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Article

Effect of air pollution with *Aspergillus* spp.in occurrence of Aflatoxin M1 in milk and its products

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Abstract: Aspergillus fungi produce a family of mutagenicity and carcinogenicity mycotoxins called aflatoxins, which has been shown to cause hepatocellular carcinoma (HCC) in human tissues and can form derivatives with several cellular macromolecules. The study was conducted in Hawija, Kirkuk City, from January to June 2021. The feedlot storages are used for a stored ration that is used for cow nutrition, which is the source of milk. In the current study, we used 80 cow raw milk samples and their products (40 soft white cheeses and 40 yogurts). The results showed fungal growth was recorded in 71 feedlot storages out of 80 feedlot storages at a rate of 88.75%, and *Aspergillus* spp detection in 59 out of 71 feedlot storages at a rate of 83.1%. Also, the current study showed that 54% of milk samples, 62% of yogurt samples, and 86% of cheese samples were contaminated with Aflatoxin M1. Pollution of feedlot storage environments by *Aspergillus* spp. led to contamination of rations with *Aspergillus* spp. and its toxin. Animals fed with contaminated rations containing toxin will have produced this toxin in the milk.

Keywords: hepatocellular carcinoma; toxicity; molecular mechanisms; *Aspergillus flavus*

1. Introduction

Aflatoxin's interaction with proteins and nucleic acids, as well as its role in the development of disease and the advancement of hepatocellular carcinoma (HCC) [1], the anti-mutagenic qualities of a number of dietary variables. Without a doubt, Aflatoxin's putative involvement in the formation of human cancer, especially HCC, is its most significant mechanism of action [2], aflatoxin plays a significant role in the genesis of human HCC [1]. Milk is a nutrient-rich medium highly susceptible to microbial contamination, including molds. Mold contamination in milk can occur at various stages of its production, processing, storage, and distribution. Molds are filamentous fungi that thrive in conditions with sufficient moisture and nutrients, making milk an ideal environment for their growth [1,2]. The presence of mold in milk not only degrades its quality but also poses significant health risks to consumers. Certain molds can produce harmful secondary metabolites called mycotoxins, such as aflatoxins, which are known to be carcinogenic and toxic. These contaminants can lead to economic losses in the dairy industry due to spoilage and reduced product shelf life. Mold contamination can occur due to poor hygiene practices, improper processing, or inadequate storage conditions. Identifying and preventing mold contamination in milk is crucial to ensuring food safety, protecting public health, and

maintaining the quality of dairy products. This highlights the importance of implementing stringent hygiene protocols, effective storage methods, and regular microbial testing in the dairy industry [3,4].

Widespread in the air, soil, plants, and saprophytic materials is the saprophytic fungus *Aspergillus*. the production and storing of agricultural grains, silage, and animals [5,6]. There are more than 150 species of *Aspergillus*, 20 of which are harmful to humans and animals, primarily *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus parasiticus* [7,8]. *A. flavus* poses a danger due to its capacity to manufacture Aflatoxin B1 geographically. The aflatoxin production process may be controlled by the AFLR gene from *A. flavus*. A binuclear zinc finger DNA-binding domain of the GAL4 type is present in the aflR gene product, AFLR. It is believed that albutoxin, which has a low molecular weight and is heat-resistant, is a typical carcinogen for hepatocells and causes hormonal abnormalities [9,10].

Along with the 28S rRNA region (D1-D2), the internal transcript spacer (ITS1 to ITS4), a distinct region found in rDNA, is frequently utilized to distinguish *Aspergillus* species [11]. Other studies used sequencing of other target genes in the genetic determination of *A. flavus*, including tubuline, tpoisomerase II, and genes of calmodulin for species that are distantly associated due to low variability in those regions. These genes are used to differentiate between closely associated species like *A. flavus* and *A. oryzae*, and *A. parasiticus* and *A. sojae*. The most varied and trustworthy target region for *A. flavus* molecular identification is still 18S rRNA, nevertheless [12]. Aflatoxin is detected using a variety of techniques, including ELISA, High-Performance Liquid Chromatography (HPLC), and Thin Layer Chromatography (TLC) [13,14].

The current study aimed to determine air pollution of feedlot storage with *Aspergillus flavus* and the effect of this pollution on the occurrence of Aflatoxin in milk and its products [15,16].

2. Materials and methods

2.1. Sample collection

The study was conducted in Hawija, KirkukCity in period January to June 2021. The cow nutrition supplied from feedlot storages, which are considered as main source of milk and their production chiefed in the current study.

