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Review

Identification and quantification of mycotoxigenic fungi by PCR

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Abstract

This review was inspired by an apparent oversight. A report claimed that a gene probe based on a regulatory gene for aflatoxins could be used selectively for screening foodstuffs. However, *aflR* also regulates sterigmatocytin production so that many other fungi could provide a positive result. I suggest that *aflP*, or *aflQ* are more logical choices. Other aspects are reviewed including why it is valid to screen for the metabolic pathway rather than marker DNA, and emphasising that the current state of fungal taxonomy does not permit absolute confidence in delineations of taxa. Also, the gene sequences determined from very few strains may not represent the situation in nature. Common genes for a wide range of important mycotoxins (e.g. polyketide synthetase) may not be able to be used with authority, and more specific ones are desirable (e.g. isoepoxydon dehydrogenase). Metabolomics may challenge PCR analysis under certain circumstances and the most appropriate technology needs to be considered. Negative PCRs can be false. Quantifying fungi is a surprisingly inaccurate science, and also in relation to mycotoxin concentration. It is noticeable that few strains of taxa have been investigated in many cases. PCR of mycotoxigenic *Aspergillus*, *Fusarium*, and *Penicillium* in particular are reviewed in the present paper.

Keywords: Fungi; Aflatoxin; Ochratoxin A; Patulin; *Fusarium* toxins; PCR

Contents

1. Introduction

This review was inspired by an apparent oversight. A method was described using primers for the regulatory gene (*aflR*) for aflatoxins biosynthesis to detect fungi in food [1]. However, the same gene is involved in sterigmatocystin production: more on this later. Now to a review of the polymerase chain reaction (PCR) identification of mycotoxigenic fungi where some novel points are raised. These will indicate the advantages and pitfalls of the process ranging from the specificity of particular genes in taxa to what constitutes a fungus species [2] in this wide-ranging review.

Mycotoxins are compounds from fungi that contaminate foodstuff and have detrimental effects on humans and animals [10]. They are referred to as secondary metabolites which are

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low molecular weight compounds that do not contribute to the bioenergetics of the producing fungi: The metabolites require energy to be produced. Production is restricted to certain taxa or strains within taxa. They often exert their effects external to the organism, but not exclusively. On the other hand, primary metabolites are the well-known and almost-universal compounds involved in life such as (a) DNA, (b) proteins, and (c) the small compounds involved in glycolysis, and the hexose monophosphate shunt mechanisms.

The use of PCR to identify mycotoxin fungi is attracting considerable attention [3,4]. These methods are based on genes separate from mycotoxin biosynthesis. However, there are only a few mycotoxins about which the biochemistry has been determined sufficiently to enable the development of gene probes of the pathway [5]. It is worth mentioning that many of the methods are intended ultimately to be used on ''virgin'' samples, i.e. those which otherwise have not been examined [6]. The concept ''Fungi'' [7] is not as straightforward as it might first appear.

''Fungi'' can be used to refer to the monophyletic ''true fungi'' in contrast to ''fungi'' which are organisms traditionally studied by mycologists. This is perhaps not the place to enter into the details, except to state that mycotoxigenic fungi are true fungi. Fungi form a separate kingdom and it has been estimated that there are 1.5 million species although ''only'' 70,000 species have been named [8]. So any implication that a particular PCR method has screened representative samples of fungi is immediately open to scepticism. The question is raised as to which species are actually being analysed in environmental samples by PCR if so few are known. Inherent difficulties with fungal taxonomy must be considered [2,9]. However, it is known is that some fungi produce toxic compounds, which are often associated with food and feed and are referred to collectively as mycotoxins [10].

