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Detection of gliotoxin in *Aspergillus fumigatus* using molecular techniques

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ABSTRACT

Gliotoxin is an important virulence factor in *Aspergillus fumigatus*. The biosynthesis of this mycotoxin is regulated and expressed by the presence of *gliP* and *gliZ* genes. This study aimed to identify *Aspergillus fumigatus* isolates in tandem with gliotoxin genes using conventional polymerase chain reaction (PCR). To achieve this, DNA was isolated from twenty *A. fumigatus* isolates using commercial kit. The yield of the DNA extracted was in the range of 65-210 ng/µl with a purity of 1.5-1.9. Species identification of the *A. fumigatus* isolates was achieved to a high specificity using tailored primers. The results showed that all isolates had positive results to the *Afumi* primer. The ability of twenty *A. fumigatus* isolates to produce gliotoxin was screened using thin layer chromatography (TLC). The results showed that eight (40%) isolates were able to produce gliotoxin. However, polymerase chain reaction (PCR) was detected the gliotoxin genes, *gliA* and *gliJ*, in all the isolates; *gliZ* in 17 isolates, and *gliP* in 15 isolates. The absence of *gliZ* and *gliP* was indicative of the inability to produce gliotoxin when the results were compared with results of TLC.

Keywords: *Aspergillus fumigatus*, gliotoxin, thin layer chromatography (TLC).

1. INTRODUCTION

The filamentous fungus *A. fumigatus* is highly pathogenic and is responsible for approximately 90% of all invasive aspergillosis infections [1-8]. *A. fumigatus* causes pulmonary clinical forms of Aspergillosis disease spatially in immunocompromised patients or those undergoing immunosuppressive therapy prior to organ transplantation and may also causes invasive disease. Moreover, the forms of pulmonary clinical are saprophytic, allergic and invasive aspergillosis. Invasive aspergillosis (IA) is the most risky form of the disease, however, since it involves the invasion of viable tissue and may produce a mortality rate of 40–90% in immunosuppressed patients [9, 11, 17]. Many of researchers found that the virulence of *A. fumigatus* due to finding of several secondary metabolites produced by *A. fumigatus* that play important roles in the pulmonary infection process [3, 10, 12, 20, 29].

Gliotoxin is a particularly important secondary metabolite of *A. fumigatus* [22], belonging to the chemical types of epipolythiodioxopiperazines (ETPs) which have immunosuppressive abilities through series of processes: (i) induction of apoptosis in macrophages and lung epithelial cells, (ii) inhibition of nuclear factor κ-B activation, and (iii) inhibition of phagocytosis [22, 28]. Another study indicated that the gliotoxin has a major role in the invasive aspergillosis process [28]. Furthermore, the researcher [19] reported that the GT biosynthetic cluster which directs gliotoxin production in the process of *A. fumigatus* infections via 13 of its genes [27]. Of these, *GliZ* responsible for transcriptional regulator of gliotoxin biosynthesis [5]. Wherease *GliP* induces GT synthesis through catalysing the first biosynthetic step by encoding a non-ribosomal peptide synthesis [7]. In addition, *GliA* has the main role in establishing tolerance to GT in *A. fumigatus*, and protection from extracellular metabolite spatially through exporting the toxin. This also allows the fungus

to evade the harmful effect of its own gliotoxin production. *GliA* contributes to the virulence of *A. fumigatus* through gliotoxin secretion [30]. Finally, the *GliJ* gene encodes metal-dependent dipeptidase, which is one part of a four-enzyme cascade in the gliotoxin biosynthesis pathway, which converts glutathione conjugates into transannular disulphide bridges [26]. Therefore; the objective of the present research was to identify *A. fumigatus* to a high specificity and to detect some of the gliotoxin genes using specific PCR.

2. MATERIALS AND METHODS

2.1 *Aspergillus fumigatus* growth conditions

Twenty of *A. fumigatus* isolates (obtained from the University of Baghdad / Department of Biotechnology) were used in this study; 18 of these were clinical and two from the environmental source. The *A. fumigatus* cultures were grown in potato dextrose agar (PDA) (Himedia-India) at 37 ˚C for between seven and ten days and preserved on Sabouraud dextrose agar (SDA) (Oxoid-UK).

