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RESEARCH ARTICLE

Detection of gliotoxin in patients with pulmonary mycosis

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Abstract

Objective: This study was designed to detection of gliotoxin in patients with pulmonary Aspergillosis .

Method: A total of 100 samples (sputum and blood) were selected from 300 outpatients who attended to Al-Qadissiyia Centre of Tuberculosis and Chest Diseases, , during the period from 2014 to May 2015. All patients had clinical manifestation in addition to 100 sample (sputum and blood) as control group.

Results: all patients infected with fungi and three different genus isolated from sputum of patients ,these genus are *Aspergillus* sp 60 Isolates(60%)with three species were *A.fumigatus* (50%)and *A.flavus* (33.3%)*Cryptococcus neoformans* (14%) *Penicillum*(8%) and *Rhizopus* sp(6%).So (71%) from patients had gliotoxin in their sputum and serum and (5%) had GT in their serum while (3%) of them had GT in their sputum. while 21 (21%) from patients didn't have GT in serum nor sputum. So this toxin was found in40(40%)in sputum and serum of control group , 6(6%)in sputum and 4(40%)in serum of control group serum

the concentration of GT in sputum of patients was (40-63µg/kg) and in sputum of control group was (16-23µg/kg) and in serum of patients was (33-47µg/kg) and (10-21µg/kg) in serum of control group. and all *A.fumigatus* isolates 30 (100 %)were have *gliz* gene.

Conclusion: several species of *Aspergillus* and other fubgi as well as possibly yeast, produced gliotoxin both in vitro and in vivo. More attention should be paid to this mycotoxin because of its multi-faceted toxic properties

INTRODUCTION

Pulmomary Aspergillosis (PA) is the most common mycotic infection of the respiratory tract caused by *Aspergillus*, a common mold (fungus) that lives indoors and outdoors. Most people breathe in *Aspergillus* spores every day without getting sick. However, people with weakened immune systems or lung diseases are at a higher risk of developing health problems due to *Aspergillus*.(1) The two major agents causing aspergillosis are *A.fumagatus* and *A. flavus* as show in more studies (2) .

Asergillus fumigatus is an ubiquitous saprophytic fungus which plays an important role in recycling environmental carbon and nitrogen ,but also it may be an opportunistic pathogen ,Human constantly inhale high amounts of conidia from this fungus ,which may affected their respiratory tract after long exposure (3). *A. fumigatus* consider the leading cause of mold infections worldwide, is an opportunistic pathogen that causes severe problems in immune-compromised populations,These populations include: AIDS patients, cancer patients receiving chemotherapy, solid organ transplant/skin graft patients and victims of chronic granulomatous disease(4). *A.fumigatus* is also capable of producing secondary metabolites ,which can be harmful, One of the most studied

secondary metabolites produced by *A. fumigatus* is gliotoxin, which is also produced by several other *Aspergillus* species, *Trichoderma* species, and *Penicillium* species **(5).** Gliotoxin is a member of the epidithiodioxopiperazine (ETP) class of toxins, which are characterized by a disulfide bridge across a piperazine ring with low molecular weight (326 Da) (6,7). The oxidized form of gliotoxin travels into host immune cells where it is able to affect cellular functions essential to the immune response. These include impediment of phagocytosis and NF-κB activation, as well as induction of apoptosis(8, 9). As with other secondary metabolites, most of the genes responsible for the production and transport of gliotoxin exist within a gene cluster. The gliotoxin biosynthesis cluster was first identified based on its homology to the sirodesmin PL biosynthesis gene cluster in the ascomycete *Leptosphaeria maculans(* 10,11) Within this cluster lies a Zn_2Cys_6 binuclear finger transcription factor, GliZ, thought to be responsible for general gliotoxin induction and regulation. Indeed, over-expression of *gliZ* leads to an increase in gliotoxin production and deletion of *gliZ* results in a loss in gliotoxin production.(12, **5,** 6**)** .