The estimated cost of control of mycotoxins in the United States of America alone is $ 1.4 (US) billion. Such fungi are also gaining credibility as sources of health problems in houses. Furthermore, in the current state of world concerns with bio safety and bioterrorism, the use of mycotoxins as weapons cannot be ignored possibly in relation to food and/or water supplies. Indeed, the unambiguous identification of mycotoxigenic fungi remains the most critical area of mycological taxonomic research because of the importance of mycotoxins and the somewhat confused state of the systematics. Despite this, a chapter was not devoted to fungal classification in the recent, otherwise comprehensive, Council for Agricultural Science and Technology (CAST) report on mycotoxins [10].

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 [11]. This assumes there is a close link between fungus and toxin. However, conventional methods for isolating and identifying fungi are time consuming and require admirably dedicated taxonomists [9]. The classification of fungi is an immature science from lack of attention compared to other groups of organisms [2,12]. However, fungi are unique as indicated by the placement of them in a separate kingdom as mentioned previously. For a practical identification of use to the food industry, and to avoid misidentification, it may be more useful to think of a collection of isolates grouped as a single taxon which produces a particular mycotoxin [9,13]. This would support the view that a species name could be used to predict features of the species.

Mycotoxins can be neurotoxic, nephrotoxic, hepatotoxic, immunosuppresive, teratogenic, and/or carcinogenic. A demand exists for rapid and reliable techniques to detect mycotoxins and mycotoxin-producers within foodstuffs, homes, etc. It is worth emphasising that PCR methods do not detect mycotoxins. The producing fungi are microscopic filamentous fungi observed commonly on substrates as ''mould'' for example. Important genera are *Penicillium*, *Aspergillus* and *Fusarium*. *Claviceps* spp. with their ergot alkaloids are often considered separately as plant pathogens. However, the involvement in human disease of ergots in the middle ages is fascinating not least because it indicates the importance of mycotoxins in general.

Mycotoxins have been responsible directly for deaths in animals including humans. Most countries have statutory limits for these compounds in some foods [10]. It is now worth indicating the information that PCR and alternatives will provide to assist users in deciding what is required for their own particular work (Table 1).

Conventional plating out methods will indicate specific viable fungi from a sample. Analysis of the pure culture (e.g. by chromatography) will inform if it can produce particular mycotoxins. PCR analysis will detect relevant genes in a sample (and it is possible to determine whether, or not, such genes are expressed). Chromatography, for example, will determine if a sample actually contains particular mycotoxins and by implication that a producing fungus was present at some stage. This is the field of metabolomics. The present author has

Table 1
Some merits and demerits of various identification methods particularly as they relate to mycotoxigenic fungi

Identification method	Merit	Demerit
Traditional colony isolation	Viability, relates to taxonomy	Unrepresentative, slow
Chromatographic analyses of cultures	Mycotoxin detected	Do not know if mycotoxin is simply below detection limits if not detected, slow
PCR of non-mycotoxin genes	Identity of taxa	Unclear how relates to mycotoxin production
PCR of mycotoxin genes	Indicates potential for toxin production	Unclear how relates to taxonomy
Culture independent PCR	Detects some unculturable fungi, rapid	Ditto
Metabolomic analysis of environmental samples	Historical identification possible, determine if complete mycotoxin pathway is expressed, rapid	Possibly less sensitive than PCR

referred it as ''secondary metabolomics'' as secondary metabolites are being considered [47]. The levels of detection for mycotoxins are extremely sensitive and now concentrations of as low as 10^{-15} (10^{-6} is the mg kg^{-1} level) can be conceived of with NMR and mass spectroscopy. Furthermore, it is possible to determine if all the genes of a pathway can be expressed by incubating a sample and assessing whether concentrations of the mycotoxin increase. It is certainly worth mentioning that taking representative samples [10] from the environment is a science in its own right that has been studied particularly for mycotoxin analysis but applies equally to all the above areas and is often overlooked. Finally, does high resolution DNA melting have a role in systematics [2], or has this been overlooked in the hegemony of PCR? To ensure that future mistakes are minimised there follows a review of the role of PCR in detecting mycotoxigenic fungi and some apparent misconceptions are addressed.

