IDENTIFICATION OF AFLATOXIGENIC FUNGI USING POLYMERASE CHAIN REACTION-BASED ASSAY

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As the aflatoxins represent a health-risk for humans because of their proven carcinogenicity, food-borne fungi that produce them as secondary metabolites, mainly Aspergillus flavus and Aspergillus parasiticus, have to be isolated and identified. The best argument for identifying problem fungi is that it indicates control points within the food system as part of a hazard analysis critical control point (HACCP) approach. This assumes there is a close link between fungus and toxin. Conventional methods for isolation and identification of fungi are time consuming and require admirably dedicated taxonomists. Hence, it is imperative to develop methodologies that are relatively rapid, highly specific and as an alternative to the existing methods. The polymerase chain reaction (PCR) facilitates the in vitro amplification of the target sequence. The main advantages of PCR is that organisms need not be cultured, at least not for a long time, prior to their detection, target DNA can be detected even in a complex mixture, no radioactive probes are required, it is rapid, sensitive and highly versatile. The gene afl-2 has been isolated and shown to regulate aflatoxin biosynthesis in A. flavus. Also, the PCR reaction was targeted against aflatoxin synthesis regulatory gene (aflR1) since these genes are nearly identical in A. flavus and A. parasiticus in order to indicate the possibility of detection of both the species with the same PCR system (primers/reaction).

KEY WORDS: aflatoxigenic fungi, polymerase chain reaction

INTRODUCTION

Aflatoxins are mycotoxins that are belonging to the group of polyketide-derived furanocoumarins. They present one of the most cancerogenic substances. Aflatoxin B1 (AFB1) has been classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (group 1A) (1). They are having nephrotoxic, carcinogenic, mutagenic and teratogenic effect (2). Although there are at least 16 structurally different chemical compounds related to aflatoxins, 4 aflatoxins (AB1, AB2, AG1 and AG2) are the most often contaminating food and agricultural products. Due to that, they are presenting

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the highest risk to human and animal health. Aflatoxins are produced mainly by two species from genus *Aspergillus*, section *Flavi* - *A. flavus* (AB1, AB2) and *A. Parasiticus* (AB1, AB2, AG1 and AG2). The B and G nomenclature derive from the blue and green fluorescent colours produced under UV light on thin layer chromatography plates (3). This section also includes other non-aflatoxigenic species that are traditionally used in Asian food industry for some fermented products - *Aspergillus oryzae*, *Aspergillus sojae* and *Aspergillus tamari* (4).

It is also reported that potential producers of aflatoxins could be some other species from genus *Aspergillus* (*Aspergillus nomius*, *Aspergillus pseudotamarii*, *Aspergillus bombycis* and *Aspergillus ochraceoroseus*) and *Emericella venezuelensis* (5).

After big turkey plague in 1960 in England, investigations on aflatoxins gained significant attention. Since then, big efforts are made to determine occurrence, structure, biochemical pathways, potential producers of this hazardous mycotoxin, as well as elimination methods and prevention measures. Other significant mycotoxin that belongs to this group is aflatoxin M1, which is one of the oxidative forms of aflatoxin B1. Aflatoxin M1 is formed in digestive tract of ruminants as a product of aflatoxin B1 metabolism, and it can be found in milk, urine and faeces (5).

Mycotoxicoses are acute food borne diseases caused by fungal contamination, presenting reactions to the toxins. Persons that are more susceptible to mycotoxicoses are one that are already have some diseases (HIV, hepatitis C, diabetes etc.) or special group of people, that are including infants, children, pregnant, old, young and immunocompromised ones (6). Aflatoxicosis are caused by intake of food or feed contaminated with aflatoxin, which is in general strongest known naturally occurring cancerogen. Long-term consumption of aflatoxin – contaminated food is associated with the development of liver cancer (5). Due to aflatoxin, and other mycotoxins in general, strong chemical stability to high temperatures, they are often detected in processed food (6).

**Aflatoxin in food and feed**

Infection of commodities with aflatoxigenic moulds can occur during growth, harvesting, transportation or storage, especially in regions with warm climate (7). These moulds are often contaminating maize, cotton, peanuts, tree-nuts, figs and spices (7).

Great efforts are made to exclude food and feed pathogens from the beginning of production of raw material. Using principles of Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP), and as well HACCP, prevention of contamination with hazardous moulds is made. In order to overcome problems with mould contamination and to overcome economic losses and health problems caused by toxigenic moulds, constant monitoring in all processing stages is necessary (6).

