SURVEY ON OCCURRENCE OF AFLATOXINS IN CHICKEN FEEDS FROM PENINSULAR MALAYSIA

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ABSTRACT. This study was conducted to observe the occurrence of aflatoxin in chicken feed from Peninsular Malaysia. A total of 336 samples of chicken feed from Peninsular Malaysia were conveniently collected in this survey. The chicken feed represented the following three categories which are starter, grower and finisher. All samples were collected from local poultry farms in East Coast Region (Kelantan, Terengganu, and Pahang), Northern Region (Perlis, Kedah, Penang, and Perak), Southern Region (Malacca, Johor) and Central Region (Selangor, Negeri Sembilan) of Peninsular Malaysia for a period of six months (July-December 2015). Enzyme-linked immunosorbent assay (ELISA) was used for screening of total aflatoxin (TA) in the samples. High performance liquid chromatography (HPLC) with fluorescence detector was used for determination of aflatoxin B and G. Moisture content of samples was determined using the hot air oven method (AOAC International, 2011). Overall, the incidence of positive TA >20 µg/kg in chicken feed is 14.9% (50 samples). The average level of TA was found significantly different between different states at $p<0.05$ for both broiler grower and finisher. The chromatograph results showed that positive samples were found in broiler finisher from Kedah (94.6 µg/kg and 42.1 µg/kg) and Penang (56.4 µg/kg) with aflatoxin B1. In this study, the range of moisture content were around 6.5 -27.3%. About 40% samples have more than 12% moisture content. One of the predisposing factors for aflatoxin accumulation in chicken feed is moisture content. The results warrant the need for surveillance and constant monitoring programmes for the prevention of aflatoxin incidence in poultry farms.

Keywords: aflatoxins, occurrence, chicken feeds, ELISA, HPLC

INTRODUCTION

Mycotoxins are natural contaminants in raw materials, food and feeds (Binder *et al.*, 2007). Mycotoxins can be toxic secondary metabolites produced by fungi growing on crops in the field, during handling and in storage. More than 500 different mycotoxins are known (Nemati *et al.,* 2014; New, 1987) Aflatoxins, a class of mycotoxins produced by the fungi *Aspergillus parasiticus* and *Aspergillus flavus*, are major contaminants of common

feed ingredients used in poultry rations (Joffe *et al*., 1969; Khalil *et al*., 2015). These fungi are found in many countries, especially in tropical and subtropical regions, where the temperature and humidity conditions are optimal for the growth of moulds and the production of toxins. Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2). *Aspergillus flavus* typically produces AFB1 and AFB2, whereas *Aspergillus parasiticus* produces AFG1 and AFG2 as well as AFB1 and AFB2 (Khalil *et al.,* 2015).

Contamination by aflatoxin can take place at any point along the food or feed chain from the field, harvest, handling, shipment and storage under a wide range of climatic conditions. When aflatoxin-contaminated feed is consumed by poultry, important production parameters including weight gain, feed intake, feed conversion efficiency, and reproductive performance are compromised. Aflatoxicosis in poultry and animal also causes changes in biochemical and hematological parameters liver and kidney abnormalities, and impaired immunity, which may enhance susceptibility to infectious diseases (Ghulam *et al.,* 2014). In general, most fungi need at least 1-2% oxygen and usually grow at temperatures between 20 and 30 °C (Khalil *et al*., 2015). It is important to note that if the grain is at high temperature at harvest, it can maintain that high temperature for several days or weeks after harvest unless the storage facility has cooling capabilities. As temperature and moisture levels are key factors for fungal growth and subsequent mycotoxin production, the climate plays a key role in the occurrence of mycotoxins (Schindler *et al*., 1967; Zinedine *et al*., 2007).

Mycotoxins are not only dangerous for the health of consumers, they also deteriorate the marketing quality of the contaminated products; thus, involving strong economic losses (Schindler *et al.,* 1967). Mycotoxicological control of feed is a procedure aiming to protect human and animal health, avoid the adverse effects of these undesirable substances (Khalil *et al*., 2015). Different countries have imposed different legal limits on various food items and animal feeds. The aflatoxin level in animal feed is generally higher than for human consumption (Nemati *et al*., 2014). US Food and Drug Administration (FDA) and European Commission have set the maximum tolerable levels (MTL) for AFB1 and TA in cereals for human consumption as 2 and 4 µg/kg, respectively and recommend 20 µg/kg TA as the worldwide range of MTL/permissible levels for poultry (FAO, 2004). In Malaysia, the permissible levels proposed by Department of Veterinary Services (DVS) under Feed Quality Assurance Programme using HACCP concept are 20 µg/kg for finished feeds while Malaysian Feed Regulation are in progress to be enforced.

