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ARTICLE

Detection of Foodborne Pathogens and Mycotoxins in Eggs and Chicken Feeds from Farms to Retail Markets

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Abstract

Contamination by foodborne pathogens and mycotoxins was examined in 475 eggs and 20 feed samples collected from three egg layer farms, three egg-processing units, and five retail markets in Korea. Microbial contamination with *Salmonella* species, *Escherichia coli*, and *Arcobacter* species was examined by bacterial culture and multiplex polymerase chain reaction (PCR). The contamination levels of aflatoxins, ochratoxins, and zearalenone in eggs and chicken feeds were simultaneously analyzed with high-performance liquid chromatography coupled with fluorescence detection after the post-derivatization. While *E. coli* was isolated from 9.1% of eggs, *Salmonella* species were not isolated. *Arcobacter* species were detected in 0.8% of eggs collected from egg layers by PCR only. While aflatoxins, ochratoxins, and zearalenone were found in 100%, 100%, and 85% of chicken feeds, their contamination levels were below the maximum acceptable levels (1.86, 2.24, and 147.53 μg/kg, respectively). However, no eggs were contaminated with aflatoxins, ochratoxins, or zearalenone. Therefore, the risk of contamination by mycotoxins and microbes in eggs and chicken feeds is considered negligible and unlikely to pose a threat to human health.

Keywords: eggs, contamination, foodborne pathogens, mycotoxins, safety

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Introduction

Contamination of chickens and eggs by *Salmonella* species and *Escherichia coli* has caused many outbreaks (Greig and Ravel, 2009). As handling fecal material, dust, and dirt in nests and layer cages can contaminate eggs from layer farms, the World Health Organization (WHO) performed a risk assessments for the presence of *Salmonella* species in eggs and broiler chickens (WHO, 2002). *E. coli*, *Salmonella* species, and *Staphylococcus aureus* are commonly found in eggs; hence, the microbial safety of eggs needs to be ensured with refrigeration at all stages, from farms to retail markets. Previous studies identified *Arcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii* in patients with chronic enteritis or septicemia, and traced the contamination sources to chicken carcasses, chicken intestinal contents, pork, beef, and drinking water (Douidah *et al*., 2014; Lee *et al*., 2010). However, the prevalence of *Arcobacter* species in eggs has not been examined, to our knowledge. Therefore, it is necessary to investigate microbial contamination in eggs collected at each stage of commercial production, to enhance their microbial safety.

Contamination by mycotoxins occurs frequently in chicken feeds containing maize and other cereals (Greco *et al*., 2014; Jang *et al*., 2007a, 2007b; Thirumala-devi *et al*., 2002). Ingestion of mycotoxin-contaminated feed by chickens causes several health problems, which leads to large economic losses in terms of egg quality and quantity. The common mycotoxins found in eggs are aflatoxins (AFs), ochratoxin (OTA), zearalenone (ZEA), and fumonisins (Greco *et al*., 2014; Iqbal *et al*., 2014; Jia *et al*., 2016). In addition, a combination of AFs and ZEA in feeds was shown to synergistically reduce the laying performance,

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egg quality, and feed intake of laying hens (Jia *et al*., 2016). However, despite the importance of eggs as a nutritious and beneficial commodity, there is little information on co-occurring mycotoxin contaminations in eggs and chicken feed in Korea.

Therefore, this study aimed to examine the contamination levels of foodborne pathogens (*E. coli*, *Salmonella* species, and *Arcobacter* species) and mycotoxins (AFs, OTA, and ZEA) in eggs collected at various stages from chicken farms to retail markets.

Materials and Methods

Collection of eggs and feeds

In total, 475 eggs and 20 kinds of commercial chicken feeds were collected from three egg layer farms, three egg-processing units, and five retail markets. One egg farm was located in Jeollabuk-do; the other facilities were located in Gyeonggi-do. Thirty eggs were collected from the egg layer farms, 180 eggs were obtained from the egg-processing units, and 265 eggs from 25 different brands were sampled at the retail markets. Twenty chicken feeds comprised 8 layer feeds for chick, 5 layer feeds for pullet, and 7 layer feeds for hen, which were commercially available in Korea (Daehan Feed Co., Daejoo Co., Hanil Feed Co., and Nonhyup Feed Co.).

Microbial examination

The surface of each eggshell was swabbed with a sterile cotton stick. The swabbed cotton sticks were vortexed and suspended in 0.1% (w/v) peptone water. *E.coli* and *Salmonella* species were isolated using the standard protocols of the food code established by the Ministry of Food and Drug Safety (MFDS) in Korea. Briefly, EC broth (Oxoid) and MacConkey agar (Oxoid) were used to isolate *E. coli*. Rappaport Vassiliadis broth (Oxoid) and XLD agar were used to isolate *Salmonella* species.

