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Screening of aflatoxin B_1 and M_1 in feed, commercial, and Desi chicken eggs using high-performance thin layer chromatography.

Detección de aflatoxinas B1 y M1 en huevos de gallina para piensos, comerciales y Desi mediante cromatografía de capa fina de alto rendimiento.

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ABSTRACT

Background: Egg consumption in India has increased due to dietary and lifestyle changes. Thus, there is a need to screen for any contamination hazardous to public health. The objective was to evaluate the presence of aflatoxin B_1 (AFB_1) and aflatoxin M_1(AFM_1) in feed and egg samples collected from commercial and desi farms in and around Chennai. Method: To determine the compartment where aflatoxin (AFT) accumulates, egg samples were fractionated into three: whole, yolk, and albumen. Matrixes were screened for AFB_1 and AFM_1 residues using the AOAC method and quantified using high-performance thin-layer chromatography (HPTLC). Result: Eighty percent of the feed samples from desi chicken farms and 86.67% from commercial farms were positive for AFB_1. The highest value of AFB_1 was 152.75 ppb in commercial feed samples, which was more than the MRL (20 ppb) by the European Union (EU). Five percent of the commercial and 11.67% of the desi chicken whole egg samples were positive for AFB_1. Other samples (yolk and albumen) were below the detection limits. AFB_1concentration was more in egg samples collected from commercial chicken farms. Further stringent measures, control, and monitoring programs must be considered to reduce AFT levels in Chennai.

Keywords: laying hens, egg production, feedstuffs, mycotoxins, residual effect, albumin, yolk.

RESUMEN

Antecedentes: el consumo de huevos en la India ha aumentado debido a cambios en la dieta y el estilo de vida. Por lo tanto, es necesario detectar cualquier contaminación peligrosa para la salud pública. El objetivo

era evaluar la presencia de aflatoxina B1 (AFB1) y aflatoxina M1 (AFM1) en muestras de piensos y huevos recogidas en granjas comerciales y desi en Chennai y sus alrededores. Método: Para determinar el compartimento donde se acumula la aflatoxina (AFT), se fraccionaron muestras de huevo en tres: entero, yema y albúmina. Las matrices se examinaron para detectar residuos de AFB1 y AFM1 utilizando el método AOAC y se cuantificaron mediante cromatografía de capa fina de alto rendimiento (HPTLC). Resultado: El ochenta por ciento de las muestras de alimento de las granjas de pollos desi y el 86,67% de las granjas comerciales fueron positivas para AFB1. El valor más alto de AFB1 fue de 152,75 ppb en muestras de piensos comerciales, que superó el LMR (20 ppb) de la Unión Europea (UE). El cinco por ciento de las muestras comerciales y el 11,67% de las muestras de huevos enteros de gallina desi fueron positivas para AFB1. Otras muestras (yema y albúmina) estaban por debajo de los límites de detección. La concentración de AFB1 fue mayor en muestras de huevos recolectadas de granjas avícolas comerciales. Se deben considerar medidas y programas de control y seguimiento más estrictos para reducir los niveles de Ayuda para el Comercio en Chennai.

Palabras clave: gallinas ponedoras, producción de huevos, piensos, micotoxinas, efecto residual, albúmina, yema.

INTRODUCTION

Foodborne diseases, including aflatoxin (AFT) contamination, affect 600 million people annually, contributing to the global health crisis (FAO,2017). The warm and humid climates are the prerequisite for AFT production, which is prevailing in tropical countries like India. The initial outbreak in India was reported in 1974, causing the death of 106 people consuming maize contaminated with AFT (Reddy and Raghavender,2007). When animals consume the contaminated crop, AFT gets metabolized, causing residual accumulation in milk, egg, and meat. Aflatoxin B_1(AFB_1), the most prevalent form of AFT categorized as a Class 1 carcinogen by IARC, contributes to hepatocellular carcinoma, which is said to be the second most common cause of cancer (WHO, 2017). The acceptable level of AFT in human food varies in different countries. There are several studies about AFB_1 in food crops though very little screening is done using meat and eggs (Khaneghah *et al*.,2020).

