

## New Diagnostic Tool for Mycotic Pneumonia in Calves Using Autofluorescence Character of Fungi

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**Abstract:** A total number of 200 calves' lung tissues was collected from Giza abattoirs, Egypt during 8-months. The total mycological isolations were 116 cases (58%) and the isolated fungi were *C. albicans* (23%), *Cr. neoformans* (12%), *Mucor spp.* (7%), *Asp. spp.* (4%) and *Prototheca spp.* (12%) as single and mixed infections. According to histopathological examination, three different pneumonia types were detected in relation to the isolated mycotic agents. Firstly, nodular eosinophilic granulomatous pneumonia which characterized by multiple large granulomes of caseous necrotic center. Bronchial epithelial metaplasia and increased mucous production were noticed and demonstrated using PAS stain. Second type was acute fibrinonecrotizing bronchopneumonia in which there was coagulative necrosis accompanied with dense neutrophilic infiltrations within alveolar lumen. The last type was chronic interstitial pleuropneumonia with pyogranulomes formation. Calcium oxalate crystals were observed as irregularly angulated polarizable material within necrotic tissues in most of that cases revealed *Asp. spp.* The fungal elements were positively stained by using PAS and GMS stains. *C. albicans*, *Cr. neoformans*, *Mucor spp.* and *Aspergillus spp.* gave bright green-to-yellow green fluorescence within tissue but prototheca didn't exhibit fluorescence. They exhibited strong enough fluorescence that the technique could be helpful. Autofluorescence was found in 114 out of 116 cases studied with a sensitivity of 98.3% and specificity of 100%.

**Key words:** Histopathology % Calves % Mycotic Pneumonia % Granulomatous Pneumonia % Prototheca % Yeast %Aspergillus % Autofluorescence % Oxalate Crystals

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### INTRODUCTION

Calf pneumonia is a widespread multifactorial respiratory problem that frequently occurs and causes by many pathogens and other conditions as stress and high stocking density predispose for this affection [1]. It is economically damaging disease that has increased by 34 % in the last 20 years and causing morbidity and nearly 21 % of newborn calf losses [2]. The fibrosis and loss of functional lung capacity in animals that recover from pneumonia has a negative impact on daily live weight gains [3, 4]. There is an association between respiratory diseases and air quality (wet weather and poor ventilation) in confinement environments as raising calves in barns in which warm air contain potentially harmful gases as ammonia, dust and microorganisms (e.g. fungal spores, viruses and bacteria). Ammonia with dust particles which oftentimes carry microbes, can reach

respiratory tissues, whereas they can multiply and cause irritation and inflammatory reactions [5].

Other factors that increase the risk of respiratory disease are shared housing with cows during the first week of life, more than 2 months difference in age within a group and previous episodes of diarrhea [6]. Fungal infections can occur in healthy individuals but are more common as opportunistic infections in debilitated and immunocompromised hosts whose normal defense mechanisms are impaired. A fatal outcome is possible in these individuals, as fungal infection may remain undiagnosed [7]. Mycotic infection is mainly caused by inhalation of spores, which can lead to haemo-lymphatic dissemination. *Aspergillus spp.*, *Cryptococcus neoformans* and *Candida spp.* are identified as the main causative agents of mycotic pneumonia [8]. In addition to, there is another form of uncommon pneumonia causes by prototheca algae [9]. Protothecosis is an infectious

condition of humans and animals caused by unicellular achlorophyllic algae which belong to the family Chlorellaceae. It is a wide-spread in the environment and can be found particularly in damp areas contaminated with manure or other organic matter which provides external sources of organic carbon and nitrogen. Their spores release takes place every 5 to 6 h in the presence of adequate nutrients [10, 11].

Elston [12] and Mathai *et al.* [13] reported that many pathogenic fungi as *Candida*, *Cryptococcus*, *Aspergillus* and *Zygomycetes* have been shown to exhibit autofluorescence when H and E stained tissue sections are examined under a fluorescent microscope without adding any immunoreagents. This test is considered as a rapid screening technique for diagnosis of fungal infections without the delay associated with special stains [14].

In order to prevent this costly problem, it is important to address mycotic agents. So the aim of the present study, was to study the mycotic causes of pneumonia in relation to histopathological examination of lung tissues of slaughtered calves in Egypt. In addition to, assess the value of autofluorescence as a screening method for detecting fungi and to compare the results of autofluorescence with special stains of fungi.

