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Microbial and Pathological Investigation of Lambs Deathes in Sheep Flock at Hosh-Issa , El- Bohaira Governorat

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ABSTRACT

An investigation of early lamb mortality at 20- 30 days of age was conducted in sheep flocks at Hosh-Issa in El- Bohaira Governorate, Egypt. Study undertaken in 2020/21 in which lamb deaths were repeated in the same flock at the same time of year during July–September and January–March, particularly in lambs average month of age .Knowledge of actual causes of death are important to know the causes of lambs mortality, and for taking preventive measures at the flock. This paper reports on the postmortem findings in 6 lambs out of 18 lambs that died at 20-30 day of age during July–September and January–March in the same flock (n=70).The most frequently identified causes of death were infectious diseases as septicemia, pneumonia and gastrointestinal infections., The results showed that different species of fungi were isolated *Aspergillus flavus*, *Aspergillus niger, Candidia albicans, Candida famata and Candida tropicalis* were isolated from all septicaemic cases. the most common bacterial agents obtained from all cases of infection were *Closteridium species, Coagulase negative Staphylococcus (CNS) and Escherichia coli* were isolated. Antibacterial and antifungal sensitivity of different bacterial and fungal isolates were done by using disc diffusion test for measurement of the susceptibility of bacteria and fungi to antibacterial and antifungal drugs. Histopathological changes in the internal organs were recorded.

In this study, the main causes of lamb mortality were bacteria accompanied by fungal infection especially *Candida albicans* which may cause gastrointestinal candidiasis in animals. Most deaths occurred at 20-30 days of age, suggesting that events related to lambing and the immediate post-lambing period are critical for lamb survival.

1- INTRODUCTION

Small ruminants (sheep and goats) play a role in the economy of most people **(Thornton 2010)**. Lamb mortality attributed to a major losses in sheep production and a major factor in reducing the profitability of sheep farming. Young animal diseases that cause morbidity and mortality are the results of the complex

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interaction of the management practices, the environment, infectious agents , and the animal it self **(Lema et al. 2001)** Different management and environmental factors such as colostrum feeding, housing ,calving assistance, production system, herd size, season, and hygiene of micro-environment were reported to affect lamb morbidity and mortality **(Shiferaw et al. 2002).**

Mortality of neonates of ruminants was mainly attributed to conditions such as diarrhea and pneumonia **(Lema et al. 2001**) joint problems, umbilical diseases, trauma, congenital abnormalities, nutritional deficiencies, dystocia, and other infections **(Svensson et al . 2003 Singla et al. 2013**) associated with poor housing, hygiene and nutrition **(Lema et al . 2001)**.

One of the most important production factors that adversely affect small ruminant production is the high pre-weaning mortality of young lambs. Studies indicate that up to 50% of the lambs born can die mainly due to diseases and other causes such as adaptation failure, dystocia, cold stress, starvation, sudden change in feed and miss mothering **(Tibbo 2006)**. In more severe cases infection occur and symptoms of a viral, fungal or bacterial disease were observed.

Diarrheal disease seems to be one of the major community health hazards both for man and animals in most countries of the world. It is resulted from enteritis, which is the inflammation of the intestinal mucosa, characterized by abdominal pain, loose feces, increase in stool mass and defecation frequency or stool fluidity (dehydration) that contains 70-95% water, the chronic form of diarrhea may last for days or week and may lead to death **(Radostits et al. 2007) .** Morbidity and mortality are of great concern for the dairyman **(Wudu et al. 2008 Gitau et al. 2010).**

Pneumonia is the most serious problem in a migratory of flocks of sheep in India and causes significant mortality in young lambs leading to considerable economic losses. It is estimated that pneumonia alone causes at least 10% mortality in sheep population. Pneumonia is caused by a complex interaction between the environment, which produces stress, microorganisms, and the host's immune response. There is an association between respiratory diseases and air quality (wet weather and poor ventilation) .Raising newborns in animal sheds in which warm air contain potentially harmful gases as ammonia, dust and microorganisms (e.g. fungal spores, viruses and bacteria). Ammonia with dust particles and microbes, can reach respiratory tissues , whereas they can multiply and cause irritation and inflammatory reactions**(Garcia and Daly 2010).**

