Ochratoxin contamination in pig feed from pig farming centres in Indonesia

Variena Intansari, Agustin Indrawati*, Sri Murtini

Department of Animal Disease and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia

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Abstract. Ochratoxin A (OTA) is the most common toxin found in nature. Ochratoxin is a metabolic product by *Aspergillus* spp. and *Penicillium* spp. OTA produced by many *Aspergillus* or *Penicillium* species that contaminate animal feeds can cause diseases. This study aims to determine the incidence of ochratoxin contamination in pig feed such as pellets, meat bone meal (MBM), and tofu by products. The sampling method used the disease detection formula to collect 36 samples taken from several regions. North Sumatra, East Nusa Tenggara, Bali, Bulan Island, Solo, Lampung, Banten, Bogor and Jakarta. Ochratoxin contamination in animal feed was detected based on the Enzyme Linked Immunosorbent Assay (ELISA) using Agraquant® ochratoxin assay following the manufacturer's protocol. The analysis showed that 6 of the 36 samples (16.7%) were tested positive for ochratoxin contamination. Pellets and MBM were the feed and feedstuff which were found to contain ochratoxin. The concentration of ochratoxin in MBM was below the limit set by the National Agency of Drug and Food Control of the Republic of Indonesia (5 ppb). The high contamination of ochratoxin was found in pellets from Lampung (19 ppb). The fungi found to dominate the culture media and come from pellet feed were *Aspergillus flavus* and *Aspergillus niger*.

Keywords: *A. niger*, feed, ochratoxins, pig

INTRODUCTION

Indonesia has a number of pig farms located in the regions of Bali, North Sumatra, Kalimantan, East Nusa Tenggara, Sulawesi, and Papua. East Nusa Tenggara, North Sumatra and Bali have the highest number of pig populations compared to other regions (Ditjen, 2018). Pig productivity is determined by the management and feed quality. Pig feed is classified according to its purpose into three categories: starter, grower, and finisher. These three categories contain protein and carbohydrate components such as fish meal, corn, groundnut meal, soybean meal, tofu by product and livestock forage. Standard pig feed containing fish meal, rice bran, and soybean meal produces

profitable growth compared to commercial feed (Rumerung, 2015).

Small farms generally prepare feed by making their own rations. Feeding material containing grains can easily be contaminated by pathogenic fungi (Bahri, 2015). Contamination can occur from feed material or material that has been formulated. Fungal contamination in animal feeds can cause damages to the feed structures. Damage during storage can occur physically, chemically, and biologically (Bryden, 2012). Fungal contamination can reduce nutrient content in feed and may threaten animal safety, leading to food safety problems.

*Author for correspondence: Agustin Indrawati, Department of Animal Disease and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia. Email – titin.seta@gmail.com

Feeds contaminated with fungi can cause serious problems in animal safety and health. Fungi that contaminate feed can produce toxins such as animal mycotoxicosis and leave residues in livestock products, which can also be dangerous to human health. Residues have been found in meat and milk from livestock which consumed feed containing mycotoxins (Bryden, 2012; Widiastuti, 2014). Mycotoxins are secondary metabolites produced by *Penicillium*, *Fusarium*, *Aspergillus*, and *Alternaria* (Repeckiene *et al.*, 2013). Mycotoxins commonly found in feed include aflatoxin, ochratoxin, fumonisin, zearalenone, deoxynivalenol, and T2 toxin (Bahri, 2014).