The best way to eliminate aflatoxin is to prevent mould contamination at first place, with application of appropriate agro-technical measures. In order to determine food quality and potential contamination with aflatoxins, it is important to identify main fungal species. Because of that, development of a rapid, sensitive method for detection and differentiation of potential aflatoxigenic species in foods and feeds is necessary in order to estimate associated health risk (8, 9).
METHODS FOR IDENTIFICATION OF AFLATOXIGENIC FUNGI

Conventional methods

The detection of moulds in substrate is usually done using selective mycological media or by immunological methods. The success of detecting fungi from food by any culture-based method depends on appropriate media, techniques for homogenizing of the particular food, filter systems for fluids and the strain composition of the food. There is no single media appropriate for growth of acidophilic, xerophilic, proteinophilic and osmophilic fungi, and because of that there is more than 37 different culture media for cultivating of filamentous fungi suggested by Centraalbureau voor Schimmelcultures (CBS), depending on different species and genus requirements for aw and pH values and nutrient composition.

The most common technique for determination of total mould count in food and feed is dilution method by Koch. It considers homogenisation of samples, preparing 10-fold dilutions cultivating on appropriate culture media. Colonies are counted and analysed after agar plates incubation for 5 to 14 days on 25°C.

This method is time consuming, labour – intensive, costly and require certain mycological expertise and facilities. It is relying on micro- and macromorphological characters and with identification keys most of the species cannot always be successful identified.

Alternative methods

Today, antibody and DNA methods, such as ELISA assay, PCR and PCR-ELISA, respectively, and Immunomagnetic Separation (IMS) are the most widely used technologies in food diagnostics today. Advantages especially in PCR-based methods lie in the specific detection of small amounts of target organisms by amplifying their DNA in a considerable short time frame. Other methods used in food diagnosis for detection fungal spoilage are based on detection of different volatile metabolites derived from primary and secondary metabolism (2-methyl-1-propanol, 3-methyl-butanol, 1-octene-3-ol, 3-octanone, ethyl acetate, 3-methylfuran, 2-methylisoborneol and geosmin). DNA based fingerprinting methods such as DGGE (Denaturing Gradient Electrophoresis), TGGE (Temperature Gradient Gel Electrophoresis), SSCP (Single Strand Conformation Polymorphism), TRFLP (Terminal Restriction Fragment Polymorphism) and ARISA (Automated R-DNA Intergenic Spacer Analysis) are also commonly used.

Mycotoxins occur at the end of the exponential growth phase of moulds, so that gives importance of the detection of viable fungi in food. Growth factors (substrate composition, temperature, aw, pH, atmosphere, redox potential) and microbial competition, influence mycotoxin production.

Immunological methods and diagnostic media have limited application, and sometimes can give false results. Because of that need for development of rapid methodologies, that are highly specific and optional to named methodologies is rising.

In the last few years, methods for identification of moulds are significantly simplified. One of suitable methods is the polymerase chain reaction (PCR) that allows in vitro amplification of the target sequence. These method is rapid, sensitive, versatile and usually
do not require prior cultivation of microorganism. Isolation of targeted DNA can be done in complex mixture, without radioactive probes (6).

**PCR INTRODUCTION**

Polymerase Chain Reaction (PCR) is robust, speedy and flexible method. It has enormous number of variations that are described in journals and books (10). PCR is based on enzymatic amplification of a target DNA region defined by two oligonucleotides, called primers, which bind opposite to complementary DNA strands (3). Primers are designed based on the sequences in public databases or alternatively by sequencing the target gene. PCR uses DNA polymerases for amplification of specific pieces of DNA, using short, sequence-specific oligonucleotides added to the reaction to act as primers. The most commonly used enzyme is Taq DNA polymerase (from Thermus aquaticus). Also is used Pfu DNA polymerase (from Pyrococcus furiosus) because of its higher fidelity when copying DNA. DNA polymerases extend a short primer at the free 3'-OH, which is hybridised to a single strand target DNA. Cycle profiles like denaturing of target DNA, binding of primers and extension of new strands are repeated until a detection of amplification with suitable methods is possible. Assuming that the PCR starts with 2 DNA target molecules, during exponential amplification (2n) millions of molecules can be detected after 20-50 cycles. The resulting PCR product can be observed after gel electrophoresis and staining with a DNA binding fluorescent dye such as ethidium bromide (EtBr) (3).

