



Detection of changes in genes related to antioxidant and biotransformation function in broiler birds fed aflatoxin by real time PCR

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Occurrence of mycotoxins in foods and feeds is a major concern all over the world (Celyk *et al.*, 2003). Consumption of such contaminated feed by poultry results in hepatotoxicity, nephrotoxicity, feed refusal, growth retardation, immuno-suppression and mortality, thereby the toxins besides affecting the health of birds, also causes economic losses in poultry. Aflatoxin is a potent liver toxin causing hepatocarcinogenesis, hepatic necrosis, cirrhosis and acute liver damage in affected animals. (Oguz *et al.* 2000). Aflatoxins cause a variety of effects in poultry, including poor performance, liver pathology, immuno-suppression, and changes in relative organ weights (Edds and Bortell, 1983; Kubena *et al.* 1990,1993). Aflatoxin B₁ causes lipid peroxidation in liver (Shen *et al.* 1994), induces oxidative damage in the liver cells, forms DNA adducts, and thus acts as a potent carcinogen (Imlay and Linn 1988).

Few *in vitro* studies and *in vivo* studies in rats and broilers suggested oxidative stress as the main mechanism by which aflatoxin exerts its toxic effects (Shen *et al.* 1995, Meki *et al.* 2001 Eraslan *et al.* 2005). Aflatoxin B₁ (AFB₁) increased the levels of caspase-3 (apoptotic marker) activity, tissue levels of lipid peroxidation products and reduced the levels of antioxidants in male rat liver (Meki *et al.* 2004). As very few reports are present on the changes if any, caused by aflatoxins at the molecular levels the objective of the present study was to evaluate the effects of aflatoxin on the expression of a few hepatic genes involved in antioxidant function [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S transferase- α (GST α)], and biotransformation [epoxide hydrolase (EH), cytochrome P450 1A1 and 2H1 (CYP1A1 and CYP2H1)] in broiler chicks fed AFB₁ employing Real time PCR.

AFB₁ for the feeding trials was produced in the laboratory from *Aspergillus parasiticus* (IMTECH 2797) by solid

substrate fermentation. The purity of the toxin was tested by the certified Animal Feed Analytical and Quality Assurance Laboratory, Veterinary College and Research Institute, Namakkal (T.N.). Commercial, day-old male broiler chicks (Cobb) vaccinated for Newcastle disease were purchased from a commercial hatchery. All broilers were fed *ad lib.* with a basal diet, fed in 2 stages: starter diets (0–3 weeks) and finisher diets (4–5 weeks), formulated according to the standards prescribed in Bureau of Indian Standards (BIS 1992). No antibiotics or liver stimulants were added to the diet and the diet was free from AFB₁. Forty-eight birds were divided into the following 2 dietary treatments with 3 replicates having 8 birds in each: Control designated as “C” in which birds were fed the basal diet alone and the treatment group designated as “T” in which birds were fed the basal diet contaminated with 1.0 ppm AFB₁. The experimental broilers were vaccinated and the experiment was conducted for 42 days (one broiler period). The temperature of the bird facility was 34°C on arrival of the chickens, was gradually decreased to 25°C after 3 wk, and then was kept constant. Continuous lighting was used throughout the 42 d experimental period. The body weight gain (BWG) of chicks individually and feed intake (F_I) of each replicated groups allotted for different treatments were recorded at weekly interval. The feed conversion ratio was calculated for each replicate separately on the basis of unit feed consumed to unit body weight gain. Daily monitoring of chicks on individually basis was carried out to record the morbidity/mortality. The postmortem examination of dead birds was done to explore the probable cause of death.

qPCR of antioxidant function and biotransformation related transcripts

Sample collection and RNA purification: At the end of the experiment, birds were slaughtered and liver tissue samples were collected randomly from one bird from the control and aflatoxin fed birds each run in four replicates. Immediately after salughtering, approximately 30 mg of tissues were

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dissected from each liver, placed into a 1.5 ml tube, snap chilled into liquid nitrogen and stored at -80°C until processed for the purification of total RNA. The purification of total RNA was performed for group 1 (control) and group 2 (AFB1 fed) using commercially available kit that included DNase-I digestion during the purification process. Following purification, the quantity and quality of the RNA samples was determined using a Nano Drop Spectrophotometer and the concentration and 260/280 ratio ranged from 311.60 to 744.9 ng/ μL and 2.10 to 2.13 for the control birds and from 675.8 to 952.0 ng/ μL and 2.13 to 2.16 in the AFB1 treatment group. The quality of the purified RNA samples was also checked on agarose-formaldehyde gel.

