Identification of toxigenic *Aspergillus* species from diet dairy goat using a polyphasic approach

**ABSTRACT**

Some species of filamentous fungi that infest agricultural commodities are able to produce mycotoxins, contaminating feed and animal products. The aim of this research was to identify the mycoflora present in the feed and forage for dairy goat and to isolate and characterize the *Aspergillus flavus* and *A. parasiticus* strains based on a morphological and molecular characterization and mycotoxigenic ability. The goat dairy diets were collected monthly from 11 goat milk farms, totaling 129 and 106 samples of concentrate and forage, respectively. For the isolation of the mycoflora the surface plating method was used. *Aspergillus*, *Penicillium*, and *Fusarium* were the main fungi producing mycotoxins isolated. The morphological and molecular characterization and mycotoxigenic ability were used for *A. flavus* and *A. parasiticus* identification. The *Aspergillus* spp. from feed produced aflatoxins B1 and B2 (39%), cyclopiazonic acid (CP A) (17%), 18% produced both toxins, and 42% had no toxigenic ability. Only 2.0% of the strains produced aflatoxins B1, B2, G1, and G2. The strains from forage were producers of aflatoxins B1 and B2 (37%), CP A (14%), 14% of both mycotoxins, whereas 49% have shown no toxigenic ability. The aflD and aflR genes were used by PCR and PCR-RFLP, respectively. The presence of toxigenic species in samples of feed for lactating goats indicates a potential risk of contamination of dairy products, if they are exposed to environmental conditions favorable to fungal growth and mycotoxin production.

**Key words:** toxigenic fungi, aflatoxin, cyclopiazonic acid, feed, molecular characterization.

**INTRODUCTION**

The complex diet of ruminants, consisting of forages, concentrates, and silages, can be a source of diverse mixture of mycotoxins that contaminate individual feed components (SMITH & KOROSTELEVA, 2012). Both, feed grains (e.g. *parasiticus*, com base em uma caracterização morfológica e molecular e capacidade micotoxigênica. Os alimentos foram coletados mensalmente em 11 fazendas produtoras de leite de cabra, totalizando 129 e 106 amostras de concentrado e volumoso, respectivamente. Para o isolamento da micoflora, foi utilizado o método de plaqueamento de superfície. *Aspergillus*, *Penicillium* e *Fusarium* foram os principais gêneros de fungos produtores de micotoxinas isolados das amostras. A caracterização morfológica e molecular e capacidade micotoxigênica foram utilizadas para identificação de *A. flavus* e *A. parasiticus*. Das cepas *Aspergillus* spp isoladas do concentrado, 39% produziram aflatoxinas B1 e B2, 17% produziram ácido ciclopiazônico (ACP), 18% produziram ambas as toxinas e 42% não tinham capacidade toxigênica. Apenas 2.0% das cepas produziram aflatoxinas B1, B2, G1 e G2. As cepas de *Aspergillus* spp. isoladas do volumoso foram produtores de aflatoxinas B1 e B2 (37%), ACP (14%), sendo que 14% produziram ambas toxinas e 49% não foram produtoras. Os genes aflD e aflR foram utilizados para a PCR e a PCR-RFLP, respectivamente. A presença de espécies toxigênicas em amostras de alimentos destinados a caprinos em lactação indica um risco potencial de contaminância dos produtos lácteos por aflatoxinas e ACP, caso estes sejam expostos a condições ambientais favoráveis ao crescimento de fungos e produção de micotoxinas.

**Palavras-chave:** fungos toxigênicos, aflatoxinas, ácido ciclopiazônico, ração, caracterização molecular.
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**MATERIAL AND METHODS**

Samples

The samples of dairy goat feed (129) and forage (106) were collected from May, 2010 to September, 2011, once a month from 11 farms producing goat milk located in the cities of Ibiúna, Piedade, Alambari, Capão Bonito, Guareí, and Porto Feliz, in São Paulo State, Brazil. In the properties, feed and forage were packed in barrels with a capacity of 20 kg each. Samples of the feed and forage were collected at nine different points of each barrel: three points of the upper third, three points of the middle third and three points of the lower third (SASSAHARA et al., 2003). Each sample contained a minimum of 500 g. The collected material was homogenized and stored in plastic packaging and sent to the laboratory for isolation and identification of the mycoflora and water activity analysis.