2.2. Detection of air contamination with *Aspergillus* spp. in feedlot storages

The distribution of *Aspergillus* spp. was determined on 80 feedlot storages using the Gravity Settling Plate Method and Potato Dextrose Agar (HiMedia-India). The plate was opened in the feedlot storages for 10 min before being closed and sent to the lab. the seven-day plate culture at 25 ℃.

2.3. Detection Aflatoxin in feedlot storages

At a rate of 500 gm/ton, feedlot samples were gathered. Ten grams were ground again, put through a fine screen, and then extracted with ethanol. Total Aflatoxin as

measured by ELISA for aflatoxin Italian ELISA Kite Euro Clone. Five plates were distributed for 25 feedlot storage rooms only to measure aflatoxin production by using ELISA kit.

2.4. Production of cheese and yogurt from raw milk

40 soft white cheeses and 40 yogurts made from 80 cows' raw milk. From the raw milk sample, fat must be removed by centrifuging the milk and aspirating the lower layer, or by storing the milk overnight in the refrigerator before removing the fatty milk layer.

Cheese production from raw milk involves several stages including, coagulation using a starter culture ferment lactose, producing lactic acid. This lowers the pH and, combined with enzymes like rennet, leads to the coagulation of casein proteins followed by Curd processing by cut and stirred to release whey. The cheese is aged under controlled conditions [17,18].

2.5. Detection Aflatoxin in milk productions

A competitive enzyme immunoassay for measuring aflatoxin M1 in raw cow milk samples, cheese and yogurt using MaxSignal® Aflatoxin M1 ELISA Kit. It was created in the United States by PerkinElmer.

2.6. Identification of *Aspergillus flavus* isolates by PCR

Using established techniques, genomic DNA was extracted from isolates. Agarose gel electrophoresis was used to detect the purity of the DNA (1.0%). After the DNA specimens were loaded onto the gel with loading buffer (PlatinumTM II Green PCR Buffer 5X, Thermo Fisher Scientific, Waltham, MA, USA), banding was subsequently observed under UV light in a photodocumenter (Bio-Rad Molecular Imaging®-Gel Doctm XR, Hercules, CA, USA) with Quantity One software (version 4.6.7). Polymerase chain reaction (PCR) was used to amplify a portion of the internal transcript spacers (ITS1-5.8S-ITS2-ITS2-rRNA) in accordance with methods previously published [19]. The following primers were used for ITS1 and ITS4: 5′-TCCGTAGGTGAACCTCTGCGG-3′, 5′-TCCTCCGCTTATTGATATATG-3′, (Thermo Waltham, Massachusetts, USA: Fisher Scientific. A molecular weight marker (1.0 L, DNA Ladder 100 bp, 0.5 g/L, Thermo Fisher Scientific) and buffer (1.0 L, BlueJuice gel loading buffer 10X, Thermo Fisher Scientific) were present in the first lane of the gel. PCR products were cleaned up using ExoSAP-IT® PCR Product Cleanup Reagent (Afflymetrix, Thermo Fisher Scientific Inc., Santa Clara, CA, USA) [20].

3. Results

3.1. Identification of *Aspergillus* spp

Fungi growth was seen in 71 feedlot storages out of 80 feedlot storages in the current study, with an occurrence rate of 88.75%, with *Aspergillus* spp. detection occurring in 59 out of 71 feedlot storages with an occurrence rate of 83.1%.

Aspergillus fumigatus 23, *Aspergillus niger* 16, and *Aspergillus flavus* 20 were the three types of *Aspergillus* that were isolated. **Figure 1**.