2. PCR

This is an elegant, and well-known, technique that has brought previously inconceivable areas of research into reach. There is a large literature on the subject. However, the basic process involves denaturing (separating the individual strands) DNA by heat applied for specific lengths of time. A small segment of DNA which can be referred to as a probe, is targeted to anneal with the piece of DNA of interest (the target). This is extended to equal the number of kb of the product to be amplified and to yield doubled DNA. The process is cycled approximately 40 times to give the desired quantity of DNA product. The use of the thermophilic polymerases involved in the reactions is of course revolutionary. The DNAs are separated on gels often of agar and the negatively charged DNA is separated through the gel on the basis of size. Above certain sizes the DNA does not separate and so techniques such as pulse field electrophoresis are required. However, this does not need to concern us for the present topic which is large in scope in any case. The technique has had great utility in fungal phylogenetics where determining the DNA structure holds the promise of attaining ''true'' evolutionary relationships. However, the DNA sequences do not mean a great deal in isolation and need to be linked to some other relevant attribute(s) [12]. The status of a catholicon has been achieved by PCR, and there are few proposals for funding that would be brave enough not to include a reference to the reaction. It is a breakthrough that small pieces of DNA can be amplified and detected routinely.

However, it has pushed some appropriate technology to the background. There has been a renaissance in the field of metabolites (via metabolomics) which could conceivably compete (see [14]). An embryonic metabolomic approach is provided in [15,16] where more fungal secondary metabolites were detected from poor than better quality food. Each metabolite is capable of being produced by a limited number of fungi, and so fungal identifications of a limited sort can be made. Of course, more sophisticated analytical methods [17] would greatly assist this type of work; routine methods are also available for individual mycotoxins [10]. What do PCR methods offer?

It is common to analyse pure cultures of fungi by PCR. This is often undertaken for taxonomic, or more specifically phylogenic, purposes [2]. However, in a review such as this it is sometimes necessary to emphasise the potential pitfalls: two bands of equal size on a gel may not represent the same DNA fragment and as the relationship between two analysed fungi increases the chances of this occurring increases; one of the biggest disadvantages with PCR is the risk of (invisible) contamination of the reaction with alien DNA [18,19]. Very small amounts of contamination will be amplified if the correct sequence is present. Conversely, ''false'' negative reactions have to be carefully assessed and control PCRs need to be mandatory [20]. It is possible to (re) amplify an apparently negative sample and obtain a positive result [6]. Whether this relates to contaminants, freeze/thaw cycles and/or detection limits needs to be determined. Also, the gene may be present but is it expressed? Apparently single bands on gels may not be: issues of inhibition and nucleic acid damage need to be carefully considered [21]. Finally, does the in vitro work ''translate'' to real-life situations?

As indicated previously, a logical step is to apply the methodology to environmental samples [6]. This has been referred to culture independent PCR [54] and has been recognised for a number of years previously for fungi (see [21]). Questions of the minimum quantity of propagules that can be detected must be considered (not just conidia) as well as issues of gene copy number in the cells (e.g. some genes may exist as multiple copies in individual cells). As discussed, the number of detected cells is lower than the genome equivalents, and false negative reactions need to be considered [20]. In addition, inhibition may be problematic, as in pure culture [21]. However, this technology gives at least an approximation to the true situation. For mycotoxigenic fungi there are two approaches: (a) the analysis of genes not involved with mycotoxin production and (b) the converse.

