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# Identification study some virulence factors of invasive mold infections isolated from patients undergoing chemotherapy in Tikrit teaching Hospital

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**ABSTRACT**

Sixty two (62) samples were collected from out and inpatients admitted to Tikrit teaching hospital in Tikrit city. these specimens were collected from sputum of immunocopromised patients of both sexes and different ages.

19(30.6%) isolates from patients sputum, were infections more *A. fumigatus* (12.9%), *A. terreus* (6.5%), and *P. sp* (3.3%) while were (1.6%) of *A. niger, A. flavus, F. solani, P. chrysogenum* and *A. alternata*. The isolated were identified according morphological and Cultural characteristic.

It emerges from the study that fungal isolates produced various virulence factors since, hemolysin, protease and phospolypase production were produced in *A. fumigatus* (62.5%) hemolysin and phospholipase production, and (87.5%) protease production, while *A. terreus* protease and phospholypase production were (50%) and (25%) of hemolysin production, while *A. flavus, P. chrysogenum* and *A. alternata* isolates were no produced protease. *P. chrysogenum* were produce only hemolysin (100%). All isolates of *A. niger*, *F. solani* and *A. alternata* were phospolypase production (100%).

## INTRODUCTION

Fungi are ubiquitous in the environment. Fungi that are part of the normal flora, or that are wide spread in the environment we are constantly exposed to fungal spores (conidia), and we inhale on a regular basis and are normally harmless to immunocompetent individuals. Some groups of fungi are pathogenic to humans and require control measures. Human fungal pathogens belong to four main groups, namely zygomcetes, ascomycetes, deuteromycetes, and basidiomycetes (Chakrabarti, 2005; Reedy *et al.*, 2007).

Invasive fungal infection (IFI) is increasingly being recognized as a significant cause of morbidity and mortality in immunosuppressed patients (Garcia-Vidal *et al.*, 2013).

Immunocompromised patients who are less capable individuals of battling infections because of an immune response that is not properly functioning (Christoph, 1991).

Invasive infections due to filamentous fungi, such as *Aspergillus* spp., pencillium sp., *Fusarium* spp. and *Alternaria* sp. cause significant morbidity and mortality in immunocompromised patients with hematological malignancies, recipients of hematopoietic stem cell transplants and those with chronic granulomatous disease (Antachopoulos *et al*., 2012).

Fungi are able to cause a disease and to overwhelm the host defense systems because of possessing several genes and proteins associated with their pathogenicity, called virulence factors (Tomee & Kauffman, 2000). Many of the putative fungal virulence factors have developed naturally during organism evolution and originally acted as a defense against unfavorable environmental conditions, and then, in this way, many of them became important as virulence factors facilitating infection (Casadevall & Pirofski, 2000), this study aimed to:

- Isolation and identification of mold infection from immunocompromised patients.
- Screening the ability of mold isolates to produce, protease, hemolysin and phospholypase as a virulence factors.

## MATERIAL AND METHOD

1- Samples Collection: Sputum and blood samples were collected from 62 patients undergoing chemotherapy within age range "11-83 years old", were during chemotherapy for different types of cancer attending Tikrit Teaching Hospital from January 2013 to Augusto 2013 were enrolled in this study.

2- Direct Examination: Specimen were placed on a microscopic slide, with the few drops of 10% KOH, a cover slip added and warmed over a small flame just before boiling. The slide was examined under the low power and high dry objectives to detect fungi and their septet hyphae (Emmons *et al*., 1977).

3- Samples Culturing: Sputum samples were cultured on sabouraud dextrose agar (SDA) supplemented with 0.04 mg/ml chloramphenicol to inhibit the growth of bacteria, then incubated at 28°C and 37°C and examined for 10 days (Midgley *et al*., 1997).

4- Criteria Of Growth Identification: All isolates were identified to the species level on the basis of macromorphological and micromorphological characteristics using SDA, and Scotch tape preparation.

**Identification of the growth depends on the following:**

1- Colony characteristics (Color, Consistency and Topography).

2- Colony Reverses (Color, Significant pigment).

