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A bacteriological and histopathological study of sheep pneumonia in Salah Aldeen Governorate

Noor W. AL-Yousif , Nihad Abdul-Hussain Jafar , Bader Khatlan Hameed

Department of Medicine , College of Veterinary Medicine, University of Tikrit, Tikrit , Iraq

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Corresponding Author:

Name: Noor W. AL-Yousif

E-mail: Nwaleed136@gmail.com

Tel:

ABSTRACT

This study aimed to isolate and diagnose the bacterial pathogens that causes pneumonia in sheep, study the biochemical and histopathological changes in lung tissue of infected animals and determining the most effective types of antibiotics for treating sheep diseased with pneumonia. 150 samples (50 nasal swabs, 50 tracheal samples, 50 lungs samples from slaughtered sheep) were collected randomly from different areas of Salah Aldeen governorate during the period (September 2019 till January 2020) .

The results showed that no significant differences in the values between positive and negative results from nasal, tracheal and infected lungs at a probability level $P< 0.05$. And showed that *Staphylococcus aureus* and *Klebsiella pneumonia* are two main bacterial causes of pneumonia in sheep .

In macroscopic examination the prominent lesion in most lung samples was congestion and presence of emphysema with an expansion of the bronchitis and found that the most common type of pneumonia infection is broncho pneumonia.

All the isolated bacterial species; however, show sensitivity to the antibiotics chloramphenicol and trimethoprim + sulfamethoxazole except *Pasteurella Spp.*, that it shows resistance to trimethoprim+ sulfamethoxazole.

Introduction

Pneumonia is an inflammation of the lung tissue with multiple causes, It may be accompanied by inflammation of the larger airways (bronchioles) and referred to as bronchopneumonia, or by inflammation of the pleura (outer surface of the lung , adjacent to the chest wall) and referred to as pleuropneumonia [1].

The disease is multifactorial in origin, which may be microorganisms such as bacteria, viruses, parasites, as well as Rickettsia and Mycoplasma, environmental factors such as cold or very hot weather, management factors such as poor health care, stress, overcrowding and the immune state of animals[2] .

Respiratory diseases in sheep result in poor live weight gain and mortality, economic losses include unthrifitness, coast of treatment and preventive measures of non-fatal cases [3]. Progressive disease may also result in endotoxaemia and septicaemia, death may occur when there is sufficient lung compromised to cause anoxia (usually when more than 70 % of the lung is affected), or from over whelming systemic infection including toxaemia[4].

There are three types of pneumonia: subacute, acute and chronic pneumonia, the majority of mortality in sheep in the form of subacute or chronic pneumonia, because of the clinical economic importance of the disease in sheep, it was a topic of interest of many researchers in the field of small ruminant practice, but in many instances, most studies were critically focused on the causes of the disease[4,5].

The most important bacterial etiology in Iraq are *Streptococcus* spp, *Pasteurella* spp, *Escherichia coli* and *Staphylococcus* spp. [6]. When the disease outbreaks into sheep flocks, the selection of effective antibiotic therapy is necessary, treatment shall be done at the beginning of the disease because of the rapid progression of lung damage and endotoxin release[7].

The present investigation aimed to isolate and diagnose the bacterial pathogens that cause

pneumonia in sheep , study the biochemical and histopathological changes in lung tissue of infected animals and determining the most effective types of antibiotics for treating sheep with pneumonia.

Material and Method

Samples

Samples were collected during the period (September 2019 till January 2020) from different regions of Salah Aldeen governorate. Samples were collected from slaughtered places, 150 samples were taken from 50 sheep of different ages and from both genders as follows (50 lungs, 50 tracheal samples and 50 nasal swabs was placed inside container tubes with the nourishing medium brain heart infusion broth), nasal swabs were collected before slaughtering the diseased animal, while the lung tissues and tracheal samples were collected after slaughter . Lungs and trachea were placed in sterile plastic containers and transferred in ice cooled box at 4 ºC to the microbiology laboratory at the College veterinary medicine, Tikrit University, and it placed in the incubator at a temperature 37° C for 24 hours and cultured on appropriate culture media. After culturing of lung samples by using cotton swabs, the lungs preserved at 10% formaldehyde solution for histopathological studies.

