Journal of Advances in Microbiology

13(4): 1-9, 2018; Article no.JAMB.40075
ISSN: 2456-7116

Detection of Aflatoxigenic Moulds and Aflatoxins in Maize and Millet's Grains Marketed in Zaria Metropolis

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Authors' contributions

This work was carried out in collaboration between all authors. All Authors designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All Authors managed the analyses of the study. All Authors managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2018/40075
Editor(s):
(1) Dr. Nurullah Akcan, Assistant Professor, Department of Biotechnology, Siirt University, Siirt, Turkey.
(2) Dr. Akpaka, E. Patrick, Professor, Unit of Pathology & Microbiology,Faculty of Medical Sciences,The University of the West Indies St. Augustine, Trinidad & Tobago.
Reviewers:
(1) Fatih Kalyoncu, Manisa Celal Bayar University, Turkey.
(2) Samuel Y. Gazuwa, University of Jos, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/27876

Original Research Article

Received 05 January 2018
Accepted 29 March 2018
Published 19 December 2018

ABSTRACT

Aims: The present study was aimed at detecting of aflatoxigenic moulds and aflatoxins in maize and millet grains commonly marketed in Zaria Metropolis.
Study Design: The study was a laboratory-based research aimed at Detecting aflatoxigenic moulds and aflatoxins in the grains samples marketed in Zaria Metropolis
Place and Duration of Study: The samples were purchased from six different markets in Zaria metropolis between July and August 2016.
Methodology: Representative samples were subjected to proximate analysis and cultural isolation by grounding of each sample of (maize and millet) and separately added to 90 ml of sterile distilled water to form a stock suspension. An aliquot of 0.5 ml was spread on already prepared sweet potato yeast extract agar plate and the inoculated plates were incubated at room temperature. The

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suspected aflatoxigenic moulds were sub cultured and incubated at room temperature to obtain a pure culture and subjected to slide culture technique for microscopic identification of the moulds from the entire samples. The main objective of this study was to detect aflatoxin-producing moulds in maize and millet grains commonly sold in Zaria metropolis markets. The isolates were then screened for aflatoxin production using desiccated coconut agar and viewed under UV light (365 nm). Enzyme-linked Immunosorbent Assay (ELISA) technique was used to determine total aflatoxin concentration of the samples.

Results: The results revealed that the maize (Zea) and millet samples analyzed contain organic and inorganic nutrients that can support the growth of aflatoxigenic moulds and production of aflatoxin. Sixteen (25) isolates from the 60 samples were contaminated which account for 41.7%. The percentage of *A. flavus* isolates in maize was 26.7% and that of *A. parasiticus* was 15%.In maize, the occurrence of *A. flavus* was 23.3% and *A. parasiticus* 13.3% and the millet had 30% *A. flavus* and 16.7% *A. parasiticus.* The isolates demonstrated ability in aflatoxin production by emitting very bright fluorescent colouration under UV light (365nm). Total aflatoxin concentration in maize sample obtained from Samaru Market was found to be 18.10 µg/Kg while millets obtained from Zaria recorded 52.0 µg/Kg. The contamination levels within the grains were found to be statistically significant (*p* value< 0.05) using analysis of variance (ANOVA)

Conclusion: This study, therefore, revealed that the contamination level in millet samples analyzed calls for concern as it exceeded the standard limit set by NAFDAC and SON.

Keywords: Aflatoxin; contamination; fluorescent; aflatoxigenic mould; screening.

1. INTRODUCTION

Mycotoxins which are relatively large, diverse group of compounds produced by moulds are secondary metabolites that are toxic to human and animals. The three main genera of fungi that produce mycotoxins in foods in the world are *Aspergillus, Penicillium and Fusarium,* and the five agriculturally significant mycotoxins are aflatoxins, fumonisins, ochratoxin A, trichothecenes and zearalenone [1].

Aflatoxins are mycotoxins produced by *Aspergillus flavus and Aspergillus parasiticus.* Even when grains are well dried, wetting due to leak in stores, insect damage and activity lead to a prerequisite for the growth of aflatoxin-producing fungi [2].

The aflatoxin-producing moulds can grow on crops in the field, poorly dried harvested crops in storage and processed food and feed products [3].