Fungal infections can occur in healthy individuals but are more common as opportunistic infections in debilitated and immuno-compromised hosts whose normal defense mechanisms are impaired. A fatal outcome is possible in these individuals, as fungal infection may remain undiagnosed **(Randhuwa 2000).** Mycotic infection is mainly caused by inhalation of spores, *Aspergillus spp*., *Cryptococcus neoformans,*

Pseudallescheria boydii and Candida spp. have been identified as the main causative agents of mycotic pneumonia **(Daniel et al. 2006)** Pneumonic lesions are frequently seen in necropsy in sheep of all ages**(Goodwin et al 2005).**

Mycotic diarrhea and respiratory affection were also detected during examination of fecal samples and nasal swabs of affected cattle which had several members of genus *Aspergillus* at the rates of (47.0 %) and yeast of *C. albicans* was also recovered from 20% of cases of diarrhea (fecal samples) in sheep and goat, while it was only recovered from 4% of cases of both apparently healthy sheep and goat **(Hassan et al. 2011)**.
Therefore, Aim of this work is the determination of the bacterial and mycotic agents causing lamb deathes in this age, pathological changes accombined this infection ,anti bacterial and antifungal sensitivity test for deterimination drug resistant.

2. MATERIALS and METHODS

The causes of lambs mortality **at Hosh-Issa in El- Bohaira Governorate** during 2021-22 were investigated in sheep flocks. Postmortem, pathological and laboratory examinations were used to identify the causes of lambs mortality. In which 18 lambs at 20-30 days of age were died. All were suffering from respiratory signs ,mild fever and diarrhea. Post mortem examination was carried out within 24 hours on six of dead lambs at Animal Health Research Institute, Damanhour branch . Samples of the internal organs and fecal samples from the intestine were taken for bacterial isolation and pathological investigation

2.1.Bacteriological analysis:

Was carried out according to the stander methods which recommended by (**Quinn et al. 1994).** Each sample was cultured on mannitol salt agar for isolation of Staphylococcus. on MacConkey agar and Levine's eosin-methylene blue (EMB) agar for the isolation of Gram-negative bacteria. The cultures were incubated at 37°C for 24 h also each specimen was streaked onto cooked meat broth for 24 hr. and cultured on bovine blood-agar (BA) plate and were incubated anaerobically for 48 hr. followed by Gram staining and biochemical tests (Oxidase , catalase, DNase, coagulase tests as well as IMViC tests **(Kateete et al. 2010).**

2.2. Antibacteria sensitivity test for isolated bacteria. according to (Kirby bauer method (Disc diffusion test):

The isolated bacteria was sub-cultured on nutrient agar (NA) and incubated at 37C for 24 hr. loopfull from pure culture from each isolate was mixed well with 5ml of nutrient broth, the concentration of bacteria was standardized by comparing the turbidity of bacteria suspended in saline or broth to McFarland standards solutions whose turbidity is equivalent to that of a suspension containing a given concentration of bacteria. Once an appropriate concentration (most commonly an 0.5 McFarland standard) then spreading over the surface of NA plates then suction excess fluid. antibacterial discs were spread on the surface of inoculated plate. Plates were incubated at 37 C for 24 hr and The diameter of inhibition zone of each disc was measured (mm) and judged and compared with standard chart and interpreted to sensitive , intermediate and resistant.

2.3. Mycological examination

All organs samples minced with sabourad dextrose broth were cultured on Sabouraud Dextrose Agar (SDA) containing chloramphincol (0.05gm/ml), then incubated at 37 C◦ and 25 C◦ and examined daily for one week after which the plates showing no growth were considered negative. Fungal isolates were characterized by morphology and identified using standard procedures **(Quinn et al. 2002, Qvirist et al. 2016).** Mold and yeast were identified by their colonial morphology and microscopic characteristics. The fungal isolates were mounted in lacto phenol cotton blue stain solution on slides with cover slips and microscopically examined for spores and vegetative bodies according to the method described by **Barnet and Hunter (1972).**

The identification was based on colonial features, pigment production and the micro morphology of the spores produced. Cultures were examined at 4 or 5 days intervals from the onset. Some characteristics were also noted on the texture, color, shape and the production of pigment on the underside. Fungi were identified according to **Nelson et al. (1983**) , **Samson et al. (2004) Pitt and Hocking (2009) and Simmons (2009).**

Yeast isolates were identified according to **Tibor and Larry (1996**). by Gram stain, the yeast was identified on the basis colony morphological character (blastocondia and chlamydospores according to **Raper and Fennel (1965)** and **Frey et al. (1979).** Germ tube test was used for differentiation between *Candida* species in which a very light suspension of yeast like organisms in 0.5-1.0ml of sterile rabbit serum can be used. Incubation was occurred at 37 ^{o}C for no longer than 3 hrs. then one drop of yeast- serum mixture was placed on a slide slip and was examined microscopically for germ tube production.