Culture media preparation and sterilization

The culture media was prepared according to the manufacturer's instructions and adjusted the pH according to the need for it, then sterilized it in the autoclave at a temperature of 121 ° C for 5 minutes. The culture media that were used are the following: MacConkey Agar.(Hi media), Mannitol Salt Agar. (Hi media), Blood Agar Base.(Hi media), Brain heart infusion broth.(Hi media), Nutrient Agar.(Hi media), Eosin-methylene blue agar (EMB). (Hi media) and Nutrient Broth. (Hi media).

Reagents and Solutions Preparation

The following tests reagent were prepared: Oxidase test reagent, Catalase test reagent, and Kovac's reagent [10] .

The Used Stains

Methylene blue, Gram Stain and Hematoxylin and eosin stain. (Sigma, USA)

Bacterial isolation

The collected swabs were cultivated under aseptic condition into mentioned culture media. All inoculated media were incubated aerobically at 37^{o}C for 24 hours. Suspected colonies onto the media were identified morphologically according to its staining reaction, shape, size and arrangement and were confirmed by full biochemical identification (catalase test, oxidase test, coagulase test, urease activity test, indole production test, sugar fermentation and gas production test) according to the researcher [10].

Histopathological study

For histological sections, the collected lung samples were rapidly fixed in 10% neutral buffered formalin solution at least 18-24 hours After that, the fixed samples dehydrated in ethyl-alcohol in ascending concentration, then cleared in xylene and embedded in paraffin wax. Cut the section in microtome (6) µm, processed and stained with haematoxylin and eosin according to [11].

Antimicrobial susceptibility testing

Antimicrobial susceptibility test for isolated colony by Kirby-Bauer disc diffusion method according to the CLSI guidelines was carried out [11].

Statistical analysis

The data was analyzed using Chi- square analysis (x2) to know the percentage of bacterial isolates at 5% probability level[13].

Results

Bacterial isolation

The use of three types of samples for the isolation of bacterial causes of pneumonia showed that the lung samples were the best accurate samples, since, 98% of lung samples showed positive culture, while 94% and 88% of nasal and tracheal swabs respectively revealed positive culture (Table 1)

Table1: The positive and negative results for bacterial culture of samples used in the study

Samples						results
%	Lungs Samples	**%**	Tracheal Samples	**%**	Nasal swabs	
98	49	88	44	94	47	Positive results
2	1	12	6	6	3	Negative results
100	50	100	50	100	50	**Total**

*No Significant differences in the values between positive and negative results from nasal, tracheal and infected lungs at a probability level P< 0.05.

Staphylococcus aureus were responsible of the majority of pneumonia diseases in sheep (Table 2 ,3 and 5). Also it is clear from (tables 1, 2, and 3) that gram negative bacteria specially *Klebsiella pneumonia* and *Escherichia coli* were important causative agents of pneumonia. Mixed infection with more than one bacterial pathogens were common in the current study.

Table 2: Bacterial species isolated from lung samples of sheep with pneumonia

Isolated bacteria	Number of isolates	%
Staphylococcus aureus	16	26.23
Klebsiella pneumonia	12	19.67
Escherichia coli	12	19.67
Streptococcus pneumonia	8	13.11
Proteus mirabilis	6	9.83
Pasteurella multocida	3	4.92
Pseudomonas aeruginosa	3	4.92
Staphylococcus epidermidis	1	1.64
Total isolates	61	100

*37 lung samples with single infection.
*12 lung samples with mixed infection (two bacterial isolates).

Isolate bacteria from healthy lungs
No bacteria were isolated from 10 healthy lungs.

Table 3 : Bacterial species isolated from tracheal samples of sheep with pneumonia

Isolated bacteria	Number of isolates	%
Staphylococcus aureus	14	25
Klebsiella pneumonia	11	19.64
Escherichia coli	10	17.86
Proteus mirabilis	6	10.71
Staphylococcus epidermidis	5	8.93
Streptococcus pneumonia	4	7.14
Pasteurella multocida	3	5.36
Pseudomonas aeruginosa	3	5.36
Total isolates	56	100

*32sheep diseased with single infection&12 sheep diseased with mixed infection

Isolation of bacteria from the10 tracheal samples of apparently healthy lungs
Two types of bacteria were isolated from 10 healthy tracheal swabs(*Streptococcus pneumonia* and *Staphylococcus epidermidis*). (Table 4)

