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# PCR Assay and its Application to Identify Mycotoxigenic Fungi

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**Abstract**

Mycotoxins are the secondary metabolites of fungi which are related to specific issue in animals and human and recognize an overall issue influencing staple harvests. The accurate identification of mycotoxigenic fungi stills one of the most basic zones for mycological ordered explorations on account of the significance of mycotoxins and to some degree befuddle condition of the systematic. The utilization of PCR to distinguish mycotoxigenic fungi is drawing into extensive consideration. These techniques depend on the isolation of genes responsible for mycotoxin biosynthesis. In general, only a couple of mycotoxins which their natural chemistry was resolved adequately to empower the improvement of the probes of the gene pathway. The basic of PCR analysis was described as the alternative assay because the microbiological and chemical methods for detection and identification of mycotoxigenic fungi are time cumbersome and time-consuming. For a viable distinguishing proof of utilization to the food manufacture and to avoid misidentification, probably it is more valuable to think about a collection of isolates grouped as a single taxon that can produce mycotoxin. So, this review is aiming to explore the PCR as a promising, specific, and reliable technique for the identification of mycotoxigenic fungi e.g., aflatoxigenic, ochratoxigenic, and mycotoxigenic Fusarium species.

*Keywords*: Mycotoxins; PCR; Toxigenic Fungi

## 1. Introduction

Problems of food safety and health hazards associated with the occurrence of toxigenic fungi producing mycotoxins in food and feedstuffs have been recognized as a serious public health concern, particularly in developing countries. Presence of fungi on susceptible foodstuffs can be identified visually or indirectly by their effects on foodstuffs as they grow. Traditional techniques to identify pathogenic fungi depending on cultivation and morphological examination required man-power and time consuming. In addition, traditional methods may be not suitable to identify fungi species in grains with heavy infection of moulds [1]. So, rapid sensitive and cost effectiveness technique for the detection of these toxigenic fungi and their secondary metabolites is urgently needed.

The prepared accessibility of DNA sequence information has prompted major developments in the investigation of the systematics, biochemistry and the biology of fungi. The presentation of molecular techniques has permitted critical strides to be made in genomics, and in numerous territories of connected mycology molecular based methodologies have gotten to be standard for strain characterization, pathogen recognition and recognizable proof. Molecular strategies are currently beginning to give significant bits of knowledge into how fungi function in the environment, where they are available, and how they interact with related living organisms. The PCR technique is an intense strategy with far reaching applications of molecular biology. Thus the enzymatic response permits *in vitro* the amplification of a specific target DNA sequence, allowing for the isolation, sequencing and cloning of a single sequence among many. The nucleic acid arrangement can be cloned, adjusted or analyzed or even uncommon groupings can be recognized using PCR

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amplification. Kjell Kleppe who was working in the laboratory of H. Gobind Korana (recipient of the Nobel Prize in 1968) initiate a description of the PCR fundamental concepts and describe a process of *in vitro* DNA amplification involving oligonucleotide primers and DNA polymerase [2]. He published the first description of the technique in Journal of Molecular Biology in 1971 [3]. Fourteen years Later, PCR assay was invented by Kary B. Mullis from biotech company Cetus Corporation of Emeryville, California, who shared the 1993 Nobel Prize in Chemistry for this work. Since the improvement of this technique in 1985, the affectability, specificity and pace of this innovation have prompted the advancement of numerous techniques for an extensive variety of natural exploration ranges and all classes of life forms. Broad applications for PCR were found in numerous fields of mycology and including systematic, contagious hereditary qualities, nature and plant pathology, soil microbiology, restorative mycology, fungi biotechnology among numerous others. PCR based analysis was conducted as an alternative assay of time consuming and cumbersome chemical and microbiological traditional methods for most mycotoxigenic fungi.

## 2. PCR principle

PCR technique is based on *in vitro* replication of specific DNA sequences using enzymatic reaction mostly containing two synthetic oligonucleotide DNA primers [4, 5]. The capability of PCR for amplifying can be done from tiny of template DNA within a large background of irrelevant sequences (genome). The main three steps for PCR can be summarized as shown in Fig. (1) [6] and as follows: I. Denaturation: in this step the double strand of DNA-template melts open to single strand DNA at high temperature ranged between 90 and 95 $^{o}$C. The Hot-star taq-polymerase is activated by this step. II. Annealing: hydrogen bonds are formed between single stranded primer and single stranded bases of template, which conducted at a specific annealing temperature. III. Extension, which is conducted at 72 $^{o}$C at the end of the annealed primers to add nucleotide to create a complimentary copy strand of the target DNA [5, 7]. The products are separated after amplification by the agarose gel electrophoresis then directly visualized after ethidium bromide staining.

## 3. PCR reaction basic components

The DNA template, DNA polymerase, oligonucleotide primers, and deoxy- ribonucleotide triphosphates (dNTPs) are mixed together in an appropriate buffer containing magnesium ions (such as $MgCl_2$). This reaction mixture volume is generally ranging from 25 to 100 µl [8].

