Isolation and identification of fungi associated with chronic respiratory infections in human and bovine

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Abstract

In order to determine the fungi that association with respiratory infections in human and bovine, sixty human sputum samples were collected from Al-Yarmook educational hospital, and fifty samples were collected from bovine which showed clinical signs of chronic respiratory disease from alkarhk slaughterhouse in Baghdad city during a period from January to April 2012. The isolation results in human showed that 34 out of 60 (56.66%) sputum were positive for fungal isolation. The highest percentage of infection was seen in January (66.66%), While the lowest percentage seen in March (42.85%). The main fungal species that isolated included *Candida albicans* 12 out of 34 (35.29%), followed by *C,tropicalis* 5 out of 34 (14.7%), *Aspergillus niger* and *A.fumigatus* 3out of 34(8.82%) for each one, *A. flavus, Rhizopus spp, Mucor spp, Penicillium spp and Saccharomyces cerevisiae* 2 out of 34 (5.88%) for each one as well as the lower percentage of *Alterneria alternate* 1 out of 34 (2.94%). The results also revealed 26 out of 50 (52%) bovine lung samples were positive for fungal isolatation, February expressed high fungal isolation (58.82%), while April showed low percentage of fungal isolation (40%), the main fungal isolates from bovine lungs included *Candida spp* in high percentage (38.46%) especially *C.albicans* (23.07%) followed by *C.tropicalis* and *C.stellatoidea* (7.69%) for each one, *Aspergillus fumigatus* and *Rhizopus spp* 3 out 26 (11.53%) for each one, *Mucor spp* and *Penicillium spp* 2 out of 26 (7.69%) for each one, as well as the lower percentage of *Aspergillus niger, Botrytis aclada, Cladosporium herbarum, Fusarium spp, Cryptococcus neoformans and Alterneria alternate* 1 out of 26 (3.84%).

عزل وتشخيص الفطريات المصاحبة لإصابات الجهاز التنفسي المزمنة في الإنسان والأبقار

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الخلاصة

من اجل معرفة العزلات الفطرية المصاحبة لإصابات الجهاز التنفسي في الإنسان والأبقار تم اخذ 60 عينة قشع من الإنسان من مستشفى اليرموك التعليمي و50 رئة أبقار من مجزرة الكرخ للفترة من كانون الثاني إلى نيسان من عام 2012. بينت الدراسة بان 34 من 60 عينة قشع (56.66%) كانت موجبة للعزل الفطري، وكانت نسب العزل الأعلى في شهر كانون الثاني بنسبة (66.66%) واقل نسبة عزل سجلت في آذار 42.85%، وشملت العزلات الفطرية *Candida albicans* والتي سجلت أعلى نسبة من العزل الفطري 12 من 34 (35.29%) ثم تلتها *C. tropicalis* 5 من 34 (14.7%) *Aspergillus niger* و*A. fumigatus* 3 من 34 (8.82%) لكل منهما ثم *A.flavus,Rhizopus spp,Mucor spp* و *Penicillium spp and Saccharomyces cerevisiae* 2 من 34 (5.88%) لكل منهما، كذلك اقل نسبة عزل كانت لـ *Alterneria alternate* 1 من 34 (2.94%). أظهرت 26 عينة من عينات الأبقار نتيجة موجبة للعزل الفطري، نسبة العزل الفطري الأعلى كانت في شهر شباط(58.82%)، بينما كانت اقل نسبة عزل في شهر نيسان (40%)، وشملت العزلات الفطرية كل من *Candida sp* الأعلى نسبة

Aspergillus fumigatus (38.46%) *C.albicans* 23.07% ثم *C.tropicalis* و *C.stellatoidea* (7.69%) لكل منهمـــا *Rhizopus spp* (11.53%) ثم *Mucor spp* و *Penicillium spp* 2 مـــن 26 (7.69%) لكـــل منهمـــا ثـــم النســـبة الأقـــل لـــــ *Botrytis aclada* و *Aspergillus niger* و *Cladosporium herbarum, Alterneria alternate Fusarium spp, Cryptococcus neoforman* 1 من 26 (3.84%).