Table 4: Prevalence rate of bacterial isolates from trachea from apparently 10 healthy sheep

Isolated bacteria	**Number of isolates**	**%**
Staphylococcus epidermidis	2	66.67
Streptococcus pneumonia	1	33.33
Total isolates	3	100

Table 5 :Isolation of bacteria from nasal swabs taken from sheep suffering from respiratory infections

Isolated bacteria	**Number of isolates**	**%**
Staphylococcus aureus	17	26.98
Klebsiella pneumonia	12	19.05
Proteus mirabilis	9	14.28
Escherichia coli	8	12.69
Staphylococcus epidermidis	6	9.52
Streptococcus pneumonia	4	6.35
Pasteurella multocida	4	6.35
Pseudomonas aeruginosa	3	4.76
Total isolates	63	100

*31 sheep diseased with single infection&16 sheep diseased with mixed infection

Isolation of bacteria from 10 nasal swaps of apparently healthy sheep
4 types of bacteria were isolated from 10 nasal swabs of sheep without respiratory infection (Table 6).

Table 6: Prevalence rate of bacterial isolates from 10 nasal swaps from apparently healthy sheep

Isolated bacteria	**Number of isolates**	**%**
Staphylococcus epidermidis	4	57.13
Escherichia coli	1	14.29
Pseudomonas aeruginosa	1	14.29
Streptococcus pneumonia	1	14.29
Total isolates	7	100