## MATERIALS AND METHODS

**Collection of Samples:** A total of 200 calves' lung tissues was collected from El-Mounib and El-Warak abattoir at Giza governorate, Egypt throughout 8-months from January to August (2011). Animals were randomly selected among the slaughtered calves. The samples were visually examined for gross lesions. Each tissue sample was divided into two parts, one part was put in a sterile polyethylene bag in an ice box under aseptic conditions for mycotic isolation and the second part was immersed in 10% neutral buffered formalin saline for histopathological evaluation.

**Mycological Examination:** This was carried out according to Anaiscie *et al.* [15]. Sabouraud's dextrose agar (Difco) plates containing 0.05 mg/ml Chloramphenicol (to inhibit bacterial growth) were prepared.

**Identification of Isolated Moulds:** The inoculated plates were incubated at 25°C / 7 days and examined daily. Identification of isolated moulds was based on their growth rate and colonial morphology. Then it was confirmed microscopically by the type of hyphae and fruiting heads.

**Identification of Yeasts:** The inoculated plates were incubated at 37°C / 24-48 hours. The morphology and staining reaction of isolates were observed after staining by the Indian ink stain, Gram's Method, Anniline cotton blue stain and wet preparations. Yeast colonies grown on blood agar at 37°C / 24-48 hours and the detected colonies were cultured on Rice extract agar with polysorbate 80 for identification and characterization of *Candida spp.* and Trypan blue agar media [16] for selective confirmation of *C. albican* and *Cr. neoformans*.

**Identification of Prototheca Spp.:** Identification of *Prototheca Spp.* was carried out according to Dubravka *et al.* [17] and Asfour and El-Metwally [18]. Streaked plates were incubated under aerobic conditions at 25-37°C/ 24-72 h and monitored daily. From the growing colonies, wet microscopic smears, Gram and methylene blue stains were done. The preparations were examined using light and phase-contrast microscopy according to Cosmina *et al.* [19]. Urease activity also applied to differentiate between *Prototheca spp.* and other yeasts.

**Tissue Preparation for Histopathological Studies:** Specimens from collected lungs were immediately taken from the slaughtered calves 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 [20].

**Detection of Fungi in H and E Stained Sections Using a Fluorescent Light:** All tissue sections were examined with an Olympus Provis microscope with a fluorescent light source (334 and 365nm) and dichroic mirror with filters (DM40-DM 455). No immunoreagents were used in processing of the specimen.

## RESULTS AND DISCUSSION

**Mycological Examination:** Radostits *et al.* [21] reported that the mycotic pneumonia is uncommon in farm animals but a high prevalence can be expected in calves and lambs kept in intensive housing units.

*Prototheca spp.* is oval or spherical in shape and differs from bacteria and fungi in its size and shape [22, 23]. Reproduction is asexual and during cell maturation, the cytoplasm undergoes a process of cleavage to form 2 to 20 irregular endospores.

Table 1: Isolated yeasts, moulds and algae from examined calf's lung tissues

Types of isolated fungi	No.	%
(No. = 200)		
A-Isolated yeast:		
1- <i>Candida albicans</i> ( <i>C. albicans</i> )	46	23%
2- <i>Cryptococcus neoformans</i> ( <i>Cr. neoformans</i> )	24	12%
B-Isolated moulds:		
1- <i>Mucor spp.</i>	14	7%
2- <i>Aspergillus fumigatus</i> ( <i>Asp. fumigatus</i> )	4	2%
3- <i>Aspergillus niger</i> ( <i>Asp. niger</i> )	4	2%
C-Isolated algae:		
<i>Prototheca spp.</i>	24	12%
Total	116	58%

Table 2: types of pneumonia in relation to isolated fungi

Types of pneumonia	No.	%	Isolated fungi
(No.=200)			
1-Nodular eosinophilic granulomatous pneumonia	40	20%	<i>prototheca spp.</i> (24), <i>Cr. neoformans</i> (24), <i>C. albicans</i> (18) and <i>Mucor spp.</i> (2)
2-Acute fibrinosuppurative bronchopneumonia	28	14%	<i>C. albicans</i> (28) and <i>Asp. fumigatus</i> (4)
3-Chronic interstitial pleuro pneumonia with granulome formation	16	8%	<i>Mucor spp.</i> (12) and <i>Asp. niger</i> (4)
Total	84	42%	

These spores increase in size upon release from the mother cell and the sporangia (mother cells) break under the pressure from the enlarging spores [24]. *Cryptococcus* is a unique environmental fungus and *Cr. neoformans* commonly cause disease. Soil contaminated with pigeon droppings is the major environmental source for these yeasts [25]. *Aspergillus spp.* is prevalent worldwide and their spores are frequently present in the biosphere so they are part of routinely inhaled particles [26].