3-Microscopic Morphology (Microconidia and Macroconidia: their Size, Shape, Arrangement, and Hyphal Structures). (Atlas *et al.*, 1995).

4. Haemolysin Activity test: Determination of haemolysin activity was evaluated with a blood plate assay according Manns *et al.,* 1994

5. Protease Production test: The protease production was determined according to Aoki 1990 using a test medium consisting of SDA plates containing bovine serum albumin (BSA).

6. Phospholipase Production: Phospholipase activity assays were performed according to (Price *et al., 1982*).

## RESULTS AND DISCUSSIO

Results fungal isolates in patients undergoing chemotherapy at tikrit teaching hospital were 19(30.6%) positive from total examined patients.

Sputum samples results showed that *A. fumigatus* were most frequently isolated (12.9%), followed by *P.* sp. (9.6%), *A. terreus* (9.%), and *Penicillium* sp. (3.3%) all each for them , *A. niger, A. flavus* and *P. chrysogenium F. solani* and *A. alternata* (1.6%) were frequently less isolated recoding , as illustrated in Table (1).

Table 1: Number and percentage of identified fungal isolates from sputum culture samples.

| Fungal isolates | Number of isolates | % |
|---|---|---|
| *fumigatus* | 8 | 12.9 |
| *niger* | 1 | 1.6 |
| *terreus* | 4 | 6.5 |
| *flavus* | 1 | 1.6 |
| *P. chrysogenium* | 1 | 1.6 |
| *F. solani* | 1 | 1.6 |
| *alternata* | 1 | 1.6 |
| *P. sp* | 2 | 3.3 |
| *No growth* | 19 | 63.4 |
| *Total* | 62 | 100 |

Our results for sputum culture showed the *Aspergillus* spp. were more 14(22.5%) out of 62 specimen.

There were many studies which have been conducted on opportunistic fungi in immunocompromesid patients. One of these studies was Manahil *et al.*, (2011) in mosul who was isolated Four species of the genus *Aspergillus* were detected from sputum in immunocompromised patients. The total isolates were 9(4.5%), 4 of them were *A. flavus*, 2 *A. fumigatus*, 2 *A. niger*, and 1 *A. terreus*. *Penicillium* spp. 3(1.5%), were *Fusarium* spp. 1(0.5%).

As repoeted Al-Khalidi and Faraj (2012) (56.66%) isolates of sputum were that isolated included *Aspergillus niger* and *A. fumigatus* 3out of 34(8.82%) for each one, *A. flavus, Penicillium* spp. 2 out of 34 (5.88%) for each one and *A. alternate* 1 out of 34 (2.94%).

In other study were 26 (27%) of *C. albicans* and were 29 (30%) non albicans spp., *Aspergillus* spp. 6.25% were *A. fumigatus* and *A. niger* each of them, were 1% of *A. flavus* and followed by *Penicillium* spp. in 6 (6.25%) including two *P*. mar *neffei isolates* (Bharathi and Usha., 2011).

The reason for these variations in all studies may be due to sample size, environment factors, nutrition requirements and virulence factors of this fungi (Cooke *et al.*, 2009), and methods used for isolation.

It is not easy to determine the pathogenic role of fungal isolates from the respiratory tract, to differentiate between infections, colonization and contamination (Paradowski, 1997). However, the prevalence and prognosis of pulmonary fungal infection is difficult to be evaluated since diagnosis was seldom confirmed (Chen *et al.*, 2001).

An important factor contributing to the increasing incidence of infection by fungi that have not been previously described to be pathogenic, is the rise in numbers of immunocompromised patients who are susceptible hosts for the most uncommon microbial agents (Disalvo., 2005).

Mentioned that Invasive *Aspergillus* is an opportunistic fungus, the immunocopromised patients are more susceptible for its colonization. Whether infection (aspergillosis) could happen, it is a matter of balance between the immunity of the person versus the pathogenicity of the organism. Denning and Coworker *et al*. (2003).

