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Rapid detection of aflatoxin -associated genes in *Aspergillus* species using colony PCR based method

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ABSTRACT

Monitoring and controlling of food healthy is an indicator of scientific progress and social health in every country. Food contamination is caused by aflatoxin of saprophyte fungus associated with a number of pathological conditions including cancer and chromosomal anomalies. Aspergillus is defined as a group of fungi with various species. Identification of these fungi is of importance in terms of pathogenicity, toxicity and industry application. There are a number of laboratory methods for differentiation between Aspergillus species including physical macro and microscopic characteristics of colony. These methods are time-consuming and require highly trained personnel. In the present study, 27 samples of contaminated food and 15 samples of intact food were collected. *Aspergillus flavus* and *Aspergillus parasiticus* were selected as positive controls and examined using colony PCR reaction. All samples were re-cultured on medium culture of potato dextrose agar. Initial identification was performed based on microscopic features. Genomes of all strains were extracted using lyticase enzyme and lysis buffer containing: EDTA, Tris HCL, and NaCl. Then, aflatoxin genes of each sample were amplified through PCR. The results of colony PCR in intact food samples demonstrated that there is no used primer related to aflatoxin genes. In suspected contamination food samples, one sample with *aflr* and two samples with *omt-1* were observed.

Conclusion: The results of our study demonstrated that lyticase enzyme and lysis buffer are potential candidates for extraction of *Aspergillus* DNA.

Keywords: *Colony PCR; Aflatoxin, Aspergillus; Detection; Gene.*

Abbreviations: *PCR, polymerase chain reaction; MT-PCR, multiplex-tandem PCR; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.*

1. INTRODUCTION

A variety of fungi are present in the air, soil and our surrounding environment. Fungal growth in food is one of the most challenging barriers and potential threat to the human health [1-3]. Many parameters such as nutrition importance, and human and food healthy should be considered in food storage [4]. Mycotoxin is a secondary toxic metabolic produced by fungi. Primary metabolites (such as amino acids, acetate and pyruvate) are essential factors in the growth of fungi. However, secondary metabolites that are produced at the end of growth phase after the accumulation of primary metabolites, are not associated with fungal growth. Aflatoxin is one of the fungal metabolites produced by *Aspergillus (A). flavus* and *A. parasiticus* [5, 6]. The most dangerous type of Aflatoxin is B1 that is associated with carcinogenic, teratogenic and toxic effects [7]. In contrast to bacterial toxins that are frequently in the form of macro-molecules (poly peptides, proteins, Lipopolysaccharide), mycotoxins often have a low molecular weight. The presence of mildew in food is not a definite reason for the presence of mycotoxin. On the other hand, its absence does not imply the lack of toxins in food, because mycotoxins remain for a long time after disappearance of mold in the food. It has been demonstrated that a total of 25% of world foods are annually contaminated with mycotoxins. Aflatoxins exist in corn, peanuts, beans, eggs, dried fruits, and dairy products used at large amounts by people and food industry worldwide [8-11].

A rapid, highly sensitive, and specific method requires for primary identification of aflatoxin fungi in food due to the toxic and carcinogenic features of aflatoxin (presence of this substance in food plays a significant role in generation of pathological conditions such as cancer, especially in growing children) [4, 12]. Recently, powerful DNA-based methods have been introduced for identification of the fungi producing aflatoxin. Polymerase chain reaction (PCR) is a superior method for this purpose that a unique sequence of genes involved in aflatoxin biosynthetic pathway can be selected for primer design [11, 13, 14].

Gene clusters contribute to the biosynthesis pathway of aflatoxin include structural, regulative and unassigned genes. *Omt-1* is one of the structural genes encoding of a key enzyme, whereas *aflr* is a regulatory gene that plays a key role in the biosynthesis of aflatoxins. *Aflr* affects the structural genes and stimulates their transcription. Enzymes encoding genes along with special regulatory genes of this pathway, known *afls* and *aflr* as a large cluster of genes with size of approximately 70 kb, have been located near the telomere of chromosome 3 of Aspergillus genome. The transcription path is unique for each gene [15-17]. The structure of aflatoxin gene cluster of A. *parasiticus* and *A.*

flavus is basically homologous. Generally, the position of involved genes in the biosynthesis of aflatoxin is a little variable between aflatoxigenic fungi. *Aflr* gene encodes a protein containing zinc finger functional domains and has the ability of binding to specific sequences of DNA. The product of this gene increases the expression by binding to palindromic sequence of 3'-TCG_5CGA-'5 (also known as *aflr* binding motif) in the promoter region of biosynthesis structural genes of aflatoxin, as a positive transcription factor [18-23]. *Omt*-1 and *aflr* genes have a key role in producing toxin, so that the production of toxin is halted by their inactivation. PCR has aimed to detect these genes before toxin production. In the present study, the colony PCR was used for detection of the aflatoxigenic aspergilli based on the mediating enzyme in the production of aflatoxin, 0-methyl transfrase, that is encoded by gene *omt-1* and the regulatory gene *aflr*. Note that this method is also applicable to the detection of clinical fungal infections [12, 24].