Fig1: *Staphylococcus aureus* golden yellow pigment on MSA

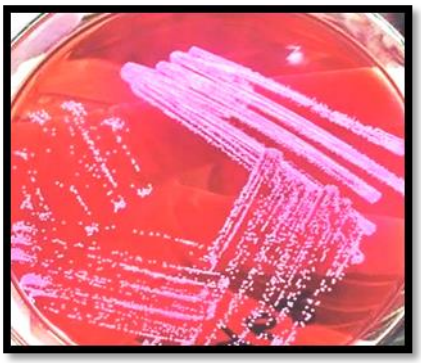

Fig 2: *Staph.epidermidis* on the macconky agar

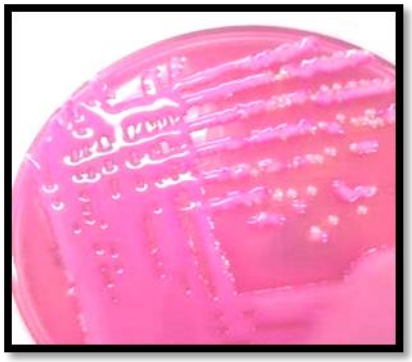

Fig. 3: *Klebsiella* on the macconky agar

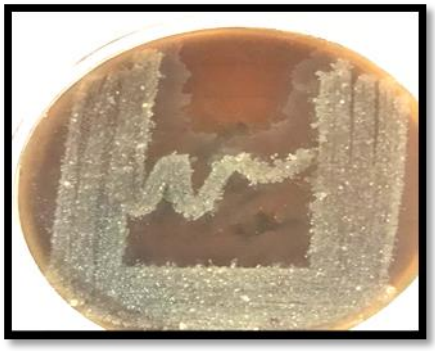

Fig 4:*Streptococcus pneumonia* with pinpoint shape on blood agar

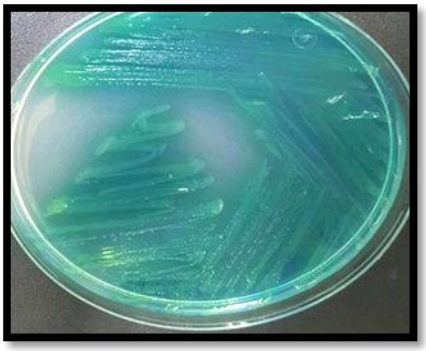

Fig. 5: *Pseudomonas auerginosa* on nutrient agar

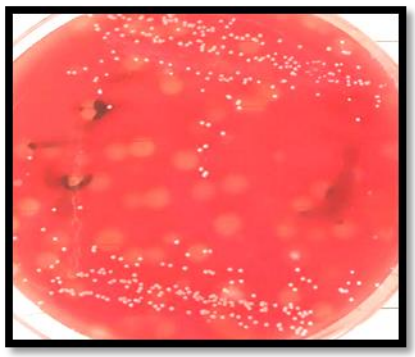

Fig6:*Pasteurella* on blood agar

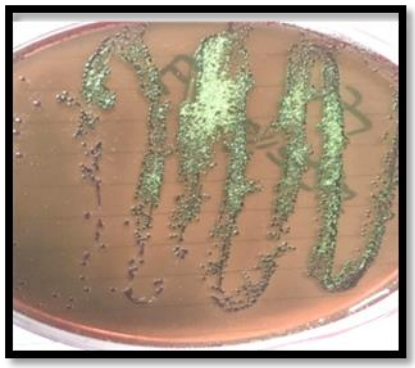

Fig7:*E.coli* showing metallic sheen on EMB agar

Fig8: *Proteus* with brown pigent on macconky agar

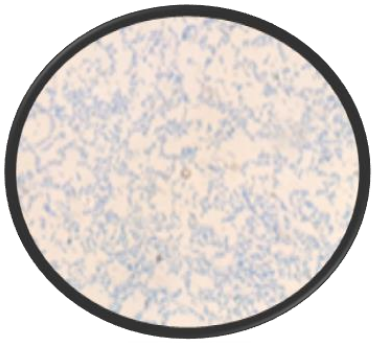

Fig. 9: *Pasterella spp* (Bipolar Staining) stained with Methylene blue

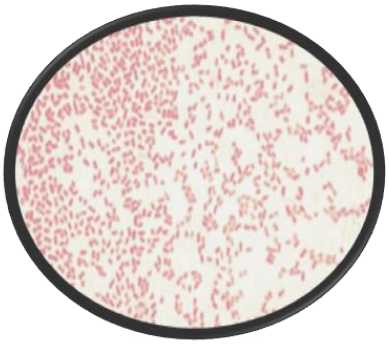

Fig. 10: *Pseudomonas aeruginosa* stained with Gram stain and examined under a light microscope

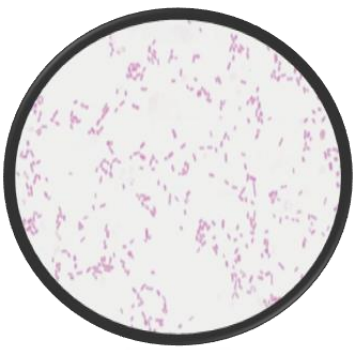

Fig11:*Klebsiella pneumonia* stained with Gram stain and examined under a light microscope

Biochemical tests

Biochemical tests were done for the purpose of confirming germs diagnosis, as shown in (Table 7)

Table 7 : Biochemical properties of bacteria isolated from Respiratory passages

Tests / Bact	Indole	Catalase	Oxidase	coagulase	Urea	T.S.I*	Motility
E.coli	+v	+v	-v	-v	-v	Y Y	+v
Pasteurella multocida	-v	+v	+v	-v	-v	R R	-v
Klebsiella pneumonia	-v	+v	-v	-v	+v	Y Y	-v
Proteus mirabilis	-v	+v	-v	-v	+v	R Y	+v
Pseudomonas aeruginosa	-v	+v	+v	-v	-v	/	+v
Streptococcus pneumonia	-v	-v	-v	-v	-v	/	-v
Staph.aureus	-v	+v	-v	+v	+v	/	-v
Staph.epidermidis	-v	+v	-v	-v	-v	/	-v

* T.S.I Triple Sugar Iron Agar

Antimicrobial Sensitivity test

The sensitivity test was performed on the isolated germs using 7 different types of antimicrobials (**Table8).** All bacterial species were sensitive to chloramphenicol, while the germs showed varying sensitivity to the other antibiotics used.

Table 8 : Antimicrobial Sensitivity test for isolated bacterial

antibacterial / Bacteria	*E.coli*	*staph.aureus*	*Streptococcus pneumonia*	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Klebsiella pneumonia*	*Pasteurella multocida*
Chloramphenicol	S	S	S	S	S	S	S
Tetracyclin	R	S	I	R	R	S	S
Penicillin	R	S	R	R	R	R	R
Erythromycin	R	R	R	R	R	R	S
Aztreonam	S	R	R	S	S	S	R
Ampicillin	R	S	R	S	R	R	R
Trimethoprim/ Sulphamethoxazol	S	S	S	S	S	S	R

Macroscopic examination

A total of 50 lungs of sheep was examined macroscopically ,the lungs showed different lesions: the prominent lesion in most lung samples was congestion and presence of emphysema with an expansion of the bronchitis , mucosal exudate in the bronchi and in some lungs noticed thickening of the pleura with the presence of adhesions between the pleura and the ribs. These lesions were seen mostly in the apical lobes of the lung, and the hepatization was observed in some lungs.

