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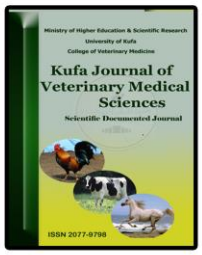

The Role of *Klebsiella Pneumonia* for Effect on Pneumonia in The Sheep

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Abstract

The study investigated the pathology changes associated with *klebsiella pneumonia* in lungs of sheep slaughtered. The objective of this study was focus on molecular detection and genotyping were done by polymerase chain reaction and gel-agarose electrophoresis. The molecular detection of capsular polysaccharide gene (cps) was investigated using PCR specific primers. PCR was performed with the primer that target the *16S rRNA*. PCR was performed with the primer that target the *16S rRNA* . Genotyping of these isolates were also carried out by using K1, specific PCR primers. When using the primer specific for the capsule cluster gene magA (K1serotype). A total of 700 lungs of slaughtered sheep were examined individually and out of which 220/700 (31.4%) affected lungs were collected for histopathology and bacterial isolation, during the period from October 2015 to February 2016. 30 / 220 isolates were identified as *klebsiella pneumonia* (13.6%) ,according to characterization of morphology and biochemical for this microorganism, VITEK 2 system and confirmed by using the primer that target the 16SrRNA. Grossly, the lung lesions were categorized into: (1) solidation of lung (40%) ,(2) hepatization in lung 30% ,(3) abscesses in lung (10%) , (4) edema and fibrosis (10%) and congestion and Edema (10%). In histopathology examination, lung lesions were categorized into: (1) Suppurative bronchopneumonia (36.6 %) ,(2) interstitial pneumonia (30%) ,(3) bronchointerstitial pneumonia (13.3%) , (4)pleuritis with pulmonary edema (10%) and (5) pulmonary abscesses (10%).Special stains were used to verify the lesions. Collagenous fibers were stained red color with Van Gieson's stain, polysaccharide were stained pink color with PAS stain. PCR technique showed that 19 isolates were positive to K1 serotype and 11 isolates were negative for K1. These results suggest that magA genotype might be a useful marker to identify K1 serotypes of K. pneumoniae.

Key word: Pathology, klebseilla pneumonia, lungs ,K1 serotype.

دور الكليبسيلا الرئوية في الالتهابات الرئوية في الاغنام

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الخلاصة:
حققت الدراسة من التغيرات النسيجية المرضية المرتبطة بالتهاب الرئوي للكليبسيلا الرئوية في الرئات الأغنام المجزورة . الهدف من هذه الدراسة كان يتمحور على تحديد النمط الجيني والجزيئي بواسطة تفاعل البلمرة المتسلسل والترحيل الكهربائي. تم تحديد الجزيئي للجينات المحفظة متعددة السكريات للكليبسيلا الرئوية بواسطة برايمرات خاصة وكذلك حدد16SrRNA واستخدام برايمر خاص لجينات المحفظة التجمع العنقودي للجين magA .
الفحص الكلي للرئات الاغنام كان 700 رئة منها كان 200 /700 رئة (31.4%) , الرئات المرضية كانت تجمع للتقطيع النسيجي وللزرع البكتيري خلال الفترة من اوكتوبر 2015 ال فبراير 2016. تم تشخيص 30 / 220 عينة (13.6%)هي كليبسيلا الرئوية , اعتمادا على الشكل المستعمرات في الزرع وكذلك اختبارات الحيوية وجهاز الفايتك . عيانيا صنفت الآفات الرئوية من 30 عينة كالتالي (1) تصلب الرئة (40%) ,(2) تكبد الرئة (30%) , (3) خراجات الرئة (10%) , (4) وذمة مع تليف (10%) , (5) احتقان مع وذمة (10%). في دراسة التغيرات النسيجية ، صنفت آفات الرئة إلى: (1)التهاب القصبي الصديدي (36.6٪)، (2) الالتهاب الرئوي الخلالي (30٪)، (3) الالتهاب الخلالي القصبي الرئوي13.3)٪(، (4) الجنب مع وذمة رئوية (10٪) و (5) الخراجات الرئوية (10٪). هنالك صبغات خاصة استخدمت لمعرفة الآفات . الألياف الكولاجين صبغت باللون الاحمر مع صبغة الفانكيزن, polysaccharidesصبغت باللون الوردي مع صبغة PAS. اظهرت تقنية التفاعل البلمرة المتسلسل 19 عينة هي موجبة للجين المرضي K1 ولكن 11 عينة هي سالبة للجين المرضي K1 ومن هذه النتيجة بان النمط الجيني magA هو يعتبر هوية تعريفية للجين المرضي K1 للكليبسيلا الرئوية.

Introduction:

The ovine respiratory system is frequently exposed to potentially pathogenic organisms , but most sheep remain healthy because of the pulmonary defenses which labor the effective clearance of these organisms. When obtain disruption of the mucociliary mechanism of lung and the pulmonary defenses function is become impaired or pulmonary tissues are damaged, allow the organism to gain entrance to the lower respiratory tract. then the organisms can entrench a effectiveness to initiation of an inflammatory or the disease, and there are many of infectious agents appear due to the interaction such as (viruses ,bacteria, parasite, fungi, host defense and environmental factors (1).

Pneumonia is regarded a respiratory disease originate from an inflammatory reactions of the bronchioles and alveoli in the lung to infective agents and resulting in the consolidation of lung tissue. Pneumonia is caused by a complex interaction, involving interactions between host (physiological and immunological), various agents (viral, bacterial,parasitic and mycoplasmal) ,environmental factors and poor management .It is have several histological forms are mild to severe, acute to chronic, and exudative to proliferative interstitial. (2).

klebsiella pneumonia is belong to the Enterobacteriaceae group and considered as one of the most important opportunistic pathogens that are repeatedly isolation from various infection in humans and animals. Particularly, is an important clinical pathogen that is highly associated with immunosuppression and secondary infection that consider mortality and morbidity. It lives in soil, on plant and as commensal habitant of the mammalian nasopharynx and gastrointestinal tract. *klebsiella pneumonia* found in the mouth, skin and intestines, besides in hospital settings and medical appliances. It is causes a wide multiple of infections including bacterial pneumonia ,urinary tract infection ,septicemia, wound infection , meningitis and purulent abscess at various sites such as liver abscesses (3 and 4). The Capsule of *k.*

pneumonia plays a very important role in virulence and pathogenicity, the determinants of the better characterized virulence of K. pneumoniae include the capsule, lipopolysaccharides, types 1 and 3 fimbriae . Also capsular serotype-specific genes like the magA gene for the K1 serotype and the k2A gene for the K2 serotype, these K1 and K2genes are regarded as predominant virulent and resistant to phagocytosis than non-K1/K2 strains of *K. pneumoniae*. (5 and 6). The *magA* gene was first described in 2004 by Fang *et al.* who reported that hypermucoviscosity and *magA* were more prevalent in invasive strains of *K. pneumoniae* and *magA*-negative mutant strains lost their exopolysaccharide web (7).