Our results clarified that the total mycological isolations were 116 cases that represented 58% and the isolated fungi were *C. albicans*, *Cr. neoformans*, *Mucor*, *Asp. fumigatus*, *Asp. niger* and *Prototheca spp.* as single or mixed infections as showed in table (1). *Aspergillus spp.* represented 4% which was lower than that recovered by Chihaya *et al.* [27] who isolated it at 52.6% from calves.

**Histopathological Examination:** Recognizing the patterns of lesions of various types of pneumonia is important for correct diagnosis and interpretation of these lesions. So in this work, we studied the pneumonic lesions in naturally infected calves. There were three different types of pneumonia in relation to isolated mycotic agents as showed in table (2).

**Nodular Eosinophilic Granulomatous Pneumonia:** Out of 200 lung tissue samples, 40 samples showed this type of pneumonia and represented 20 % of total examined cases. *Prototheca* algae was isolated as the main organism singly or mixed with *C. albicans* and *Cr. neoformans* or *Mucor spp.* as showed in table (2).

Roesler *et al.* [28] reported that, there are 2 pathogenic species, *Prototheca zopfii* and *Prototheca wickerhamii* as the etiologic agents of protothecosis in human and animals. Most human protothecosis are caused by *P. wickerhamii* (small sporangia) and *P. zopfii* (large sporangia) mainly affects animals [29]. The pathogenesis of protothecosis is largely unknown. It is believed that *Prototheca spp.* may infect through contact with potential sources or by traumatic inoculation with the algae [30]. The incidence of infections depends on predisposing factors such as poor environmental conditions or immunosuppressed animals. It is believed that in the systemic form of the disease the organism is ingested, enters the body via the intestinal mucosa and spreads throughout the body hematogenously or through the lymph system [31]. Since *P. zopfii* is highly resistant to all known chemotherapeutics and chronically infected animals become intermittent shedders so infected animals should be removed from the herd [28].

Nimrichter *et al.* [32] mentioned that cryptococcus is packed in a rigid, pore-containing cell wall consisting of polysaccharides, proteins and pigments. In this respect, Poeta [33] and Monari *et al.* [34] reported it is the only eukaryotic pathogen that produces a polysaccharide capsule, which serves as the major virulence factor as it has antiphagocytic character that interferes with the uptake of organism. Also, the melanin pigment production provides protection against oxidative mechanisms of host defense [35].

Roy and Pyane [36] referred the pathogenicity of *C. albicans* to the role of acid proteinase enzyme which may facilitate the entry of the organism and spread of infection. On the other hand, candida infection should be suspected, when there was a history of unsuccessful antibiotic treatment which might aggravate the infection as it utilizes penicillin and tetracycline as a source of nitrogen [37].

In the present study, the gross examination of the lung tissues revealed multifocal large (1.5-2cm) circular nodules among whole lung which containing creamy caseated material and the intervening tissue was atelectatic. Pleura were thick and adhered with lung tissue. The histopathological examination of lung parenchyma was characterized by severe granulomatous reaction with necrosis in the form of multiple large foci of caseous necrosis that clearly separated from non necrotic parts (Fig. 1). These necrotic foci were surrounded by granulocytes mainly recognizable eosinophiles and neutrophils as well as foamy macrophages containing rounded bodies, plasma cells and multinucleated giant cells, encircled by fibroblasts (Figs. 2 and 3). Yeast and prototheca cells were observed within necrotic tissues, lung alveoli and activated macrophages as rounded bodies of different shapes and sizes (Figs. 4 and 5). The pulmonary blood vessels were severely engorged with blood, some of them showed fibrin thrombi with perivascular lymphocytosis and wall hyalinization. The bronchiolar lining epithelium showed necrotic changes with epithelial desquamation within bronchial lumen accompanied with loss of cilia and perbronchial lymphocytosis. The peribronchial associated lymphoid tissue (BALT) showed marked proliferations and hyperplasia (Fig. 6). There was prominent fibrin accumulation with mononuclear cellular infiltrations noticed in pleura.