Introduction

Fungi are common in nature, and they are present low intrinsic pathogenicity health individual although they can cause very aggressive infection in certain clinical condition (1). There are over 250000 different species of fungi in which approximately180 are known to be pathogenic to human and animals, these which are pathogenic have been classified into four broad categories, superficial, cutaneous\ subcutaneous and systemic mycosis (2). Most systemic mycosis are form opportunistic that are usually innocuous, but become pathogenic when the host becomes abnormality susceptible to infection (3). Pulmonary fungal infection are considered a major problem in immunocompromised host (4), pulmonary mycosis can be causes by yeast like fungi (*Cryptococcus spp*), dimorphic fungi and can be categorizes according to the patient risk factor, changes in T lymphocytes (genera *blastomyces, coccidioides, Cryptococcus, histoplasma spp and pneumocystis*) or neutopenic (genera Aspergillosis, candidacies and Fusarium, as well as zygomycetes (5). However, the pulmonary opportunistic mycosis are associated with chronic respiratory diseases as Mycobacterium tuberculosis in human and animals, and these iterance may be fatal if not well diagnosed and treated. during the last several decades have been alarming increases in Aspergillosis, Candidiasis, Cryptococcosis, and Zygomycosis which of some degree appear to be related to medical treatment such as chemotherapeutic agent, irradiation, immunosuppressive agents (6). In Iraq, there are little information about the fungal isolation from human sputum and lung tissue of bovine. In the present study, an attempt to determine the fungal species associated with chronic respiratory disease in human and pulmonary lesions in the bovine.

Materials and Methods

- **Preparation of cultural and biochemical media:**

1. **Sabouraud Dextrose agar (SDA):** The medium was prepared according to the manufacturers' directions.
2. **Sabouraud Dextrose broth (SDB):** The medium was prepared according to the manufacturers' directions

- **The Samples Collection**:

1. **Human samples:** Sixty human sputum samples were collected from Al-Yarmook educational hospital, these samples were collected by sterile cotton swabs from patients of both sexes (males and females) in different ages suffering from chronic respiratory symptoms. These samples were collected during four months, which began in January 2012 till April, all these samples were transmitted under aseptic conditions to the laboratory in the college of veterinary medicine/ University of Baghdad.
2. **Bovine samples:** Fifty samples were collected from bovine which shows clinical signs of chronic respiratory disease, emaciation, weakness. From alkarhk slaughterhouse in Baghdad city. These samples were taken from the lungs and trachea immediately after slaughter and transmitted to the laboratory under aseptic conditions, by making several deep incisions in both right and left lung tissue and take small pieces to grind it in sterile morter and grinder, so that the samples were collected by cotton swabs.

3. **Samples culture:** each sample from (human and bovine) were cultured directly on six sabouraud dextrose agar media with chloramphenicol, three of them incubated in the incubator at 25±1º C to assist growth of moulds and another three at 30±1ºC to assist growth of yeasts for (1-8) days with intermittent observation of the fungal growth, and when the growth appeared and complete the identification test was done

Identification of fungi

Mould identification

A. **Macroscopic examination:** The colonies morphology including shape, color, consistency, texture and reverse plate color and other apparent characteristics of the colonies were examined according to (7).

B. **Microscopic Examination:** One drop of lactophenol cotton blue stain had been put on the slide and then mixed with a colony of mould then covered with a cover slip and examined under 40X lens to determine the shape of mycelium and shape of spores.

Yeast identification: Biochemical kit: Remel RapID™ Yeast Plus System is a qualitative micro method employing conventional and chromogenic substrates for the identification of medically important yeast, yeast-like, and related organisms isolated from clinical specimens., The principle of tests used in the RapID™ Yeast Plus System are based upon the microbial degradation of specific substrates detected by various indicator systems. The reactions employed are a combination of conventional tests and single-substrate chromogenic tests.