2. MATERIALS AND METHODS

2.1. Sample collection.

Fungal strains, *A. flavus* NO. 5004 PTCC and *A. parasiticus* NO.5018 PTCC were purchased from Mycology Research Center of Tehran Science. These samples, after culturing in potato dextrose agar medium, were incubated for 48 to 72 h at 27°C and used as a positive control in the experiment. Suspected samples were collected from different food sources based on their morphological and microscopic characteristics. Morphological identification of species was performed using characteristics of colonies in terms of growth rate, shape, color, texture, topography, as well as macroscopic features of colonies including length and diameter of conidiophores, shape and size of vesicles, shape and size of conidia [25, 26]. After the isolation of *Aspergillus* strains, they were kept at -20°C in potato dextrose broth medium. Sampling from suspected materials was performed using a sterile swab. The samples were immediately incubated in potato dextrose agar medium. Seven samples of local dairy products (were produced by non-industrial technique), fourteen days after their production was transferred to laboratory in sterile containers. Ten samples of Pistachio and ten samples of peanut, with three years of storage, were randomly collected from a market in Chaharmahal and Bakhtiari province and transferred to the laboratory. Fungal samples were re-cultured in potato dextrose agar medium containing ampicillin (50 μg mL^{-1}) and incubated at 25-30°C. Healthy food samples were as following: five samples of dairy products, three days after production, five samples of pistachio and five of peanut with year storage were isolated (Table 2). All the intact samples like suspicious samples were cultured on potato dextrose agar medium containing ampicillin [27]. The numbers of contaminated samples were as follows: 7 dairy products, 10 peanuts and 10 pistachios.

2.2. Colony PCR protocol.

The spores were isolated from colony with diameter of 1 mm using micropipette tip and then, were transferred to potato dextrose broth medium and incubated for 48 h at 27°C after culturing of fungal samples in potato dextrose agar medium. For each sample, mycelium mass was transferred into a sterile tube using a toothpick, mixed with normal saline, and centrifuged at 3000 rpm for 5 min at 4°C to remove the culture medium (culture medium is an inhibitor factor in PCR). The supernatant was discarded and the pellet was carefully resuspended in 50 μl of KC solution (0.8 M potassium chloride, 10 mM citric acid). 50 U of lyticase *ArthrobacterLuteus* (*ArthrobacterLuteus*, Sigma-Aldrich) in H_2O was added and the reaction carried out at 37°C under gentle shaking for 60 minutes. Lyticase enzyme hydrolyzes linear polymers of glucose with beta 1-3 bonds in fungi wall. Then, 150 μL of fungal lysis buffer (10 mMTris-HCL with pH 8.0, 1 mM EDTA with pH 8.0,100 mMNaCl) and 100 mg of K-proteinase (Fermentas, Canada) per mL were added to the tubes and incubated for 60 minutes at 75°C. The tubes were boiled for 5 min at 95°C, placed on the ice for 5 min, and centrifuged for 10 min at 12000 rpm at 4°C. This is for the thermal shock and better destruction of wall. After reaching a homogeneous suspension, 5 μL of supernatant was used for colony PCR as a DNA sample. The quality and quantity of extracted DNA were approved using electrophoresis on 1% agarose gel (Fig.1) and optical absorption of spectrophotometer (Fisher, Scientific, Ottawa,ON) [27].

2.3. PCR.

Specific primers were designed as forward and reverse primers, based on the sequence of *A. flavus* and *A. parasiticus* strains. The primers were *aflr* and *omt-1*. The forward and reverse primers for *aflr* were respectively as following: 5-′TATCTCCCCCGGGCATCTCCCGG-3′ and 5′-CCGTCAGACAGCCACTGGACACGG-3′ [12]. Also, the forward and reverse primers for omt-1 were respectively as following: 5′-GTGGACGGACCTAGTCCGACATCAC-3′ and 5′-GTCGGCGCCACGCACTGGGTTGGGG-3′ [28]. After BLAST and specialty confirmation, target genes were constructed by Takapuzist Company, Iran. The *omt-1* and *aflr* fragment sizes were 757bp and 1032bp, respectively.

Table 1. The number of intact and contaminated samples and their fragment sizes.

