Characterization of aflatoxigenic *Aspergillus flavus* and *A. parasiticus* strain isolates from animal feedstuffs in northeastern Iran

Davari, E.[1]; Mohsenzadeh, M.[2*]; Mohammadi, Gh.[3] and Rezaeian-Doloei, R.[4]

[1]Graduated from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [4]Department of Agronomy and Plant Breeding, Mashhad Branch, Islamic Azad University, Mashhad, Iran

[*]***Correspondence:*** *M. Mohsenzadeh, Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: mohsenza@um.ac.ir*

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Summary

Aflatoxins are secondary toxic metabolites produced by some *Aspergillus* spp. particularly, *Aspergillus flavus* and *A. parasiticus* that contaminate food and feed. The objective of this study was to evaluate the contamination of feedstuffs with *Aspergillus* spp. and detect genes involved in the aflatoxin biosynthesis pathway of *A. flavus* and *A. parasiticus* isolates. A total of 110 cow feed samples (comprised of silage, concentrate, hay and total mixed ration) from 30 industrial and semi-industrial dairy farms of Khorasan Razavi province, northeastern Iran, were examined using cultural and PCR methods. 68 (61.82%) *Aspergillus* spp. were isolated from 110 samples of feedstuff. The predominant *Aspergillus* isolates were *A. fumigates* (21.81%), followed by *A. flavus* (17.27%), *A. niger* (10%), *A. parasiticus* (8.18%), and *A. oryzae* (4.54%). Fungal contamination levels of industrial and semi-industrial dairy farm samples were not significantly different (P>0.05). Using four sets of primers, a quadruplex PCR was developed to detect genes (*nor1*, *ver1*, *omtA* and *aflR*) at different loci coding enzymes in the aflatoxin biosynthesis pathway of *A. flavus* and *A. parasiticus* strains. Out of 28 strains of *A. flavus* and *A. parasiticus*, 10 isolates (35.71%) showed a quadruplet pattern indicating the important genes involved in the aflatoxin biosynthesis pathway, encoded for functional products. These isolates were confirmed to be aflatoxigenic by Thin Layer Chromatography. 18 isolates (64.29%) had three, two and single molecular patterns. The results obtained by this study show that rapid and specific detection of aflatoxigenic molds is important to ensure the microbiological safety of feedstuffs.

Key words: Aflatoxin, *Aspergillus* spp., Feedstuff, Multiplex PCR

Introduction

Mold and mycotoxin contamination of animal feedstuffs is largely a feed management problem. Most species of *Aspergillus* are able to grow on wide ranges of feed. They are essentially saprophytic and particularly associated with stored moldy plant products (Shapira *et al*., 1996). *Aspergillus* genera is the most important aflatoxigenic fungi (Rodrigues *et al*., 2009). Aflatoxins, are highly toxic secondary metabolites produced predominately by *A. flavus*, *A. parasiticus* and *A. nomius*. Food and feed commodities are usually contaminated by a range of different fungi during growth, harvesting and storage. Local weather conditions as well as environmental conditions in storage facilities, especially temperature and relative humidity, contribute to the growth of *A. flavus* and *A. parasiticus* and are, therefore, potential risks for aflatoxin production (Michael *et al*., 1999; Rahimi *et al*., 2008). *Aspergillus parasiticus* are able to produce aflatoxins B1, B2, G1 and G2, whereas *A. flavus* is only able to produce aflatoxin B1 and B2 (Yu, 2012). Only about 40-50% of *A. flavus* strains are able to produce these mycotoxins. Aflatoxin B1 is widely regarded as the most potent liver carcinogen, affecting a large number of animal species and humans (Michael *et al*., 1999). Aflatoxin M1 and M2 are hydroxylate derivatives of AFB1 and AFB2, which are formed and excreted in the milk of lactating animals and humans that have consumed aflatoxin-contaminated foods (Michael *et al*., 1999). Aflatoxins are highly toxic and carcinogenic in animals and humans, leading to hepatotoxicity, immunotoxicity (Mehrzad *et al*., 2011), teratogenicity and even death (Erami *et al*., 2007; Ghiasian and Maghsood, 2011).