In Malaysia, there has been no comprehensive study regarding contamination of chicken feed with

aflatoxin. Based on uncertainties which have been shown in previous DVS laboratory reports and the huge concerns of practitioners in animals and poultry, further investigations on feed ingredients and products are required to keep our food and feed fairly safe. Thus, this study is to observe the contamination of chicken feed with aflatoxin in the Peninsular Malaysia.

MATERIAL AND METHODS

Sample collection

A total of 336 chicken feed were collected from local poultry farms in the east coast regions (Kelantan, Terengganu, and Pahang), northern regions (Perlis, Kedah, Penang, and Perak), southern regions (Malacca, Johor) and central regions (Selangor, Negeri Sembilan) of Peninsular Malaysia for a period of six months (July-December 2015). The chicken feed represented the following three categories which are starter, grower and finisher.

Samples and sampling procedure

Samples of chicken feed were taken directly at poultry farms and analysed in the period from July 2015 to January 2016. Samples were collected by appointed officers *Pegawai Diberi Kuasa* (PDK). PDKs were strongly encouraged to follow the principles of the Akta Makanan Haiwan 2009 guidelines on "Sampling and Sample Preparation for Feed Analysis". PDKs were also asked to indicate origin and type of material sent in. The quantity of samples sent to laboratory was 500 g each by random selection from a whole lot.

Reagents

Aflatoxin standard mix (B1, B2, G1 and G2) were purchased from Biopure®Referenzsubstanzen GmbH, Austria. Organic solvent and other chemicals were purchased from Merck AG, Germany. Water was purified by the Milli-Q Biocel (Bedford, MA, USA).

Moisture analysis

Moisture content of samples was determined using the hot air oven method (AOAC International, 2011). The samples were dried for 4 hours at 103±2°C. The moisture content of each sample is expressed as a percentage by weight, of the dry sample.

Aflatoxins Total Analysis by ELISA

All chicken feed samples were ground and homogenized using Ultra Centrifugal Mill, Retsch, to pass through 1 mm sieve. About 5 g of ground sample was extracted in 25 ml of methanol 70%. The extract was filtered through a Whatman filter (No. 1) and 1 ml from the filtered sample was then diluted by 1 ml of deionized water. 50 µl of diluted filtrate per well was used for the ELISA test (Aflatoxin Total Fast, ELISA Kit), R-biopharm. The optical density was measured at 450 nm using ELISA 96-well

plate reader (Biotek, USA). Absorbance percentages were taken and the calibration curve obtained with standards at different concentrations. The standard range is 0, 1.7, 5, 15 and 45 µg/kg (ppb). The ELISA data and the aflatoxin concentrations for samples were evaluated using software program R-biopharm (Ridasoft win, version 1.78, R-biopharm, Germany). The LOD for the total aflatoxin kit is < 1.7 µg/kg.

Aflatoxin B and G Analysis by HPLC

Positive samples from ELISA were further confirmed with HPLC. HPLC analyses were performed using HPLC Waters System With Fluorescence Detector (model 2475), Waters Auto Sampler (model 717), Waters Pump Controller (model 600), HPLC mobile phase filtering and degassing, and photochemical post column derivatization (UVE LC Tech, Germany). Chromatograph separation of aflatoxin was done by Waters Symmetry C18 µm, 3.9×150 mm column at ambient temperature. The mobile phase applied was acetonitrile:methanol:water (15:30:55). The flow rate was 1 ml/min with injection volume 10 µl. A UVE was used for post-column derivatisation, fluorescence detector setting were 365 nm (excitation), 455 nm (emission). A 20 g ground and homogenized sample was extracted in 100 ml of methanol 70%. The extract was filtered and diluted before proceeding to sample clean-up procedures. Sample clean-up was performed by AflaStar™ (Romer Labs) immunoaffinity column for the purification of aflatoxin in conjunction with HPLC. Quality control of analytical processes was performed by spiking uncontaminated feed at two levels 5 µg/kg and 10 µg/kg using aflatoxin mix standard. A six-point calibration standard (5, 10, 15, 20, 25 and 30 µg/kg), blank, and control samples were performed with the same batch of unknown samples.

Data analysis

Data were summarized and analyzed using SPSS (version 12.0) and Duncan's multiple range was used to determine differences in the means among samples obtained from the different states ($p = 0.05$).

