Detection of Gliotoxin in Patients with Pulmonary Aspergillosis

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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 100 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%) *A.flavus*(33.3%) and *A.niger* (16.6%)*Cryptococcus neoformans* (20%) *Penicillum* sp.(14%) and *Rhizopus* sp.(6%). Seventy nine (79%) from patients had gliotoxin distributed in their sputum and serum by ratio (71%) and 5 (5%) had GT in their serum while 3 (3%) of them had GT in their sputum.. So this toxin was found in50(5%) in control group distributed in sputum and serum by (40%) , 6(6%)in sputum and 4(4%)in 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 fungi 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.
Keyword: Aspergillosis ,Gliotoxin, *Aspergillus fumigatus.*

الخلاصة

صممت هذه الدراسة للتحري عن سم الجليوتوكسين لدى المرضى المصابين بداء الفطار الرئوي.

تضمنت الدراسة جمع 100 عينة من المرضى المراجعين الى مركز الصدرية والتدرن الرئوي في محافظة القادسية للفترة من اذار 2014 ولغاية اذار 2015 علما ان جميع المرضى كانوا يعانون من اعراض سريرية بالاضافة الى جمع 100 عينة كمجموعة سيطرة .

اظهرت نتائج الدراسة ان جميع المرضى مصابون بالفطريات وتم عزل ثلاث اجناس من الفطريات من القشع ساد فيها الفطر *Aspergillus* sp. بنسبة 60% وظم ثلاث انواع هي *Aspergillus fumigatus* بنسبة50% و *A.flavus* بنسبة (33.3%) و*A.niger* بنسبة (16.6%) وخميرة *Cryptococcus neoformans* بنسبة 20%والفطر *Penicillium* sp بنسبة 14%ومن ثم الفطر *Rhizopus* بنسبة6 %.

تسع وسبعون بالمائة من المرضى المصابون كانوا حاملون لسم الجليوتوكسين في القشع والدم بنسبة 71% اذ ان 5% منهم كانوا حاملين لهذا السم في الدم و3 (3%) كانوا حاملين للسم في القشع كذلك وجد هذا السم في 5% من مجموعة السيطرة توزع بنسب 40% في القشع والدم و6% في القشع و4% في الدم .

تراوح تركيز سم الجليوتوكسين في قشع المرضى المصابين بين (40–63)مايكروغرام /كغم من وزن الجسم ومن(16–32)مايكروغرام /كغم في قشع مجموعة السيطرة في حين بلغ تركيزه (33–47%)مايكروغرام /كغم في دم مجموعة المرضى ومن (10–21)مايكروغرام /كغم في دم مجموعة السيطرة .وكانت جميع عزلات الفطر *A.fumigatus* (100%) حاوية على جين *gliz* .

هناك بعض انواع الفطر Aspergillus وفطريات اخرى بالاضافة الى الخمائر لها القابيلة على انتاج سم الجليوتوكسين ويجب الانتباه الى هذا السم لما له من تاثيرات متعددة على الجسم .
الكلمات المفتاحية: سم الجليوتوكسين ،الفطر *Aspergillus fumigatus* والفطار الرئوي .

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*. (Smith and Denning, 2011) The two major agents causing aspergillosis are *A.fumagatus* and *A. flavus* as show in more studies (Kradin and Mark, 2008) .

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 (Latge , 1999). *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 (Brand, 2012). *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 (Kwon-Chang and Sugui, 2009). 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) (Scharf *et al.,* 2012, Cramer *et al.,* 2006). The oxidized form of gliotoxin travels into host immu ne 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 (Waring *et al*., 1994; Yoshida *et al*., 2000). 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* (Fox and Howlett, 2008) 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. (Bok *et al*.,2006).

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 (Gardiner *et al*., 2004). Several genes (*gli* genes) have been identified related to the biosynthesis of GTX (Balibar and Walsh, 2006) 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 (Schrettle *et al*., 2010). Additionally mutation in the gliP gene resulted in failure to make GTX (Bok *et al*., 2006). These genes are activated when secondary metabolism

commences in fungi (Rementeria *et al.*, 2005). *Lea A* is a methyltransferase that possibly is involved in regulation of these genes (Stack *et al.*, 2007).

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 100 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 100 were also studied as control as clinical examination showed by clinician specialist, sputum and blood samples were taken from each subject.

Sputum sampling (Ellis, 2004).

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 chloroform ,after shaking the content of tube will be separated into two portion ,one portion chloroform layer which contain toxin and another potion was discharge the sediment was obtained, and was inoculated in Sabouraud Dextrose Agar and incubated at 25C˚ for one weeks for mycotic examination, The isolated fungus was suspended in Lacto phenol and examined microscopically by slide (Kubica *et al*.,1993).