Traditional culture techniques for monitoring foods and animal feed for fungal contaminations are extremely labor-intensive and require several days to complete. During the past years, a number of molecular based detection methods have been developed to characterize aflatoxigenic and non-aflatoxigenic *Aspergillus* spp. isolates in human foods and animal feeds (van der Vossen, 1996; Konietzny and Greiner, 2003). Aflatoxin biosynthesis is a complex process involving many intermediates and enzymes. The regulation of aflatoxin gene expression occurs at multiple levels and by multiple regulatory components. Twenty seven enzymatic steps are estimated for an aflatoxin biosynthesis (Ehrlich and Yu, 2010), and as many as 30 genes are potentially

involved in the process. The genes and corresponding enzymes have been extensively studied (Yu *et al*., 2004a, b). In *A. flavus* and *A. parasiticus*, aflatoxin pathway genes are clustered within a 75-kb region of the fungal genome on chromosome III, roughly 80 kb away from the telomere (Yu *et al*., 2004a, b; Chang *et al*., 2005). *Nor-1*, *ver-1* and *omt-A* are three structural genes in the cluster genes of the biosynthesis aflatoxin pathway that are coded for key enzymes in the production of aflatoxin, thus they are essential for the production of aflatoxin (Erami *et al*., 2007). Norsolorinic acid (NOR) was confirmed to be the first stable aflatoxin precursor (Yu *et al*., 2004b). The *ver-1* gene was predicted to encode a ketoreductase, similar to *nor-1* (Keller *et al*., 1994). Aflatoxin pathway genes were found to be clustered in the genome of *A. flavus* and *A. parasiticus* (Yu *et al*., 2004a, b). These genes are expressed concurrently except for the regulatory gene *aflR*. In this gene cluster, a positive-acting regulatory gene, *aflR*, is located in the middle of the gene cluster. The *aflR* gene, encoding a 47 kDa sequence-specific zinc-finger DNA-binding protein, is required for transcriptional activation of most, if not all, structural genes of the aflatoxin gene cluster (Ehrlich *et al*., 1998; Chang *et al*., 1999a, b). Several studies were carried out regarding the use of genes involved in aflatoxin biosynthesis (Shapira *et al*., 1996; Criseo *et al*., 2001) for the identification of aflatoxin-producing *A. flavus* and *A. parasiticus*.

The aims of this study were to determine *Aspergillus* spp. contamination of animal feed samples and to specifically detect genes involved in aflatoxin biosynthesis in A. *flavus* and *A. parasiticus* isolated from feedstuffs of Khorasan dairy industries, Iran. To this end, a multiplex-PCR method was used to detect important genes involved in aflatoxin synthesis using four set of primers, namely aflR, omtA, ver1, nor1.

Materials and Methods

Feed samples

A total of 110 animal feed samples comprising silage (n=28), concentrate feed (n=30), total mixed ration (n=27) and hay (n=25) were randomly collected from 30 industrial and semi-industrial dairy farms of Khorasan Razavi province (Table 1). All samples were transported to the laboratory under cold (4°C) conditions. All samples were intended for animal consumption and none showed any visible sign of mold contamination.

Identification of *Asprgillus* spp.

Samples were homogenized and stored at 4°C and protected against light until the day of analysis. A 10 g portion of each sample was homogenized in 90 ml of 0.1% peptone water solution for 30 min in an orbital shaker. Serial dilutions of up to 10^{-6} were made and 0.1 ml of each dilution was inoculated in duplicate onto potato dextrose agar (PDA, Merck). The plates were incubated at 25°C for 5-7 days. To identify*Aspergillus* species, the spores were transferred on the Czapek-Dox agar medium (CZA, Merck) after fungal colony formation and incubated for 5-7 days at 27°C. Wet mount smears and slide cultured colonies were stained with lactophenol cotton blue. Taxonomic fungi identification was made based on macroscopic and microscopic features according to appropriate keys proposed by Klich (2002).