Similar findings were described by Roth and Weber [38], Torres *et al.*, [39], Chen *et al.* [40], Lass-Florl and Mayr [29], Kelly [41] and Macedo *et al.* [11] in animals

and human. The previously mentioned giant cells infiltrations, was demonstrated also by El-Naggar *et al.* [42] Who suggested that they play a very important role in the phagocytosis of the fungal elements in tissues. The alveolar foamy macrophages were the most prominent inflammatory cells involved in the mycotic lesions. Although, they are very numerous and able to phagocytize yeasts, these macrophages did not appear to be effective in destroying the yeast bodies or in controlling their spread. This failure in turn could have facilitated the development of the observed extensive pneumonia [43].

Epithelial cell metaplasia and increased mucous production by bronchial epithelium were noticed in cases of *Prototheca spp.* and *Cr. neoformans* infection in this study and was demonstrated using PAS stain. Abundant PAS-positive goblet cells with PAS-positive granules was showed within the cylindrical bronchial epithelial layer (Fig. 7). Goblet cells were absent in the bronchial epithelium of control mycological negative lungs. These changes are consistent with epithelial cell metaplasia and increased mucous production observed in allergic airway responses that observed by Chen *et al.* [40].

**Acute Fibrinonecrotizing Bronchopneumonia:** 28 out of 200 lung tissue samples showed this type of pneumonia (14 %) and the isolated fungi were *C. albicans* and *Asp. fumigatus* as single or mixed infection as showed in table (2).

The macroscopic appearance of collected lungs was hepatized, congested oozing bloody exudates with cut section. Microscopically, lung parenchyma showed coagulative necrosis accompanied with dense fibrinocellular exudates mainly neutrophilic in nature and macrophages filled the alveolar lumen. Severe congestion observed in pulmonary blood vessels and some of them showed fibrin thrombi (Figs. 8 and 9). Bronchiolar epithelium showed necrosis and desquamation within the lumen as well as peribronchial edema and mild cellular infiltrations mainly neutrophilic and macrophages. Pleura showed oedema, vascular congestion and cellular infiltrations mainly neutrophils, macrophages and few lymphocytes.

Our histopathological results were inconstant with those of Green *et al.* [44], Chihaya, *et al.* [27], Pérez *et al.* [45], Vaideeswar *et al.* [46], Breshears *et al.* [47], Katherine Hughes and Karin Mueller [48], Hanaa *et al.* [49] and Kousha *et al.* [50]. Washburn *et al.* [51] and