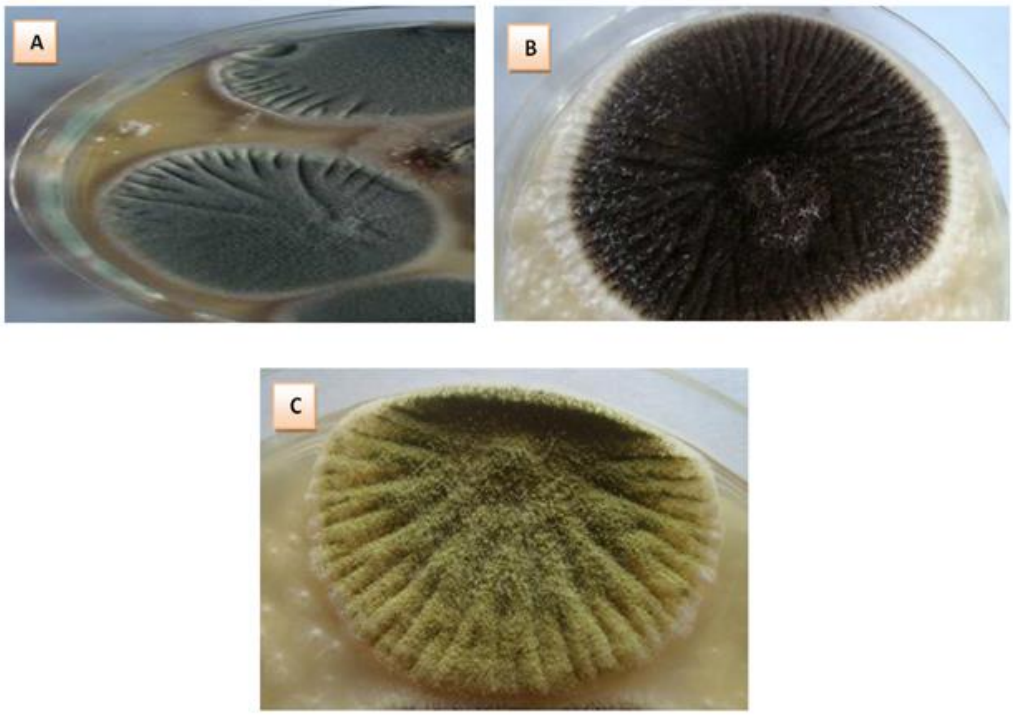

Figure 1. (A) Colony of *A. fumigatus* after 5 days of cultivation, *Aspergillus fumigatus*: characterized by bright bluish-greento gray; **(B)** Colony of *Aspergillus niger* after 5 days of cultivation characterized by black colony figure; **(C)** colony of *Aspergillus flatus* after 5 days of cultivation characterized by yellowish green colony.

3.2. Evaluation Aflatoxin production on feedlot storges and on raw milk & its production using ELISA kit

Aspergillus spp. was successfully isolated from feedlot storages. The geographical region, storage method, season, types of feedlots, and the humidity may all have an impact on the fungal growth and its toxin production capability. The current study reveals all feedlot was contaminated with Aflatoxin in different concentration as in **Table 1**.

Table 1. Aflatoxin concentration on feedlot storges.

Concentration of Aflatoxin [ng/gram]	No. of sample [25 feedlot measured by ELISA KIT]	Rate
1–50	13	48%
51–100	5	20%
101–200	3	12%
201–300	1	4%
301–400	2	8%
401–500	1	4%

The detection of aflatoxin in the feedlot was consistent while there were differences in the concentration that were brought on by the content of the feedlot and the storage method. The contamination rate with the Aflatoxin M1 toxin was

elicited in raw cow milk, yogurt samples, and cheese samples, according to **Table 2**. The findings of the present investigation showed that the toxin was present in milk samples in 54% of cases, yogurt samples in 62% of cases, and cheese samples in 86% of cases at levels greater than the permitted limit of 50 ng/kg as required by European law.

Table 2. concentration of Aflatoxin M1 toxin in raw milk and its products.

Results		**Sample types**		
		Raw cow milk sample	**Yogurt sample**	**Cheese sample**
Contaminated sample	No/rate	50(100%)	50(100%)	50 (100%)
	Mean ± SD	39.5 ± 31.2	45.3 ± 39.4	47.3 ± 22.3
	Rage	0.92–103.4	9.5–132.3	24.2–173.8
Positive sample	No/rate	27 (54%)	31(62%)	43(86%)
	Mean ± SD	63.2 ± 28.1	71.6 ± 21.7	96.5 ± 41.2
	Range	52.1–103	51.3–125.6	53.6–183.6
Negative	No/rate	23(46%)	19(38%)	7(14%)
	Mean ± SD	26.21 ± 18.5	29 ± 19.3	35 ± 17.3
	Range	0.92–44.2	12.2–46.3	23.2–46.5

Mean and range measured in ng/kg, Positive samples which are more than 50 ng/kg.

3.3. Identification of aflatoxin using HPLC

According to the retention times for each of them, **Figure 2A** displays the identification of Aflatoxin produced from isolates of *A. flavus* by HPLC in comparison to the standard (Aflatoxin B1) (**Figure 2B**).

