Sequencing of Gliotoxin Genes in Clinical and Environmental *Aspergillus fumigatus* isolates in Iraq

تتابع الحمض النووي لـ جينات Gliotoxin في فطر*Aspergillus fumigatus* المعزولة من مصادر سريرية وبيئية في العراق

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Abstract

Gliotoxin is an important virulence factors in *Aspergillus fumigatus*. The biosynthesis of this mycotoxin is regulated and expressed by the presence of *gliP* genes. This study aimed to identify *Aspergillus fumigatus* isolates in clinical and environmental sources with glip genes using conventional PCR and sequence. To achieve this, DNA was isolated from twenty *A. fumigatus* isolates using commercial kit. The range of the DNA extracted was 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 by using tailored primer. The results showed that all isolates had positive results to the primer and all isolates were able to produce gliotoxin. PCR detected the gliotoxin genes, *glip* in five isolates. The five PCR product samples were sent for sequence analysis and 25 µl (10 pmol) from the forward primer. The results of all the samples indicated have a single band of the desired product of *gliP* gene of *A.fumigatus* and the samples sent for sequencing related to molecular weight 190 bp.

Key words: *Aspergillus fumigatus*, gliotoxin, PCR Polymerase Chain Reaction, bp base pairs, DNA Deoxyribonucleic Acid

الملخص

Gliotoxin هو عامل سمي مهم في *Aspergillus fumigatus*. وينظم التخليق الحيوي لهذا السموم الفطرية ويعبر عنه وجود الجينات Glip. هدفت هذه الدراسة إلى التعرف على عزلات *A. fumigatus* في السلالات السريرية والبيئية مع جينات Glip باستخدام pcr التقليدي وارسلت لتحليل التسلسل . لتحقيق ذلك، تم عزل الحمض النووي من عشرين عينة *A. fumigatus* باستخدام عدة التجارية. كان العائد من الحمض النووي المستخرجة في نطاق 65-210 نانوغرام / ميكرولتر مع نقاء 1.5-1.9. تم التعرف على الأنواع لعزل*A. fumigatus* إلى خصوصية عالية باستخدام بادء خاص . وأظهرت النتائج أن جميع العزلات كانت نتائج إيجابية على بادء *Afumi*. وأظهرت النتائج أن خمس عزلات كانت قادرة على إنتاج Gliotoxin باستخدام pcr تم الكشف عن وجود جينات Gliotoxin و Glip في جميع العزلات. وأظهرت النتائج أن عائد من مجموعة واحدة من المنتج المطلوب من جين Glip من*A. fumigatus* العينات أرسلت للتسلسل المتعلقة الوزن الجزيئي 190 bp. تم إرسال خمسة عينات من المنتج pcr لتحليل التسلسل.

الكلمات الدالة: الفطر *Aspergillus*، gliotoxin، البلمرة، الدنا

Introduction

The filamentous fungus *A. fumigatus* is highly pathogenic and is responsible for approximately 90% of allinvasive aspergillosis infections [1] *A. fumigatus* cause pulmonary clinical forms of Aspergillosis disease spatially in immunocompromised patients or those undergoing immunosuppressive therapy prior to organ transplantation and may also cause invasive disease, the forms of pulmonary clinical forms are sap rophytic, allergic and invasive, 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 [2,3,4 and 5]. And [6] found the reasons for the virulence of *A. fumigatus* virulence to be linked to several secondary metabolites produced by *A. fumigatus* that play important roles in the pulmonary infection process.

Gliotoxin is a particularly important secondary metabolite of *A. fumigatus* [7,8], belonging to the chemical types of epipolythiodioxopiperazines (ETPs) which have immunosuppressive abilities through:

(i) Induction of apoptosis in macrophages and lung epithelial cells, (ii) inhibition of nuclear factor κ-B activation, and (iii) inhibition of phagocytosis [7,9]. [10] reported the Gliotoxin (GT) biosynthetic cluster which

directs gliotoxin production in the process of *A. fumigatus* infections via 13 of its genes [11]. Of these, *gliP* induce GT synthesis through catalysing the first biosynthetic step by encoding a non-ribosomal peptide synthase [12].

The aims of the work presented here were to identify *A. fumigatus* and to detect some of the gliotoxin genes using specific PCR and identify sequence of peptide synthase *gliP*.