Reverse transcription and real time PCR: Purified total RNA was reverse transcribed into cDNA using commercially available kit. RNase inhibitor was included into the reaction for synthesizing cDNA. Real time PCR amplification was performed using SYBR Green assay. The PCR amplification was performed using previously reported (Yarru *et al.* 2009) primers (Table 1) for the genes related to antioxidant function (Catalase: CAT, superoxide dismutase: SOD; glutathione peroxidase: GPx; glutathione S-transferase- α : GST- α) and biotransformation (epoxide hydrolase: EH; cytochrome P450 1A1: CYP1A1; cytochrome P450 2H1: CYP2H1). Additionally, the amplification of GAPDH (reference gene for relative quantification) was also tried using previously reported primer, but could not be amplified. PCR amplification was conducted in a total reaction volume of 20 μL containing 20 ng total RNA equivalent cDNA with the following amplification protocol: (50°C , 2 min; 95°C , 10 min; and 40 cycles at 95°C , 15 s; 60°C , 1 min). Statistical analyses of the data was performed by comparing birds fed AF with control birds for each gene using a 2-tailed *t*-test with unequal group variance (SAS 2008).

Apart from a reduction in growth performance and mortality as also evidenced in the present study (data not presented), oxidative stress is the main mechanism by which aflatoxins exert their toxic effects and thus to evaluate the changes in genes related to antioxidant function and biotransformation RNA was purified and its quality checked on agarose-formaldehyde gel (Fig. 1). The concentration of RNA in respect of the control and aflatoxin fed birds averaged 619.3 ± 110.33 ng/ μL and 779.03 ± 122.76 ng/ μL while the 260/

280 ratio was 2.12 ± 0.015 and 2.14 ± 0.012 respectively.

The results for real time PCR amplification and agarose gel electrophoresis of the PCR products of different transcripts are given below in plate 2. Agarose gel electrophoresis (1%) of the PCR products (Yarru *et al.* 2009) of catalase (CAT, 130 bp) did not reflect any significant variation ($P > 0.05$) as evident from lanes 2 and 3, between the control (G1) and AFB1 fed birds (G2) in our study (Fig. 2A) and are in agreement with the findings of Yarru *et al.* (2009) who observed no statistical difference in gene expression among the treatment groups for CAT.

However, cytochrome P450 1A1 (CYP1A1, 125 bp) and cytochrome P450 2H1 (CYP2H1, 137 bp) showed significantly higher expression ($P < 0.05$) as evident from lanes 4, 5, 6 and 7 (Fig. 2A) a finding in agreement with the results of Yarru *et al.* (2009). No significant difference ($P > 0.05$) was obtained in the expression of epoxide hydrolase (EH, 128 bp), glutathione peroxidase (GPx, 140bp), superoxide dismutase (SOD, 122bp) and glutathione S-

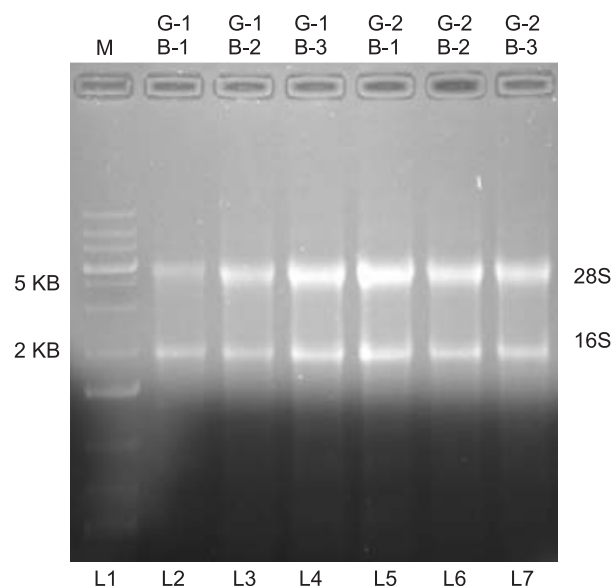


Fig. 1. Agarose-formaldehyde gel electrophoresis of purified RNA of the control (G-1) and aflatoxin fed (G-2) birds

Lane 1-DNA Step Ladder of 1 Kb; Lane 2,3 and 4- RNA from the 3 replicates of birds B1,B2,B3 of group 1 and Lane 5,6 and 7- RNA from the 3 replicates of birds B1,B2,B3 of group 2.