Biosynthetic ETPs are derived fromat least one aromatic amino acid. GTX is derived from phenylalanine and serine as precursor amino acids .Secondary metabolites of fungi that have more than one amino acid are generallyproduced by non-ribosomal peptide synthetases. The complete genome sequence of A. fumigatus showed that the non-ribosomal peptide synthetase enzymes that synthesise GTX usually have genes clustered in the genome (11). Several genes (*gli* genes) have been identified related to the biosynthesis of GTX (13) including gliZ, atranscriptional regulator of GTX production that encodes the Zn(II) 2Cys6 binuclear transcription factor. Substitution of the gliZ gene with a marker gene caused no detectable GTX biosynthesis and failure to express the other gli cluster genes (12, 14). Additionally mutation in the gliP gene resulted in failure to make GTX (12). These genes are activated when secondary metabolism commences in fungi (15). *Lea A* is a methyltransferase that possibly is involved in regulation of these genes (16).

However ,studies focusing on the relation between mycotoxins and its pathogenesis have been limited and significance of mycotoxins in the virulence of *Aspergillus fumigatus* has not yet been demonstrated in Iraq .

So,until recently the relationship between mycotoxins and the pathogenicity of the fungi that produce them has received little attention ,therefore this study was designed to detection of gliotoxin in patients with pulmonary Aspergillosis .

Material s and methods

- A total of 100 samples (sputum and blood) were selected from 300 outpatients who attended to Al-Qadissiyia Centre of Tuberculosis and Chest Diseases, , during the period from 2014 to May 2015. All patients had clinical manifestation
- signs such as fever, weight loss, cough, anorexia, and some of them with bloody sputum., and clinical examination by a specialist clinician. Those patients already diagnosed as not tuberculosis patients and did not respond to treatment. Diagnosis was established by clinical picture, chest X-ray examination . Samples of blood and sputum were taken from all patients. So The healthy person 30 were also studied as control as clinical examination showed by clinician specialist, sputum and blood samples were taken from each subject.

Sputum sampling (17).

Patients were advised to wash their mouths with antiseptic mouth wash and then three times with water. To obtain a sputum sample, the patient was given a labeled sputum container and was asked to :

- Take a deep breath.
- Open the container ,bring it close to the mouth and bring the sputum out into it.
- Not to put saliva or nasal excretion into the container .
- Not to have sputum in the mouth but immediately spit into the container.
- Close the container.

Processing of sputum samples

The sputum samples were decontaminated and digested by treatment with an equal volume of sputolysin/sodium hydroxide (4%) for 30 minutes at room temperature with rocking. After neutralization with 10 ml of PBS (pH 7.4), the mixture was centrifuged at 3000 rpm for 30 minutes. the supernatant put in sterile tube and added to it equal amount choroform ,after shaking the content of tube will be separated into two portion ,one portion choroform layer which contain toxin and another potion was discharge the sediment was obtained, and was inoculated in Sabouraud Dextrose

Agar and incubated at 25C̊ for one weeks for mycotic examination,The isolated fungus was suspended in Lactophenol and examined microscopically by slide . (18).

Blood samples

Four ml of blood were collected by vein puncture into two sterile test tubes, in one of them 3 ml of blood were putted and left for about 2-4 hours, then the upper layer (serum) was collected in clean test tube and add to it equal amount chloroform after shaking the content of tube will be separated into two portion one portion chloroform layer which contain toxin and another portion was discharge ,the tube which contain chloroform layer stored at -20˚C until use.

Determination of Gliotoxin in serum and sputum by Thin Layer Chromatography

By capillary tube 10 µl from chloroform layer which separated from each sputum and serum of patients in above step was took and spotted on the TLC plate(Silica gel G60) adjacent to 1 µl of standard GT which dissolved in 200 mixture of dichloromethane :methanol (97:3 v/v) were also spotted on the same plate , and the gliotoxin were separated by TLC method using chloroform :methanol (70:30 v/v) as the developing solvent, TLC plate was dried after the end of development and front was marked . Location of GT spot on TLC plates was accomplished by illumination under shortwave UV light (254 nm) and by comparing the sample spots to the standard GT(same shape, retention factors (R*f*) and color) (19).

Standard curve of Gliotoxin

Standard curve was prepared by measuring the absorbance of the following prepared pure standard GT concentration (10, 20, 40,80,160 and 320 µg/ml) .Each of standard concentration was plotted against its absorbance values and a linear standard curve was achieved from which the GT quantity of any sample was determined according to its absorbance in comparison with that of standard curve values.

