

**Original Paper****Cryptococcosis in small ruminants with special references to conventional and molecular methods of diagnosis**Ashraf A. Abd El-Tawab<sup>1</sup>, Eman M. El-diasty<sup>2</sup>, Ahmed A. Fawaz<sup>3</sup> and Emad E. El-Mougy<sup>1</sup><sup>1</sup> Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Benha University, Egypt<sup>2</sup> Department of Mycology, Animal Health Research Institute (AHRI), Ministry of Agriculture, Dokki, Giza, Egypt<sup>3</sup> Tanta lab branch, Animal Health Research Institute (AHRI), Ministry of Agriculture**ARTICLE INFO****ABSTRACT****Keywords**

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This work intended to detect yeast species involved in sheep and goats respiratory infections using culturing method, biochemical auto-fluorescence technique and PCR assay. To realize this, 50 lung specimens and 50 samples of nasopharyngeal swabs were collected from clinically diseased sheep and goats freshly slaughtered animals in Kalyobia Governorate. These samples were subjected to fungal examination and the result showed that from the 50 examined sheep and goats lung, 21 lung (42%) had yeast infection and out of 50 nasal swab from sheep and goat 16 (32%) had positive yeast. The architectural detail of bright green-to-yellow green auto-fluorescence spherules, yeast forms *Cryptococcus* gave stronger and brighter fluorescence it is known to be fast test used in detection to mycotic pneumonia with any late accompanied by haematoxylin and eosin stain (H&E) staining. PCR results proved that *Cryptococcus* isolates were positive, where for the results of PCR proved that only 3 (17.6%) isolates were positive for *Cryptococcus albidus*. Considering of this fact this study suggest screening and detecting fungal species using of auto-fluorescence methods and PCR technique in pneumonic sheep and goats.

**1. INTRODUCTION**

Respiratory disorders represent one of the most important causes of great economic losses among animals. Lung affections in farm animals constitute serious problem that hinders animal production and may result in great losses in animal husbandry. The primary causes of sheep and goat pneumonia are bacteria, followed by viruses and fungi whereas poor hygienic measures and climate disorders are the most predisposing factors to infection (Mahmoud et al., 2005).

*Cryptococcus* species is yeast like fungus with worldwide distribution; it causes medical problems in humans and animals. The organism occurs as a saprophyte in nature, usually in soil contaminated with pigeon and chicken manure, and is considered an opportunist pathogen. Sporadic cases of mastitis have been also described in other domestic ruminants, including goats; *Cryptococcosis* has been previously reported in cats, pigeons, and goats in association with caprine pleuropneumonia (Refai et al., 2017).

El-Metwally et al., (2011) reported that Trypan blue agar media for selective confirmation of many pathogenic fungi as *Candida*, *Cryptococcus*, *Aspergillus* and *Zygomycetes* have been shown to exhibit autofluorescence when H and E stained tissue identification of *Prototheca* Spp. Identification of sections are examined under a fluorescent microscope without adding any immune-reagents. This test is considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains. Molecular techniques utilizing implication of

target DNA provide alternative methods for diagnosis and identification. PCR-based detection of fungal DNA sequences can be rapid, sensitive, and specific. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships. Between coding regions there are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2, respectively) which evolve more rapidly and may therefore vary among different species within a genus. Thus, PCR implication may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be easily identification to its regions (Chen *et al.*, 2000).

This study targets for isolation and identifying possible causative yeasts pathogen of such respiratory disease conditions as direct methods of their diagnosis, beside evaluation the results of auto-fluorescence in identification of fungi.

**2. MATERIAL AND METHODS****2.1. Collection of samples**

A total of 50 lung samples of small ruminant (sheep and goats 25 samples for each) were collected from abattoirs at Kalyobia Governorate. Samples were collected from freshly dead diseased animal suffering from respiratory manifestation.. The samples were visually examined for gross lesions. Each tissue sample was divided into two parts, one part was kept in a sterile polyethylene bags in an ice box under aseptic conditions for mycotic isolation and the second part was immersed in 10% formalin saline for

histopathological evaluation. Nasopharyngeal swabs were aseptically taken from affected animals (50 in number) and transferred into icebox directly to the laboratory with minimum of delay. All samples were subjected to mycological examinations.