Table 1. Primer sequences (5' $\rightarrow$ 3') used in real-time PCR (Yarru *et al.* 2009)

Name	Symbol	Forward primer	Reverse primer	Product size (bp)
Catalase	CAT	GGGGAGCTGTTTACTGCAAG	TTCCATTGGCTATGGCATT	139
Cytochrome P4501A1	CYP1A1	CACTTTCTGCCTGCTCCTG	GGTCCTTCCTCAGCTCCAG	125
Cytochrome P4502H1	CYP2H1	ATCCCCATCATTGGAAATGT	TCGTAGCCATACAGCACCAC	137
Epoxide hydrolase	EH	AAAGGGACAGAAGCCTGACA	CCTCCAGTGGCTCAGTGAAT	128
Glutathione peroxidase	GPx	TGTAAACATCAGGGGCAAA	TGGGCCAAGATCTTTCTGTAA	140
Superoxide dismutase	SOD	AGGGGGTCATCCACTTCC	CCCATTGTGTGTGTCTCCAA	122
Glutathione S-transferase-a	GSTa	GCCTGACTTCAGTCCTTGGT	CCACCGAATTGACTCCATCT	131

transferase- α (GST- α) of the control and AFB1 fed birds as evident from lanes 2 to 9 (Fig. 2B).

There are numerous studies in poultry on the effects of AFB1 with regard to growth performance, liver pathology, immuno-suppression, changes in relative organ weights (Edds and Bortell 1983, Kubena *et al.* 1990,1993), lipid peroxidation in liver (Shen *et al.* 1994), oxidative damage in the liver cells (Imlay and Linn 1988). Apart from the study of Yarru *et al.* (2009) who studied the effects of turmeric (*Curcuma longa*) powder (TMP) on the expression of hepatic genes associated with biotransformation, antioxidant, and immune systems in broiler chicks fed aflatoxin, there is a dearth of literature on changes in these genes in AFB1 toxicity to compare our results.

Primary hepatic detoxification processes include xenobiotic biotransformation (phase I metabolism) and the subsequent conjugation of the resulting metabolites (phase II metabolism), making them more water-soluble and available for excretion from the body. The microsomal cytochrome P450 (CYP)-dependent monooxygenase system in the liver plays an essential role in phase I metabolism (Akahori *et al.* 2005).

The CYP enzymes are associated with several biological interactions involving hydroxylation, epoxidation, oxygenation, dehydrogenation, nitrogen dealkylation, and oxidative deamination (Hari Kumar and Kuttan 2006). Cytochrome P450-mediated reactions can also generate ROS.

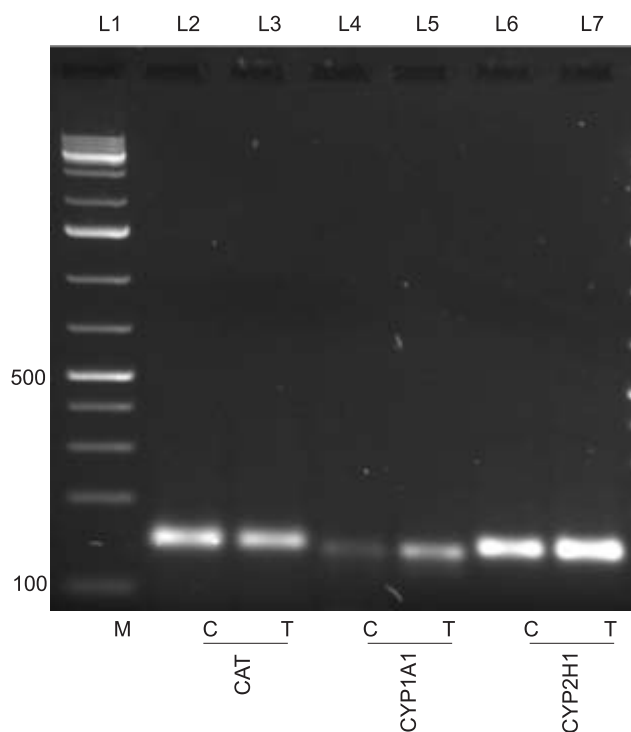


Fig. 2A. Agarose gel electrophoresis of the PCR products of CAT, CYP1A1 and CYP2H1. Lane 1- Molecular marker; Lane 2, 4 and 6 - control group 1 and Lane 3,5 and 7-treatment group 2.

