Molecular Typing of *Malassezia* and *Histoplasma* in Bronchoalveolar Lavage Fluid from Patients with Pulmonary Respiratory Infections

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Abstract

Pulmonary infections caused by fungal infection is one of the generality serious lung disease. Its symptoms and risk vary from controlled to fatal if not treated early. The aim study isolation and identification of *Histoplasma* and *Malassezia* inhabitant lung of patients pulmonary respiratory infections. A total of 103 clinical samples were collected from the patients who attenuated the Thoracic and Respiratory Diseases Center, Tebba National Hospital and Morjan Educational Hospital in Babylon province . The samples were taken under the supervision of the physician after diagnosis of pulmonary diseases such as Tuberculosis , Pulmonary fibrosis , lung cancer , asthma , Thoracic Allergy. Lop full of bronchoalveolar lavage fluid (BAL) were Direct cultured on Sabouraud's Dextrose Agar (SDA), microscopically and molecular identification was performed based on used specific primer pairs for *Histoplasma* and *Malassezia*. The results shown identification many yeast and molds based on cultural and molecular assay shown: A 438 out of 500 isolates of yeast were identified as *Candida* spp. , 27 isolates of *Malassezia* spp. and other filamentous fungi such as *Zasmidium cellare, Aspergillus* spp. , *Penicillium* spp. , *Fusarium* spp. , *Mucor* sp., *Alternaria* spp., *Acremonium* sp. and *Cochliobolus kusanoi* .

Key wards: *Pulmonary diseases , lavage samples, molecular assay, Histoplasma, Malassezia.*

Introduction

The lung exposes of chronic infection with some fungi such as *Candida* ,I*Histoplasma* ,*Malassezia* and others molds, these fungi are capable of causing self-limiting pneumonia when inhaled. *Malassezia* species and pneumonitis have been described in children and adults with central venous catheters and in patients with preceding surgery [8]. Thus, they exist at the vary interface between commensal and pathogen such as, their interaction with the human immune system is of great interest. [3]. Histoplasmosis is a pulmonary fungal disease that inhales the airborne infectious conidia of the dimorphic fungus, *Histoplasma capsulatum* [6]. This yeast may causes acute pulmonary disease, disseminated disease, chronic pulmonary disease and fibrosing mediastinitis [10]. The aim of the study was to determine the lung fungal infections, particularly pathogenic yeast, in patients with chronic pulmonary diseases based in lavage samples and molecular diagnosis of fungal isolates based on culturing sample methods .

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Material and Method

A total of 103 samples of sputum and bronchoalveolar lavage fluid (BAL) (39 sputum , 64 lavage) were collected over an approximate 6 months era from Oct-2018 to April-2019. Clinical samples was collected from patients who were consulted for chest respiratory diseases from Morjan educational hospital, Tebba respiratory center , advisory clinical for respiratory disease in Babylon province, Iraq. All samples were taken

from the patient under the supervision of the concerned physician. After diagnosis of respiratory infection of the patient, an aspiration sample was taken from patients with pulmonary allergies, asthma, cancer, pulmonary fibrosis , Tuberculosis (Tb) patients. The questionnaire was used for each patient and included some important information. Questioners: Age, Gender, Nature of work, Housing, Chronic diseases , Smoking , drugs uptake were performed. The samples were collected in sterile containers and the samples were directly transferred to the laboratory for culture. Each specimen was inoculated into Sabouraud's Dextrose Agar ,by stricken on SDA, with chloramphenicol , streptomycin and erythromycin then incubated at 28 °C for 2 - 7 days [7]. The gDNA was extracted according to [11] .The PCR procedure were as previously described by [12],The universal primer pair used in this study was ITS5/ITS4 Forward: (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and Reverse (5′-TCCTCCGCTTATTGATATGC-3′), the specific primer pairs for *Malassezia* : Malup (5′-AGCGGAGGAAA AGAAACT-3′), Mal down primer (5′-GCGCGAAGGTGTCCGAAG-3′), and For *Histoplasma*: HistoAF 5'-CACGCCGTGGGGGGCTGGGAGCCT-3', HistoAR: 5'-CGGGTGTCCCCGGCGGACACGGGCCC-3' [20].

Results and Discussion

The majority of patient samples were females 53.39% (55/103) , while 46.60% (48/103) males samples. The average of patients age were ranged from (11-90) in both genders. The high infection percentage was 28.72% of the total positive samples occur between (51-60 years) and the lowest percentage of infection were 1.06% occur between (81-90 years),the high incidence percentage of fungus infection was calculated in Table (1).

Table (1): Show the percentage of fungal pneumonia based on ages and genders of patients.

Ages(years)	No of female samples	No of male samples	Total Percentage %
11-20	3	3	6.38%
21-30	2	5	7.44%
31-40	8	2	10.63%
41-50	11	7	19.14%
51-60	16	11	28.72%
61-70	7	10	18.08%
71-80	4	4	8.51%
81-90	1	-	1.06%
Total	52	42	100%

The result shown a total 500 isolates of yeast (85.61%), *Candida* spp. were the most frequent yeast with 438 isolates (83.05%) , followed by *Malassezia* spp. with 27 isolates (5.4%) , with 1 isolate of *Histoplasma* spp . and 21 isolates of *Zasmidium cellare* in its yeast form. While the fungi isolates were 84 isolates (14.38%) Table(2).

