Research Article

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Isolation and Identification of Fungi from Lungs and Rumens of Small Ruminants

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Abstract

The current study aimed at the isolation and identification of some fungal types that infected the lungs and rumens of small ruminants (non-healthy animals). To achieve this goal, 100 samples from sheep, and goat lungs and rumen tissues (50 for each), were collected from the Basrah slaughterhouse. Fungi were isolated from the samples and diagnosed based on the morphology of colonies cultured on Sabraud Dextrose Agar (SDA) media, then detected microscopically after staining with methylene cotton blue and Indian ink stain. PCR and DNA sequencing of the18S rRNA ribosomal gene confirmed the important and common fungal isolates. The current study showed the presence of fungal isolates in lung samples of sheep including, *Cryptococcus* spp. (29.43%) represented the highest percentage. While the lowest percentage was *Coccidioides* spp. accounted for (4.878%). However, those isolated from the rumen of sheep in high percent was *Cryptococcus* spp. (48.571%), and finally, the lungs contained *Coccidioides* spp. (4.878%). The result of fungal isolates from the lungs of the goats in high numbers was *Aspergillus* spp. (42.5%), the fungal isolate that appears in low numbers, *Candida* spp. (7.5%).

Key words: Basidiomycetes Yeasts, Sheep lung, Sheep Rumen, Goat, *Cryptococcus adeliensis*, Basrah

Introduction

Sheep and goat breeding provides a small but important supply of animal protein in the form of milk and meat. This is especially useful for low-income farmers and landless workers. In certain areas, sheep/goat production is the only source of income for producers. Sheep offers three main products: meat, wool, and milk. In many parts of the world, especially in temperate regions, meat is the main product and meat is becoming more important in the production of sheep (1).

Sheep and goats contribute significantly to the economy of farmers in different countries including Iraq (2). Numerous biological diseases such as fungal diseases can infected small

ruminants. About 1.5-5 million species of fungi worldwide can grow in soil, plants, and animal, only a small number of these species are considered pathogens for humans and animals (3, 4). Fungal disease is an important active infection that associated with mycotoxins secretion when the fungi grow in tissues of animals and humans. These pathogenic agents disorders specific fungal disorders such as bovine mastitis, respiratory disorder, fungal diarrhea in calves, superficial, subcutaneous, mycotoxicosis, and systemic infection (5, 6).

There are many types of fungi that causes fungal pneumonia and rumenitis in immunocompromised and non-immunocompromised hosts including small ruminants. Inhalation of spores is the most common form of infection, which can lead to hemolymphatic dispersion. For these organisms, pulmonary tissues and secretions provide an ideal environment. Cryptococcosis, *Coccidioides immitis*, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Pneumocystis jiroveci*, *Aspergillus* spp., *Candida* spp. and other less common fungi cause infection in the lungs (6). Systemic mycoses are typically suspected based on clinical symptoms and macroscopic lesions. However, final diagnosis requires microscopic identification, organism culture, or polymerase chain reaction PCR conformation (7). Cryptococcosis is an important invasive fungal disease in humans and animals induced by *Cryptococcus* particularly *C. neoformans* and *C. gatti* that cause a global incidence of infection in Human Immunodeficiency Virus (HIV) infected patients with approximately 1 million cases and 620 000 mortality in each year (8, 9).

In Iraq, there are few research's papers about the isolation and identification of fungi from lungs and rumens of small ruminants (sheep and goat); therefore, the aim of the present study was to investigate the main fungal species isolated from lung and rumen tissues of sheep and goats. In addition, molecular detection of the most prevalent fungal isolates using PCR followed by nucleotide sequencing.

Materials and Methods

Samples collection

During the slaughtering of sheep and goats at Basrah slaughterhouse, 200 spacemen from sheep and goats (50 rumen tissues and 50 lung tissues from each) were collected randomly. The samples were examined visually for gross lesions, direct examination of specimens provides a presumptive diagnosis and allows for the selection of downstream analysis in diagnosing diseases. The main methods for direct examination are Direct wet mount, India Ink, KOH/calcofluor mounts, Lactophenol cotton blue mounts, Gram stain, and the Acid-Fast stain (10).