**2. Identification of molds isolates:**

The isolates identified performed a morphological characterization using the coloration on SDA as indicating that each species has a specific color and For descriptions of species, and additional information see Samson and Pitt (1990), de Hoog *et al.* (2000)and Ellis *et al*.(2007).

***A: Identifection of A. alternate:***

***A. alternate*** was able to grow and form colonies on SDA media in a moderate growth rate. The colony was felt , the color at the first seem off-white to be to then turning to dark brown and changed to grey-brownish, reversed side of the colonies appeared pale yellow to tan Figures (4-5A,B,C).

Microscopic examination as shown in Figure (1) multicelled, septate and irregularly branched. Conidiophores arised singly or in clusters, usually were long or short. They were pale olivaceous to, conidia were brown with a short, cylindrical beak, and form long and profusely branched chains (ten or more conidia). Similar results were reported by Wellman (1949), Ghosh (1998) and Nagrale *et al.* (2012).

**B: Identification of *Aspergillus* species:**

The isolates showed different apparent morphological characteristics, mainly in the color of the culture medium and For descriptions of species showed Table (2) and the Figures (2 to 5) shows image which *Aspergillus species* isolates during this study The present findings are in conformity with the result of earlier workers Nelson *et al*. Nelson *et al*. (1983), Samson and Pitt (1990), de Hoog *et al.,* (2000) and Ellis *et al.*, (2007).

Table 2: Morphology and Microscope feature of *Aspergillus* species.

| Species | Surface | Reverse | Conidiophore | Phialides | Vesicle |
|---|---|---|---|---|---|
| ***A. flavus*** | Yellow-green | Coldish to red brown | Colourless, rough | Uniseriate or biseriate | Round, radiate head |
| ***A. fumigatus*** | Blue-green to gray | White to tan | Short, smooth, colourless or greenish | Uniseriate | Round, columnar head |
| ***A. niger*** | Black | White to yellow | Long, smooth, colourless or brown | Biseriate | Round, radiate head |
| ***A. terreus*** | Cinnamon to brown | White to brown | Short, smooth, colourless | Biseriate | Round, compactly columnar head |

**C: Identification of *F. solani*:**

Morphotype produced aerial to abundant cottony mycelium with pale white to cream, brown-greenish to white-greenish aerial mycelium, and the pigmentations were pale brown to yellowish brown with a dark brown zonation.

Microscope examinations showed Figure (6) Microconidia are usually abundant, cylindrical to oval, one-to two-celled and formed from long lateral macroconidial septation from 3 to 5 from short multi-branched.

**D: Identification of *P. chrysogenum:***

The colonies on SDA Medially grown. The colonies were velvety and sulcate, with blue-green in colour. The reverse side of the colonies was yellow, as shown in Figure (7).

Microscopically, penicilli were terverticillate and the conidia were spherical to elliptical in shape. Conidia were smooth and had a green colour reflection. The results agree with other studies such as, Justen *et al.*, (1998) and we found ability isolate to growth at 37$^{o}$C that was in a agreement with Rafi and Rahman (2002) but do not agree with Florey *et al*. (1949) and Singh *et al*. (1991).

1- **Screening of some fungi isolates for virulent factors.**

In order to select the efficient isolate for some virulent factors production, screening of fungal isolates had been achieved on specific media.

**A: Screening of some fungi isolates for protease production.**

In this study, the extracellular protease production of *A. fumigtus* (87.5%), (50%) of *A. turreus* and (100%) of *A. niger* while none *A. favus* isolate produce protease , which is in agreement with (Theeb *et al*., 2013) who stated that hemolysin is produced by (95.1%) from 41 *A. fumigatus* isolates. Also, In Another study connected by Aboul-Nasr *et al*. (2013) who found 20 (80%) of *A. falvus*, 16(62%) of *A. fumigatus*, 19(79%) and 17(65%) of *fusarium solani*, as shown in Table (3).