Ochratoxin can be produced by several fungi, such as *Aspergillus ochraceus* and *A. niger*. *A. ochraceus* naturally grows on decaying seeds and plants. Studies have shown that one ochratoxin-producing fungus known as *A. ochraceus* can be found in animal feed such as corn, wheat, barley, and beans (Denli, 2015). Another fungus that can produce ochratoxin is *A. niger*. A study using 73 samples of *A. niger* showed that approximately 14% of the samples produced ochratoxin A (Pfohl-leszkowicz, 2007). Ochratoxin one type of mycotoxins, consists of three types, ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC). The most common type of ochratoxin found in nature is OTA. OTA is one of the most toxic and widely found in agricultural products and animal feed products. Animals that consume feeds contaminated with OTA can have nephrotoxins and hepatoxin (Denli, 2015). Other studies have also shown that in pigs OTA can cause nephritis, necrosis of the liver and lymphatic tissue (Pfohl-leszkowicz, 2007; Zhang *et al.*, 2015). Approximately 25 µg.kg⁻¹ of OTA added in pig feed can leaves a residue of more than 1 mg.kg⁻¹ in the liver, heart, and muscles, exceedings the safe limit set by European Union (Malagutti *et al.*, 2005).

The prevalence of mycotoxicosis due to ochratoxin contamination in North Asia, Southeast Asia and South Asia in feed samples was 24%, 9%, and 50%, respectively (Binder, 2007). The prevalence of mycotoxicosis due to ochratoxin in pigs from Indonesia has not been reported. This is perhaps due to the lack of information regarding ochratoxin contamination in pig feed. The ochratoxin contamination that

has been reported is limited to chicken feed. Therefore, the study aims to detect the content of ochratoxin contained in pig feed.

MATERIALS AND METHODS

The materials were pig feed samples obtained from several livestock centres in Indonesia, 70 % alcohol, *Dichloran 18 Glycerol Agar* (DG18) media, *Sabouraud Dextrose Agar* (SDA), methanol, and ochratoxin standard (Agraquant® ochratoxin assay). The tools were petri dishes, autoclave, Bunsen, incubator, plastic containers, microscope, digital scales, shakers, 250 mL Erlenmeyer, aluminium foil, high speed blender with a capacity of 500 mL, Whatman no. 1, and ELISA reader.

Sampling method and sample size

The pig feed samples were obtained from livestock centres in North Sumatra, East Nusa Tenggara, Bali, Bulan Island, Solo, Lampung, Banten, Bogor and Jakarta (Table 1). The sample size was determined at 95% confidence level using the Thrusfield method (Thrusfield, 2006). The formula used was: $n = 4PQ / L^2$, where n = sample size, P = prevalence assumption, $Q = 1 - P$ and L = the desired error magnitude. The amount of prevalence is 10%, while the desired error is 10%.

Sample preparation

The samples were ground and passed through a 20-mesh sieve (0.84 mm). Then, the subsample portion was thoroughly mixed. Approximately 20 g of ground samples was placed into a clean jar, which was then tightly sealed. Following this, approximately 100 mL of 70/30 (v/v) methanol solution was added into a jar, and the jar was then sealed. The sample and extraction solution was 1:5. The samples were shaken or blended for three minutes. Lastly, the samples were allowed to settle and filtered from the top layer of extract through a Whatman No.1 filter. The filtrate was collected afterwards.

Ochratoxin A assay by ELISA

The basic principle of the ELISA test is a reaction between an antigen and antibodies. The

procedure was performed according to Agraquant® ochratoxin assay instruction manual. An appropriate number of blue/green bordered dilution strips were placed in a microwell strip holder. Next a serial dilution process was performed as follows: 0, 2, 5, 20 & 40 ppb and 36 filtrate samples. Following this, an equal number of antibody-coated microwell strips were placed in a microwell holder. Approximately 200 µl of conjugate was dispensed into each blue/green-bordered dilution well. After that 100 µl of each standard or sample was added into the appropriate dilution well containing the 200 µl of conjugate. The solution was mixed by pipetting it up and down three times, and 100 µl content from each dilution well was immediately transferred into the corresponding antibody coated microwell, which was then incubated at room temperature for 10 minutes. After that the content of the microwell strips was removed. The washing steps were done by filling each microwell with distilled water and then dumping the water from the microwell strips. These steps were repeated four times for a total of five steps afterwards. Several layers of absorbent paper towel were laid on a flat surface, and the microwell strips were tapped on the towels to remove as much residual water as possible after the fifth wash. Following this, 100 µl of substrate was added into each microwell strip, and it was incubated at room temperature for 5 minutes. Approximately 100 µl of stop solution was added into each microwell strip afterwards. The absorbances were detected using a microplate reader in a 450 nm.

