. |
| 8. | 2e | 28 | 1 | *Cunninghamella* sp. |
| 9. | 6e | 40 | 7 | *Fusarium*, *Cephalosporium* sp., *Trichophyton* sp., *Acremonium*, *Chrysosporium* sp. |
| 10. | 2a | 13 | 5 | *Scopulariopsis* sp., *Syncephalastrum* sp., *Chrysosporium* sp. |
| 11. | 7e | 21 | 6 | *Rhizopus* sp., *Scopulariopsis* sp., *A. flavus* |
| 12. | 9e | 53 | 3 | *Absidia* sp., *Emmonsia* sp., *A. niger* |

**Tabel 5.** Results of aflatoxin B1 contamination test with competitive ELISA method, HHPLC and culture.

| No. | No. of samples | Feed faw materials | Country of origin | Test | | |
|---|---|---|---|---|---|---|
| | | | | ELISA (ppb) | HPLC (ppb) | Kultur |
| 1. | 12 | MBM | Australia | - | NT | - |
| 2. | 36 | MBM | New Zealand | - | NT | - |
| 3. | 54 | PPM | USA | - | NT | - |
| 4. | 3 | MBM | New Zealand | (3.51) | NT | - |
| 5. | 9 | MBM | USA | (1.79) | NT | - |
| 6. | 33 | PPM | USA | (1.23) | NT | - |
| 7. | 5 | MBM | Australia | (5.56) | NT | - |
| 8. | 2e | MBM | USA | (4.12) | NT | - |
| 9. | 6e | PPM | USA | (6.70) | - | - |
| 10. | 2a | MBM | New Zealand | (7.45) | - | - |
| 11. | 7e | PPM | USA | (11.9) | (0.03) | + |
| 12. | 9e | PPM | USA | (8.13) | (0.18) | - |
| 13. | 5e | MBM | New Zealand | (5.68) | (0.08) | NT |
| 14. | 10e | PPM | USA | (2.21) | - | NT |
| 15. | 11e | PPM | USA | (4.40) | - | NT |

*NT: not tested, (-): negative, (+): positive.