Detection of *gliz* gene in *A.fumigatus* isolates

1- **Isolation of Fungal DNA**

Template DNA was extracted from fungal mycelia according to(20) as follows: fungal mycelia grown in Potato Dextrose Broth (PDB) under stationery conditions for 21 days was harvested by filtration. The mycelium was washed twice with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) followed by centrifugation. The mycelium was transferred to a mortar and ground well. Freshly prepared, sterile Lysis buffer (50 mM Tris, 150 mM EDTA, 1% (w/v) SDS, pH 8.0) was added to the pulverized mycelia and incubated at 65°C for 1 h. The suspension was centrifuged and supernatant was then extracted twice with phenol: chloroform: isoamylalcohol (25:24:1) and the aqueous layer was washed twice with chloroform and then precipitated with two volumes of isopropanol. The precipitate was resuspended in 200 µL of TE buffer (10 mM Tris-Cl, 1.0 mM EDTA, pH 8.0.

2-Polymerase chain reaction (PCR)

The primer GZ5(R 5´GGAGAGAATTCATTTAACCTTCTATCGCAG3´,GZR5 F5´AGTGACCGACCGTCCAAGAACCGTAG3´) were synthesized by American Geneoids Company. The PCR conditions were optimized by varying the concentration of above primer, the number of units of Taq polymerase and annealing temperature of the reaction. The PCR reaction mixture (25 µL) contained 100 ng of genomic DNA, deoxyribonucleoside triphosphates at 0.025 nmol each, primer at 4 nmol each and reaction buffer. Each reaction mixture was heated to 95°C for 10 min before adding 0.3 units of Taq DNA polymerase. Amplification conditions used consisted of 4 min at 94°C followed by 35 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 75 sec. The reaction was completed with incubation for 10 min at 72°C. PCR products were analyzed by electrophoresis in a 1% agarose gel in TAE buffer. Ethidium bromide (0.5 µg μL^{-1}) stained gels were visualized under UV light with Digital camera (21).

Results and Discussion

All cases studied where didn't respond to antituberclosis treatment and didn't isolated any bacterial agents from pulmonary infection ,therefore this study attention in isolated the fungal etiological agents of pulmonary infection thus, the results of study showed that all patients infected with fungi and three different genus isolated from sputum of patients ,these genus are *Aspergillus* sp 60 Isolates(60%)with three species were *A.fumigatus* (50%)and *A.flavus* (33.3%)*Cryptococcus neoformans* (14%) *Penicillum*(8%) and *Rhizopus* sp(6%).Table (1)

The predominant fungal species in this study was *A. fumigatus* (50 %). This results agree with (17,22) whom found *A.fumigatus* was the most common cause pulmonary mycotic infection .This fungus causes many human lung diseases and a major factor in determining the pathogenicity of the fungus is the size of the spores (around 3µm in diameter) which are present ubiquitously in the air.(8).

Mullins *et al*.,(23) have show that many more spores of *A.fumigatus* present in the lung at necropsy than would be anticipated from their presence in the air .So ,this fungus can produce toxins such as gliotoxin which is an important factor in allowing the hyphae to grow in tissue such as lung .

Aspergillus fumigatus is a saprophytic and opportunistic pathogenic fungus with a widespread occurrence. *A. fumigatus* is known to produce several secondary metabolites, including mycotoxins (e.g. gliotoxin). Increasing evidence supports a significant role of gliotoxin in hampering various defence mechanisms of the host, leading to virulence enhancement (24, 5). The level of gliotoxin production by *A. fumigatus* isolates can vary or even be completely absent (25). This fungus is known to cause allergic reactions and mycotoxicoses, and is believed to be responsible for more than 90% of invasive aspergillosis in humans (26, 3).

Table (1) Number of mycotic isolates and their percentage throughout the study

Specimens	Type of specimens	Agents of mycotic infection	No. of isolates	%
Patients group (100)	Sputum	*Aspergillus* *A.fumigatus* *A.flavus* *A.niger*	60/100 30/60 20/60 10/60	60 50 33.33 16.66
		Cryptococcus neoformans	20/100	20
		Penicillium sp	14/100	14
		Rhizopus sp	6/100	6
	Blood	No growth		
Control group(100)	Sputum	No growth		
	Blood	No growth		
total			100	

Detection of gliotoxin in serum and sputum of study groups

In the solvent system chloroform and methanol (70:30V/V), gliotoxin had an R*f* of 0.97 and visualized as orange /brown spots under UV light.

