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EFFECT OF AFLATOXIN RESIDUE ON CHICKEN EGG QUALITY OF LAYER CHICKENS IN LAMONGAN DISTRICT EAST JAVA PROVINCE

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ABSTRACT

This study was aimed to evaluate the effect of aflatoxin residue on chicken egg quality of layer chickens in the district of Lamongan, East Java Province, Indonesia. Aflatoxine is the result of secondary metabolism produced by fungus *Aspergillus flavus*, well known as maize contaminates, the main ingredient of chicken feed. When layer chickens consume aflatoxine-contaminated feed, the aflatoxine residues on eggs will also cause decreasing of egg quality. The samples used in this study were 40 eggs collected from 20 chicken farms that have been screened by UV light with positive result showed fluorescent. The egg samples then were observed according to the physical qualities which includes the egg weight, eggs measurements and egg shell weight, and internal egg quality such as egg yolk color index, egg albumin index, egg yolk index, and Haugh Unit (HU). Aflatoxine residue in eggs were tested using ELISA. Significant result ($P<0.05$) was obtained using Structural Equation Modelling (SEM). An increase of aflatoxine residue of 1 ppb in egg will cause decreasing of 0.624 of its physical quality and 0.891 of its internal quality. Hence, it is concluded that the higher the aflatoxin residue content is the lower the egg quality.

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INTRODUCTION

Aflatoxin is one of the most poisonous mycotoxins produced by Aspergillus species. This toxin has contaminated food and animal feed such as maize, wheat, and beans (World Health Organization, 2008). Fungus *Aspergillus flavus* contaminates maize and other crops during pre-harvest, transportaion, and processing (Bennert Klich, 2003). Aflatoxin contamination of feed often occurs in the barn. The critical point of fungus growth occurs at 27 ^{o}C, 62% humidity level, while aflatoxin may be synthesized if the feed humidity level reaches 14% with temperature range from 12^{o}C to 41^{o}C (Royes and Yanong, 2002). Aflatoxin distribution in poultry feed leads to decreased production and egg quality, impaired immune response, increased mortality, liver and intestinal damage (Pandey and Chauhan, 2007). Based on study by Cesaria (2017), it is reported that 100% of feed ingredients used in poultry farming of egg-laying chickens in Blitar, East Java, Indonesia are aflatoxin contaminated with the highest content found in maize of 176.54 ppb. Study on AFB1 contamination in chicken's feed has been conducted and is reported that AFB1 in the feed causes residue in liver, meat, and eggs (Hussain *et al*., 2010; Herzallah, 2013). A study by Oliveira *et al.* (2000) on eggs in Brazil showed an AFB1 residue in the egg of 0.05-0.16 µg / kg. Aflatoxin residue in the egg causes the decreasing of egg yolk's and whole egg weight, Haugh Unit (HU Unit), fading of egg color, thinning of eggshells (Jia *et al*., 2016). Residue aflatoksin will appear in eggs chicken only in a day (24 hours after the provision of feed contaminated (Hassan and Ahmad, 2015). Poultry is able to biotransform and detoxify AFB1, but if the contaminated feed

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is consumed in long-term, though in low dosage, the ability to detoxify will be decrease which means that the metabolite result will be deposited in the liver and transmitted to various organs and tissues (Del Bianchi *et al*., 2005; Rauber *et al*., 2007). People consuming aflaxtoxin-contaminated food are prone to human hepatic cell carcinoma (HCC) (United Stated Department of Agriculture, 2006). Indonesian government through Indonesian National Standard (SNI): 3148-3-2009 establishes the maximum limit of aflatoxin content in the feed of egg-laying chickens of 50 ppb (National Standardization Agency, 2009). The regulation stipulated by National Agency of Drug and Food Control, The Ministry of Health, Republic of Indonesia (BPOM) no. HK.00.06.1.52.4011 states that the maximum limit of aflatoxin in food is 0.5 ppb (BPOM, 2009). Meanwhile, the maximum limit of aflatoxin in food defined by Food and Drug Administration is 20 ppb (National Grain and Feed Association, 2011). Up to now, there is a little information about the study specifically in evaluating aflatoxin contamination in feed or feed ingredients as well as aflatoxin residue in eggs and its effect in terms of quality. The study conducted in the district of Lamongan with 546.714 population of egg-laying chickens, evaluated the aflatoxin residue in egg using the ELISA, and it's effect on the egg quality.