Objectives of The Research:

- study the etiology (bacterial) as well as clinicopathological and histopathological changes of pneumonia in the sheep lungs to determine the correlation between the bacterial agent and its pathological characters.
- To investigate the prevalence and pathology of *klebsiella pneumonia* in the sheep,to investigate the macroscopic and microscopic lesions of condemned the sheep lungs.

Materials and Methods:

Samples Collection:

A total of 220 affected lungs out of 700 examined were collected for bacterial isolation .Samples collected, 220 specimens taken from the different lobes of the affected lungs were the sheep different ages, sexes and breeds . Lungs apparently affected in the lesion which divided into two portions: One portion was fixed in 10% neutral buffered formalin for histopathological examination ; The other portion ,these lungs were placed in sterile plastic bags and transported within little from two hours under cooled conditions to laboratory for bacteriological examination (8 and 1).

Isolation and Identification of *K. pneumonia*:

Samples were cultured on MacConky's agar plate (Oxoid) and cultured plates were incubated overnight at 37 C˚. After incubation suspected *Klebsiella* colonies were selected and stored in pure form for further identification.

A number of morphological, physiological and biochemical tests were performed for identifying the bacterial isolates as recommended by (9). The isolates were confirmed for VITEK 2 system were also used as a confirmation of characterization.

plasmid DNA Extraction and Purification:

It has been prepared according to recommendation company product (Bosphore).

PCR Amplification

Primer name		Sequences (5́ -3́)	size (bp)	Reference
16S rRNA	F	ATT TGA AGA GGT TGC AAA CGA T	130	(Turton et al., 2010)
	R	TTC ACT CTG AAG TTT TCT TGT GTT C		
K1	F	GGTGCTCTTTACATCATTGC	1283	(Fang et al., 2004)
	R	GCAATGGCCATTTGCGTTAG		

Results And Discussion:

Pathological results:

Prevalence:

Study on bacteriological and pathological investigation of lungs sheep was conducted at examined in different slaughters, during the period from October 2015 to February 2016. A total from 700 lungs were examined, 220 (31.4%) had one or more gross lesions appeared pneumonia . The lungs with macroscopic pneumonic lesions were obtained (the lesion showing parasitic lesions were excluded). 220 lungs lesion were found to be apparently abnormal in naked eye to were subjected to bacteriological and histopathological study .

In the present study, 220 sample are subject to bacterial isolates from the lung lobes randomly, 85/220 (38.6%) were culture positive for gram negative bacteria , only 30/85 (35.2%) klebsiella pneumonia isolations . 30 / 220 isolates (13.6%) were identified as *klebsiella pneumonia*.

the clinical signs of respiratory infections of sheep:

The common clinical signs were shallow rapid respiration ,dyspnea, nasal discharge (purulent and thick mucopurulent may be tinged with blood, redness of ocular mucous membrane and conjunctiva, crusts around nasal orifice, intermittent cough with moist and dry cough, depression and anorexia compared with apparently healthy sheep.

Gross lesions examination of the lungs:

The gross lesions were observed distribution various in the lung lobs and between right and left lungs. Grossly lesions (Table1) were categorized into following types: (1) solidation of lung 40% ,(2) hepatization 30% (3) abscesses in lung 10%, (4) edema and fibrosis 10% and congestion and edema 10%.

Table1: Gross pathology of lungs sheep (N = 30)

Lung lesions		**No. of lung affected**	**Affected(%)**
1	Solidation of lung	12	40
2	Hepatization in lung	9	30
3	Abscesses in lung	3	10
4	Edema and Fibrosis	3	10
5	Congestion and Edema	3	10

Histopathological changes:

Different types of pneumonia were observed in histopathology examination of lung tissues, the prevalence of lung lesions (Table 2) was categorized into: (1) suppurative bronchopneumonia (36.6 %) ,(2) interstitial pneumonia (30%) ,(3) bronchointerstitial pneumonia (13.3%) , (4)pleuritis with pulmonary edema (10%), (5) pulmonary abscesses (10%).

Table2: Histopathological findings and most frequent isolates of 30 pneumonic lungs sheep

Lung lesions		No. of lung affected	Affected(%)
1	Supportive bronchopneumonia	11	36.6
2	Interstitial pneumonia	9	30
3	Bronchointerstitial pneumonia	4	13.3
4	Pleuritis with pulmonary edema	3	10
5	Pulmonary abscesses	3	10

(1) Suppurative bronchopneumonia:

A total of 11/30 percentage 36.6% Suppurative bronchopneumonia was the most commonly affected with frequency. Distribution of bronchopneumonia in different lung lobes. Grossly affected lungs were characterised by irregular consolidations of their cranio-ventral regions with present congestion . The pulmonary parenchyma was firm in texture. The consolidated lungs varied from dark red in acute to grey-pink and grey in chronic form (figure 1) . In the acute phase, the cut surface of the consolidated lobules appeared moist and purulent exudate leaked from small airways .In the chronic phase, abscesses of different sizes, with fibrous capsules surrounding them, were observed in the affected lobes. Histologically, present various amounts of cell debris, mucus, fibrin, neutrophils, macrophages in the alveolar spaces and lumens of the bronchioles and bronchi. In severe cases, fluid or purulent exudates completely filled the entire lumen of alveoli and bronchioles(figures2). complete or partial obstruction of the airways some of the lobules showed atelectasis and/or emphysema.Varying degrees of bronchiolar lymphatic tissue hyperplasia was another common finding. In some cases, extensive peribronchiloar lymphoid accumulation narrowed the bronchiolar Lumina.