Table 8: Gross appearance of lung lesions.

Gross lesions	Lungs affected numbers	%
Hemorrhage & congestion of the trachea and bronchi	18	36
Emphysema	12	24
expansion of the bronchitis	9	18
adhesion between the pleura and the chest wall	7	14
lung hepatization	4	8

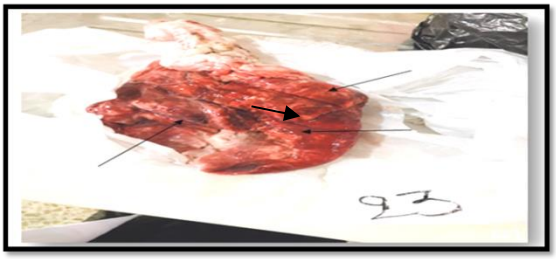

Fig12:Lung of a sheep afflicted with pneumonia, noting the lesions represented by red hepatization in its different stages

Fig13:adherence of the lung to the chest wall completely

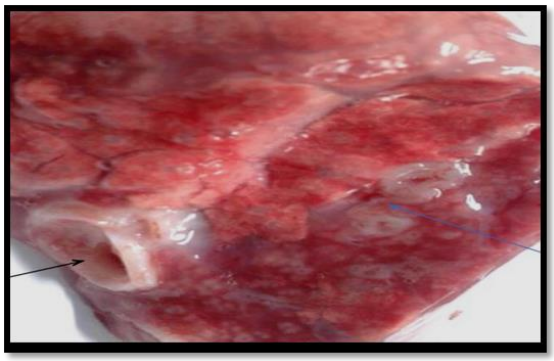

Fig13: Fibrin deposition between lobules and expansion of the bronchitis

Histopathological changes of lung

Histopathological examination of lungs revealed Alveolar emphysema, twisted alveolar walls observed With shedding of some cells that lining the alveolar cavity and the interstitial tissue between the alveoli contained fibrinous edema Fig(14).

Shedding of the epithelial cells lining the trachea was clearly visible and its presence within the bronchial lumen in the form of complete and curved stripe .The walls of these bronchioles are surrounded by infiltration of inflammatory white blood cells. Thickening the walls of the peripheral pulmonary blood vessels has also been observed Fig(15).

bronchiolar lumen contained epithelial cell debris and mucus. Also, the bronchiolar epithelium has degenerates some of their cells. Its walls are surrounded by dense fibrous tissue infiltrated by lymphocytes and some macrophages surrounding the walls of the alveoli as well Fig(16).

The pulmonary bronchi showed pseudostratified ciliated columnar epithelium shedding. Thickening the fibers of the interstitial tissue was also observed, The blood vessels contained Congested and hemolyzed blood Fig(17).

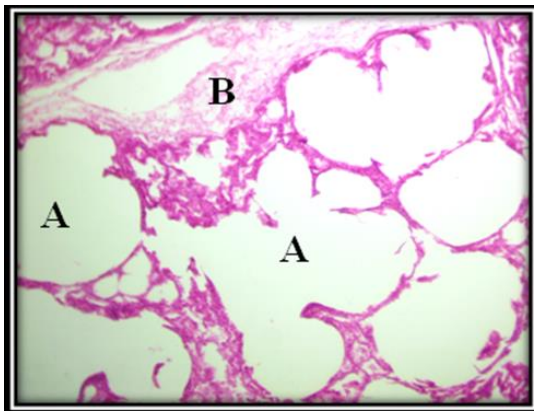

Fig14:A-Alveolar emphysema and twisted alveoli. B-fibrinous edema in lung tissue (H&E; 40X).

