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Mixed infection of Mycoplasma and bacteria in the respiratory tract of sheep with reference to the histopathological picture in Sharkia Governorate

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ABSTRACT:

The current study deals with the prevalence of Mycoplasma mixed with *Escherichia coli*, Klebsiella sp. and Pseudomonas sp. causing respiratory diseases in sheep from different farms at Sharkia Governorate. A total of 110 different samples including; 30 nasal swabs and 80 lungs and tracheas were aseptically collected. The prevalence rates differ from one bacterial agent to another. *M. ovipneumoniae* was detected by 31.8%, *E. coli* by 21.8%, Klebsiella sp. by 11.8% and finally, *P. aeuroginosa* by 9.1% from the collected samples. Biochemical and serological identifications revealed the presence of *M. ovipneumoniae, E. coli* of different serotypes, *K. pneumoniae, K. oxytoca* and *P. aeuroginosa*. Molecular characterization using PCR test targeting the *16S rRNA* genes of these bacterial agents revealing their specific DNA amplicon sizes of each bacterium except for *Klebsiella* sp. which was targeted by *gyr*A gene. Various pictures of tracheal lesions in infected sheep were observed and recorded. Different types of pneumonia were observed macroscopically and microscopically among the different causative bacterium. Antibiotic profiles show variety of sensitivity. Ciprofloxacin is the drug of choice for most isolates. *P. aeuroginosa* shows high level of antibiotics resistance.

INTRODUCTION

Sheep production is encouraged in Egypt as it can improve the daily protein intake of humans; it is constituted as an important component of Egypt food security plan **(Elshazly and Youngs 2019)**. In 2017, 2.34 million head was produced representing approximately 7.4% of all red meat production in Egypt **(FAO STAT 2018)**.

Sheep has occupied an advanced position within the Egyptian livestock sector because of their suitability to different agricultural condi-

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tions in the country **(Elshazly and Youngs 2019)**, However sheep diseases represent major limiting factor for such huge industry where pneumonia and neonatal diarrhea represents the more leading health problems **(Tarabees et al. 2016).**

The respiratory problems particularly different types of pneumonia are common in all species of domestic animals. The causative agents are multifactorial and the disease appears due to the interaction of the infectious microorganisms (bacteria; Pasteurella and Mycoplasma, viruses; PI3, reovirus, and adenovirus and fungi), host defense, environmental factors and stress **(Lacasta et al. 2008; Azizi et al. 2011)**.

Different species of *Mycoplasma* are associated with many pathological problems in small ruminants including respiratory manifestation, this problem results in significant losses, especially in African countries **(Pálma et al. 2018)**. It is highly fastidious microorganisms that required very precise media to develop in vitro **(Abdel-Halium et al. 2019)**.

Mycoplasma species commonly associated with pneumonia in small ruminants are *M. ovipneumoniae*, *M. arginini*, *M. capri*, *M. capripneumoniae*, and *M. capricolum* **(Chinedu et al. 2016)**. *M. arginini* is frequently isolated with *M. ovipneumoniae* from cases of atypical pneumonia in sheep and goats **(Kumar et al. 2013)** and other cases with lung consolidation **(Fernández et al. 2016)**. In Egypt, different *Mycoplasma* species have been isolated including *M. arginini*, *M. ovipneumoniae*, and *M. agalactiae* **(Ammar et al. 2008)**.

Although the role of *M. ovipneumoniae* in sheep respiratory infection is often overlooked, it may be the primary agent with various manifestations (chronic soft persistent cough with ocular and nasal discharges) but the real problem lies in that the disease condition become exaggerated by other bacterial infections **(Ozturkler and Otlu 2020)**.

E. coli is the most common member of family Enterobacteriacea frequently isolated from respiratory affection in sheep either from nasal swabs or from pneumonic lungs **(Zaghawa et al. 2010)**. Also *Klebsiella pneumoniae and Klebsiella oxytoca* of the same family are mostly associated with ovine pneumonic cases **(Patel et al. 2017; Franco et al. 2019)**.

Pseudomonas aeruginosa induces various disease conditions in sheep and goats with respiratory illness considered as one of the major issues mainly represented by pneumonia leading to significant mortalities and increased economic losses **(Dapgh et al. 2019).**

Mixed infection with different bacterial causes in the same sheep was common and this was attributed to the respiratory problems which were considered as multifactorial diseases where interaction between different microbial agents as bacteria leads to increase the incidence of those problems **(Thrusfield and Robert 2018)**.

There has been some degree of resistance of pathogens isolated from pneumonic sheep to some of the commercially used antibiotics **(Goodwin-Ray 2006)**. However, there are some effective antimicrobial agents as ciprofloxacin, ceftriaxone and oxytetracycline for the treatment of sheep pneumonia **(Kumar et al. 2018)**.

Severe congestion of the nasal sinus with catarrhal mucous, bilateral pneumonia with pulmonary congestion of cranial lobes with progression to the caudal lobes, fibrinous pleurisy, adhesions to the chest. Histological sections of the lung revealed suppurative bronchopneumonia with alveoli and bronchioles filled with variable proportions of neutrophils, macrophages, serofibrinous exudation, degenerated leukocytes and necrotic debris, and multifocal presence of aggregates of bacteria. Fibrinous pneumonia and severe distension of the interlobular septa by fibrin and edema was also observed and occasional peribronchiolar lymphocytic accumulation **(Rosário et al. 2010)**.