(2) Interstitial pneumonia:

A total of 9/30 (30%) showed interstitial pneumonia. Grossly, the lungs affected with interstitial pneumonia had diffused lesion distribution often with more severe involvement of dorsocaudal regions of the lungs . There are no signs of evidence of exudates could be detected in cut surfaces and air passages.The affected lungs had dark-red to pale and meaty in appearance, heavy and

rubbery in consistency (figures 3). The affected lung failed to collapse if pressed .Note some of the lungs affected with interstitial pneumonia, rib impressions were seen on the costal surfaces of the diaphragmatic lobes . Histologically, lungs with interstitial pneumonias showed thickened interstitial tissues because infiltration of lymphocytes, macrophages and plasma cells also present severe inflammatory cell infiltration in the alveolar septa and perivascular region (figures 4) , the alveolar lumen contained past positive material properly mucin (neutralism polysaccharide) (figure 5). The present collagen fibers in the alveolar septa .Hyperplasia of pneumocyte type II was seen.

(3) Bronchointerstitial pneumonia:

The prevalence was recorded 4/30 (13.3%) .Grossly, The affected lungs were diffuse red, wet, and failed to collapse, the affected lungs showed red consolidation (figure 6) . Histopathologic findings revealed mixed characteristic features of suppurative bronchopneumonia and interstitial pneumonia. The bronchi ,bronchioles and alveoli lumen filled with cellular debris and exudate due to bronchopneumonia . The inflammatory infiltrate consisted of numerous neutrophils, macrophages, lymphocytes, and plasma cells in peribronchi, peribronchioles and alveoli septea. The present positive collagen fibers in alveolar septa (figure 7).

(4) Pleuritis with pulmonary edema:

The prevalence was recorded 10% (3/30),also often seen in sections of chronic pneumonia. Grossly, the affected lungs were distended (non-collapsed when thorax was opened), wet, relatively heavy, the interlobular septae were notably distended and foamy fluid was coming out when the tissues were incised (figures 8).Fibrinous exudate cover the surface of the right lung .

Histologically, the alveoli were filled with acidophilic edema fluid and neighboring alveolar septae were congested and condensed and also rarely emphysematous alveoli were seen in the examined tissue sections, appear inflammatory cells infiltration (figure9). Sever Pleural fibrosis (the collagenous fibers reacted with Van Gieson's stain in red color), (figure10).

(5) Pulmonary abscesses:

In this study, the prevalence of pulmonary abscess 3/30(10%) was considered without concurrent bronchopneumonia. The abscesses occurred as single or sometimes multiple instances in one or more lobes.

Some of them were very large and involved an entire pulmonary lobe. Lung abscesses containing viscous white-yellow odourless pus were found in the affected lung and mediastinal lymph nodes (figure 11). Chronic abscesses were often surrounded by reactive fibrous walls. Histologically, necrotic pneumonia (necrosis, oedema, thickening in the alveoli and leukocytic infiltration dominantly with neutrophils) (figure 12).

Bacterial results:

Morphological and Cultural Characteristics:

The bacterial isolates were from the lung deep tissues identified their appearance on specific media (the MacConkey agar).The characters of the bacterial colonies grown on MacConkey agar were studied; the lactose fermenter have been taken in consideration, since *Klebsiella pneumoniae* . typically produced large, rounded, mucoid (due to thick

polysaccharide capsule) and pink color colonies on MacConkey agar (figures 13). In addition single pure isolated colony was transferred to nutrient agar medium (figure 14).

Identification by using ViTEk2 system:

Complete identification by biochemical profile with use VITEK2 compact system for the purpose diagnosis of *Klebsiella pneumoniae* subspecies *pneumoniae* and for the purpose to make sure final diagnosis of the bacteria with accura isolation up 98% after the diagnosis, the number of isolates are 30 after been 35 isolations are not pure.

Prevalence of *magA* gene(K1 capsular serotypes):

The prevalence of *magA* gene and their association with capsular serotype. Result showed that capsular serotypes K1(*magA gene*) appear 63.3% (19 of 30 isolates) and non-K1/ K2 appear 36.6% (11 of 30 isolates) table 4.

Table 4: Prevalence of *magA* gene in relation to serotype in *Klebsiella pneumoniae* isolates causing ovine pneumoniae and pulmonary abscesses.

Type lesions	Isolates, no 30. (%)	Prevalence (K1)*magA* isolates
Supportive bronchopneumonia	11 (36.6%)	6
Interstitial pneumonia	9 (30%)	5
Bronchointerstitial pneumonia	4 (13.3%)	2
Pleuritis with pulmonary edema	3 (10%)	3
Pulmonary abscesses	3 (10%)	3
Total	30 (100%)	19 (63.3%)

Molecular Detection *16S rRNA*, *magA* gene by PCR:

In order to molecular typing of *K. pneumonia* isolates DNA was extracted from all isolates. Results showed that the recorded range of DNA concentration was 20.9-164.1 ng/µl and the DNA purity was 1.4-2.19 . The obtained quantities and purity of DNA are fair enough for amplification by

PCR. Higher amounts of DNA template increase the risk of generating of Non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification.

All isolates were subjected to molecular identification through PCR amplification of *16S rRNA* using K 16S-F and K 16S-R primers which represents specific primers for the PCR amplification of *K. pneumonia 16S rRNA.* Results showed that the amplified fragments were 130 bp in size as shown in (figure 16), which the same size is obtained by (10) when they used the same primer. 30/33 isolates gave positive results (130 bp bands), and identified as *K pneumoniae* ,but (3) isolates gave negative results. Results of PCR amplification confirmed that (30) isolates were *K pneumoniae.*

K. pneumoniae serotype K1 was diagnosed with PCR by using a primer pair magA-F and magA-R specific for amplification *magA* gene. Thirty *K. pneumoniae* isolates were subjected to amplification using this primer, 19 isolate (63.3%) was positive for *mag*A gene. These results demonstrated that these pathogenic (19 isolates) have a K1 serotype ,(figure 15) shows that PCR product was 1283 bp in size, which is the same size obtained by (11) when they used the same primer.