Seventy one (71%) from patients had gliotoxin in their sputum and serum and (5%) had GT in their serum while (3%) of them had GT in their sputum. while 21 (21%) from patients didn't have GT in serum nor sputum. So

this toxin was found in40(40%)in sputum and serum of control group , 6(6%)in sputum and 4(40%)in serum of control group serum. table (2).

There are no similar study for detection of gliotoxin in human cases so that we can compare our results with it.

Table (2) Distribution of GT in serum and sputum of patients and control group

Study groups	Serum	Sputum	Sputum +serum	Total
Patients	5(5%)	3(3%)	71(71%)	79(79%)
Control	4(4%)	6(6%)	40(40%)	50(50%)

Gliotoxin may play an important role in the establishment and development of an infection with *A. fumigatus* and has immunosuppressive properties in vitro, but little is known about its in vivo activity . In term of the detection of GT in vivo ,only few cases of gliotoxin being detected in infected tissues have been reported .Bauer *et al.*(27) reported the presence of gliotoxin in Cows udder. Richard *et al*.,(28) made in animal model using Turkeys and found significant amount of gliotoxin in the poults of infected animals .Reeves etal.,(2004) detected gliotoxin in the bodies of Larvae of experimentally -infected *Galleria mellonella*, so (25) showed a detectable level of gliotoxin in the sera of aspergillosis mice.

Sutton *et al*.,(30) referred to Pretreatment of normally resistant mice with a single injection of a sub lethal dose of gliotoxin was sufficient to make them susceptible to infection and subsequent death, after challenge with *A. fumigatus* spores. Animals infected with the non-gliotoxin producing strain survived significantly longer than those infected with a gliotoxin producer. We propose that the release of gliotoxin by *A. fumigatus* hyphae during infection can exacerbate the pathogenesis of aspergillosis .

Sutton *et al.*(31) report that gliotoxin has increased toxicity in mice after irradiation. A single injection of gliotoxin delayed the recovery of immune cells after immunosuppressant by sub lethal irradiation by 2 weeks. Study of the morphology of cells of the thymus, spleen, and mesenteric lymph nodes by light microscopy and electron microscopy and agarose gel electrophoresis of DNA from these organs showed that the injection of gliotoxin induced apoptosis in cells of the immune system in vivo. Thus, gliotoxin does have immunosuppressive activity in vivo and could potentially play a significant role in the pathogenesis of aspergillosis and other fungal diseases.

Gliotoxin production was found to be dependent on the oxygen concentration of the environmental ,the primary target of infection by *A.fumigatus* is the lung ,the most well-aerated organ .in this sense ,the high concentration of oxygen in the lung provides an optimal condition for the production of gliotoxin by *A.fumigatus* .SO it is able dramatically to modulate lung cell functions such as attachment of epithelial cells and fibroblasts as well as inhibitory phagocytosis by macrophages ;other important functions of the host immune defense are also impaired by gliotoxin ,including induction of cytotoxic and all reactive T .cell (32). Eichner et al.,(33) referred to a small molecular weight (`10 KD) that released from isolate of*A.fumigatus* within minutes of deposition in the right place of lung ,which is capable of inhibiting the oxidative burst of macrophage.

Gliotoxin induces apoptosis in monocytes and dendrite cells, resulting in the suppression of AF-specific T cell responses.(34) examined the ability of GT to induce apoptosis in polymorph nuclear leukocytes (PMN) and assessed GT effects on important neutrophil functions, including phagocytic function, degranulation, myeloperoxidase activity, and the production of reactive oxygen species (ROS). So Gliotoxin contains an epipolythiodioxopiperazine (ETP) ring that is believed to be involved in redox reactions. The reactive oxygen species produced interact with DNA to form hydroxylated and other altered DNA products.(35).

Different histological changes found in tissues of lung included necrosis in lung alveoli with inflammatory cells infiltration and abuses formation in addition to thickness in lung alveolar sac wall and bronchioles with hemorrhage.(36).

Quantification of Gliotoxin

Spectrophotometric method was used for quantification of GT in Serum and sputum of study groups after its extraction, isolation and identification by TLC method.

