RESEARCH ARTICLE

Detection of *Aspergillus fumigatus* by Polymerase Chain Reaction

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SUMMARY

Aspergillosis refers to fungi infections of the respiratory tract caused by Aspergillus species, especially *Aspergillus fumigatus*. Infection of *A. fumigatus* was increased in the last few years due to either resistances to antibiotics or the influence of other factors such as other fungal infections. The present study aimed to review the impact of *Aspergillus fumigatus* in Aspergillosis cases, and study the role of Singleplex Polymerase Chain Reaction (PCR) for amplification of internal transcribed space (ITS1), ITS4 of ribosomal RNA (rRNA) gene in the detection of fungal isolate. In this study, One hundred sputum samples were collected from patients admitted to the specialize chest and respiratory diseases center / Baghdad who were suffering from respiratory problems. During these studied, molds were isolation and identification based on Conventional method (Direct microscopy by using 10% KOH, and fungal culture was done on Sabouraud Dextrose agar supplemented with chloramphenicol and on Czapek-Dox agar incubated at 37°C and examined for 3–7 days then macroscopic, microscopic examination of the colony by(lactophenol cotton blue stain) and molecular methods by using Polymerase chain reaction (PCR) technique for identification. The 10% KOH examination was positive for 35 cases, while laboratory culturing was positive for 53 cases. *Aspergillus sp* were isolated from 44 (83%) patients; A. *fumigatus* was isolated in 23 (42.4%) patients while *A. flavus, A. niger,* and *A. terreus* were isolated from 11 (20.08%), (13.2%) and 3 (5.7%) patients respectively, also isolated *Penicillium* spp. at percentage 1(1.9%). In this study. The ages of participants ranged from 10-70years with a mean age of 34years, the males were more susceptible to fungal infection, were recorded 35/53 (66.3), compared to females were 18/53 (33.96). The infection of fungi was more prevalent in ages 30-40recorded 26(53.06%) followed by ages 40-50, 13(26.5), while the lowest infection recorded in the age group 10- 20 years was 2(2.04%). DNA isolated from twenty-three A. fumigatus isolates was used as a template, and the specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of internal transcribed spacer (ITS) region of the rRNA gene for *Aspergillus fumigates*. The results of the PCR amplification of the rRNA gene showed that this gene was present in 19 samples out 23 positive samples which isolation with a PCR product size of approximated 385 bp, while 4 samples out 23 positive samples showed negative results for the presence of this gene as indicated by the absence of the PCR products in their relevant lanes. Statistical analysis revealed that the PCR to have a sensitivity of 95.1% in the detection of *Aspergillus fumigatus* in Aspergillosis cases. Polymerase chain reaction (PCR) is a rapid, specific, and sensitive method to detect *Aspergillus fumigatus* in aspergillosis cases of humans.

Keywords: Aspergillosis*, Aspergillus fumigatus*, PCR.

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INTRODUCTION

Aspergillosis is a pulmonary disease found worldwide that results from hypersensitivity to *Aspergillus* antigens. It is known to occur in 1–2% of patients with asthma and 6–25% of patients with cystic fibrosis.[1] This disease may be caused by a number of *Aspergillus* species, which are saprophytic, thermotolerant fungi that are ubiquitous in the air and environment.[2] The genus *Aspergillus*, is a member of the Trichocomaceae family, Order Eurotiales, Class Eurotiomycetes, Phylum Ascomycota. *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in humans and animals. *Aspergillus* causes diseases of the respiratory system by the inhalation of *Aspergillus* conidia. The clinical manifestations of pulmonary Aspergillosis are many ranging from harmless saprophytic colonization to acute invasive disease.[3-6] *Aspergillus* spp. are rapidly growing mold with septate hyphae. Many have highly colored colonies ranging from bluish-green through yellow to black due to the profuse production of pigment spores conidia.[7] Among these*, Aspergillus fumigatus* is the most common species isolated, followed by *Aspergillus flavus*, *Aspergillus niger, and A. terreus*.[8-10] *Aspergillus fumigates* was firstly described in 1863 by physician Georg W. Fresenius from his studies on isolates obtained from human lung infections.[11] This mold is a filamentous saprotrophic widespread, typically found in soil and decaying organic matter where it plays an essential