Eggs are the cheapest source of protein available to people of different economic strata, and any form of contamination to the same is a public health concern. Moreover, the per capita consumption of eggs has increased tremendously due to their nutritional value, current lifestyle changes, and diet trends (Deeb *et al.,*2016). With this background, the following research was aimed to evaluate the AFT levels in egg and feed samples collected in and around Chennai, Tamil Nadu.

MATERIALS AND METHODS

The study was conducted in PLAFFS (Pharmacovigilance laboratory for Animal Feed and Food Safety Laboratory, DCAHS, MMC, Chennai-600051). Thin Layer Chromatography (TLC) was used for screening, and the High-Performance Thin layer chromatography (HPTLC) method was used for quantification because of its

sensitivity, improved precision, and accuracy (Scussel *et al.,*2003). The extraction was done based on a validated method to evaluate the presence of AFT in feed samples using HPTLC (Ramesh *et al.,* 2013) as per the AOAC guidelines. The standard used for AFB_1 and $AFM_1$was from SIGMA-ALDRICH. Other chemicals like acetone, chloroform, and acetonitrile were from Merck (AR. Grade)

Sample collection: Sixty egg samples were collected from commercial farm outlets and 60 from desi layer farm outlets in and near Chennai, Tamil Nadu, from the peak layers (26-30 weeks). Up to the time of the examination, the egg samples were stored in the refrigerator at 4°C. Fifteen layer-feed samples from the same commercial farms and 15 layer feed samples from the same desi farms were procured, which were given to the layers during the week of egg collection.

Sample preparation: Each egg sample had its surface washed with 70% (v/v) alcohol. A tiny hole was created using sterile forceps at the egg's tip, and the albumen and egg yolk were drained out separately (egg fractionation). The three fractions are collected from the same egg sample by taking 10mg each for AFT estimation. The feed samples were ground thoroughly and passed through the number 14 sieve and split sample. Reground the sample in the number 20 sieve and mix thoroughly. 25 g of feed was taken for aflatoxin estimation, and levels of AFB_1 and AFM_1 were quantified.

Extraction of aflatoxin: A known quantity (25g of feed and 10g of egg) of the powdered material was placed in a 250 ml flask, and it was then treated with 19 ml of distilled water and 106 ml of acetone. On a shaker, this mixture was agitated for 30 minutes at 200 rpm. Following that, it was filtered via the Whatman paper (No1). 1.5g of cupric carbonate was added to 75 ml filtrate. Another beaker containing a mixture of 15 ml of 0.4M $FeCl_3$ and 85 ml of 0.2N NaOH was prepared. The contents in both beakers were mixed thoroughly and filtered through Whatman No.1 filter paper.100 ml of filtrate was transferred to a 500 ml separating funnel, to which 100 ml of 0.03% H_2SO_4 and 25 ml of chloroform were added and vigorously shaken. After 30 minutes, the bottom layer was transferred to another 100 ml separating funnel treated with 40 ml of 1% KCl in 0.02M KOH solution. After the layers separated, the lowest layer was filtered through a funnel containing anhydrous Sodium sulfate into a vial. Under a fume hood, the extract was evaporated on a hot plate. To use the dried extract for TLC and HPTLC spotting, the extract was redissolved in 0.2 ml of chloroform (Ramesh *et al.,*2013).

TLC method: The redissolved residue was spotted on to TLC plate as 5 µl drops with a thickness of about 0.5mm. The AFB_1 standard was spotted in three different volumes 1,3, and 5µl. It was developed in a development chamber containing chloroform and acetone in a ratio of 1:9 and later air-dried. On observing under UV light, the fluorescence from the sample spot was compared with that of the standard.