	Number of contaminated samples	Number of intact samples	*aflr* PCR fragment size	*omt-1* PCR fragment size
dairy products	7	5	1032 bp	757bp
Peanut	10	5	-	757 bp
Pistachio	10	5	-	-

PCR was performed in a reaction volume of 25μl containing 2.5μl of 10X PCR buffer, 0.75μl of 25mM $MgCl_2$, 0.5μl of 0.1mM dNTPs, 1μl each primer, 1U *Taq*DNA polymerase (Fermentas), 1μl of extracted DNA as template and 17.25μl of sterile distilled water. Totally, 35 cycles were started by heating at 95 ºC for 10min and continued by denaturing 1min at 94ºC, annealing 1min at 57 º C, elongating 1min at 72 ºC and a final extension 10min at 72 ºC**.** The PCR products were analyzed by gel electrophoresis on 1% agarose and using 100 bp and 1000 bp DNA size markers (Life Technologies, Grand Island, NY). The gels were stained at GelRed Nucleic Acid Gel Stain, 10,000X in water (0.5μg mL^{-1}),

visualized on an ultraviolet (UV) transilluminator and photographed using an Alpha Imager (Fisher Scientific) gel documentation system [12].

3. RESULTS

Isolated *A. flavus*, *A. parasiticus* and Aspergillus strains from food materials were cultured on potato dextrose agar medium. After 3 days, the colonies were observed in the forms of viridian, fluffy, soft, and wavy. Mycelia with the septum contained short conidiophores and spherical conidia which formed vesicles at the end. Colony PCR was applied using isolated DNA samples of *A. flavus, A. parasiticus* and isolated samples from food material and environment along with specific primers of *omt-1*& *aflr* genes. In PCR, *omt-1* and *aflr* region of positive control samples were successfully proliferated. Figures 2 and 3 demonstrate the PCR products obtained from each primer. A band of approximately 757 bp and 1032 bp were observed for *omt-1* and *aflr*, respectively (Table 1). As shown in Figure 3, *omt-1* gene indicated the same band and molecule weight in *Aspergillus* isolated from dairy products and *Aspergillus* isolated from the peanut. *Aflr* gene was successfully amplified in one dairy sample and showed a band of approximately 1032 bp (Fig. 2). *Omt-1* gene was successfully proliferated in a peanut and dairy sample and revealed a band of approximately 757 bp. Negative control was used for all PCRs (Fig. 2 and Fig. 3). In all intact and pistachio samples, PCR results were negative for both primers and no band were observed. Totally, PCR was positive in 3 out of 27 suspicious contaminated food samples.

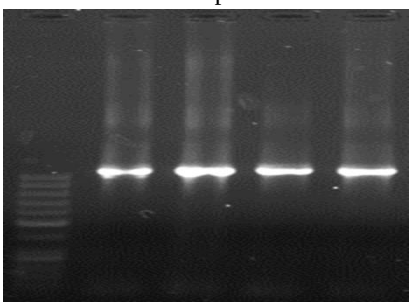

Figure 1. Agarose gel electrophoresis image. Extracted genome of isolated Aspergillus strains from food and environment samples by buffer solution and enzyme treatment (colony PCR method).

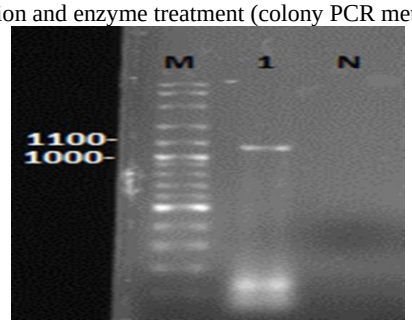

Figure 2. Products of PCR amplification of *aflr* gene. Lane 1: isolated *Aspergillus* from dairy Products; lane M: Size of DNA ladder100bp; lane N: negative control.

Discussion.

Due to the increased level of toxicity cases, much attention has been directed towards this field and molecular identification is of interest in terms of prevention of toxicity [29-39]. Aspergillus species, particularly *A. parasiticus* and *A. flavus* are considered as the most challenging factors in food storage. In this study, we used colony PCR as a potential tool in identification of Aspergillus species. Various methods have been developed for DNA extraction that is time-consuming. Moreover, the results of DNA extraction are not desirable due to using toxic and chemical materials in some protocols [40]. Colony PCR is associated with a number of benefits including high-accuracy by passing through the extraction process, rapid and simple reproduction of desired DNA fragments and efficiency in cloning [41]. Colony PCR is an effective method not only due to time-saving but also because of early detection of fungi in clinical laboratories. In previous studies, colony PCR was used and some advantages achieved including time-saving, using simple equipment and materials for DNA extraction, and a minimum amount of culturing materials [42].