Fungal growth may begin on agricultural produce at moisture content lower than 18.0%. The aflatoxigenic moulds grow best on crops at 18.0-18.5% moisture level [4]. Then as the fungus grows, respiration occurs releasing heat and moisture into the surrounding environment in the grain mass. This results in an increase in the moisture content and temperature of the surrounding crop, causing a hot spot. If moisture content and temperature continue to rise, the environment for aflatoxigenic moulds becomes more favourable. At 20% moisture content and above, other fungi grow better and crowd out aflatoxigenic moulds. [4]. Drought stress can lead to cracks in crops surfaces, providing additional entry sites for hyphae of aflatoxigenic moulds [4]. There is strong evidence for drought stress alone to be a contributor to elevated aflatoxin levels. According to [3], moisture levels in crops below 12 to 13% inhibit the growth of the fungus at any temperature.

2. MATERIALS AND METHODS

2.1 Description of the Study Area

Zaria metropolis is located at latitude 11°07' N and longitude 07°42' E, and is presently one of the most important cities in Northern Nigeria [5]. It has a total area of 300 km^2. As at 2007 Census, Zaria metropolis had a population of 1,018,827 [6].

2.2 Collection of Samples

Sixty samples; 30 each of maize and millet were collected from the major grains sellers from six different public markets comprising; Zaria city, Sabon Gari, Danmagaji, Kwangila, Tudun Wada and Samaru Markets in Zaria metropolis, Kaduna State, Nigeria in July 2016.

Five vendors were selected at different points in each market and 2 samples (1 maize and 1

millet). Approximately 100 g samples were purchased from each vendor. A total of 60 samples were collected; 30 samples of maize and 30 of millet and each was placed separately in clean, sterile container, labelled appropriately and transported immediately to the Laboratory for analyses.

2.3 Proximate Compositions of the Samples

Proximate composition of the grain samples was carried out following standard procedures of proximate analysis using Association of Analytical Chemist [7].

2.4 Mycological Analysis

2.4.1 Media preparation

Sweet potato yeast extract (SPIE) Agar was compounded as follows; 250 g of fresh sweet potato was cut into smaller pieces to increase the surface area and boiled in 1000 mL of distilled water for 30 minutes. The extract was collected in a clean container by passing the suspension through a clean muslin cloth.

To the total volume (500 ml) of the extract obtained in an Erlenmeyer flask, 1.3 g of yeast extract and 20 g of agar-agar was added and the pH was adjusted to 5.5 and boiled until the agar dissolved. The contents of the flask were sterilized in an autoclave at 121°C for 15minutes. After cooling, streptomycin antibiotic was added using a clean spatula and poured into sterile Petri-dishes and allowed to set. The plates were dried in an incubator at temperature 40°C for 35 minutes.

Slants of the SPYE agar was also prepared as described above but in test tubes.

Desiccated Coconut Agar (DCA) was prepared by modification of the method of Davis et al. as reported by [8] as follows: Two hundred grams of desiccated coconut was soaked in 1 l of hot distilled water for 30 min., blended aseptically in a Warring blender (Torrington, CT, USA) for 5 min and filtered through two layers of cheese cloth. Two percent agar (Oxoid) was added to the filtrate, heated to boiling, cooled to 50°C. To the filtrate 0.1% neutral red liquid stain was added and the pH was adjusted to 4.5. The media were then sterilized at 121°C for 15 min, cooled and poured uniformly into sterile Petri dishes.

2.4.2 Sample processing and inoculation

Ten gram (10 g) of each ground sample of (maize and millet) was separately added to 90 ml of sterile distilled water and homogenized for 2 minutes to form a stock suspension. An aliquot of 0.5ml was spread on already prepared sweet potato yeast extract agar plate and the inoculated plates were incubated at room temperature for 3- 5 days [9].

2.4.3 Isolation and identification of aflatoxigenic moulds

The Suspected Aflatoxigenic moulds isolate cultured on SPYE was sub-cultured on sweet potato yeast extract agar plate and incubated at room temperature for 3- 5 days and pure isolates was obtained. The observed colonies were identified based on morphology and microscopic features using standard reference by Pitt and Hocking [10] and the pure isolate was sub-cultured into fresh sweet potato yeast extract agar slant until required.