### *3.1 DNA template*

The DNA template contains the region of the DNA fragment to be amplified. The quality of DNA template affects the outcome of the PCR. For example, Great amounts of RNA can chelate $Mg^{2+}$ and decrease the PCR amplification yield. Moreover, contaminated DNA may include PCR inhibitors that can reduce the reaction competence [9]. Furthermore, the template should be high molecular weight. This can be checked by running an aliquot on an agarose gel.

### *3.2 Primers*

The primers are single-stranded consist of four nucleotides that bind to any strand of genetic material [10]. PCR application mostly success is dependent on primer specificity, concentration and the sequence thereof. For designing primers, some important characteristics should be taken into consideration, which include: the length of primer should be 15-30 bases, G/C content should range between 40 and 60 % showed balanced distribution of G/C and A/T rich domains which are not complementary to each other at the 3' ends and have melting temperature that allow annealing temperature of 52-58 °C, which can be reached 45-65 °C. Primer concentration levels between 1 and 6 µM are optimal. Moreover, higher primer concentrations may enhance the miss priming and gathering of the nonspecific product. Also the decrease of concentrations affects the reaction in lower yields [9, 11].

### *3.3 DNA polymerase*

It considers the enzyme key in the whole process that links individual four bases nucleotides to generate the resultant PCR product. For more regular PCR applications *Taq* polymerase considered the standard PCR enzyme. The enzyme was basically

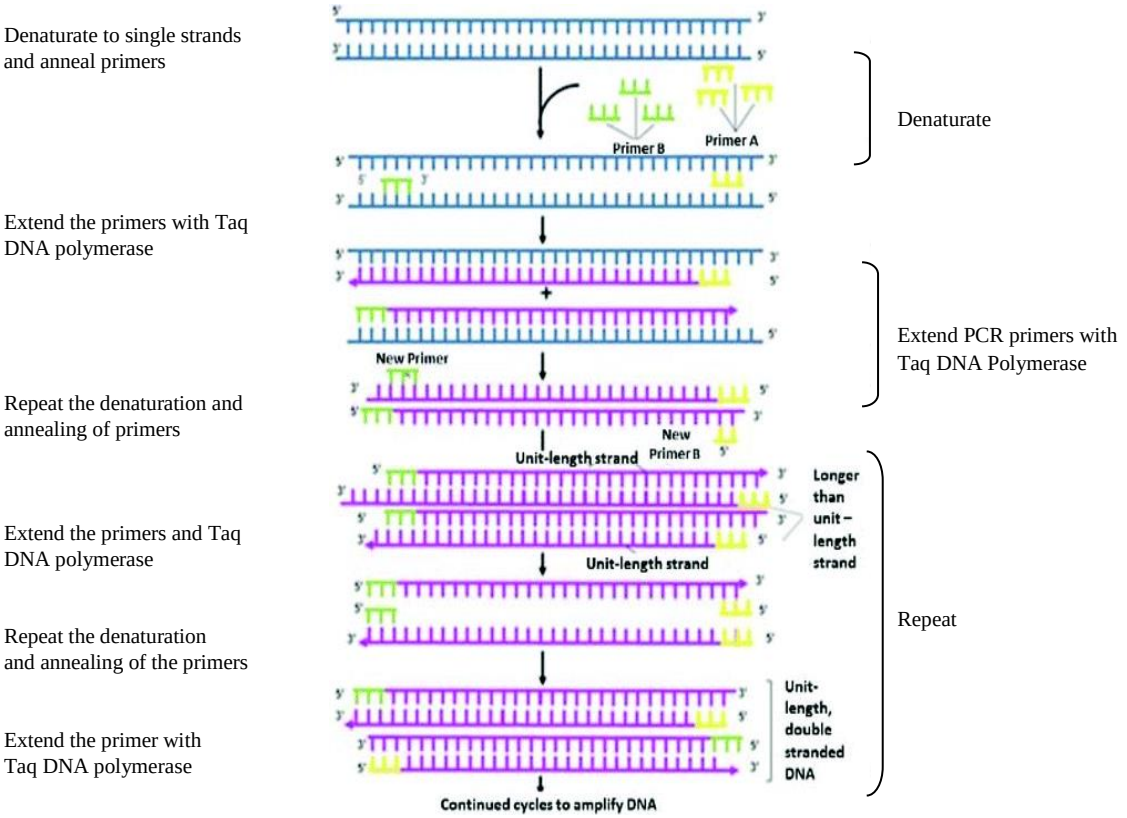


Fig. 1. Principle of PCR amplification (Rajapaksha et al., 2019) [6]

isolated from thermophilie eubacterium *Thermus aquaticus* [12]. Lately it is supplied as a recombinant enzyme from *E. coli.* The enzyme is free on nonspecific endo-or exo -nuclease. Also it consists of a single polypeptide chain with a MW of approximately 95 kDa. It is a 5' →'3' DNA polymerase which loss 3'→5' activity. The enzyme is stable during prolonged, repetitive high temperature incubations.