3. Primers for general sequences

Papers cited in [5] appear to assume that non-mycotoxin biosynthetic pathways will indicate adequately mycotoxin production potential. However, inadequate taxonomy and identifications [9] imply there may not be a direct relationship. A great reliance is made on current taxonomies of the target fungi in the cases where general genes are employed. For example, the polygalacturonase gene is claimed to identify *Penicillium expansum* [3]. This fungus is a member of the terverticillate penicillia which is one of the most variable groups of fungi, and many more representative strains and taxa need to be examined before it can be reliably employed. A partial β-tubulin sequence was used to separate many fungi within this group, although the two ochratoxin A (OTA) ''species'', *P. verrucosum* and *P. nordicum*, were virtually identical [22]. So a probe based on β-tubulin could not separate these different species. Peterson [23] considered the whole group to be monophyletic. However, the work of Karolewiez and Geisen indicate that the two species were quite different [24]. This indicates how important it is to use multi-phasic

approaches. A ''good tool'' is described for separating *A. niger* and other aspergilli based on PCR of the ITS sequence [50]. It is described as an early detection tool for OTA-producing *Aspergillus* species. However, effectiveness is dependant again on an association with species and OTA production, which is seldom exact and where primers for the metabolic pathway would be more useful. This was extended to include *A. carbonarius* and *A. ochraceus* in Patiño et al. [51]. It is noted that some strains produced detectable OTA but were negative for the PCR products. The authors provided information on the detection limits of the ITS amplification product which was also interesting.

The most extensive single general sequence work is [4], where a generic assay for target species was developed. The tests varied in specificity from species or subspecies to closely related species groups. All the issues concerning the number of genes in fungal structures and how this relates to quantity apply. As does using a gene not involved in mycotoxin production unlike the situations cited below. Of course, the assays were developed on conidial suspensions and how that relates to hyphae and other spores is not recorded but could be significant nevertheless: Spores of all kinds can be uni- or multinucleate. The nuclei of the latter may be derived from a single parent nucleus or from several nuclei and so may be homokaryotic or heterokaryotic (see also [24]). Therefore PCR analysis of these spores will overestimate the number of fungi (depending upon how fungal quantity is defined which is, in itself, not straightforward). Obviously, environmental samples may contain all structures. The conidial suspensions tested were ''only'' 95% pure, and one wonders how many genes are equal to 5% hyphae. Some ''conidial'' suspensions were combinations of ascospores and conidia (in addition to hyphae). Further, species assignments were sometimes made from sequences published in Gene Bank which may be problematic although such statements cannot be made from superficial studies (see [25] and correspondence in the same volume of the journal). This of course puts an extremely high premium on existing taxonomies and identifications. Finally, non-orthologous genes could affect results. This means that more than one gene in a strain could code for enzymes that perform the same function (e.g. in a metabolic pathway) and so would overestimate the number of fungi present in the sample.

Other issues of using the procedures as an estimation of mycotoxin levels is severely problematic as (a) the toxins could be present without the fungi and (b) quantification is not ''one gene one conidium''. It would be optimal to relate a PCR method with quantification of the mycotoxin of interest (see [26] for in vitro comparisons), or a more quantitative non-mycotoxin technique such as ergosterol [9]. Even here the correlation was not direct at all stages as is discussed.

Some observations are made in [4] concerning the relative merits of an identification of ''AspPen'' compared with their methods. ''AspPen'' was the term used to describe a general identification of a fungus that belongs to the aspergilli and/or penicillia. One could argue that an identification based on a sequence which is unrelated directly to mycotoxin genes could be equally misleading (see later). Also, it would be entirely possible to simply analyse the sample of interest for offending mycotoxins by chromatography, spectroscopy and/or NMR, with or without an incubation period, where quantification can be achieved using well documented protocols. Similar arguments can be made for [3] for *P. expansum* where the polygalacturonase gene is employed. Two further specific examples are now provided.