### 2.2. Culture of sampling (Anaissie et al., 2003)

The nasopharyngeal swabs were directly cultured by streaking on the surface of two plates of Sabouraud dextrose agar media (Oxoid) containing chloramphenicol 0.05mg/ml, inoculated plates were incubated at 37°C for 2 days. While, the lung samples were immersed in 70% ethyl alcohol for 3 minutes to remove the external contamination and then the samples were opened and the contents were inoculated onto Sabouraud dextrose agar media.

### 2.3. Identification of Yeasts

The isolates were picked up by sterile loop from primary culture and sub cultured on Sabouraud dextrose agar slopes and was incubated at 37°C / 24 - 48 hours. The morphology and staining reaction of isolates were observed after staining wet mount preparations by the Indian ink Gram's, and lactophenol cotton blue stain according to (Ellis et al., 2007), Microscopical examination to the isolates grew on rice agar (Ajello et al., 1966), in addition to the germ tube test (Koneman et al., 1992) were done.

### 2.4. Tobacco agar (Tendolkar et al., 2003)

The isolated colonies were streaked on Tobacco agar plates, by platinum loop. Plates were incubated at 28 °C for 48 -72 hours. Tobacco agar was showing the yellowish-brown colonies of *C. dubliniensis*, the white-to-cream-colored colonies of *C. albicans* and the brown colonies growth of *C. neoformans*.

### 2.5. RapID Yeast plus System

The Rap ID Yeast plus System uses a qualitative micro method with 18 conventional and chromogenic substrates were used for completing the diagnosis following Espinel-Ingroff et al., (1998).

### 2.6. Tissue Preparation for auto-fluorescence (H&E Stain)

Specimens from collected lungs were immediately taken from the slaughtered animals and immersed in 10 % formalin. The fixed specimens were trimmed, washed, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. The embedded samples were sectioned at 3-5 µm thickness, stained with H and E stain. Periodic Acid Schiff stain (PAS) and Gomori Methenamine Silver (GMS) stains were used as special stains. All the formentioned procedures were applied according to Bancroft and Marilyn (2002).

### 2.7. Antifungal susceptibility testing

The isolates were sub-cultured (on SDA) and incubated at 37°C for 48 hrs. In vitro, the sensitivity of the isolate to antimicrobials was determined according to standards of National Committee for Clinical Laboratory (NCCLS, 2002). Loopfull from pure culture of Cryptococcus was mixed well with 9 ml of sodium chloride solution then spreading over the surface of SDA plate then suction the excess fluid. Six antifungal discs ( HIMEDIA Company (Nystatin 100µg/ml, Clotrimazole 10 mcg, Itraconazole 10µg/ml, Voriconazole 200mg, Amphotericin B100µg/ml and Fluconazole 10µg/ml) were spread on the surface of inoculated plates. Plates were incubated at 37 °C for 2 days. The diameter of inhibition zone of each disc

was measured (mm) and judged according to (NCCLS, 2002).

### 2.8. PCR Amplification

DNA extraction from three strains *Cryptococcus albidus* strains using Patho Gene-Spin™ as described by DNA/RNA Extraction kit iNtRON cat. No. 17154 Korea. The PCR primers used Forward CNa-70-S 5 'ATTGCGTC CACCAAGGAGCT-3 and Reverse CNa-70-A 5 'ATTGCGTCCATGT TACGTGGA -3 oligonucleotide primer used in PCR reactions were synthesized by Sigma Company, (Germany) at bp 500 (Hideo Aoki et al., 1999). DNA samples were tested in 50 µl. reaction volumes in a 0.2 ml. eppendorf tube, containing 25 µl PCR Mix which was composed of (10X buffer, 10mM d NTPs mixture, Taq polymerase), 1 µl of each primers, 2 µl target DNA, complete to a final volume of 50 µ with sterile deionizer water. PCR amplification conditions for *C. albidus* strains were: 5 min initial denaturation step followed by 38 cycles at 94 °C for one min, 38 cycles at 590C for 1 min and a final extension step at 72 °C for 5 min. Amplification products were electrophoresed in agarose gels (1.5% w/v) (Agarose, Sigma, USA) and stained with ethidium bromide using Gene Ruler 100bp DNA Ladder for 10 min. for 30 sec and 35 cycles for 72 °C for 1min.