### *3.4 The concentration of $MgCl_2$*

Free $Mg^{2+}$ concentration be contingent on the concentration levels of the compounds which bind with the ion, including free pyrophosphate, dNTP, and EDTA. All forms of $Mg^{2+}$ are soluble complexes with the dNTPs and provide the real substrate of the polymerase recognize. Excess $MgCl_2$ may enhance nonspecific primer binding and increase the non-specific background of the reaction. $Mg^{2+}$ should be empirically optimized for each new application [4].

### *3.5 Deoxynuceoside triphosphate concentration (dNTP)*

Deoxynucleoside triphosphate (dNTP) is the substrate for DNA polymerizing enzymes known to be limited in their concentration level in cells because the enzyme specified to synthesize deoxynucleotides from ribonucleotides, ribonucleotide reductase (RNR), is synthesized and enzymatically activated as cells enter the S phase [13]. The most commonly concentration used for dNTP in PCR reaction is 200 µM for each dNTP. It is important to note that when you increase the dNTP concentration that the $MgCl_2$ concentration should also be increase.

## 4. Factors affect the success and PCR limitation

The whole chemical and physical factors can be modified for a potential increase in yield, sensitivity or specificity although all these factors are not independent. Because PCR is so sensitive, any form of sample contamination by any DNA trace amount can serve as templates resulting in false positive [14,

15]. Some prior sequence data is needed to design PCR primers. Therefore, PCR mainly used for identification the occurrence or absence of a known pathogen or gene. Moreover, in a rare case, incorrect nucleotides can be incorporated into the PCR sequence by the DNA polymerase [15]. Another problem that can affect the application successful is that the specificity of PCR may be changed by nonspecific binding of the primers that are similar but not completely identical to target DNA [15]. Products from previous reactions, even in aerosol, cross contamination, pipettes, bench surfaces, could all act as sources of contamination.

## 5. DNA isolation from microorganisms (bacteria, fungi, biofilms)

A variety of DNA extraction procedures are available and could be attributed to the heterogeneity of the sample composition [16]. Sample from environmental sources differ in terms of chemical composition and may contain substances that could be co-extracted with the DNA that may interfere with the subsequent PCR analysis. It may have critical implications for the subsequent analysis and validity of the deductions, especially when the analysis involves PCR based DNA profiling [16]. To avoid introduction of false negatives or reduced PCR amplifications it is imperative that high-quality with DNA, free from any polymerase inhibitors, is extracted. On other hand it is easier to extract DNA from pure cultures and it is easier from young cultures than from older ones. Cell thickness, secretion of secondary metabolites such as certain polysaccharides could interfere with the extraction procedure [17]. The existing techniques to extract DNA from bacteria and viruses showed limited release of DNA when applied to fungi [18]. This is due to the structure of the cell wall, which characterized as rigid, laminated, and complex. This structure makes the cell wall of fungi resistant to lysis by the common methods employed for other microorganisms [19]. For DNA extraction, the microorganisms are lysed either by chemical, enzymatic, physical means or a combination thereof. DNA may be purified by phenol/chloroform extraction followed by alcohol/salt precipitation. The cationic detergent, hexadecyltrimethyl ammonium bromide (CTAB) is commonly of DNA from plants [20, 21] and microorganisms [22] that produce large quantities of polysaccharides. To eliminate the remaining polysaccharides during DNA extraction, the precipitation of DNA is modified and performed by Aboul-Maaty and Oraby [23] via increasing the concentration of NaCL and $CH_3COOK$.

## 6. Detection of mycotoxins producing fungi by PCR

About quarter of the annually production of foods is contaminated with fungi, which have the ability to produce toxins. Till now, more than 300 different types of mycotoxins are identified, however about 20 mycotoxins produced by different species are relevant to human and animal health. For example, the estimated loss of wheat yields due to the presence of pathogens ranged between 12 and 20% around the world [24]. The influences of mycotoxins on human and animal health range from immunosuppressive, teratogenic, tremorgenic, nephrotoxic, hepatotoxic to carcinogenic effects due to their molecular structure. Some mycotoxicoses are documented by epidemiological studies [25-27]. It is important to use appropriate methods to control the mycological infection of food and feed commodities to reduce the risk of mycotoxigenic fungi to human and animals. Traditional methods for the mycotoxigenic fungi detection are considered culture methods, which require well trained fungal taxonomist as well as their time-consuming. Selective media can be simplified use for taxonomic classification, but the time required is long [28]. Therefore, uses of the PCR-based technologies can reduce the detection time to several hours instead of several days. The diagnostic of PCR protocol to detect the mycotoxigenic fungi is an indirect method. Positive PCR can, along these lines is taken as a sign for the sample possibly contains mycotoxins. Moreover, the PCR approach, nonetheless, has a detriment due to the measurement of the fungi biomass is difficult and albeit certain PCR-based evaluation strategies were described earlier and they are excessively relentless for routine investigation [29]. The accessibility of one of kind target successions, which are particular for the mycotoxin-producing fungi, is essential for the improvement of asymptomatic PCR. These objective sequences may not be available in strains of the same species which cannot produce mycotoxins. The gene that code for the enzyme of the mycotoxin biosynthetic pathway is the partners for mycotoxigenic fungi (mycotoxin biosynthetic gene). Up till now, only some of

mycotoxin biosynthetic pathways genes were identified. At the genetic level, the best biosynthetic pathways are those from the trichothecens [30], the aflatoxins [31], sterigmatocystin [32], patulin [33]. Different PCR-based methods were created to distinguish and measure the mycotoxigenic fungi which were distinguished based on the specific target DNA from mycotoxigenic genes, the ribosomal DNA or remarkable DNA band from random amplified polymorphic DNA (RAPD) examination.