Problems from using RAPDs are demonstrated in the case of toxigenic *Fusarium* spp. where ''species specific'' primers were not tested against closely related species [5]. This is a general problem and needs to be addressed with any PCR work and could be controlled at a prepublication stage. An extensive study by Jurado et al. [27] claims to distinguish mycotoxin producing species one from the other. However, no attempt was made to relate the results to actual production of the mycotoxins as part of the experimental procedures. Better was the correlation observed for RAPD patterns and secondary metabolite formation patterns in *P. roqueforti* [28]. β-Tubulin sequences were used to separate *P. expansum* from *P. solitum* in Sholberg et al. [48]. However, the two are quite different from morphology and of course *P. expansum* is associated with patulin production and *P. solitum* is not: it would be interesting to test *P. solitum* for the isoepoxydon dehydrogenase (*idh*) gene as discussed later. The four subclusters indicated for *P. expansum* merit further investigation. Incidentally, PCR methodology is available for *Claviceps* [29] although they tend to be treated historically from a plant pathogenic, rather than a mycotoxin perspective.

What is the situation regarding the genes controlling the biosynthetic pathways involved in the production of mycotoxins?

4. Primers for mycotoxin pathway sequences

In these cases, the metabolic pathway is being assessed of direct relevance to mycotoxin potential. There is no reliance on taxonomy whatsoever if the gene is specific for the chosen mycotoxin (see later). A chance contamination may also represent a problem but this would only be intermittent, and an overall assessment could still be made. Of course the fact that PCR analysis can be coupled to mycotoxin production is a large advantage [30]. (Indeed, the benefits of reverse transcriptase PCR are somewhat negated by the possibility of undertaking mycotoxin analysis per se.) This technology will be useful for determining associations between detection of a gene at critical control points in food production (e.g. apple orchards) and quantification of the mycotoxin in the final product (e.g. apple juice) (see [6]). It has implications for a functional taxonomy of the species involved, and will distinguish between genetically different strains of the same species (cryptic species). So which specific probes are available?

4.1. Aflatoxins

The biosynthetic pathways for the aflatoxins have been determined [31]; they are typical polyketide compounds. Many potential probe sites are available. However, which reaction

steps are unique to the aflatoxins, and would not include sterigmatocystin, another potent mycotoxin? The ''dozens'' of fungi which are known to produce the precursors within the aflatoxin biosynthetic pathway are provided in [32]. The shared metabolic pathways for aflatoxins and sterigmatocystin is what was not realised by [1], and apparently in [33,34]. In [1], only the single *aflR* gene was used which was not tested with recognised sterigmatocystin producers per se. Interestingly, *A. sojae* contains the gene although it is defective [31]. Chang et al. [35] describe a system using the *aflR* regulatory gene which can distinguish aflatoxigenic fungi from other fungi found commonly on grains. However, I would argue that the system could create false positives from sterigmatocystin fungi on the same substrate. Although an intriguing result was presented almost as an aside that ''F1 and R1'' primers did not generate PCR products in *A. nidulans*, *A. versicolor*, *Chaetomium thielaviodeum* single strains, and ''some'' *A. oryzae*. It concluded that if counterparts of *aflR* are present they exhibit low similarity to the sequence for *aflR*. Further, Yu et al. [36] stated that the *AflR* homologs in *A. flavus* and *A. parasiticus* are almost identical, whereas the *A. nidulans* one is 31% similar. However, the zinc binuclear region is the most highly conserved having 71% identity amongst the aspergilli. So one of my conclusions is simply that any probe for aflatoxin species in food should be tested carefully with numerous exclusively sterigmatocystin fungi. The current author has tested a much wider range of taxa for false positives for the *idh* gene (see later). Of course *idh* is not part of the aflatoxin pathway and would not be relevant to the situation under discussion above.

Furthermore, an enrichment procedure could have been employed to analyse for the aflatoxins themselves hence removing any doubt as to whether the genes were expressed. Enrichment is also suggested in [1]. However, clearly *aflP* and probably better *aflQ*, are not relevant to sterigmatocystin biosynthesis [31] and would be a valid choice for specific aflatoxin primers for food, etc. If the gene sequences for the specific conversions to individual aflatoxins were available they too would be of great utility in this respect. There is more than one aflatoxin and there is not as yet a set of primers for one specific compound, the most obvious choice being aflatoxin B1—the most toxic (see Table 2). Finally, Manonmani et al. [1] also claims that *A. flavus* produces the aflatoxin Gs which conventionally is not considered to be the case.