## 3. RESULTS

Out of 50 examined lung sheep and goats, 21 lungs (42%) had yeast infection and out of 50 nasal swab from sheep and goat 16 (32%) had positive yeast result illustrated in Table (1). The rate of yeast infection varied from species to another, higher infection rate of 60% (15/25) and 36% (9/25) in lung and nasal swab, respectively was recorded in sheep. However, goat was the lower infected (24%, 6/25) and 28% (7/25) in lung and nasal swab, respectively.

Table (1): Infection rate of pneumonic cases Prevalence of pneumonia from examined samples depending upon on culture growth

Animal	Lung			Nasopharyngeal swabs		
	No	+ve	%	No	+ve	%
Sheep	25	15	60	25	9	36
Goats	25	6	24	25	7	28
Total	50	21	42	50	16	32

It is obvious from results obtained in table (2) that out of 15 and 6 examined lung tissue of sheep and goats with auto-fluorescent 10 (66.7%) and 2 (33.3%) lung tissue exhibited strong enough fluorescence that the technique could be helpful.

Table (2): Infection rate of pneumonic cases depending on Auto-fluorescence examination

Animal	Lung		
	No. of samples	+ve	%
Sheep	15	10	66.7
Goat	6	2	33.3

As depicted in Table (3) yeasts belong to genus *Candida* and *Cryptococcus* was distributed among the examined animals. *C. gullermondii* and *C. albidus* were equally distributed among the examined lung samples of sheep 5 (33.3%, each), While in goats lung samples *C. tropicalis* and *C. albidus* were equally distributed 2 (33.3%, each). *C. gullermondii* and *C. tropicalis* were isolated from nasal swabs of sheep 4 (44.4%) and 5 (55.6%), respectively. In all instances, *C. tropicalis* was the predominant one.

Table (3): Estimates of isolated yeast species from examined samples

Yeast species (Isolates species)	sheep				Goat			
	Lung tissue		Nasopharyngeal swab		Lung tissue		Nasopharyngeal swab	
	No	%	No	%	No	%	No	%
<i>C. albicans</i>	1	6.7	0	0	1	16.7	1	14.3
<i>C. gullermondii</i>	5	33.3	4	44.4	1	16.7	1	14.3
<i>C. tropicalis</i>	4	26.7	5	55.6	2	33.3	5	71.4
<i>C.albidus</i>	5	33.3	0	0	2	33.3	0	0
Total	15	100	9	100	6	100	7	100

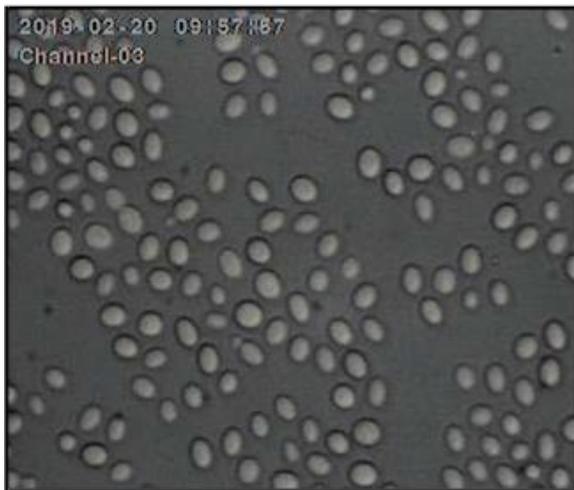


Fig. (1): *Cryptococcus albicus* with Indian ink (X 40)