These stains are used for direct examination of fungi (yeast, molds, and dimorphic fungi) and members of the actinomycete ('Mycobacterium' and 'Streptomyces). Each tissue sample was placed in a sterile polyethylene bag (containing normal saline) in an icebox under aseptic conditions for fungal isolation.

Mycological examination

Fungal culture was accomplished based on the standard mycological techniques using Petry dishes of Sabouraud dextrose agar (Himedia, India) (SDA) containing 0.05 gm/L chloramphenicol (TROUGE, Germany) and incubated at (25-30)°C for (3-5) days. Subsequently, the fungal isolates were identified at the genus level by conventional techniques, such as lactophenol cotton blue stain (LPCB)

(Fluka, Switzerland) and Indian ink stain (BDH, UK) (11,12). Cultures of all the reported species were brought to the Central laboratory, Department of Pathology and Poultry Diseases, College of Veterinary Medicine, the University of Basrah for species identification.

Molecular Diagnosis

The DNA was extracted from yeast isolates after activation on potato dextrose agar (incubated at 25 ºC for 48 h). Then, 100 ng DNA of *Cryptococcus adeliensis,* colonies were weighed and transferred to a 1.5 ml micro centrifuge tube contained sterile distilled water and centrifuged at 4500 rpm for 3 min. The supernatant was discarded completely according to the procedure of Presto ™ Mini gDNA Yeast Kit (13, 14).

The amplification of extracted DNA from clinical isolates by using PCR. Molecular identification of *Cryptoccocus* spp. in this study, with the aid of the use of conventional PCR for the amplification of a partial gene of 18S rRNA by way of specific primer sequences was used (ITS) region using primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') or ITS5 (5'-GAAGTAAAAGTCGTAACAAGG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') under standard conditions (15).

The PCR reaction mixture included the Master mix (Bioneer/ Korea), 100 ng DNA template, 10 pmol of each primer, nuclease free water up to final volume of 50 µl. The PCR components are mentioned in Table 1.

Table 1: Component of PCR reaction mixture

No.	Component	Working concentration	Volume
1	DNA template	100 ng	10 µl
2	Forward primer ITS1 ITS5	10pmol	2 µl
3	Reverse primer ITS4 or NL4	10pmol	2 µl
4	Master mix	-	10 µl
5	Nuclease free water	-	26 µl
	Total reaction volume	-	50 µl

The PCR cycling conditions involved an initial degeneration step at 94 ºC for 5 min, followed by 25 cycles of denaturation at 94 ºC for 30 sec, annealing at 56 ºC for 45 sec, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 7 min.

Agarose gel electrophoresis

The agarose gel was prepared according to (16). Briefly, 1g of agarose powder (1%) was dissolved in 100 ml of 1x TBE buffer and boiled on a hot plate until the mixture became clear. Then, 0.2 µl of ethidium bromide was added and mixed. Afterward, the gel was left at room temperature until it reached approximately 45 °C. The gel was then poured into its appropriate tray and the comb was placed in its place to make holes later in the solidified gel. The gel was left to solidify at room temperature, the comb removed carefully, and the gel tray placed into an electrophoresis tank filled with 1x TBE buffer that covered the surface of the gel.

Samples loading and running the gel

3 µl of DNA samples (with equal concentration by using free nuclease

water) was mixed with 2 µl of bromophenol blue, loaded in the well of 0.8% agarose gel, and loading the ladder DNA as slandered. The gel electrophoresis was run at 60 V for 50 minutes. Until bromophenol blue migrate to the end of the gel. Under UV transilluminator, the gel was photographed to detect DNA bands (17).

Sequencing

PCR product (20 µl) of sample was labeled with a number identical to the number of excel sheet, which is given by MACROGEN Company after sending the18S rRNA primers and PCR product. The company for sequencing (18) accomplished the purification of the product. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The sequence results were compared with other fungal sequences in NCBI's GenBank sequence database to identify species by using a blast search. An alignment of the sequences was carried out with the CLUSTAL W program (19, 20). Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov).