Table (2):list and frequency percentage of isolated clinical yeasts and their numeral based sources (BAL and Sputum).

Isolated fungi	Number of isolates / clinical case		Percentage of frequency %
	BAL	Sputum	
Candida albicans	22	53	15%
C. dubliniensis	6	5	2.2%
C. glabrata	48	3	10.2%
C. krusei	85	66	30.2%
C. tropicalis	67	3	14.2%
C.parapsilosis	55	24	15.8%
Cryptococcus cellulolyticus	9	-	1.8%
H. capsulatum	1	-	0.2%
Malassezia frufur	2	-	0.4%
M. symopadialis	2	1	0.6%
Malassezia spp.	20	4	4.8%
Saccharomyces mikatae	3	1	0.8%
Zasmidium cellare	21	-	4.2%
Total	500		100%

These molds isolated in this results Table (3) referred to roles in infection and causes Fusariosis, [18], The exsperiment had been given us that *Aspergillus* spp. were high frequently such as *A. fumigatus , A.niger , A.oryzae , A.flavus* and *A. ochraceas*, and its compatible with [9]. Several scientist [5, 17] spotted that most repeated species which effect and lead to pulmonary infection were *A.fumigatus, A.flavus, A.niger* and *A.terreus* which we disagree with them too for having *A.niger* as a second frequent species. Penicilliosis high frequency was and this is consonant with the study of [15].

Table (3):list and frequency percentage of clinical fungi and their numeral based sources (BAL and Sputum).

Isolated fungi	Number of isolates per clinical case		Percentage of frequency %
	BAL	Sputum	
Aspergillus achraceas	2	-	2.38%
A.flavus	1	2	3.57%
A.fumigatus	9	5	16.66%
A.niger	2	3	5.95%
A.oryzae	2	3	5.95%
Acremonium sp.	2	-	2.38%
Alternaria spp.	2	-	2.38%
Alternaria alternaria	-	1	1.19%
A. metachromatica	1	-	1.19%
Cochliobolus kusanoi	2	1	3.57%
Fusarium sp.	6	5	13.09%
Mucor sp.	4	5	10.71%
Penicillium chrysugenum	1 1	1	2.38%
Penicillium commue	1	-	1.19%
Penicillium sp.	-	9	10.71%
Non identified fungi	5	9	16.66%
Total	84		100%

PCR Ribotyping of fungi depending on ITS region:

The results have successfully identified the targeted fungi and yeast based on amplification part of rDNA region by the primer pair ITS5 and ITS4 as in Figure (1).

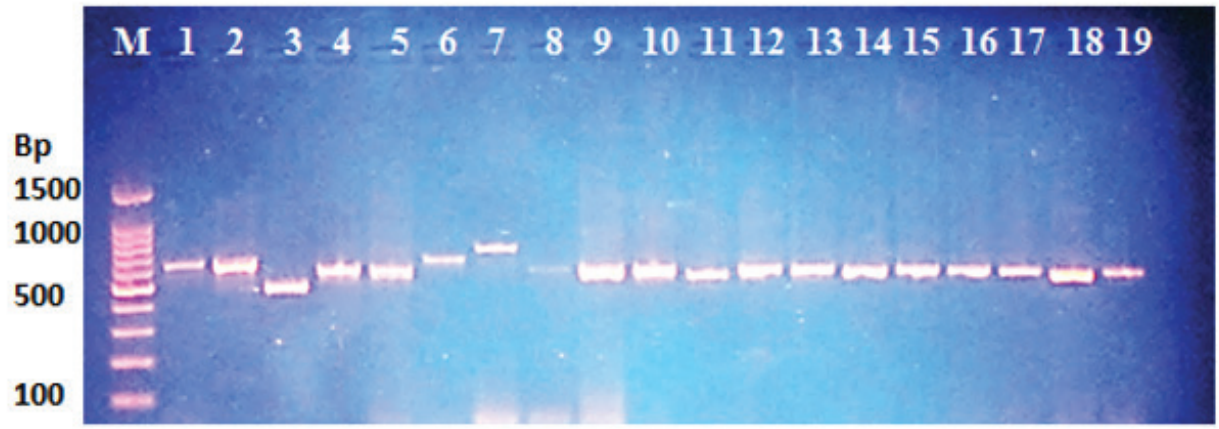

Figure (1): Profile gel electrophoresis of PCR products of 19 filamentous fungi and yeasts isolates amplified by ITS5/ITS4 primer: Lanes (left to right): (1,2,8,17) *Malassezia* **spp. =650bp , (3)** *C.krusei* **=500bp, (4,5,9,10,11,18,19)** *C.albicans* **=600bp, (6)** *C. dubliniensis***= 690bp (7)** *C. glabrata* **=700bp , (12,13,14,15,16)** *C.parapsilosis* **=610bp .Molecular M (100bp for each step.). 1.6% agarose gel at 70 volt for one hour.**

Identification results of Histoplasmosis using PCR technique

The targeted species have been successfully identified using *Histoplasma* spp. primer pair: HistoAF ,HistoAR [20]. the species have been identified on a molecular weight (516bp).(data not shown).