It is possible that genes from a morpho-species (a species defined by morphology) would have specific sequences but this cannot be assumed. In this respect it is informative to consider [37], where a multiplex PCR reaction was developed for nor-1, ver-1, omt-A aflatoxin (and sterigmatocystin) genes. Triplets were indeed observed for *A. flavus*, *A. parasiticus* and the sterigmatocystin-only producing species, *A. versicolor*. Interestingly, so-called aflatoxin producing negative strains of *A. flavus* varied in the patterns, including one from which all three bands were recorded. The system could not distinguish between aflatoxigenic and ''non aflatoxigenic'' strains of *A. flavus*. Could it actually produce small concentrations of the compounds? A consideration of the terms ''not produced'' versus ''not detected'' is provided in [9]. This has obvious negative implications for genes not even involved in mycotoxin production if the ones that are cannot differentiate (see above). What is the situation with other mycotoxins?

4.2. OTA

The steps in OTA biosynthesis have not been established and proposed pathways are hypothetical. A polyketide synthetase (PKS) is probably involved. OTA is a complex molecule combining the amino acid and the polyketide structure basically of the mycotoxin citrinin. Of course OTA is quite different from citrinin. However, would an OTA gene based on the polyketide portion differentiate OTA from citrinin fungi? The answers are ''no'' in one case and ''yes'' in another [38] where two PKS-based primers are involved, although crucially no OTA-producing penicillia were tested. Furthermore, citrinin is a co-metabolite of dihydrocitrinone and sclerin in, for example, *A. carneus* and there are other metabolites similar to citrinin which may share PKS genes. All these need to be considered as sources of false positives for OTA fungi. O'Callaghan et al. [39] report the first cloning and characterisation of a gene involved in OTA biosynthesis but reaffirm that there was no information involved in the steps from acetate and malonate to the isocoumarin group. Work is continuing on the elucidation of the other genes involved in the pathway. Again, this does not help in the search for a specific gene.

Perhaps more interestingly, Karolewiez and Geisen [24] demonstrate that a particular PKS gene can be identified only in *P. nordicum* and not in *P. verrucosum* or *Aspergillus* spp. Although, whether the PKS is involved in biosynthesis can only be surmised at present. The conclusion is that OTA PKSs are potentially useful screening genes. However, are the primers specific to OTA and not other citrinin species (e.g. *P. citrinum*)?

Table 2
Comparison of the potential problems and solutions of the non-polyketide synthetase gene probes of the principal mycotoxins

Mycotoxin	Gene	Problem	Solution
Patulin	*idh*	None. Specific. Patulin is a single compound. Precursors (e.g. isopatulin) are not mycotoxins	None required
Aflatoxins	Various (e.g. *aflR*)	Various exclusively sterigmatocystin fungi detected. All four aflatoxins are major concerns	Look for genes upstream of sterigmatocystin; probes for individual aflatoxin steps?
Ochratoxin A	Various	Complex molecule. Exclusively citrinin producers detected (e.g. *P. citrinum*)	Probe for the step governing the combination of phenylalanine to the ''citrinin'' molecule
Fusarium trichothecenes	Various (e.g. *Tri5*)	Cannot detect individual deoxynivalenol or nivalenol producers (i.e. not specific)	More work required to target specific genes

It is worthwhile pointing out that *P. nordicum* and *P. verrucosum* were considered different biochemically and genetically. However, they were virtually identical in [22] by β-tubulin sequences. The capacity of fungal strains which had been genetically manipulated to be defective in production of secondary metabolites, but which could in fact still manufacture low quantities is revealing [24]. What does ''defective'' mean? Do the genes not work or are they simply impaired?