2.4.Anti fungal Sensitivity testing for isolated moulds and yeast:

Two main groups of antifungals are used in the clinical setting to treat fungal infections polyenes represented by Amphotericin B (AP), Nystatin (NS) and azoles with several derivatives such as fluconazole (FCA) ,voriconazole

(VRC), clotrimazole (CC), and Metronidazole (MTZ) (Humid).

Antifungal sensitivity test was conducted using disc diffusion test. The in vitro sensitivity of the isolate to antimicrobials was detremined according to standards of National committee for clinical laboratory **(NCCLS 2002) and (Silva et al. 2011).**

The isolated fungi was sub-cultured on sabourad dextrose agar (SDA) and incubated at 37C for yeast and 25C for mold . Loopfull from pure culture from each isolate was mixed well with 9ml of sodium chloride solution then spreading over the surface of SDA plates then suction excess fluid. Anti-fungal discs were spread on the surface of inoculated plate. Plates were incubated at 37 C for (yeast) and 25 C for 5 days (mold). The diameter of inhibition zone of each disc was measured (mm) and judged.

2.5. Pathological examination:

A six lambs average age was 20-30 days old from private farm at Hosh-Issa in El- Bohaira Governorat, showed respiratory manifestation, diarrhea and loss of weight .the lambs submitted for postmortem examination .Following necropsy, small tissue specimens from the lungs, liver and intestine were collected and processed through 10% neutral buffered formalin as the methodology described by **Culling (2013).** Dehydration of tissues in graded of ethyl alcohol, cleared by xylene, and embedded in paraffin wax. sections of 5 µm thick stained with Hematoxylin and Eosin stain (H&E) for pathological examination and another sections stained with Periodic Acid-Schiff stain (PAS) for demonstration of fungi (**Bancroft and Gamble 2008)**,in which PAS staining demonstrate the presence of carbohydrates such as polysaccharides, mucin, glycogen and fungal cell wall components. Photomicrographs were captured with a digital camera (Labomed LC-1 CMOS, Labomed, USA) connected to a microscope (Labomed LB-212).

3.Results:

3.1.Bacteriological and Mycological results:

Table 1. Incidence of isolated bacteria from freshly dead lambs (N=6)

Bacteria	Liver (n=6)	Kidney (n=6)	Lung (n=6)	Spleen (n=6)	Intestine (n=6)	Total
E.coli	3	5	4	4	5	21
Coagulase negative Staphylococcus (CNS)	4	3	2	3	4	16
Clostrdium spp.	5	4	_-	3	6	18

Table 2. Incidence of isolated fungi from freshly dead lambs (N=6).

Fungi	Liver (n=6)	Kidney (n=6)	Lung (n=6)	Spleen (n=6)	Intestin (n=6)	Total
A.flavus	5	2	1	-	2	10
A.niger	1	1		-	1	3
Candida albicans	1	-	4	1	3	9
Candida tropicalis	2	1	2	1	3	9
Candida famata	2	1	1	2	1	7

Table 3. Anti bacterial sensitivity test of CNS (n=10) isolated from the dead lambs.

Antibacterial agents	Susceptible	Intermediate	Resistant
Cefquinom	4	3	3
Cefotaxime	4	4	2
Amoxicillin with clavulanic acid	6	3	1
Ampicillin	0	5	5
penicillin G	1	1	8
sulfamethoxazole with trime-thoprim	0	3	7
Oxytetracycline	O	3	7
Florofenicol	2	6	2
Streptomycin	0	0	10

Table 4. Anti bacterial sensitivity test of *E.coli.* (n=10) isolated from the dead lambs.