Arcobacter species were isolated as described by Lee *et al*. (2010). Each sample was mixed with *Arcobacter*-selective broth (Oxoid) including a cefoperazone, amphotericin, and teicoplanin (CAT)-selective supplement (Oxoid) at a 1:10 ratio and incubated in microaerobic conditions at 37°C for 48 h. The enriched broth was inoculated and incubated in *Arcobacter*-selective agar (Oxoid) at 37°C for 48 h. To detect *Arcobacter* species, species-specific multiplex polymerase chain reaction (PCR) was performed with boiled enriched broth, as described previously (Lee *et al*., 2010).

Mycotoxin analysis

To evaluate the occurrence of AFs (sum of AFB1, AFG1, AFB2, and AFG2), OTA, and ZEA, egg and chicken feed samples were analyzed using high-performance liquid chromatography (HPLC) coupled with fluorescence detection after post-derivatization. The extraction procedures were in accordance with the manufacturer's protocol. The samples were extracted as described by Ok *et al*. (2015) with a few modifications.

Whole egg without eggshell was homogenized with a blender for 5 min at room temperature under continuous agitation. Further, 2 g sample was transferred to a polypropylene centrifuge tube (50 mL), and 2 mL water was added and vortexed for 2 min. Subsequently, each egg sample (20 g) was placed in a 200-mL beaker with 84 mL 90% acetonitrile and 0.5 g sodium carbonate and homogenized for 5 min using a high-speed blender (Ultra Turrax, IKA). For feed samples, 25 g sample was extracted with 100 mL 80% methanol and 2.5 g sodium chloride. After extraction, both egg and feed samples were filtered through filter paper (Whatman No. 1), and 4 mL filtrate extract was diluted with 36 mL PBS containing 1% Tween 20. After filtration through a GF/B filter, 25 mL filtrate was passed through an immunoaffinity column (IAC; AOZ WB, Vicam) at a flow rate of one drop per second. The IAC was washed with 15 mL PBS and 15 mL water, and dried by passing air rapidly through the column. The toxins were eluted with 3 mL methanol containing 0.1% acetic acid. The eluent was evaporated in a water bath at 50°C. Dried residues were reconstituted with 0.5 mL methanol/water (50:50, v/v) and filtered through a syringe filter (0.2 mm).

The HPLC system (Agilent 1200 series, Agilent Technologies) consisted of an autosampler system, four pumps, a column oven, and a fluorescence detector. The analytical column was a Symmetry C18, 3.5 mm, 4.6 × 150 mm column (Waters). To enhance the fluorescence activity of AFB1 and AFG1, a PHRED photochemical derivatization system (AURA Industries, USA) was applied before fluorescence detection. The excitation and emission wavelengths were respectively 360 and 455 nm (0-17 min) for AFs, 276 and 460 nm (17-22 min) for ZEA, and 225 and 460 nm (22-35 min) for OTA. The injection volume was 50 mL. The mobile phase was pumped at a flow rate of 1.0 mL/min under gradient elution at 35°C. The optimal HPLC conditions for the mobile phase (methanol, acetonitrile, and 0.1% acetic acid) were established as follows: 0-10 min, isocratic elution (27:14:59, by vol.); 10-12 min, gradient elution (27:14:59 to 10:44:46, by vol.); 12-28

min, isocratic elution (10:44:46, by vol.); and 28-33 min, column re-equilibration (27:14:59, by vol.). For column re-equilibration, a 2-min delay was employed between injections.

Quality control parameters for mycotoxin analysis

The HPLC performance parameters such as limit of detections (LOD), limit of quantification (LOQ), recovery, and repeatability were determined for AFB1, AFG1, AFB 2, AFG2, OTA, and ZEA. The LOD and LOQ for spiked samples were determined at signal-to-noise ratios of 3:1 and 10:1, respectively. Method precision was evaluated in terms of repeatability at three concentrations on the same day with three replicate analyses of each spiked sample. The results showed a good linear response and coefficient of determination for all analyzed mycotoxins (R^2>0.998). The recoveries were ascertained by spiking 1, 2, and 5 mg/kg AFB1, AFG1, or OTA; 0.3, 0.6, and 1.5 mg/kg AFB 2 or AFG2; and 10, 20, and 50 mg/kg ZEA in non-contaminated eggs (Table 2). The method showed good recoveries for all the spiked samples in the range of 71-118% with relative standard deviation (RSD) 2-20%.

Data analysis

The AFs, OTA, and ZEA concentrations were presented as the mean of three measurements. The coefficient of determination (R^2) was obtained by regression analysis using SPSS software (IBM, PASW Statistics 19, USA). The results for toxins were not corrected for analytical recovery.