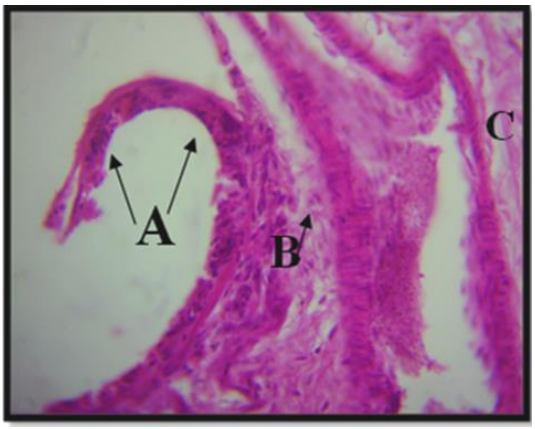

Fig15: A- Shedding of the epithelial cells lining the trachea. B- infiltration of inflammatory white blood cells. C- Thickening the walls of the blood vessels (H&E; 40X).

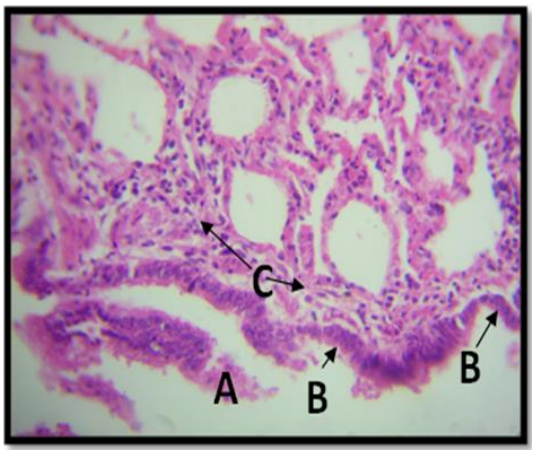

Fig. 16: A- bronchiolar lumen contained epithelial cell debris and mucus. B- degeneration the cell of bronchiolar epithelium. C- infiltration of white blood cells(H&E; 40X).

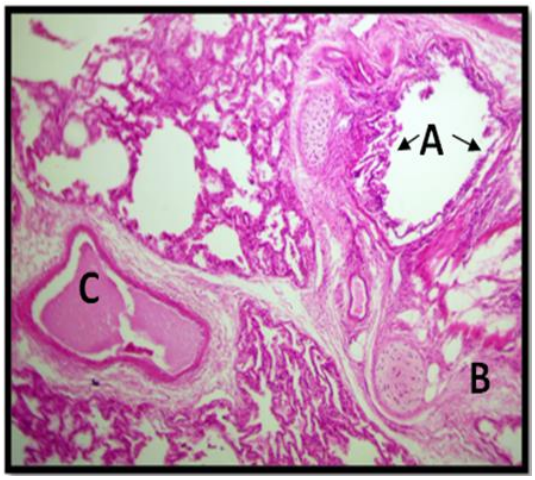

Fig17: A- pseudostratified ciliated columnar epithelium shedding. B- Thickening the fibers of the interstitial tissue. C- hemolyzed blood in a blood vessel (H&E; 40X).

Discussion

Sheep play a vital economic role as they are raised mainly for lamb production, followed by wool and milk for large section of population especially in village and desert areas, Thus they can support the survival of millions of people in many countries all over the world including Iraq[14]. Respiratory disorders are still serious problem facing sheep raring, The importance of respiratory diseases of sheep depends on their prevalence, their effect on productivity, the value of the animal and for some diseases, their international spread [14].

In this study, bacteriological examination of 30 samples(10 nasal swabs,10 tracheal samples and 10 lung samples) from apparently healthy, and 150 samples(50 nasal swabs,50 tracheal samples and 50 lung samples) from diseased sheep was carried out.

The results revealed the presence of many bacterial species as single or mixed isolates, *Staphylococcus aureus* were the most predominant bacteria isolated at a percentage of 26.23% in examined lungs and 26.98%, 25% in nasal and tracheal swabs respectively, and this result agree with the researcher [15] ,Where the highest isolation rate was for Staphylococcus aureus, who said that the increase in the incidence of infection with Staphylococcus aureus bacteria is due to the pathogenic characteristics and virulence factors for these bacteria that enhance the formation of colonies in the host's tissues, which facilitates their spread In the tissue through the inhibition of defense cells. But this result not agree with the study of [9] ,As it is isolated at the lowest rate compared to the rest of the bacterial causes, He explained the role of bacteria that found in the respiratory tract that it is not necessary to cause disease, but it may actively Involved in the inflammation, and this is what was observed in the variation in the rates of isolation of germs from the samples

Other bacteria were isolated in varying rate from the affected lungs. These bacteria are *Klebsiella pneumonia* and *Escherichia coli* by 19.67%, *Streptococcus pneumonia* 13.11%, *Proteus mirabilis* 9.83%, *Pasteurella multocida* and *Pseudomonas aeruginosa* were 4.92% as in (Table2). finally *Staphylococcus epidermidis* was 1.64%, and this is what [2,3] and [15] where they indicated the possibility of isolating many bacteria, in varying rate, from infected lungs in sheep.