Fig. (2): RapID Yeast plus System for *Cryptococcus albicus*

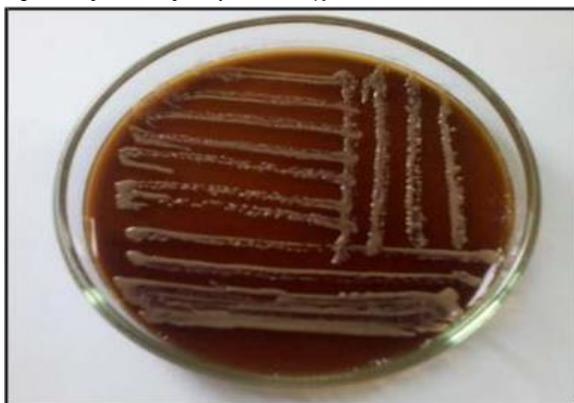


Fig. (3): *Cryptococcus albicus* on Tobacco agar (Beige small bright colony)

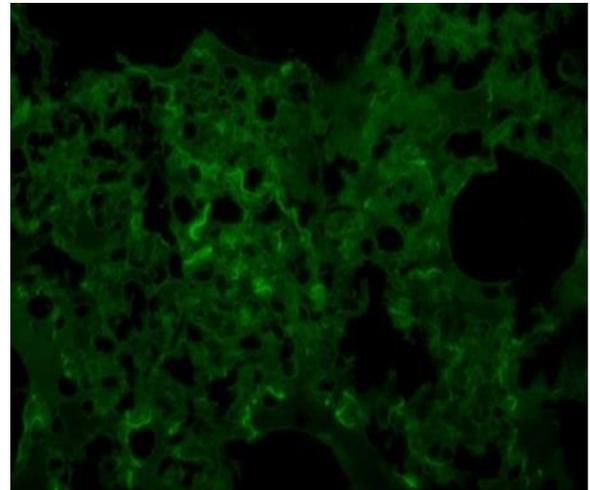


Fig. (4): *Cryptococcus albicus* showing bright green auto fluorescence (Stain H&E, X40)

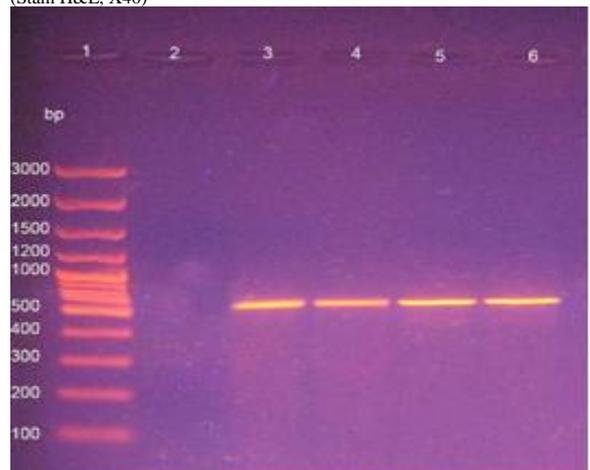


Fig (5): Agarose gel electrophoresis (1.5 %) of PCR product of *Cryptococcus albicus*, Lane 1: 100 bp ladder, Lane 2: control Negative, Lane 3: control positive and Lane 4-6: samples

Table (4): Antifungal sensitivity test results

Cryptococcus	NS100	CC10	IT10	VRG	AP100	FCA23
Type (1)	20	25	30	40	15	20
Type (2)	19	20	32	41	15	20

NS100=Nystatin 100µg/ml; CC10 = Clotrimazole 10 mcg; IT10 = Itraconazole 10µg/ml; VRG = Voriconazole 200mg; AP100= Amphotericin B100µg/ml and FCA23=Fluconazole 10µg/ml

The results recorded in Table (4) showed that Voriconazole 200mg, Itraconazole 10µg/ml and Clotrimazole 10 mcg were more effective than Nystatin 100µg/ml, Amphotericin B100µg/ml and Fluconazole 10µg/ml in inhibiting *C. albicus*.