HPTLC Method: The upper edge of the HPTLC plate was marked with a pencil to indicate the direction of development. In a 20 x10 cm twin-trough chamber (TTC), the plate was developed with 20 ml of methanol per trough to the upper edge. After development, the plates were dried (pre-wash). Samples and the working standards were applied on the TLC plate using a Linomat-5 sample applicator after diluting the dried samples via a spray-on approach in such a way that there was a bandwidth of 8mm and space between two bands was 12mm. Then, 20 mL of the 9:1 mixture of chloroform and acetone was added to each trough of TTC for plate

development. The pre-saturated TLC was used to develop the spotted samples up to 80 mm from the plate's lower edge.

Derivatization: Spraying or dipping was used to transfer reagents for derivatizing materials on an HPTLC plate. Thus, for derivatization, 20% H_2SO_4 was sprayed on the developed plates and dried. After exposure to ammonia vapours, trifluoroacetic acid was sprayed and dried. The developed TLC plate was kept on a hot plate for better visibility (Ramesh *et al.,*2013).

Scanning: To assess the contamination of AFB_1 and AFM_1 in the samples, the plates were scanned in the CAMAG HPTLC scanner-3 at a 365nm wavelength. AFB_1 requires derivatization to improve the fluorescence. TLC or HPTLC detection of AFB_1 is dependent on the fluorescence under UV light (Ramesh *et al.,*2013)

RESULTS

Qualitative analysis using TLC: Feed and egg samples were screened using TLC method for the presence of AFB_1 and AFM_1. A bluish fluorescence was seen in the positive samples and standard. The R_f value of AFB_1 was found to be 0.62 (figure 2) and the R_f value for AFM_1 was 0.57 (figure 3).

Figure 2: Qualitative analysis of feed samples for AFB_1 using TLC.

1-commercial layer feed; 2- groundnut cake; 3-deoiled rice bran; 4 – mineral mixture; 5-maize; 6- sunflower oil cake; 7- vegetable oil. AFB_1 Standard from left to right at volumes 1, 3 and 5 µl. R_f value was found to be 0.62.

Figure 3: Qualitative analysis of egg samples for AFM_1 using TLC.

E_1 and E_2 are egg samples; The AFM_1 standard was used in volumes 5 and 8 µl. The R_f for AFM_1 was found as 0.57.

HPTLC results: The mobile phase (chloroform: acetone) resulted in a symmetrical, sharp, and resolved peak at an R_f value of 0.60 ± 0.2 for AFB_1(figure 5-7), and that for AFM_1 was 0.59 ± 0.2 (figure 9-11). The calibration curve was linear for the AFB_1 concentration in the range of 2-20 ng/spot. The correlation coefficient (r) was 0.9992, which was highly significant ($p< 0.05$). The linear regression equation was y=275.697+102. 057x. For AFM_1, the calibration curve was linear in the range of 50-5000pg/ spot with a correlation coefficient (r) of 0.9999, which was highly significant ($p< 0.05$). The linear regression equation was y =-8.370+1.279x.

Aflatoxin analysis in feed samples: On analyzing 30 feed samples (15 from commercial farms and 15 from desi farms), it was observed that 12 out of 15 desi feed samples (80%) were found to be positive for AFB_1 with a minimum and maximum concentration of 6.02ppb and 35ppb, respectively (figure 9), whereas 13 samples (86.67%) out of 15 commercial feed samples had AFB_1 residues with a minimum and maximum concentration of 12.54ppb and 152.75ppb, respectively (figure 4). The average concentration of AFB_1 in feed samples from the commercial chicken farms was 65.964ppb, and that in the desi chicken farm was 24.0525 ppb. The AFB_1concentration of the rest of the feed samples was less than 2 µg/kg, which was below the detection limit (BDL).