2.4.4 Screening of the isolates for aflatoxin production

The various pure isolates obtained were screened for aflatoxigenicity using desiccated coconut agar as described by Ezekiel et al. [11]. Each isolate was inoculated on freshly prepared desiccated coconut agar and incubated at room temperature for 3-5 days and examined. Isolates that absorbed and emitted very bright, moderate and weak UV light (fluorescence) at 365 nm were confirmed to be aflatoxin producing aflatoxigenic moulds.

2.5 Detection and Quantification of Aflatoxin Level in the Samples Using ELISA

2.5.1 Sample preparation and extraction

A representative sample of maize and millet was grounded to 20 mesh sieve size. 50 g of the grounded samples was put into a conical flask and 5.0 g of NaCl was added. The samples were mixed with 100 ml of 80% (v/v) methanol and blended at high speed (250 RPM) for 3 minutes. The mixture was allowed to settle after which it was filtered. 5 ml of the filtrate was mixed thoroughly with 20 ml of distilled water and filtered through a glass fibre filter. The optical density (OD) was taken from each micro well and

the concentrations were obtained from a graph curve that was obtained from OD and the concentration of the standards [12].

2.6 Data Analysis

Data processing was carried out and the results obtained from the Proximate Compositions and Aflatoxin concentrations were analyzed by comparison of means using Analysis of Variance (ANOVA).

3. RESULTS AND DISCUSSION

The environment in which foodstuffs are displayed in the markets is not always hygienic and this is an avenue for contamination. Very often, food vendors display the food samples in an open tray or bowl beside gutters or refuse heaps, this encourages fungal attack and subsequent production of toxins.

3.1 Proximate Composition of the Grain Samples

The moisture contents observed in the grain samples analyzed in this study can favour the growth of aflatoxigenic moulds if agricultural produced are stored for a long period of time. The finding in this study is similar to the findings of Samir et al. [13] who reported a moisture content of 9 – 19% in maize sample. However, the moisture contents observed in this study contradict the finding of Fageer and El-Tinay [14] that reported 4.6 – 6.7% moisture in 12 corn genotypes. The observed differences might possibly be due to variation in grain type and environmental factors.

The percentage ash content is an indication of minerals in the grains samples analyzed. Maize is less in minerals based on their ash contents, where as millet is rich in minerals required for growth of moulds. This observed variation in the ash contents in the different grains might be due to a genetic factor, soil composition and fertilizer used [15]. The ash content observed is, however, lower than the 5.1% in maize reported by May et al. [16]. This might be due to agronomic practices like field drying and other environmental factors.
The percentage crude protein content of the grains indicated that they are good sources of nitrogen required for growth of moulds and aflatoxin production. The crude protein content observed in this study is lower than the 20.8 – 23.7% reported by Habibullah and Hamid [17] in beans. The possible reasons for the variation might be due differences in storage conditions in the study area, grain variation and perhaps genetic factor.

The carbohydrate content of the grains indicated fermentable sugars required for the growth of moulds and subsequently aflatoxin production. This finding is in agreement with the 65 – 75% and 71.7% reported by Subramanium and Metta [18] in maize. The reasons for the observed differences could be that the carbohydrate constituents such as carbon, hydrogen and oxygen components of the varietal grains used are low due to a low constituent of the two major components of starch (amylose and amylopectin) during germination or possible degradation along processing line (Tables 1 and 2).

Table 1. Percentage proximate composition of maize please arran

Parameters	% Value
Moisture content	10.40
Ash content	0.90
Crude Lipid	6.65
Crude Protein	13.13
Crude fiber	1.53
Carbohydrate	67.39

Table 2. Percentage proximate composition of millet

Parameters	% Value
Moisture content	12.70
Ash content	1.70
Crude Lipid	5.15
Crude Protein	8.17
Crude fiber	1.95
Carbohydrate	70.33