Our study aimed to throw a spot of light on the mixed infection of Mycoplasma with other bacterial agents isolated from respiratory mani-

fested sheep and studying their antibiotic susceptibility patterns for selection of the appropriate ones. In addition, the current study elucidated the macroscopic and histopathological lesions in the affected organs.

MATERIAL AND METHODS

Samples collection:

One hundred and ten samples were collected from respiratory manifested sheep (fever, nasal discharge, râles, cough, accelerated respiration, dyspnea), freshly dead and slaughtered sheep. Thirty nasal swabs and eighty tissue specimens including pneumonic lungs and tracheas from 60 freshly dead and 20 slaughtered sheep were aseptically collected. These samples were collected from different farms at Sharkia Governorate and immediately transported in icebox to the laboratory.

Bacteriological isolation:

All samples were submitted for bacteriological and mycoplasmal examinations. Bacteriological isolation was done by inoculation into brain heart infusion broth then streaked into and MacConkey, Ethylene Methelyne Blue (EMB) (Oxoid- UK) and Cetramide agars (HIMEDIA) followed by further identification for suspected isolates. The various members of family Enterobacteriacae were subjected to biochemical identification according to **Krieg and Holt (1984)**. *Pseudomonas* was identified biochemically as described by **Quinn et al. (1994)**. *Mycoplasma* isolation was done by inoculation into PPLO broth media, then plated onto PPLO agar media **(Sabry and Ahmed 1975)** and maintained at 37ºC for 3-7 days with 24-48h observation interval for "fried egg" colonies under dissecting microscope (Reichert Wien – Germany).

Identification of the bacterial isolates:

Biochemical identification of the purified *Mycoplasma* isolates was applied according to **Watson et al. (1988)**. Digitonin sensitivity, glucose fermentation, arginine deamination and film and spot formation tests were applied as mentioned by **Erno and Stipkovits (1973)** and **Razin et al. (1998).** Serological identification of the isolated *E. coli* and Klebsiella isolates was done according to **Kok et al. (1996)** and **Carter (1984)** respectively in "Food Analysis Center - Faculty of Veterinary Medicine - Benha University.

Molecular confirmation of the bacterial isolates:

The bacterial genomic DNA was obtained using the extraction kit (GeneJET Genomic DNA purification Kit Thermo-scientific) following the manufacturer's instructions. DNA concentration was determined using nanodrop. The PCR primers were synthesized by metabion international AG, (Germany), **(Table 1)**. The PCR reaction was performed in a gradient thermal cycler (1000S Thermal cycler Bio-RAD, USA). The total volume of there action mixture (50µl) contains: 25µl PCR master mix (Thermo Scientific™ PCR Master Mix (2X) Cat. no: K0171, USA.), 3µl target DNA, 1µl of each primers (10p mole/µl) and the mixture was completed to 50µl by PCR grade water.

Table (1) Primers sequences used of target genes

Bacterial Isolates	Target gene	Primer sequence (5′→3′)	Amplicon Size(bp)	Reference
Mycoplasma sp.	*16S rRNA*	GGG AGC AAA CAG GAT TAG ATA CCC T TGC ACC ATC TGT CAC TCT GTT AAC CTC	280	**van Kuppeveld *et al.* 1994**
M. ovipneumoniae	*16S rRNA*	TGA ACG GAA TAT GTT AGC TT GAC TTC ATC CTG CAC TCT GT	361	**Mcauliffe *et al.* 2003**
P. aeruginosa	*16S rRNA*	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	**Spilker*etal.*, 2004**
Klebsiella sp.	*gyrA*	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GAT CAC TTC GGT CAG G	441	**Brisse and Verhoef 2001**
E. coli	*16S rRNA*	GCT TGA CAC TGA ACA TTG GCA CTT ATC TCT TCC GCA TTA G	662	**Riffon *et al.* 2001**

After amplification as shown in **Table (2)**, the PCR products for the target genes were separated by 1.5% agarose gel electrophoresis (Sigma, USA) using Tris-boric EDTA buffer and stained with ethidium bromide using Gene Ruler 100 base pair DNA ladder (Thermoscientific Company, Cat. No. SM0243, USA).

Table (2) PCR amplification conditions for the bacterial PCR products

Bacterial genes Thermal profiles	**16 SrRNA gene of Mycoplasma sp.**	**16 SrRNA gene of *M. ovipneumoniae***	**16 SrRNAgene of *P. aeruginosa***	**gyrAgene of Klebsiella sp.**	**16SrRNA gene of *E. coli***
Initial denaturation	- -	- -	95ºC for 2 min	95ºC for 2 min	94ºC for 2 min
Denaturation	94ºC for 1 min	94ºC for 30 sec	94ºC for 20 sec	94ºC for 30 sec	94ºC for 45 sec
Annealing	55ºC for 1 min	55ºC for 30 sec	58ºC for 20 sec	55ºC for 40 sec	57ºC for 1 min
Extension	72ºC for 2 min	72ºC for 30 sec	72ºC for 40 sec	72ºC for 40 sec	72ºC for 2 min
Amplification	40 cycles	30cycles	25 cycles	35 cycles	35 cycles
Final extension		72ºC for 7 min	72 ºC for 1 min	72ºC for 10 min	72ºC for 10 min
References	**van Kuppeveld *et al.* (1994)**	**Mcauliffe *et al.* (2003)**	**Spilker *et al.* (2004)**	**Brisse and Verhoef (2001)**	**Riffon *et al.* 2004**

Pathological investigations:

All investigated eighty tissue samples including 60 freshly dead and 20 slaughtered sheep were carefully examined for gross abnormalities. The gross tissue lesions were observed and recorded carefully, and representative parts of the tissue samples were fixed in 10% neutral formalin buffer for further histopathological studies. Afterward, the preserved samples were dehydrated in alcohol, cleared in xylene, impregnated and embedded in paraffin wax, sectioned at 4µm and finally stained with hematoxylin and eosin (H&E) for histopathological examination as described by **Survarna et al. (2013)** and examined microscopically.