Table 3: test protease Production by fungal isolates.

| Fungal isolates | Number isolates | Protease | |
|---|---|---|---|
| | | No | % |
| *A. fumigatus* | 8 | 7 | 87.5 |
| *A. terreus* | 4 | 2 | 50 |
| *A. niger* | 1 | 1 | 100 |
| *A. flavus* | 1 | 0 | 0 |
| *F. solani* | 1 | 1 | 100 |
| *A. alternata* | 1 | 0 | 0 |
| *P. chrysogenum* | 1 | 0 | 0 |

In this study, the extracellular protease production of *A. fumigtus* (87.5%), (50%) of *A. turreus* and (100%) of *A. niger* while none *A. favus* isolate produce protease , which is in agreement with (Theeb *et al*., 2013) who stated that hemolysin is produced by (95.1%)from 41 *A. fumigatus* isolates Also, In Another study connected by Aboul-Nasr *et al.* (2013) who found 20(80%) of A. *falvus*, 16(62%0) of *A. fumigatus*, 19(79%) and 17(65%) of *fusarium solani*.

Salyers and Witt (1994) reported that microbial cells secrete hydrolytic enzymes that destroy the constituents of host cell membranes leading to membrane dysfunction, physical disruption as well as aid in the invasion of host tissues. Proteolytic degradation of lung tissues has been suggested as one of the key events involved in the physiopathology of *A. fumigatus* (Kothary *et al.*, 1984).

**B: Screening of fungi isolates for hemolysis production:**

Our results indicated that (62.5%) of *A. fumigatus*, (25%) of *A. terreus*, then *A. niger*, *A. flavus and P. chrysogenum were* (100%) for each of them and (50%) of *F. solani*., as shown in Table 4 and Figure (8). Which are In agreement with the study of Aboul-Nasr *et al.* (2013) who found 20(95%) of *A. flavus*, 16(75%) of *A. fumigatus*, 16(68.5%) of *A. niger* and 17(41.2%) of F.solani .However in other study it has been found that (73.1%) *P. chrysogenum* were produces Hemolysin (Rossoni *et al.,* 2013).

Table 4: hemolysin Production some by some fungal isolates.

| Fungal isolates | Number isolates | Hemolysis | |
|---|---|---|---|
| | | No | % |
| *A. fumigatus* | 8 | 5 | 62.5 |
| *A. terreus* | 4 | 1 | 25 |
| *A. niger* | 1 | 1 | 100 |
| *A. flavus* | 1 | 1 | 100 |
| *F. solani* | 2 | 1 | 50 |
| *A. alternata* | 1 | 0 | 0 |
| *p. chrysogenum* | 1 | 1 | 100 |

These differences are possible to be due to the environmental conditions, the source of isolate and the detection method that was used in the detection of the enzyme.

Hemolysin lyses RBCs by creating pores or holes in red blood cell membranes resulting in the release of iron that promotes microbial growth (Bullen, 1981; Almeida *et al.*, 2009).

**C: Screening of fungal isolates for phospholipase production:**

Results showed that (62.5%) *A. fumigatus*, (50%) of *A. terreus* and (100%) of *A. niger, F. solani* and *A. alternata* of each for them isolates were positive for phospholipase production, as shown in Table (5).

Table 5: Production of phospholypase by fungal isolates.

| Fungal isolates | Number isolates | Phospholipase | |
|---|---|---|---|
| | | No | % |
| *A.fumigatus* | 8 | 5 | 62.5 |
| *A.terreus* | 4 | 2 | 50 |
| *A.niger* | 1 | 1 | 100 |
| *A.flavus* | ١ | 0 | 0 |
| *F.solani* | 1 | 1 | 100 |
| *A.alternata* | 1 | 1 | 100 |
| *p. chrysogenum* | 1 | 0 | 0 |

Our results were relatively agreed with the results of Birinci *et al*. (2014) was detected of phospholipase activity in 30(93.3%) of *A. fumigatus*, 4(25%) and None of nine *A. flavus* isolates exhibited phospholipase activity.

Phospholipases may enhance pathogenesis of invasive fungi by hydrolysing phospholipids in the membrane lipid bilayer of epithelial and endothelial cells. Phospholipids in mammalian cell membranes and in lung surfactant are preferred substrates for secreted phospholipase B from *C. albicance* and hence are a potential site of membrane attack (Chen *et al*., 2000).