Figure 1: Macroscopic appearance of bronchopneumonia. Dark red consolidation had variable appearance from dark-red to grey.

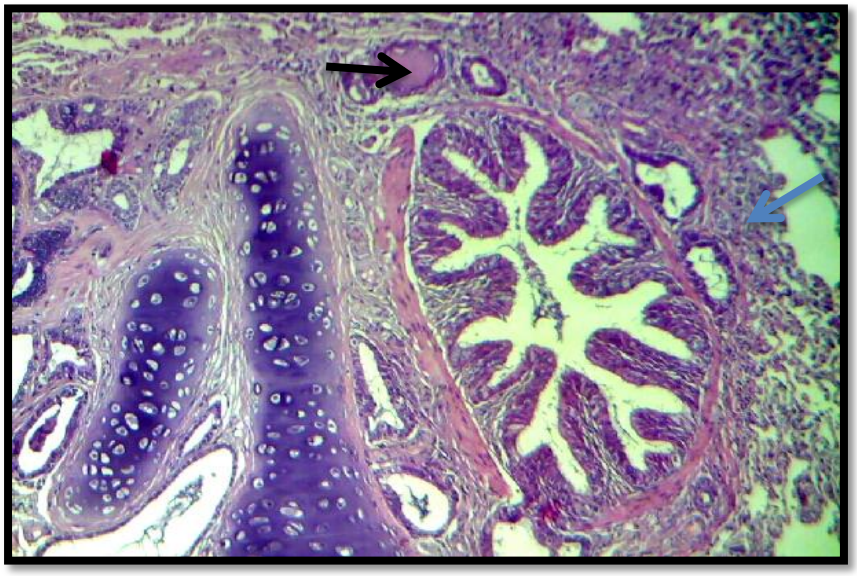

Figure2: Infection of the sheep lungs show bronchopneumonia with → aggregation of inflammatory cells in bronchus, peribronchiolar and bronchiolar and → clear edema in lumen bronchiolar(H&E,X10).

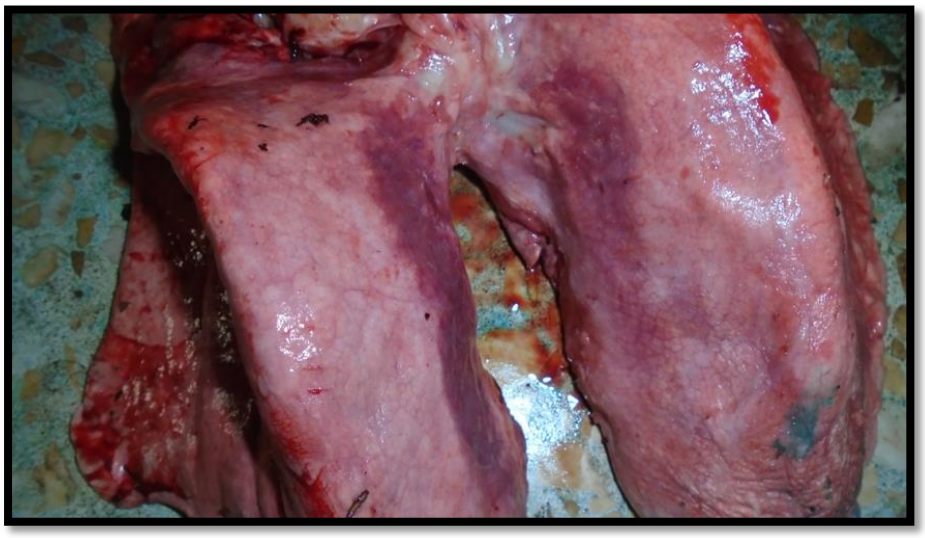

Figure 3: Diffuse lung hepatization of the caudal lung lobes interpreted as interstitial pneumonia with meaty appearance.

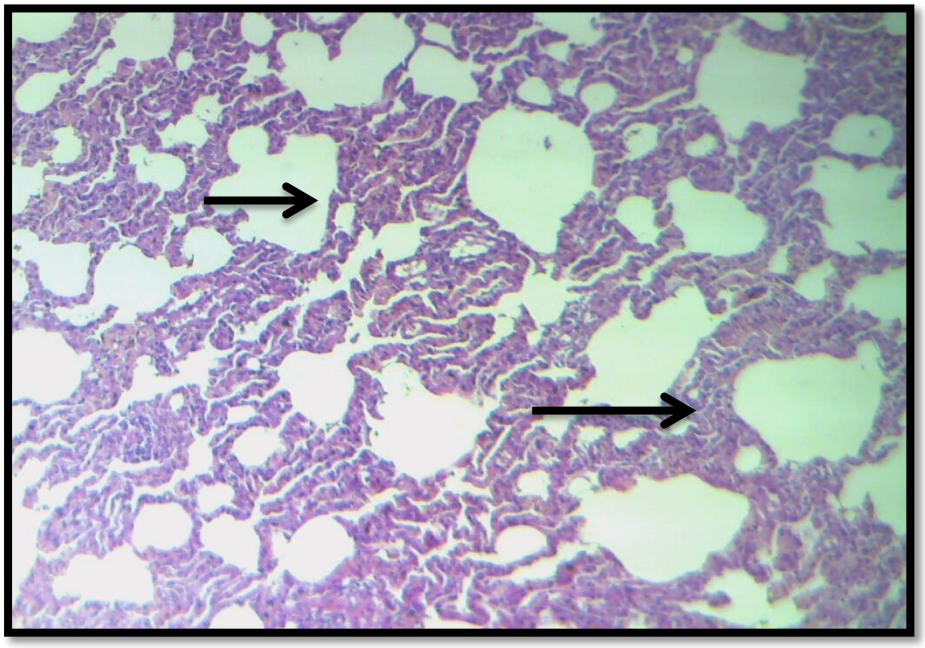

figure 4: Infection of the sheep lungs show lungs showing interstitial pneumonia characterized by ——→ thick alveolar septa due to cellular proliferation and interstitial infiltrations of lymphocytes and macrophages into the interalveolar spaces and with narrow bronchiolar lumen (H&E, X10).