Antibiotic susceptibility test:

It was performed for the obtained bacterial isolates by disc diffusion method for testing their susceptibly to ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, doxycycline, erythromycin, gentamycin, lincomycin, oxytetracycline, spectinomycin and sulfamethoxazole + trimethoprimre presenting different antibiotic classes according to **CLSI (2017)**.

RESULTS

Prevalence and identification:

M. ovipneumoniae, *E.coli*, *Klebsiella* sp. and *P. aeruginosa* were detected with recovery rates (31.8, 21.8, 11.8 and 9.1%, respectively) **(Table 3)**. Biochemical identification declared that all *Mycoplasma* isolates were positive for digitonin inhibition and glucose fermentation but negative for arginine hydrolysis and film and spot formation. Different types of pneumonia including; alveolar pneumonia, bronchopneumonia, fibrinous bronchopneumonia, hemorrhagic pneumonia and interstitial pneumonia were correlated with their causitive agents and thus, multiple types were observed in the mixed infections. Alveolar pneumonia was the most prominent type associated with *M. ovipneumoniae*, *E. coli* and *P. aeruginosa* **(Table 3).**

Table (3) Prevalence of bacterial isolates associated with type of pneumonia

Bacterial isolates	Type of infection	Swabs n= 30	Tracheas n= 40	Lungs n= 40	Total	Types of pneumonia involved with each single infection
M. ovipneumoniae n= 35 (31.8%)	Single	4	6	13	23	-Alveolar pneumonia -Bronchopneumonia
	Mixed	2	4	6	12	
E. coli n= 24 (21.8%)	Single	3	5	4	12	-Alveolar pneumonia -Bronchopneumonia -Fibrinousbroncho-pneumonia -Hemorrhagic pneumonia
	Mixed	2	4	6	12	
Klebsiella sp. n= 13 (11.8%)	Single	2	2	5	9	-Bronchopneumonia -Interstitial pneumonia -Fibrinousbroncho-pneumonia
	Mixed	1	0	3	4	
P. aeruginosa n= 10 (9.1%)	Single	1	0	5	6	-Alveolar pneumonia -Bronchopneumonia -Hemorrhagic pneumonia -Fibrinousbroncho-pneumonia
	Mixed	1	0	3	4	
Total				82 (74.5%)		

n= number

Mixed infections between different bacterial isolates were illustrated in **Table (4)**. *M. ovipneumoniae* was observed in mixed infection with *E. coli* and *P. aeruginosa* while *K. pneumoniae*. was detected with *E. coli* and *P. aeruginosa* **(Table 4)**.

Table (4) Mixed infection between the bacterial isolates

Bacterial isolates	Swabs n= 30	Tracheas n= 40	Lungs n= 40	Total n= 110
M. ovipneumoniae + E. coli	4	8	8	20
M. ovipneumoniae + P. aeruginosa	0	0	4	4
E. coli + K. pneumoniae	0	0	4	4
K. pneumoniae. + P. aeruginosa	2	0	2	4

n= number of samples

Members of family *Enterobactariaceae* were serologicaly identified and results revealed the detection of *E. coli* serogroups (O153, O44, O91, O84 and O8) where O153 was the most prominent serogroup. All Klebsiella sp. were identified as *K. pneumoniae* except for only one isolate was identified as *K. oxytoca* in a single infection **(Table 5)**.

Table (5) Serological identification of *E. coli* and Klebsiella sp.:

Bacterial isolates	Sero- group	Number of identified isolates
***E. coli* (24)**	O153	5
	O44	4
	O91	4
	O84	4
	O8	3
	Un-typed	4
Klebsiella sp. (13)	*K. pneumoniae* K1 (HVKP)	5
	K. pneumoniae K2 (HVKP)	4
	K. pneumoniae K1 (CKP)	3
	K. oxytoca	1

Molecular identification:

Conventional PCR test was carried out on the culturally positive bacterial isolates after being biochemically and serologically identified for confirmation of these isolates. The obtained results revealed the detection of 16SrRNA genes specific for Mycoplasma sp. and *M. ovipneumoniae* in seven and eight examined isolates which gave their characteristic bands at 280bp and 361bp, respectively as shown in **Fig. (1 and 2)**.