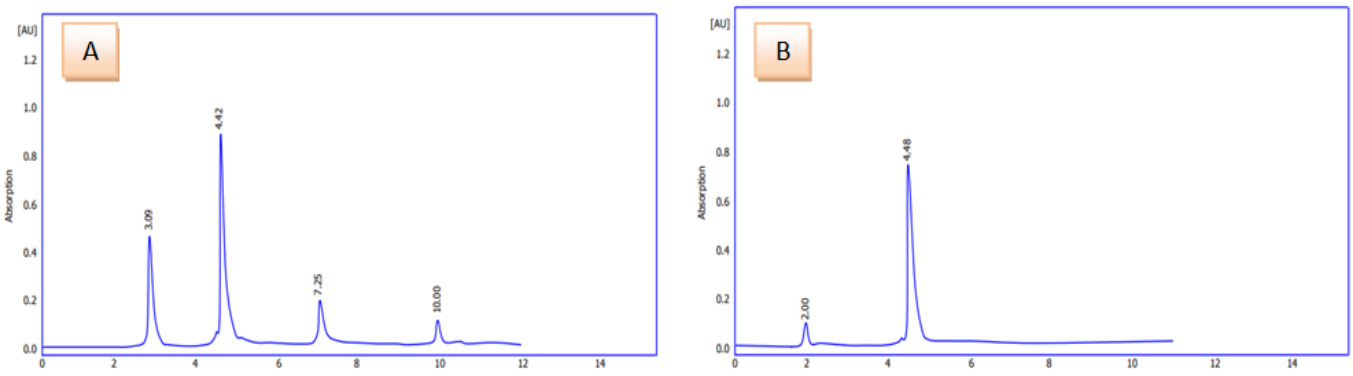

Figure 2. (A) HPLC diagram Aflatoxin B1 extracted from *Aspergillus flavus*; **(B)** HPLC diagram of Aflatoxin B1 standard.

3.4. PCR amplification

The oligonucleotide primer sequences were generated from the ribosome's DNA using the internal transcribed spacer gene. On a 1% agarose gel, every cDNA that had been amplified using traditional PCR displayed the same mobility. All 20 positive samples produced a distinctive 600 bp DNA band (**Figure 3**).

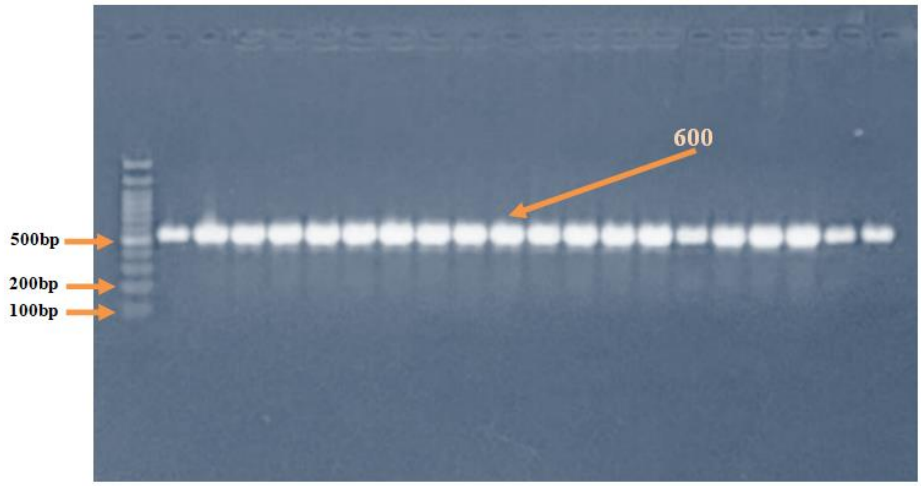

Figure 3. Twenty distinct fungal milk samples were tested using PCR. Primers ITS1 and ITS4 created a target band of 600 bp in the highly conserved (ITS) sections of ribosomal DNA. Lanes 1 through 15 represent samples from fungi. M: 100 bp marker, voltage 5 volts, for 2 h.

4. Discussion

According to the result that demonstrated cheese had higher concentrations of Aflatoxin M1 than milk and yogurt due to the function milk processing plays in stimulating the production of toxin-producing *Aspergillus* spp. In the study by Darsanaki et al. [21] they used the ELISA technique to test raw milk for the presence of AFM1 and found that in 56 out of 90 samples of raw milk, the level of AFM1 was between (2.1–131) ng/L and higher in 23 samples (31.11%) than the maximum tolerance level (50 ng/L) [21]. The current investigation was in agreement with a study conducted in Iraq by Najim et al. discovered that milk and dairy products contained 100% of the AFM1 protein. All types of milk were used in that study, including raw milk, domestically produced soft white cheese, domestically produced yoghurt, and imported pasteurized milk.AFM1 contamination ranged from 0.15 to 86.96, 31.84, 89.44, 0.16 to 42.74, and 0.18 to 85.66 ng/kg in the 30 samples for each. However, 2.5% of 40 pasteurized yogurt specimens and 10% of 10 local yogurt samples showed pollution above the limit of European Community Regulation (50 ng/L), and 10% and 30% of pasteurized and local yogurt specimens other than 25 ng/L were identified. It was found that 100% of processed and local yogurt specimens collected in (Northern Iran) were positive for AFM1 [22].