RESULTS AND DISCUSSION

The results of aflatoxin occurrence of chicken feed by screening using ELISA are presented in Table 1. Overall, the incidence of positive TA >20 µg/kg in chicken feed is 14.9% (50 samples). The highest percent of positive TA were observed in broiler starter from Malacca (50%), with average and maximum contamination levels of 49.4 µg/kg and 80.36 µg/kg, respectively. The incidence of positive TA in broiler grower and broiler finisher from different states are 24.2% and 37.9%, respectively. The range of aflatoxin occurrence were 1 µg/kg to 169.8 µg/kg and 1.6µg/kg to 167.84µg/kg, respectively. Penang has more than 50% positive samples in both types of chicken feed (grower and finisher). Out

of the total samples analysed in Penang, the average and maximum contamination levels in broiler grower were 33.4 µg/kg and 79.34 µg/kg, respectively and in broiler finisher were 38.98 µg/kg and 103.56 µg/kg, respectively. However, the highest contamination level of aflatoxin was detected in broiler grower from Pahang with 169.77 µg/kg. The average level of aflatoxin was found significantly different between different states at $p<0.05$ for both broiler grower and finisher.

A total of 50 samples which exceeded the legal limit of 20 µg/kg from ELISA was further analysed by HPLC method. Figure 1 was showed HPLC chromatogram for standard, blank sample and control sample. The percentage of recovery for aflatoxin B and G were above 80% in both spiking levels. Occurrence of aflatoxin

Table 1. Occurrence of aflatoxins in chicken feeds in Peninsular Malaysia

Chicken feeds	States	N	Range (%) Min-Max	Mean±SD (µg/kg)	Mean ± SD (µg/kg)	
					< 20 µg/kg	> 20 µg/kg
Broiler starter, n= 3	Malacca	2	18.4 - 80.4	49.4±43.8	18.4	80.4
	Pahang	1	6.3	6.3	6.3	0
Broiler grower, n=114	Penang	7	14.3 - 79.3	33.4±24.4^{c}	16.5±2.3	46.1±26.3
	Perak	10	4.5 - 48.4	22.0±17.4^{abc}	6.4±5.3	37.6±6.9
	Malacca	4	7.9 - 20.6	9.6±8.5^{ab}	8.9±1.4	20.6
	Johor	4	1.3 - 3.2	1.8±1.4^{a}	1.8±1.4	0
	Selangor	9	2.9 - 16.7	7.8±4.3^{ab}	7.8±4.3	0
	Kelantan	39	1.0 - 28.2	8.7±8.3^{ab}	5.3±4.1	24.2±2.5
	Terengganu	28	1.0 - 28.4	6.9±8.2^{ab}	5.3±6.1	27.2±1.6
	Pahang	13	5.3 - 169.8	22.8±45.2^{bc}	5.4±4.3	62.0±71.9
Broiler finisher, n= 219	Perlis	12	2.3 - 103.9	15.8±28.0^{a}	7.8±4.0	103.9
	Kedah	36	1.9 - 46.6	15.1±14.2^{a}	6.4±4.6	32.5±9.9
	Penang	9	20.2 - 103.6	39.0±28.8^{b}	0	43.9±26.5
	Perak	33	1.7 - 167.8	26.2±39.3^{ab}	7.1±5.0	59.4±50.5
	Malacca	9	5.2 - 36.9	19.9±13.8^{ab}	27.5±7.1	30.4±6.0
	Johor	32	1.1 - 70.9	9.2±13.3^{a}	6.5±5.8	50.5±28.9
	Selangor	26	2.2 - 85.6	19.3±22.5^{ab}	11.4±1.7	11.9±2.0
	Negeri Sembilan	30	1.6 - 140.0	26.5±35.4^{a}	9.3±7.0	56.2±44.9
	Kelantan	4	4.1 - 33.8	16.4±13.8^{a}	5.3±1.8	27.5±8.9
	Pahang	28	5.0 - 43.6	13.9±10.3^{a}	9.4±6.2	27.4±8.3

abc Means within a column with different superscripts are different (P<0.05)

* post hoc tests are not performed for broiler starter because there are fewer than three groups

was low with HPLC compared to ELISA. The chromatograph results showed that 3 samples were positive for aflatoxin B1 in broiler finisher (Figure 2). Two samples of broiler finisher were positive for aflatoxin B1 in Kedah with 94.6 µg/kg and 42.1 µg/kg. One sample was positive in Penang with 56.4µg/kg of aflatoxin B1. Aflatoxin B1 raises the most concern among the others as its toxicity is highest, and is regulated worldwide (Gulam *et al.*, 2014; Nemati *et at.*, 2014). The highest level of aflatoxin G1 was found in broiler finisher from Selangor with 24.5 µg/kg.