Matereals and Methods

***Aspergillus fumigatus* growth conditions**

Twenty *A. fumigatus* isolates (obtained from the University of Baghdad/ Department of Biotechnology) were used in this study; 10 of them were from clinical and 10 were from 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).

The genomic DNA was extracted from the 20 *A. fumigatus* isolates using commercial kit. ZR Fungal/Bacterial DNA MiniPrep™ ,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 [13].

key word The concentration and purity of the isolated DNA samples were measured by the NanoDrop spectrophotometer before the performance of PCR, for DNA isolated by the commercial kit technique and by the manual technique. Nanodrop is highly sensitive and directly provides us with the concentration of DNA, A260/A280 ratio, and A260/A230 ratio.

Primer selection and PCR assay

Specific sequence primer was used, synthesized by (Korea) in a lyophilized form and were dissolved in sterile deionizer distilled water to give a final concentration of (100pmol/µl) as recommended by provider. The primer and this sequence is:

F AAACCCCTGTGAATGCAGAC

R CCCCTTGAGATGAAAGGTGA

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), using the following PCR reaction programme:- cycle of 5 min at 94 ˚C for initial strand separation, followed by 40 cycles of 1 min at 94 ˚C for denaturation and 45 s at 58 ˚C for annealing and 1 min 72 ˚C for primer extension. Finally, 1 cycle of 10 min at 72 ˚C was used for the final extension. This programme was used after optimization of cycling conditions.

Approximately 7 µl of amplified PCR products were separated by electrophoresis in 1% agarose gels (1X TBE buffer. Gel was run horizontally also in 1X TBE 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 [14]. Purification and concentration the product by Nanodrop after extraction process and send 5 sample to work sequence analysis

Sequencing and alignment of NCBI

Five PCR product samples were sent for sequence analysis; and 25 µl (10 pmol) from the forward primer. The samples were treated with AB13730XL APPLIED BIOSYSTEMS machine in national instrumentation center for environmental management NICM/USA company online at (http://nicem.snu.ac .kr/main/?en_skin=index.html). Then analyses by blast in the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism.

Results and discussion

DNA Extraction

The extraction of genomic DNA was done efficiently using a Reagent Genomic DNA Kit as show in figure (1) .The purity and concentration were measured using the standard method [13]. After end the DNA extraction should measurement the concentration and purity of DNA by Nanodrop, the result showed a concentration between (65-210) ng/µl with a purity of (1.5-1.9).