PCR typing of the LSU rDNA region by using Mal primer pair

The results of PCR amplification of DNA from colonies of *Malassezia* species were successfully accomplished PCR-typing method for part of the LSU rDNA region by using the *Malassezia* specific primers All *Malassezia* spp. gave monomorphic bands, approximate 605bp of PCR products Figure (2). This results similar to same results of [14] and [2].

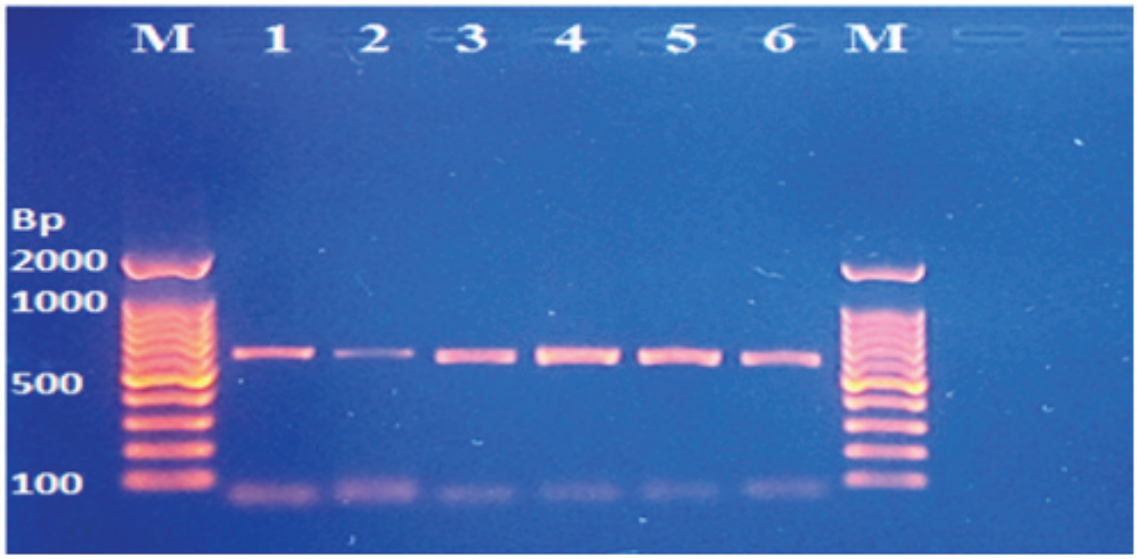

Figure (2): Profile gel electrophoresis of PCR products of *Malassezia* **spp. amplified by primer pair Malup/Maldown. All samples yielded a single band of approximately 605 bp. :1-27 isolates of** *Malassezia* **spp. Molecular M_1(100bp) and M_2marker (50bp). 1.6% agarose gel at 70 volt for one hour.**

As we mentioned that *Malassezia* spp. is not well described or discovered only view studies explained it such as

[19] as we mentioned explained it in infants and Aguirre (2011) [1] explained the fungemia of *Malassezia* spp. in Iraq there were no studies described it as a lung infection , patients with *Malassezia* spp. were in chronic conditions such as lung cancer or TB infection or were in a long term of drugs or antibiotics therapy such as antibacterial and antifungals.

Frequency of this yeast (*Malassezia*)in our patients samples were high as a second frequent yeast isolate and it was higher than the observation of the mentioned researchers. Infected with many lung infections leads to long terms of therapy which leads to weakness of patients immunity and this leads to the wide spread of fungal infection more widely and more dangerous and leads to one of the patient's death causes , beside many of these fungi causes fungemia and as the lung fluid is enrich with materials that help increase growth and infection.

At the final, this study had been faced many problems; like collecting the samples was on a level of difficulty specially for BAL samples like suffering the unavailability of samples easily and quickly, sometimes the lack of response of medical staff, the presence of patients with these diseases, waiting for long time, required the presence of surgical intervention to obtain these samples, sometimes not responding to the patient and not giving samples or information about his or her illness or health status, the risk of dealing with this type of samples in relation to patients with Tuberculosis (TB) as well as BAL samples for the presence of blood and bodily fluids that are likely to carry viruses or dangerous bacteria that causes series infections like Hepatitis etc.

Likewise the interference and coexistence between yeast species leads to the difficulty of purifying and isolating the yeasts from each other and identification or diagnosing it accurately. Finally the lung diseases that occur due to fungi need to have a higher concentration and give it more importance especially within the Iraqi medical field because all of these infections lead to serious troubles (infections) besides in many cases lead to death.

Ethical approval : Both authors hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Conflict of Interests : The authors did not declare any conflict of interest.

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