Results

Isolation of Fungi

Mycological analysis of lung and rumen samples of sheep revealed isolation of 41 and 35 fungal isolates out of 50 lung and 50 rumen samples, respectively. Mycological analysis of lung and rumen samples of goats revealed isolation 40 and 40 fungal isolates out of 50 lung and 50 rumen samples respectively (Table 2). The fungal isolates identified according to the appearance (color, shape, and nature of the growth) as well as microscopic test, of lung samples of sheep included: *Cryptococcus* spp. (29.43%) followed by *Rhodotorula* spp. (19.512%), *Pencillinium* spp. (14.63%), *Candida* spp. (12.195%), *Histoplasma* spp. (9.756%), *Aspergillus* spp. (9.756%), and *Coccidioides* spp. (4.878%). While, those isolated from rumen of sheep included *Cryptococcus* spp. (48.571%), *Aspergillus* spp. (20%), *Cladosporium* spp. (17.142%), *Cladophialophora* spp. (5.741%), (5.741%), and *Coccidioides* spp. (4.878%).

The results of fungal isolates from lung of the goats included *Aspergillus* spp. (42.5%), *Cryptococcus* spp. (40%), *Rhodotorula* spp. (10%), *and Candida* spp. (7.5%). While, those isolated from the rumen of goats included *Aspergillus* spp. (47.5%), *Cryptococcus* spp. (40%), *Rhodotorula* spp. (10%), and *Candida* spp. (7.5%). Table 2 showed the sample number, fungal spp., and it's percent that isolates from small ruminant tissues.

Table 2: Numbers and species of fungi isolated from lung and rumen-samples of sheep and goat.

	Animal Tissues	Sample number	Fungal spp.	Number	Percentage
1.	Sheep Lungs	50	*Cryptococcus*	12	29.43
			Rhodotorula	8	19.512
			Penicillium	6	14.63
			Candida.	5	12.195
			Histoplasma	4	9.7566
			Aspergillus	4	9.7566
			Coccidioides	2	4.878
			Total no.	41	
2.	Sheep Rumens	50	*Cryptococcus*	17	48.571
			Aspergillus	7	20
			Cladosporium	6	17.142
			Cladophialophora	2	5.714
			Rhodotorula	2	5.714
			coccidioides	1	2.857
			Total no.	35	
3.	Goat Lungs	50	*Aspergillus*	17	42.5
			Cryptococcus	16	40
			Rhodotorula	4	10
			Candida	3	7.5
			Total no.	40	
4.	Goat Rumens	*50*	*Aspergillus*	19	47.5
			Cryptococcus	16	40
			Rhodotorula	4	10
			Candida	3	7.5
			Total no.	40	

The primary diagnosis depends on the colony morphology, color, and size on culture media, as well as their features under the light microscope. The yeast colony of *Rhodotorula* spp. isolated `from the lung of goat appear in culture media as small pink, glossy, and mucoid colonies (Figure 1). Moldy Colony and texture on PDA medium revealed grey-olivaceous, regular, feathery. White, aerial mycelium sparsediffuse or sometimes abundantly formed (Figure 2). Margin of the colony was white to grey-olivaceous. Colony reverse was olivaceous-black and velvety. The macroscopic morphology of *Coccidioides* colonies on the Sabouraud dextrose agarwas moist, glabrous, membranous, and grayish initially, later producing white and cottony aerial mycelium (Figure 2).

Figure 1: Showing small pink, glossy, and mucoid yeast colony *Rhodotorula mucilaginosa* culture.

Figure 2: showing *Cladosporium* spp. (black arrow). *Coccidioides* (red arrow).

The macroscopic morphology of moldy colonies are usually fast growing, dark green, raised in the center, surround by white ring, mostly consisting of a dense felt of erect conidiophores (Figure 3). Figure 4 showing microscopic morphology to *Aspergillus* the conidiophore, conidial head.

Penicillium spp. that appear microscopically stained with lacto phenol cotton blue as a chains of single-celled conidia are produced in basipetal succession from a specialized conidiogenous cell called a phialide (Figure 5).The macroscopic morphology of colonies of *Cladophialophora bantiana* are moderately fast growing, olivaceous grey, suede-like to floccose that appears in SDA media (Figure 6).