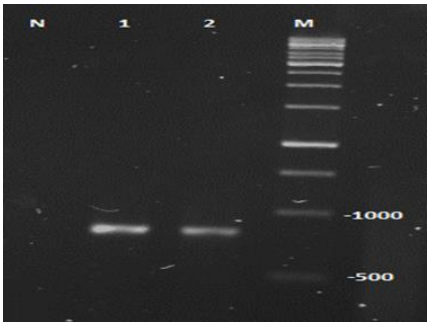

Figure 3. Products of PCR amplification of *omt-1* gene. Lane 1: isolated *Aspergillus* from dairy products; lane 2: *Aspergillus* isolated from the peanut; lane M: size of DNA ladder 1000bp; lane N: negative control.

Colony multiplex-tandem PCR (MT- PCR) has been used by Lau (2008) [43] for identification of fungal cultures. They showed that MT- PCR is in a good agreement with phenotypic identity of all of studied cultures. As a result, this method was reported as a rapid and sensitive in identification of fungal pathogens. A study on yeast conducted by Mirhendi (2007) [44] indicates colony PCR as the fastest method for yeast proliferation. They also state that colony PCR is a reliable method for identification tests. Colony PCR on *Chlamydomonas* was carried out by Cao et al. (2009) and confirmed our study [41]. In this work, the selected colony should be as minimum as possible to inhibit the possible of aggregation and correctly perform the action of primer annealing. Bonnet et al. (2013) [45] have performed the colony PCR on *Saccharomyces cerevisiae* in two procedures including treatment with Lyticase enzyme and without it. A poor band in PCR was sometimes observed by direct use of colonies without enzyme treatment, while in the presence of enzyme, a sharp band was observed. A comparison of molecular type of allergens fungi by colony PCR and PCR methods was performed by Nevisi et. al. (2010) [46]. For doing so, they used *A. fumigatus* and *Fusarium* colonies and Observed bands were different in terms of sharpness for both fungi due to the using of different amounts of colonies. Colony PCR band's quality was similar to observed bands in the PCR method. Finally, they concluded that colony PCR is an accurate and reliable test for identification of target fungi. Our method is the first simultaneous use of lysis buffer and lyticase enzyme in colony PCR method on filamentous fungi. This method is often used for fungal samples which have less pigment, because these pigments are considered as inhibitory factors in PCR. These factors must be treated by pigment removing materials (CTAB

method) before PCR [47]. In order to enhance the performance of this method, fresh colonies must be used. The best time for the use of colonies is 2-3 days after their culturing (thinner cell wall).

In a study, Sohrabi and Taghizadeh examined the potential of PCR for identification of aflatoxigenic aspergillus species in feedstuff samples [48]. The results of this study demonstrated that PCR is a beneficial method for identification of *aflr*, *aflP* and *aflD* in feedstuff samples and has a high efficacy similar to thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). In another study, a polyphasic method was used to determine the presence of aflatoxigenic aspergilla in cashew nuts [49]. It was found that using a single method for identification of aflatoxins is inadequate and a combination of TLC, HPLC, PCR and so on should be used. However, colony PCR showed highest sensitivity for identification of aflatoxins. Taking everything into account, it seems that various methods applied for identification of Aspergillus species suffer from two major problems: A) lack of precise identification, and B) high amount time needed for conduction of experiment. However, at the present study, we elucidated that our proposed method is time-saving as it decreases the time required for identification from several days to multiple hours which is in favor of units that are dealing with this problem. On the other hand, we revealed that our suggested method is reliable, as the results from PCR confirmed the presence of fungi which have been determined by morphological identification, but it provided more information about the species of fungi [50-52].

4. CONCLUSIONS

Estimates demonstrate the enhanced incidence rate of fungal infections such as aspergillosis, requiring novel methods for identification of toxins and PCR has shown great potential in terms of high sensitivity and specificity. Aspergillus strains are currently used in industry and some of them have shown resistant to drugs. One of the practical aims of this study is application of colony PCR to identify aflatoxigenic aspergillus strains in food samples and also environment. For doing so, the presence of toxin in samples is confirmed using other methods such as TLC and then, colony PCR is performed for final confirmation. Different methods have been proposed for DNA extraction which is time-consuming. In this study, we isolated the involved genes in biosynthesis of aflatoxin through amplification. For doing so, we optimized the PCR on extracted genomic DNA from strains of *A. flavus* and *A. parasiticus*. Despite the difficulty of cultivation and extraction of genomes from filamentous fungi, this study showed that the simultaneous use of lysis buffer and lyticase enzyme can provide genomic DNA for PCR. This technique is also applicable for identification of dermal fungus and tissue samples exposed to the fungus infection. This method offers some advantages including high operation speed, minimum rate of culture materials, and no need for extraction. Therefore, we offer this method as a trusty and accurate technique that has a high performance to identify all of filamentous fungi. PCR can be applied to detect this gene before toxin production occurs.

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6. ACKNOWLEDGEMENTS

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