It was observed that variations in the levels of aflatoxin in chicken feed were due to marked fluctuations in the environmental temperature and humidity during sample collections (Binder *et al.*, 2007).

One of the predisposing factors for aflatoxin accumulation in chicken feed is moisture content. In this study, the range of moisture content was around 6.5-27.3% (Table 2). Overall, about 40% of samples

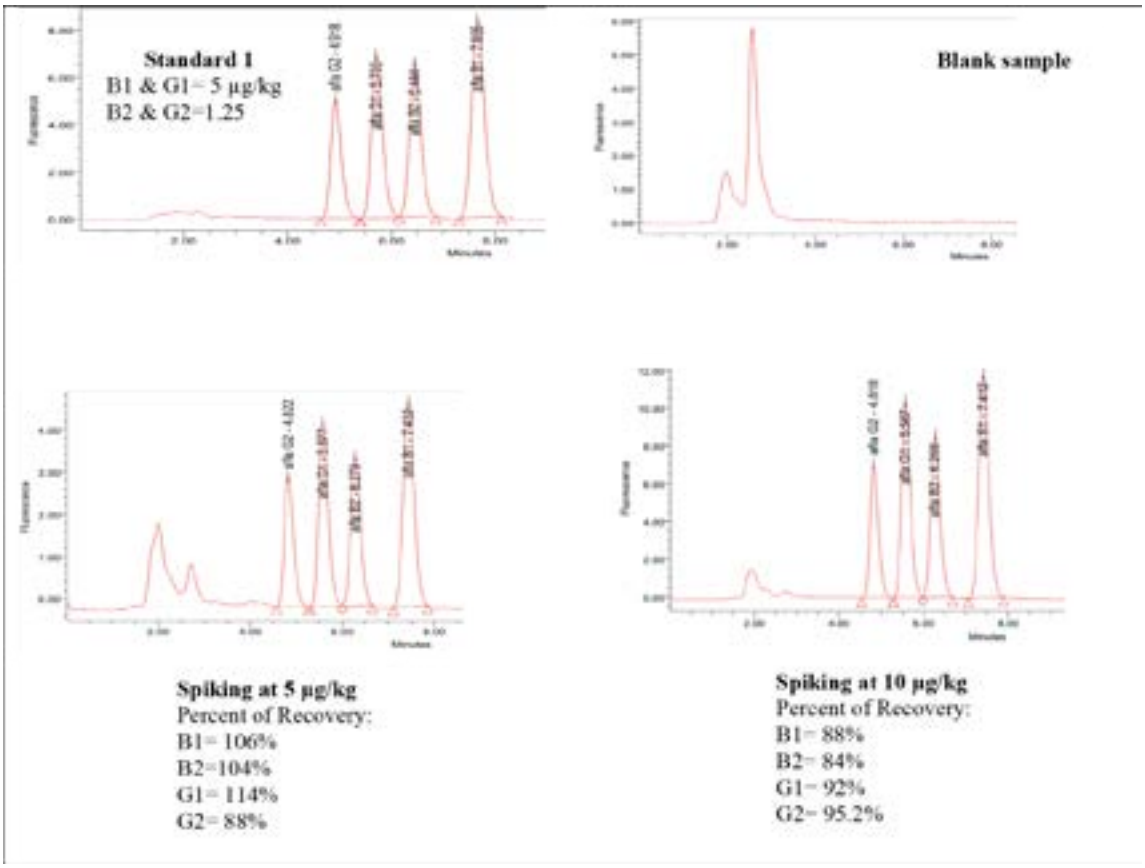

Figure 1. HPLC chromatogram for calibration standard, blank and control samples.

Table 2. Moisture content of chicken feeds in different states in Peninsular Malaysia.