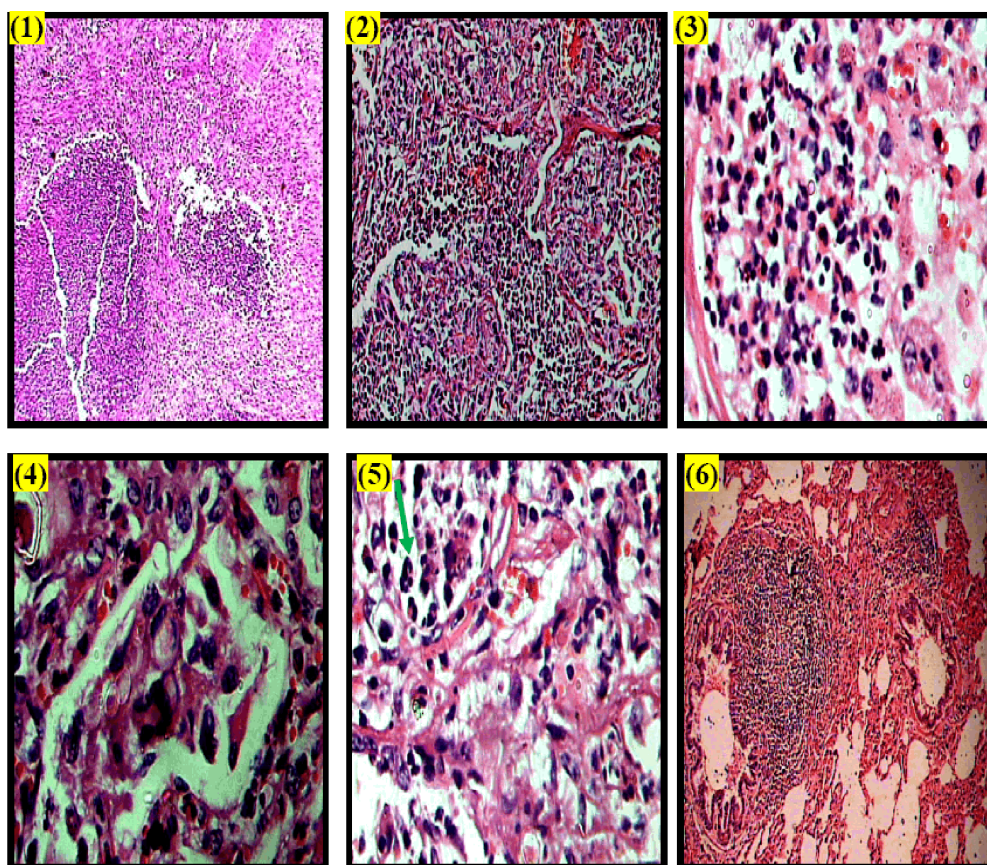


Fig. 1: Lung tissue showing multiple large foci of caseous necrosis surrounded by inflammatory cells (Stain H&E, X4).

Fig. 2: High power of the previous picture (Stain H&E, X10).

Fig. 3: Lung parenchyma showing necrotic foci were contained recognizable eosinophiles and neutrophile as well as foamy macrophages containing rounded bodies, plasma cells and multinucleated giant cells (Stain H&E, X40).

Fig. 4: Yeast cells were observed within necrotic tissue, lung alveoli and activated macrophages as rounded bodies of different shapes and sizes (Stain H&E, X40).

Fig. 5: Prototheca cells were appeared within necrotic tissue and in the lumen of alveoli as large non budding spherical, ovoid, or elliptical sporangia with a wall, containing multiple endospores in different numbers and sizes (arrow) (Stain H&E, X40).

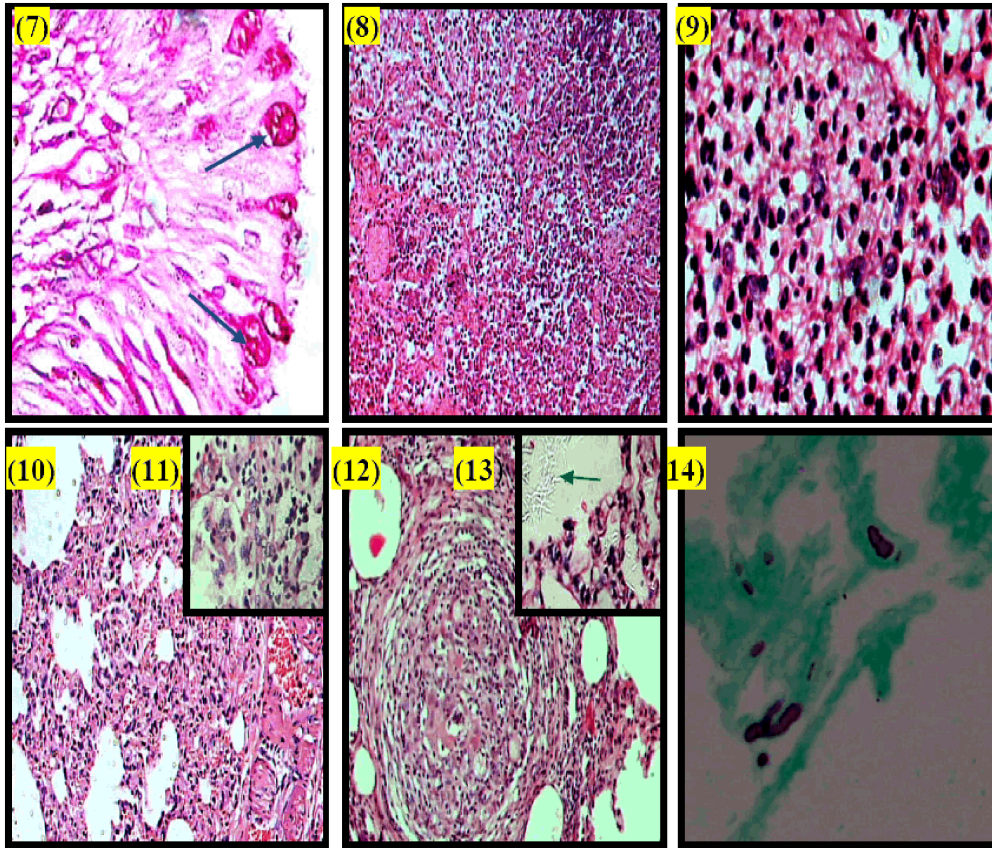
Fig. 6: The bronchiolar lining epithelium showed necrotic changes with epithelial desquamation within bronchial lumen accompanied with peribronchial lymphocytosis. BALT showed marked proliferations and hyperplasia (Stain H&E, X4).