## 7. Biosynthetic Genes of Mycotoxins

### *7.1 Aflatoxinogenic fungi*

Aflatoxins are secondary metabolites for different species of the genus *Aspergillus* such as *A. flavus*, *A. parasiticus* and *A. nomius* [34, 35]. Aflatoxins can be found in various food commodities including corn, rice, peanuts, cottonseed oil, Brazil nuts, copra (dried coconut), pistachios, figs, and spices, grown in tropical and subtropical areas of the world [36 -39]. *A. flavus* is profoundly related systematically to *A. oryzae* just like *A. parasiticus* to *A. sojae* and they share 90% nucleotide sequence homology [40].

melting temperature, and 5′3′ overlap. A G/C substance of 40-60% and a length of 23-28 nucleotides were proposed as general rules for specific annealing. The advancement of a multiplex reaction is the primer location is considered to be another critical viewpoint. They must be picked in a manner that the subsequent PCR products are effectively isolated by agarose gel electrophoresis. These primer binding sites for the discovery of aflatoxigenic fungi with the PCR system is illustrated in Fig. 2 [41]. It is clear now that the presence of introns in two of the amplicons did not impact the results. It was also demonstrated that the intron position and the length were extremely preserved in the aflatoxin biosynthetic genes. Fig. 3 showed the results of the run of the multiplex PCR investigations with aflatoxigenic fungi DNA [42]. Aflatoxin-producing strains showed the same triplet pattern after electrophoretic separation in the PCR products. The aflatoxigenic *A. flavus* strains were identical to that of the *A. parasiticus* strains and demonstrating the homology of the aflatoxin biosynthetic genes in both species [43]. The sequence of a portion of the genes is now known, distributed, and clarified [44].

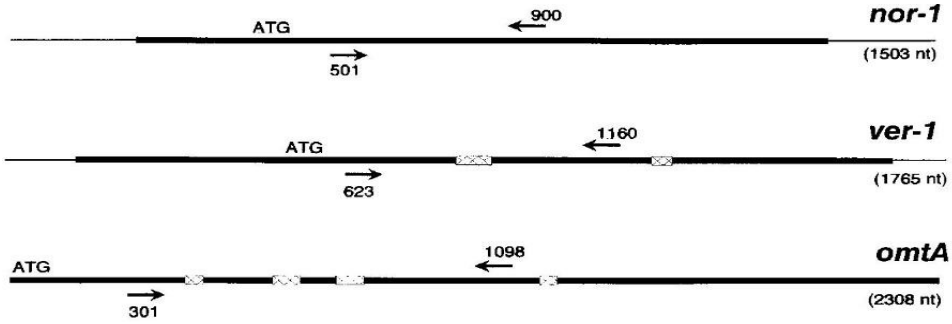


**Fig. 2. The primer binding sites of the three aflatoxin biosynthetic gene-specific primer pairs used for multiplex PCR**. Thick lines represent the aflatoxin biosynthetic gene coding sequences, cross-hatched boxes indicate intron sequences (Geisen, 1998) [41].

A technique particular to the aflatoxin-producing fungi should be fit for distinguishing between these firmly related species, aflatoxin-producing and non-producing strains. Because of the multifaceted nature of a PCR system, several requirements should be met. In addition, the reaction kinetics of the distinctive primer sets should ensure similar amounts of PCR products that delivered during a reaction. The reaction kinetics are emphatically reliant on the primer design: G/C content, secondary structures,

The aflatoxin genes located in the 54$^{th}$ cluster and are organized in 8 chromosomes which belong to aflatoxins production [45]. The aflatoxin gene cluster activation is mainly regulated by *aflR* and *aflS* [46, 47]. Curiously, the genes are sorted out in a manner that the gene encoding the principal chemical in the pathway is situated toward one end of the cluster and alternate genes follow in the same request as the enzymatic responses in the biosynthetic pathway (Fig. 4) [48]. In the multiplex PCR, the entire aflatoxin biosynthetic gene cluster can be secured and

incomplete cancellations of the cluster were identified [42].