To continue, it would have been instructive to have measured OTA after 10 days in [26]: was the apparent ''correlation'' maintained with the real-time PCR? Also, more than one analysis per day would have been beneficial. It is difficult to extrapolate as to what may occur on ''real'' food. The lack of correlation with such a crucial parameter as temperature is noted. Finally, it is possible to question the need for the real-time procedure when samples can ''simply'' be analysed for OTA and at very low concentrations, with incubation if required. Furthermore, some problems exist with other mycotoxin systems.

4.3. Fusarium

The real problem with these is that there are so many trichothecenes (see Table 2). Supposedly unique bands for *F. avenaceum* were observed also from *F. tricinctum*, and primers for ''*F. moniliforme*'' were not tested unfortunately with the related complex *Gibberela fujikuroi* (they were demonstrated subsequently to cross-react). Various other problems are reported for PCR of the fusaria [5]. However, PCR to differentiate between the chemotypes of *F. graminearum* which produce deoxynivalenol (DON), and nivalenol (NIV) is suggested in [40]. The other taxa that produce these, such as *F. culmorum*, *F. cerealis*, *F. crookwellense*, or *G. zea* were not considered. Further, there are now nine species that are reported to constitute the *F. graminearum* complex [41] and that ''species'' do not conform to mycotoxin patterns [42] making the situation regarding a general probe complex indeed. Basically, the answers to these questions have yet to be found. As suggested by [40], their method may be better utilised to determine two mycotoxin chemotypes of *Fusarium* in general rather than for *F. graminearum*.

As Edwards et al. [5] stated, any assay to distinguish DON and NIV production (or potentially producing—my addition) needs to be tested against all lineages of both chemotypes. However, to state that PCR allows the detection of trichothecene producing *Fusarium* species is misleading, and further that such tests could indicate the presence or absence of all *Fusarium* ''trichothecenes'' is untrue as this can only be achieved by non-PCR methodology (often chromatography).

Although mainly dealing with non-mycotoxin pathway genes, [43] is worth a special mention as it relates these to the Tox 5 gene in particular and mycotoxin analysis per se. For example, 18% of *F. equiseti* as defined were positive for Tox 5. However, somewhat ambiguous results were obtained for actual analysis with detection sometimes being recorded. However, to state that a PCR assay has been developed for detection of trichothecenes (p282) is simply a lack of comprehension, and one wonders why this was not eliminated prior to publication.

The biosynthesis of the polyketide compounds moniliformin, zearalenone and fumonisins have not been established. Nevertheless, various PCR methods have been developed and there has been some less-than-robust testing of related species (see above). These compounds are considered briefly in [5].

4.4. PR toxin

This is perhaps not one of the major mycotoxins but illustrates some interesting points. Some potential problems mentioned with PCR of the PR toxin genes in reality represent opportunities and provide a model for other more important mycotoxins. *Penicillium* strains produced PR toxin, although a PCR fragment was not observed. However, the gene was detected by more sophisticated methods. Some species were positive for the gene which had never been reported to produce PR toxin: this indicates that the probe can provide new information to assist with a practical taxonomy such as has been suggested for penicillia in general (see below and [13]). The genes may be present but not expressed. Interestingly, it was postulated that the *ari1* gene or similar may be involved in producing other secondary metabolites given the relatively unspecific nature of secondary metabolite genes [49]. A compound which has been investigated in various guises over the decades next will be considered.