Determination of aflatoxin production by chromatography

The isolates identified as *A. flavus* and *A. parasiticus* were tested for the production of aflatoxins based on the thin layer chromatography (TLC) method (Moubasher *et al*., 2013). The spore suspension (100 µL) of each strain containing 10^7 spores/ml prepared in 0.1% (vol/vol) Tween 20 was added to a 250 ml Erlenmeyer flask containing 100 ml yeast extract sucrose broth (YES, Merck), incubated for 7 days at 26°C and shook at 150 rpm. To extract aflatoxin from the mycelium, the cells were lysed by adding 100 ml chloroform to each flask and transferring the lower transparent phase to a new tube. Chloroform was evaporated at 100°C in a water bath and the remaining pellets were dissolved in 1 ml methanol. Silica gel TLC plates (Sigma) were used for the aflatoxin analysis. From each sample, 50 µL was spotted onto the TLC sheets, developed in a toluene-ethyl acetate-acetic acid (50:30:4) solvent system. Pure aflatoxins (Sigma) were used as standards. Aflatoxins were visualized under a UV lamp at 365 nm and their presence was chemically confirmed by spraying 50% H_2SO_4, and heating to charring. An aflatoxigenic strain was used as positive control.

DNA extraction

The isolation of fungal DNA was performed according to the method described by Yelton *et al*. (1984) with some modifications. The strains were grown for 72 h under continuous shaking conditions (150 rpm) in the PDA Broth. The mycelium was then harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground to a powder which was resuspended in a lysis buffer (50 mmol/L EDTA, 0.2% SDS, pH =

Table 1: The frequency of *Aspergillus* spp. isolated from feedstuffs of Khorasan dairy farms

Feedstuff sample	No of samples	*Aspergillus* spp.					Total (%)
		A. flavus	*A. parasiticus*	*A. fumigatus*	*A. niger*	*A. oryzae*	
Silage	28	1	2	3	2	0	8 (28.57%)
Concentrate	30	8	3	8	3	1	23 (76.66%)
TMR	27	5	2	5	0	2	14 (51.85%)
Hay	25	5	2	8	6	2	23 (92%)
Total (%)	110	19 (17.27%)	9 (8.18%)	24 (21.81%)	11 (10%)	5 (4.54%)	68 (61.82%)

Table 2: Primers used in this study, target gene, sequence and PCR product size

Primer name	Target gene	Primer sequence (5′-3′)	PCR product size (bp)	Reference
NorF	*nor-1*	ACCGCTACGCCGGCACTCTCGGCAC	400 bp	Criseo *et al*., 2001
NorR		GTTGGCCGCCAGCTTCGACACTCCG		
VerF	*ver-1*	GCCGCAGGCCGCGGAGAAAGTGGT	537 bp	Criseo *et al*., 2001
VerR		GGGGATATACTCCCGCGACACAGCC		
OmtF	*omt-A*	GTGGACGGACCTAGTCCGACATCAC	797 bp	Criseo *et al*., 2001
OmtR		GTCGGCGCCACGCACTGGGTTGGGG		
AflrF	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1032 bp	Criseo *et al*., 2001
AflrR		CCGTCAGACAGCCACTGGACACGG		

8.5) and heated immediately at 68°C for 15 min. After centrifugation for 15 min at 15000 × g, a 7-10 ml volume of the supernatant fluid was transferred to a new centrifuge tube and 1 ml 4 mol/L sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15000 × g. After centrifugation, the supernatant fluid was transferred to a fresh tube and extracted by AccuPrep® Genomic DNA extraction Kit (Bioneer, Korea).

Multiplex PCR reaction

In the present study, multiplex PCR was performed according to the method described by Criseo *et al*. (2001). All of the isolated *A. flavus* and *A. parasiticus* were examined for the presence of four important aflatoxin genes (*aflR*, *omt-A*, *ver-1* and *nor-1*) enclosed in the aflatoxin biosynthesis pathway by multiplex PCR using four published primer sets (Table 2).

PCR reaction was performed in 25 µL containing 2.5 µL 1 X PCR buffer, 0.75 µL 50 mM $Mgcl_2$, 0.5 µL 10 mM dNTPs, 2 µL of each primer, 0.2 µL *Taq* DNA polymerase (1 U/µL), 5 µL extracted DNA as template and 8.05 µL sterile distilled water. A total of 35 cycles was started with heating at 94°C for 5 min, and continued by denaturation for 30 s at 94°C, annealing for 30 s at 67°C, elongation for 30 s at 72°C and a final extension of 10 min at 72°C. Amplified products were visualized by UV illumination after electrophoresis on 1% agarose gel and ethidium bromdie staining.