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role in carbon and nitrogen recycling.[10,12] A. fumigatus is one of the most common species to cause disease in individuals with an immunodeficiency.[13] The Pathogenicity of *A. fumigatus* depends on immune status of patients and fungal strain, which can produce a wide variety of virulence factors like toxins, enzymes as well as the factors that are related to fungus structure, these factors thought to be involved to its pathogenicity.[14] The mold *A. fumigatus* has genetic variety as different strains it has, so several methods is designed to enable possible detection of this fungus traditional methods such as conidial shape, color and size.[15] However, *A. fumigatus* colonies are distinguishable from other fungi due to their Conventional studies that have highlighted the difficulties in identifying *A. fumigatus* strains based on commercial phenotypic identification systems. The identification of *Aspergillus* species has long been based on phenotypic and biochemical characteristics. In recent years identification of *A. fumigatus* strains has been based on molecular identification. The molecular-based technology that has undoubtedly had the greatest impact in the clinical diagnosis of fungi infections is PCR.[4] The most commonly used target for fungi diagnostic PCR primers is the rRNA gene operon, encoding the 18S, 5.8S, and 28S rRNA gene subunits, namely internal transcribed spacer 1 (ITS1), ITS2 and ITS4.[16-20] The PCR assay were examined for species-specific identification. The developing countries had been indicated to be the home of *A. fumigatus* infections, especially Aspergillosis, notably in India, China, Thailand, Pakistan, Bangladesh, Sri Lanka, Malaysia, Iran, Iraq, and Saudi Arabia.[1] In Iraq, few researches had been tackled relation between the human pathogen *A. fumigatus* especially after increasing interest and concern in fungal infection of lung and its complications and its relation with health problem.

MATERIAL AND METHOD

During the period of study from the beginning of April 2016 to the end of March 2017, a total of 100 sputum samples were collected from patients admitted to the specialize chest and respiratory diseases center/Baghdad who were suffering from respiratory problems. Each sputum sample were examined directly under the microscope using 10% KOH and culturing onto two plates of Sabouraud's dextrose agar supplemented with 0.04mg/mL chloramphenicol to inhibit the growth of bacteria; one was incubated at (28˚C) and the other one was incubated at (37˚C) for 3 to7 days, and then sub cukture on Czapek-Dox agar at 37°C for 4 days of incubation, all samples were cultured given that the full characterization of mycotic agents is achieved through culture.

Morphology Diagnosis

The identification was done depended on the shape and color of the other colony on the plate, and examined under the microscope. For an appearance of the fungus, small portion from the fungal growth was taken and putting in microscope slide, mixed with one drop of lactophenol cotton blue and covered with coverslip then examined under (40X) by the microscope (The identification of *Aspergillus spp* was conducted according to.[21,22] All colonies were detected; they were sub cultured on Czapeck's dox agar media for specific species identification according to colony characteristics and microscopical characteristics by using (lactophenol cotton blue stain).

DNA extraction

Genomic DNA was extracted from the *A. fumigatus* isolates using Fungi/Yeast genomic DNA purification kit according to manufacturer's instructions bio-WORLD. *A fumigatus* culture grown at 28°C in SDA(Sigma, USA) for 7 days, then loop-full from mycelium put on Eppendorf tube (1.5mL) content 1ml of phosphate buffer saline (PBS), put eppendrof tube into microcentrifuge tube and centrifuge at 14000 rpm for 1 min to pellet the cells and supernatant was removed, 600µL of Lysis buffer was added and gently pipet until the cells are resuspended. Resuspend the cells by gentle vortexing. Transfer the mixture to a Bead tube and secure the tube horizontally on flatbed vortex pad with tape. Vortex for 5 mins at maximum speed or optimize the condition for any commercially available bead beater equipment. Incubate the bead tube with lysate at 65˚C for 10 mins. Occasionally mix the lysate 2 or 3 times during incubation by inverting the tube. Transfer all the lysate Eppendorf tube. Centrifuge the tube at 14000 rpm for 2 min. Transfer clean supernatant to Eppendrof tube without disturbing the pellet. Add an equal volume of 96%–100% ethanol to the lysate collected above. Vortex to mix. 300µL of Binding buffer was added and briefly vortex to mix. Obtain a spin column assembled with its collection tube (provided), apply 650µL of the lysate with ethanol onto the column and centrifuge at 10000 rpm for 1 min. A 500µL of Wash buffer was applied to the column and centrifuge at 10000 rpm for 1 min. Discard the flow-through and reassemble the column with its collection tube, wash the column a second time. Discard the flow-through and reassemble the spin column with its collection tube. Spin the column at 14000 rpm for 2 mins to thoroughly dry the resin. Discard the collection tube. Place the column into a fresh Elution tube provided with the kit. 100µL of Elution buffer was added to the column and centrifuge at 10000 rpm for 2mins term. Then the DNA sample was stored at -20°C until use.

DNA quantification

Determination of genomic DNA concentration and purity

The DNA concentration was determined by using a spectrophotometer; 10µL of each DNA specimen was added to 990µl of distilled water and mixed well. A spectrophotometer was used for measuring the optical density (O. D) at a wavelength of 260nm and 280nm.

An O. D of one corresponds to approximately 50µg/mL for double-strand DNA. The concentration of DNA was calculated according to the formula:

DNA concentration (µg/mL) = O. D 260nm ×50× dilution factor

A spectrophotometer was used also to estimate the purity ratio of DNA according to the following formula:

DNA purity = O. D 260nm/O. D 280nm

The ratio used for detecting DNA contamination with protein preparation. DNA quality could be assessed by 0.8% agarose gel electrophoresis.[23]

The extracted DNA from the *A. fumigatus* isolates was concentration and purity by nanodrop instrument, briefly, 3µl of DNA was quantified by using spectrophotometrically at wavelength 260nm, 280nm. The DNA concentration was calculated with the OD260nm. The purity was estimated with the OD260nm/OD280nm ratio, a ratio of (1.6-1.8) was generally accepted as "pure" DNA, indicating a low degree of protein contamination.