3.2 Occurrence of Aflatoxigenic Moulds in Maize and Millet Samples

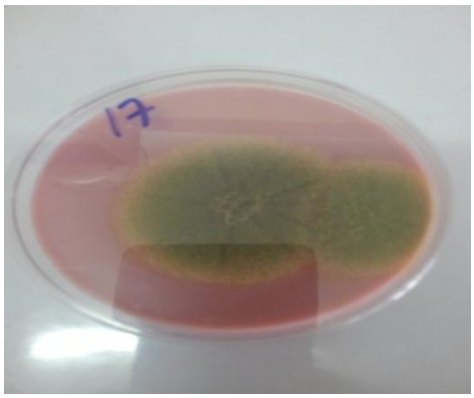
The distribution of moulds isolate in this study showed that *Aspergillus flavus* was the most dominant than the *A. parasiticus* in both maize and millet grain samples analyzed. Millet samples recorded highest occurrence of *A. flavus* and *A. parasiticus* and produced highest aflatoxin concentration than other samples analyzed, while maize samples account the least occurrence, Table 3 showed the occurrence and pictures (1; a and b and 2; a and b) presented the photo of the fungi (*A. flavus and A. parasiticus*) growing on the media and photo

micrograph respectively. The observed variation might be due to differences in storage practices. The presence of *A. flavus and A. parasiticus* however, call for concern as these and moulds are known to produce aflatoxin and have been implicated in mycoses.

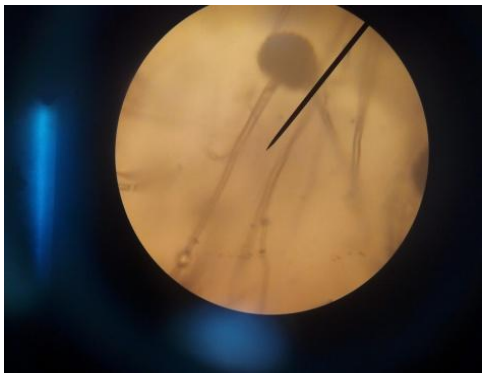
Among the two species, *A. flavus* was found to be dominant mould occurring which is in agreement with Klich [19] who reported a high incidence of *A. flavus*. Previous studies had shown that *A. flavus* frequently occurred in the field and this is attributed to agro-ecological conditions of the field. The presence of both *Aspergillus flavus and A. parasiticus* in the grain samples is very common. According to Jelena et al. [20], drought condition caused higher incidence and favours *Aspergillus flavus*. Similarly, Rossetto et al. [21] attributed this high frequency of the two moulds to the great adaptation of these fungi to the substrate, especially during storage, the high frequency of *A. flavus* observed in millet samples may be as a result of the contact of these substrates with the soil. The distribution of the aflatoxigenic moulds isolates in agricultural products observed in this study may be traced to inadequate storage and handling practices, the ubiquitous nature of these moulds and environmental factors [22].

3.3 Screening of Isolates for Aflatoxin Production using Desiccated Coconut Agar

The fluorescence production is an effective cultural method for the detection of aflatoxin-producing ability [3]. In this study, isolates of *A. flavus* and *A. Parasiticus* isolated from the grain samples analyzed were found to be capable of production of Aflatoxin. Dyer and McCammon [23] reported that the colour fluorescence under UV light is a useful tool in the differentiation of toxigenic isolates, as *A. flavus* fluoresced pastel blue in a ring around each colony, while *A. parasiticus* fluoresced bluish white. Thus fluorescence colouration could be used in differentiating *A. flavus* and *A. parasiticus*. This is an indication that neutral red desiccated coconut agar (NRDCA) enhance the aflatoxin detection ability of the medium. This finding is in agreement with the findings of Cotty [24] and Yu et al. [25] who reported that *Aspergillus* species produce aflatoxin in appreciable amounts on NRDCA by the isolates as reported by Olsen et al. [26] (Figs. 1 and 2).