Components of the RapID™ Yeast Plus System:

1-Glucose
2- Maltose
3- Sucrose (Utilization of the carbohydrate)
4- Trehalose
5 - Raffinose

6 -LIP Fatty acid ester (Hydrolysis of the fatty acid ester)

7 - p-Nitrophenyl-N-acetyl- β,D-galactosaminide
8- p-Nitrophenyl-α,D-glucoside Enzymatic hydrolysis
9- p-Nitrophenyl-βD-glucoside
10- o-Nitrophenyl-β,D-galactoside

11- p-Nitrophenyl-αD-galactoside
12- p-Nitrophenyl-β,D-fucoside (Enzymatic hydrolysis)
13- p-Nitrophenyl phosphate
14- p-Nitrophenyl phosphorylcholine

15- Urea (Hydrolysis of urea)

16- Proline-β-naphthylamide
17- Histidine β-naphthylamide (Enzymatic hydrolysis)
18- Leucyl-glycine β-naphthylamide

Procedure: Inoculum Preparation

1. The tested organisms grew in pure culture.
2. Gently transferred the entire contents of the Inoculation Fluid tube into the panel.
3. After adding the test suspension, and while keeping the panel on a level surface, the panel tilted back away from the reaction cavities.
4. The panel was slowly tilted forward toward the reaction cavities until the inoculums flows along the baffles into the reaction cavities.
5. Incubated inoculated panels at 30ºC in a non-CO2 incubator for 4 hours.
6. After that, the following reagents were added to the cavities indicated:

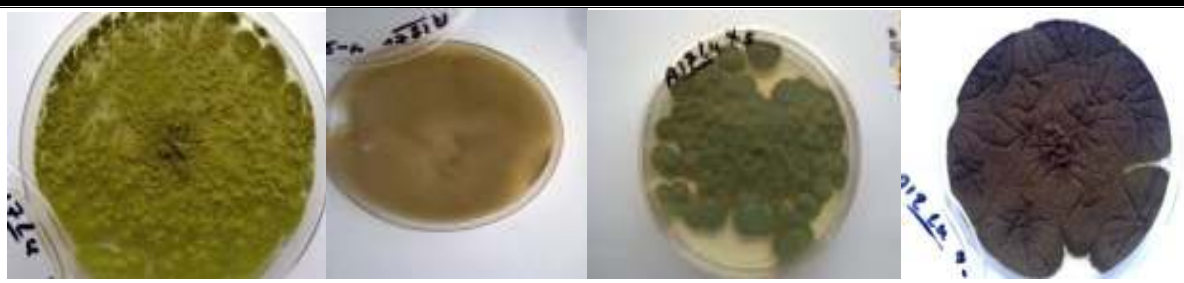
- 1 drop of RapID™ Yeast Plus Reagent A to cavities7 to 14.
- 1 drop of RapID™ Yeast Plus Reagent B to cavities 16 to 18.

7. Finally Reading and scored the test cavities from left to right using the interpretation guide presented.

Results and Discussion

Fungal isolation and identification:

Human sputum samples: The results showed clear fungal colonies at 1-3 days post-inocubation on Sabouraud dextrose agar with chloramphenicol at 30ºC for yeast growth and 3-8 days at 25ºC for moulds growth. The colonies were variable in their colors and shapes, microscopically, lactophenol cotton blue stain revealed mycelium, fruiting head, macro and microspores and chlamydospores (Fig. 1), these results were agree with (7) who explained that the best temperature for yeast and molds growth were 30ºC and 25ºC respectively.



Aspergillus fumigates *Mucor spp* *Penicillium spp* *Aspergillus niger*

Fig. (1) Morphological appearance of some isolated moulds cultures

According to the morphological and microscopical feature of fungal isolates, the obtained data demonstrated that 34 out of 60 (56.66%) from patient sputum which collected during a period between January-April 2012, showed fungal positive isolation. Highest percentage of fungal isolates was seen in January (66.66%) followed by February (65.21%), April (45.45%) and March (42.85%) (Table.1).

Table (1) The number of sputum samples, number of positive fungi and their percentage which collected from human sputum samples during January to April/ 2012

Month	Number of sample	Number of positive sample with fungi	Percentage%
January	12	8	66.66
February	23	15	65.21
March	14	6	42.85
April	11	5	45.45
total	60	34	56.66