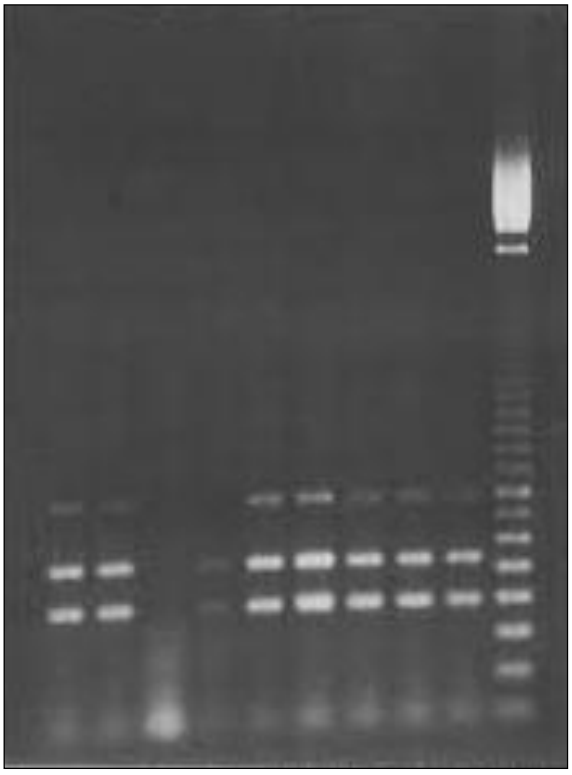


**Fig. 3. Agarose gel of the multiplex PCR products of different *A. flavus, A. parasiticus and A. versicolor* strains**. Chromosomal DNA of these strains was isolated and subjected to PCR analysis using the aflatoxin biosynthetic gene specific primer. Lane 1, *A. versicolor* BFE294; lanes 2 to 4, *A. flavus* BFE301, BFE310, BFE311; lanes 5 to 7, *A.flavus* BFE84, BFE292, BFE302; lanes 8 to 9, *A. parasiticus* BFE291, BFE293; lane 10, 100 bp ladder (Pharmacia, Uppsala) (Geisen, 1996) [42].

Traditional morphological strategies for the detection of aflatoxigenic fungi can't recognize the strains that have or not have the ability to produce aflatoxin. On the other hand, the PCR methodology can recognize both genetic modifications if the non-producing phenotype is due to the cancellation of the biosynthetic gene cluster or a section thereof or nucleotide changes at the primer binding sites. Moreover, several genes can be recognized in one reaction at once. Several primer sets were added to the reaction mixture and the reaction was done at an optimized temperature. Most of the amplicons get to be noticeable after electrophoresis of the products. Multiplex PCR with up to 18 primers has been depicted [49].

*7.2 Ochratoxegenic fungi*

Ochratoxin A (OTA) is a secondary metabolite created by *Aspergillus* and *Penecillium* fungi contaminated a wide variety of foodstuffs such as oats, beans coffee, instant coffee, beer, wins, cocoa, dried fruits, grapes as well as different tissues of animal origin [50, 51]. OTA have variety of harmful impacts, including nephrotoxic-having been connected to the Balkan Endemic Nephropathy, immunotoxic, cancer-causing, teratogenic incidences [52, 53]. OTA is a complex molecule joining the amino acid and the polyketide structure basically of the mycotoxin citrinin. Obviously, OTA is unique concerning citrinin. Specific types of fungi may deliver a few mycotoxins, but not all fungi produce mycotoxins. The most important OTA producers were surveyed by *A. ochraceus, A. niger* and *A. carbonarius* in coffee. Recent advances in molecular biology and fungal metabolite investigation brought about the depiction of some critical new OTA-creating species by European scientists (Frisvad et al., 2004) [54]. *A. westerdijkiae*, which nearly looks like *A. ochraceus*, is currently perceived as the primary OTA producer in coffee. The traditional method for identification of ochratoxigenic organisms from food tests are time-consuming and require high information of fungal taxonomy.

Indeed, even with taxonomic expertise, identification is commonly difficult in some genera of fungi that contain a substantial number of related species. The use of molecular biology techniques can conquer these issues. PCR system has permitted quick discovery of ochratoxigenic species without the requirement for isolating pure culture. In this concern the *A. westerdijkiae* and *A. ochraceus* can create OTA in culture medium. *A. westerdijkiae* and *A. ochraceus* are fundamentally the same as, and several isolates previously recognized as *A. ochraceus* are currently identified as *A. westerdijkiae*, including the original OTA-producing strain (NRRL 3174). Several Brazilian strains of both species indicated nucleotide varieties that recognize *A. westerdijkiae* and *A. ochraceus* [55]. In ITS1, all sequences of *A. westerdijkiae* varied from the *A. ochraceus* sequences