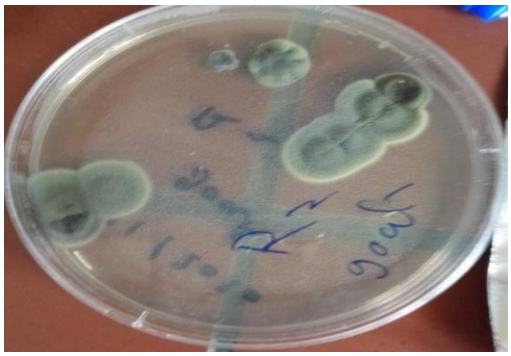

Figure 3: Showing *Aspergillus* spp. colony

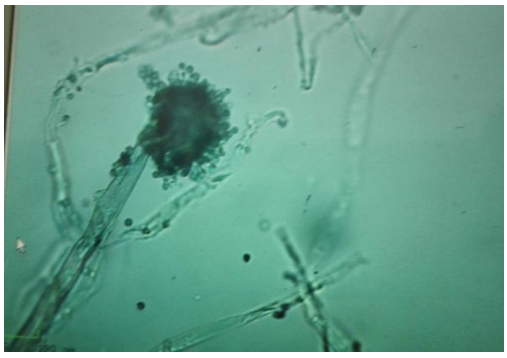

Figure 4: showing *Aspergillus* spp. conidiophore MCB stain 400X

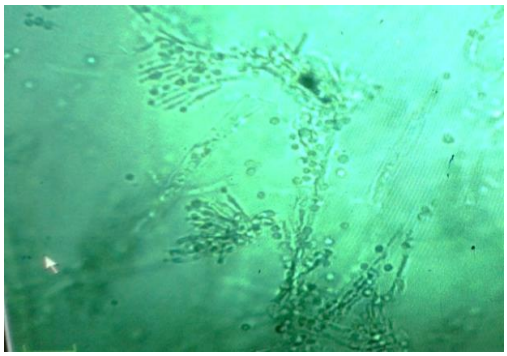

Figure 5: *Penicillium* spp. hyphomycete, flask-shaped phialides arranged in groups from branched metulae forming a penicillus MCB stain 400X.

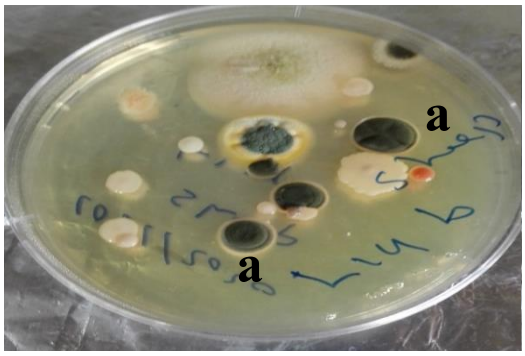

Figure 6: Showing *Cladophialophora bantiana (a)* colony on SDA media.

The macroscopic morphology of colonies of *Candida albicans* on (SDA) media as white to cream-coloured smooth, glabrous, yeast-like (Figure 7). Microscopy: Spherical to subspherical budding blastoconidia stained with LPCB (Figure 8).

The macroscopic morphology of colonies at 25° C are slow growing, white or buff-brown, cottony with a pale yellow-brown reverse (fig 9).

Figure 7: Showing *Candida albicans* showing typical cream-coloured, smooth surfaced, waxy colonies culture on SDA media.

Figure 8: showing *Candida albicans* narrow based budding spherical to ovoid blastoconidia

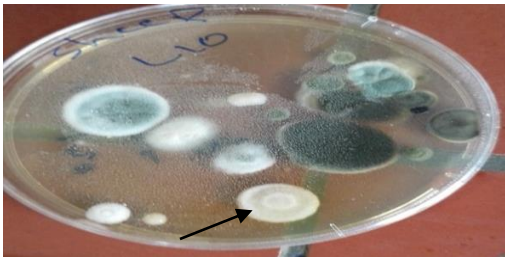

Figure 9: Showing *Histoplasma capsulatum* white or buff-brown, cottony colony (arrow) culture on SDA media.

Identification of fungi based on morphology

The present study showed a new colony of *Cryptococcus adelineisis* isolated from the lungs of sheep. The colonies appeared on SDA as white to creamy in color, after 3-4 days, with 1-2 mm in diameter, at 27°C, and had a smooth and glossy surface, soft to mucoid. The yeast cells showed a small capsule (Figures 10 and 11).