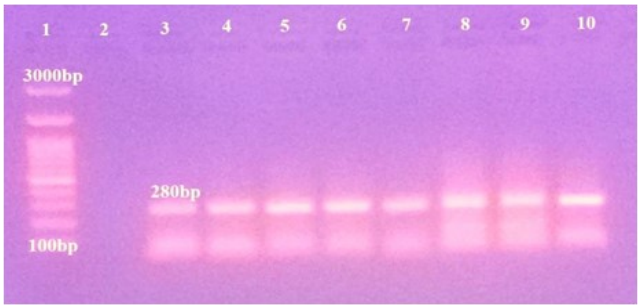

Fig (1): Agarose gel electrophoresis of the group-specific primer set of Mycoplasma 16S rRNAgene. Lane (1): 100bp DNA ladder. Lane (2): control negative. Lane (3): control positive. Lanes(4-10): positive samples for the target gene at 280bp

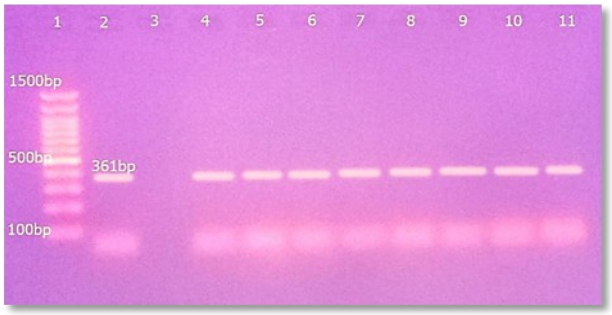

Fig (2): Agarose gel electrophoresis of *M. ovipneumoniae* 16S rRNA gene. Lane (1): 100bp DNA ladder. Lane (2): control positive. Lane (3): control negative. Lanes (4-11): positive samples for the target gene at 361bp.

16S rRNA specific gene of *P. aeruginosa* was amplified in five isolates showing its characteristic band at 965 bp as illustrated in **Fig (3)**.

E. coli 16S rRNA gene was successfully amplified in six tested *E. coli* isolates representing different serogroups at 662 bp **(Fig 4)**.

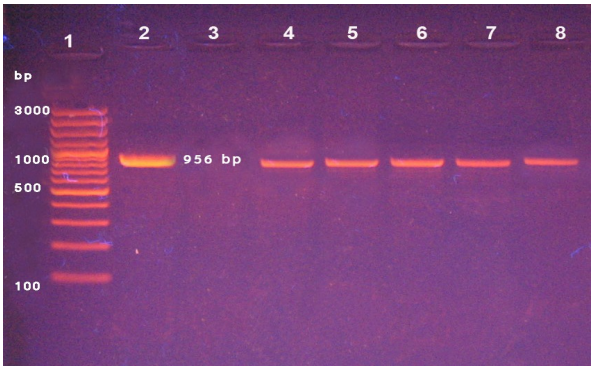

Fig (3): Agarose gel electrophoresis of *P. aeruginosa 16S rRNA* gene. Lane (1):100 bp DNA ladder. Lane (2): control positive. Lane (3): control negative. Lanes (4-8): positive samples for the target gene at 956bp.

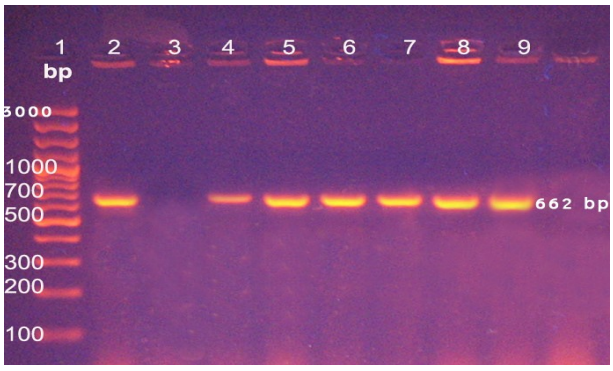

Fig (4): Agarose gel electrophoresis of *E.coli 16S rRNA* gene. Lane (1):100 bp DNA ladder. Lane (2): control positive. Lane (3): control negative. Lanes (4-9): positive samples for the target gene at 662bp.

Klebsiella gyrA gene was amplified in six isolates (5 *K. pneumonaie* and 1 *K. oxytoca*) and gave a specific band at 441bp **(Fig 5).**

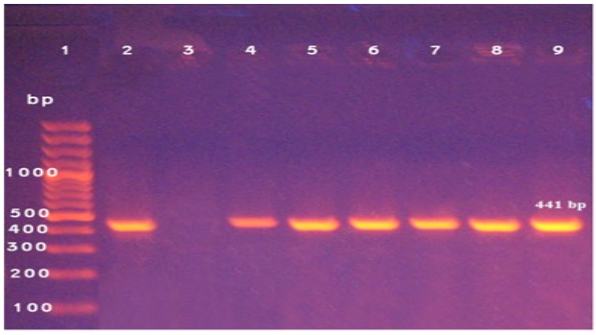

Fig (5): Agarose gel electrophoresis of Klebsiella gyrA gene. Lane (1): 100bp DNA Ladder. Lane (2): control positive. Lane (3): control negative. Lanes (4-9): positive samples for the target gene at 441bp.

Gross Findings:

Grossly, tracheas showed limited gross lesions restricted in mild to moderate congestion in some cases and petechial hemorrhage in other few cases. Lung lesions were observed and described then categorized into the following types: (1) Congestion (15%), (2) hemorrhage (20%), (3) mixed congestion and hemorrhage (35%), (4) hepatization of lungs (20%) and (5) emphysema (10%) **(Fig. 6).**

Microscopic findings:

Various pictures of tracheal lesions in infected sheep were observed. Tracheal lesions were not specific to certain pathogen. Most cases showed submucosal and periglandular leukocytic cells infiltration **(Fig. 7a and 7b)**. Only heavy infected cases revealed necrosis of some chondrocytes of tracheal rings cartilage **(Fig. 7c)**. Metaplasia of some tracheal epithelium into goblet cells common lesion **(Fig. 7d)**. Five types of pneumonia were identified, microscopically, categorized and summarized in **Table (3)** with its related pathogens. Peribronchial hyalinization of connective tissues with hyperplasia of bronchial epithelium was observed **(Fig 8a)**. Alternative red and grey hepatization of pulmonary tissue **(Fig 8b)** was the most observed lesion as common stages of pneumonia. Alveolar pneumonia was characterized by dilated and congested pulmonary blood vessels with the presence of serous alveolar exudate with leukocytic cells infiltrations. Bronchopneumonia was characterized by the pneumonia presence of inflammatory cells consisted of mainly neutrophils within the lumen of bronchiole. Fibrinous bronchopneumonia **(Fig. 8c)** characterized by peribronchial inflammatory zone with intrabronchial fibrinous exudates with visible fibrin strand, thickened interlobular septa, congestion of pulmonary blood vessels. Hemorrhagic pneumonia was also detected and characterized by excessive extravasated erythrocytes within the bronchi, alveoli and interalveolar septa related to leukocytic cells infiltration. Leukocytic cells infiltrated pulmonary tissues suffering from extravasated erythrocytes **(Fig. 8d)** were observed in some cases. Lung showed emphysema **(Fig. 8e)**. Interstitial pneumonia characterized by congestion with reactive cells in and around the bronchial wall, and sometimes intrabronchial exudate contained few fibrin threads with peribronchial inflammatory zone with fibroblast and thickened interlobar septa.

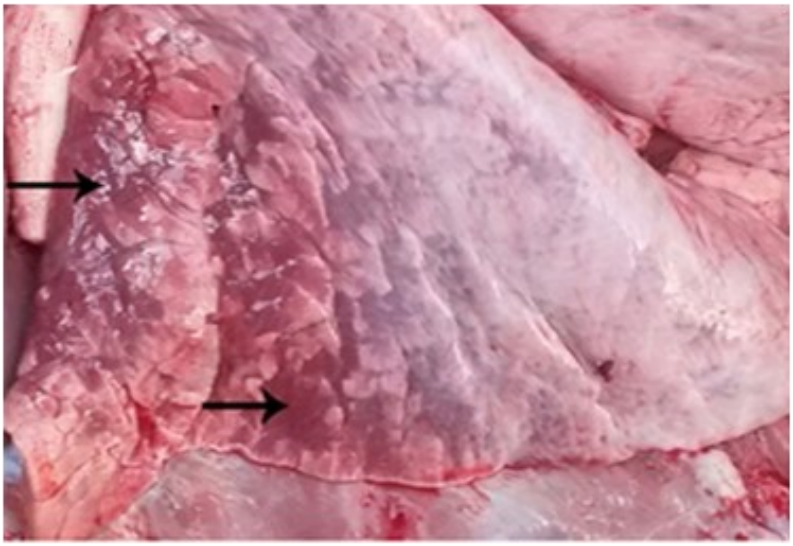

Fig (6): Lung of infected sheep with *E. coli* + *M. ovipneumoniae* showing multiple focal areas of congestion and hepatization of lung tissue (arrows).

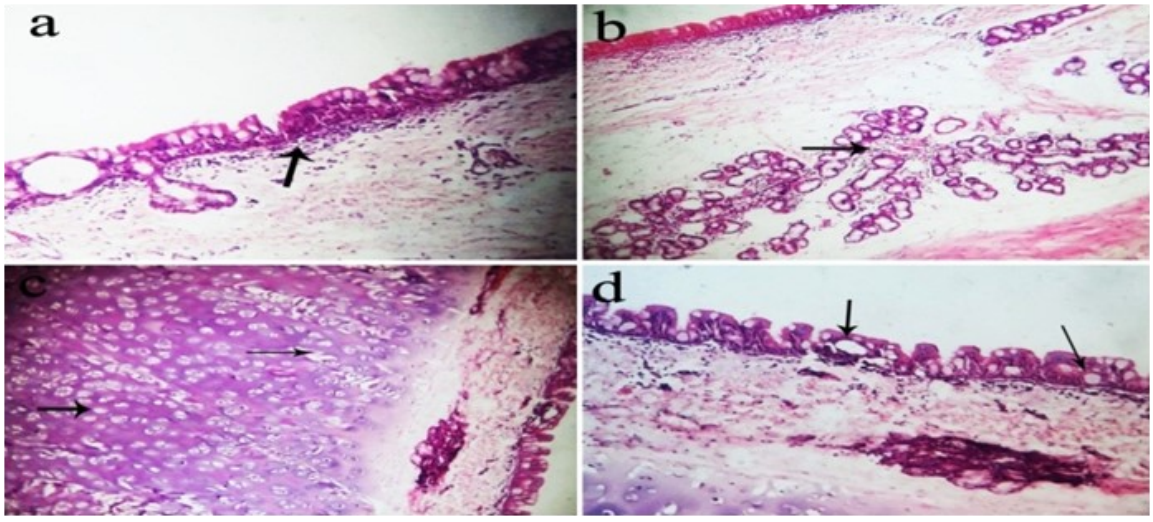

Fig (7): Photomicrograph of trachea of infected sheep. (a): trachea infected with *Klebsiella* sp. showing submucosal leukocytic cells infiltration (arrow) (H&E x 200). (b): trachea infected with *M. ovipneumoniae* showing periglandular leukocytic cells infiltration (arrow) (H&E x100). (c): trachea infected with *P. aeruginosa* showing necrosis of some chondrocytes (arrows) (H&E x 400). (d): trachea infected with *E. coli* showing metaplasia of some