by processing a C instead of a T at positions 76 and 80. Likewise, *A. ochraceus* has an erasure of a T at position 89. In ITS2, particular nucleotides at position 494-495 (AT) characterized the strains of *A. westerdijkiae*, compared with a TC at this position in *A. ochraceus*. Besides, a T at position 487 is disappeared only in *A. ochraceus* strains [50]. Different enzymes can be relied upon to catalyze key reactions in the formation of OTA. A polyketide synthase is estimated to be essential in OTA biosynthesis because the isocoumarin group of OTA is a pentaketide likely to be formed from a polyketide synthesis pathway [56]. Two sequences no preserved in the acyltransferase domain of a polyketide synthase gene, intended Ac12RL3, was utilized as an objective sequence to distinguish *A. carbonarius* by PCR [57]. The primer pair Ac12RL_OTAF/given its structure. Ac12RL-OTAR created a 141-bp PCR primer in all *A. carbonarius* isolates tested, while no different species gave a positive result with this PCR set. This primer pair was effectively utilized to specifically measure *A. carbonarius* in grape tests [58]. In the recent 5 years, several molecular methods for the distinguishing proof and quick detection of ochratoxigenic species without the requirement for disengaging isolating pure culture have been distributed. These methods include traditional PCR, real time PCR, RT real-time PCR, and microarray innovation [59]. As of recently, they have been utilized as a part of research labs to recognize putative mycotoxin-producing fungi in culture or even in food tests to get data on the epidemiology and ecology of ochratoxigenic species or to obtain fundamental data on gene expression. The utilization of molecular examines in routine analyses in the food and feed industries remains a challenge. Specificity, sensitivity and simplicity of investigation are all zones that must be enhanced before these molecular examines become helpful for practical applications. Besides, OTA biosynthesis is ineffectively comprehended concerning the combination pathways of other economically important mycotoxins. Better learning of the genes required in OTA biosynthesis is important to adequately foresee the danger of OTA production.

*7.3 Fusarium genus*

Until 10 years ago, morphological strain determination and quantification by agar-plating strategies were the main procedures to evaluate fungal diseases. These strategies were elaborate and the obtained results may not generally reflect the natural biological situation. Toward the end of the 1990s, various groups everywhere throughout the world began with the molecular characterization of the genus *Fusarium* and defined several in the genome of most conspicuous *Fusarium* species as reasonable for the discrimination of isolates. Considering these characteristic sequences initially connected for taxonomic studies, quantitative PCR measures were improved from the turn of the millennium up to this point. PCR tests for certain species were also improved.

*Fusarium* genus is mainly classified into 4 species: *Fusarium graminearum* species (FGS), the *Fusarium fujikuroi* species (FFS), the *Fusarium oxysporum* species (FOS), and the *Fusarium solani* species (FSS). FGS, *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. avenaceum*, *F. acuminatum*, *F. tricinctum*, *F. sporotrichioides*, and *F. poae*are known to be associated with common cereal diseases [24, 60, 61]. The genus *Fusarium* includes several mycotoxigenic species of economic as well as human and health significance [62]. Some of these strains have been identified as etiological agents in various human infectious diseases, particularly in immune incompetent individuals [63].

*7.3.1 Mycotoxigenic Fusarium Species*

Mycotoxins produced by various plant pathogenic *Fusarium* species include fumonisins, fusaproliferin, fusaric acid, gibberelic acid, DON, and zearalenone [64 -66] all of which were found to contaminate animal and human feed and foods. Rheeder et al. (2002) [64] reported eight *Fusarium* species in the *Liseola* section capable of producing fumonisins. Members of the *Fusarium* genus have been reported to infect a range of crop plants including barley, maize and wheat [67]. *F. verticilliodes*, *F. proliferatum* (Section *Liseola*) and *F. nygami* (Section *Dlamini*) are the most proliferative producers of FB1 [68], though *F. subglutinans*, *F. anthophilum, F. globsum* (Section *Liseola*), *F. napiform* (Section *Dlamini*), *F. oxysporum* var. *redolens* (section *Elegans*) and *F. poliphialidicum* (Section *Anthrosporiella*) produce FB1 in smaller amounts [64]. *F. oxysporum* [62] *F. graminearum*, *F. sporotrichioides*, *F. poae* and *F.*

*culmorum*, are reported as producers of trichotecenes [65]. *F. equisiti* produces Zearalenone (ZEN), fusarochromanones, [69] beauvericin and trichothecenes such as T-2 toxin, nivalenol [62].

The fumonisin biosynthetic pathway in *Fusarium* species starts with the development of a direct