Antibacterial agents	Susceptible	Intermediate	Resistant
Cefquinom	5	2	3
Cefotaxime	5	1	4
Amoxicillin with clavulanic acid	3	5	2
Ampicillin	0	4	6
penicillin G	0	0	10
Sulfa-methoxazole with trimethoprim	2	1	7
Oxy-tetracycline	0	0	10
Florofenicol			
Streptomycin	0	1	9

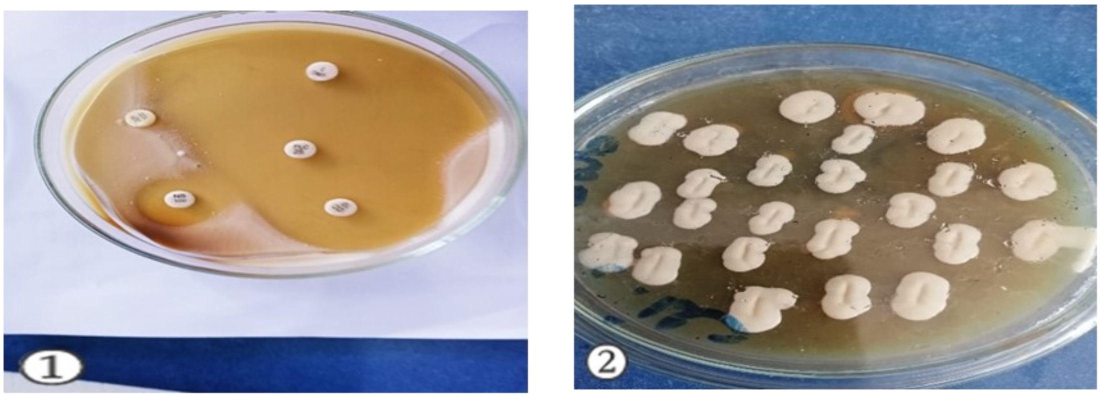
Table 5. Anti bacterial sensitivity test of *Clostridium spp.* (n=10) isolated from the dead lambs

Antibacterial agents	Susceptible	Intermediate	Resistant
Cefquinom	0	0	10
Cefotaxime	0	0	10
Amoxicillin with clavulanic acid	0	0	10
Ampicillin	5	3	2
penicillin G	4	3	3
Imipenem	7	1	2
sulfamethoxazole with trimethoprim	0	0	10
Oxytetracycline	0	0	10
Florofenicol	0	3	7

Table 6. Results of antifungal sensitivity of different fungal isolates from dead lambs.

Antifungal	*A.flavus* n=5	*A.niger* n=3	*C.albicans* Intestine n=3	*C.albicans* Lung n=3	*C.tropicalis* n=5	*C.famaat* n=5
CC10	S	S	S	R	S	S
NS100	I	S	R	R	S	S
FlC25	S	S	S	R	R	R
AP100	S	I	R	R	R	R
VRC1	S	S	S	R	S	S

NS 100 = Nystatin , C C 10 = Clotrimazole, VRC1 = Voriconazole , Ap 100 = Amphotericin B, FlC 25 = Fluconazole .
R=Resistant S=sensitive I=intermediat
*The site of isolation was identified for C.albicans because C.albicans isolated from lung was resistant for all antifungal.



(Fig.1): **1)**Antifungal sensitivity of C.albicans (isolated from intestine)
2) *C. albicans* on sabourauds dextrose agar .

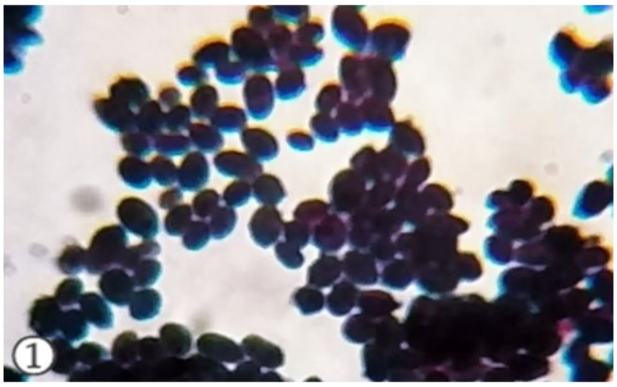


Fig 2 (1) *C. albicans* stained with Gram´s stain.