The current results explained that high percentage of fungal isolates from sputum of patients suffering from chronic respiratory disorders. These results may be due to that the majority of fungi in nature are opportunistic pathogens and may be present in healthy people and cause disease when the host immunity is lower, or defective as a result of chronic pulmonary infection, these evidence was supported the idea mentioned by (8) who explained that The incidence of pulmonary mycosis has increased over the past few decades due to the wide use of broad-spectrum antibiotics, immunosuppressive and chemotherapy agents as well as the increased incidence of respiratory diseases, including chronic obstructive pulmonary disease, lung cancer and tuberculosis, also the result of the present study was agreed with (9) who observed that 46.7% of the patients were culture positive for pulmonary fungal agents. Also (10) reported that the overall incidence of systemic fungal infection is up to 11.3%, and 60% of them involve the bronchi and lung at autopsy. The variety in the percentage of fungal isolates according to months in the present study may be due to the influence of the lower degree of temperature that may be associated with viral or bacterial infection of the pulmonary system and provide a predisposing condition for opportunistic fungal infection. The current study revealed that *Candida albicans* constituted a high percentage of fungal isolates; 12 out of 34, (35.29%), followed by *C.tropicalis* 5 out of 34 (14.7%), (Fig. 2) *Apegillus niger* and *A.fumigatus* 3 out of 34 (8.82%) for each one, *A. flavus, Rhizopus spp,Mucor spp, Penicillium spp and Saccharomyces cerevisiae* 2 out of 34 (5.88%) for each one as well as the lower percentage of *Alterneria alternate* 1 out of 34 (2.94%) (Table 2).

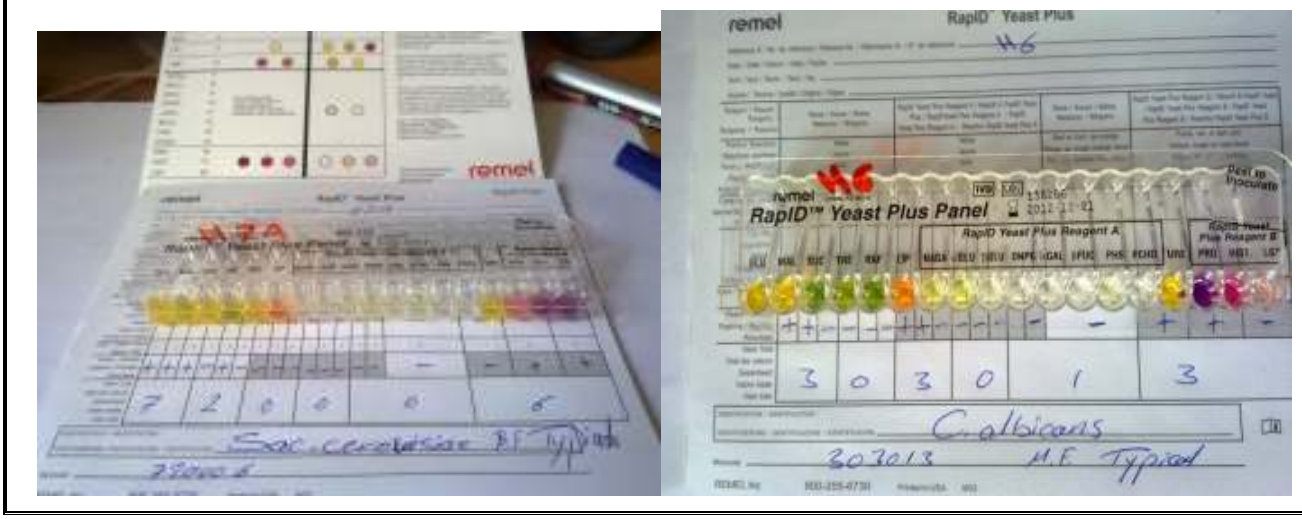


Fig (2) Bio chemicals kit RapID yeast plus system results, some yeast identifications

Table (2) Percentage of fungal isolates from respiratory infections in human

	Fungi	Number of isolates	Percentage%
1	*Aspergillus niger*	3	8.82
2	*Aspergillus flavus*	2	5.88
3	*Rhizopus spp.*	2	5.88
4	*Mucor spp.*	2	5.88
5	*Aspergillus fumigates*	3	8.82
6	*Penicillium spp.*	2	5.88
7	*Alterneria alternata*	1	2.94
8	*Candida albicans*	12	35.29
9	*Candida tropicalis*	5	14.70
10	*Saccharomyces cerevisiae*	2	5.88
	Total	34	100