Tegmteir *et al.* [52] referred those changes to a chemical substance known as diffusates that release from the spores and antagonize pulmonary phagocytosis of fungal spores and had immunosuppressive effect.

The main observable histopathological lesions in all aspergillus's lung tissues were prominent vasculitis and thrombosis. These were explained by Pérez *et al.* [45] as the fungal hyphae tend to penetrate the vascular wall causing vasculitis and thrombosis leading to ischemia and necrosis of the infected tissue. Moreover,

Vaideswar *et al.* [46] stated that proteinolytic enzymes are considered as significant virulence factor in aspergillois, as they may facilitate the entry of the organism from a colonization site to the rest of organ.

**Chronic Interstitial Pleuropneumonia with Pyogranulome Formation:** From the total cases, 16 cases (8%) showed this type of pneumonia and the isolated fungi were *Asp. niger* and *Mucor spp.* as single and mixed infection as showed in table (2).



- Fig. 7: PAS-positive goblet cells with PAS-positive granules showed abundant within the cylindrical bronchial epithelial layer (blue arrows) (Stain PAS, X40).
- Fig. 8: Lung parenchyma showed coagulative necrosis accompanied with fibrinocellular exudates filled the alveolar lumen as well as fibrin thrombi and congestion in pulmonary blood vessels (Stain H&E, X4).
- Fig. 9: High power of the previous picture showed coagulative necrosis accompanied with fibrinocellular exudates mainly neutrophilic in nature filled the alveolar lumen (Stain H&E, X40).
- Fig. 10: Lung parenchyma characterized by diffuse mononuclear cellular infiltrations mainly macrophages and lymphocytes in the interstitial tissue and within alveoli with emphysema (Stain H&E, X4).
- Fig. 11: High power of the previous picture (Stain H&E, X40).
- Fig. 12: Lung parenchyma showed pyogranuloma with necrotic center and mostly consisted of fungal elements and surrounded by inflammatory cells with prominent emphysema and perialveolar vascular congestion (Stain H&E, X4).
- Fig. 13: Calcium oxalate crystals (green arrow) were observed as irregularly angulated polarizable material within lung parenchyma (Stain H&E, X40).
- Fig. 14: *Aspergillus* hyphae were stained positively within the necrotic lung tissues and appeared as radiating branching septated hyphae (Stain GMS, X40).

Grossly, there were multiple minute pyogranulomas within the lung parenchyma with thick fibrosed pleura. Microscopic examination characterized by diffuse mononuclear cellular infiltrations mainly macrophages and lymphocytes in the interstitial tissue and within alveoli while other parts showed emphysema (Figs. 10 and 11).

Extensive multifocal coalescing pyogranulomas with necrotic center were observed. The later is mostly consisted of fungal hyphae surrounded by neutrophils, lymphocytes, macrophages, plasma cells, histiocytes, few eosinophiles and epitheloid cells with multinuclear giant cells, capsular formation was apparent at the periphery.