3.2. Pathological Examination:

Six lambs average age was 20-30 days old showed respiratory manifestation , diarrhea and loss of weight . On necropsy the lung was spotted with dark-red areas ,intestine was ballooned with few hemorrhagic spots (fig. 3), kidney and spleen were normal in appearance. Lungs showed on microscopic examination congestion of pulmonary blood vessels and presence of eosinophilic exudate within the lumen of some alveoli with presence of large round to oval yeast- like cells (*Candida albi-*

cans) cells (fig. 4, 1 & 2), and inflammatory cells (fig.4, 4) ,some of the large rounded to oval yeast- like cells (spore –like) showed clear zone surrounding them this zone called biofilm (fig.4,3) and Liver showed congestion of blood vessels, periportal edema and focal areas of coagulative necrosis of hepatocytes (fig. 5,1) , hepatic blood vessels revealed bacterial cells in its lumen with red blood corpuscles (fig. 5,2).

Intestine showed enteritis, enlarged mucus secreting cells (goblet cells) , degeneration and sloughing of intestinal epithelium (fig. 6,1) . Necrosis , degeneration and vacculation of intestinal villi (fig. 6,2) .Bacterial cells as well as *C.albicans* were present in mucosa, submucosa and blood vessels of intestinal tissue (fig. 6,3&4), *C.albicans* in intestine have no biofilm. .

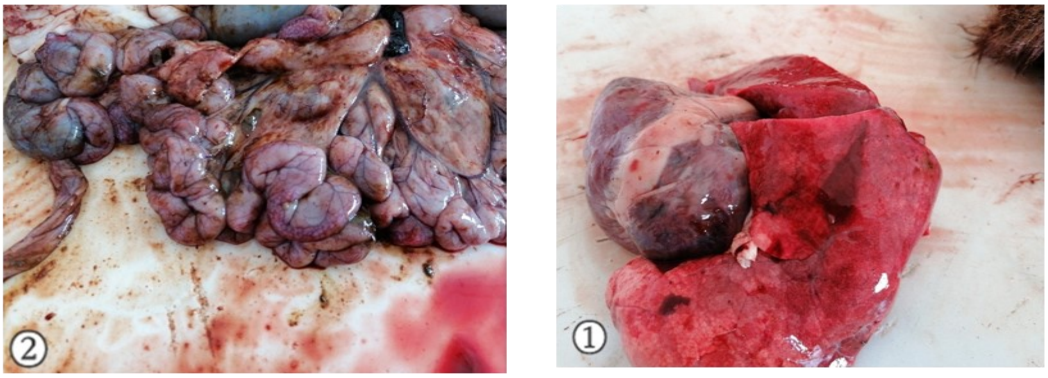


Fig. (3): 1) lung spotted with dark-red areas . 2) intestine ballooned with few hemorrhagic spots.