The present study demonstrated that *Candida spp* and *Aspergillus spp* constitute the main fungi causing pulmonary mycosis and these finding are consistent with reports of (9) who recorded that *Candida albicans* was the most frequent isolate, being recovered from 42.9% of patients, followed by *Aspergillus flavus* 21.4%, *Aspergillus fumigatus* 14.3%, *Aspergillus niger* 10.7%, *Candida tropicalis* 7.1% and *Cryptococcus neoformans* (3.6%). In a similar study on *Candida* infection in chronic pulmonary conditions, (11) reported an isolation rate of 50% from sputum specimens, with *C. albicans* and *C. tropicalis* predominating. Also in studies on pulmonary Aspergillosis occurring in chronic lung diseases, (12, 13) had reported isolation of the fungi from 16.3% and 14.7% of cases of chronic respiratory diseases, using sputum and Bronchoalveolar lavage samples respectively. Also (14) reported that among 68 patients suffering from fungal infection, 38 cases (55.9%) were identified as pulmonary aspergillosis, 19 (27.9%) as pulmonary cryptococcosis, 5 (7.4%) as pulmonary candidiasis, 4 (5.8%) as pulmonary histoplasmosis, On the base of above evidence we suggested that fungi may be colonized the respiratory tract of patients presenting with different chronic respiratory condition, particularly *Mycobacterium tuberculosis* or cancer that widespread in Iraq, these investigation was confirm result of (9) who reported that in 28 patients (46.7%) were culture-positive, with *Candida* and *Aspergillus* being recovered from 14 and 13 patients respectively and also they showed that patients with bronchogenic carcinoma showed increased predilection for colonisation with *aspergillus* while *candida* was recovered more commonly in tubercular squeal. Also our result was in consistence with the observation that mentioned by (14) who investigated that among 66 patients positive for pulmonary fungal isolates, 53 patients (77.9%) had underlying diseases, including 16 cases of tuberculosis (23.5%), 13 of chronic obstructive pulmonary disease (19.1%), 6 cases of bronchiectasis, 6 of lung cancer (8.2%), 4 cases of inflammatory pseudo tumor, 3 of pulmonary cysts, 2 cases each of lung abscess, gout and diabetes, and 1 case each of severe pneumonia, empyema, bronchopleural fistula, idiopathic thrombocytopenic purpura, systemic lupus erythematosus, drug-induced neutropenia, pemphigus, acute immunodeficiency syndrome (AIDS), cytomegalovirus infection, and asthma. Four cases had used corticosteroids for more than 6 month. It was found in this study that the mucormycosis constituted the 3rd pulmonary fungal infection (4 out 34,11.76%) as compared with *Candida spp*17 out of 34 (35.29%) and of *Aspergillus spp* 8 out of 34 (23.55%), these results may be indicated that mucormycosis are considered as one important pulmonary fungal disease that may cause invasive mycosis in immunocompramized patients, these evidence are agreed with previous observation of several authors. Kontoyiannis and Lewis, (2006) explained that the Mucormycosis is the

second most frequent mold infection in immunocompromised patients, and can progress rapidly in both immunocompromised and immunocompetent individuals, also (16) explained that *Rhizopus*, *Mucor*, and *Lichtheimia* (formerly *Absidia*) species are the most common members of the order Mucorales that cause mucormycosis, accounting for 70 to 80% of all cases affect primarily immunocompromised hosts, mostly resulting from spore inhalation, causing pulmonary and disseminated infections with high mortality rates. However, we used mucormycosis that isolated from human sputum in experimental study which revealed that these isolates are highly virulent, these finding may indicated that the mucormycosis isolates induced lung lesion in the naturally infection patients ,these evidence was supported observation that mention by (17) who said that the Pulmonary Mucormycosis is less common opportunistic fungal disease, localized in the lungs or the mediastinum. Invasion of blood vessel by fungal hyphae, results in necrosis of tissue parenchyma, which may ultimately lead to cavitation and/or hemoptysis. Most common predisposing conditions for mucormycosis are uncontrolled diabetes mellitus, malignancy, chronic illnesses and transplants. Also the present investigation were supported the idea mentioned by (18), in France, who recorded that the incidence rate of zygomycosis increased from 0.7 cases million persons in 1997 to 1.2 cases million persons in 2006 with early increase was 7.4%. Also in India, (19) reported Zygomycosis is an increasingly common infection in immunocompromised patients especially in patients with uncontrolled diabetes.