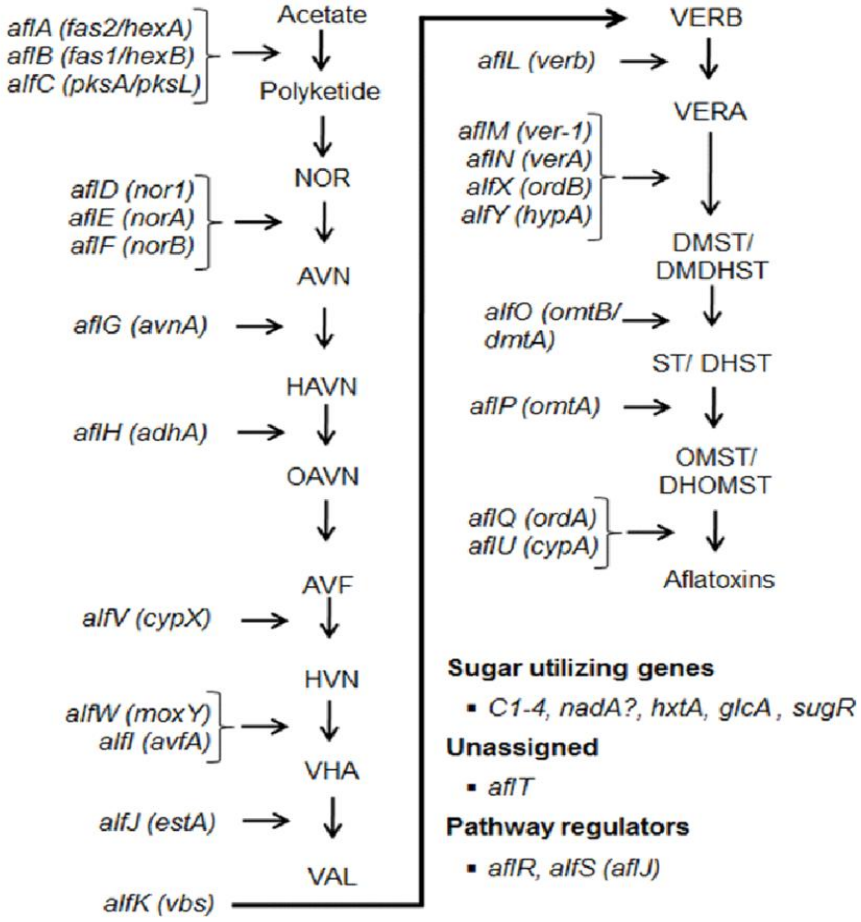


**Fig. 4. Aflatoxin biosynthetic pathway** (Dodia et al., 2014) [48]

NOR: Norsolorinic acid; AVN: averantin; HAVN: 5'-hydroxyaverantin; OAVN: oxoaverantin; AVF: Averufin; HVN: hydroxyl versicolorone; VHA: versiconalhemiacetal acetate; VAL: versiconal; VERB: versicolorin B; VERA: versicolorinA; DMST: demethylsterigmatocystin; DMDHST: demethyldihydrosterigmatocystin; ST: sterigmatocystin; DHST: dihydrosterigmatocystin; OMST: O-methylsterigmatocystin; DHOMST: dihydro-O-methylsterigmatocystin.

*7.3.2 Mycotoxegenic Fusarium (Fumonisin)*

Fumonisins, a family of food-borne mycotoxins, were first isolated in 1988 from cultures of *F. verticillioides* (Sacc.) Nirenberg then known as *F. moniliforme* [70]. The natural occurrence of fumonisins is worldwide reported [61, 71] with the highest levels of contaminated maize for human consumption occurring in Transkei, Egypt and China [36, 72].

dimethylated polyketide and buildup of the polyketide with alanine, trailed by a carbonyl decrease, oxygenations, and esterification with two propane-1,2,3-tricarboxylic acids. To date, the genes for fumonisin biosynthetic have been mapped in the F. verticillioides genome. The whole 42-kb fumonisin biosynthetic gene is truant from F. graminearum however, flanking genes guide to F. *graminearum* genomic contig 1.159. Fumonisin polyketide synthase (FUM1) was the primary fumonisin gene to be cloned and is the stay of a

group of 15 co-controlled fumonisin biosynthetic genes. Gene-disruption concentrates on have established that eleven of these genes are required for fumonisin biosynthesis. Fumonisin polyketide synthase is an iterative Type I, diminishing, polyketide synthase with seven practical domains (ketoacyl synthase, acyl transferase, acyl transorter protein, ketoacyl reductase, dehydratase, methyl transferase, and enoyl reductase). The gene clusters for the biosynthesis of fumonisin were confirmed to have 16 core genes (Fig. 5) (Proctor et al. 2013) [73]. Generally, it is thought that by quantifying genomic DNA which codes the core genes in mycotoxin biosynthesis refers to the amount of the mycotoxin in the detected samples. In recent study cases, the genes involved in mycotoxin biosynthesis and frequently used in quantifying the amount of the *Fusarium*-producing mycotoxin are *FUM6* and *FUM8* for fumonisin. In 2013, multiplex real-time PCR assay with a competitive internal amplification control to detect fumonisin-producing *Fusarium* species using the primer sets named *FUM13* is developed by Rashmi and co-workers [74]. The cluster additionally encodes an aminotransferase (FUM8), a C-3 carbonyl reductase (FUM13), and cytochromes P450 and different catalysts that catalyze oxygenations at C-5 (FUM3), C-10 (FUM2) and an undetermined site (FUM6). Four genes (FUM7, FUM10, FUM11, FUM14) are required for tricarballylic acid esterification. At the inverse end of the cluster from FUM1 are genes encoding a transporter protein (FUM19) and for two proteins (FUM17 and FUM18) with anticipated capacities in fumonisin self-protection and sphingolipid breakdown. Interruption of these genes had little or no impact on fumonisin production, showing that their capacities might be repetitive or not required for fumonisin biosynthesis.