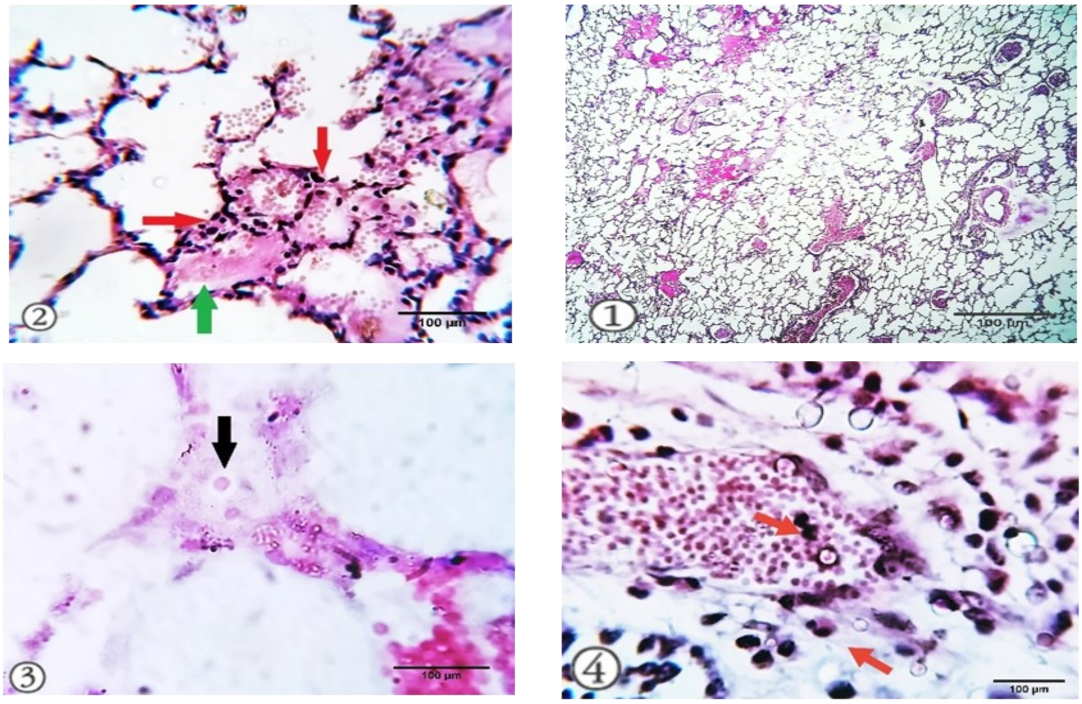


Fig. 4. 1) Lung tissue of lamb showing congestion of pulmonary blood vessels (H&E,X100). 2) Lung showing intera alveolar eosinophilic exudate within some alveoli (green arrow) with numerous intracellular and extracellular round to oval,elongated yeast –like cells (red arrows) (H&E,X250).3) Lung showing yeast cell-spore like (candida) with biofilm (black arrow) (PAS,X400).4) Lung showing infilteration of infilammatory cells in the pulmonary tissue (red arrows) (H&E,X400).

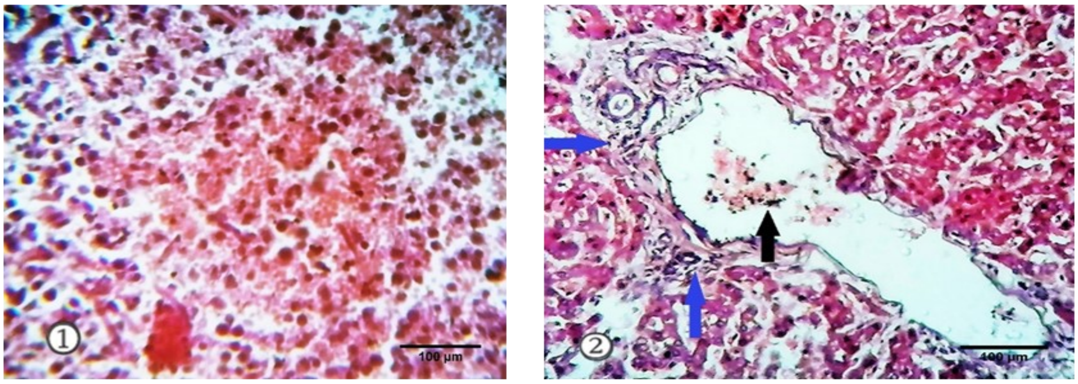


Fig. (5): 1) Focal area of coagulative necrosis of hepatocytes and precence of blood cells in between hepatocytes (H&E,X400).
2) Aggregation of bacterial cells in blood vessel (black arrow) and periportal infilteration of inflammatory cells (blue arrows) (H&E,X250)