**Fig. 1:** Isolate 1. *Aspergillus niger* (a) Colony on SDA medium (b) Microscopic 20µ.

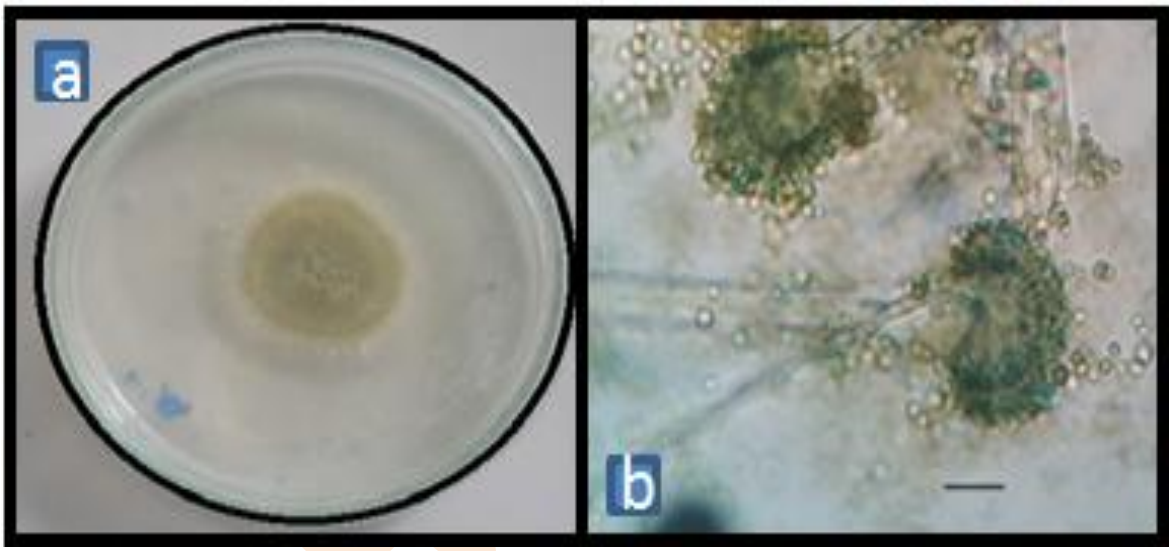


**Fig. 2:** Isolate 2. *Aspergillus flavus* (a) Colony on SDA medium (b) Microscopic 30µ.

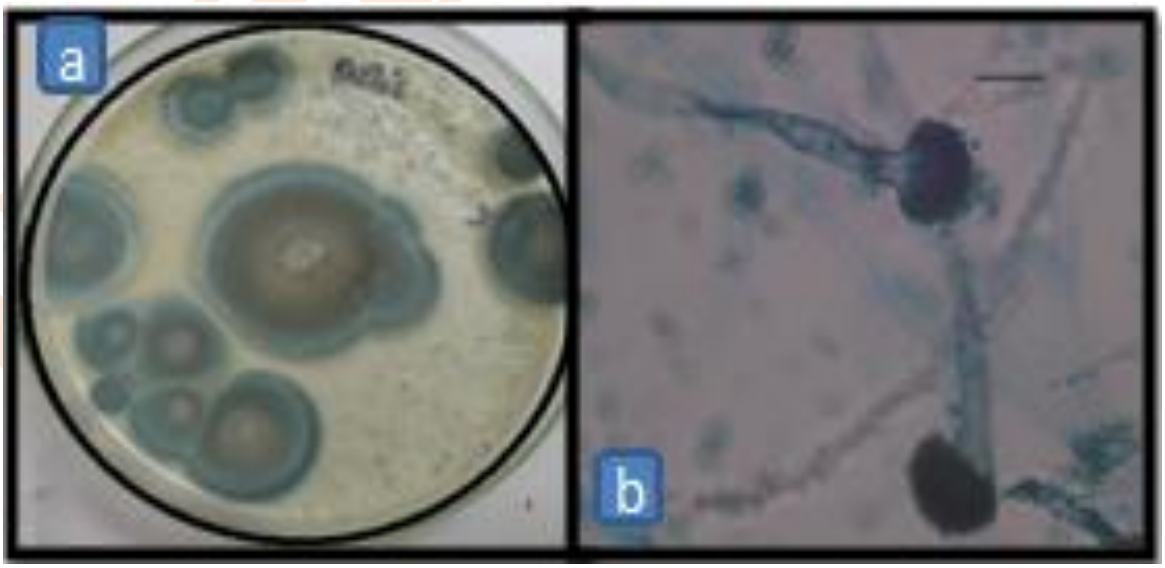


**Fig. 3:** Isolate 3. *Aspergillus fumigatus* (a) Colony on SDA medium (b) Microscopic 20µ.

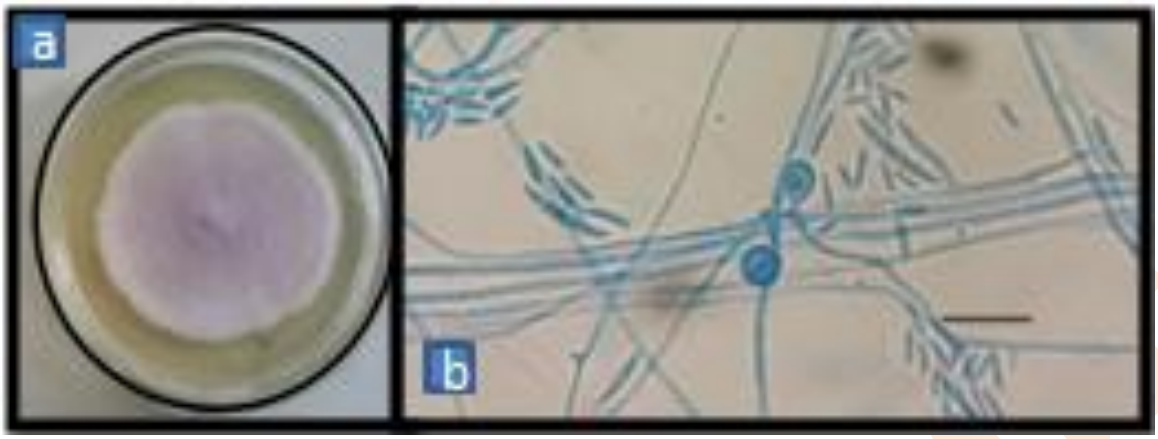


**Fig. 4:** Isolate 4. *Fusarium* sp. (a) Colony on SDA medium (b) Microscopic 20µ.

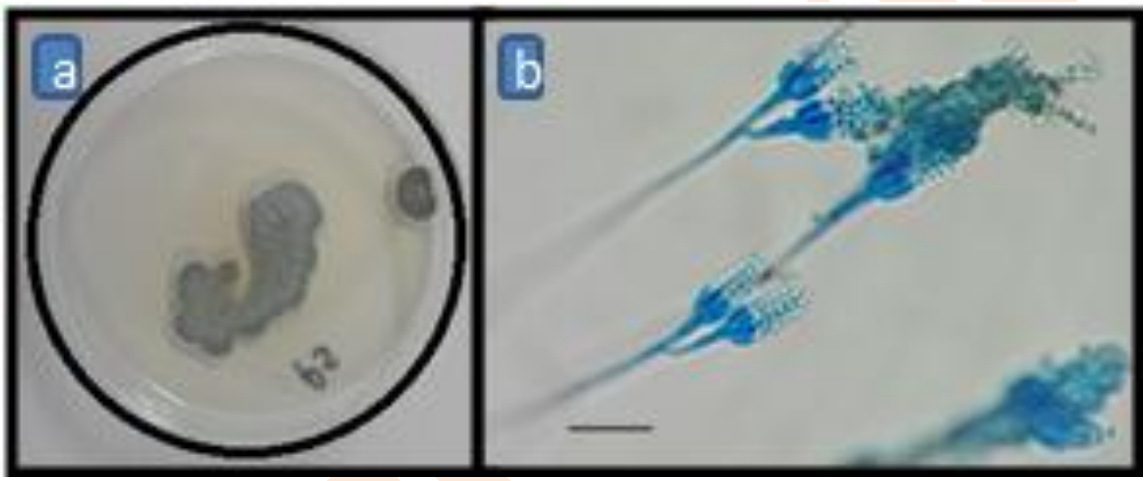


**Fig. 5:** Isolate 5. *Penicillium* sp. (a) Colony on SDA medium (b) Microscopic 20µ.

## Discussion

The incidence of aflatoxin B1 contamination is more than 50%. Feed raw materials have a potential to be contaminated by Aflatoxin caused by two things. First is from the source of the animal itself, which previously consumed fodder that has been contaminated by Aflatoxin B1. Widiasuti (2006) says that aflatoxin B1 can be accumulated and deposited in animals body then part of its body is used as the basic material of animal raw feed. The absorption of aflatoxin in the upper part of the small intestine (80-90% of what is eaten is absorbed), then further processed in liver. In fact, aflatoxin are not toxic per se, but require metabolic conversion by hepatic enzymes to the metabolically active metabolite exo-AFB1-8, 9-epoxyde (AFBO) to exert its toxicity (Chen et al., 2013).