Agarose Gel Preparation and Electrophoresis

DNA samples were electrophoresed by horizontal agarose gel electrophoresis, according to J Sambrook, *et al*.[24] as follows: Agarose(Promega, USA) at a concentration of 1% was prepared, the agarose solution was left to cool at 55°C, then (0.5µL) of ethidium bromide solution (Promega, USA) was added, Agarose solution poured into the taped plate. A comb was placed near one edge of the gel. The gel was left to harden until it became opaque; each of the comb and tape was removed gently. TBE buffer (1X) prepared was poured into the gel tank, and the slab was placed horizontally in electrophoresis tank. About 3 microliters of loading buffer prepared was applied to each 7 µL of DNA sample wells were filled with the mixture by a micropipette, PCR products were directly applied. The power supply was set at (5 V/cm (70) for 1 hr) for genomic DNA and PCR products electrophoresis. When the electrophoresis was finished, the gel was exposed to UV light using UV transilluminator at 350 wavelengths and then photographed using a digital camera. Five microliters of the 100bp DNA ladder(Promega, USA) were mixed with two microliters of blue loading dye(Promega, USA) and subjected to electrophoresis in a single lane. The gel was stained with ethidium bromide solution (0.5 µg/mL) for 15-30 minutes; finally, bands were visualized on UV transilluminator at 350 wavelength and then photographed by using photo documentation system.

Primers selection and preparation

To get specific amplification for (ITS1, ITS4) region of the rRNA gene in the DNA samples of *Aspergillus fumigates* isolates by using conventional PCR, The specific of oligonucleotide primer sequences were used according to (18) The primers were supplied by Alpha-DNA company as lyophilized products of different picomoles concentrations. These primers were provided in lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmol/µL as recommended by the provider and stored in the deep freezer until used in PCR amplification. The primers sequences and their size of the product are shown in Table 1.

PCR Master Mix

Optimization of Singleplex PCR master mix for amplification of ITS1 ITS4 of rRNA gen**e** was accomplished after several trials; thus, the Singleplex PCR reactions were performed in 25 µL volumes containing 5.0 µL of nuclease free water, 12µL of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2 µL of 10 pmol F primer and 2µL of 10 pmol R primer and 2 µL of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

PCR program

Optimization of Singleplex PCR program for amplification of ITS1, ITS4 of rRNA gen**e** was accomplished after several trials; thus including initial denaturation at 94°C for 5 minutes, followed by 40 cycle *A. fumigatus* of denaturation at 94°C for 20 sec, annealing at 55°C for F *A. fumigatus* and R *A. fumigatus* primers for 5 min, and extension at 56°C for 20 sec. The thermal cycles were terminated by a final extension at 72ºC for 20 sec. Positive control and Nuclease free water as negative control were used too reaction tubes were holding at 4ºC as final steps of PCR amplification.

Singleplex PCR products analysis

The analysis of Singleplex PCR products were performed on 2% agarose gel. The 1 kb DNA ladder (Promega, USA) was used. The gel was stained with ethidium bromide and run at 100 volt for 45 minutes at room temperature then exposed to UV using UV light transilluminator and then photographed using a digital camera (Sony-Japan).

RESULTS

Conventional methods

Out of hundred samples were subjected to 10% KOH examination, 35 (35%) of the samples showed positive result by 10% KOH examination and only 53 (53%) of the samples revealed positively by culture. Examination by direct microscopy failed to detect 18 samples, which were later found to be positive with culture Table 1.

Distribution of Aspergillus sp. and Other Fungal Isolates

Only 44 specimens (83%) revealed positive culture for Aspergillosis, which belonging to genus Aspergillus. The isolation rates were as follows: *A. fumigatus* was the most common species 23/53 (42.4%) among Aspergillosis were

Table 1: The primers sequences of Its gene of *A. fumigatus* and their product size.

Name of Primer	*Sequence of Primer (5'-3') Size of Product*
F F *A. fumi*	CGC CGA AGA CCC CAA CAT GAA CGC ≈385
R *A. fumi*	TAA AGT TGG GTG TCG GCT GGC

Table 1: Direct microscopic examination KOH and laboratory culture of the studying samples.

Total Number	*Direct examination by 10% KOH*				*Culture on SDAC*			
100	Positive result	%	Negative result	%	Positive result	%	Negative result	%
	35	35. 0	65	65. 0	53	53. 0	47	47. 0

isolated from patients followed by *A. flavus* 11/53 (20.8%), *A. niger* 7/53 (13.2%) and *A. terreus* 3/53 (5.7%.) with lowest levels. Other fungal species recorded included; *Penicillium species* 1(1.9%). Three species of yeast organisms were isolated as follow: Among these were *Candida species* 8 (15.1%) comprising of 5(9.4%) *C. albicans*, 2 (3.8%) *C. tropicalis* and 1 (1.9%)*C. krusei* as in Table 2.