Genomic DNA of the following organisms was used to test the sensitivity of primers listed in Table 2: *A. parasiticus* ATCC 15517, *A. oryzae* IMI 126842, *Penicillium purpurogenome* PTCC 5212, *A. fumigates* PTCC 5009, *Fusarium oxysporum* PTCC 5115, *A. niger* ATCC 9142, *Alternaria alternata* PTCC 5224.

Statistical analysis

Statistical analysis was performed using SPSS version 19. Chi-square and Fisher exact tests were used to assess the possible differences between fungi incidence in feeds and types of dairy farm. The significance level was set at $P<0.05$ for all tests.

Results

Among 110 feed samples, 68 (61.82%) were contaminated with *Aspergillus* species. The predominant isolate was *A. fumigates* (21.81%), followed by *A. flavus* (17.27%), *A. niger* (10%), *A. parasiticus* (8.18%) and *A. oryzae* (4.54%) (Table 1).

The most frequent isolated fungi were found in the hay samples (92%) and the lowest fungal contamination frequency was in the silage (28.57%). The incidence rates of fungal contamination in industrial and semi-industrial dairy farm samples were (62.5%) and (64.81%), respectively, with no significant differences between farm types ($P>0.05$).

The molecular patterns obtained for 28 examined *A. flavus* and *A. parasiticus* isolates are shown in Fig. 1 and Table 3.

DNA fragments of *aflR*, *omt-A*, *ver-1* and *nor-1* genes were visualized at 1032, 797, 537 and 400 bp, respectively (Fig. 1). Five isolates (CAF1, CAF5, CAF7, CAP1 and CAP2) from the concentrate (Fig. 1a), 2 isolates (SAF1 and SAP2) from the silage (Fig. 1b), 2 isolates (TAF4 and TAP1) from the TMR (Fig. 1c) and 1 isolate (HAP2) from the hay (Fig. 1d) had a quadruplet pattern, indicating the presence of the four genes involved in the aflatoxin biosynthesis pathway. Other strains, however, showed different molecular patterns (Table 3). The results obtained by the TLC method indicated that all aflatoxigenic strains of *A. flavus* and *A. parasiticus* (10 strains) contained the four structural genes (Table 3).

No DNA amplification was observed with *A. niger* ATCC 9142, *Penicillium purpurogenome* PTCC 5212, *Fusarium oxysporum* PTCC 5115, *A. oryzae* IMI 126842, *A. fumigates* PTCC 5009 and *Alternaria alternata* PTCC 5224, even at the highest level, thus indicating the high specificity of the PCR. To determine the PCR's sensitivity, lower concentrations of spores were tested. The DNA was only amplified in *A. parasiticus* ATCC 15517, even at the lowest spore level.

Discussion

In this study, *A. fumigatus* (21.81%) and *A. flavus* (17.27%) were the predominant *Aspergillus* spp. isolated from feedstuffs. These results differ from reports describing *A. niger* as the most predominant followed by *A. flavus* (Saleemi *et al*., 2010), and vice versa (Accensi *et al*., 2004; Somashekar *et al*., 2004). In a study conducted in Brazil on raw materials and finished cow feed samples by Rosa *et al*. (2008), eight fungal genera were isolated. The predominant *Aspergillus* isolated from finished cow feed samples were *A. flavus* (31.6%) followed by *A. niger* (22.4%). The predominance of *A. flavus* isolates showed that it can easily adapt itself to