Results and Discussion

Contamination by foodborne bacteria

The microbial contamination results for eggs are shown in Table 1. *Salmonella* species were not isolated from any of the 475 eggs collected from egg layers, egg-processing units, and retail markets. *E. coli* was isolated from 43 eggshell swabs, of which 16 and 27 obtained from egg-processing units and retail markets, respectively. Interestingly, *E. coli* was not isolated from eggs obtained from egg layers. *Arcobacter* species were not isolated from eggshell swabs, because they are fastidious bacteria. However, species-specific multiplex PCR detected *A. butzleri* 16S ribosomal DNA in *Arcobacter*-enriched broth prepared from four eggs collected from egg layers.

Table 1. Microbial contamination of eggs collected from farms to retailers

Sampling site	*Salmonella* species	*E. coli*	*Arcobacter* species
Egg layer farms (3)	0/30 (0%)	0/30 (0%)	4/30 (13.3%)
Egg-processing units (3)	0/180 (0%)	16/180 (8.9%)	0/180 (0%)
Retail markets (5)	0/265 (0%)	27/265 (10.1%)	0/265 (0%)
Total	0/475 (0%)	43/475 (9.05%)	4/475 (0.84%)

Table 2. HPLC parameters for analyzing aflatoxins, ochratoxin A, and zearalenone in eggs and chicken feed

Parameter / Mycotoxin	Concentration spiked (μg/kg)	Recovery (%)	RSD [a] (%)	LOD [b] (μg/kg)	LOQ [c] (μg/kg)
Aflatoxin B1	1	108.7	15.81	0.07	0.26
	2	117.8	8.60		
	5	102.4	2.41		
Aflatoxin B2	0.3	118.3	4.44	0.02	0.08
	0.6	112.5	11.40		
	1.5	100.7	2.58		
Aflatoxin G1	1	118.1	2.85	0.13	0.32
	2	98.2	13.79		
	5	88.3	7.94		
Aflatoxin G2	0.3	110.4	6.50	0.02	0.07
	0.6	96.3	15.65		
	1.5	76.4	6.50		
Ochratoxin A	1	71.4	9.38	0.10	0.65
	2	71.6	2.97		
	5	78.9	6.06		
Zearalenone	10	74.9	16.40	1.30	8.00
	20	76.8	9.42		
	50	105.4	19.74		

[a]RSD, relative standard deviation. Precision is statedas mean percent RSD. [b]LOD, limit of detection. [c]LOQ, limit of quantification.

The first isolation of *Salmonella* Enteritidis was reported from eggs at a grocery market in Korea in 2013; the annual microbiological survey was negative for *Salmonella* contamination of chicken eggs distributed from farms to markets between 2000 and 2011 (Kim *et al*., 2013). As the high risk of *Salmonella* contamination was addressed in outbreak data, risk assessments of eggs, performed by the US Department of Agriculture and Health Canada, showed that the overall contamination frequency of *Salmonella* species was as low as 0.03% (Greig and Ravel, 2009; Health Canada, 2000; USDA-FSIS, 1998; WHO, 2002). In the WHO risk assessment of *Salmonella*, the attack rate of *Salmonella* serovars and the dose-response of *Salmonella* outbreak were analyzed in various models and cases (WHO, 2002). Various *Salmonella* serotypes including Enteritidis, Typhimurium, Heidelberg, Cubana, Infantis, Newport, and Oranienburg were associated with the outbreaks of *Salmonella* species contaminating chicken, eggs, and other foods (Greig and Ravel, 2009; Health Canada, 2000; WHO, 2002). Because *S.* Enteritidis is frequently isolated from slaughtered chickens in Korea, there is a high possibility of egg contamination (Lee *et al*., 2007). Although this study did not isolate *Salmonella* species, the number of eggs examined herein was too limited to conclude that there was no contamination. Therefore, intensive risk assessment and microbial monitoring of eggs should be undertaken in Korean farms and markets.

The statistics on foodborne outbreaks by the MFDS in Korea indicate that pathogenic *E. coli* outbreaks occur more frequently than those of *Salmonella* or other foodborne bacteria. Although an association between raw eggs and *E. coli* outbreaks was not officially reported, eggs could be a source of contamination. In this study, the overall isolation rate of *E. coli* in eggs was as high as 9.05%. Interestingly, *E. coli* was isolated only from egg-processing units and retail markets. This may imply that contamination with *E. coli* occurs during egg processing or packaging.

Although *Arcobacter* species were not isolated in bacterial culture, multiplex PCR identified the contamination of *A. butzleri* in eggs collected from layer farms. When *A. butzleri* and *A. cryaerophilus* were isolated from chickens and intestinal contents (Lee *et al*., 2010), the isolation rate from enriched broth was lower than the detection rate by multiplex PCR. Thus, detection of *A. butzleri* by multiplex PCR indicated that four eggs were contaminated with *Arcobacter* species in this study. Because *Arcobacter* species can grow at room temperature and penetrate a micropore structure similar to the eggshell, bacterial internalization under various storage conditions should be examined in further studies.