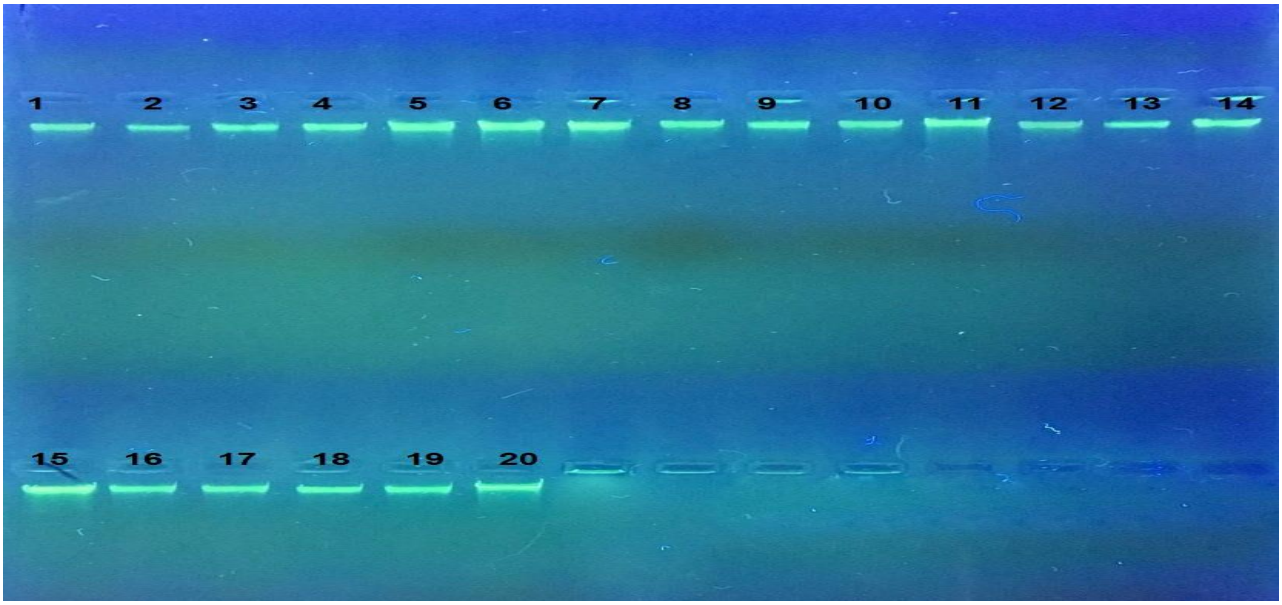

Fig. (1): Agarose gel electrophoresis of the total genomic DNA for *A. fumigates* isolates. Fragments were fractionated by electrophoresis on a 0.8% agarose gel visualized by U.V. light after staining with ethidium bromide.

PCR analysis

Polymerase Chain Reaction (PCR) for identification of gliotxine regulatory gene *gliP* in clinical and environmental *A. fumigates* isolates.

Through the use of PCR kit according to the company's instructions the interaction was done in volume 25µl. Taken 12.5 from Master Mix which consist of $MgCl_2$, dNTPs and Taq polymerase and this was used constant volume for 20 samples and complemented materials by optimal condition for *gliP* primer.

After a work several experiments to reach optimal condition for (Initial denaturation and annealing) and this condition show that, the temperature has changed through the work of (Gradient PCR) for all samples to select the optimal condition, and also the changed in the concentration for DNA template between (1.5-2µl) where is considered these two factors from important factors in primer annealing with complement. It has been added 1µl from Forward primer (F) 10 Pico mole concentration and same amount from Reverse primer (R), the same concentration and 2µl from DNA temple. During the work the temperature was used in PCR steps (95°C) to initial denaturation for (3) minutes to one cycle as for annealing temperature has adopted on gradient PCR were used several temperature in same time to shorten the time the temperatures were (57,58,59, and 60C) for 0.35 second depending on Garcia (2010) which 40 cycle, after two hours the reaction ended and the results deports electrically where have appeared the best result at the temperature 58°C.

Detection of gliotxine regulatory gene *gliP* in clinical and environmental *A. fumigates* isolates.

Polymerase Chain Reaction (PCR) was done for the detection of *gliP* gene region in 20 isolates of clinical and environmental *A. fumigatus*, all sample show positive for *gliP* gene region with PCR product 190bp, as show in figure (2).

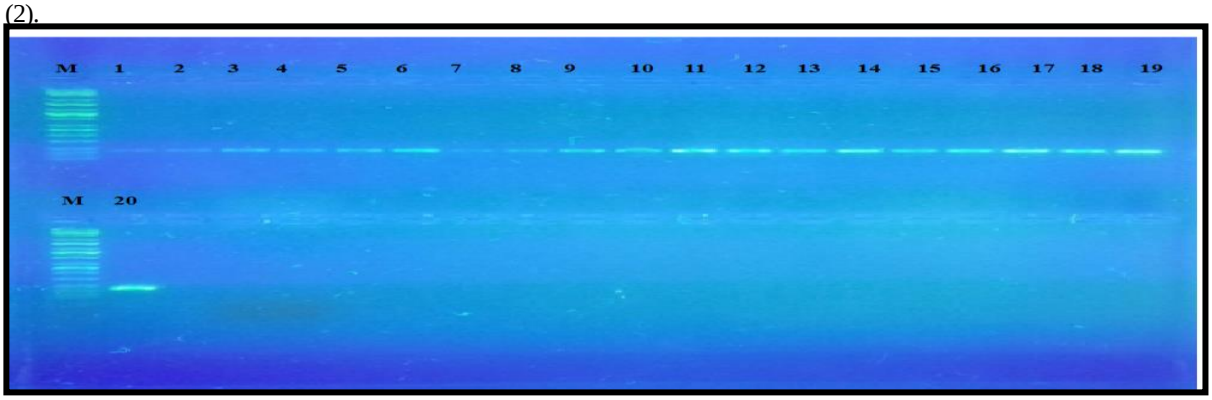

Fig. (2): PCR product for *gliP* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel visualized by U.V. light after staining with ethidium bromide. M: 100 bp DNA ladder.