Blood samples

Four ml of blood were collected by vein puncture into two sterile test tubes, in one of them 3ml 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 (TLC)

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.Sigma Aldrich Z) 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) (Vander Merwe *et al*.,1965).

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(Lee *etal.*,1998) 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 re suspended 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 n mol each, primer at 4 n mol 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% a garose gel in TAE buffer. Ethidium bromide (0.5 µg μL^{-1}) stained gels were visualized under UV light with Digital camera (Sambrook *et al*.,1989).

Results and Discussion

All cases studied where didn't respond to anti tuberculosis 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%), *A.flavus* (33.3%) and *A.niger* (16.6%) *Cryptococcus neoformans* (20%) *Penicillum* sp.(14%) and *Rhizopus* sp.(6%). While didn't isolate any fungus from blood of patients and blood and sputum of control group. Table (1).

The predominant fungal species in this study was *A. fumigatus* (50 %). This results agree with (Al-Taee, 2009; Ellis, 1994) whom found *A.fumigatus* was the most common cause pulmonary mycotic infection .This fungus responsible for causes especially lung diseases . the size of the spores (around 3µm in diameter) which are present ubiquitously in the air considers the major factor in determining the pathogenicity of this fungus. (Waring *et al*., 1994).

Mullins *et al*., (1976) have shown that the inhalation of *A.fumigatus* spores which present in present in the air lead to precipitate in lung as he shows that in lung in necropsy. So ,this fungus can produce toxins such as gliotoxin which is an important factor in helping the fungus mycelium to grow in tissue such as lung .

Aspergillus fumigatus is a saprotroph widespread in nature commonly found in soil and decaying organic compounds. this fungus cause invasive in the lung of immunocompromised patients and responsible for morbidity and mortality in above individuals (Kupfahl *et al*., 2006). So *A. fumigatus* can cause allergic bronchopulmonary aspergilosis and allergic reactions in immunocompetent hosts. (Latge, 1999).

Aspergillus fumigatus produce mycotoxins ,(secondary metaboites) one importants of these mycotoxins is gliotoxin which is affect on host deffenses through suppresser of immune system. (Kwon-Chung and Sugui ,2009). Gliotoxin production by *A. fumigatus* isolates can vary from isolat to another (Denning , 1998; Lewis *et al*., 2005).

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
Control group(100)	Blood	No growth		
	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 nine (79%) from patients had gliotoxin distributed in their sputum and serum by ratio (71%) and 5 (5%) had GT in their serum while 3 (3%) of them had GT in their sputum.. So this toxin was found in50(5%) in control group distributed in sputum and serum by (40%) , 6(6%)in sputum and 4(4%)in 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 effects on the function of leukocytes by inhibiting migration and production of superoxide and causes apoptosis in macrophages. So Gliotoxin inhibition of NF-κB thus disrupts the proinflammatory response and play an important role in the establishment and development of an infection with *A. fumigatus* . We research about studies which designed to the detection of GT in vivo ,and found only few cases of gliotoxin being detected in infected tissues have been reported such as Bauer *et al.* (1989) who detected of gliotoxin in Cows, Richard *et al*.,(1998) designed in animal model by using Turkeys and detected detectable level of gliotoxin in the poults of animals infected with *A. fumagatus*. Reves *et al*., (2004) found gliotoxin in the bodies of larvae of *Galleria mellonella,* which expermintally infected , so (Lwies *et al*.,2005) showed a significant amount of gliotoxin in the sera of mice infected with *A. fumagatus*.

Sutton *et al*.,(1994) referred to treatment of Rats with a one injection of a sub lethal dose of gliotoxin was suitable to make above animals critical to infection and ensuing death, after challenge with *A. fumigatus* spores .So showed if infected animals with the strain that non-gliotoxin producing ,these animal survived safely longer than the animals infected with strains that produced gliotoxin..

After injection of animals with sub lethal dose of GT and Study of the morphology of cells of the thymus, spleen, and mesenteric lymph nodes by electron microscopy and agarose gel electrophoresis of DNA from these organs showed that the gliotoxin induced apoptosis in macrophage in vivo. Thus, gliotoxin has immunosuppressive effectiveness in vivo and may be play a critical role in the pathogenicity of *A.fumagatus*. (Sutton *et al*., 1996)