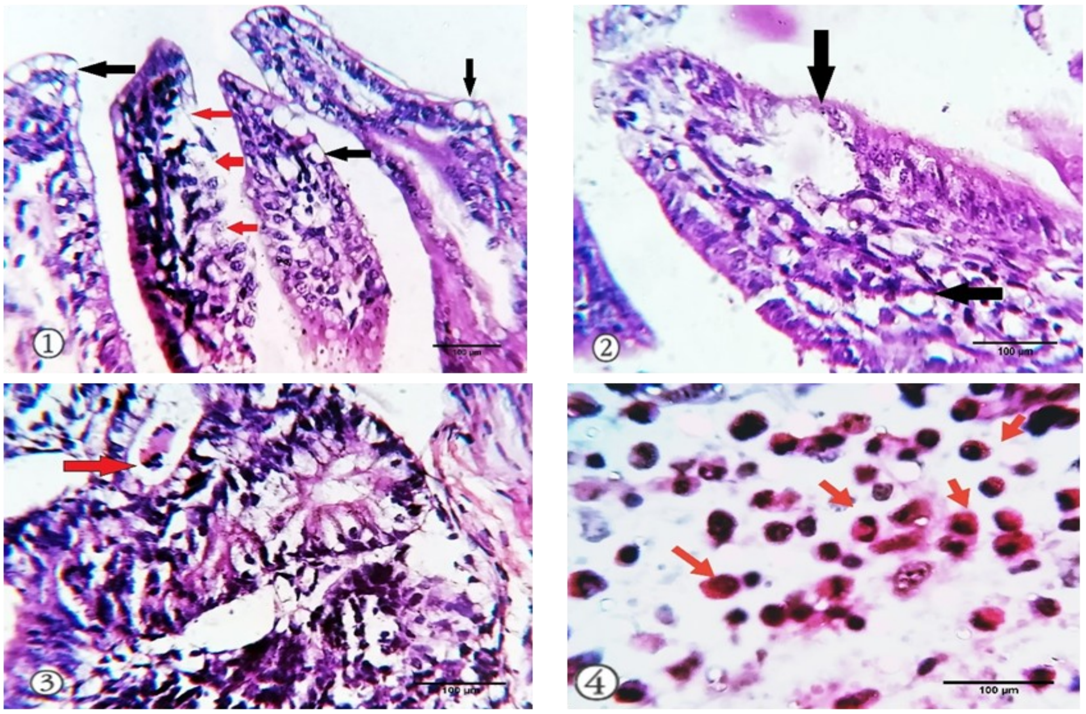


Fig. 6: (1) Intestine of lamb showing enlargement of mucus-secreting cells (goblet cells)(black arrows) and degeneration and sloughing of intestinal epithelium(red arrows) (H&E,X250) . 2) Necrosis , degeneration and vacculation of intestinal villi (black arrows) (H&E,X400). 3) presence of bacterial cells in intestinal blood vessel (red arrow) (H&E,X250).
4) presence of different inflammatory cells in intestinal submucosa (red arrows) (H&E,X400).

DISCUSSION:

The most frequent cause of early neonatal death in our study was infection, in which bacteria and fungi were isolated as a cause of deaths in most examined cases. Bacteriological examination revealed the isolation of 21 isolates of E. coli, 16 isolates of Coagulase negative Staphylococcus (CNS) and 18 isolates of Clostridium bacteria . Septicaemia and enterotoxaemia were most prevalent causes of infection in which it was isolated from the lungs , kidney ,liver ,spleen and intestine of the six lambs when examined bacteriologically . *E. coli* accounted for 42% of deaths caused by infection and 14% of all early neonatal death **(Holomy et al. 2016)** thus must be considered as an important infectious agent in this examined cases. Coagulase negative *Staphylococcus* (CNS) and *Clostridium* bacteria were isolated with or without *E.coli* so there mixed bacterial infection (table 1).Providing new bedding for lambing pens daily has been associated with lower flock level mortality rates in the first 24 h **(Binns et al. 2002),** probably mediated through prevention of build-up of high pathogen burdens in the environment. where daily cleaning of claiming pens was or was not practiced.

Table (2) shows the incidence of isolated fungi from internal organs of examined dead lambs In the current study, mycological examination revealed isolation of 10 isolates of *A.flavus* and 3 isolates of *A.niger* while the most prevalent yeast were *C.famata*, *C.tropicalis* and *C.albicans*. They were isolated 9,9 and7isolates respectively. Candida was the most common yeast species isolated (fig.1,2), nearly similar results were recorded **by Abd El-Tawab et al. (2020)**.while these results disagree with **Refai et al. (2010) and Bassiouny et al. (2019)** who reported high recurrence of mold isolates.

Candida sp. may cause gastro- intestinal candidiasis in animals **Hassan et al. (2010)** noticed that, the yeasts isolated from the diarrheic cases of sheep and goats were at higher rates than that from apparently healthy animals. Fungi are opportunistic pathogens it can infect the digestive tract of animals through setting animals on contaminated soils with yeast pathogens (**Donskey 2004**). The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to decrease host defences, adherence, biofilm formation and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin **(Silva et al. 2011)** (**Marak and Dhanashree 2018).** *Asperigillus sp*. is a cause of gastroenteritis in ruminant (**Tell 2005, Abou-Elmagd et al. 2011).**
In our study there was a mixed infection between bacteria ,between fungi and between bacteria and fungi in which all cases were positive for fungal and bacterial isolation