Figure 10: showing *Cryptococcus adeliensis* colony on SDA media.

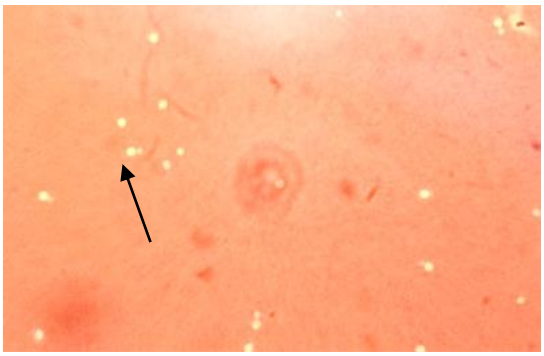

Figure 11: Showing India ink preparation of *C. adeliensis* surrounded by a characteristic small capsule 400 X.

Genetic analysis of *Cryptococcus* spp.

Molecular Detection

The results of gel electrophoresis using (18S rRNA) gene for detection yeast samples showed the PCR product with amplification region (600bp) (Figure 12).

Nucleotide sequencing and phylogenetic tree analysis

The of sequencing revealed that 99% (Table 3) of our isolates done compatible with reference strain which recorded as new strains that deposits in GenBank by using the BLAST program (http://www.ncbi.nlm.nih). This table was also demonstrated the level of identity of local strain (*C. adeliensis).*

In the present study, the phylogenetic tree was constructed based on neighbor-joining analysis of 18S rRNA gene to see the relationship of local samples with the higher query cover (above 99%) of national samples(Table 3 and Figure 13). Figure 13 recognizes the Iraqi local samples and national samples from NCBI information. The Iraqi isolates were appeared in green colors. The Iraqi *Cryptococcus* sp. was extreme likely to the national sample from Greece (Figure 13).

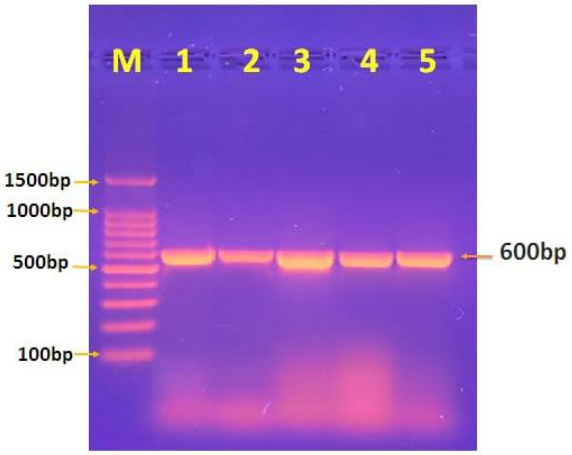

Figure (12): Conventional PCR amplification of 18S rRNA ribosomal gene for isolates. Lane M: DNA ladder (100bp), Lane M: DNA ladder (100bp), lane 1-5 positive result for *Cryptococcus* spp. (600bp.)

Table (3): The NCBI-BLAST Homology Sequence identity between local *Cryptococcus* spp. isolates and other NCBI-BLAST submitted for C*ryptococcus* spp. isolates.

local *Cryptococcus* sp. isolates	Accession number	Homology sequence identity (%)			
		NCBI related species	Accession number	Identity (%)	Country
***Cryptococcus sp.* No.1**	MZ031280	*Cryptococcus adeliensis*	KC254019.1	99.15%	Greece
***Cryptococcus sp.* No.2**	MZ031278	*Cryptococcus adeliensis*	KC254019.1	99.17%	Greece
***Cryptococcus* sp. No.3**	MZ031279	*Cryptococcus adeliensis*	KC254019.1	99.16%	Greece