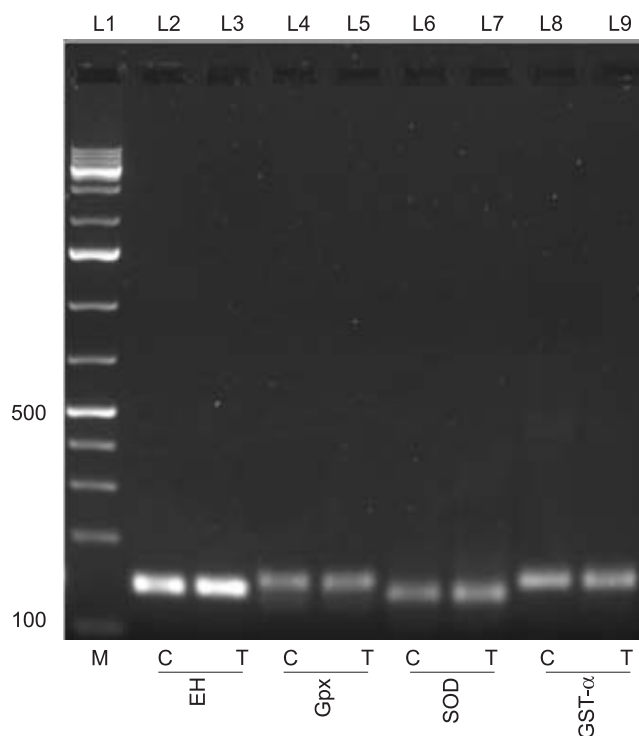


Fig. 2B. Agarose gel electrophoresis of the PCR products of EH, GPx, SOD, and GST- α . Lane 1- Molecular marker; Lane 2, 4,6 and 8 - control group 1 and Lane 3,5,7 and 9-treatment group 2.

The major CYP enzymes involved in hepatic metabolism of AF in poultry are the CYP1A1 (Klein *et al.*, 2003) and CYP2H1 isoforms (Hamilton *et al.* 1993). The gene CYP1A1 is known to activate certain promutagens to their carcinogenic forms (Haas *et al.* 2006, Hari Kumar and Kuttan 2006). Over expression of these CYP isoforms has been shown to induce chronic oxidative stress by generating more ROS, possibly leading to hepatocellular injury and death (Hari Kumar and Kuttan 2006). Yarru *et al.* (2009) determined changes in gene expression using the quantitative real-time PCR technique and observed no statistical difference in gene expression among the 4 treatment groups for CAT and IL-2 genes. However, increased expression of IL-6, CYP1A1 and CYP2H1 genes due to AFB1 was also alleviated by TMP.

It is evident from the results of the present study that transcriptional activation of CYP1A1 and CYP2H1 isoforms, in response to AFB1, has the potential to increase oxidative stress. In the present study, an increase in the expression of hepatic CYP1A1 and CYP2H1 genes was observed in chicks fed AFB1 (Fig. 2B).

Superoxide dismutase catalyzes the conversion of superoxide anions to hydrogen peroxide and is one of the primary enzymatic defenses against ROS. In the present study, no change was obtained in the hepatic gene expression of SOD in chicks fed a diet containing AFB1 versus the control diet (Fig. 2B). Antioxidant enzymes, such as CAT

within the peroxisomes and cytosolic GPx, are involved in the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, into water and molecular oxygen (Liska 1998). Compared with controls, the expression of the GPx gene was not significantly decreased in birds fed AFB1.

Conjugation of reactive xenobiotic metabolites with glutathione is an important step in detoxification and is mediated by GST α . An overload of xenobiotics may deplete glutathione through conjugation activities, thereby contributing to oxidative stress (Percival, 1997). Decreased expression of SOD and GST genes in chicks fed AFB1 is additive with respect to oxidative damage but no alteration in hepatic gene expression of these genes, was observed in the present study in birds fed AFB1.

SUMMARY

Current findings suggest that the AFB1 failed to influence hepatic gene expression of SOD, CAT, EH, GPx and GST α but increased the gene expression of the two biotransformation genes CYP1A1, and CYP2H1 at the levels used. Results show that changes in genes related to antioxidant and biotransformation function could be detected by real time PCR. Further work to study the expression of antioxidant and biotransformation genes with higher levels of aflatoxin along with beneficial effects of phytochemicals in ameliorating the same in broiler birds are under progress.

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