Detection of mycotoxins in eggs and chicken feeds

In the present study, 475 eggs, collected from farms to retailers, and 20 chicken feeds were analyzed for the incidence of AFs, OTA, and ZEA (Table 3). The results showed that no egg samples were positive for AFs, OTA, or ZEA; however, these mycotoxins were detected in 100, 100, and 85%, respectively, of the 20 commercial chicken feeds analyzed. The maximum levels of AFs (sum of AFB 1, AFG1, AFB2, and AFG2), OTA, and ZEA were 1.86, 2.24, and 147.53 μg/kg, respectively. Of the AFs, the level of AFB1 in chicken feeds was 0.09-1.70 μg/kg with a mean of 0.38 μg/kg.

Several studies reported limited data for AFs, OTA, or ZEA contamination in eggs. Iqbal *et al*. (2014) reported that 28, 35, and 32% of eggs (n=80) collected from Pakistan were contaminated with AFs, OTA, and ZEA, respec-

Table 3. Contamination of eggs and chicken feeds by aflatoxins, ochratoxin A, and zearalenone

Number of Samples	Sampling site	Mycotoxin	Positive sample	Mean (μg/kg)	Range
Egg (n=30)	Egg layer farms (3)	Aflatoxins	0/30	-[a]	-
		Ochratoxin A	0/30	-	-
		Zearalenone	0/30	-	-
Egg (n=180)	Egg-processing units (3)	Aflatoxins	0/180	-	-
		Ochratoxin A	0/180	-	-
		Zearalenone	0/180	-	-
Egg (n=265)	Retail markets (5)	Aflatoxins	0/265	-	-
		Ochratoxin A	0/265	-	-
		Zearalenone	0/265	-	-
Feed (n=20)	Egg layer farms (3)	Aflatoxins	20/20 (100%)[b]	0.56 (0.38)[c]	0.10-1.86 (0.09-1.70)[c]
		Ochratoxin A	20/20 (100%)	0.77	0.14-2.24
		Zearalenone	17/20 (85%)	35.02	5.17-147.53

[a]Not detected. [b]The data in parenthesis represent the percentage of positive samples. [c]The data in parentheses represent aflatoxin B_1.

tively. A report by Amirkhizi *et al*. (2015) showed that 58% of egg samples from Iran were contaminated with AFB1 at 0.30-16.36 μg/kg. Herzallah (2009) demonstrated AFs contamination levels of 0.15-6.36 μg/kg in analyzed food products, with a mean of 1.23 μg/kg from 10 eggs. However, no egg samples in this study were contaminated with AFs, OTA, or ZEA. These data were consistent with the previous finding that ZEA in feed are not transferred to eggs (Danicke *et al*., 2002).

Jang *et al*. (2007a, 2007b) reported that 85% of 41 poultry feeds in Korea were contaminated with OTA, and detected levels ranged from 0.27 to 3.39 μg/kg. In Pakistan, 24% of 123 poultry feeds were contaminated with AFB1, and detected levels ranged from 0.43 to 2.54 μg/kg (Iqbal *et al*., 2014). When AFs and OTA were analyzed in Indian poultry feed ingredients using indirect competitive enzyme linked-immunosorbent assay, their levels in groundnut cake, maize, mixed feed, sorghum, and millet were 10-3500 μg/kg and 0-400 μg/kg, respectively (Thirumal-devi *et al*., 2002).

Mycotoxin contamination in eggs could be attributed to feed quality, and in animal feed might induce sanitary disturbances, animal mortality, and secondary contamination in human consumers via eggs, meat, or milk (Greco *et al*., 2014). Therefore, regulation of mycotoxin levels in feed is important. The commission regulation of the European community set maximum levels for AFB1, OTA, or ZEA in various animal feeds (European Commission, 2006), whereas the Korea Ministry of Agriculture, Food and Rural Affairs (MAFRA) set maximum acceptable levels only for AFs (10 μg/kg) and OTA (200 μg/kg) in chicken or broiler feeds. However, no maximum acceptable level for ZEA has been established for animal feed in Korea (MAFRA, 2010). We found that the levels of AFs and OTA in all domestic chicken feeds were below the maximum acceptable levels. Consequently, the occurrence and incidence of AFs, OTA, and ZEA in eggs and chicken feeds are considered negligible and unlikely to pose a threat to human health in South Korea.

Conclusions

This study demonstrated that *E. coli* and *A. butzleri* contaminated eggs from farms to retail markets. Even though AFs, OTA, and ZEA were found to be under the maximum acceptable level in chicken feeds, they were not detected in eggs in Korea. However, continuous surveillance is recommended, given the high consumption and importance of eggs as a nutritious food in Korea.

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