Figure (1) shows that the genomic DNA of all isolates was recognized and complementary to *gliP* primer sequence and represented by presence of single band in molecular weight 190bp. The PCR yield was sharp intense single band of the desired product without primer dimmer. The results of this study demonstrated that *gliP* primer based PCR method had high sensitivity and specificity in detecting gliotoxin regulatory gene *gliP* in *A. fumigatus* isolated from clinical and environmental sources.

The glip gene (regulators gene) is 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 [9]. Since gliotoxin is known as the non-ribosomal peptide toxin [12]. The presence of this important gene is considered to underpin gliotoxin production in the most studied isolates the absence of gliP genes leads to the loss of gliotoxin production, as also shown by [9] who reported that deletion of the gliP gene in A. fumigatus resulted in abrogation of gliotoxin synthesis. Disruption of the *gliP* gene resulted in elimination of gliotoxin production, confirming the role of *gliP* in the biosynthesis of gliotoxin [12]. [15] Confirmed that deleting *gliP*, 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 [7], who reported that the Δ*gliP* mutant showed reduced virulence in two different mouse strains. [9] Likewise indicted the failure to induce apoptosis in mammalian cells, and a reduced ability to inhibit the oxidative burst in human neutrophils *in vitro* in the deleted *gliP* isolates.

While the absence of *gliP* genes seems to be strongly associated with loss of gliotoxin production capability [15].

Wang, D. (2014) [16] Suggested that, through its capacity to export gliotoxin extracellular, *glip* functions to protect the fungus from the harmful effects of extracellular gliotoxin, which strongly suggests that *glip* also contributes to protection from its own produced gliotoxin by constantly exporting the toxin. The disruption of *glip* caused the fungus to be highly susceptible to extracellular gliotoxin. Furthermore, the amount of gliotoxin was reduced, both in extracellular and intracellular spaces, which suggests that gliotoxin production was greatly reduced by the *glip* disruption. The *glip* gene, meanwhile, 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 [17].

Sequencing and alignment of NCBI

The results shown in Table (1) and Figure (3) indicated that a yield of single band of the desired product of *gliP* gene of *A. fumigatus* was obtained from 5 samples sent for sequencing related to molecular weight 190b. Five PCR product samples were sent for sequence analysis; of *A. fumigatus* isolated from clinical and environmental and 25 µl (10 pmol) from the forward primer. The result of the sequence analysis was analysed by blast in the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism and mutation in *gliP*, found 6 mutations in the five *A.fumigatus* isolates between one transversion (refers to the substitution of a (two ring) purine for a (one ring) pyrimidine) and five translation (a point mutation that changes a purine nucleotide to another purine (A↔G) or a pyrimidine nucleotide to another pyrimidine (C ↔ T)) and 0 deletion nucleotide.

Showed clinical *A.fumigatus* isolates 1 and 2 (100%) compatibility as shown in Table (1), and score (252 at 255) and expect (7e-63 at 6e-64) respectively with the wild type of *gliP* gene from Gene Bank as shown in Table (1) and Figure (3) and isolated from clinical source. While the clinical *A.fumigatus* isolate 3 showed (95%) compatibility as shown in Table (1), and score (100) and expect (2e-17).

The environmental *A.fumigatus* isolates number (12 and 13) showed 99% and 98% compatibility as shown in Table (1), and score (198 at 193) and expect (9e-47at 4e-45) respectively with the wild type of *gliP* gene from Gene Bank as shown in Table (1) and Figure (3) and isolated from clinical source. With the wild type of *gliP* gene from Gene Bank.

Table (1): Represent type of polymorphism in *gliP* Gene in clinical and environmental *A.fumigatus* isolates.