PCR-based methods sensitive have been improved to detect the toxigenic *Fusarium* species, and especially to identify nonproducing sub-populations or nontoxigenic strains within the toxigenic species. DNA markers were used in conjunction with phylogenetic methods to distinguish between groups of toxin producers and nonproducers. González-Jaén et al. (2004) [75] developed an intergenic spacer - restriction fragment length polymorphism (IGS-AFLP) assay which could identify a polymorphism associated with toxigenic strains of *F. verticillioides* [75]. Other authors have highlighted the presence of intraspecific polymorphism for such assays. The application of AFLP and IGS/EF-1asequence variation led to the definition of two *F. verticillioides* sub-groups, based on a contrast between efficient producers of fumonisin, and nontoxigenic strains from Central and South American banana fruits [76]. The same nontoxigenic population was also exploited by Patiño et al. (2006) [77] to generate an IGS-RFLP assay diagnostic for toxigenicity [77]. The non-toxigenic isolates were crossable *in vitro* with MP-A testers (corresponding to *Gibberella moniliformis*), but showed only about 50% genetic similarity.

*7.3.3 Trichothecenes-producing Fusarium spp*

Trichothecenes are produced by *Fusarium spp.* including *F. graminearum, F. sporotrichioides, F. poae, F. culmorum* and *F. langsethiae* [65, 78, 79] and can be produced by species from other genera, for example, Myrothecium and Trichothecium [80]. Trichothecenes mycotoxins include T-2 toxin, deoxynivalenol, nivalenol, and diacetoxyscirpenol (DAS). The trichothecene are naturally occurred worldwide and has been accounted in North America, Asia, Africa, South America, and Europe in corn,

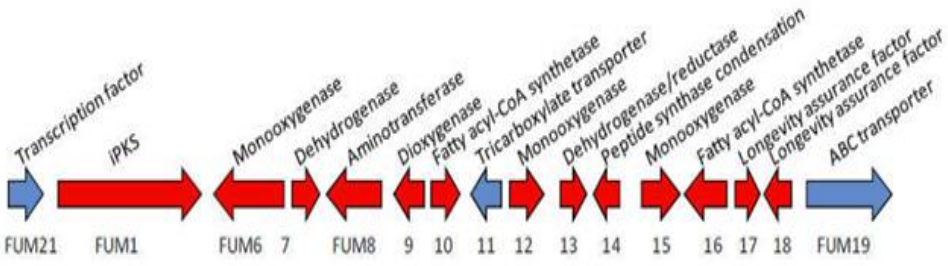


**Fig. 5.** Fumonisin gene cluster in *Fusarium moniliformis* (Proctor et al., 2013) [73].

wheat, grain, oats, rice, vegetables, and different products [81, 82]. The gene clusters for the biosynthesis of tichothecenes were confirmed to have 12 core genes. Bluhm et al. (2002) [83] focused on the gene TRI6 to deliver two PCR diagnosis for trichothecene-producing species. These methods were utilitarian only from template of *F. culmorum, F. graminearum, or F. sporotrichioides*, all of which are known to be good producers of trichothecenes [83]. One assay directed to biosynthetic genes have been designed for the same purpose was TRI5, encoding the catalyst of the isomerization and cyclization of farnyl phosphate to trichodiene [84], and this assay could identify trichothecene-producing *Fusarium spp*. both from *in vitro* and from infected grains [85, 86]. A second test abused the trichodiene synthase relative TOX5, and this was not just ready to distinguish the presence of *F. graminearum and F. culmorum* DNA in grain tests [85]. Eventually, a method has been improved based on TRI13, and used to investigate the toxigenic capability of several Iranian isolates of *F. graminearum* [87].

## 8. Conclusions

Definition of species may be difficult in the fungi due to morphological variation between species is regularly missing or difficult to perceive, as many species lack a known teleomorph. Phylogenetic investigations are along these lines especially significant to allocate disconnects to their right species. We have talked here about a variety of DNA-based tools that consider a quick and reliable finding of *Fusarium spp*. inside the Liseola and Discolor sections, and for the identification and quantification of toxin-producing isolates. PCR-based techniques are moderately direct and quick yet are not mistake free. Their precision should increase as the informative loci from more isolates of different provenance are sequenced. During the most recent 5 years, several molecular assays for the identification and quick detection of ochratoxigenic species without the requirement for isolating pure culture have been distributed. These assays include conventional PCR, real-time PCR, and microarray innovation. Now, they have been utilized as a part of research laboratories to recognize putative mycotoxin-producing fungi in culture or even in food samples to get data on the study of disease transmission and nature of ochratoxigenic species or to secure fundamental data on gene expression. The utilization of molecular methods in routine examinations in the food and food industries remains a challenge. Specificity, affectability and effortlessness of investigation are all areas that must be enhanced before these molecular assays get to be valuable for handy applications. Besides, OTA biosynthesis is poorly understood relative to the synthesis pathways of other economically important mycotoxins.

## 9. Conflicts of interest

No potential conflict of interest was reported by the authors.

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