2.2 Genomic DNA extraction

The genomic DNA was extracted from the 20 of *A. fumigatus* isolates using a Reagent Genomic DNA Kit (Geneaid-Taiwan). After extraction namedrop used to determine the purity and concentration of extracted genomic DNA and then integrity was detected by running 0.8% agarose gel electrophoresis, followed by staining with ethidium bromide and visualization under UV light [26].

2.3 Primer selection and PCR assay

Five oligonucleotides primer sequences were used in a lyophilized form and were dissolved in sterile deionized distilled water (DDW) to give a final concentration of 10 pmol/µl. One primer set was used for identification of *A. fumigatus* and the other four primer sets were used for gliotoxin detection. The primers and their sequences are showed in (Table 1).

PCR amplification was performed in a volume of 25 µl (PCR PreMix (Promega), (final reaction volume = 25 µl) carried out with a thermo cycler (Eppendorf-Germany). The following PCR reaction programme: - cycle of 5 minutes at 95 ˚C for initial strand separation, followed by 40 cycles of 1 minute at 95 ˚C for denaturation and 45 minutes at 58 ˚C for annealing and 1 minute 72 ˚C for primer extension. Finally, 1 cycle of 10 min at 72 ˚C was used for the final extension. This programme resulted in optimization. Approximately 7 µl of amplified PCR products were separated by electrophoresis in 1.2% agarose gels (1.5 hrs, 5V/cm, 1X Tris-borate buffer). The gels were stained with ethidium bromide; PCR products were visualized with a UV transilluminator and then imaged with a gel documentation system. The amplified products usually consist of one discrete band and their size was estimated by comparing them with the marker DNA ladder (100-2000) bp (Bioneer).

Table 1: The names and sequences of the specific primers

No.	Primers	Sequence (5'-3')	Purpose
1	AfumiF1	GCCCGCCGTTTCGAC	Identification of *A. fumigatus*[a]
	AfumiR1	CCGTTGTTGAAAGTTTTAACTGATTAC	
2	*GliAf*	TTTGCGATCAACGAACTCTG	Gliotoxin detection[b]
	GliAr	CCCTTGACGGACTGGAAGTA	
3	*GliJf*	CTCTGATCGACGGCCATAAT	
	GliJr	TCGAGCTGTTGGAGTGTCTG	
4	*GliPf*	AAACCCCTGTGAATGCAGAC	
	GliPr	CCCCTTGAGATGAAAGGTGA	
5	*GliZf*	TCCAGAAAAGGGAGTCGTTG	
	GliZr	ACGACGATGAGGAATCGAAC	

a [29]; b [18]

2.4 Extraction and purification of gliotoxin

Gliotoxin was produced by *A. fumigatus* under the following conditions: *A. fumigatus* was inoculated on a yeast-extract-sucrose broth mainly containing 20 g of yeast extract Ph; 40 g of sucrose dissolved in 1000 ml DW, and was adjusted to be 5.8±0.2 for 7 days at 37 ˚C. Whatman No.1 filter paper was used to filter the hyphae of *Aspergillus* from the YES culture medium. The filtrate was extracted three times by adding an equal volume (30 ml) of chloroform before mixing for 30 minute continually, the collected fraction of chloroform was evaporated to dryness at 50 ˚C, and then dissolved in 250 µl methanol and stored at -70 ˚C until they were analysed.

The TLC plates were activated in an oven at 100 °C for 1 hour before use [6]. Dried extracts were redissolved in 200 µl methanol. 20 µl of each extract was spotted on a silica gel 60 plate; the plate developed 10 cm in a TLC tank with a solvent system toluene: ethyl acetate: formic acid (5:4:1) as the mobile phase. The plates were air-dried and gliotoxin was visualized under the UV box at wavelength 365 nm [25].