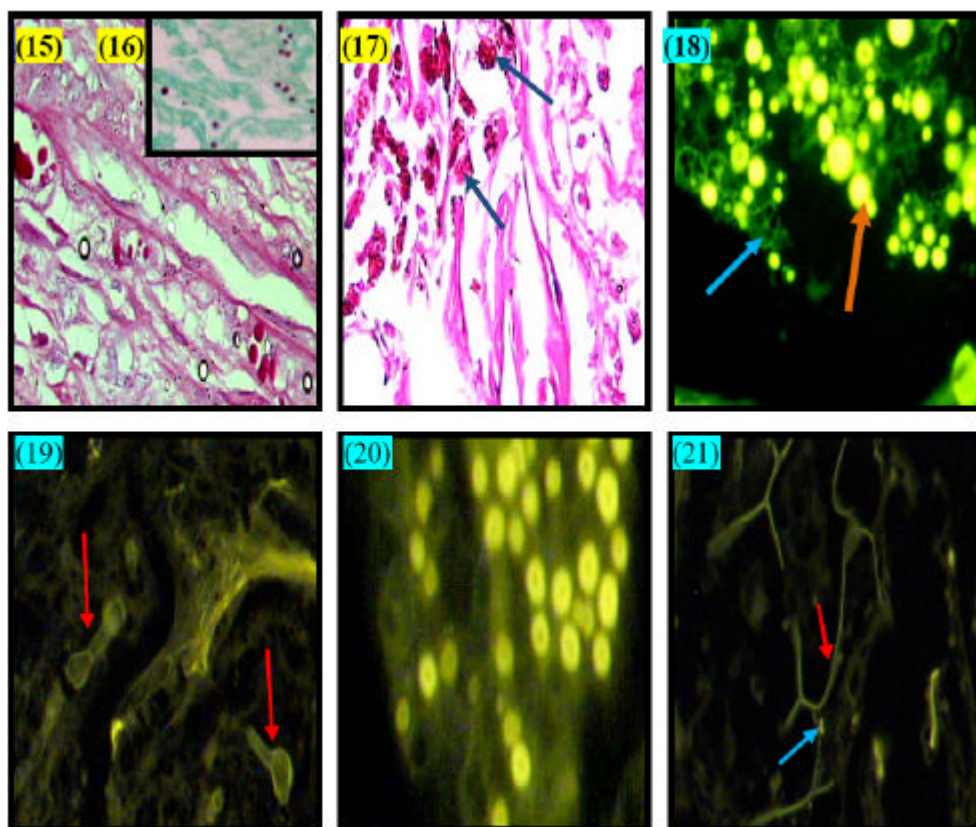


Fig. 15: Positive reaction of *C. albicans* which appeared as light reddish purple ovoid noncapsulated cells (Stain PAS, X40).

Fig. 16: *C. albicans* cells were stained positively within the necrotic lung tissues (Stain GMS, X40).

Fig. 17: Prototheca algae were appeared in the lumen of alveoli as large non budding spherical, ovoid, or elliptical sporangia with thick wall, containing multiple endospores in different numbers and sizes (blue arrows) (Stain PAS, X40).

Fig. 18: Candida and the budding yeast forms of Candida showed bright yellow green autofluorescence (orange arrow) but prototheca didn't exhibit any fluorescence (blue arrow) (Stain H&E, X40).

Fig. 19: Candida and pseudohyphal forms of candida showed bright yellow green autofluorescence (red arrow) (Stain H&E, X40).

Fig. 20: *Cr. neoformans* showed bright yellow green autofluorescence with prominent capsule (Stain H&E, X40).

Fig. 21: Mucor species has wide-angled, non-septated hyphae and showed bright green autofluorescence (red arrow) with yellow green budding yeast forms of candida (blue arrow) (Stain H&E, X40).

Perialveolar vascular congestion accompanied with mononuclear cellular infiltration was noticed (Fig. 12). Necrotic and desquamated bronchiolar epithelium with activation of lymphoid follicles were detected. Pleura showed prominent diffuse fibrosis with cellular infiltrations and granulation tissue formation.

Similar findings were recorded by Kurup and Sheth [53], Sumi *et al.* [54], Clercx *et al.* [55], El-Hallwany [56], Callister *et al.* [57], Al-Alawi *et al.* [58] and Katherine Hughes and Karin Mueller [48].

Pulmonary oxalosis is often secondary to an aspergillus infection. Calcium oxalate crystals considered as a mycotoxin that released by *A. niger* and sometimes by *A. fumigatus* [59]. In this study, calcium oxalate crystals were observed and identified microscopically as irregularly angulated, polarizable material within necrotic tissue in most of that cases revealed *A. fumigatus* and *A. niger* (Fig. 13). These crystals mentioned before by Vaideeswar *et al.* [46] and El-Hallwany [56], as they produced through tricarboxylic acid cycle.