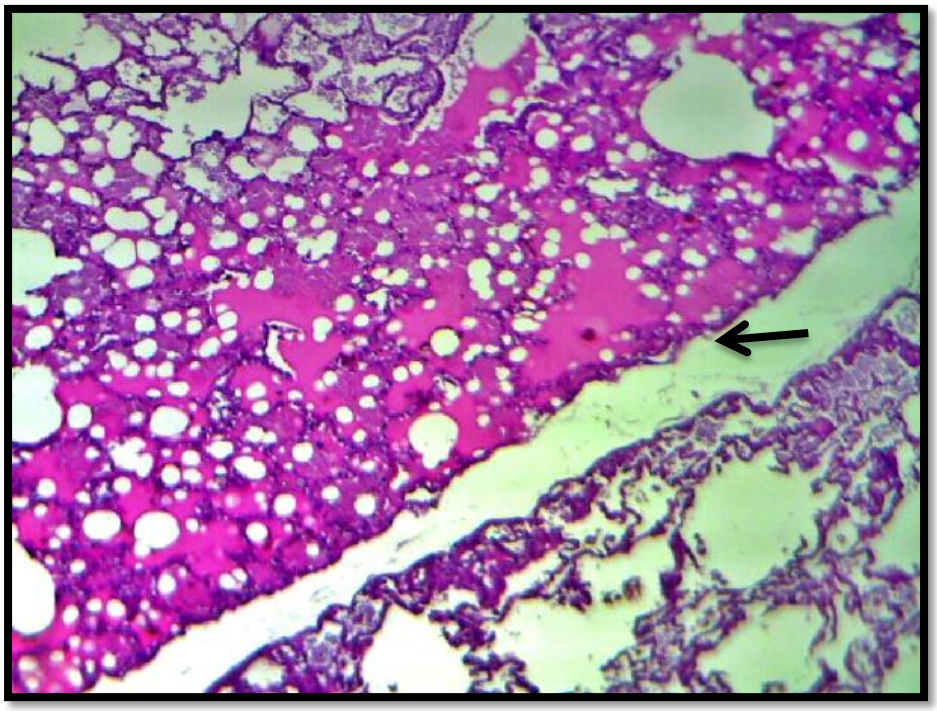

Figure 5: Infection of the sheep lungs show interstitial pneumonia characterized by ——→ alveolar edema with pas positive material and condensed alveolar septae (PAS stain,X10).

Figure 6: Hepatization with Rib impressions were on left lung appeared as Bronchointerstitial pneumonia.

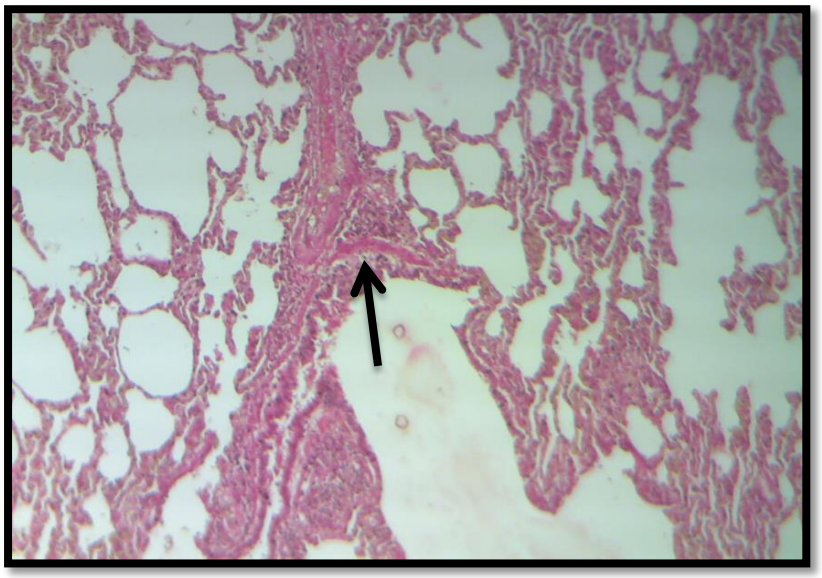

Figure 7: Infection of the sheep lungs show bronchointerstitial pneumonia characterized by ⟶ peribronchiolar and alveolar septa positive collagen fibers ,present exudate in bronchioles lumen (Van Gieson's stain ,X10).

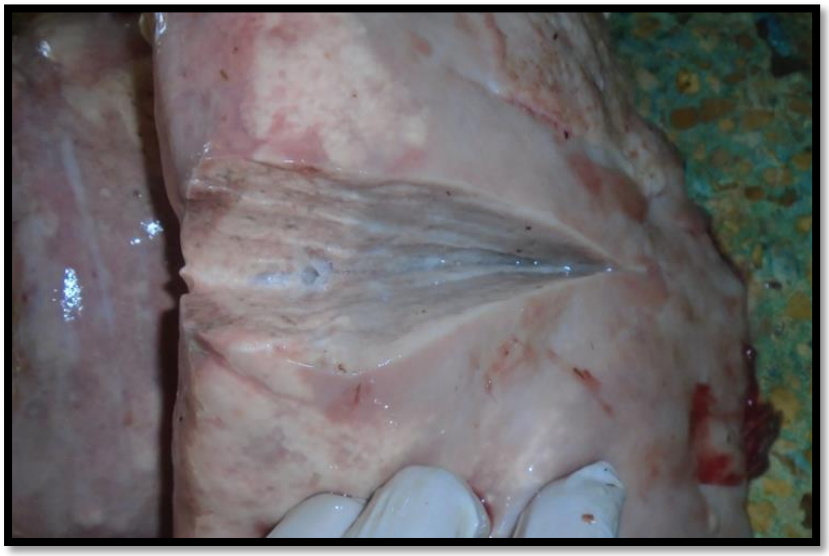

Figure 8: Foamy fluid oozed out from odematous lung and distended interlobular septae.