MATERIALS AND METHODS

Sample

This study was conducted using purposive sampling. Feed samples were taken from 40 poultry farming sites in the district of Lamongan which meet the criteria of high poultry population (>300 chickens), self-built feed barn, applying first in and first out method, using commercial or self-mixing feed and the products are marketed in the area of Lamongan. Feed samples were taken from three places: barns, mixers, and feeding containers. The egg samples on each farm were taken according to positive result by UV screen test.

Materials and equipments

The materials used in this study are paper bags, Whatmann filter paper no. 1, 70% methanol, hexane, aquadest, and ELISA Kit from Neogen: Veratox for Total. Equipments required in sampling process were scales, gloves, 365 nm UV lamp, egg tray. Equipments used in egg quality observation were as follows: glass, scales, spherometer, caliper and color fan index. Equipments needed for extraction of egg samples were as follows: 50 ml organ tube, 15 ml centrifuge tube, test tube, 8-hole centrifugation, test tube rack, and 1 ml pipette. Equipments required for ELISA test were as follows: vortex-mixer, 8-channel micropipette, microtiter plate reader 450 nm, micropipette 25-1000 µl, blue tip and yello tip, ELISA washing machine plate, and stopwatch.

Procedure

Feed testing using uv light

One hundred gram of feed samples were taken each from barn, mixer, and feeding container, then was examined using UV light (Evaco, T5, 220V, Taiwan) in a dark room. The positive result was shown when there is fluorescent light in greenish blue (Fente *et al*., 2001). The amount of fluorescent light indicates the level of aflatoxin contamination. Positive feed samples with the criteria of 4 fluorescent dots (estimated to contain ± 20 ppb aflatoxin) were given as feed towards egg-laying chickens (Munkvold *et al*., 2005; Cesaria, 2017). Aflatoxin residue appears on the eggs within one day (24 hours) after being given contaminated feed, so that egg sampling was collected 24 hours after feeding (Hassan and Ahmad, 2015). Two egg samples were randomly taken for each poultry farming site then were labeled and placed on the egg tray.

Egg quality observation

Factors observed in the physical appearance of the egg includes: weight, length and width to be measured by caliper (Three-Cycle, 6 inch, China), egg color (white, slightly brownish, brown), wholeness, shell weight, shape (normal or abnormal), and cleanliness of the shell (clean or dirty). The criteria for egg quality observed includes: egg yolk index, egg white index, Haugh Unit (HU) value, egg color index using BASF's egg yolk color fan (National Standardization Agency, 2008). The results were then compared to the standard of egg quality by SNI and FDA.

Egg extraction

Egg extraction was aimed to obtain aflatoxin contained in the egg. The first stage for extraction is to mix the egg yolk and white until homogeneous then is weighed for 5 grams and put into a 50 ml falcon. Then methanol 70% (SAP chemicals R: 11-23/25) of 25 ml is added to the mixture and is shaken at high speed until homogenous. The solution, then, is filtered using Whatmann filter paper no. 1 (Neogen, 2015). The next stage is to move the filtered solution to a 15 ml centrifuge tube. The sample was centrifuged for 10 minutes at a speed of 4000 rpm. In order to separate the extract from fat contained in the egg, n-hexane (PRA 60750LC100) can be added. The supernatant containing aflatoxin of 1 ml was taken using a pipette and was transferred to a sterile test tube, 1 ml sterile diluted aquadest was added and is mixed until homogenous. The result should be tested immediately using ELISA method (Salwa and Anwer, 2009; Hussain, 2011; Neogen, 2015).

ELISA test

Content of aflatoxin residue was determined through ELISA test. First, the labeled extraction sample was arranged on the test tube rack. Next, each component of ELISA kit Veratox® for Aflatoxin USA-8030 (redwell, antibodiwell, blue label conjugate, standard (0, 5, 15, 50 ppb), substrate, and red stop solution) was removed from the refrigerator to the room temperature. Then, red-well and antibodiwell were taken and placed on the well holder. 100 µL conjugate was taken and put on each redwell. Then, control (0 ppb, 5 ppb, 10 ppb, and 50 ppb) was set up in the vortex. 100 µL of control as well as 100 µL of the egg extract were added on each redwell. The control and egg extract were added to the mixture for 3 – 4 times using 8-channel micropipette then 100 µL was taken and transferred to antibodiwell. It was, then, incubated for five minutes at room temperature. The solution was removed from antibodiwell. The next stage is to wash it using Microplate Strip Washers Labtech LT-3500 UK for five times using deionized water and then was dried using paper towel. 100 µL of the substrate was transferred to antibodiwell using 8-channel micropipette. After being incubated for five minutes, the microwell was then shaken for 30 seconds, starting slowly. 100 µL of red stop was transferred to antibodiwell. Testing

using ELISA reader (The Benchmark Bio-rad. 170-6909, 450 nm) was conducted for not more than 20 minutes after the addition of red stop. The optical density (OD) was converted to parts per billion (ppb) using Neogen log/logit program (Veratox Software Manual V3.3) (Neogen, 2015).