Reports from different countries show that there are more different phospholipase activities in different regions **(**Zarei *et al*., 2010) Borst and Fluit (2003) believed that virulence factors could be associated with geographical region and infection type.

Probably variable levels of phospholipase activity in our study are related to source of fungal isolates (Oksuz *et al*. 2007), Borst and Fluit (2003) also found different phospholipase activity between samples originated from urine, blood and wound.

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Fig. 1: Morpholpogy of *A.alternata* grown on SDA at 37°C A: the Colony morphology after 10 days top view B: Colony morphology after 10 days reversed view C: Microscopic morphology (40x).

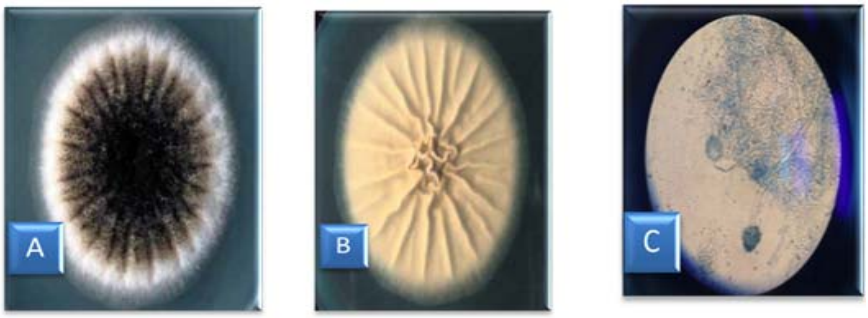


Fig. 2: Culture morphology of *A. niger* grown on SDA after 5 days at 37C A: Top view B: Reversed view C: Microscopic morphology of *A. niger* stained by gram stain (40x)


Fig. 3: Morphology of *A. terreus* grown on SDA at 37C A: The colony morphology after 5 days Top view B: The colony morphology after 5 days Reversed view C: Microscope morphology of *A. terreus* staining by LPCB stain (40x).

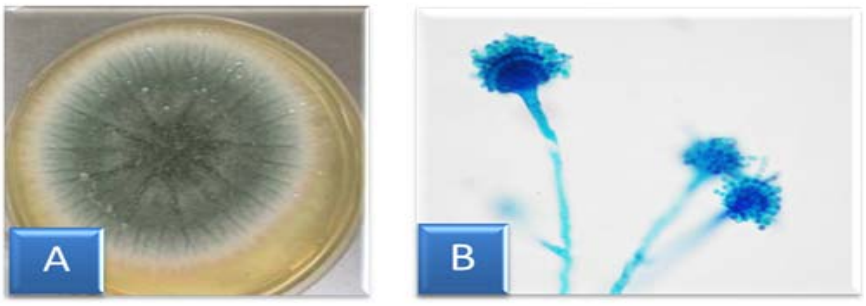


Fig. 4: A: the colony morphology of *A. fumigatus* grown on SDA at $37^0$C B: microscope feature of *A. fumigatus* staining by LPCB stain (40x).

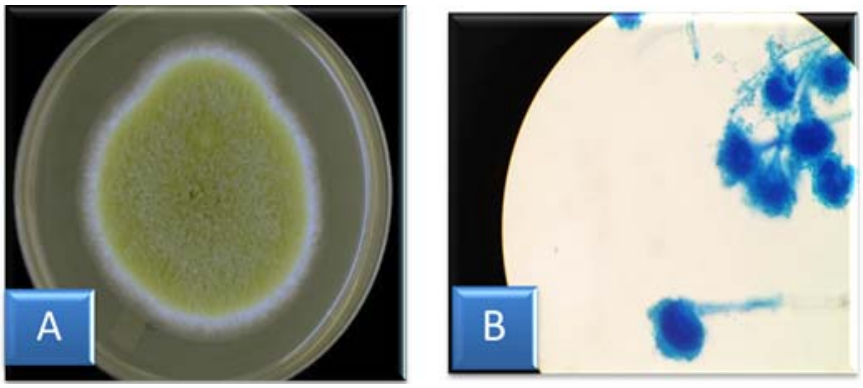


Fig. 5: A: the colony morphology of *A. flavus* grown on SDA at $37^0$C B: Microscope morphology of mycelium staining by LPCB stain (40x).