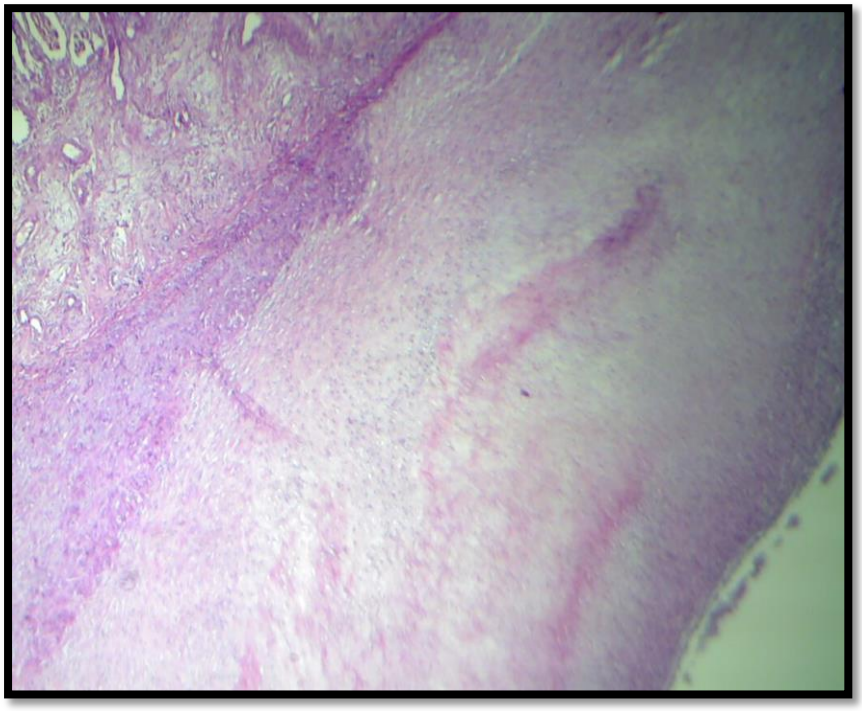

Figure 9: Infection of the sheep lungs show sever Pleural fibrosis (H&E, X 4).

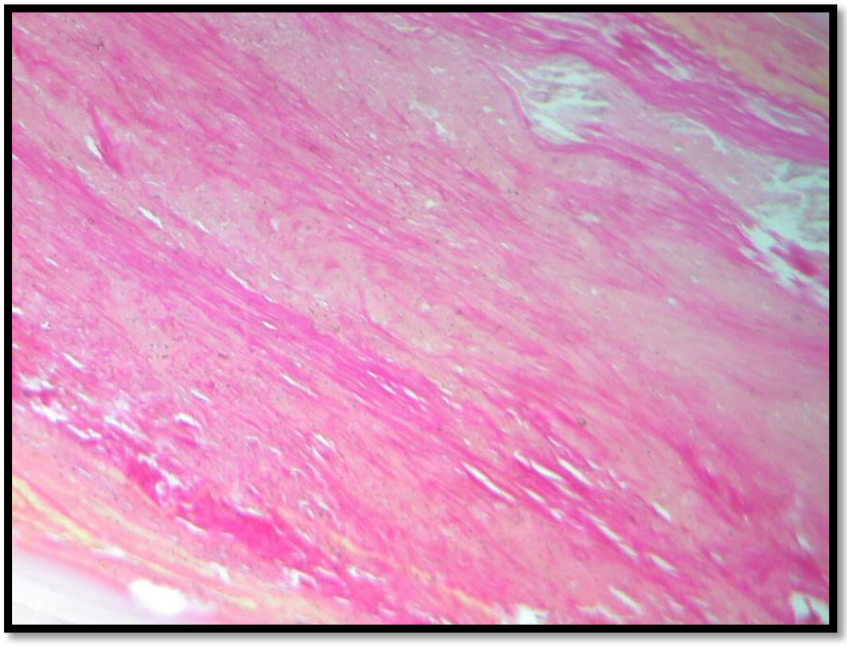

Figure 10: Infection of the sheep lungs show pleuritis appear the collagenous fibers reacted with Van Gieson's stain in red color (Van Gieson's stain , X 10).

Figure 11: Abscesses in lung.

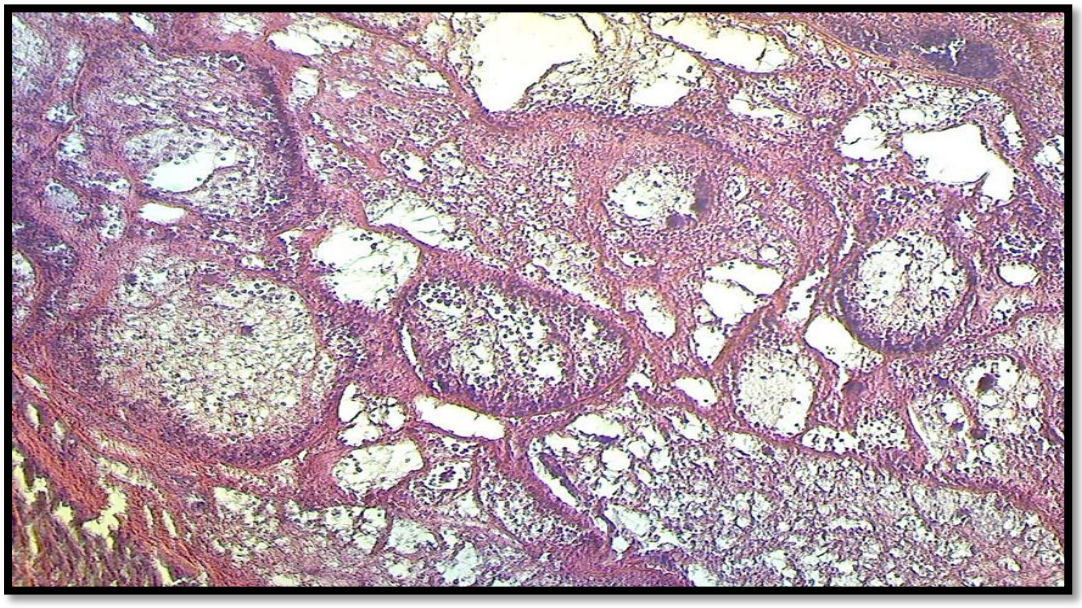

Figure 12: Infection of the sheep lungs show necrotic pneumonia (necrosis, oedema, thickening in the alveoli and leukocytic infiltration dominantly with neutrophils (H & E ,X10).