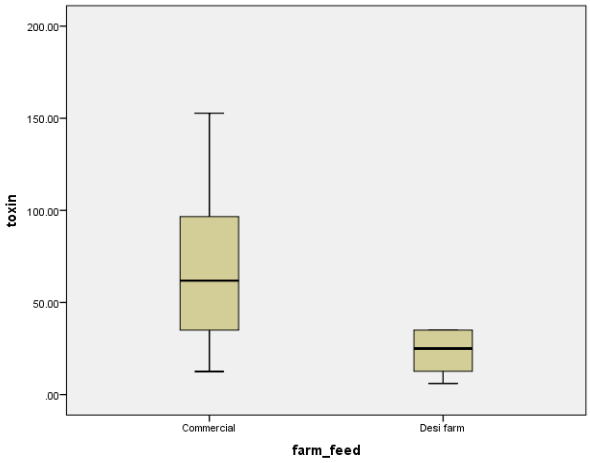

Figure 4: The concentration range of AFB_1 (in ppb) in commercial and desi layer feed samples

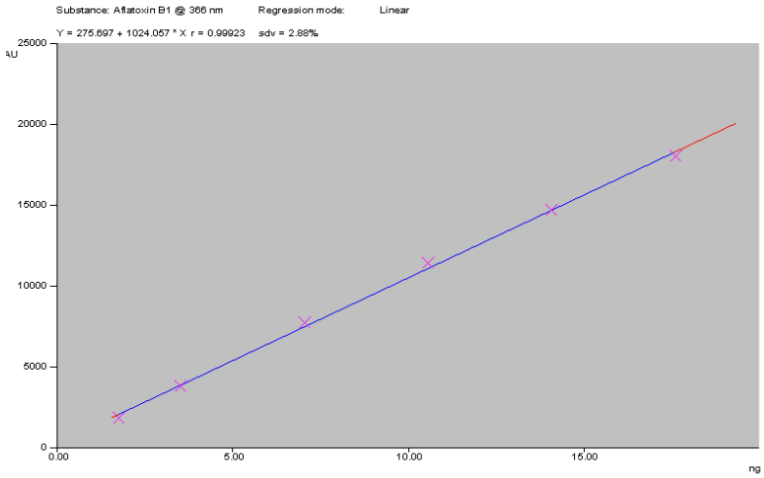

Figure 5: Linear Regression curve of AFB_1

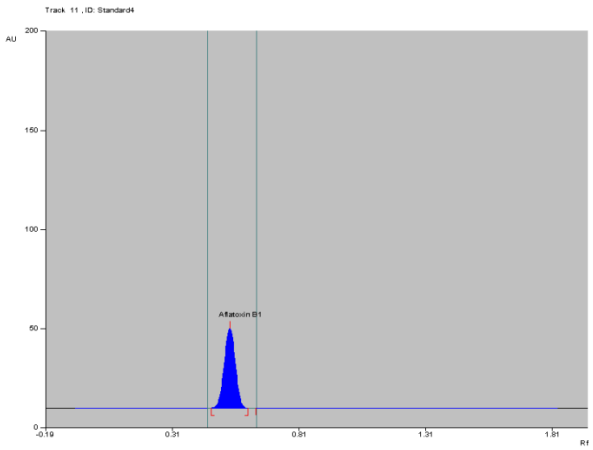

Figure 6: AFB_1 standard peak

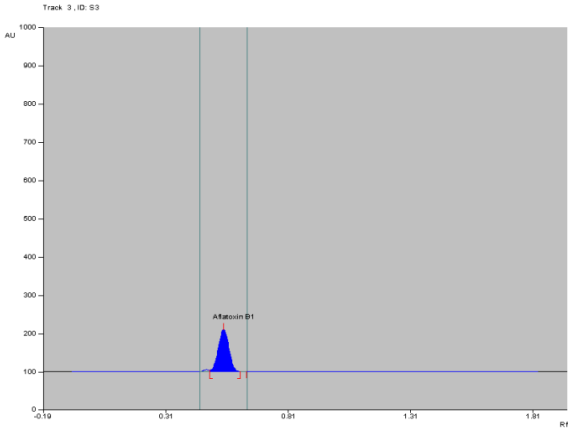

Figure7: AFB_1 positive sample peak

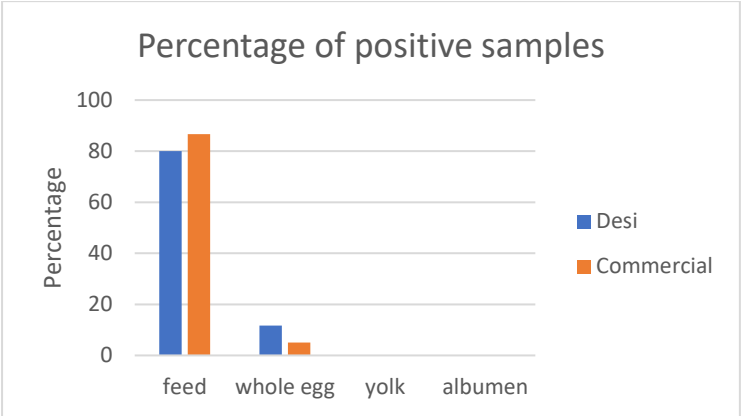

Figure 8: The Percentage of positive feed and egg samples from commercial and desi farms compared using HPTLC results.