Fungal isolation

The fungal isolation step was conducted using serial dilutions ranging from 10^{-1} to 10^{-5} (Dharmaputra *et al.*, 2013). Approximately 25 g of pig feed was diluted in 250 mL sterile distilled water. 1 mL dilution was then poured into a petri dish containing Dichloran 18 Glycerol Agar (DG18) media.

Fungal macroscopic identification

Each fungal colony grown on Dichloran 18 Glycerol Agar (DG18) media was purified, and incubated for 7 days. The macroscopic observations included colony colour, colony diameter, radial lines from the centre of the colony to the edge of the colony, and concentrations of circles. The fungal macroscopic and microscopic identification process referred to Pitt (1997) and Ganjar *et al.* (1999).

Fungal microscopic observation

The fungal microscopic identification used the Riddel method of slide culture. The glass object and cover glass were inserted into a petri dish which had been sterilised using an autoclave. The sterile SDA media on petri dishes were cut into cubical shapes and placed using an inoculating needle on a glass slide that had been sterilised. Spores that had been grown were inoculated on each side of the SDA in the form of cubes using an inoculating needle, and then covered with a glass cover and incubated at 35°C for 48 hours. The glass cover after incubation was then transferred to the glass of a sterile object which had been given a Lactophenol cotton blue dye and was then observed under a microscope.

Table 1. Quantity of sample from areas in Indonesia.

No	Area	Pellet	Tofu By-Product	Meat Bone Meal	Total
1	North Sumatera	2	-	-	2
2	East Nusa Tenggara	5	-	-	5
3	Bali	3	-	-	3
4	Bulan Island	3	-	-	3
5	Solo	2	-	-	2
6	Lampung	2	-	-	2
7	Banten	1	1	-	2
8	Bogor	-	2	-	2
9	Jakarta	-	-	15	15
Total					36

Table 2. Ochratoxin assay for pig feed by ELISA.

Type	Total	Positive	Negative	Percentage
Pellet	18	4	14	28.6
Meat Bone Meal	15	2	13	15.4
Tofu By-product	3	0	3	0
Sample total	36	10	26	16.7

RESULTS

OTA in feed

The test on ochratoxin contamination in pig feed material using ELISA showed that 6 of 36 feed samples (16.7%) contained ochratoxin contaminant. Approximately 28.6% of the pellets from several areas showed higher results as compared to the MBM material (15.4%) (Table 2).

The MBM results in a positive incidence of ochratoxin contamination were 15.4%. This could have been caused by the animals used as raw materials which contained ochratoxin A (OTA). The concentration of OTA contamination in the MBM sample tested using a competitive ELISA ranged from 2.22 to 2.89 ppb.

Ochratoxin A producing fungi

Variable results were shown in the culture tests DG18 media. The isolation and identification of the total 36 samples total showed that 19 samples contained *Aspergillus flavus*, 24 samples contained *A. fumigatus* and *A. niger*, three samples contained *Penicillium* spp., and three samples contained *Rhizopus* sp. The presence of the fungi could be seen both macroscopically and microscopically. The samples in each dilution were also grown by several other fungi (Table 3).

DISCUSSION

The pellet feed showing positive results came from Bali, Bulan Island, Lampung, and Banten. The highest concentration of ochratoxin contamination in pellets came from Lampung at 19 ppb (Table 4). Ochratoxin contamination may have occurred during storage due to the humid storage location, which promotes fungal growth. Feed contamination may be affected by several environmental conditions during storage such as

water activity, temperature, specificity of fungal strains, and unstable toxigenic properties (Atanda *et al.*, 2011).