Statistic analysis

Data analysis using Structural Equation Modeling (SEM) was carried out to find out the correlation and effect of aflatoxin residue within the egg on egg quality, both physically and internally using SmartPLS 3, Boenningstedt, Germany (Hox and Bechger, 1998).

RESULTS

The result of this study includes feed sample of: maize, concentrate, commercial feed being taken from three sites: barns, mixer, and feeding container at 40 poultry farming in the district of Lamongan using UV light screening method. The result is available in Table 1. The result of feed sample test including: maize, concentrate, and commercial feed indicates the presence of bluish green fluorescent light during UV light testing in dark room (Figure 1). Based on ELISA test, it is obtained the content of aflatoxin residue 0.00-0.84, 1.02-1.76, 2.05-2.94, 3.53, 4.23-4.72, 5.48-5.62, 6.17-6.77 and

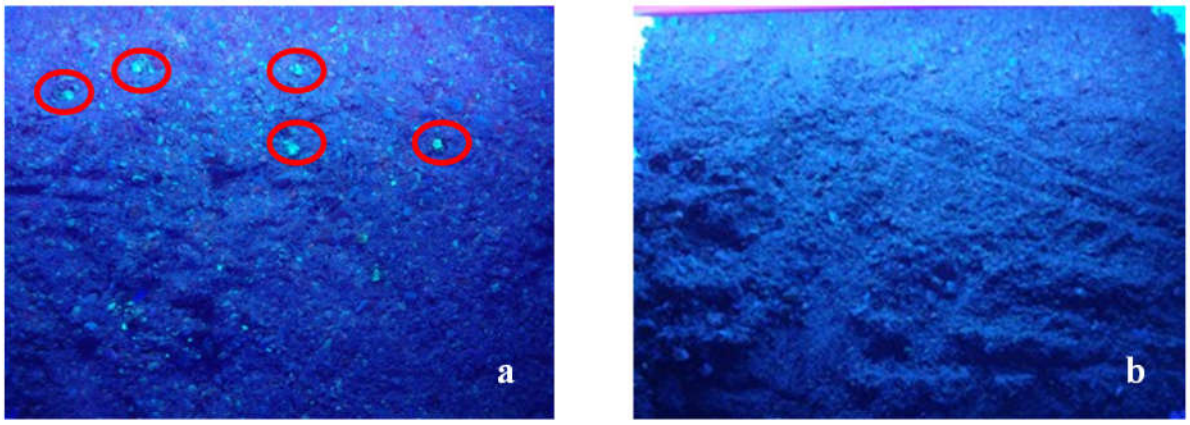


Figure 1. The feed test using UV light shows positive result (a) containing aflatoxin. Greenish blue fluorescent lights under the UV light are visible. The negative result (b) does not show any fluorescent light