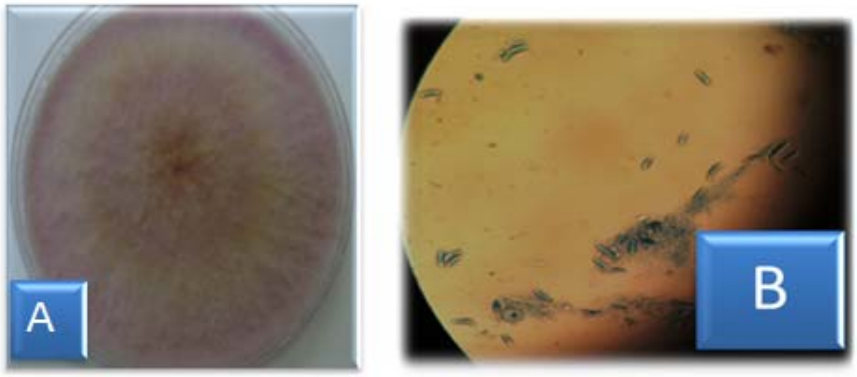


Fig. 6: A: Culture morphology of *F. solani* grown on SDA after 7 days Microscope morphology of *F. solani* grown on SDA at $37^o$C on SDA staining by LPCT stain (40x).

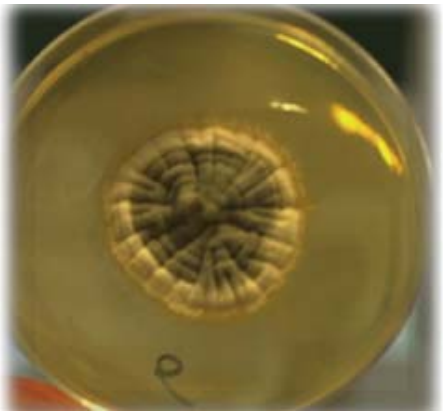

Fig. 7: Culture morphology of *P. chrysogenum* grown on SDA after 7 days.

## ARABIC SUMMARY

**دراسة تحري عن بعض عوامل الضراوة من الاعفان الغازية المعزولة في المرضى الخاضعين للعلاج الكيمياوي في مستشفى تكريت التعليمي.**

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تم جمع ٦٢ عينه قشع من المرضى الراقدين والمراجعين لمستشفى تكريت التعليمي من كلا الجنسين ومن مختلف الاعمار ضمن هذه الدراسة.

كانت نسبه الإصابة 19(30.6%) عزل من القشع ، كانت اعلى نسبه اصابات هي *A. fumigatus* بنسبه (12.9%) وكانت *A. terreus* بنسبه (6.5), %) و*P. sp* بنسبه (٣.٣%) . بينما كانت العزلات من *A. niger* و *A. flavus* , *F. solan* و *P. chrysogenum* و *A. alternata* بنسبه (١.٦ %) لكل منها حيث تم تشخيص العزلات وفقا لخواصها الشكلية والمز رعيه.

تم دراسة عوامل الضراوة متنوعه للفطريات المعزولة مثل انتاج hemolysin و protease و phospolypase كانت الانتاج في *A. fumigatus* بنسبه (62.8%) لانتاج كل من hemolysin و phospholipase وبنسبة (87.5%) منتجة لل hemolysin ، بينما *A. terreus* كانت منتجه لل phospholipase و protease في بنسبه (٥٠%) و انتاجها hemolysin كانت بنسبه (٢٥%) ، بينما عزلات *A. flavus* و *P. chrysogenum* و A. alternata كانت غيلر منتجه لل protease بينما كل عزلات *A. niger* و *F. solani* و *A. alternata* كانت منتجه phospolypase بنسبه (١٠٠% ).