No.Of sampe	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities
1				5751 to 5886	ID: XM_745762.1	252	7e-63	100 %
2				5749 to 5886	ID: XM_745762.1	255	6e-64	100 %
3	Transition	5852	T>C	5824 to 5886	ID: XM_745762.1	100	2e-17	95%
	Transversion	5863	A>C					
	Transition	5873	T>C					
12	Transition	5789	C>T	5777 to 5886	ID: XM_745762.1	198	9e-47	99%
13	Transition	5789	C>T	5777 to 5886	ID: XM_745762.1	193	4e-45	98%
	Transition	5865	G>A					

1

Aspergillus fumigatus Af293 nonribosomal peptide synthase GliP (AFUA_6G09660), partial mRNA
Sequence ID: XM_745762.1Length: 6408Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
252 bits(136)	7e-63	136/136(100%)	0/136(0%)	Plus/Plus

```
Query 12   GTCTACGCGTGCAGCGAGCCAGGCAGCCGTCTGCAATTCCATTCCATTCGAGCAGGTCCT   71
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5751 GTCTACGCGTGCAGCGAGCCAGGCAGCCGTCTGCAATTCCATTCCATTCGAGCAGGTCCT 5810
Query 72   GAACCTCCTCCACCTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTCAC   131
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5811 GAACCTCCTCCACCTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTCAC 5870
Query 132  CTTTCATCTCAAGGGG 147
           ||||||||||||||||
Sbjct 5871 CTTTCATCTCAAGGGG 5886
```

2

Aspergillus fumigatus Af293 nonribosomal peptide synthase GliP (AFUA_6G09660), partial mRNA
Sequence ID: XM_745762.1Length: 6408Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
255 bits(138)	6e-64	138/138(100%)	0/138(0%)	Plus/Plus

```
Query 10   CAGTCTACGCGTGCAGCGAGCCAGGCAGCCGTCTGCAATTCCATTCCATTCGAGCAGGTC   69
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5749 CAGTCTACGCGTGCAGCGAGCCAGGCAGCCGTCTGCAATTCCATTCCATTCGAGCAGGTC 5808
Query 70   CTGAACCTCCTCCACCTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTC   129
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5809 CTGAACCTCCTCCACCTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTC 5868

Query 130  ACCTTTCATCTCAAGGGG 147
           ||||||||||||||||||
Sbjct 5869 ACCTTTCATCTCAAGGGG 5886
```

3

Aspergillus fumigatus Af293 nonribosomal peptide synthase GliP (AFUA_6G09660), partial mRNA
Sequence ID: XM_745762.1Length: 6408Number of Matches: 1

See 1 more title(s)

Related Information
Gene-associated gene details
Map Viewer-aligned genomic context
Range 1: 5824 to 5886GenBankGraphicsNext Match Previous Match

Score	Expect	Identities	Gaps	Strand
100 bits(54)	2e-17	60/63(95%)	0/63(0%)	Plus/Plus

```
Query 1    CTGCCGCGGACCATCCGGCAACACCCGCCGTTCGAAGCCCTGGTCACCTCTCATCTCAAG  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5824 CTGCCGCGGACCATCCGGCAACACCCGCTGTTCGAAGCCATGGTCACCTTTCATCTCAAG  5883
Query 61   GGG  63
           |||
Sbjct 5884 GGG  5886
```

12
Aspergillus fumigatus Af293 nonribosomal peptide synthase GliP (AFUA_6G09660), partial mRNA
Sequence ID: XM_745762.1Length: 6408Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
198 bits(107)	9e-47	109/110(99%)	0/110(0%)	Plus/Plus

```
Query 1    CCGTCTGCAATTTCATTCCATTCGAGCAGGTCCTGAACCTCCTCCACCTGCCGCGGACCA  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5777 CCGTCTGCAATTCCATTCCATTCGAGCAGGTCCTGAACCTCCTCCACCTGCCGCGGACCA  5836
Query 61   TCCGGCAACACCCGCTGTTCGAAGCCATGGTCACCTTTCATCTCAAGGGG  110
           ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5837 TCCGGCAACACCCGCTGTTCGAAGCCATGGTCACCTTTCATCTCAAGGGG  5886
```

13
Aspergillus fumigatus Af293 nonribosomal peptide synthase GliP (AFUA_6G09660), partial mRNA
Sequence ID: XM_745762.1Length: 6408Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
193 bits(104)	4e-45	108/110(98%)	0/110(0%)	Plus/Plus

```
Query 1    CCGTCTGCAATTTCATTCCATTCGAGCAGGTCCTGAACCTCCTCCACCTGCCGCGGACCA  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5777 CCGTCTGCAATTCCATTCCATTCGAGCAGGTCCTGAACCTCCTCCACCTGCCGCGGACCA  5836
Query 61   TCCGGCAACACCCGCTGTTCGAAGCCATAGTCACCTTTCATCTCAAGGGG  110
           ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 5837 TCCGGCAACACCCGCTGTTCGAAGCCATGGTCACCTTTCATCTCAAGGGG  5886
```