3. RESULTS AND DISCUSSION

3.1 Genomic DNA Extraction

The extraction of genomic DNA was done efficiently using a Reagent Genomic DNA Kit. The purity and concentration of DNA were measured using the standard method [26]. The yield of the extracted DNA

was in the range of 65-210 ng/µl with a purity of 1.5-1.9.

3.2 PCR Analysis

3.2.1 Identification of *A. fumigatus*

In this study, the PCR technique was applied to confirm identification of *A. fumigatus* isolates. The results showed that the *Afumi* primer, based on PCR technique, was sensitive and specific in the identification *A. fumigatus*. An amplicon corresponding to 136 bp in size was seen after agarose gel electrophoresis. PCR was tested with all subjected of A*. fumigatus*. Additionally, the genomic DNA of all isolates was recognized and is complementary to the *Afumi* gene sequence, being represented by the presence of a single band in molecular weight 136 bp as shown in (Fig.1).

3.2.2 Screening of gliotoxin production in *A. fumigatus* isolates

The screening experiment was conducted to show that the ability of the examined isolates to produce GT, using the YES medium as a liquid state fermentation at 37 °C after 7 days of incubation. Chromatographic analysis using TLC indicated that only eight isolates (AF11, AF2, AF3, AF4, AF12, AF16, AF19, and AF20) showed the ability to produce GT (Fig. 2).

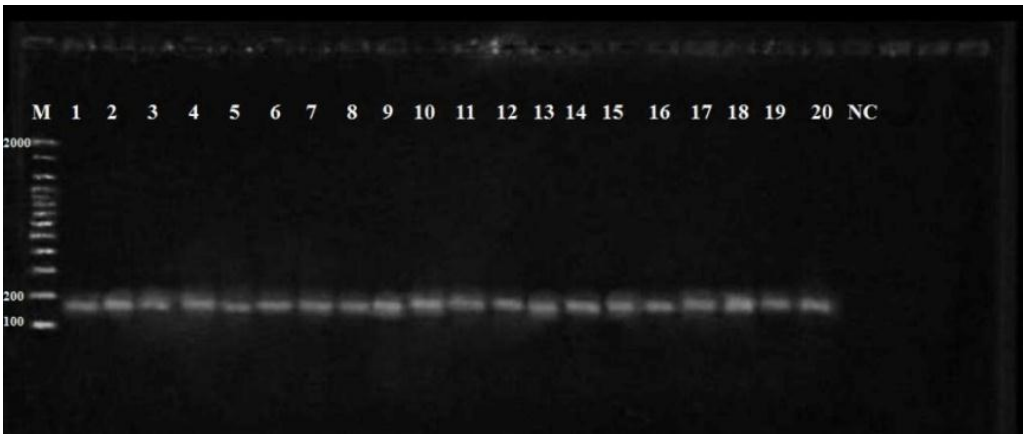

Figure 1: PCR product for Afumi primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.