The metabolism of aflatoxin B1 is associated with efficient hepatic cytochrome P450-mediated bioactivation and deficient detoxification by glutathione S-transferases (GST) (Rawal et al., 2010). The second, aflatoxin contamintion can occur during the storage and transportation. Atanda (2011) reports that environmental conditions related to storage, climate or intrinsic factors such as fungal strain specificity, strain variation, and instability of toxigenic properties could influence the presence of mycotoxins in feeds. The increased aflatoxin contaamination is an accumulation of aflatoxin contaminants from harvest to storage. Storage conditions that produce environments with abundant oxygen contents spur mold to produce aflatoxin (Rahmianna et al., 2007).

MBM samples from USA showed a high incidence of aflatoxin B1. It may be derived from animal feed raw materials which already have been aflatoxicosis, or can also occur when feed ingredients is on the way to Indonesia. As we know, USA is the farest importer

country and the ship board trips to Indonesia can be taken within weeks. The long journey or travel time and the long build up process in the home country can be a factor of *Aspergillus* spp. growth and produced aflatoxin. The quantity of aflatoxin is higher in commodities which comes from subtropical and tropical countries, where the environmental conditions are more suitable for mold growth and production of aflatoxin (Dani et al., 2012). Even the *Aspergillus* spp. has already gone by the treatment, the aflatoxin will still exist on the product.