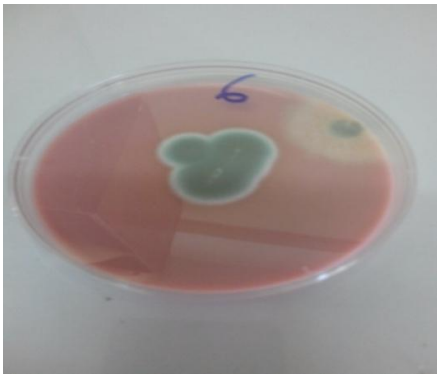
Picture 1a. Culture growth of *Aspergillus flavus* on DCA



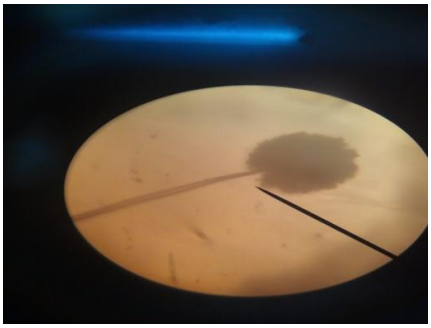
Picture 1b. Photo micrograph of *Aspergillus flavus* (40x)

Table 3. Occurrence of aflatoxigenic moulds in maize and millet grain samples

Grain samples	No. of samples analyzed	Occurrence of (%)		Total No. of samples contaminated
		A. flavus	*A. parasiticus*	
Maize	30	7(23.3)	4(13.3)	11
Millet	30	9(30)	5(16.7)	14
Total	60	16(26.7)	9(15)	25



Picture 2a. Culture growth of *Aspergillus parasiticus* on DCA



Picture 2b. Photo micrograph of *Aspergillus parasiticus* (40x)

3.4 Aflatoxins Concentration of the Grains Samples

Maize is one of the most widely distributed food plants in the world [27]. Maize samples analyzed in this study revealed that the mean aflatoxin concentration and the range are within the acceptable limit, except maize sample from Samaru and Tudun Wada markets that had the highest level of contamination which exceeded the limits sets by NAFDAC and SON as presented in Table 4. The observed differences between the markets might be due to a method of storage and handling. The high susceptibility of maize to mycotoxin contamination as observed in this study has also been reported by Jelinek et al. [28] and Okoye [29] in Nigeria. However, Atehnkeng et al. [30] reported that the mean values of aflatoxin contamination in samples of maize ranged between (30.9 µg/kg-507.9 µg/kg) from 11 districts across three agro-ecological zones of Nigeria, which were far beyond acceptable limits. Manjula et al. [31] reported mean aflatoxin levels of 0.55 µg/kg and 0.46 µg/kg in maize samples. The traditional method that involves the removal of pericarp from maize to make maize flour could simply mean the removal of fungi associated with mycotoxin production [32]. The difference in the level of contamination in the maize samples

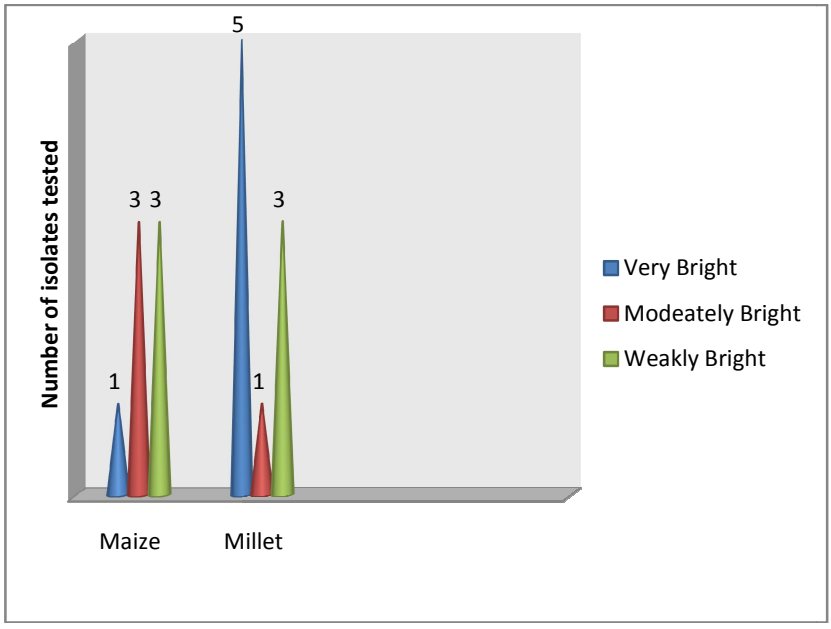


Fig. 1. Aflatoxins production ability of *A. flavus* isolates on dessicated coconut agar under UV light (365 nm)