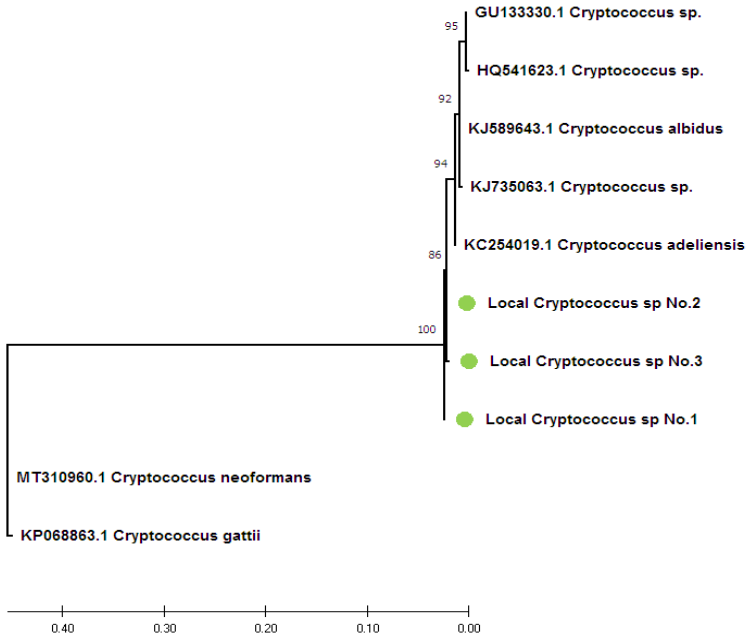

Figure (13): Phylogenetic tree analysis local *Cryptococcus* spp. sheep isolates that used for *Cryptococcus* species genetic identification analysis. The local *Cryptococcus sp.* sheep isolates (No.1- No.3) showing genetic variant related to NCBI-BLAST *Cryptococcus adeliensis* (MZ031278) at total genetic changes (0.40-0.10%).

Discussion

Small ruminants including sheep and goats are important sources of milk and meat in Iraq, respiratory tract infection is one cause of animal deaths, so studying fungal isolated from the respiratory tract of sheep and goats is important. In the present study, the fungal isolates from the lungs sheep and goats were demonstrated, out of 100 lung samples from both sheep and goats, 50 for each one, 42 and 40 fungal isolates were recorded through the mycological examination of the samples. Healthy animals may carry more opportunistic fungus, which may cause fungal infection in the debilitated and immunocompromised hosts, which may be the fatal outcome in undiagnosed infection (21).

The current findings revealed that the primary fungal cause of respiratory mycosis in sheep, *Cryptococcus* spp. due to the high percent incidence, whereas *Aspergillus* spp. considered the primary fungal cause of respiratory tract mycosis in goats. These findings were consistent with those reported by (22, 23), who discovered that the prevalence of aspergillosis in goats and sheep was 4.9 %. The percentage of *Aspergillus* spp. isolates in the current study was lower than that reported by (24), which might be attributed to the number of samples, the region of isolation, and environmental conditions. In the current study, high percentage of *Aspergillus* spp. isolates was lower than that reported by (23), possibly due to prolonged use of prophylactic antibiotics. This finding is consistent with (25), who demonstrated

that prolonged use of antimicrobials may predispose to pulmonary aspergillosis in ruminants. The current study discovered *Rhodotorula* spp., fungal isolates from sheep and goat lung samples. These findings suggest that these fungi may be responsible for an important fungal lung mycosis in these animals; however, there have been few studies on the pathogenicity of *Rhodotorula* spp. in animals. Has received little attention (25, 22), recorded lung infection in sheep by *R. mucilaginosa*, as well as several outbreaks of skin infection in chickens, (26) isolated these fungi from cattle ear canal. *Cladosporium* spp. was isolated from rumen sheep, but these fungi were not isolated from lung samples. These findings may indicate that this organ can become infected via the gastrointestinal tract of animals (27). *Cladosporium* spp. are plant pathogens that can affect animals and humans. According to Mercier and colleagues (28), the current study found that *Pencillinium* spp. form third fungal isolates from sheep lung samples. These findings are consistent with those of (23), who isolated 12.5 % of *Pencillinium* spp. from sheep nasal swaps.