The following standard curve was plotted for different standard GT prepared concentrations that used for quantification of GT in different samples.

the concentration of GT in sputum of patients was (41-63µg/kg) and in sputum of control group was (16-23µg/kg) and in serum of patients was (33-47µg/kg) and (10-21µg/kg) in serum of control group .(table 3,figure1)

Table (3) Concentration of GT in serum and sputum of patients and control group

Study groups	Concentration of GT(µg/kg) in	
	Serum	Sputum
Patients	33-47	41-63
Control	10-21	16-23

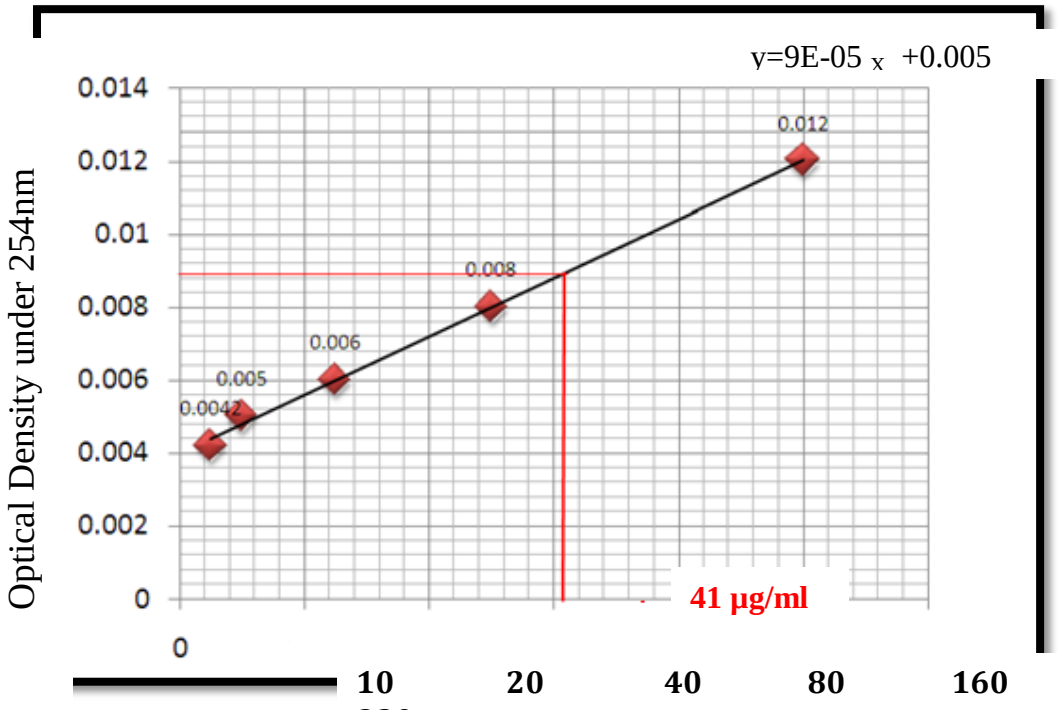

Figure 1: Gliotoxin standard curve.

Bauer *et al.*,(27) was isolated GT for the first time from naturally infected tissue The gliotoxin concentration analyzed (9.2 mg/ kg udder) and referred to this concentration was approximately 100 times higher than the concentration known to produce morphological changes of cells.

Detection of *gliz* gene in *A.fumigatus*

The results of detection of *gliz* gene showed that all *A.fumigatus* isolates 30 (100 %)were have *gliz* gene as show in figure 2.