Table(3) shows the anti bacterial sensitivity of coagulase negative *staphylococci* in which it more resistant to streptomycin followed by pencillin, sulfamethoxazole with trimethoprim, Oxytetracyclines, and Ampicillin.and more sensitive to Amoxicillin with clavulanic acid, Cefequinom ,Cefotaxime and Florofenicol.While E.coli was resistant to pencillin G ,Oxytetracycline, Streptomycin, Sulfamethoxazole with Tri- methoprim and Ampicillin. 5 out of 10 tested strains were sensitive to Cefequinom and Cefotaxime (table 4). so when *Escherichia coli* is the most likely causative bacterium, it may be sensitive to combination of two antibiotic (**Leekha et al. 2011)** However, bacteria can be resistant to several antibiotics, because of resistance classes of antibiotics. This resistance might be because a type of bacteria following past exposure to antibiotics, or because resistance may be transmitted from other sources such as plasmids (**Partridge et al. 2018).**

Table (5) shows the sensistivity test of 10 isolated strains of Clostridium species in which all strains were completely resistant to cefequinom, cefotaxime, sulfamethoxazole with trimethoprim, Amoxicillin with clavulanic acid and Oxytetracycline, while it was sensitive to imipenem followed by Ampicillin then pencillin G (7, 5, 4, out of 10) tested samples respectively.

The activity of antifungal drugs sensitivity against different fungal isolates from samples of dead lambs is summarized in Table (6).

C. albicans isolated from intestine showed resistance to Ns100 and Ap100 while it sensitive to other antifungal drugs used (fig.1,1). Some studies have also shown that biofilms of Candida develop same level of resistance to fluconazole and amphotericin B **(Seneviratne et al. 2008 Sardi et al. 2011).** while *C.albicans* isolated from lung show complete resistance to all tested antifungal drugs. Where as *C. tropicalis* and *C. famata* showed sensitivity to all antifungal drugs tested except for Flc25 and Ap100 drug. The mold spp. (*A. flavus* and *A. niger*) was sensitive for all antifungal drugs used except Ns100 and Ap100 show intermediat effect for *A. flavus* and *A. niger* respectively.

Ability of *Candida* spp. to form biofilms get it resistance to antifungal drugs and ability to reinfect various tissue of the lung. A biofilm has been defined as a community of microorganisms organized at interfaces, enclosed in a self-produced polymeric matrix and adhered to an inert or living tissue. This detected in *C.albicans* isolated from lung (fig.4). While *C.albicans* isolated from intestine without biofilm formation (fig.6) **Van leeuwen et al. 2016**. Found that in the presence of *C. albicans, C. difficile* can persist and grow under aerobic conditions. Furthermore, p-cresol, produced by *C. difficile,* is involved in inhibiting hypha formation of *C. albicans*, directly affecting the biofilm formation and virulence of *C. albicans.* Biofilms is an important factor in causing disease and lead to drug-resistant in the majority of microbial biofilms **(Rajendran et al. 2010).**

C. albicans biofilms are less susceptible to antimicrobial agents The sensistivity test for the same organism and antibiotic may differ based on the site of infection **(Kuhn & Ghannoum 2004).**

Eosinophilic exudate in the alveoli of lung tissue may related to disturbance in cellular permeability and inflammation due to enterotoxemia .*C.albicans* in lung had biofilm which help candida to be invasive. Hepatocytes showed coagulative necrosis, hepatic blood vessels were congested, increase number of bile ductule in portal area as reported by **Kumar et al. (2015) .** Desquamation of intestinal villi and increase of goblet cells were due to enterotoxemia by *E .coli* **Kumar et al. (2013).** The *C. albicans* in intestine without biofilm, the majorty of *C. albicans* are associated with its ability to form biofilms as reported by **Mathe and Van Dijck (2013).**

5.CONCULSSION

Finally our study proved that the main causes of lamb mortality were bacteria combined by fungal infection especially *Candida albicans* which may cause disease affecting lamb stock, particularly after prolonged exposure to adverse environmental conditions. The histo pathological identification of *Candida albicans* in lung and intestine with typical mixed inflammatory cellular reaction was indicative of pneumonia due to candida in lambs the immediate post-lambing period are critical for lamb survival awareness should be created for the livestock owners regarding husbandry practices that can reduce the loss of the young stock. Proper veterinary service and disease identification mechanisms have to be designed and implemented.
All these can help to reduce and minimize lambs deathes.

Conflict of interest: Authors declare they have no confliction of interest.

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