Furthermore, the isolation of *Candidia* spp. from sheep and goat lung samples suggests that these fungi can cause lung infection. (29) Came to an agreement, exploring that candidiasis and aspergillosis were the most common mycotic infections in ruminants. As stated by (30), the respiratory and gastrointestinal tracts are the most common sites of fungal infection. The current study found that fungal isolates from goat rumen were *Candida* spp., which is similar to the findings of (31), who discovered that *Candida* spp. might cause gastrointestinal candidiasis in animals. *Histoplasma* spp., particularly *Histoplasma capsulatum*, cause a non-contagious fungal disease, and these fungi may reach the alveoli via inhalation of hyphal elements, and microconidia are then disseminated to other parts of the body via the bloodstream (32, 33, 34). The current findings suggest that *Histoplasma* spp. isolates may be the first to be reported from sheep lungs. Since (24), isolated *Coccidioides immitis* from aborted placental sheep and discovered that these fungi make up 20% of fungal isolates from aborted placentas; the current result may be considered a second fungal isolate from sheep samples in Iraq.

The morphological features of *Cryptococcus adeliensis* which diagnose in the present study showed similar characterization of *Cryptococcus* done by (15), and in other cases, *C. adeliensis* was isolated from medical samples, such as the case of a 40-year-old female suffering from acute myeloid leukemia. After aspirating some cerebrospinal fluid and incubating it for three days on Sabouraud glucose (2 percent) agar, tiny colonies with a yeast-like appearance were visible at 30°C but not at 37°C (21). For the Iraqi isolates with those listed in GenBank and given an accession number for the nearest national isolate, three strains of *Cryptococcus* spp. were isolated and identified from local sheep, finding that had over 99% with identified *Cryptococcus adeliensis* (35). Previously registered and listed in the GenBank. Consequently, when compared with traditional identification methods 18srRNAgene sequence analysis method is considered more accurate and more reliable, with obvious advantages in the identification of yeast at the level of species. Gene sequencing enables a rapid diagnosis without the need for viable organisms or spores (molds). Gene targets for identifying medically important yeast and fungi are not as well codified as those for bacteria (23). ITS1 and ITS2, encoding the genes for 18S, 5.8 S and 28S rRNA, the optimal objectives of many medically important fungi can be identified by ITS region, including *Candida, Cryptococcus, Trichosporon* (36, 37), *Aspergillus* species; *zygomycetes*; and

dematiaceous molds (38, 39). Analysis of (phylogenetic) patterns, i.e., patterns of gene presence/absence in a particular set of genomes, is a valuable approach both for the detection of evolutionary trends and for function prediction (40,41).

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عزل وتحديد الفطريات من الكرش والرئتين في المجترات الصغيرة

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الخلاصة

هدفت الدراسة الحالية إلى عزل وتحديد بعض الأنواع الفطرية التي أصابت رئتي وكرش المجترات الصغيرة (حيوانات غير سليمة). لتحديد هذا الهدف، تم تحديد جمع 200 عينة من كرش ورئة الأغنام والماعز والتي كانت بواقع 50 عينة لكل عضو، وتم جمع هذه العينات من مجزرة البصرة. وبعد ذلك قمنا بتشخيص الفطريات المعزولة من العينات اعتمادا على الشكل الخارجي للمستعمرات المستزرعة على وسط سابرود دكستروز اكار(SDA)، بعدها تم فحص العزلات مجهريا بعد تصبيغها بصبغة قطن الميثيلين الأزرق وصبغة الحبر الهندي. العزلة التي كانت من الأنواع المهمة والأكثر نسبة تكرار تم تأكيد نوعها او تشخيصها عن طريق PCR وgene sequence باستخدام 18S rRNA.أظهرت الدراسة الحالية ان نتائج العزل الفطري من عينات الرئة في الاغنام وجود الأنواع الفطرية التالية: *Cryptococcus* spp. بنسبة (9.43٪) والتي كانت تمثل اعلى نسبة تكرار اما الفطريات التي أظهرت اقل نسبة هي *Coccidioides* spp. بنسبة (4,878 %). في حين تلك المعزولة عن كرش الأغنام شملت الأنواع التالية: *Cryptococcus* spp. بنسبة (48.571٪)، اما التي كانت اقل *Coccidioides* spp. وبنسبة (4,878 %). وأخيرا كانت نتيجة العزلات الفطرية المعزولة من رئة الماعز كانت *Aspergillus* spp. بنسبة (42.5 %) الأكثر نسبة تكرار، *Candida* spp. (7.5 ٪).