Several conditions such as humidity and temperature during storage may cause growth, which in turn lead fungi to produced ochratoxin. The optimum temperature during storage ranges between 28°C and 34°C, while 82-90% humidity is optimum for the growth of fungi producing ochratoxin. The optimum temperature for *Aspergillus* sp. to produce toxin is between 25°C and 32°C, humidity 85%, and moisture content 18% (Mousa, 2013).

The MBM samples were tested positive for containing OTA contamination. This was allegedly due to the raw materials containing OTA. The presence of OTA was found in dairy products derived from cows with a concentration of 5.0-6.6 ng/L (Boudra *et al.*, 2007). The high incidence rate was suspected because the livestock used as animal feed ingredients containing OTA residues. OTA was found in the serum of pigs consuming contaminated feed with a concentration in the kidney higher than that in the liver (Zhang *et al.*, 2015). Pigs have been shown to be more sensitive to ochratoxin than other animals. The OTA concentration in the MBM samples was below the limit determined by the National Agency of Drug and Food Control of the Republic of Indonesia which was 5 ppb for raw materials (BPOM, 2009). Feed with OTA concentration below the maximum limit was deemed safe for consumption by livestock.

Our study has shown that only one samples contaminated with OTA in the feed derived from tofu by-products. The concentration does not exceed the maximum contamination limit, meaning that the pulp remained safe to be consumed by livestock. The incidence of OTA contamination found in tofu by-products was lower in concentration than that in MBM. It is perhaps because tofu by-products are not stored

for a long time and commonly given in a fresh condition.

MBM cultures collected from January to March were, dominated by *Aspergillus* species including *A. flavus*, *A. fumigatus*, and *A. niger*. Fbd sample was dominated by *A. niger* and also *Penicillium* sp. This was thought to result in the positive ochratoxin contamination results in the MBM at 2.89 ppb. *A. niger* has the potential to produce OTA even though its concentration is not as large as *A. ochraceus* (Abarca *et al.*, 2003). Fungal contaminant in addition to *Aspergillus* also has *Penicillium* sp., which has the potential to produce OTA. *P. verrucosum* and *P. citrinum* are

fungi from the *Penicillium* group that can produce OTA (Taniwaki, *et al.*, 2002).

A. flavus (Figure 1) and *A. niger* (Figure 2) are fungi that dominate culture media and come from the pellet feed. *A. flavus* is commonly found in feed material (Ahmad, 2009). Feed from Lampung contained *A. flavus*, *A. niger*, *A. fumigatus*, and *Penicillium* sp. The feed also produced the highest ochratoxin contamination compared to the other feed samples. *Aspergillus* sp. and *Penicillium* sp. are fungi that are able to produce ochratoxin and can cause nephrotoxic, hepatotoxic, carcinogenic, teratogenic, and immunosuppressive (Wang *et al.*, 2016).

Table 3. Fungal contamination with negative Ochratoxin Assay using ELISA.