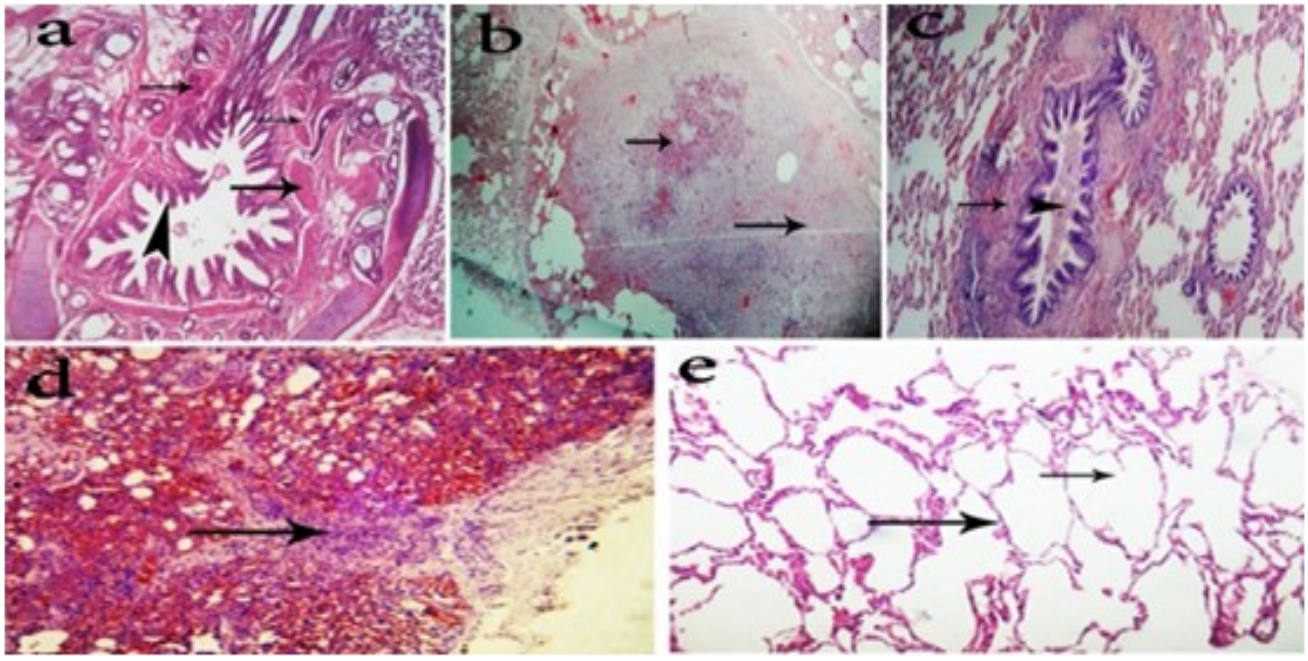

Fig (8): Photomicrograph of lung of infected sheep. (a): lung infected with *M. ovipneumoniae* + *P. aeruginosa* showing peribronchial hyalinization (arrows) with hyperplasia of bronchial epithelium (arrow head) (H&E x400). (b): lung infected with *Klebsiella sp.* + *M. ovipneumoniae* showing alternative red and grey hepatization of pulmonary tissue (H&E x100). (c): lung infected with *P. aeruginosa* + *E. coli* showing intrabronchial exudate and peribronchial inflammatory zone with fibrin threads and mild thickened interlobar septa (H&E x200). (d): lung infected with *P. aeruginosa* showing leukocytic cells infiltrated pulmonary tissue (arrow) suffering from extravasated erythrocytes (H&E x100). (e): lung infected with *M. ovipneumoniae* showing emphysema (arrow) (H&E x400).

Anti-biogram susceptibility:

Results revealed absolute resistance to some antibiotics and variable resistance levels to other ones that vary from one bacterium to another. *M. ovipneumoniae* were sensitive to doxycycline lincomycin, oxytetracycline and spectinomycin. *E. coli* isolates showed highest susceptibility to colistin and ciprofloxacin with absolute resistance to oxytetracycline and ceftriaxone and variable resistance levels to rest of the used antibiotics. Klebsiella isolates represented susceptibility to ciprofloxacin, gentamycin and chloramphenicol and variable susceptibility to other antibiotics *P. aeruginosa* was only sensitive to ciprofloxacin and gentamycin and highly resistant to other tested antibiotics. Highest susceptibility levels were recorded for ciprofloxacin on all examined bacteria **(Table 6)**.

Table (6) Anti-biogram susceptibility results of the isolated bacteria

Antibiotic / symbol	Potency (µg)	*M. ovipneumoniae* (n=35)		*E. coli* (n=24)		Klebsiella sp. (n=13)		*P. aeruginosa* (n=10)	
		S	R	S	R	S	R	S	R
Ampicillin (AMP)	10	-	-	4	20	6	7	1	9
Ceftriaxone (CTX)	30	-	-	0	24	2	11	0	10
Chloramphenicol (C)	30	10	25	6	18	8	5	1	9
Ciprofloxacin (CIP)	10	30	5	20	4	13	0	8	2
Colistin (CT)	10	-	-	23	1	-	-	-	-
Doxycycline (Do)	30	29	6	2	22	7	6	0	10
Erythromycin (E)	15	20	15	2	22	2	11	0	10
Gentamycin (CN)	10	12	23	12	12	8	5	7	3
Lincomycin (MY)	15	29	6	-	-	-	-	-	-
Oxytetracycline (OT)	30	30	5	0	24	6	7	0	10
Spectinomycin (SH)	100	33	2	-	-	-	-	-	-
Sulfamethoxazole + Trimethoprim (SXT)	25	-	-	5	19	4	9	1	9

S= sensitive R= resistant

DISCUSSION

Sheep respiratory diseases are sometimes acute and fatal. Early rapid and specific diagnosis allowed designing appropriate prevention and control strategies to alleviate the economic losses **(Chakraborty et al. 2014)**, consequently the present study aimed to correlate the histopathological findings in respiratory diseased sheep with their bacterial etiologies.