Distribution of fungi isolated from patients according to gender and age groups.

In this study, the ages of participants ranged from 10–70 years with a mean age of 34 years. The highest incidence 26(53.06%) was found in patients aged 30–40 years from the studied specimens followed by age groups 40–50 years and constitute 13(26.5), then (50–60) and (60–70) years old which recorded (10.27%, 7.54%) respectively while the lowest infection recorded in age group 20–30 years was 6.1% and in 10–20 years old which recorded 2(2.04) from the infected peoples as summarized in Table 3. The results indicated that the distribution of Aspergillosis is more prevalent in males compared to females the ratio constitute in males 35/53 (66.3) while in females was 18/53 (33.96**)** as shown in Table 1.

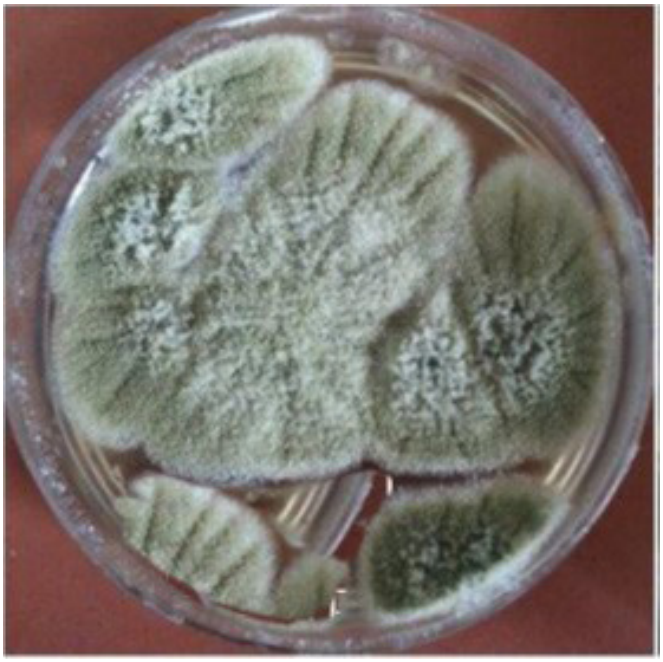

Figure 1: *Aspergillus fumigatus* grown on Czapek-Dox agar at 37°C after 4 days of incubation.

Table 2: Distribution of Aspergillus spp. isolated and Other Fungal Isolates from patient

Fungal isolated	*No.*	*%*
A. fumigates	23	42. 4
A. flavus	11	20. 8
A. niger	7	13. 2
A. terreus	3	5. 7
Penicillium	1	1. 9
C. albicans	5	9. 4
C. tropicalis	2	3. 8
C. krusei	1	1. 9
Total	53	53%

Morphological identification

The Morphological characteristics of *A. fumigatus* are based predominantly upon the microscopic features of the conidia and conidiophores.

A. fumigatus is a fast grower; the colony size can reach 4 ± 1 cm within a week. The colony powdery, green echinulate conidia, 2.5 to 3 µm in diameter, produced in chains from greenish phialides, 6 to 8 by 2 to 3 µm in size. when grown on Czapek-Dox agar at 25°C. Colonies are usually gray green color with a woolly to cottony texture. Reversed side of the colonies appeared pale yellow to tan (4) Figure 1.

Microscopic examination as shown in Figure 2 appeared as clavate vesicles conidia, phialides arrangeed uniseriate upper vesicle conidia and parallel to axis of conidophore, produced in chains of spores basipetally from phialides. The chains of spores were borne directly in absence of metulae and represented by septat and branching hyphae. Fruiting heads rarely occur in clinical specimens in sites exposed to air.[25]

Molecular identification

PCR assay was used to identification of *A. fumigatus* by using specific primer *A. fumigates*.[26]

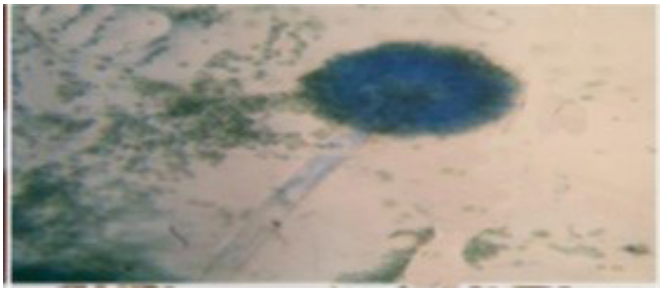

Figure 2: Microscopic feature of *Aspergillus fumigatus* stained with Lactophenol cotton blue(40x)

Table 3: Distribution of Aspergillosis in patients according to gender and age groups.