Identification and enumeration of the mycobiota from goat feed and forage

The animal feed and forage samples were apportioned in 10g aliquots and homogenized for 30 minutes in bottles containing 90mL of sterile distilled water. Aliquots with 0.1mL of the dilutions in serial from 10^{-1} to 10^{-6} of the samples were plates in duplicate using the surface methods in potato agar dextrose (PDA) medium. The plates were incubated at 25°C for 5 days, but the observations were made daily. The colonies were identified at the genus level, and those belonging to the genus *Aspergillus* were identified at the species level according to PITI & HOCKING (1997).

Molecular Characterization of *A. flavus* and *A. parasiticus*

DNA extraction

The strains characterized as the genus *Aspergillus* (233) were submitted to molecular identification. The strains of *A. flavus* and *A. parasiticus* were maintained in a tube containing PDA at 25°C at 7 days, and a loop full of spores from each strain was transferred to 1.5mL of lysis buffer with 1.0g of sterile acid-washed 0.4 to 0.6mm diameter glass beads and vortexed for 5.0min at maximum speed. Proteins and polysaccharides were precipitated by 750µL of cold 3M sodium acetate, pH 5.5. The solution was mixed, placed at -20°C for 10min and centrifuged at 5000g for 10min at 4°C for twice. The supernatant was precipitated with one volume of cold isopropanol. The solution was mixed gently, incubated for 1.0h at -20°C and centrifuged at 5000g for 10min at 4°C. DNA pellet was washed twice with 1.0mL of cold 70% ethanol, centrifuged at 5000g for 5min at 4°C. DNA was diluted in 100µL of ultrapure water and stored at -20°C (RODRIGUES et al., 2009). The DNA was quantified by measuring the absorption at 260nm in a Hitachi U-2000 spectrophotometer.

PCR amplification of *aflD* gene

The gene *aflD* was tested for all strains and the *aflD* primers were specifically designed according

Ciência Rural, v.45, n.8, ago, 2015.

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PCR amplification of *aflD* gene

The gene *aflD* was tested for all strains and the *aflD* primers were specifically designed according
to the described by RODRIGUES et al. (2009). The PCR amplifications were performed using about 1µg of fungal template DNA, 200 pmol of each primer (Nor1-Forward: 5’-ACC GCT ACG CCG GCA CTC TCG GCA C-3’ and Nor2-Reverse: 5’-GTT GGC CGC CAG CTT CGA CAC TCC G-3’), MgCl₂, free reaction buffer, 2.0mM MgCl₂, 2.5U of Taq polymerase and 0.2mM of each dNTP. PCR was carried out under the following conditions: one cycle at 94°C for 3min; 30 cycles at 94°C for 1min, at 55°C for 1min and at 72°C for 1min; and at 72°C for 10min in a final extension.

Restriction site analysis of PCR products

After analysis of PCR products by amplification of aflID gene, only the samples positive for Aspergillus fungi were used in the restriction site analysis. First, a new PCR was used to amplify two target fragments on A. flavus and A. parasiticus, and the aflIR primer sequences were designed according to the described by SOMASHEKAR et al. (2004) to amplify a fragment of 796pb (aflR1-Forward: 5’-AAC CAC ATC AAT CTC AT-3’ and aflR2-Reverse: 5’-AGT GCA GTT CGC TCA GAA CA-3’). The reaction mixture consisted of 1.0µg of fungal template DNA, 50pmol of each primer, MgCl₂-free reaction buffer, 2.0mM MgCl₂, 0.5U of Taq polymerase and 0.2mM of each dNTP. PCR was carried out under the following conditions: one cycle at 94°C for 3min; 30 cycles at 94°C for 1min, at 55°C for 1min and at 72°C for 1min; and at 72°C for 10min in a final extension.

Aflatoxin analyses

The aflatoxins identification and quantification were performed by TLC using an aliquot (40µL) of each sample, which was spotted on silica gel-G thin layer plate (Merck, Germany) and then developed with chloroform:acetone 9:1 (v/v) as a solvent system. The concentration of aflatoxins was determined by photodensitometry (Shimadzu, CS 9000) comparing the area and density of the spot samples with aflatoxins B₁, B₂, G₁, and G₂ standards (Sigma Aldrich, USA) (GONÇALEZ et al., 2001). The detection and quantification limits were established how the lowest fluorescence detectable signal and the lowest concentration measured, respectively. The detection and quantification limits for AFB₁ were 0.8ng g⁻¹ and 1.6ng g⁻¹, respectively. Recovery for standard aflatoxin B₁ should be at least 94%.