Prevention and control of fatal infectious respiratory diseases of small ruminants require many diagnostic strategies to be adopted and routine antibiotic sensitivity tests to be done. The diagnostic tests include combination of conventional and advanced diagnostic investigations. However, the initial suggestive diagnosis involves clinical signs observation accompanied by postmortem examination followed by isolation, serological and molecular detection of etiological agents **(Chakraborty et al. 2014).**

The obtained data of the bacteriological examination indicated the role of *M. ovipneumoniae*, *E. coli*, Klebsiella sp. and *Pseudomonas* sp. in occurrence of respiratory diseases. The total bacterial recovery rate was 82/110 (74.54%) which is in accordance with **Zagwa et al. (2010)** (78.3%).Furthermore, results revealed the presence of these bacterial species as single or mixed infection as reported in the previous studies **(Kumar et al. 2018; Mona 2019; Nahed and Allam 2019)**. Mixed infections with different bacterial etiologies in respiratory affection are common and that can be explained by the fact that infection with one bacterial agent can increase the chance of infection with another **(Mona 2019)**.

Myccoplasma is considered a part of the respiratory tract micro biota in sheep that can turn pathogenic under certain conditions. Primary

infection with *M. ovipneumoniae* can lead to secondary infection by other micro-organism **(Franco et al. 2019)**. Isolation rates showed that *M. ovipneumoniae* was the highly detected bacterium (31.8%). Our findings are in accordance with **Ibrahim et al. (2018)** who isolated Mycoplasma from 29% of pneumonic sheep where *M. ovipneumoniae* was the main detected species but higher than that reported by **Ozturkler and Otlu (2020)** who isolated *Mycoplasma* form 10.4% of sheep pneumonic lungs showing that *M. ovipneumoniae* was the most predominant specie and the isolation rate variation may be attributed to the different circumstances in the geographies studied.

Our study clarified the potential role of *E. coli* in the occurrence of respiratory disease in sheep, its incidence rate was 21.81% which was nearly similar to that reported by **Ertan (2006)** (24.56%) and **Kumar et al. (2018)** (25%), but higher than reports of **Garedew et al. (2010)** (11.41%) and **Nehra and Jakhar (2018)** (12.5%). However, **Azizi et al. (2013**) failed to isolate *E. coli* from trachea and lung of pneumonic goats.

Klebsiella sp. isolation emphasized its importance as an etiological pathogen in respiratory disorders in sheep with total recovery rate (11.8%).Isolation rate of Klebsiella sp. varied greatly among studies **(Azizi et al. 2013** (15.09%)**; Obaid and Khudair, 2016** (13.6%) and **El-Mashad et al. 2020** (3.84%)**.** Herein, we isolate *K. pneumonia and K. oxytoca*, both species were detected in former studies **(Patel et al. 2017** and **Franco et al. 2019)** in the contrary many previous studies did not isolate *Klebsiella* sp. from ovine pneumonic lungs **(Garedew et al. 2010)**.

P. aeruginosa involvement was obvious in the present study as it was isolated with recovery rate (9.09%). This was somewhat similar to that reported by **Nahed and Allam (2019)** who isolated *P. aeruginosa* from sheep with 10% and **Mona (2019)** where its isolation rate was 11.4% from the examined lung tissues. In the contrary **Dapgh et al. (2019)** found that *P. aeruginosa* was isolated from 5.8% of the examined sheep and **Zagwa et al. (2010)** isolated *P. aeruginosa* with low rates 5.85% from both nasal swabs and pneumonic lung tissues.

Molecular techniques have proved beneficial impact in bacterial diagnosis as it overcomes some disadvantages of conventional methods in addition to its sensitivity and rapidity **(Kaber et al. 2004)** and in spite of that cultured based methods remain to the forefront of clinical microbial detection **(Srinivasan et al. 2015).** Bacteria identification by PCR is founded mainly on target gene amplification that should be highly conserved **(Gangwal and Kashyap 2017).** 16SrRNA gene, a molecular marker for identification of bacterial species, is ubiquitous to members of this domain **(Srinivasan et al. 2015).**

Therefore this gene gave positive amplifications at 280 and 361bp for *Mycoplasma* sp. and *M. ovipneumoniae*, respectively **(Besser et al. 2008)** which was in accordance to **Mona (2019**) who proved that *16SrRNA* PCR based assay for Mycoplasma species identification in sheep is more accurate than other methods.

The results of conventional method was confirmed by PCR applied on different *E. coli* isolates representing different serotypes indicating that the primer set used herein is highly specific to *E. coli* species and could be used in the future in direct *E. coli* detection from various clinical cases without need to time consuming culturing methods as mentioned before **(Saei et al. 2012)**.