Age group (y)	*Male No. %*	*Female No. %*	*Total No. %*
10-20	2(5. 71)	-	2(2. 04)
20- 30	1(2. 85)	2(7. 1)	3(6. 1)
30-40	16(45. 71)	10(35. 7)	26(53. 06)
40-50	9(25. 71)	4(12. 9)	13(26. 5)
50-60	3(8. 57)	2(7. 1)	5(10. 2)
60-70	4(11. 42)	-	4(7. 54%)
Total	35(66. 3)	18(33. 96)	53(53%)

Analysis of extracted DNA of A. fumigatus isolates

After performing of the DNA extraction from *A. fumigatus* isolates, purity and concentration measured using the standard method.[24,27] The yield in range of (65–210) ng/µL with purity of (1.5–1.9), agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1 % agaros gel at 7 volt/ cm for 1-hour Figure 3.

Analysis of PCR products of A. fumigatus ITS rRNA gene

The PCR products and 1 kb DNA ladder were resolved by electrophoresis. 5 µL of the PCR product was loaded on 1% agarose gel and run at 7 volt/cm for 1 hour. PCR result was considered positive for *A. fumigatus* when there was presence of ~385 bp PCR product band on agarose gel electrophoresis, no amplification was observed with negative control Figure 4

DISCUSSION

Aspergillosis constitutes one of the health problems among people in worldwide including Iraq. Aspergillus fumigatus is most common species found in human infections all over the world.[42]

Number of studies about the prevalence of Aspergillosis conducted in worldwide and in Bagdad and South provinces of Iraq.[23] Aspergillosis were more prevalent in males compared to females the ratio constitutes in males 35/53 (66.3)while in females was 18/53 (33.96), with the highest frequency of Aspergillosis was observed in the patients with age 30–40 years old and this is maybe associated with problems in public health such as immunocompromised by systemic infection such as diabetes, tuberculosis, and AIDS.

In this study, the prevalence of *Aspergillus spp* in the sputum of patients suspected of pulmonary Aspergillosis was (83%). Our findings relating to the prevalence is accordance with previous published report by M. Shahid,[30,31] however, it is much lower than the prevalence reported by (4, 25) and high incidence.[30,31] In a recent study 44 of the 100 participants were infected with *Aspergillus sp.* Preliminary detection of samples was done by the direct examination of sputum before culturing. The results obtained by direct microscopy 10% KOH (35%), were comparable to those obtained by culture, (53%), examination by direct microscopy failed to detect 18 samples, which were later found to be positive with culture. These results showed similarity with the results of[32,33] found that 10% KOH test was less sensitive than the laboratory culturing of Aspergillosis.[34,35] Direct microscopy can lead to the diagnosis of fungal infections using specimens such as urines, aspirates, and bronchoalveolar lavages by the presence of thread-like hyphae or spores.[11, 36-38] KOH is very simple, fast, and the most cost-effective mycological technique but it does not allow species identification.[22,28] While the technique of culturing specimens is inherently simple and low cost, an enhanced method of sensitively, rapidly, and specically detecting of *mold*.[30] reported the be able to increase the number of positive cultures by 17% by extending incubation time from 2 to 5 days. The advantages of conventional methods were not costly, but the disadvantages of those methods were consuming time, contamination present, false-positive result and require a large amount of sample 6.

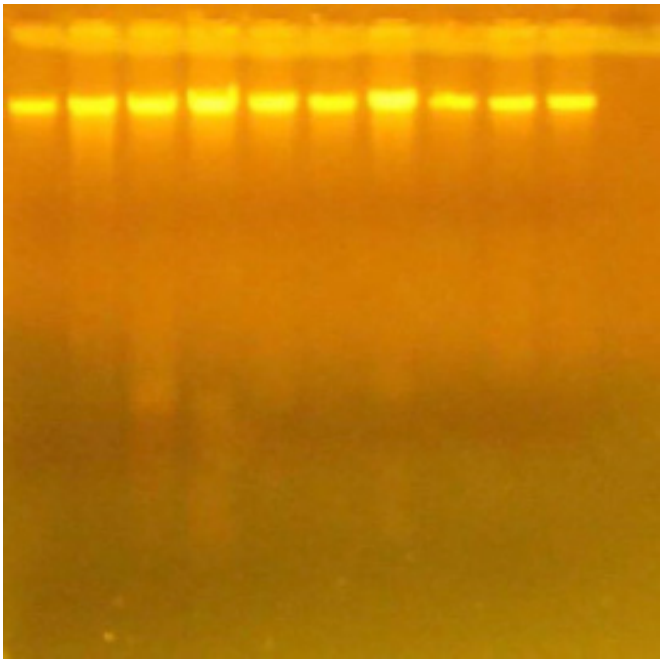

Figure 3: Gel electrophoresis of extracted DNA of *A. fumigatus* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 1-9: Extracted DNA.