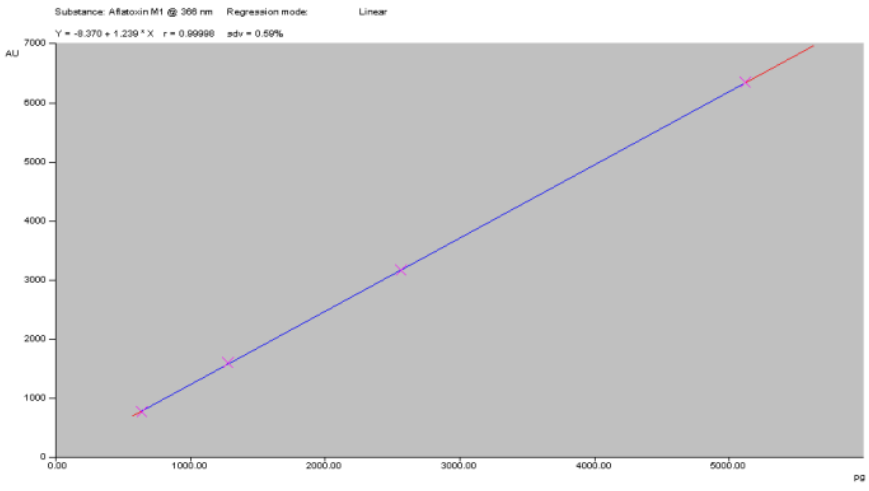

Figure 9: Linear Regression curve of AFM_1

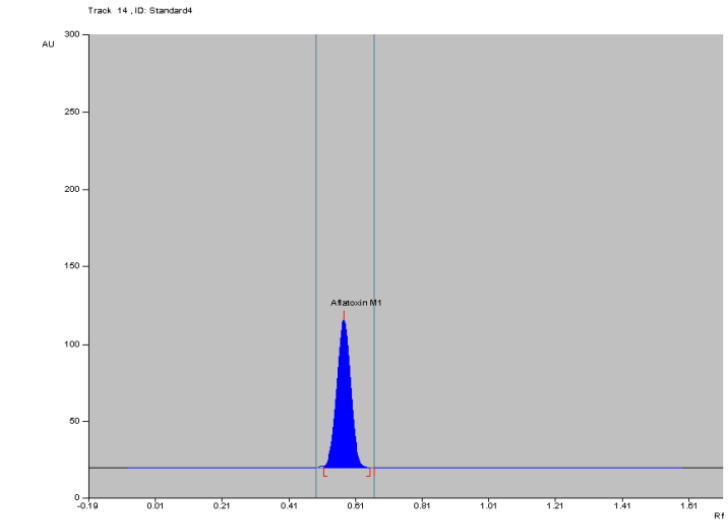

Figure 10: AFM_1 standard peak

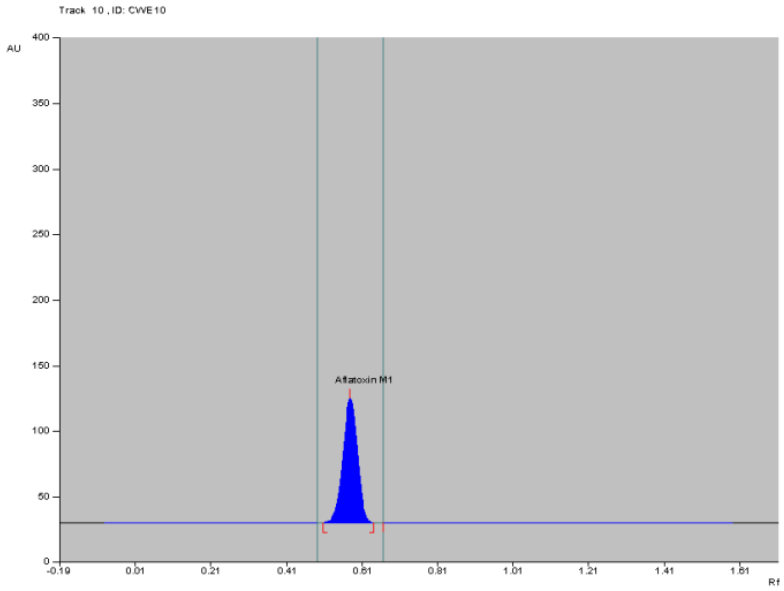

Figure 11: AFM_1 positive sample peak

Aflatoxin residual analysis in egg samples: hundred and twenty commercial and desi chicken eggs collected from various farms were further fractionated into the whole egg, yolk, and albumen, totaling 360 samples. The observed levels of $AFB_1$are given in table 1. AFB_1 was detected in the whole egg samples. Out of 60 desi whole egg samples, 7 samples (11.67%) were found positive for AFB_1 with a minimum and maximum concentration of 2ppb and 4ppb, whereas, in commercial chicken whole egg samples, 3 samples (5%) out of 60 were found to have AFB_1 with a maximum and minimum concentration of 5ppb and 2ppb respectively (table 2). The AFB_1 concentration in commercial egg samples was 3 ± 0.22, and that in desi eggs was 2.57 ± 0.10 (*p>0.05)*.

Table 1: The percentage of positive egg samples collected from commercial and desi farms using HPTLC.

Chicken eggs		Sample Number (N)	AFB_1	
			No. of positive samples	Percentage of positive samples
Commercial	Whole egg	60	3	5
	Albumen	60	-	-
	Yolk	60	-	-
Desi	Whole egg	60	7	11.67
	Albumen	60	-	-
	yolk	60	-	-
Total		360	10	2.78