The PPM sample from USA results seen as much as 15 of 20 (75%) positive samples contained aflatoxin B1. Based on the composition or content of the PPM itself, which comprises parts of the poultry such as the neck, legs, eggs, intestines and so forth, which is where aflatoxin deposits, it is probably that aflatoxin has been established in the country of origin USA. PPM also contains higher methionine and tryptophan than MBM, both of them are amino acids that can push aflatoxin produced by *A. flavus* and *A. parasiticus* (Payne and Hagler 1983). The results of research conducted by Galuh (2016), reports that aflatoksin B1 has been found on raw materials of animal feed imported through port of Tanjung Priuk, Jakarta. The aflatoxin B1 contamination comes from Australia (MBM), Canada (MBM), and United States (PPM).

The concentration of aflatoxin B1 contaminants in samples examined by competitive ELISA method value ranged from 1.01 to 11.9 ppb. According to SNI 7652.3: 2011, the value of the result does not exceed the maximum limit of aflatoxin contamination (40 ppb). It means aflatoxin contaminant levels still be tolerated to enter Indonesia.

There are also other fungi that grow in the culture medium, but most are the fungus that contaminate the MBM and PPM during transport, hoarding, or in the laboratory. Khotimah et al. (2015) found some potential contaminant fungus such as *Aspergillus flavus, A. fumigatus, A.niger, Penicillium citrinum, Cladosporium cladosporioides,* and *Fusarium verticillioides* in layer feed.

AFB1 contamination in animal feed raw is probably occur in the country of origin or at the time of transportation and stockpiling in the destination country. Although processing has been done in the country of origin, distant travel and container conditions may be a predisposition factor for the occurrence of aflatoxin fungus. The degree of humidity and temperature is crucial for *Aspergillus* spp. All containers used to transport animal feed raw materials are not equipped with temperature control and humidity devices. Temperature during transport and build up ranges from 28-35°C and 80-92% moisture, which is suitable for *Aspergillus* spp. growing and producing toxin. *Aspergillus* spp. grows and produces toxin at optimum temperature 25-32°C, 85% humidity, and water content 18% (Mousa et al., 2013).

The fungal identification showed *Aspergillus flavus* in 1 sample only (PPM sample from USA). It was the sample with highest ELISA (11.9 ppb) and positive value on HPLC test. Of the 7 positive samples of competitive ELISA tested, only 3 samples showed positive HPLC. This is probably due to the influence of protein content, pigment, and amino acids that estimated could increase toxin levels in testing by competitive ELISA methods (Rossi et al., 2012). Comparison of the ELISA method, ability to detect aflatoxins to be rugged, sensitive, accurate, precise and effective comparable to HPLC for measuring total aflatoxins ranging from 4 to 40 ppb (Zheng et al., 2005).

According to all samples, PPM is the most widely tested with competitive ELISA method which shows the highest incidence of Aflatoxin B1 (75%), while MBM from all samples tested 50% positive for Aflatoxin B1. Both types of animal feed raw materials (PPM and MBM) comes from the United States. Rodrigues et al. (2007) reports that the incidence rate of Aflatoxin contamination in animal feed in the USA tested by ELISA method was 20%.

Aflatoxin in animal feed raw amounts <40 ppb actually does not cause major problems, because the body will try to remove through the mechanisms of bioactivation, conjugation and deconjugation. This metabolism occurs in liver, but some are metabolized in blood and other organs. In these three stages, the body attempts to reduce the toxic effects of aflatoxin. Symptoms that may arise usually include decreased appetite, delayed growth and mild irritation due to tissue death (necrosis). Aflatoxin will be released by the body through bile, milk, egg, and urine. When aflatoxin can not be excreted from the body, there will be pathological changes and cause some symptoms

such as birth defects (teratogenic effects), cancer (human and animals) and the death (Wu et al., 2014).

## Conclusion

Competitive ELISA and HPLC methods have been used to determine aflatoxin B1 contamination in feed raw materials (MBM and PPM) imported through Tanjung Perak Port Surabaya and its association with *Aspergillus flavus*. There are 47 samples (15 PPM samples and 32 MBM samples) containing Aflatoxin B1. Of the 47 samples, there were *Aspergillus flavus*, the main fungi producer of aflatoxin B1. There are also *Aspergillus niger*, *Aspergillus fumigatus* and *Fusarium* sp. which can also produce aflatoxin.

## Conflict of interest statement

Authors declare that they have no conflict of interest.

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