In this study, The predominant *Aspergillus species* isolated from sputum was *Aspergillus fumigatus* (42.04%) and correlated well with results of other similar studies[34,39,9,8] and is lower than those obtained by Shahid *et al.*[11] followed by *A. flavus*. This wide variation in the incidence and frequency of isolation of various *Aspergillus* species colonization may be due to geographical differences.,[40] The identification of *A. fumigatus* by using conventional methods which agreed with result of the same study conducted by Ellis, *et al.*[22,25] showed that the *A. fumigatus* colonies on Czapek-Dox agar appear a grey green color as a result of the conidia pigmentation. It forms septate mycelia, which reproduces asexually by the production of conidial spores. Microscopic examination of the organsim appears the conidia (2–3 µm in diameter), each one conidia contain haploid nucleus and are developed from specialized cells called phialides, which are part of the conidiophores

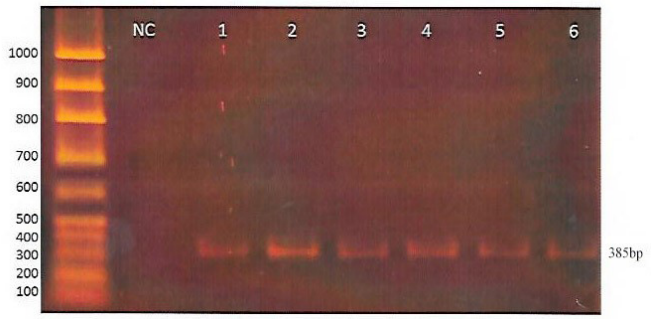

Figure 4: Gel electrophoresis singleplex PCR products of ITS1-ITS4 of *A. fumigatus* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 100 bp DNA ladder, lane 1-6: ITS1-ITS4 PCR products of *A. fumigatus isolates.* NC=negative control.

spore-producing structures, while millions of conidia are attached in chains of eight spores.[29] Conidia are released from the conidophore and easily become airborne.[13,41] The released conidia undergo germination to form septate hyphae, in addition, the result of *A. fumigatus* goes together with the result of Ellis, *et al.*[15] exhibited. The classical morphology criteria, such as, conidia shape, color and size, are very difficult to differentiate and identification *Aspergillus* species in clinical laboratories.[17] The advantages of conventional methods were non costly but the disadvantages of those methods were consuming time, contamination present, false positive result and require a large amount of sample.[8] In the current study, specific oligonucleotide primer sequences were used in singleplex PCR to detect the presence of (ITS1-ITS4) region of the rRNA gene for Aspergillus fumigatus. PCR product size of approximated 358 bp when compared with a 100-bp DNA ladder. The same results were found in the study deals with the identification of *Aspergillus* spp. by the same primer ITS1 and ITS4 (1) In addition to the identification of *Yarrowia lipolytica* DNA by using the same primers ITS1 and ITS4, the isolate is isolated from raw and processed poultry ITS 1 and 4 was widely used for identification fungi, the amplicon may vary among different species. with the product size is 385bp.[1] The result showed *A. fumigatus* found in 19 out of 23 samples that were positive by the conventional methods. While the other 4 was not A. *fumigatus* may be another species of Aspergillus, so we achieved that we can′t depend on culture morphology or microscopy in classify microorganisms with the development of biotechnology. The most common cause of false positive culture was belonged to the error of sampling process or inadequate specimen, which subjected to splitting to perform a microscopic examination and laboratory culturing, or maybe due to inappropriate use of a drug which had been taken from the patient.[33] While the molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods.[7,17,34,42] Also, the result of this study goes together with results of other studies which indicated that the ITS region of the *A. fumigatus* rRNA gene has often been used to identify this fungus in molecular biology-based identifications, such as PCR, In addition, the finding of this study in agreement with findings of other studies which referred that the choosing of universal fungal primers are known not only to amplify high-copy-number RNA genes, but to amplify DNA from most, if not all, fungi.[43,44]

CONCLUSION

Aspergillus. fumigatus was the most dominant molds isolated from sputum in aspergillosis patients. PCR method has a high degree of specificity for the identification of *Aspergillus spp*