N- the number of samples analyzed; n- the number of positive samples; %-percentage of positive samples detected.

Table 2: The concentration of AFB_1 detected in the positive egg samples using HPTLC.

Samples		Range of values (ppb)	Average(ppb)	SEM	EU MRL (ppb)
Commercial	Feed	12.54-152.75	65.96	10.109	20
	Whole egg	2-5	3	2.993	2-12
Desi	Feed	6.02-35	24.05	0.223	20
	Whole egg	2-4	2.57	0.102	2-12

ppb- partsper billion; EU- European union; MRL- Maximum Residual Limit; SEM- Standard error.

DISCUSSION

In both the desi and commercial feed samples, the concentration of $AFM_1$was less than 5µg/kg, which was below the Limit of Detection (LOD) of the instrument. The estimated percentage of positive samples was more than the value observed in the study conducted by Kotinagu *et al.* (2015) using HPTLC, where AFB_1 was detected in 97 samples of animal feed (48) and feed additives (49), of which 29 samples (30%) were contaminated.AFB_1 was identified in 16 of 48 samples of compound feed for livestock, or 33% of the total, whereas in 13 of 49 samples of components for feed for animals, or 24.5% of the total. This is lesser than the scenario observed from 1983 to 1993, where out of 4818 feed samples collected in India, about 96.35% of groundnut cake samples were positive for AFB_1 with the highest concentration of 8260 ppb in maize samples (Dhavan *et al.,*1995).