Real-time polymerase chain reaction (PCR), based on the revolutionary method of PCR, developed by Kary Mullis in the 1980s, which allows investigators to amplify specific pieces of DNA more than a billion-times (8). Real-time PCR is regarding on amplification of DNA (by PCR) that is monitored during the amplification process. This method allows investigator to precisely determine amount of starting DNA in the sample before the amplification by PCR. Real-time PCR involves usage of fluorogenic probes that have ability to “light up” and show the amount of DNA present at each cycle of PCR. “Kinetic PCR” refers to this process as well. Quantitative PCR has ability to quantify the starting amount of a specific sequence of DNA. This term refer to any PCR procedure, including earlier gel-based end-point assays, that attempts to quantify the starting amount of nucleic acid.

In methods where reverse transcriptase enzymes are used, than before PCR amplification in any of the earlier mentioned situations, “RT-PCR” replaces “PCR” in the term. Real-time, and quantitative, are often used interchangeably or in combination, because real-time PCR also can quantify nucleic acids.

Real-time PCR distinguish and measure specific nucleic acid sequences in samples even if they are present in very small quantity. Real-time PCR amplifies a specific target sequence in a sample then monitors the amplification progress using fluorescent technology (8).

The key to real-time PCR is the ability to monitor the progress of DNA amplification in real time. The ability of real–time PCR to monitor the process of amplification is accomplished using specific chemicals and instrumentation. Basically chemicals consist of special fluorescent probes in the PCR, which includes DNA-binding dyes like EtBr or SYBR green I, hydrolysis probes (5'-nuclease probes), and hybridization probes, molecular-
lar beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) light-up probes. Each type of them has specific characteristics, and usage depends on sample that is analysed. They are linking a change in fluorescence to amplification of DNA.

Real-time quantitative PCR (qPCR) provides a method for accurate and sensitive quantification of target DNA that could be applied to quantify aflatoxin producing moulds. qPCR greatly simplifies the procedure comparing to conventional culturing techniques, because gives opportunity of continuously monitoring of samples through amplification which allows for their easy identification using either the fluorescence of non-specific dyes or a sequence specific hydrolysis probe (TaqMan) (11).

Great efforts are made in order to create accurate method for determination of aflatoxigenic moulds in food and feed samples. Multiplex PCR applies in the few genomes that are structural and on the other hand ones that are regulatory for aflatoxin production (12).

There are a few limitations to real-time PCR methods. Real-time PCR can be inhibited by compounds present in certain biological samples. Many parameters can affect polymerase chain reaction (PCR). Some of them are essential for PCR: selection of appropriate thermostable DNA polymerases, programming of polymerase chain reaction (denaturation, annealing of primers to template DNA and extension of oligonucleotide primers and number of cycles required for amplification) (10). Amplification efficiency in PCR-based detection of microorganisms can be significantly reduced in complex biological samples, such as food and feed, by presence of numerous substances (13). Isolation of DNA in food and feed samples, and preparation of samples for PCR is described by many authors, where s also reported difficulties in this field (6, 7, 14, 15). Also many components that naturally occur in food and feed can inhibit DNA amplification, and give incorrect results. Many published results on methods for fungal DNA extraction is developed using mycelium grown in liquid media, which is far from real fungal surroundings (16).

PCR inhibitors may act by interference with the cell lysis necessary for DNA extraction, by nucleic acid degradation or capture and by inhibition of the polymerase activity necessary for amplification of the target DNA (17, 18). The inhibition of the amplification reaction is one of the most limiting factors and can cause complete reaction failure, leading to false negative results or reduced sensitivity of specific detection of the mycotoxigenic producers (16).

Biochemical pathway of aflatoxin production

In fungi essential metabolites are produced from intermediate metabolic pathways like glycolysis and the citric acid cycle. Secondary metabolism removes products from intermediate metabolic pathways when growth is temporarily restricted. Secondary metabolites such as mycotoxins are often specific for individual genera, species or strains, and in food industry they are highly unwanted (6).

Biosynthetic pathway of aflatoxin production involves around 15 different intermediary compounds (Figure 1.)
Figure 1. Biosynthetic pathway of aflatoxin biosynthesis (5).

The aflR regulatory gene was initially named afl-2 in *A. flavus* and apa-2 in *A. parasiticus*. The regulatory gene was later named aflR in both *A. flavus* and *A. parasiticus* as well as in *A. nidulans* for its function as a transcription activator (5).

Two other genes associated with aflatoxin biosynthesis in *A. parasiticus* were identified by complementation: the nor-1 gene, associated with the conversion of norsolorinic acid to averantin, and the ver-1 gene, associated with the conversion of versicolorin A [VERA] to sterigmatocystin (2).

Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path: acetate -> polyketide ->anthraquinones->xanthones->aflatoxins (5).

Many isolates of *A. flavus* strains are not aflatoxigenic because of mutation in one or more genes belonging to the biosynthetic gene cluster (12).
Identification and quantification of aflatoxigenic fungi using PCR

PCR is presenting promising tool for detection and quantification of fungi in food and feed industry. Maximum tolerance of aflatoxins levels of between 5 and 20 \(\mu g \cdot g^{-1}\) (5-20 ppb) in foodstuffs are common in most countries, with Japan one of the world’s largest importers of agricultural products having a legal limit of AFB1 in foodstuffs of zero. Aflatoxin M1 is a mono-hydroxylated derivative of AFB1 that is formed and excreted in the milk of lactating animals including humans that have consumed AFB1 contaminated material. Consumption of aflatoxin M1 contaminated infant milk and milk products is of concern and in European Union very low limits have been set (0.01-0.05 \(\mu g \cdot kg^{-1}\) for infant foods; given their relatively high consumption rate of these products and the possible higher susceptibility of younger children to aflatoxins (19).

Viable fungi contain both DNA and RNA, where DNA is often stable during food processing it is not a specific marker of viable cells (20). On the other side, only under extreme heat rRNA was destroyed experimentally. Using DNA or rRNA as target molecules for viable cells, nucleic acids from/or in dead cells in food have to be destroyed selectively by enzymatic digestion before running a PCR to avoid false positive signals. One of the options is to detect only mRNA, because it is found only in living organisms, and can be marker of viable cells. One of disadvantages for usage of mRNA is its instability (6).

Various PCR-based techniques have been developed to detect and quantify mycotoxigenic fungi. These fungi have been detected based on specific target DNA from mycotoxigenic genes, other genes, ribosomal DNA or unique DNA bands from random amplified polymorphic DNA (RAPD) analysis (3). Commonly target molecules in diagnostic PCR are 18S rDNA genes, internal transcribed spacer (ITS) regions of ribosomal genes, mitochondrial DNA and specific proteins (6).

Detecting airborne filamentous fungi was successfully done by a PCR based method developed for detection of aflatoxigenic moulds in grains in 1996 by Shapira et al. (2). Targets were key enzymes and regulatory factors in aflatoxin biosynthesis: ver-1, omt1 and apa-2, respectively.

Using a multiplex PCR approach, by targeting three aflatoxin biosynthetic genes namely norsolorinic acid reductase (nor-1), versicolorin A dehydrogenase (ver-1) and sterigmatocystin O-methyltransferase (omt-A), was able to observe a triplet banding pattern in aflatoxin producing strains of Aspergillus flavus, A. parasiticus and sterigmatocystin producing strains of A. versicolor (3).

Multiplex PCR that amplifies structural genes such as nor1, omtA, ord-1, omtB, ver-1, avnA and avfA, and/or regulatory genes such as aflR (apa-2) and aflS, in the aflatoxin biosynthesis pathway has been successfully applied to the detection of aflatoxigenic fungi in a variety of foods and feeds (2, 14, 15, 21-23).

The sequence of the forward and reverse primers aflR1 of the aflatoxin regulatory gene was (5'-AACCGCATCCACAACTCTCAT-3') and (5'-AGTGAGTTGCTGCTAGACAA-3'), and were designed based on the published sequence strand for A. flavus and A. parasiticus (7). They have investigated aflatoxigenic moulds in raw food materials, spiked with other fungal cultures. Investigation had revealed that using aflR1 primer based
PCR method had high sensitivity and specificity in detecting aflatoxigenic Aspergilli in pure and mixed culture systems.

qPCR assay that can quantify aflatoxin producing moulds, is so far only developed for the moulds from genus *Aspergillus*. Big efforts are made to design primers essential in new qPCR protocols for detection and quantification of aflatoxin-producing strains that are belonging to other mould genera (11).

In order to determine number of colonies using qPCR, food samples can be spiked with certain number of fungal colonies to make calibration curves. Investigations have shown that in general, the *omt-1* gene copies and the fungal load obtained by qPCR correlates well with the CFU data always obtaining R² values around 0.98. In all foods, both qPCR methods were able to quantify *omt-1* gene copies just after inoculation and before mycelium growth. In the most of the cases, the number of *omt-1* gene copies determined by qPCR was higher than the cfu data. Nevertheless there were no significant differences between fungal load determined by qPCR and by counting on PDA throughout the 14 days of incubation time (11).