Aflatoxin B1 (AFB1) produced by the fungus isolate *A. flavus* that was recovered from milk samples had a mean concentration of (219.3) ng/L, although it has been found that *A. flavus* has a strong ability to synthesize Aflatoxin B1 at high concentrations that range from 18.6–740 ppm. All local isolate-produced toxins had retention times that were identical to those of the Aflatoxin B1 standard. Yogurt showed a greater prevalence of AFM1 than dairy products and milk, while local cheese had a higher concentration (300.7) ng/L when tested using HPLC, according to a study by AL-Mossawei et al. the limit detected by the HPLC technique was (10 ng/mL) [23].

All of the ITS region isolates evaluated in the current investigation using traditional PCR were effectively amplified using the primers ITS1 and ITS4.

According to sequence analysis, PCR results can be broken down into fragments that can be used to identify specific strains using the restriction enzymes EcoRI, HaeIII, and TaqI. There are numerous techniques, including PCR primer-based diagnostics randomly amplified polymorphic DNA, and sequencing, have been developed for the systematic examination of fungus. The methods employed, however, often rely on universal rRNA (or rDNA) gene analysis sequences that contain conserved and variable areas and enable the differentiation of fungi at various taxonomic levels [24]. One of the simplest methods for finding any microorganism in samples was the PCR. We discovered that all 20 *A. flavus* isolates responded favorably to the primer set as a result (**Figure 3**). According to a DNA ladder that was electrophoresed on the same gel and in the same circumstances, the *Aspergillus* DNA PCR fragment that was obtained was determined to be 600 bp in size. These results are consistent with prior studies that used PCR amplification of genomic DNA obtained from *A. flavus* isolates with ITS1 and ITS4 primers, which caused the amplification and predicted the target band, to show a high level of specificity and sensitivity. Due to evolutionary restrictions, the internal transcribed spacer (ITS) in the gene rRNA is thought to be one of the best places to detect strains and can therefore be utilized to accurately differentiate between species and strains [25–28].

Studies indicate that over 60% of aflatoxin contamination in milk is directly linked to contaminated feed [29,30].

Innovations in biosensors and rapid detection methods are improving the ability to monitor milk for fungal toxins during production [31–33].

The primary way that people are exposed to aflatoxin is via eating tainted milk [16,34]. When ducklings were exposed to aflatoxin orally, the first evidence of aflatoxin -induced acute hepatotoxicity [7,35], aflatoxin can additionally harm DNA by covalently attaching to it. This could intensify the genotoxicity that AFB1 already direct hazardous potential in human cell lines, even in the absence of metabolic activity [9,36]. Additionally, a number of in vivo investigations have demonstrated that aflatoxin suppresses both innate and adaptive immune responses [18].

5. Conclusions

Pollution ration of the environment storage feedlot related to contamination with *Aspergillus* spp and its toxin. Animals fed awith a contaminated ration containing toxin will have produced this toxin in the milk.

Author contributions: Conceptualization, SMA, ASH, SHM, BID and NBJ; methodology, SMA, ASH, SHM, BID and NBJ; software, SMA, ASH, SHM, BID and NBJ; validation, SMA, ASH, SHM, BID and NBJ; formal analysis, SMA, ASH, SHM, BID and NBJ; investigation, SMA, ASH, SHM, BID and NBJ; resources, SMA, ASH, SHM, BID and NBJ; data curation, SMA, ASH, SHM, BID and NBJ; writing—original draft preparation, SMA, ASH, SHM, BID and NBJ; writing—review and editing, SMA, ASH, SHM, BID and NBJ; visualization, SMA, ASH, SHM, BID and NBJ; supervision, SMA, ASH, SHM, BID and NBJ; project administration, SMA, ASH, SHM, BID and NBJ; funding acquisition, SMA, ASH,

SHM, BID and NBJ. All authors have read and agreed to the published version of the manuscript.

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