Fig (3): Sequencing of sense flanking the partial *gliP* gene in *Aspergillus fumigates* compared with standard *gliP*, obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

References

1. Dagenasis, A.O., Keller, N. (2009). Pulmonary aspergillosis. Clin Infect Dis. 33: 740-752; quiz 806-795.
2. Denning, D.W., (1998). Invasive aspergillosis. Clinical Infectious Diseases. 26: 781–803.
3. Denning, D.W. (1996a). Aspergillosis: diagnosis and treatment. Int. J. Antimicrob Agents. 6, 161–168.
4. Denning, D.W. (1996b). Therapeutic outcome in invasive aspergillosis. Clin Infect Dis. 23, 608–615.
5. Walsh, T. J., and Groll, A. H. (2001). Overview: non-*fumigatus* species of *Aspergillus*: perspectives on emerging pathogens in immunocompromised hosts. Curr. Opin. Investig. Drugs 2:1366–1367.
6. Ben-Ami, R., Lewis, R.E., Leventakos, K., Kontoyiannis, D.P. (2009). *Aspergillus fumigatus* inhibits angiogenesis through the production of gliotoxin and other secondary metabolites. Blood; 114.
7. Kupfahl, C., Michalka, A., Lass-Fl, ¨ orl C. (2008). Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. Int J Med Microbiol. 298: 319–327.
8. Nouri, M. A. (2015). Study Toxicity effects of Gliotoxin produced by *Aspergillus fumigatus* using solid state fermentation. Msc. Thesis. College of Science, University of Baghdad. Iraq.
9. Sugui J. A., Pardo, J., Chang, Y.C. (2007). Gliotoxin is a virulence factor of *Aspergillus fumigatus*: *gliP* deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot Cell. 6: 1562–1569.

10. Gardiner, D. M., Cozijnsen, A. J., Wilson, L. M., Pedras, M. S. and Howlett, B. J. (2004). "The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus Leptosphaeria maculans." Mol Microbiol. 53(5): 1307-18.

11. Schrettl, M., Carberry, S., Kavanagh, K., Haas, H., Jones, G.W., O'Brien, J., Stephens, J., Fenelon, O., Nolan, A., and Doyle, S. (2010). Self-protection against gliotoxin—a component of the gliotoxin biosynthetic cluster, GliT, completely protects Aspergillus fumigatus against exogenous gliotoxin. PLoS Pathog. 6, e1000952.

12. Cramer, R.A., Jr., Gamcsik, M.P., Brooking, R.M., Najvar, L.K., Kirkpatrick, W.R., Patterson, T.F., et al. (2006). Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. Eukaryot. Cell 5, 972–980.

13. Sambrook, J., Friteg, E. F. and Maniatis, T. (1989). Molecular cloning: alaboratory. New York, USA.

14. Barrios-González, J., Fernández, F. J., Tomasini, A. and Mejía, A. (2005). Secondary metabolites production by solid-state fermentation. Malays J Microbiol. (1):1–6.

15. Bok, J.W., Balajee, S.A., Marr, K.A., Andes, D., Nielsen, K.F., Frisvad, J.C., Keller, N.P. (2005). Lae A., a regulator of morphogenetic fungal virulence factors. Eukaryotic Cell 4: 1574–1582.

16. Wang, D., Toyotome, T., Muraosa, Y., Watanabe, A., Wuren, T., Bunsupa, S., Aoyagi, L. K., Yamazaki, M., Takino, M. and Kamei, K. (2014). GliA in Aspergillus fumigatus is required for its tolerance to gliotoxin and affects the amount of extracellular and intracellular gliotoxin. Med Mycol. 52: 504–516.

17. Scharf, D. H., Chankhamjon, P., Scherlach, K., Heinekamp, T., Willing, K., Brakhage, A.A., Hertweck, C. (2013). Epidithiodiketopiperazine Biosynthesis: A four-enzyme cascade converts glutathione conjugates into Transannular Disulfide Bridges. Angew. Chem. Int. Ed.52: 1 – 5.