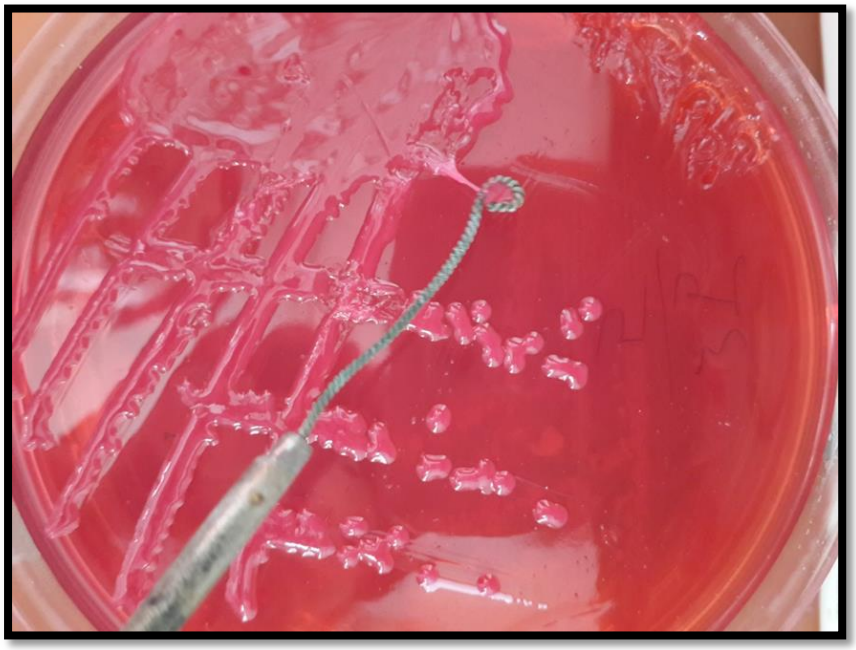

Figure 13: *Klebsiella pneumonia* appeared pink color colonies and mucoid (2-3) cm on MacConkey agar Petri dish.

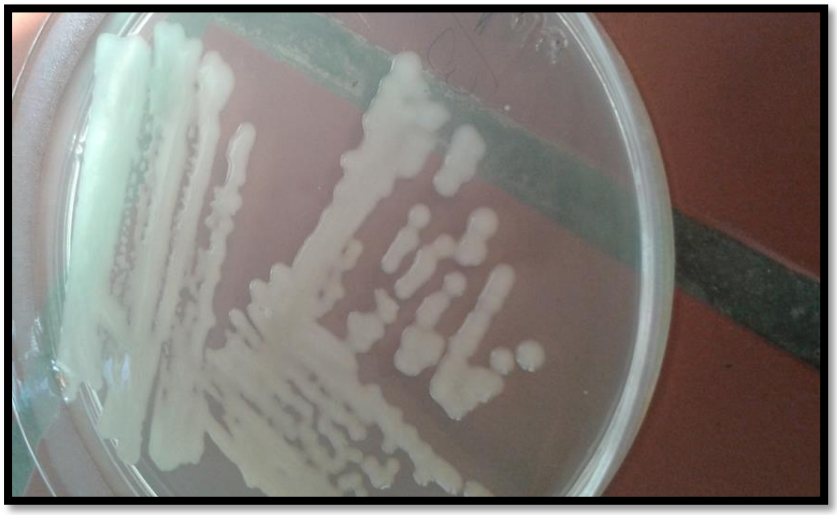

Figure 14:*Klebsiella Pneumoniae* on nutrient agar.

