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Research Article

Detection of *Aspergillus fumigatus* by Polymerase Chain Reaction (PCR)

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ABSTRACT

Aspergillosis refers to fungi infections of the respiratory tract caused by Aspergillus species especially *Aspergillus fumigatus*.Infection of *A. fumigatus* were increased in last few years , due to either resistances to antibiotics or to influence of other factors such as other fungal infections.The aims of the present study were to review the impact of *Aspergillus fumigatus* in Aspergillosis cases, and study the role of Singleplex PCR for amplification of ITS1,ITS4 of 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 foridentification. 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 for fungal infection were recorded 35/53 (66.3), compared to females were 18/53 (33.96).The infection of fungi were more prevalent in ages 30-40recorded 26(53.06%) followed by ages 40-50 ,13(26.5), while the lowest infection recorded in age group 10- 20 years was 2(2.04%). DNA isolated from twenty three A.fumigatus isolates was used as template and the specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of internal transcribed spacer region (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 human.

Keywords: Aspergillosis, *Aspergillus fumigatus*, PCR.

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 in 6-25% of patients with cystic fibrosis[10]. This disease may be caused by a number of *Aspergillus* species which are saprophytic, thermo tolerant fungi that are ubiquitous in the air and environment[34]. 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 human and animal. *Aspergillus* causes diseases of respiratory system by the inhalation of *Aspergillus* conidia. The clinical manifestations of pulmonary aspergillosis are many ranging from harmless saprophytic colonization to a cute invasive disease[24,38,43,49]. *Aspergillus* spp. are rapidly growing mould 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[17]. Among these, *Aspergillus fumigatus* is the most commonly species isolated, followed by *Aspergillus flavus*, *Aspergillus niger and A.terreus*[31,33,45]. *Aspergillus fumigates* was firstly described in 1863 by physician Georg W. Fresenius from his studies on isolates obtained from human lung infections[40]. This mold is a filamentous saprotrophic widespread in nature, typically found in soil and decaying organic matter where it plays an essential role in carbon and nitrogen recycling[16,45]. A. fu migatus is one of the most common species to cause disease in individuals with an immunodeficiency[13]. The

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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	

Pathogenicity of *A. fumigatus* depends on immune status of patients and fungal strain which can produce 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[35]. 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, colour and size[12]. However, *A.fumigatus* colonies are distinguishable from other fungi due to their Conventional studies have highlighted the difficulties in identifying *A. fumigatus* strains based on commercial phenotypic identification systems. Identification of *Aspergillus* species has long been based on phenotypic and biochemical characteristics. In recent years identification of *A. fumigatus* strains has been based molecular identification. The molecular-based technology that has undoubtedly had the greatest impact in the clinical diagnosis of fungi infections is PCR[32,39]. 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[3,8,11,29,47]. 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[10]. In Iraq, few research 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[23].

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 othe colony on the plate and examined under the microscope. For appearance of the fungus, small portion from the fungal growth was taken and putting in microscope slide, mixed with one drop of lacto phenol cotton blue and covered with cover slip then examined under (40X) by the microscope (The identification of *Aspergillus spp* was conducted according to (9,14). All colonies were detected; they were sub cultured on Czapeck's dox agar media for specific species identification according to colony characteristics and microscopical characteristic 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 eppendrof 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 supernstsnt 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℃ for 10 mins. Occasionally mix the lysate 2 or 3 times during incubation by inverting the tube. Transfer all the lysate eppendrof 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 .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 in order 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 spectrophotometer; 10µl of each DNA specimen was added to 990µl of distilled water and mixed well. Spectrophotometer was used for measuring the optical density (O.D) at wave length of 260nm and 280nm.

Table 2: 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

Table 3: 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%

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

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 (30).

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 wave length 260nm, 280nm. 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 (37). as follows: Agarose (Promega, USA) at a concentrations 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 were 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. 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 wave length and then photographed using digital camera. Five microliters of the 100bp DNA ladder (Promega, USA) were mixed with two microliter 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 transiluminator at 350 wave length and then photographed by using photo documentation system.

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

Primers selection and preparation

To get specific amplification for (ITS1, ITS4) region of 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 provider and stored in deep freezer until used in PCR amplification.The primers sequences and their size of product are shown in (Table 1).

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 a 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

Table 4: 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%)

temperature then exposed to UV using UV light transilluminator and then photographed using 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 positive 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 follow: *A.fumigatus* was the most common species 23/53 (42.4%) among Aspergillosis were 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).

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[20]

Molecular identification

PCR assay was used to identification of *A. fumigatus* by using specific primer *A. fumigates*[18].