Toxigenic potential of A. flavus strains for CPA

The methodology of GONÇALEZ et al. (2013) was employed to evaluate the CPA production by A. flavus strains. The A. flavus strains isolated from goat feed (130) and from forage (70) were maintained in a tube containing PDA at 25°C for 7 days. Spore solutions (1.0mL) of each sample were inoculated into 25mL of Czapec-Dox broth (Difco) and incubated for 12 days at 25°C. The cultures were filtrated, and the CPA was extracted twice with 25mL of chloroform. The chloroform was evaporated and the extract was diluted in 1.0mL of methanol HPLC grade and then submitted to high performance liquid chromatography (HPLC).

HPLC conditions

The mobile phase consisted of methanol: water/zinc sulfate 4.0mM 7.3 (v/v) at a flow rate of 0.6mL min⁻¹. A C₁₈ column, 250mm x 4.6mm (Shimadzu, Japan) was used, and the analyses were done with a UV detector at 284nm. The calibration curve was established by the external standard method with five concentrations: 0.611; 1.223; 2.446; 4.892, and 9.798µg mL⁻¹ (r²=0.998). The HPLC quantification and detection limits for a standard CPA were 0.030µg mL⁻¹ and 0.005µg mL⁻¹, respectively. Recovery for standard CPA was 97%.
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**RESULTS AND DISCUSSION**

An important component of efforts to control mycotoxin contamination problems is the study of the morphological, molecular genetics, metabolic and plant pathological diversity of mycotoxigenic fungi (MORETTI et al., 2013).

The mycoflora of the animal feed and forage samples are shown in Table 1. Toxigenic fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* were isolated from most samples (Table 1). Other studies also reported the presence of *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and *Eurotium* spp. in animal feed (PEREYRA et al., 2010; ASTORECA et al., 2011). Among representatives of the genus *Aspergillus*, were isolated from animal feed: *A. flavus* (69%), *A. fumigatus* (2.30%), *A. niger* (1.50%) and *A. parasiticus* (0.80%). The highest incidence of *A. flavus* was shown in animal feed and in their ingredients (PEREYRA et al., 2010 and 2011; ASTORECA et al., 2011). The *Aspergillus* species isolated from forage were: *A. flavus* (49%), *A. fumigatus* (5.70%), and *A. niger* (1.0%). The forage samples showed highest incidence of *Aspergillus* spp. than in feed. Same results were found by ACCENSI et al. (2004). The identification of *Aspergillus* Section Flavi has been traditionally based on morphological and biochemical characterization (RODRIGUES et al., 2009).

The morphological and molecular characterization and mycotoxigenic ability were used for *A. flavus* and *A. parasiticus* identification. Morphologically, *A. flavus* has finely roughened conidia mostly produced from heads bearing both metulae and phialides, whereas conidia of *A. parasiticus* are usually conspicuously roughened and most heads bear phialides alone (PITT & HOCKING, 1997). Based on these morphological characters, it was possible to identify *A. flavus* in 64% of the *Aspergillus* spp. isolated from feed and 37% from the forage. The identification was confirmed by molecular characterization and mycotoxigenic ability of the *A. flavus* and *A. parasiticus* strains.