In this research, AFB_1 was found in the range 12.54-152.75 ppb in the feed samples collected from the commercial farms and 6.02-35 ppb in the feed samples collected from the desi chicken farms. The average levels of AFB_1 found in the commercial feed samples (65.96 ± 10.85) was 3.25 times higher than the EU-recommended Maximum Residual Limit (MRL). The desi feed sample at an average level of 24.05 ± 11.59, which was 1.2 times more than the MRL and was statistically significant ($p < 0.05$) (figure 4). The EU-recommended MRL for animal feed is 20ppb.

Aflatoxin residual levels found in the egg samples were found to be within the Maximum residual limit (MRL) established by various agencies. The toughest regulatory limit is found in the European Union, where products intended for direct consumption cannot include more than 2 µg/kg of AFB_1 and 4 µg/kg of total AFT, respectively (EC, 2007, 2010). It is acceptable up to 20µg/kg in the US (Mahato *et al.,*2019). Though adult human beings are tolerant to AFT, children are susceptible to these toxins causing delayed development, stunted growth, and recurrent infections due to immunosuppression. Chronic exposure in adults can cause hepatitis, cancer, and even death (Williams *et al*., 2004).

Wolzak *et al*. (1985) estimated AFT deposition and its Clearance in chicken eggs; it was evident that the egg production dropped considerably during week 3 of AFT feeding, reaching about 82 percent of the control value, and continued to drop until it reached a minimum of 39 percent by day 2 -4 after the AFT-contaminated diet was discontinued. It was noted that an average value of 0.03 µg AFB_1/kg eggs was consumed at a 3 mg AFB_1/kg diet. However, average quantities of 2.2 and 3.6 µg AFB_1/kg within albumen and yolks of eggs from chickens fed only a 100 µg AFB_1/kg diet have been documented. Zaghini*et al.* (2005) mentioned that AFT and its metabolites may or may not be carried over from feed to eggs at various ratios ranging from 5,000:1 to 66,200:1 and even 125,000:1. This variation is due to feeding naturally contaminated feed with different levels of toxicity. Residues of AFB_1 were isolated in breast muscles, and eggs of hens fed AFB_1 were in the same research. Similarly, Deeb *et al.* (2016) found that the two (4 percent) samples of table eggs tested had AFB_1 in them in amounts of 1.1 ppb and 1.7 ppb, whereas the other samples were below the method's detection limit of 1 ppb.

Pourelmi *et al.* (2013) evaluated the levels of AFB_1 in local and industrial eggs using ELISA, where eggs from the local area had the highest AFT contamination (0.107 ng/ml). In contrast, industrial eggs had the lowest contamination (0.050 ng/ml). These readings were below the maximum permitted level (12 ng/ml). Comparing local eggs to industrial eggs revealed that the local eggs were numerically more contaminated with AFT. AFT levels in local eggs were not less than 0.080 (ng/ml), whereas, in industrial eggs, the maximum level was 0.081 (ng/ml).

A similar result was observed in this research that the percentage of positive samples was more in the commercial feed samples compared to the desi feed samples. The corresponding egg samples collected from the same farms were expected to have a similar trend, but the desi egg samples had lower concentrations and the highest percentage of positive samples. The commercial egg samples showed highest concentration of AFB_1 with lowest percentage of positive samples. This could be due to insufficient feed intake by the commercial chicken

or inconsistency in the consumption of naturally contaminated feed in varying levels to facilitate the residues to get transmitted to the eggs.

The eggs were collected specifically from the peak layer (25-32 weeks) to get maximum feed-to-egg transmission reflected in the egg. The ability of the bird to tolerate toxins is greatly reduced during this period. In commercial poultry farms, there exists a systematic deworming and vaccination protocol that could have helped the birds to tolerate the high levels of AFT in the feed.

In this study, the detection of AFB_1in the eggs are a public concern, though not statistically significant. Moreover, contaminated eggs can lead to chronic AFT exposure in a person who consumes more than one egg per day, putting him to the risk of hepatocellular carcinoma. With the current trend in change of diet, the number of eggs consumed per day increases, and the toxin exposure may also cross the recommended MRL levels. Thus, more stringent measures and monitoring programs must be conducted on specific food items to ensure the safety of human beings.

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