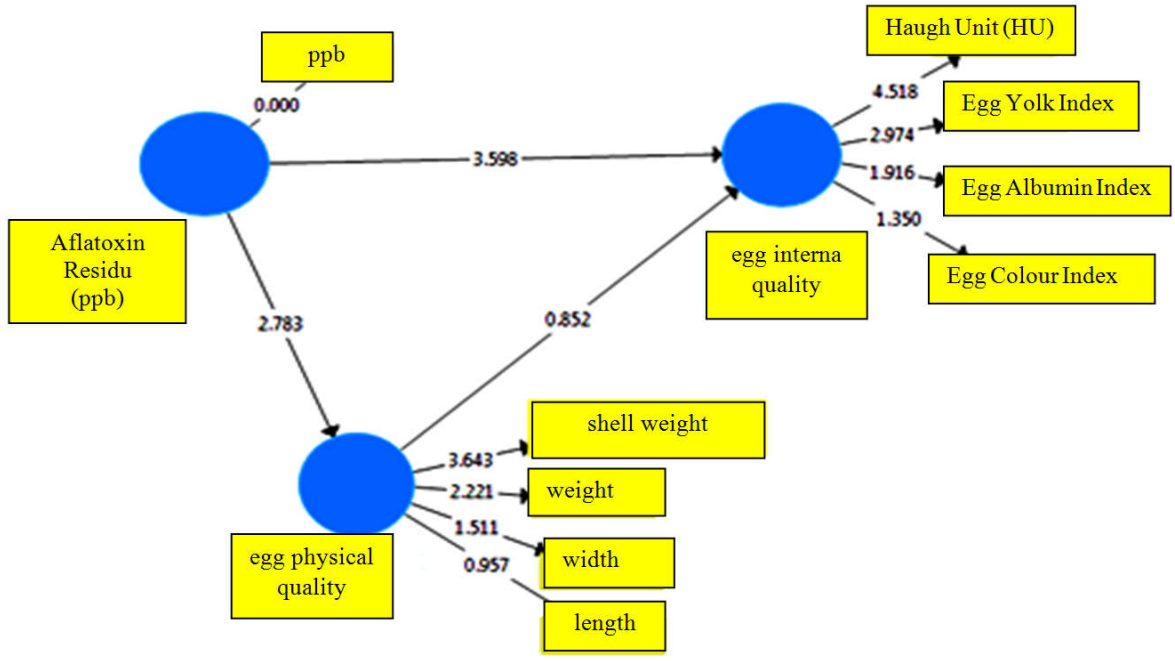
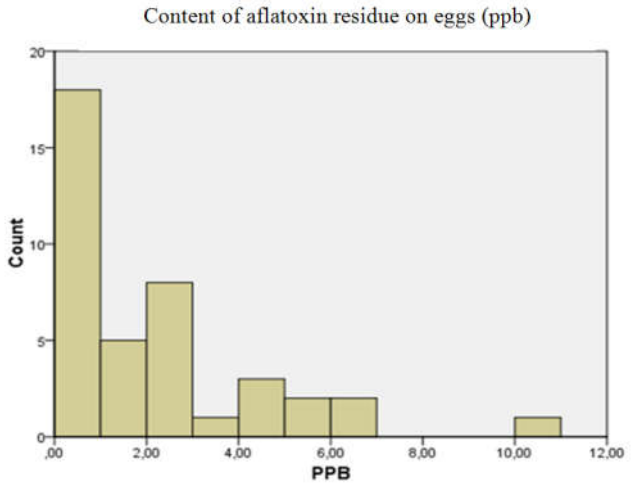


Figure 2. Structural Equation Modeling (SEM) analysis shows the result (P<0.05) in which aflatoxin residue directly affects the physical and internal quality, while physical quality does not directly affect the internal quality

Table 1. Feed test result using UV light

Sites	Sample type	Amount (n)	Positive Sample Percentage (%)
Barn	Maize	16	62.5
	Concentrate	15	0
	Commercial feed	18	11,1
Mixer	Feed	11	9,1
Feeding Container	Feed	40	2,5
Total Sample		100	85,2

10.74 (Graph 1). One sample contains the highest content of aflatoxin residue of 10 ppb, while the lowest content is 0.034 ppb. Analysis on the effect of aflatoxin residue within the egg on egg quality shows significant result ($P < 0.05$). SEM result can be seen in Figure 2. Based on SEM analysis, the parameter coefficient for aflatoxin residue within the egg on egg internal quality is (-0.891). It is interpreted that 1 ppb increase of aflatoxin residue decreases 0.891 of egg internal quality. The value of t-Statistics is 3.598 and is considered to be significant (t table significant $p<0.05$).



Graph 1. Graph on aflatoxin residual content found on eggs based on ELISA test

Meanwhile, the parameter coefficient for aflatoxin residue within the egg on egg physical quality is (-0.624) meaning that 1 ppb increase of aflatoxin residue decreases 0.624 of egg physical quality. The value of t-Statistics is 2.783 and is considered to be significant (t table significant $p<0.05$). The parameter coefficient for egg physical quality towards egg internal quality is (-0.157) which indicates the presence negative effect between physical and internal quality. In other words, physical quality affects 15.7% towards the decrease of internal quality. The value of t-Statistics is 0.852 and is considered to insignificant (t table insignificant $p<0.05$). The output from testing on the ability of aflatoxin residue (independent variable) in determining dependent variable with most influence in terms of physical and internal quality is known based on R-square. The most suitable test output in measuring egg internal quality is Haugh Unit (HU) (4.518) and Egg Yolk Index (2974). Meanwhile, the most suitable test output in measuring egg physical quality is the shell weight (3.643) and Egg weight (2.221).