#### 4. DISCUSSION

Pneumonia is regarded as a disease complex, involving interactions between the host (Immunological and physiological), multiple etiological agents (viral, bacterial, mycoplasma, parasitic and mycotic), beside environmental

The auto-fluorescent yeast species identified were *C. albicus* (Fig. 4), within lung tissues and they exhibited strong enough fluorescence that the technique could be helpful. The architectural detail of bright green-to- yellow green auto-fluorescence spherules, yeast forms *Cryptococcus* gave stronger and brighter fluorescence.

In the present study, PCR was used for the identification of three isolates (2 strains from sheep and one from goats) from seven *C. albicus* strains representative isolates. Using the described primer amplify DNA from all three tested yeasts representing a broad range of clinically relevant yeasts. The primer used gives an amplicon (500 bp) from all isolates tested

factors like temperature, humidity, dust levels, etc (Taylor et al., 2010 and Dar et al., 2014). Regardless of the causative agents, respiratory disease adversely affects the farms, health condition of animals, it represents the main causatives of deaths in lambs and low productivity of old animals in different countries (Bell, 2008). Mycotic pneumonia in some cases is very dangerous due to lack of quick laboratory diagnosis and usually pneumonia is produced as the result of mixed infection with bacteria.

Mycotic pneumonia in goats due to *C. gatti* was reported in Spain by (Baro et al., 1998). The strains were isolated from lung (10 samples), liver (1 sample), and brain (2 samples) tissue specimens from six goats suffering from predominantly severe pulmonary disease that were autopsied.

Mycotic pneumonia in some cases is very dangerous due to lack of quick laboratory diagnosis and usually pneumonia is produced as result of mixed infection with bacteria. Elston (2001) and Mathai et al. (2008) reported that many pathogenic fungi as *Candida*, *Cryptococcus*, *Aspergillus* and *Zygomycetes* have been shown to exhibit auto-fluorescence when H and E stained tissue sections are examined under a fluorescent microscope without adding any immune-reagents. This test is considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains (Gutierrez and Garcí, 1999; Rao et al., 2008; El-Metwally et al., 2011).

Results of effective antifungal against *C. albidus* agree with Gomez-Lopez et al., (2005) who reported that Fluconazole, Itraconazole and Voriconazole are inactive in vitro against the majority of clinical isolates of fungi species, while Amphotericin B exhibit a good activity. While, Beltaire et al. (2012) who reported that fungal strains isolated from animal suffering pneumonia showed resistance rates of 19% and 2% for itraconazole and fluconazole, respectively. An early diagnosis and prompt therapy can reduce the morbidity and mortality of *Cryptococcus* infection. The complex interactions between the agent, host and environment need to be elucidated. More studies are required to investigate the role of *Cryptococcus* in immunocompetent as well in immune-suppressed subjects. Phenotyping methods not reach to a high grade in specificity of *Cryptococcus* spp. identification while, PCR plays a confirmative role in and more accurate in detection of *Cryptococcus* spp. in diseased and apparent healthy animals. We suggest more sanitary conditions in rearing of sheep and goats to avoid predisposing factors leads to occurrence of pneumonia.

Three isolates of the *C. albidus* strains which had previously been identified biochemically with RapID yeast plus system were molecularly identified using The PCR primers used Forward CNa-70-S and Reverse CNa-70-A oligonucleotide primer used in PCR reactions. The size of the resulting amplicons at bp 500. The detection of *Cryptococcus* species DNA by PCR has been studied by a small number of groups (Evertsson et al., 2000 and Bialek et al., 2002). Bialek et al. (2002) concluded that PCR assay could be broadly applicable. Our PCR results compared favorably with the reported detection limits of 10 cells ml and could be used for regular routine diagnosis because of the high sensitivity

## 5. CONCLUSION

In the present study, *Cryptococcus* was the most pathogenic cause of pneumonia from sheep and goats. Traditional ways are not sufficient for diagnosis of large number of

yeasts. Genotypic way is good for yeast diagnosis. Auto-fluorescence way is known to be quick test to identify mycotic diseases with no retard accompanied without using any specific strains. However, molecular identification is considered to be fast to detect fungi and strengthen the epidemiological data that reveal higher number of mycotic pathogens.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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