The results of the molecular analysis showed that 97% of the *Aspergillus* spp. isolated from feed was PCR positive for *A. flavus* and *A. parasiticus*. The molecular identification using the gene *aflD* (PCR) was a good maker for differentiating the *A. flavus* and *A. parasiticus* species (fragment of 400pb) from the other *Aspergillus* spp., but it was not possible to differentiate them from each other. Of 131 strains isolated from feed and confirmed by PCR, 65.60% were *A. flavus* and 0.76% was *A. parasiticus*, using the gene *aflR* (PCR-RFLP). Among the *Aspergillus* spp. isolated from forage (98 strains) 71.40% were positive by PCR analysis and 51.40% of them were identified with *A. flavus*, however *A. parasiticus* was not isolated. The results obtained in this work are in agreement with SOMASHEKAR et al. (2004), who showed that PCR-RFLP patterns obtained with *HincII* can be used to distinguish the two species. *A. flavus* cleaved into 3 fragments of 385, 250, 161bp whereas *A. parasiticus*, having one restriction site for the *HincII*, produced 2 fragments of 546 and 250bp. The *aflD* and *aflR* genes were not able to identify the ability of the strains to produce aflatoxins (SOMASHEKAR et al., 2004; RODRIGUES et al., 2009). In addition, the genomic structural genes involved in aflatoxins biosynthesis do not guarantee the production of aflatoxins by all isolates of *A. flavus* and *A. parasiticus* (LEVIN, 2012), so their mycotoxigenic ability was investigated. The 131 *Aspergillus* spp. strains confirmed by PCR from feed and 70 strains from the forage were evaluated as having ability to produce aflatoxins and CPA (Table 2). *A. flavus* is able to produce aflatoxins...
and CPA, and *A. parasiticus* can produce only aflatoxins. The results showed that of the *Aspergillus* spp. from feed 39% produced aflatoxins B₁ and B₂, 17% produced CPA, 18% produced both toxins, and 42% had no toxigenic ability. Only 2.0% of the 17% produced CPA, 18% produced both toxins, and 42% had no toxigenic ability. Only 2.0% of the strains produced aflatoxins B₁, B₂, G₁ and G₂ but no CPA, and, based on all characteristics analyzed, they were identified with *A. parasiticus*. Almost all *A. parasiticus* isolated produce both aflatoxins B and G, but not CPA (HORN & DORNER, 1999). The strains from forage were producers of aflatoxins B₁ and B₂ (37%), CPA (14%), 14% of both mycotoxins, and 49% did not show toxigenic ability. The concentrations of aflatoxins B₁ and B₂ and CPA were showed in the table 2. The results showed the great variability in the mycotoxins producing potential by *A. flavus* in the culture conditions. Our results agree with the literature that also found difference in the mycotoxigenic ability of the *A. flavus* (VAAMONDE et al., 2003; ASTORECA et al., 2011). VAAMONDE et al. (2003) proposed five chemotypes for *A. flavus* species, based on the mycotoxins produced by them: chemotype I for aflatoxin B and CPA producers; II for AFB, AFG and CPA producers; III for AFB producers; IV for CPA producers and V for non mycotoxin producers. The *A. flavus* strains isolated from feed and forage belong to the following chemotypes, respectively: type I (18% and 14%); type III (39% and 37%); type IV (17% and 14%) and type V (25% and 35%). The chemotype II was not isolated. ASTORECA et al. (2011), also isolated *A. flavus* belonging to chemotypes I, III, IV and V, but no type II from poultry feed. By comparing the chemotypes isolated from feed and forage, there was no statically significant difference between them (P<0.05).

The average aₙ values of the samples ranged from 0.51 to 0.75 for feed and from 0.46 to 1.0 for forage. The minimum aₙ values required for growing *A. flavus* and *A. parasiticus* as well as for producing aflatoxins are 0.80 and 0.83, respectively (PITT & HOCKING, 1997). The aₙ did not influence the *A. flavus* and *A. parasiticus* isolation from feed and also forage (P<0.05) since these species were isolated in all samples analyses. Therefore, the feed did not have water enough for the fungal activity. On the other hand, the forage is not a good substrate, because it has high fiber concentration and low nutrients to the fungi.

According to SMITH & MOSS (1985), feed with good microbiological quality should present a maximum of 10⁵ colony-forming units g⁻¹ (CFU g⁻¹). The results showed that only 17% of the feed and 2.8% of forage samples had more than 10⁵CFU g⁻¹, therefore most of goat feeding had good quality in all farms studied.

### CONCLUSION

The results obtained in this study indicate that, to distinguish the *A. flavus* and *A. parasiticus* species, it is necessary more than one technique of identification. The presence of *A. flavus* capable of producing CPA and aflatoxins indicates a risk of contamination in dairy goat feed and forage if they are exposed to environmental conditions that allow fungal growth.

### ACKNOWLEDGEMENTS

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support.

### REFERENCES


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