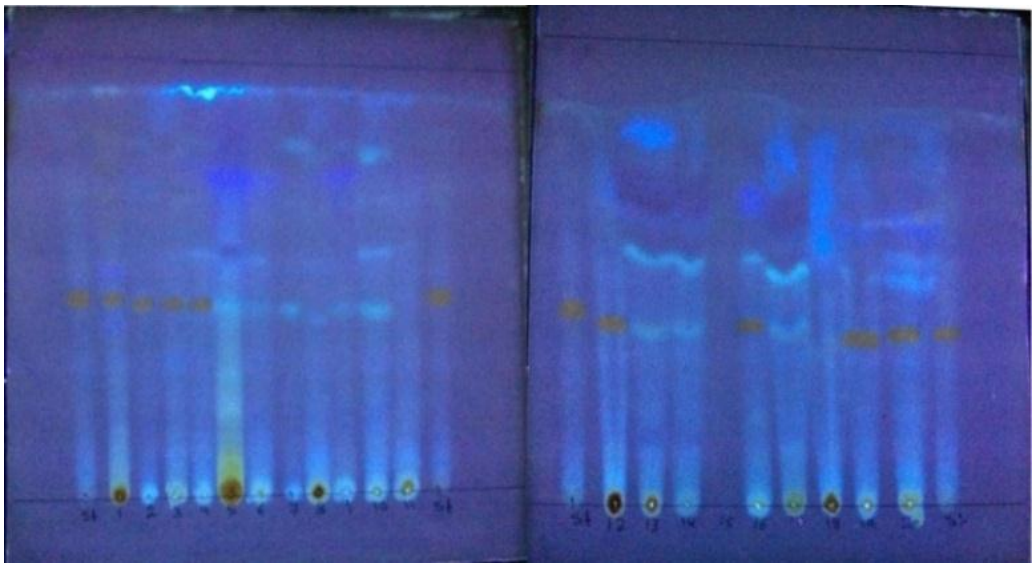

Figure 2: Detection of the gliotoxin production ability of 20 of *A. fumigatus* isolates for on YES medium. Using TLC analysis, the mobile phase was toluene: ethyl acetate: formic acid (5:4:1), gliotoxin appeared as a brown colour, No.1 to 20: *A. fumigatus* isolates, St: Standard gliotoxin

Additionally, PCR results for primer *GliP*, which amplified genomic DNA extracted from spores of *A. fumigatus* isolates showed that isolate numbers 1,2,3,4,5,7,9,10,11,12,13,14,16,17,18,19 and 20 had positive results for the *Glip* primer, as represented by the presence of a single band at molecular weight 168 bp, but isolates 6, 8 and 15 had negative results for this primer, as represented by the absence of a band at molecular weight 168 bp (Fig. 3).

Concerning, PCR results for primer *GliZ* showed that isolates 1,2,3,4,5,6,7,8,9,10,12,14,16,19 and 20 were positive for the *GliZ* primer, as represented by the presence of a single band at molecular weight 162 bp, but isolates 11, 13, 15, 17 and 18 were negative for this

primer (lacked a band at this molecular weight (Fig. 4). All the tested isolates were positive for the *GliA* primer, represented by the presence of a single band at molecular weight 155 bp (Fig. 5). Moreover, all isolates were also positive for the *GliJ* primer, represented by the presence of a single band at molecular weight 258 bp (Fig.6). All the PCR results in respect to gliotoxin genes are summarized in (Table 2).

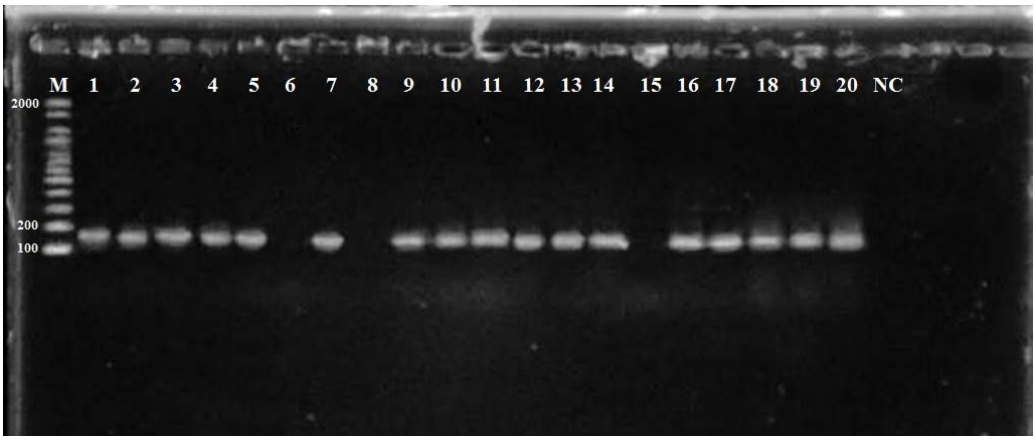

Figure 3: PCR products for the GliP primer for DNA samples of *A. fumigatus* spores on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control