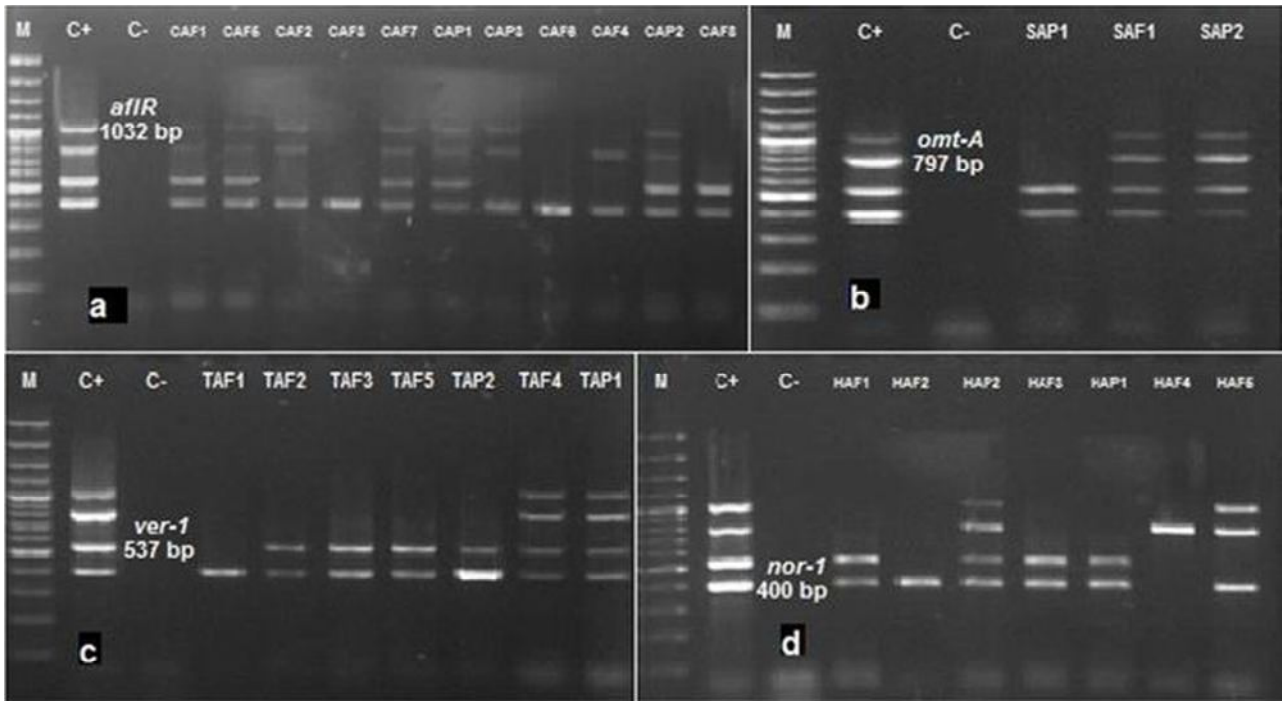

Fig. 1: Agarose gel electrophoresis of multiplex PCR products amplified from *A. flavus* and *A. parasiticus* isolated from feedstuffs. M: 100 bp DNA molecular size marker, C+: Positive control (*A. parasiticus* ATCC 15517), and C-: Negative control (without genomic DNA). (a): DNA banding patterns of *A. flavus* and *A. parasiticus* isolates of concentrate samples, (b): DNA banding patterns of *A. flavus* and *A. parasiticus* isolates of silage samples, (c): DNA banding patterns of *A. flavus* and *A. parasiticus* isolates of TMR samples, and (d): DNA banding patterns of *A. flavus* and *A. parasiticus* isolates of hay samples

Table 3: Results obtained by multiplex PCR and conventional methods (TLC and PCR)

Isolate code	Aflatoxin biosynthesis gene				Aflatoxin production by TLC method
	aflR	*omt-A*	*ver-1*	*nor-1*	Aflatoxin production
SAF1	+	+	+	+	+
SAP1	-	-	+	+	-
SAP2	+	+	+	+	+
CAF1	+	+	+	+	+
CAF2	+	+	-	+	-
CAF3	-	-	-	+	-
CAF4	-	+	-	+	-
CAF5	+	+	+	+	+
CAF6	-	-	-	+	-
CAF7	+	+	+	+	+
CAF8	-	-	+	+	-
CAP1	+	+	+	+	+
CAP2	+	+	+	+	+
CAP3	+	+	-	+	-
TAF1	-	-	-	+	-
TAF2	-	-	+	+	-
TAF3	-	-	+	+	-
TAF4	+	+	+	+	+
TAF5	-	-	+	+	-
TAP1	+	+	+	+	+
TAP2	-	-	+	+	-
HAF1	-	-	+	+	-
HAF2	-	-	-	+	-
HAF3	-	-	+	+	-
HAF4	-	+	-	-	-
HAF5	+	+	-	+	-
HAP1	-	-	+	+	-
HAP2	+	+	+	+	+
A. niger ATCC 9142	-	-	-	-	-
F. oxysporum PTCC 5115	-	-	-	-	-
A. parasiticus ATCC15517	+	+	+	+	+
P. purporogenome PTCC 5212	-	-	-	-	-