Type	Sample code	Fungal Contamination	Fungal Population CFU/mL
Pellet	1. Nta	<i>A. flavus</i> , <i>Syncephalastrum</i> sp.	1.5×10^4
	2. Ntb	<i>A. flavus</i> , <i>A. fumigatus</i>	4×10^4
	3. Ntc	<i>A. flavus</i> , <i>A. niger</i>	6.5×10^4
	4. Ntd	<i>A. flavus</i> , <i>A. niger</i> , <i>Fusarium</i> sp., <i>A. fumigatus</i>	8×10^4
	5. Nte	<i>A. niger</i> , <i>A. fumigatus</i>	1.5×10^4
	6. Sla	<i>A. niger</i> , <i>A. flavus</i>	2.5×10^4
	7. Slb	<i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	1×10^3
	8. Mda	<i>A. niger</i>	8.5×10^3
	9. Mdb	<i>A. niger</i> , <i>A. fumigatus</i>	2×10^4
	10. Bna	<i>Cladosporium</i> sp., <i>Fusarium</i> sp., <i>A. niger</i> , <i>A. flavus</i>	5×10^3
	11. Bnc	<i>Fusarium</i> sp., <i>A. niger</i> , <i>Cladosporium</i> sp., <i>A. fumigatus</i>	6×10^4
	12. Blb	<i>A. niger</i> , <i>A. fumigatus</i>	18×10^4
	13. Blc	<i>A. niger</i> , <i>A. flavus</i>	5×10^3
	14. Tpa	<i>A. flavus</i> , <i>A. niger</i> , <i>Fusarium</i> sp., <i>A. fumigatus</i>	114×10^4
Meat Bone Meal	1. Jna	<i>A. flavus</i> , <i>Fusarium</i> sp.	5×10^3
	2. Jnb	<i>A. fumigatus</i> , <i>Fusarium</i> sp.	5×10^3
	3. Fba	<i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	5×10^2
	4. Fbb	<i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	3×10^3
	5. Fbc	<i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	3.5×10^3
	6. Fbe	<i>A. niger</i> , <i>A. fumigatus</i> , <i>Fusarium</i> sp.	1×10^4
	7. Mrb	<i>A. flavus</i>	5×10^3
	8. Mrc	<i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	5×10^3
	9. Mrd	<i>A. fumigatus</i>	2×10^4
	10. Mre	<i>A. flavus</i> , <i>A. fumigatus</i>	5×10^3
	11. Mrf	<i>A. flavus</i> , <i>A. fumigatus</i>	5×10^2
	12. Mrg	<i>A. niger</i> , <i>A. fumigatus</i>	1×10^4
	13. Mrh	<i>A. fumigatus</i> , <i>A. flavus</i>	5×10^2
Tofu By-Product	1. Bga	<i>Rhizopus</i> sp.	91×10^4
	2. Bgb	<i>Rhizopus</i> sp.	51.5×10^4
	3. Tpb	<i>Rhizopus</i> sp.	4.5×10^4

Table 4. Fungal contamination with positive Ochratoxin Assay using ELISA.

Type	Sample code	Positive OTA	Contaminated fungi	Fungal Population CFU/mL
Pellet	1. Bla	4.33	<i>A. niger</i> , <i>A. fumigatus</i>	5×10^2
	2. Bnb	3.44	<i>Fusarium</i> sp., <i>A. niger</i> , <i>A. flavus</i> , <i>A. fumigatus</i>	1×10^3
	3. Lpa	2.22	<i>A. niger</i> , <i>A. flavus</i> , <i>Penicillium</i> sp.	1×10^3
	4. Lpb	19	<i>A. fumigatus</i> , <i>A. niger</i> , <i>Penicillium</i> sp	2.5×10^3
Meat Bone Meal	1. Fbd	2.89	<i>A. niger</i> , <i>A. fumigatus</i> , <i>Penicillium</i> sp.	1×10^3
	2. Mra	2.29	<i>A. niger</i> , <i>A. fumigatus</i>	2.5×10^3

Figure 1. *Aspergillus flavus*. **Figure 2.** *Aspergillus niger*.

Ochratoxin concentration in the MBM and tofu by-products was less than 5 ppb and therefore may not cause serious problems. Ruminants can detoxify ochratoxin, unlike pigs that can leave residues in livestock products. OTA residues in pigs can be found in the kidney, liver, and muscle tissues (Bataccone *et al.*, 2010). Symptoms that may be caused include decreased appetite, diarrhoea, polyuria, and weight loss. Detoxification failure by animals can result in reproductive disability and even death (Wang *et al.*, 2016).

CONCLUSION

The incidence rate of OTA contamination produced by *A. niger* and *Penicillium* spp. in the feed pellets was 28.6 % higher than that in the MBM and tofu by-products. *A. niger* was the most common fungus producing OTA, and was detected in 24 feed samples, while *Penicillium* spp. Was present in three pellet feed samples. Other fungi that produce mycotoxins were also found in feeds such as *A. flavus*, *A. fumigatus*, and *Fusarium* spp.

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