The later generated oxalic acid which combines with the host derived calcium in an alkaline or neutral environment, in the animal's tissue fluid or blood and produce calcium oxalate crystals. Then deposited in the host tissues. Oxalic acid exerts a toxic effect on tissues and causes marked pulmonary tissue necrosis which can be extensive enough to cause fatal pulmonary hemorrhage.

At times those fungi cannot be visualized in H and E-stained sections, special stains as PAS and GMS become mandatory.

From our results, the fungal elements were stained positively within the necrotic lung tissues by using PAS and GMS stains. The fungal hyphae appeared as radiating branching septated hyphae (*Aspergillus spp.*) (Fig. 14) or non-septated (*Mucor*). Yeast cells appeared ovoid possessed a thick capsule (in case of *Cr. neoformans*) or noncapsulated (in case of *C. albicans*) (Figs. 15 and 16) and reproduced by narrow-based budding. In this respect, *Prototheca spp.* algae were appeared in the lumen of alveoli as large non budding spherical, ovoid or elliptical sporangia with a prominent thick wall, containing multiple endospores in different numbers and sizes (Fig. 17). These results coincide with those previously described by El-Naggar *et al.* [42], El-Metwally and Asfour [62], Cosmina *et al.* [19] and Asfour and El-Metwally [18].

Lack of rapid diagnostic techniques results in delayed diagnosis, which may even culminate in a fatal outcome. The fact that many pathogenic fungal organisms produce autofluorescence in H and E-stained sections under ultraviolet illumination led us to evaluate the role of autofluorescence as a rapid screening technique for fungal infections [12, 14]. The advantages of this method was reported by Mann [61] as no special staining procedures required, no time delay, the ability to scan sections at a relatively low power and the ability to tentatively identify the fungus. On the other side, the disadvantages may include lack of a fluorescent microscope.

In the present work, most of the demonstrated organisms gave at least weak fluorescence except *Prototheca* which didn't exhibit any fluorescence. The four autofluorescent fungal species identified were *C. albicans* (Figs. 18 and 19), *Cr. neoformans* (Fig. 20), *Mucor* (Fig. 21) and *Aspergillus* within granulomas, or necrotic tissue debris and they exhibited strong enough fluorescence that the technique could be helpful. The architectural detail of bright green-to-yellow green autofluorescence spherules, yeast forms

and hyphae was very well delineated. *Candida*, *Cr. neoformans* and *Mucor* gave stronger and brighter fluorescence than *Aspergillus* species. The control cases (negatively mycotic isolation) did not show any autofluorescence.

Graham [62], Mann [61], Graf *et al.* [63], Hettlich *et al.* [64], Mathai *et al.* [13] and Rao *et al.* [14] reported that the technique was most helpful for identifying candidiasis, cryptococcosis and aspergillosis. On the other side, Mann [61] failed to demonstrate *Mucor* hyphae autofluorescence. Graham [62] and Elston [12] noted that *Prototheca* exhibited strong enough fluorescence which disagrees with our results.

A green autofluorescence was noted in our study differed as compared to bright green-to-yellow green autofluorescence in earlier studies. This explained by Graf *et al.* [63] and Rao *et al.* [14] as due to mild variation in the wave length, fixing method or mounting medium used.

When our autofluorescence findings was compared with the results of PAS and GMS stains, we found that this character in 114 out of 116 cases studied. This test was, with a sensitivity of 98.3% and specificity of 100%. The same results obtained by Rao *et al.* [14].

## CONCLUSION

- C *Prototheca algae* causes uncommon form of pneumonia and it is highly resistant to all known chemotherapeutics and chronically infected animals become intermittent shedders so infected animals should be removed from the herd.
- C In case of nodular eosinophilic granulomatous pneumonia, *Prototheca algae* was isolated as the main organism singly or mixed with *C. albicans* and *Cr. neoformans*.
- C Abundant PAS-positive goblet cells with PAS-positive granules were showed within the metaplastic cylindrical bronchial epithelial layer as a result of allergic airway responses in *Prototheca algae* and *Cr. neoformans*.
- C Autofluorescence is a simple and quick can be done on H and E-stained slide using fluorescent microscope without addition of any immunoreagents and has high sensitivity and specificity. *Candida*, *Cr. neoformans* and *Mucor* gave stronger and brighter fluorescence than *Aspergillus* species while *Prototheca* didn't exhibit any fluorescence.



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