Production of gliotoxin was found to be based on the oxygen concentration of the environmental , the lung considers as first target for infection by *A.fumigatus* ,the most well-aerated organ ,in this meaning ,found high level of O_2 in the lung supply an best environment to production of gliotoxin by *A.fumigatus* .SO it is able dramatically to alter function of lung cell such as attachment of epithelial cells and fibroblasts as well as inhibitory phagocytosis by macrophages ;so others critical functions of the individuals immune defense are also impaired by gliotoxin ,including induction of cytotoxic and all reactive T .cell (Reijula *et al*., 1992). Eichner *et al*., (1986) 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 stimulate apoptosis in cellular components of immune system which are monocytes and dendrite cells, leading to the inhibition of cellular immune responses.(Suen *etal*.,2001) studied of the capability of gliotoxin to cause apoptosis in polymorph nuclear leukocytes (PMN) and refers to gliotoxin was effect on neutrophil functions, which include phagocytic function, degranulation, myeloperoxidase activity, and the production of reactive oxygen species (ROS). So GT contains an epipolythiodioxopiperazine (ETP) ring that is consider to be take part in redox reactions. The sensitive oxygen radicals yield interaction with DNA to form hydroxylated and other foreign DNA components .(Golden *et al.*,1998).

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. (Korbel *et al.*,1993).

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

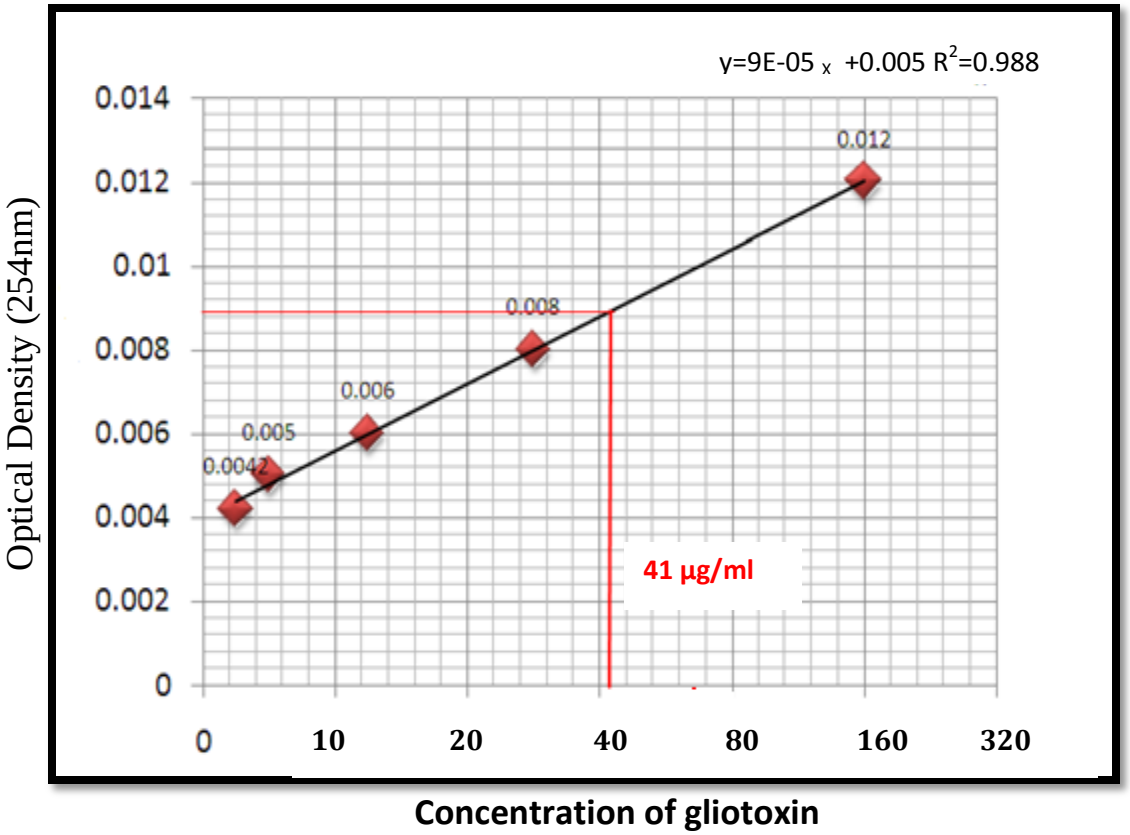

Figure 1: Gliotoxin standard curve.

Bauer *et al*.,(1989) had isolate Gliotoxin for the primary time from the naturally infected tissue, The GT level was (9.2 mg/ kg) and mention this level was about 100 times higher than the level that known to cause apoptosis 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.