S: Silage, C: Concentrate, T: TMR, H: Hay, AF: *A. flavus*, and AP: *A. parasiticus*

various geographical regions. Another important point to consider is that *A. flavus* can grow at low (11-14%) humidity levels (Pitt and Hocking, 1997; Macioro *et al*., 2007).

In the present study, similar to Khosravi *et al*. (2004), the most frequent isolated fungi were found in concentrate samples rather than other feedstuffs. In another study carried out in Iran by Ghiasian and Maghsood (2011), concentrate feed was reported to be the most contaminated, with mean colony counts of 7.25 × 10^2 and 7.50 × 10^2 CFU/g for *A. flavus* and *A. parasiticus*, respectively.

In our study, similar to Khosravi *et al*. (2004), the lowest fungal contamination frequency was found in silage. The incidence rate of fungal contamination in both dairy farm samples were (63.31%) and (67.18%), respectively, and no significant difference was detected between farm types (P>0.05). Similar results regarding Iranian farm types were reported by Ghiasian and Maghsood (2011).

PCR is a method of choice for the diagnosis of aflatoxigenic molds (Shapira *et al*., 1996; Erami *et al*., 2007). In the present work, using a set of four primers aflR, omtA, ver1 and nor1, a multiplex PCR procedure was used to detect genes involved in the aflatoxin biosynthesis pathway. Primers *omt-1*, *nor-1*, *ver-1*, are three structural genes used for the biosynthesis of aflatoxin. The *afl*R gene, that codes for a regulatory factor, seemed to be involved in the activation of the transcript of pathway genes (Woloshuk *et al*., 1994). It was also found to regulate aflatoxin biosynthesis. The *omt* gene was found to be involved in the conversion of sterigmatocystin to o-methylsterigmatocystin in the aflatoxin biosynthetic pathway. The results indicated that all aflatoxigenic strains of *A. flavus* (5 isolates) and *A. parasiticus* (5 isolates) contained the four tested: *nor-1*, *ver-1*, *omtA*, and *aflR* genes. In our study, 10 isolates (35.71%) of 28 strains of tested A. *flavus* and *A. parasiticus* showed a quadruplet pattern, indicating the presence of all genes which encode for functional products and their involvement in the aflatoxin biosynthetic pathway (Yu *et al*., 2004a, b). These strains had a quadruplet DNA banding pattern, indicating the presence of the four genes in the aflatoxin biosynthetic pathway. In contrast, non-aflatoxigenic strains showed different band patterns comprising of 1, 2 and 3 bands. These results complement those of the TLC aflatoxin detection method, and were in agreement with the findings of Rashid *et al*. (2008), who concluded that aflatoxigenic isolates of *A. flavus* and *A. parasiticus* had all four *nor1*, *ver1*, *omtA*, and *aflR* genes.

In our study, we found that conventional methods of using cultural media for aflatoxin production distinguished perfectly between aflatoxin-producing and non-producing strains. Unfortunately, these methods are time-consuming and labor-intensive, and can fail to detect some aflatoxin-producing strains due to the fact that aflatoxin production instability may occur in certain aflatoxigenic strains growing in culture media (Criseo *et al*., 2001). Using multiplex PCR utilizing primers targeting the *aflR*, *nor-1*, *ver-1* and *omt-A* genes appears to offer some promise in detecting aflatoxigenic molds. This study confirms the importance of further surveillance of mycotoxigenic fungi and mycotoxin occurrence in feedstuffs in Iran.

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