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REFERENCES

1. Chatterjee, S. S.; Das, A. and Shivaprakash, M. R. (2011). Invasive Aspergillosis in developing countries. Medical Mycology; 49(1): S35–S47.
2. Prakash, V.; Mishra, P. P.; Verma, S. K.; Sinha, S. and Sharma, M. (2014). Prevalence and fungal profile of pulmonary Aspergillosis in Immunocompromised and immunocompetent patients of a tertiary care hospital. Int J Med Res Health Sci 3: 92-97.
3. Klich, M. A. (2009). Health effects of Aspergillus in food and air. Toxicol Ind Health, 25:P: 657-667.
4. Samson, R.A.; Hong, S.; Peterson, S.W. p; Frisvad, J.C. and Varga, J. (2007). Polyphasic taxonomy of *Aspergillus* section Fumigati and its teleomorph Neosartorya. Stud. Myco. 59:147–203.
5. Seyedmousavi S, Guillot J, Arné P, de Hoog GS, Mouton JW, Melchers WJ, et al. (2015). Aspergillus and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease. Med Mycol. 2015; 53(8):765-97.
6. Zhao Y., Park S., Warn P., Shrief R., Harrison E., Perlin D. S. (2010). Detection of *Aspergillus fumigatus* in a rat model of invasive pulmonary Aspergillosis by real-time nucleic acid sequence-based amplification. J. Clin. Microbiol. 48, 1378–1383. 10. 1128/JCM. 02214-09
7. Fragi, M. E.; Santana, D. M. N.; Gatti, M. J.; Direito, G. M.; Cavalieri, L. R.; Alberto, C. (2008). Characterization of *Aspergillus* species based on fatty acid profiles. J. Mem. Inst Oswaldo. 103(6): 540-544.
8. Patterson, T. F.; Kirkpatrick, W. R.; White, M.; Hiemenz, J. W.; Wingard, J. R. and Dupont, B. (2000). Invasive Aspergillosis. Disease spectrum, treatmentpractices, and outcomes. I3 Aspergillus Study Group. Medicine(Baltimore)., 79(4): 250–260.
9. Peterson, S. W. (2008). Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* 100:
10. Steinbach, W. J., Marr, K. A., Anaissie, E. J., Azie, N., Quan, S. P., Meier Kriesche, H. U., ***et al. (***2012). Clinical epidemiology of 960 patients with invasive Aspergillosis from the PATH Allianceregistry. *J. Infect.* 65, 453–464. doi: 10. 1016/j. jinf. 2012. 08. 003 27-46
11. Shahid, M., Malik, A. and Bhargava, R. (2007). Secondary Aspergillus in Bronchoalveolar Lavages (BALs) of Pulmo-nary Tuberculosis Patients from North-India., Ameri-can-Eurasian Journal of Scientific Research, 2 (2), 97-100
12. Frisvad, J. C. and Larsen, T. O. (2015). Extrolites of *Aspergillus fumigatus* and other pathogenic species in *Aspergillus* section fumigati. *Front. Microbio.* 6:1485
13. De Almeida, M. B.; Bussamra, M. H. F. and Rodrigues, J. C. (2006). Allergic bronchopulmonary Aspergillosis in paediatric cystic fibrosis patients. Paediatric respiratory reviews. 7(1):67-72.
14. Rementeria, A; Lopez-Molina, N; Ludwig, A; Vivanco, A; Bikandi, J; Ponton, J and J Garaizar, J (2005). Genes and molecules involved in *Aspergillus fumigatus* virulence. Rev Iberoam Micol; 22; 1-23.
15. Dagenais, T. R. and Keller, N. P. (2009). Pathogenesis of Aspergillus fumigatus in invasive Aspergillosis. Clin. microbio. Rev. 22. (3): 447-465.
16. Aufauvre-Brown, A., Cohen, J. and Holden, D. W. (1992). Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol, 30(11): 2991-3.
17. Buchan, A.; Newell, S.; Moreta, J.; Moran, M. (2002). Analysis of Internal Transcribed Spacer (ITS) regions of rRNA genes in