Passone et al. (1) had investigated presence of aflatoxin producing species in peanuts. They used real time PCR to determine and quantify these moulds using primer/probe set with following nucleotide sequence: nortaq-1, 5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2, 5′-TCGTGCATGTTGGTGATGGT-3′; norprobe, 5′-TGTCTTGATCGGCGCCG-3′ according to the published sequence of nor-1 for the isolate AF36 of *A. flavus* (AY510455) (24). Results of their investigations showed that number of CFU received with PCR correlate with one received from plate counting method (r=0.613; p<0.0001), which proved that real-time PCR can be used to quantify aflatoxigenic moulds.

The sterigmatocystin O-methyltransferase gene (*omt-1*), that converts sterigmatocystin to O-methylsterigmatocystin or dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (5) has been reported as structural gene of the aflatoxin gene cluster together with norsolorinic acid reductase (*nor1*) and versicolorin A dehydrogenase (*ver1*) and is activated by the *aflR* gene product. Considering these facts, this gene can be used only to detect aflatoxin producing moulds, but without determination whether is B or G type (13).

**False positive results**

In multiplex RT-PCR, set of primers is used in order better to define tested fungal strains. (25) have used as target genes in aflatoxin cluster - *aflD, aflS, aflQ, aflP* together with β-tubulin as IAC. Housekeeping genes (e.g., cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein 36B4, _α-actin, 18S rRNA, transferrin receptor, etc.), that are not expected to change under the experimental conditions serve as a convenient internal standard (26).

Previous analyses showed that some of tested strains are not reported after 4 days to produce aflatoxin. But they have observed that one of isolates expressed same genes as the strains that were positive on aflatoxin production. They tested *A. flavus* isolates from corn that are reported to be slow producer of aflatoxin, i.e., accumulation is observed after 10 days of cultivation and also depends whether is cultivated on solid (CAM) or liquid media (YES). These results are pointing out possible underestimation of contami-
nation risk by aflatoxin-slow-producing strains, that are expressing aflatoxigenic character on synthetic media in laboratories after longer period of incubation (10 days), where in field or on production line (environmental conditions) may be expressed faster. PCR is helping to reveal this gene before toxin production occur (27).

CONCLUSION

PCR based methods obtain increasing acceptance for testing the microbiological safety of food, because of their high sensitivity and specificity. Despite the apparent problems of fungal contamination in food and the demand of reliable test systems, today, only a few general obligations exist for fungal testing in food. Validated and standardised use of the polymerase chain reaction (PCR) can facilitate the implementation of fungal diagnostic PCR in routine. Thus, using primer designed to aflatoxin regulatory pathway, for example gene aflR, presents a rapid method of detecting the aflatoxigenic fungi in selected raw food samples, compared to conventional plating techniques.

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REFERENCES


RETRACTED

ИДЕНТИФИКАЦИЈА АФЛАТОКСИГЕНИХ ПЛЕСНИ И ПОЛОЖАЈ МЕТОДА ЗАСНОВАНИХ НА ПРИНЦИПУ ЛАНЧАНЕ РЕАКЦИЈЕ

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Због своje доказане карциногености, афлатоксини представљају ризик по људско здравље, те плесни које генерирају ове токсичне елементе, морају бити изоловане и иденитификоване. Утврђивањем присуства плесни током процеса производње хране, указује се на потенцијално критичне контролне тачке процеса у оквиру „HACCP“ приступа. Овим се такође утврђује и могућа везаност између плесни и токсина. Традиционалне методе за изолацију и идентификацију плесни су дуготрајне и захтевају искусне стручњаке из области таксономије. Из тог разлога постоји изузетна потреба за развојем метода које су брзе, прецизне и представљају алтернативу постојећим методама. Ланчана реакција полимеразе (PCR енгл.) омогућава in vitro умножавање одређеног дела генома микроорганизма у принципу „PCR“-а су те се микроорганизми претходно не култивирани, али у комбинованим смешама, тако да дискусија о потребе радиоактивне пробе, те је метода брза, осетљива и изузетно применива. Ген afl-2 је изложен у врсте A. flavus и утврђено је да регулише синтезу афлатоксина. Регулаторни ген који је такође често праћен у циљу утврђивања афлатоксигене способности одређених врста је aflR1, јер је заједнички за врсте A. flavus и A. parasiticus, па се праћењем овог гена омогућава праћење обе врсте у једном „PCR“ систему.

Кључне речи: афлатоксигене плесни, ланчана реакција полимеразе

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