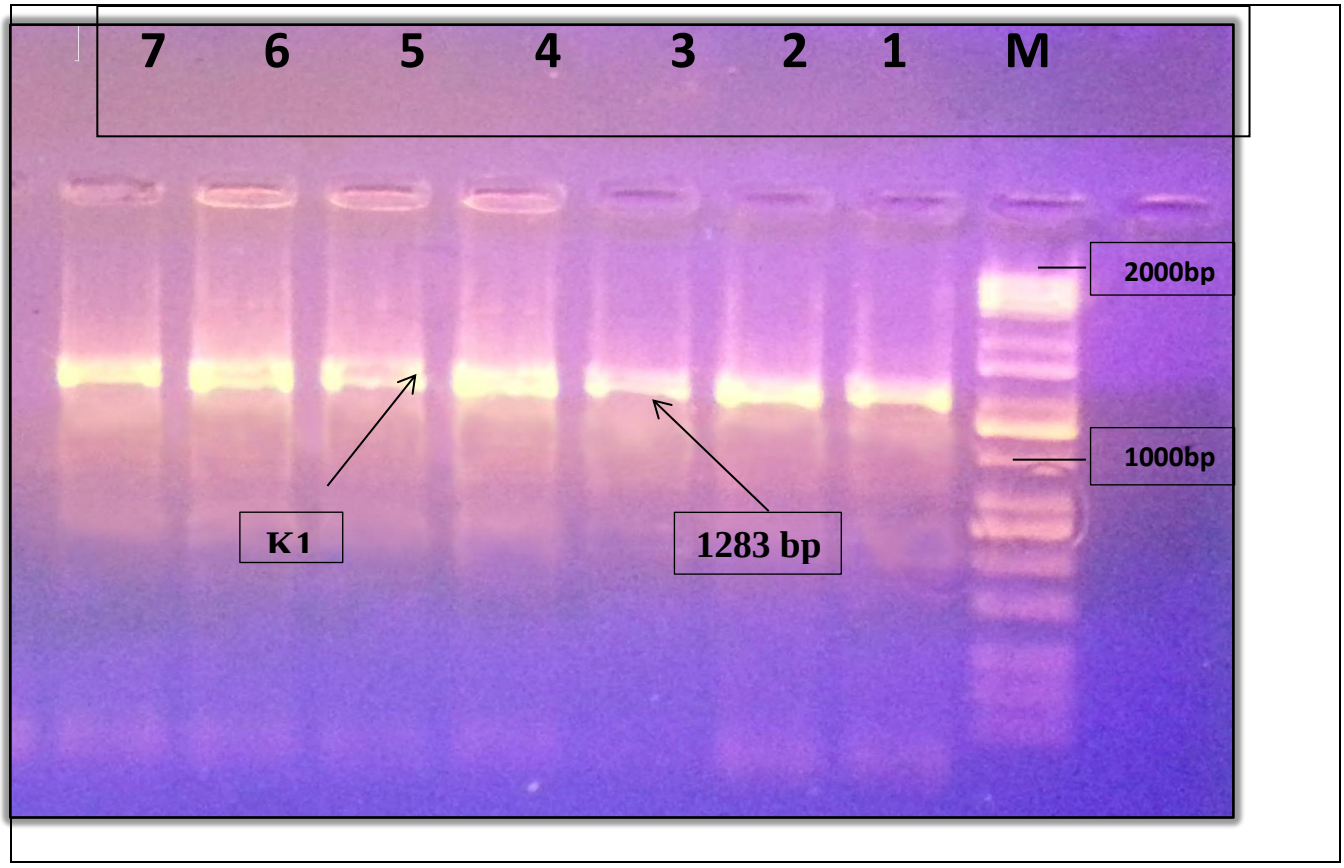

Figure 15: Ethidium bromide stained agarose gel showing PCR amplification has with *k1* (1283 bp) primer for *Klebsiella pneumonia* extracted DNA.

●M: Marker (2000bp),

●Lane (1-7) positive *k1* gene.

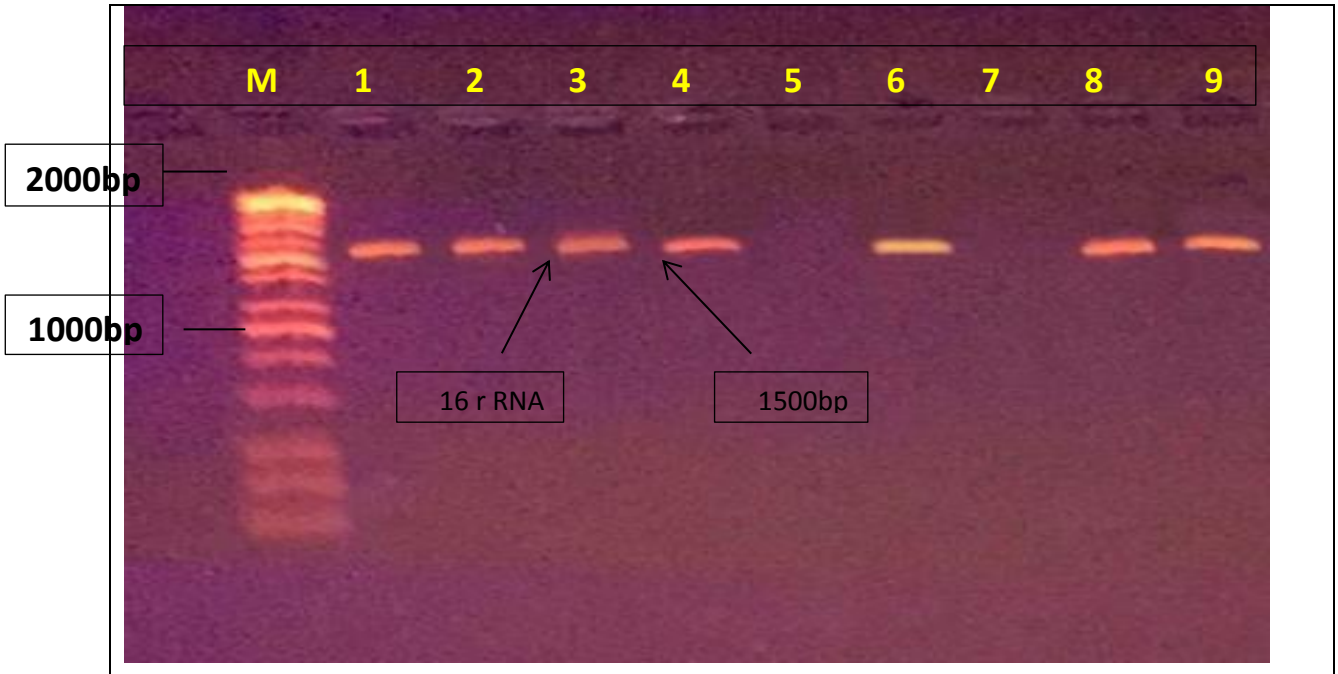

Figure 16: Ethidium bromide stained agarose gel showing PCR amplification has with 16 rRNA (1500 bp) primer for *Klebsiella pneumonia* extracted DNA.

●M: Marker (2000bp),

●Lane (1,2,3,4,6,8 and 9) positive16 rRNA gene.

●Lane (5 and 7) negative 16 rRNA gene.

Discussion:

The present study was designed to refine and correlate the histopathological pattern of ovine pulmonary lesions with their bacterial aetiologies particularly *klebsiella pneumoniae*. Of 700 lungs examined, 220 (31.4%) had one or more gross lesions cases showed gross lesions of various types of pneumonia and pulmonary abscesses, of 30/220 (13.6%) isolations are *klebsiella pneumoniae* . These results were the incidence of bacterial isolation nearly

similar to that obtained by previous studies (12 , 13 and 14), recorded a percentage of its isolation of 8%, 15.09% and 8% , respectively. But disagree with **(**15), recorded *klebsiella pneumoniae* isolations (48%) .The differences between the records were mainly due to the geographical distribution at which the investigator was adopted. In camels, in Sudan and Jordan (16and 17) were isolation of klebsiella pneumonia 15.4% ,14%, respectably .

The results of this study show that three lungs showed multiple abscesses , which were *K. pneumoniae* isolated. These results agree with (13).But disagree with finding (17) studied bacterial aetiologies together with histopathological changes of pneumonia in 284 lungs of slaughtered camels in the northern parts of Jordan with a different geographical position.

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