DISCUSSION

Feed detection using UV light is used to determining aflatoxin contamination in feed. Aflatoxin has the ability to absorb UV light with a maximum wavelength of 360 nm (Akbas and Ozdemir, 2006). The presence of fluorescence is associated with the amount of aflatoxin contained in the feed, so that the test is performed in the dark room in order to know the contrast of fluorescence sufficiently (Espinosa-Calderón *et al*., 2011). Feed sample were obtained from three sites: barns, mixer, and feeding containers. The result of UV light shows 62.5% of maize stored in barns is allegedly contaminated by aflatoxin. Barn is a place which becomes the critical point of overgrowth fungus. If storage does not use any covering and the humidity level reaches 62%, the place will be overgrown by fungus (Royes and Yanong, 2002). However, feed isolated from feed container shows a positive result of 2.5% since only little amount of feed remains on the container every morning. The poultry farmer is accustomed to feeding in daytime, so that feeding time is longer; and the feeding container is routinely cleaned. The result of UV light test on mixer shows a positive result of 9.1%. According to the poultry farmer, the mixer used has never been cleaned meaning that remaining self-mixing feed can possibly be contaminated by the fungus. The use of UV light is unable to directly determine aflatoxin contamination in the feed, its use is only as a quantitative screening since the presence of fluorescence remains unstable and may be lost after 4 – 6 weeks (Espinosa-Calderón *et al*., 2011). Based on ELISA test, the content of aflatoxin residue in the egg is considerably high. The highest content of aflatoxin residue found in the eggs is 10.774 ppb; 6.77 ppb; and 6.17 ppb (Graph 1). When referring to regulation by BPOM, the limit of aflatoxin in food is 0.5 ppb (BPOM, 2009). On the other hand, the limit of aflatoxin in food set by FDA is 20 ppb (National Grain and Feed Association, 2011). Aflatoxin contamination found in feed reduces the egg quality both physically and internally. The presence of aflatoxin residue in eggs gives significant effect ($p < 0.05$) on egg physical quality. Parameters used in determining and shell weight. Based on SEM result ($p < 0.05$), the most affected factors in terms of aflatoxin residue are weight and shell weight of eggs. Thus, both of these factors can be used as parameters in egg physical quality are weight, length and width, measuring the decrease of egg quality due to aflatoxin residue. Based on the analysis of SEM showed decreased physical quality of 0,624 so Aflatoxin content of 1 ppb in an egg may decrease 0,624 of its physical quality. This is in accordance with Zaghini *et al*. (2005) which state that aflatoxin-contaminated feed decreases egg weight after 3 weeks and eggshell weight after 4 weeks of feeding. Parameters of internal egg quality may also decrease significantly ($p < 0.05$) due to aflatoxin residue in eggs; decrease in egg yolk index, egg white index, and Haugh Unit (HU). Similarly, a study by Lee *et al*. (2012) with the parameters evaluated decrease ($p < 0.05$) in Haugh Units (HU), egg weight, egg white and egg yolk index, and relative weight of egg yolk. There is correlation between aflatoxin residue in egg and internal quality. Based on the analysis of SEM showed decreased internal egg quality of 0,891, with the increasing of 1pbb aflatoxin content in eggs caused the decreasing of Haugh Unit (HU) and Egg Yolk Index of 0,891. Aflatoxin-contaminated feed is able to maintain its metabolite up to 5.000:1 within the egg and it results in residue (Oliveira *et al*., 2000). These data indicated that aflatoxin in feed reduces egg quality, especially on the parameters of weight, shell weight, Haugh Unit (HU), and Egg Yolk Index. Aflatoxin content in feed also affects carry over ratio in which aflatoxin metabolite content is not only deposited in liver, but in long term detoxification ability decreases and results in aflatoxin residue becoming residue in egg through transovarial transfer by binding into albumin within the egg; aflatoxin leads to a complex reduction in egg quality both physically and internally (Del Bianchi *et al*., 2005; Rauber *et al*., 2007; Salwa and Anwer, 2009).

Conclusion

In this study, aflatoxin residue found in egg might be reduced the egg quality both physically and internally. Such parameter signifies the effect of aflatoxin residue on eggs and that

affected in decreasing of 0,624 in physical quality and 0,891 of eggs internal quality. Among other parameters that determined the decreasing in egg quality due to aflatoxin residue of were whole egg weight, egg shell weight, Haugh Unit (HU), and Egg Yolk Index. The effects of aflatoxin contamination in feed resulted in various aspects, one of which is egg quality. This study showed that the decrease of egg quality due to aflatoxin residue is considerably high, monitoring of feed as a source aflatoxin contamination is necessary.

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