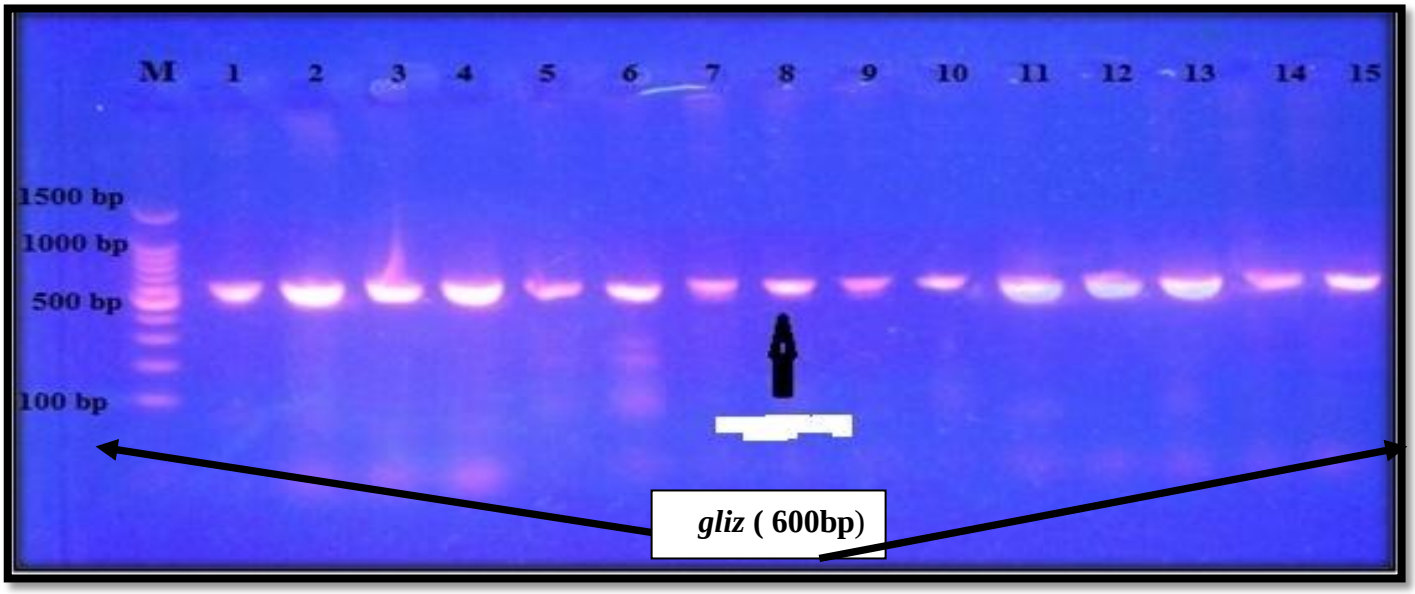

Figure (2) Electrophoresis of the amplified product of *gliz* in a 1 % agarose gel. Lane M, 1500bp DNA ladder; lanes 1 to 15, *A.fumigatus* isolates that contain *gliz* gene in size (600 bp) .

Recently, a predicted gliotoxin biosynthetic gene cluster was identified in *A. fumigatus* (37) In an attempt to assess the contributions of gliotoxin to the role of LaeA in virulence, Bok etal.,(2006) have created a null mutant in *gliZ* encoding a putative Zn_2Cys_6 binuclear finger transcription factor. He show that *gliZ* is required for gliotoxin biosynthesis and expression of other genes in the *gli* gene cluster and that placement of two or more copies of *gliZ* in the genome results in increased gliotoxin synthesis.. Two concurrent studies, where *gliP* encoding a nonribosomal peptide synthase required for gliotoxin synthesis was deleted from the *A. fumigatus* genome, yielded similar results where the authors report no difference in mouse survival (**7;** 24). However, in both studies loss of gliotoxin resulted in decreased toxicity as measured either by mast cell degranulation (**Cramer etal.,2006**) or macrophage/T-cell viability (**24),** thus leading to speculation that this metabolite can play a role in disease development. Here, cytotoxicity assays with polymorphonuclear leukocytes (PMNs) support a role for gliotoxin in apoptotic but not necrotic cell death. Taken together, they posit that gliotoxin is one factor that can be involved in disease development and that its effects may not be readily measured by the current animal model systems.

GliZ, thought to be responsible for general gliotoxin induction and regulation. Indeed, over-expression of *gliZ* leads to an increase in gliotoxin production and deletion of *gliZ* results in a loss in gliotoxin production so the culture supernatants of wild-type *A. fumigatus* induced apoptosis of neutrophils in vitro. However, culture supernatants of a *gliZ* deletion mutant did not contain gliotoxin and did not induce apoptosis in neutrophils. (12; 6) A DNA binding site has been proposed for GliZ ($TCGGN_3CCGA$), but has not been experimentally proven. This site is present within the promoter region of every gene within the gliotoxin cluster, except *gliZ* and *gliA*, which encodes an efflux pump within the cluster(10). Gliotoxin itself positively regulates expression of the genes within the gliotoxin cluster, as deletion of *gliz*, the non-ribosomal peptide synthetase (NRPS) required for the first step in the synthesis of gliotoxin, virtually eliminates expression of the other genes in the cluster. This loss in gene expression can be reversed by the addition of exogenous gliotoxin to culture medium **(38; 39, 40**).

In conclusion, several species of *Aspergillus* and other fubgi as well as possibly yeast, produced gliotoxin both in vitro and in vivo. More attention should be paid to this mycotoxin because of its multi-faceted toxic properties.

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