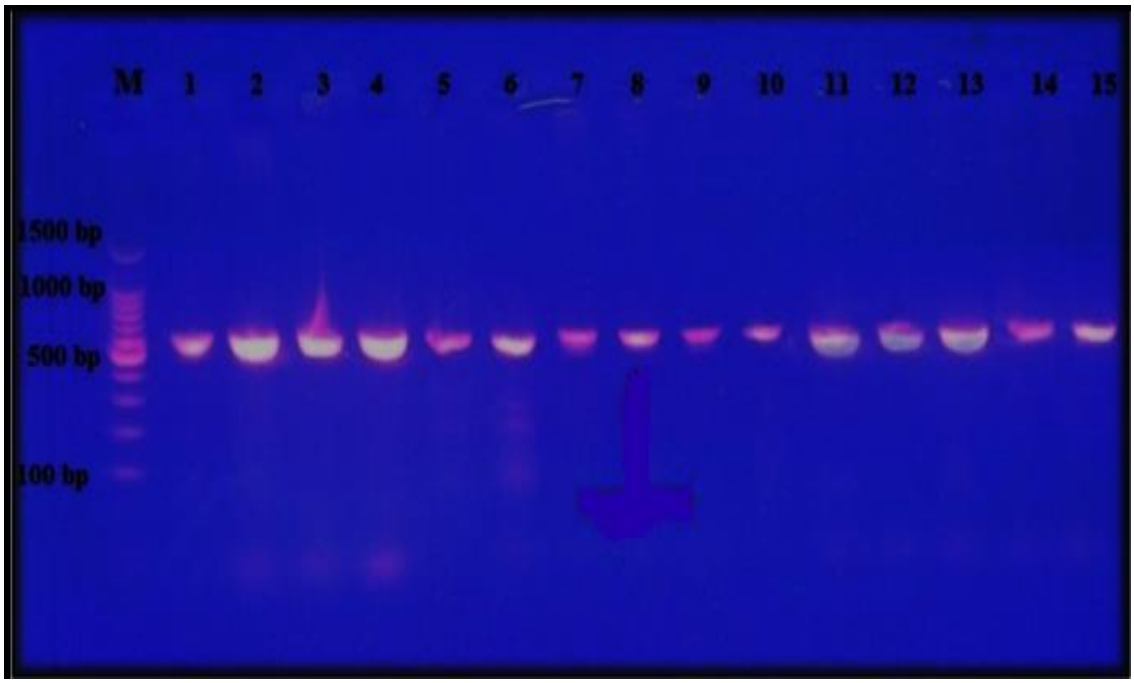

Figure (2) Electrophoresis of the amplified product of *gliz* in a 1 % agarose gel. Lane M, 1500bp DNA ladder; lanes 1 to 15, *A.fumigatus* isolates that contain *gliz* gene in size (600 bp) .

gliZ, a transcription factor which regulates gliotoxin biosynthesis, encoding a $Zn(11)_2$ cys_6 protein and located within the gliotoxin gene cluster(*gli* cluster) and is likely to be contributory in gliotoxin production (Gardiner and Howlett,2005) .Deletion of *gli*Z result in inability of *A.fumigatus* to produce GT and loss of gene expression of other *gli* cluster genes (Bok *etal*.,2006).

gliZ gene involved in metabolic pathways related to virulence of *A .fumigatus* appeared to be Up-regulated at 37C̊ when above fungus had high ability to grow in this temperature, this finding is critical factors to confirm the found of GT in human body who infected with *A .fumigatus* where human body temperature 37C˚. (Rementeria *etal*.,2005).

Sugui *et al*.,(2008) referred to *gliZ* is a positive regulator of *gliP* gene (which is a nonribosomal peptide synthase act on catalyses the first step of gliotoxin biosynthesis) and the over regulation of *gliZ* inhance the ability of fungus to produce GT ,So deletion of *gliZ* lead to loss the ability of fungus to produce of above toxin.

The study of Scharf *et al*., (2012) which amid to Biosynthesis and function of gliotoxin in *Aspergillus fumigatus* and detection the role of GT in pathogencity of this fungus reached to the culture supernatant of Wild-type of *A.fumagatus* enhanced apoptosis process in neutrophils of treated animals ,while supernatant of *gliZ* deletion mutants didn't induce apoptosis in neutrophils of another group of animals ,this result confirms an important role of GT in increase pathogenicity of *A. fumigatus* . However, loss of gliotoxin resulted in decreased toxicity as measured either by mast cell degranulation (Cramer *etal*.,2006) or macrophage/T-cell viability (Lewis *etal*.,2005), 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.

$TCGGN_3CCGA$ is the region which considers as A DNA binding site and has been proposed for *GliZ* , but has not been experimentally certain. This site is found within the promoter region of every gene within the gliotoxin cluster, Gliotoxin itself positively regulates expression of the genes within the gliotoxin cluster, (Gradiner and Howlett,2005).

In conclusion, More attention should be paid to *Aspergillus fumigatus* which had ability to produced gliotoxin that have multi toxic properties.

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