This differs with [10] which the results of his study showed that *Pasteurella multucida* is the highest percentage, followed by *Pasteurella haemolytica*.

The results of the bacterial isolation from tracheal swabs that taken from sheep suffering from pneumonia also showed that *Klebsiella pneumonia* rate 19.64% which is the second highest percentage after *Staphylococcus aureus*, then *Escherichia coli* bacteria was 17.86%, *Proteus mirabilis* 10.71%, *Staphylococcus epidermidis* 8.93% then *Streptococcus pneumonia* 7.14% and the lowest percentage was *Pseudomonas aeruginosa* and *Pasteurella multucida* 5.36% as in (Table3).

These results agree with [16],[17] where *Klebsiella pneumonia* showed a high percentage, as it was isolated by 48%, which is the most common. The difference of bacterial isolation might be due to sample size, the presence of contamination sources, as well as pathogenic properties and virulence factors of these bacteria, which enhance the formation of colonies in the host tissue, which facilitates their spread in the tissue by inhibiting the defense cells[2].

The results of bacterial isolation of nasal swabs taken from sheep suffering from respiratory signs showed the presence of different types of bacteria .The *Klebsiella pneumonia* bacteria formed the second highest percentage after *Staphylococcus aureus*, it reached 19.05%, followed by *Proteus mirabilis* 14.28%, *Escherichia coli* 12.69%, then *Staphylococcus epidermidis* 9.52%, *Streptococcus pneumonia* and *Pasteurella multocida* 6.35%, finally *Pseudomonas aeruginosa* percentage 4.76% (table 5).

Similar results more or less were reported by previous study [18], where the rate of *Escherichia coli* reached 20.78%, while other bacteria formed different proportions of *Pseudomonas aeruginosa* 11.76%, *Pasteorella haemolytica*and the *Pasteorella multocida* were 2.35% and 4.31%, respectively The differences between the records were mainly due to the geographical distribution at which the investigator was adopted.

And these results differ with the results of [5] the results of his study show that *Pasteurella spp*. are the most important causes of pneumonia in sheep, in which the infection rate reached 41.6% in nasal swabs.

The indiscriminate use of antibiotics leads to high rate of resistant pathogenic germs infection, and in the event that the infection is transmitted from animal to human, it exposes the person to the risk of disease and as a result of the indiscriminate use of antibiotics complicates the treatment of human diseases[19].

study the sensitivity of the isolated strains to antibiotics and sulfa compounds that we determines the most effective antibiotic for the proposed and effective treatment.

Through the results of this work, it is clear that all the isolated strains were very sensitive to Chloramphenicol, and most of them were sensitive to Trimethoprim/ Sulphamethoxazol, as shown in (Table 8).

The effectiveness of these antibiotics was clear and significant towards the isolated bacteria, and this is agree with the findings of the researchers [19] and [20] where they referred to the sensitivity of the germs toward antibiotics, especially chloramphenicol, ciprofloxacin and trimetheprim / sulfamethoxazole, the sensitivity rate was 100%.

The isolated germs varying in sensitivity to the rest of the antibiotics, such as Tetracyclin, Aztreonam, and Ampicillin, this agree with the studies [21], which confirmed the effectiveness of tetracycline, oxytetracycline and ampicillin against *E.coli* and *Klebseilla spp*. And *Pseudo. aerogenosa* , *Pasteurella spp*. ,*Streptococcus* and *Proteus* by inhibiting the bacterial DNA gyrase type 11 topoisomerase which is necessary during the stationary phases of the bacterial growth.

Most germs showed resistance to penicillin and erythromycin, as the results of the study showed that all germs were resistant to penicillin except for *Staphylococcus aureus*, and all germs were resistant to erythromycin except for *Pasteurella multucida*, and this differs with[19], which indicated the effectiveness of erythromycin in sheep against pneumonia.