In most laboratories, the detection of *P. aeruginosa* is still accomplished by culturing and biochemical tests. Although this result is reliable, they are time consuming **(Deschaght et al. 2009)**. Moreover, in some cases in which the bacterial count is low it gave false-negative results. Thus, access to rapid and specific methods that have a high sensitivity as PCR is of a great importance. Suspected *P. aeruginosa* isolates gave positive amplification for 16SrRNA at 956bp as mentioned by **Spilker et al. (2004).**

DNA-based method has been developed for the detection of pathogenic Klebsiella sp. **(Jonas et al. 2004)**. In the present study tested isolates for gyrA gene amplified a region of 441 bp. This is in accordance to **Brisse and Verhoef (2001)** who mentioned that subunit A of DNA gyrase is encoded by *gyr*A gene that is considered the main target of fluroquinololnes in Klebsiella and also used for confirmation of genus Klebsiella.

Concerning the antibiotic sensitivity test results, *M. ovipneumoniae* declared susceptibility to both oxytetracycline and ciprofloxacin. **Maksimović et al. (2020)** mentioned that oxytertacycline had the highest values of minimal inhibitory concentration (MIC) but ciprofloxacin showed the lowest ones.

E. coli exhibited high susceptibility to colistin and ciprofloxacin, moderate sensitivity to gentamycin and high resistance level to others. These results are similar to some extent with **Nehra and Jakhar (2018)** who stated that *E. coli* showed high sensitivity to gentamycin and ciprofloxacin and high resistance to tetracycline. **Seai et al. (2014)** mentioned that *E. coli* isolated from sheep showed absolute resistance to penicillin and erythromycin, low resistance level to tetracycline and gentamicin. Our results also detected multiple drug resistance in *E. coli* isolates and that highlighted that ovine host can act as reservoir of resistant isolates which may transfer to human.

Klebsiella isolates showed susceptibility to ciprofloxacian, gentamycin and doxycycline and chloramphenicol which was in agreement with **Wassif and El-Kattan (2015)** as they proved the susceptibility of Klebsiella isolated from sheep to cephalosporin, gentamycin, ciprofloxacin and chloramphenicol.

Infection caused by *P. aeruginosa* is one of the major issues in sheep and associated with significant mortality rates, consequently early diagnosis and correct medical treatments are the best strategies for saving animal life **(Bangar et al. 2016)**. Our results showed that *P. aeruginosa* displayed elevated resistance level to all tested antibiotics except for ciprofloxacin and gentamycin as mentioned by **Dapgh et al. (2019)** who detected their susceptibility to both ciprofloxacin and norfloxacin and complete resistance to ampicillin, erythromycin, tetracycline, sulfamethoxazole +trimethoprim and chloramphenicol. *P. aeruginosa* is commonly resistant to antibiotics are consequently difficult to eliminate when they infect a compromised site **(Kadhim 2020)**. Antibiotics resistance property among *P. aeruginosa* isolates could be attributed to the existence of the unusually restricted outer membrane permeability which acts as a safeguard barrier for antibiotics also there is other second -ary intrinsic factors as multidrug efflux pumps **(Xu et al. 2014)**.

Grossly, our study showed limited gross lesions restricted in mild to moderate congestion and petechial hemorrhage in the trachea which were in partial accordance with those described by **Radostits et al. (2002)** who described tracheal congestion, hemorrhage, edema, obstruction and pustular. In the present study, the lung exhibited several gross forms as congestion, hemorrhages, emphysema and pulmonary tissue hepatization similar to those described by many authors **(Akbor et al. 2007** and **Sukanta et al. 2018).** On the other hand, **Jubb et al. (1993)** observed pus and cyst in the pulmonary tissue which could be explained on the base of differences in pathogen nature and severity of the disease. Our current study could not find out any pathognomonic lesions related to any of the previously isolated pathogen in both lungs and tracheas and this was in accordance with that recorded by **Damassa et al. (1992)** who mentioned that clinical mycoplasmosis often lacks pathognomonic characteristics and symptoms can be shared by or can mimic other clinically significant infections. Pneumonic lesions in our study were summarized in five types including; alveolar pneumonia, bronchopneumonia, fibrinous bronchopneumonia, hemorrhagic pneumonia and interstitial pneumonia; with their related frequencies in each pathogen. Similar pneumonic lung tissues were described by many authors **(Collie et al. 2013; Lindstrom et al. 2018; Sukanta et al. 2018)**.The most pneumonic lesion recorded was; alveolar pneumonia followed by fibrinous bronchopneumonia and hemorrhagic pneumonia then interstitial pneumonia. No purulent pneumonianor purulent bronchopneumonia were detected, which was opposite to that recorded by **Akbor et al. (2007)** and **Sukanta et al. (2018)** in spite of the isolation of *E .coli* which is one of the pyogenic pathogens having the ability to progress the purulent lesion. This could be attributed to the virulence of the isolated strains and the response of the sheep immune system. Mixed infections have obvious pneumonic lesions than those declared in single ones similar to that mentioned by **Novert (2002)**. Histopathology of bronchopneumonia and hemorrhagic pneumonia de-

scribed in this investigation corresponded to the lesions of other investigators **(Ashok et al. 2004)**.

CONCLUSION:

Mixed bacterial infection including *M. ovipneumoniae*, *E. coli*, *K. pneumonaie*, *K. oxytoca* and *P. aeruginosa* causing different degrees of respiratory disease in sheep is observed. They are accompanied with general respiratory manifestations and five characteristic types of pneumonia are related to these pathogens. Molecular investigations are beneficial in confirming the isolated bacterial agents. High level of antibiotics resistance especially in *P. aeruginosa* is alarming.

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