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 (36,37). 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 were 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 constitute 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 may be 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[40], however, it is much lower than the prevalence reported by[4,25] and high incidence[44,48]. 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

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

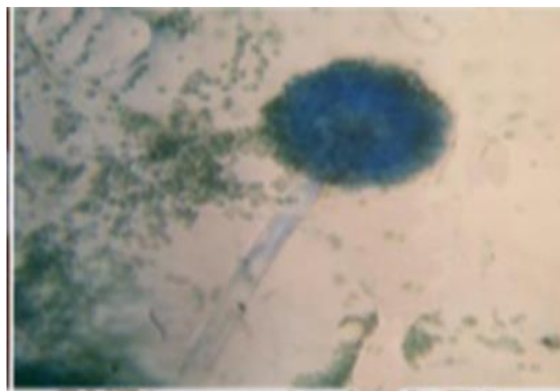

Figure 2: Microscopic feature of *Aspergillus fumigatus* stained with Lactophenol cotton blue(40x)

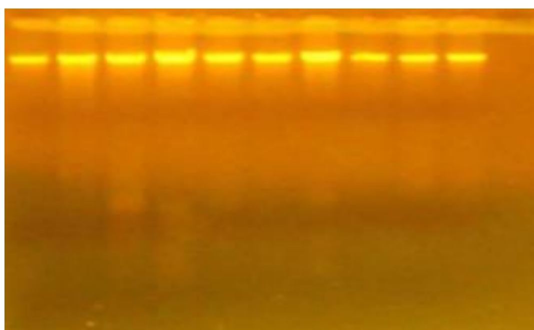

Figure 3: Gel electrophoresis of extracted DNA of *A. fumigatus* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 1-9: Extracted DNA.

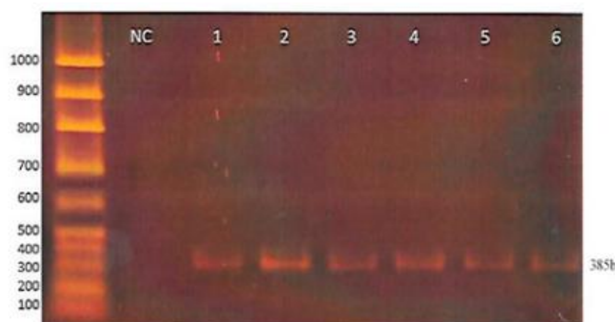

Figure 4: Gel electrophoresis singleplex PCR products of ITS1-ITS4 of *A. fumigatus* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 100 bp DNA ladder, lane 1-6: ITS1-ITS4 PCR products of *A. fumigatus isolates.*NC=negative control.

culture. These results showed similarity with the results of[22,28] found that 10% KOH test was less sensitive than the laboratory culturing of aspergillosis[5,7]. Direct microscopy can lead to diagnosis of fungal infections using specimens such as urines, aspirates and bronchoalveolar lavages by the presence of thread-like hyphae or spores[27,39,40,41]. 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.*[44] reported the be able to increase the number of positive cultures by 17% by extendingincubation time from 2 to 5 days. 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 (6)

In this study, The predominant *Aspergillus species* isolated from sputum was *Aspergillus fumigatus* (42.04%) and correlates well with results of other similar studies[5,26,29,31] and is lower than those obtained by (40) 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[26]. The identification of *A. fumigatus* by using conventional methods which were agrees with result of the same study conducted by[14,20] showed that the *A. fumigatus* colonies on Czapek-Dox agar appear a grey green colour as a result of the conidia pigmentation. It forms septate mycelia which reproduce asexually by the production of conidial spores. Microscopic examination of the organsim appears the conidia (2–3µm in diameter), each one conidia contain haploid nucleus and are developed from specialized cells called phialides, which are part of the conidiophores spore producing structures, while millions of conidia are attached in chains of eight spores[23]. Conidia are released from the conidophore and easily become airborne[13,15]. The released conidia undergo germination to form septate hyphae in addition, the result of *A. fumigatus* goes togther with result of[15] exhibited. The classical morphology criteria ,sush as, conidia shape, colour and size are very difficult to differentiate and identification *Aspergillus* species in clinical laboratories[8]. 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 current study, specific of 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 it compared with a 100-bp DNA ladder. The same results was found in the study

deals with the identification of *Aspergillus* spp. by the same primer ITS1 and ITS4 (1) In addition to identification of *Yarrowia lipolytica* DNA by using the same primers ITS1 and ITS4, the isolate is isolated from raw and processed poultry (Deak *et al.,* 2000). ITS 1 and 4 was widely used for identification fungi, the amplicon may vary among different species. With the product size is 385bp[10]. The result showed *A.fumigatus* found in 19out of 23samples 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 postive culture was belonged to the error of sampling process or inadequate specimen which subjected to splitting to perform microscopic examination and laboratory culturing ,or may be due to inappropriate use of drug which had been taken from the patient[28] 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[5,8,19,46]. 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[1,21].

CONCLUSION

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

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