fungal communities in a southeaster U. S. Salt Marsh. *Microbiol. Ecolo. J.* 43, 329-340.
18. Chen, Y.; Eisner, J.; Kattar, M.; Rassoulian-Barrett, S.; LaFe, K.; Yarfitz, S.; Limaye, A. and Cookson, B. (2000). Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 3 region of the rRNA genes. *J. Clin. Microbiol.* 38: 2302-2310.
19. Luo, G.; Mitchell, G. (2002). Rapid identification of pathogenic fungi from cultures by using multiplex PCR. *J. Clinical Microbiol*. 40(8), 2860-2865.
20. Williams, D.; Wilson, M.; Lewis, M. and Potts. A. (2001). Identification of *Candida* species by PCR and restriction fragment polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J. Clin. Microbiol*. 33: 2476-2479.
21. Chandler, F. W.; William, K. and Libero, A. (1980). A colour atlas and textbook of the histopathology of mycotic Disease. Wolfe Hous. London
22. Ellis, D. H. (1994). Clinical mycology: The human opportunistic mycoses. Pfzor, New York.
23. Maniatis, T.; Fritsch, EF and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
24. Sambrook J and Russell D W. (2001). Molecular Cloning: A Laboratory Manual Cold Spring Harbor. New York, USA, Cold Spring Harbor Laboratory Press, N. Y.
25. Jawetz, Melnick, and Adelberg's. (2007). Medical Microbiology, 24th Chapter 45. Medical Mycology :Edition by Vishal Geo. F. Brooks, San Francisco,; Janet S., Butel Houston, Stephen A.,
26. Guizhen, L. and Thomas, G. (2002). Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. *J. of Clinic. Micrbiol*. 40(8): 2860-2865.
27. Sambrook, J.; Friteg, E. F. and Maniatis, T. (1989). Molecular cloning :alaboratory. new york. USA.
28. Shrimali, G. P.; Bhatt, J. K.; Rajat, R.; Parmar, R. V. and Nayak, S. et al. (2013) Isolation of Aspergillus species from sputum samples: A study conducted in a tertiary care hospital, National Journal of Medical Research 3: 289-291.
29. Kawathar, J. K. (2016). Pathogenicity Genes of *Aspergillus fumigatus*" Report of higher diploma. p:61.
30. Singh, R.; Singh, G. and Urhekar, A. D. (2015). Incidence of Aspergillus Infections in Patients in a Tertiary Care Hospital in NaviMumbai. 5(2).
31. Xess, I.; Mohanty, S.; Jain. N. and Banerjee, U. (2004). Prevalence of Aspergillus species in clinical samples isolated in an Indian tertiary care hospital. Indian J Med Sci 58: 513-519.
32. Kaur, R;. Kashyap, B. and Bhalla, P. (2008). Onychomycosis – epidemiology, diagnosis and management. Indian J Med Microbiol.; 26:108–16
33. Lilly, K. K.; Koshnick, R. L.; Grill, J. P.; Khalil, Z. M.; Nelson, D. B. and Warshaw, E. M. (2006). Costeffectiveness of diagnostic tests for toenail onychomycosis: a repeated-measure, single-blinded, cross-sectional evaluation of 7 diagnostic tests. J Am Acad Dermatol. 55:620–6.
34. Barnes, P. D.; Kieren A. and Marr, K. M (2006). Aspergillosis: Spectrum of Disease, Diagnosis, and Treatment Infect. Dis. Clin. N Am20: 545–561
35. Bonifaza, A.; Rios-Yuila, J. M.; Arenasb, R.; Araizaa, J.; Ramn Fernandez, R.; Patricia Mercadillo-Péreza, P. and MarA. R. (2013). Comparison of direct microscopy, culture and calcofluor white for the diagnosis of onychomycosis. 30(2):109–111.
36. Law D. (2010). Clinical Mycology. In M. Ford, *Medical Microbiology.* (pp. 253-255).
37. Schmidt, A. Schmidt, D. I.; Georg, J. B. and Fresenius, W. (1999). the description of the species Aspergillus fumigatus in 1863. Contrib Microbio. 2:1-4.
38. Shenoy M. M., Teerthanath S., Karnaker V. K., Girisha B. S., Krishna Prasad M. S., Pinto J. (2008). Comparison of potassium hydroxide mount and mycological culture with histopathologic examination using periodic acid-Schiff staining of the nail clippings in the diagnosis of onychomycosis. *Indian Journal of Dermatology, Venereology and Leprology*, *74*(3), 226-9
39. Larone, D. H. (2002). Medically important fungi -4th edition, ASM press, Washington, D. C.
40. Ellis, D.; Davis, S.; Alexiou, H. Handke, R. andBartely, R. (2007). Description of medical fungi. 2nd ed. Mycology unit, Australia. PP:9-127.
41. Walsh, T. J.; Wissel, K; Ruta, P.; Vidmantas, P.; Miki, K.; Andera, F. et al., (2011). Molecular Detection and Species Specific Identification of Medically Important Aspergillus Species by Real –Time PCR vin Expermental Invasive Pulmonary Aspergillosis. Clinical Microbiology. P. 4150-4157.
42. Ahmed, D., Al-Khafaji, N. J, and Ahmed, L. T. (2017). Isolation and Molecular Identification of *Aspergillus* spp. Collected from Different Sources of Animals Feed. Int. J. Curr. Microbiol. App. Sci. 6(6): 1792-1797
43. Kamal, S. A.; Awadh, R. M. and Al-Marzoqi, A. H. (2015). Phenotypic and genotyping study of aspergillus niger: molecular detection of calmodulin, 18srrna and pepsin like protease genes based on multiplex pcr. Food Sci. Qual. Manag., 35: 1-6. Direct Link |. 19:. 35
44. Bakare, N., Rickerts, V., Bargon, J., Just-Nübling, G., (2003) Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis, Mycoses, 46(1-2).
45. Barton, R. C. (2013). Laboratory diagnosis of invasive Aspergillosis from diagnosis to pridiction of autcome scientifica. Article 459405, 29.
46. Horvath, J. A. and Dummer, S. (1996). The use of respiratory-tract cultures in the diagnosis of invasive pulmonary Aspergillosis. Am J Med.; 100(2):171–8. 3.
47. Kurhade, A. M., Deshmukh, J. M., Fule, R. P., Chande, C. and Akulwar, S., (2002). Mycological and serological study of pulmonary Aspergillosis in central India., Indian Journal of Medicine. Microbiology, 20, 141-144
48. Pattron, D. D. (2006). *Aspergillus*, Health Implication &Recommendations for Public Health Food Safety. Internet Journal of Food Safety. 8: 19
49. Alshareef, F. O. (2012). Genetic and virulence variation of the population of environmental and clinical isolatea of the pathogenic Aspergillus fumigatus, Thesis, university of Manchester.