Source of fungal isolate

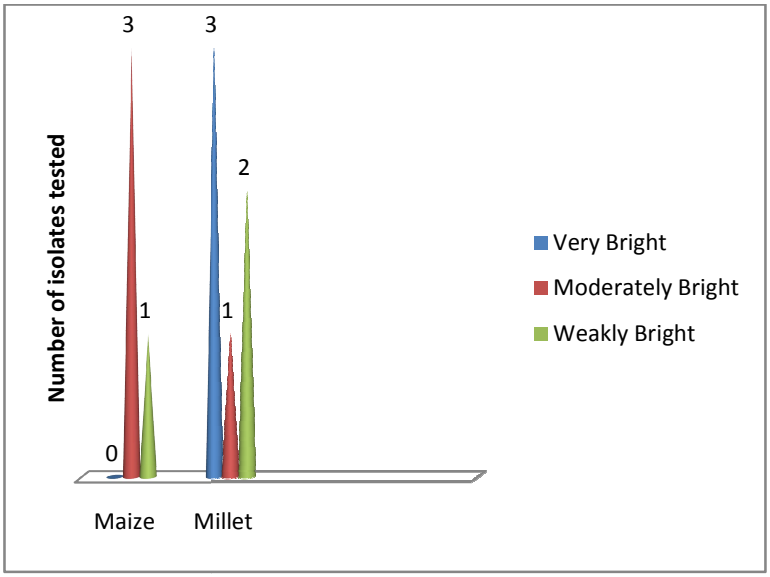


Fig. 2. Aflatoxins production ability of *A. parasiticus* isolates on dessicated coconut agar under UV light (365 nm)
Source of fungal isolate

might be due to agricultural practices and storage conditions.

Millet is a major staple food in northern Nigeria. In this study, the highest level of aflatoxins found in millet obtained from Zaria, Sabon Gari, Kwangila and Samaru markets are far beyond the acceptable limit of 10 µg/kg set by National Agency for Food and Drug Administration Control (NAFDAC) and Standard Organization of Nigeria (SON). This could be due to differences in storage conditions between the markets. The concentration of aflatoxins level obtained from this study is not in agreement with the findings of Ezekiel et al. [11] who reported 0.08-1.40 µg/kg in millet from Plateau State, Nigeria. The difference in contamination level might be due to the difference in environmental factors (temperature and relative humidity) that favours the growth of aflatoxigenic moulds and also agricultural practices between the two study areas.

Table 4. Aflatoxins concentrations in maize from six different markets in Zaria metropolis

Location	Number of samples		Mean concentration (µg/Kg)
	Analyzed	Positive	
Zaria City	5	1	2.60
Tudun Wada	5	2	11.50
SabonGari	5	1	3.50
Kwangila	5	1	3.00
Samaru	5	1	18.10
Dan-Magaji	5	1	0.40

Table 5. Aflatoxins concentrations in millet from six different markets in Zaria metropolis

Location	Number of samples		Mean concentration (µg/Kg)
	Analyzed	Positive	
Zaria City	5	1	52.00
Tudun Wada	5	1	5.00
Sabon Gari	5	2	23.15
Kwangila	5	2	23.75
Samaru	5	1	39.6
Dan-Magaji	5	2	4.20

4. CONCLUSION

From the observations obtained from this study, the following conclusions were drawn;

The maize and millet grain samples analyzed in this study contain organic and inorganic nutrients in sufficient amounts to support the growth of aflatoxigenic moulds and subsequent production of aflatoxin. *Aspergillus flavus* was the major fungal contaminants of the grains. All the isolates o*f Aspergillus flavus* an*d A. parasiticus* demonstrated the ability for aflatoxin production. Furthermore, the study revealed that Aflatoxins were found to contaminate the samples but in varying degrees. Highest concentration occurred in millet samples in the range of 4.2-52.0 µg/kg, while maize samples have the least concentration in the range of 0.4-18.1 µg/kg. This survey also revealed that most of the vendors are not handling the agricultural commodities properly at the point of sale and during storage as observed during sampling resulting in contamination of these samples analyzed and poor human and animal health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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