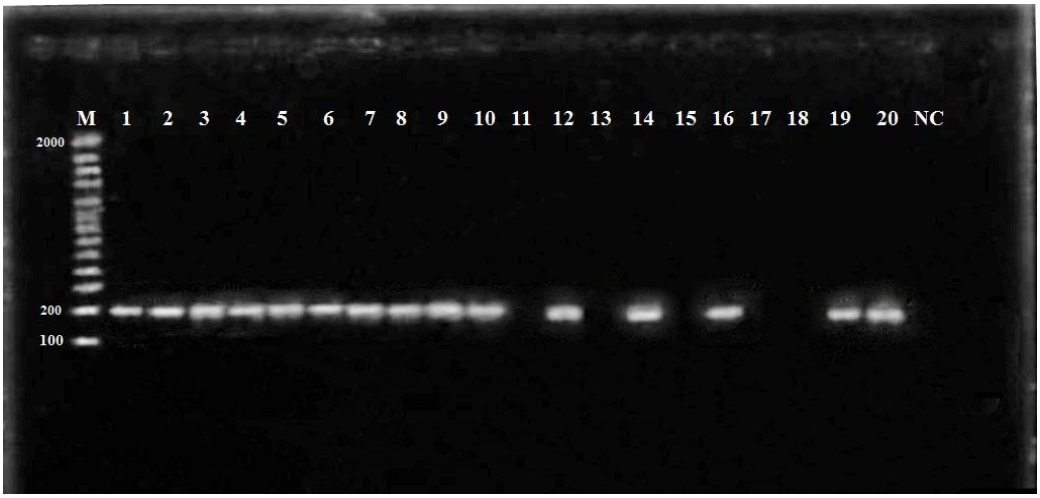

Figure 4: PCR products for the GliZ primer for DNA samples of *A. fumigatus* spores on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control. GliA primer

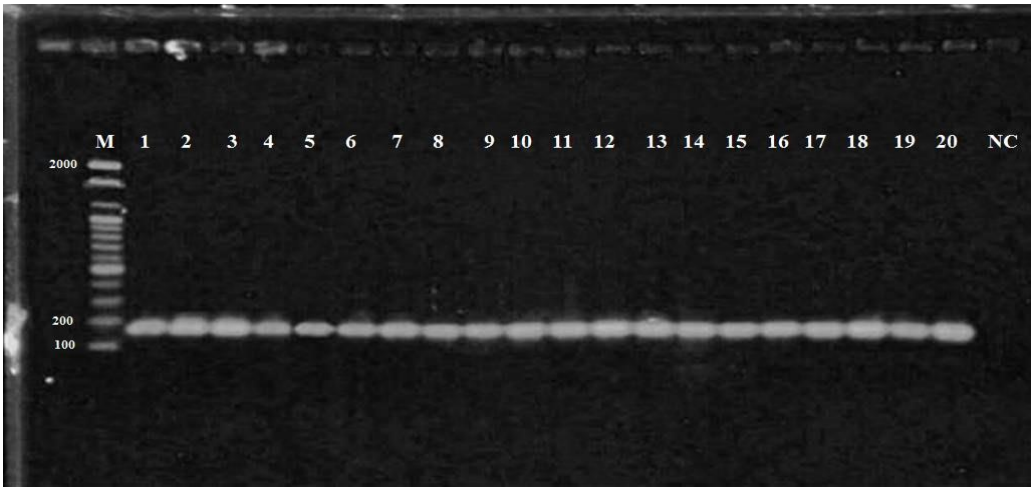

Figure 5: PCR products for the GliA primer for DNA samples of A. fumigatus spores on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control