The gross lesions of 50 infected lungs varied in the rates, with a variation in the rates of germs isolation from these lesions, Where the prominent lesion in most lung samples was a large abscess in the lungs with congestion of the trachea and bronchi 44% from the examined lungs, while the other lesions included the presence of pulmonary emphysema 36%, and expansion of the bronchi with the presence of mucous exudation in the bronchi by 32% , and thickening of the pleura in some lungs with the presence of adhesions between the pleura and the ribs 28%, and the observation of red hepatization in all its stages 20%, and these lesions were seen mostly in the apical lobes of the lung

This is agree [1], which stated that most lesions were in the upper parts of the lung, and this may be attributed to the anatomical region of the lung, as the branches of the trachea enter first in the frontal lobes, so these lobes are more vulnerable to infection then after that transmitted to the rest of the lobes [1].

The histopathological study that we conducted showed the presence of different types and degrees of pneumonia, which included: fibrous bronchopulmonary pneumonia, interstitial bronchopulmonary and lymphoproliferative pneumonia and proliferative pneumonia with lung abscesses and purulent bronchopulmonary pneumonia, as was the presence of necrotizing bronchiolitis, which is characterized by the presence of necrosis In the epithelium lining the mucous layer of the trachea, it is accompanied by thickening of the alveolar wall due to its infiltration of single-core and multi-core inflammatory cells, as well as congestion in the blood vessels between the pulmonary alveoli and fibrous hyperplasia, and this studey agree with [3].

Conclusion

1. *Staphylococcus aureus* was dominant bacteria isolated from the lung abscesses of the sheep.
2. The results showed the sensitivity of the isolated bacteria to the antibiotic chloramphenicol, followed by Trimethoprim / sulphamethoxazol. The results of the study also showed the bacteria's resistance to the antibiotic penicillin and Erythromycin.
3. It was found from the anatomical characteristic of the affected sheep the most common type of pneumonia is broncho pneumonia were manifested by the presence of exudate in the pulmonary alveoli, hemorrhage, blood vessel congestion

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دراسة بكتريولوجية ونسيجية لالتهاب الرئة عند الأغنام في محافظة صلاح الدين

نور وليد صالح يوسف ، نهاد عبد الحسين جعفر ، بدر ختلان حميد

كلية الطب البيطري ، جامعة تكريت ، تكريت ، العراق

الملخص

هدفت هذه الدراسة إلى عزل وتشخيص مسببات الأمراض البكتيرية المسببة للالتهاب الرئوي في الأغنام، ودراسة التغيرات البيوكيميائية والنسيجية في أنسجة الرئة للحيوانات المصابة وتحديد أكثر أنواع المضادات الحيوية فعالية في علاج الأغنام المصابة بالالتهاب الرئوي. تم جمع 150 عينة (50 مسحة أنف ، 50 عينة من القصبة الهوائية، 50 عينة رئة من الأغنام المذبوحة) بشكل عشوائي من مناطق مختلفة من محافظة صلاح الدين خلال الفترة (سبتمبر 2019 حتى يناير 2020). أظهرت النتائج عدم وجود فرق معنوي للنتائج الموجبة والسالبة للمسحات الانفية والرغامي والرئات المصابة عند مستوى الاحتمالية $P< 0.05$. كما بينت النتائج ان بكتريا المكورات العنقودية الذهبية والكلبسيلا الرئوية هما المسببان المرضيان الرئيسيان لمرض الالتهاب الرئوي في الأغنام في محافظة صلاح الدين, وفي الفحص المجهري ، كانت الآفة البارزة في معظم عينات الرئة هي خراج كبير في الرئتين مع احتقان الرغامي والشعب الهوائية ووجود نفاخ رئوي مع انخماصات رئوية. ووجد أن أكثر أنواع عدوى الالتهاب الرئوي شيوعًا هو الالتهاب الرئوي القصبي.

كما أظهرت جميع الأنواع الجرثومية المعزولة إنها حساسة للمضادات الجرثومية الكلورامفينيكول وتريميثوبريم + سلفاميثواكسازول عدا الباستوريلا فانها كانت مقاومة للمضاد الجرثومي تريميثوبريم + سلفاميثواكسازول.

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