4.5. Patulin

The useful thing about patulin is that the pathway is so specific without other mycotoxins being involved (see Table 2). The compound is second only to aflatoxins in terms of the amount of data that exist because of the antibiotic and model secondary metabolite roles of the compound. It has the clear advantage as a model mycotoxin of its singularity rather than being one of many ''patulins'' as end products. Patulin is conceived of as being formed from isoepoxydon → phyllosphyllostine → isopatulin → ascladiol → patulin where the precursors are naturally similar to patulin. There are dimeric compounds which can be conceived of as being formed from two patulin molecules but (a) the formation of these structures is not well established, (b) they are not co-produced with patulin, and (c) they are not mycotoxins. In comparison, all four important aflatoxins mycotoxins have a common precursor and any potential dimeric compounds simply would not be considered within the same category. The *idh* gene catalyses the conversion of isoepoxydon to phylostine: Much of this work is based on the data from Gaucher's group [55]. Similar phenomena to PR toxin of previously unrecorded patulin producers containing the isoepoxydon dehydrogenase (*idh*) gene of the patulin biosynthetic pathway were not considered to be false claims [30,44,45]. Incidentally [5], ascribed wrongly the possibility of patulin being translocated through soil into apples to Mantle, rather than Paterson et al. [44]. Mantle provided evidence for OTA moving from soil to coffee beans as discussed in [44] and the current author made a simple

comparison for patulin and apples. Again Paterson et al.'s conclusions appear to have been misinterpreted as the *idh* primers do not simply identify *P. expansum* and *P. brevicompactum* from environmental samples as suggested in [5] but is certainly conceived of as being capable of identifying all organisms that possess the *idh* gene.

A new identification procedure of fungi by reducing all *idh* positive fungal strains to pen idh^+ [13] has been suggested, which was based on a premise in [9] for OTA. There are no similar reactions from isoepoxydon to phyllostine described leading to another end-point metabolite, although epoxydon and phyllostine have been reported in *Phyllostica* spp. [46] which are associated with infection of leaves. It would be interesting to determine if representative strains produce patulin. Of course, other important secondary metabolites could be described in the future in fungi that are produced via isoepoxydon to phyllostine but this is speculation. Paterson et al. [21,30] extended the work to a wide range of penicillia indicating that the gene is widespread. Importantly, negative *idh* strains and positive patulin detection were not observed. When strains were demonstrated to be *idh* positive but patulin negative it was concluded logically that (a) some part of the patulin metabolic pathway was not functioning although the *idh* gene was intact or (b) patulin was being produced at below the detection limits of the chromatography system. Also, the gene and production from other genera were demonstrated [21,44].

There are increasing amounts of information on the sequence of the *idh* gene [52,53]. Dombrink-Kurtzman [53] observed differences between the *idh* sequences of *P. expansum* and *P. griseofulvum* which were correlated to the quantities of patulin that each species could produce. In White et al. [52] various other genes were also cloned in the search for a molecular based detection method for *P. expansum*. They demonstrated that patulin was regulated at the transcriptional level and represented the first report of this phenomenon.

The *idh* gene method was also used by Varga et al. in *Aspergillus* where it was suggested that the occurrence of patulin in taxa was subjected to evolutionary influences without considering the possible effects of preservation (see [45]). Furthermore, Paterson [21] introduced the concept of secondary metabolites affecting the analysis by inhibiting PCR. Results from environmental samples indicated that the gene was detected in orchard soils in comparison to a non-orchard soil and HACCP procedures were proposed [6,44]. It is emphasised that the *idh* gene is not a PKS type: equivalent sites are required to be investigated for some of the other main toxins.

5. Conclusions

The promise of PCR to identify fungi has blinded some to its potential pitfalls. Contamination is a real problem for all PCR. DNA from fungi related to the target fungus may interfere with the analysis in the environment. Morphologically identical species to mycotoxigenic fungi without the ability to produce a particular mycotoxin may result in false positives if general genes are employed. In addition, genes which are shared for different mycotoxins (e.g. sterigmatocystin and aflatoxins) would tend to overestimate contamination problems. Non-viable cells or pieces of DNA may provide results. On the other hand, the benefits obtained from undertaking mycotoxin analysis per se are often not fully appreciated. Potential mycotoxin production can be detected by PCR which may permit the establishment of critical control points and is a significant advantage. The detection of unculturable fungi is a large advantage of PCR. In general, a more critical assessment of the benefits of PCR methodology would be advantageous. Finally, I have been struck by how few strains of each taxon have been studied in most of the papers. It is possible to inquire just how representative the results are of the situation in nature.

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