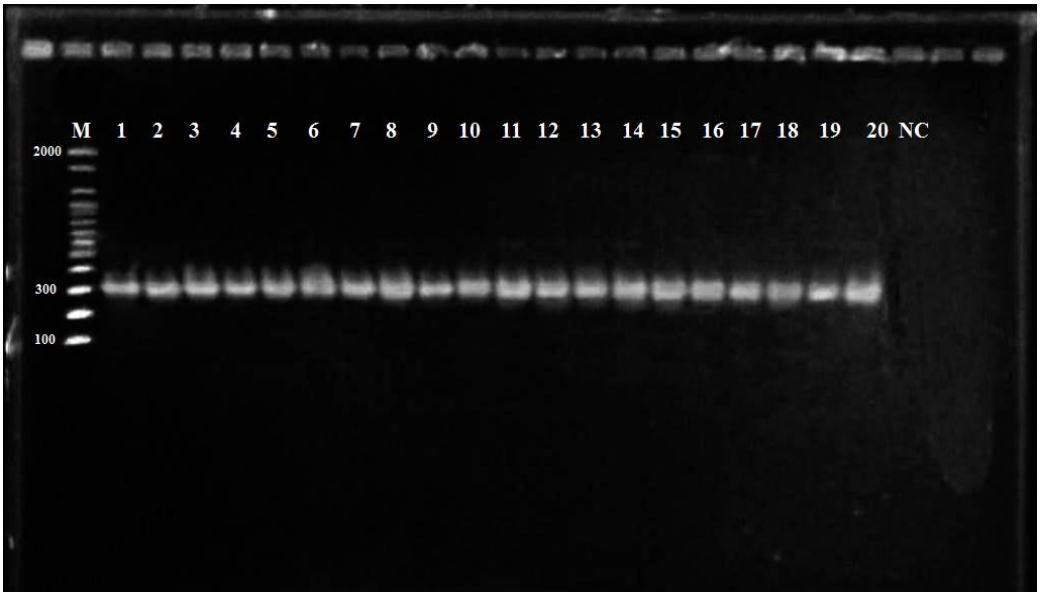

Figure 6: PCR products for the GliJ primer for DNA samples of *A. fumigatus* spores on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control

Table 2: PCR analysis for gliotoxin genes of *A. fumigatus*

Primers	Samples																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	NC
GliA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GliZ	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	-	+	+	-
GliP	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-
GliJ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

The efficiency of fungal isolates for mycotoxin production may be affected by environmental and genetic factors and it is therefore important to conduct production in optimum conditions for secondary metabolites and to optimize nutrients to stimulate mycotoxin production [2].

These results support the findings of [21] who documented considerable differences in gliotoxin production abilities among *A. fumigatus* strains. Out of the fifteen examined clinical isolates, only 18% gave positive results for gliotoxin production, although this may be attributed to the geographic region and/or the type of patients from whom isolates were collected. In our results, however, there appear to be more gliotoxin producing *A. fumigatus* strains among clinical isolates than among environmental isolates, and this agrees with [22]. Out of 41 of *A. fumigatus* isolates, only five showed the ability to produce gliotoxin using solid state fermentation [13], whereas [15] showed that a small percentage of the clinical *A. fumigatus* strains in the Azores were gliotoxin producers. Anthor study, [23] reported that over 90% of the clinical *A. fumigatus* strains isolated from invasive aspergillosis cases in tertiary-care cancer centres were gliotoxin producers. Furthermore, the amount of GT produced by *A. fumigatus* was based on strains and not every strain was a GT producer [13, 23].

The research [18] discovered a 12-gene cluster in the *A. fumigatus* genome sequence that resembles the genes responsible for gliotoxin biosynthesis; mutations in this gene cluster gene are associated with reduced virulence factors in *A. fumigatus*. Another research, [32] used a rice medium for the highest GT production from environmental *A. fumigatus* isolates. The gliotoxin on TLC plates appeared as a brown colour in visible light and as a yellow colour under UV at 365 nm before spraying with silver nitrate reagent. However, after spraying it appeared as brown in visible light and dark brown under UV at 365 nm. These results were similar to the results which obtained by [1]. Another study indicated that the production of gliotoxin on a rice medium was higher than production of gliotoxin on barley, corn or wheat media, and also indicated that the optimum conditions for gliotoxin production were

included: - a moisturizing ratio of 5:1 (w: v) with distilled water, an inoculum size of 6×106 spores and incubation at 37 ºC for 10 days [24].