Chicken feeds	States	N	Range (%) min - max	Mean ± SD
Broiler starter, n= 3	Malacca	2	11.8 - 15.7	13.75±2.76
	Pahang	1	10.6	10.6
Broiler grower, n=114	Penang	7	7.4 - 12.0	10.21±1.71[a]
	Perak	10	8.1 - 12.7	11.54±1.31[a]
	Malacca	4	10.5 - 11.5	11.03±0.5[a]
	Johor	4	8.6 - 15.4	11.55±3.30[a]
	Selangor	9	9.3 - 19.2	12.22±3.05[a]
	Kelantan	39	6.5 - 22.4	12.23±3.2[a]
	Terengganu	28	10.0 - 18.0	12.17±2.07[a]
	Pahang	13	10.3 - 19.2	12.72±2.67[a]
Broiler finisher, n= 219	Perlis	12	8.3 - 12.4	10.75±0.94[a]
	Kedah	36	8.3 - 12.3	10.84±1.05[a]
	Penang	9	8.8 - 15.0	11.53±1.74[ab]
	Perak	33	10.4 - 27.3	14.19±3.98[c]
	Malacca	9	10.7 - 15.4	11.97±1.44[ab]
	Johor	32	8.6 - 19.4	13.05±3.00[bc]
	Selangor	26	8.5 - 16.0	11.58±1.78[ab]
	Negeri Sembilan	30	9.2 - 17.0	11.85±1.63[ab]
	Kelantan	4	11.0 - 12.6	11.58±0.71[ab]
	Pahang	28	9.4 - 18.3	12.33±2.28[abc]

abc Means within a column with different superscripts are different ($p<0.05$)

* post hoc tests are not performed for broiler starter because there are fewer than three groups

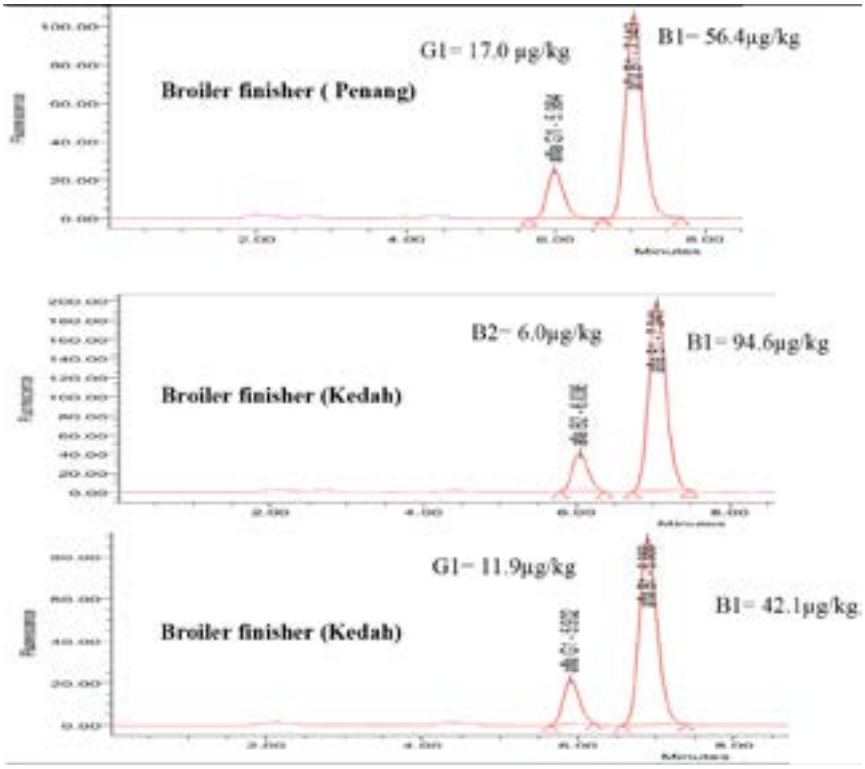

Figure 2. HPLC Chromatogram for positive samples.

have more than 12% moisture content. According to Ghulam F. *et al.* (2014), high moisture content may increase the level of TA. Normally, the safe storage level is around 10-12% moisture (Joffe *et al.*, 1969). There are many factors effects the moisture content. Climate changes among different states could be one of the reason. Normally, contamination of aflatoxin will influence by moisture content, pH and relative humidity. Some fungi to be capable of growing on a dry surface on feeds containing 12-13% moisture (5, 11). However, the pH and relative humidity was not include in this study.

CONCLUSION

In conclusion, the present study provides information about the occurrence of aflatoxin in chicken feed in Peninsular Malaysia. From 336 samples measured, about 14.9% was found positive in total aflatoxin. Three samples (6%) had quantities of more than 20 µg/kg in aflatoxin B1. The results warrant the need for surveillance and constant monitoring programmes

for the prevention of aflatoxin exposure in poultry farms. For recommendation, awareness of proper storage for chicken feedstuffs at the farm management level should be increased to lessen the occurrence of aflatoxin. Implementation of good storage management techniques can minimise mycotoxin contamination of agricultural crops. Proper ventilation, uniform loading, reducing insect infestation and proper temperature control are the most important factors to minimize mycotoxin contamination at the storage level (Afsah-Hejri *et al.*, 2013).

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