Bovine samples: We demonstrated that 26 out 50 (52%) bovine lung samples were positive fungal isolation and these isolates were variable according to months in which samples were collected, and the result showed high percentage of fungal isolated in February 58.82% followed by January 52.63%, March 44.44% and April showed lower percentage of fungal isolates 40%. (Table.9).

Table (3) The number of lung samples, number of positive samples and their percentage which collected from bovine lung during January to April/2012

Month	Number of sample	Number of positive sample with fungi	Percentage(%)
January	19	10	52.63
February	17	10	58.82
March	9	4	44.44
April	5	2	40
total	50	26	52

The present study explained high percentage of fungi that isolated from lung tissue that collected aseptically from bovine at slaughter house specially in cold month, these may be due to predisposing condition that induced by low temperature, poor nutrition or prolong using antibiotics. *Candida spp* was isolated in high percentage (38.46%) from the lungs of the bovine especially *C.albicans* (23.07%) followed by *C.tropicalis* and *C.stellatoidea* (7.69%) for each one, *Aspergillus fumigatus* and *Rhizopus spp* (11.53% for each one), *Mucor spp* and *Penicillium spp* 2 out of 26 (7.69%) for each one, as well as the lower percentage of *Aspergillus niger, Botrytis aclada, Cladosporium herbarum, Fusarium spp, Cryptococcus neoformans and Alterneria alternate* 1 out of 26 (3.84%) for each one. (Table.4). The current results explained that *C.albicans* constitute high percentage of fungal isolates, these observations may be indicated that *C.albicans* form important cause of bovine mycotic infection these evidence was agree with (20) who recorded that *C.albican* in 50% of vaginal and respiratory swabs that collected from cattle. Also the present investigation are supported the result that mentioned by (21), Who explained that animal

Candidiasis induce economic problems in dairy cattle via cause of abortion and mastitis, in addition, *Candida albicans* induce depression of immune response through its mannan cell wall which have ability to suppress cellular immunity as well as *C.albicans* avoid host defense as a result of antigenic variation on its cell surface (23).

Table (4) Percentage of fungal isolates from respiratory infections in bovine

1	*Aspergillus fumigatus*	3	11.53
2	*Rhizopus spp*	3	11.53
3	*Aspergillus niger*	1	3.84
4	*Mucor spp.*	2	7.69
5	*Penicillium spp*	2	7.69
6	Botrytis aclada	1	3.84
7	Cladosporium herbarum	1	3.84
8	Fusarium spp.	1	3.84
9	*Alterneria alternate*	1	3.84
10	*Cryptococcus neoformans*	1	3.84
11	*Candida albicans*	6	23.07
12	*Candida tropicalis*	2	7.69
13	*Candida stellatoidea*	2	7.69

It was found in the present study other *Candida* species from the lung of examined bovine where *C. tropicalis* (7.69%) and *C.stellatoidea* (7.69%) were isolate, these results may indicated increasing incidence of non *C.albicans* infection in bovine, these findings confirm the results of (21) who isolated *C.tropicali* (22%), *C.kruesi* (19%). *C.parapsilosis* (4.9%) and *C.rugosa* (2.4%) from vaginal and nostril swabs of cattle. The present revealed that *Aspergillus sp* were consistute the 2nd lung fungal isolates (15.38%), these finding was in agreement with (23) who found that the Pulmonary aspergillosis was found in 10 (52.6%) of 19 calves. Mucorales also isolated from lung tissues of the bovine (23.07%) in the present study, these percentage may indicated that the mucormycosis form a third etiological agents of bovine fungal pneumonia, (23) reported that Systemic mycoses were found in 19 (4.7%) of 406 calves less than 6 months old, and in alimentary mycosis, mucormycosis recorded in 11 out of 12 (91.7%) of calves. Also (24) explained that pulmonary mucormycosis is an uncommon disease caused by fungi of class Zygomycetes, occurs predominantly in an immunodeficient host, most common risk factor being diabetes mellitus and the lesions are localized in the lungs or the mediastinum.

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