In addition, the two transcriptional gene regulators for the biosynthesis of gliotoxin have been described: transcriptional regulator *LaeA* and transcriptional regulator *GliZ* [5]. These two regulators are very important in gliotoxin biosynthesis and work hand-in-hand with the enzyme that catalyses the first step of this pathway. This enzyme is encoded by the gene *gliP* and is called the non-ribosomal peptide synthetase [28]. Therefore; gliotoxin is known as the non-ribosomal peptide toxin [7]. The presence of these important genes is considered to underpin gliotoxin production in the most studied isolates, since 85% had the *gliP* gene, 75% had the *gliZ* gene and 75 % had both genes. Conversely, the absence of one or both of these genes leads to the loss the ability of production of GT as shown by [28] who documented that the deletion of the *gliP* gene in *A. fumigatus* resulted in abrogation in the synthesis of GT as well as that the elimination of GT production related to *gliP* disruption. Another researcher confirmed the role of *gliP* in the biosynthesis of gliotoxin [7]. However, [5] confirmed that deleting *gliZ*, a putative transcription factor located in the gliotoxin gene cluster, resulted in the loss of gliotoxin production *in vivo* and *in vitro*. This loss was associated with the absence of transcription of a biosynthetic gene in the gliotoxin gene cluster. Gliotoxin is a virulence determinant of *A. fumigatus* according to [22] who demonstrated that the Δ*gliP* mutant reduced virulence in two different mouse strains. The research [28] reported in the *in vitro* experiment in deleted *gliP* isolates failure to induce apoptosis in mammalian cells, and reduced in its ability to inhibit the oxidative burst in human neutrophils. While the absence of *gliP* and/or *gliZ* genes seems to be strongly associated with loss of gliotoxin production capability, some isolates were unable to produce gliotoxin, even when screening in TLC demonstrated the presence of *gliP* and *gliZ*. There may be a number of reasons, genetic and environmental, why these genes are not expressed, thereby resulting in the non-production of gliotoxin. It may be that some other genes have a role in regulating the biosynthesis of gliotoxin, such as the *LaeA* gene, which is a nuclear protein known to regulate several putative fungal virulence factors, including synthesis of gliotoxin and other secondary metabolites, as well as a role related to gliotoxin in *A. fumigatus* pathogenesis and the development of invasive aspergillosis [4, 5]. Moreover, [14] reported that gene expression levels of *gliZ* were greater in the *veA* strain, in spite of the observed low levels of *gliP gene* expression and reduced gliotoxin accumulation. When *gliZ* was overexpressed in a *veA* wild-type background, high levels of both *gliP* and concomitant gliotoxin accumulation were observed. This suggests that *veA* in *A. fumigatus* may influence the expression of structural genes in the gliotoxin clusters by other unknown mechanisms in addition to the effect on the gene expression of *gliZ*. Another research suggested that it is necessary to protect *A. fumigatus* from harmful during process of invasive *GliA* may serve to protect *A. fumigatus* from the harmful effects of extracellular gliotoxin [30]. This strongly implies that *GliA* also contributes to protection from its own produced gliotoxin by constantly exporting the toxin. The disruption of *gliA* caused the fungus to be highly susceptible to extracellular gliotoxin. Furthermore, the amount of GT was reduced, both in extracellular and intracellular spaces, which suggests that gliotoxin production was greatly reduced by the *gliA* disruption. Meanwhile, the gene *GliJ* is encoded metal-dependent dipeptidase which is one of the four-enzyme cascades that converts glutathione conjugates into transannular disulphide bridges in the gliotoxin biosynthesis pathway [26].

Concerning TLC experiments, the results showed that only 40 % of isolates had the ability to produce gliotoxin which attributed to several environmental factors that affect on gliotoxin production such as (culture media, temperature, incubation period, moisturizing ratio, inoculums size and extraction method) [24]. As explained previously, these may result in the genes that are responsible for production not being expressed.

4. CONCLUSION

Although *GliA* and *GliZ* genes were present in all isolates, the absence of *GliP* and/or *GliZ* genes is indicative of the inability of isolates to produce gliotoxin. Moreover, 40 % of *A. fumigatus* isolates have the ability to produce gliotoxin when screened by TLC.

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