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Evaluating the Role of Capsular Polysaccharide K and Lipopolysaccharide O antigens in the pathogenesis of Klebsiella pneumoniae Using a Rat Model of Pneumonia

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ABSTRACT

Klebsiella pneumoniae is one of the commonest bacterial causes of hospital acquired pneumonia and accounts for 5 to 7.5% of all nosocomial infections in intensive care units, lower respiratory, urinary tract, and burn wound infections. The objectives of this study are to compare total polysaccharide concentrations extracted from K. pneumoniae isolates. Secondly, to investigate the cellular events using rat model of infection leading to pneumonia with the highest capsular polysaccharide contents as well as evaluating the contributions of each capsular polysaccharide K & lipopolysaccharide O-antigens in this process. For this purpose, thirty-nine strains of K. pneumoniae were isolated from nosocomial infections at Sulaimaniyha hospitals. Capsular polysaccharides and total polysaccharide in all isolated K. pneumoniae strains, were extracted by heating method and quantification were determined on the bases of standard glucose curve. Results revealed that K. pneumoniae was significantly different in their capsular polysaccharide concentrations with regard to their site of isolation. The highest polysaccharide content was found in a clinical isolate of blood specimens (80µg / 1010 CFU/ ml). Histopathological examinations were performed on rats suffering from induced pneumonia with whole bacterial cell antigens, extracted capsular polysaccharide (K-antigens), and (O-antigens). In the case of whole bacterial cells, lung sections showed severe effects; the outpouring of polymorphonuclear and mononuclear leukocytes to the interalveolar septa, accompanied by destruction of alveolar walls (emphysema) with severe congestion of blood vessels, peribronchiolar and perivascular infiltration of inflammatory cells. K-antigens exhibited relatively whole bacterial cells-like histopathological effects whereas O-antigens did not reveal such profound effects.

Keywords: *Klebsiella pneumoniae, Capsular polysaccharide, Lipopolysacharide O side chain, pneumonia, K-antigens, O-antigens*

INTRODUCTION

K. pneumoniae species are opportunistic Gram-negative rod-shaped pathogens involved in the outbreaks of nosocomial infections.[1] They account for 5 to 7.5% of all hospital-acquired infections in intensive care units including lower respiratory, urinary tract, and burn wound infections.[2] Studies have emphasized K. pneumoniae clinical importance as one of the major causes of nosocomial infections.[3,4] More importantly, the emergence of multidrug-resistant (MDR) strains of K. pneumoniae and other MDR healthcare-associated infecting bacteria has been regarded as one of the main urgent microbial threats to the world. As a result of limited choices to treat MDR K. pneumoniae infections, the length of hospital stay and mortality has increased, and the cost of overall healthcare has inflated.[5,6] Therefore, the prevention of K. pneumoniae infections has become vital to control the emergence of MDR K. pneumoniae isolates, by developing vaccines targeting their capsular polysaccharide (CSP), O polysaccharide, outer membrane proteins, and extracellular vesicles.[7] Several Klebsiella components like fimbriae and siderophores have been reported as virulence factors as well as the two major factors CPS and lipopolysaccharide (LPS).[8,9] K. pneumoniae CSP (K-antigen) and LPS (O-antigen) can activate the innate immune system of the host.[10,11] K. pneumoniae are distinguished by the presence of CPS, of which there are currently more than 77 K antigenic types whereas there are 10 LPS O-antigen serotypes.[12,13] Although K. pneumoniae can cause severe pneumonia; both CPS and LPS are found essential elements for the bacterial ability to spread in the blood leading to sepsis. Nevertheless, their contribution in the development of pneumonia infection have not been fully understood despite the experimental data that suggests that at least CPS play a key role in the establishment of pneumonia. This stems from the fact that capsule-host immune system interactions often determine the outcome of infection.[14,15] The organism is carried in the respiratory tracts of approximately 10% of healthy individuals, who may become prone to pneumonia once their defense systems are impaire.[16] LPS, on other hand, may contribute to the progress of necrotic lesions, however, its other potential pathological effects have not been fully studied. K. pneumoniae is known by its abilities of producing huge amounts of CPS a combined with endotoxin, which increases the virulence of K. pneumoniae, and may neutralize bacterial opsonizing antibodies.[17,19] Although, roles of both CPS and LPS in resistance to complement cells as well as opsonophagocytic polymorphonuclear immune cells are well documented,[20] their role in the development of pneumonia is not fully understood. Knowing which K. pneumoniae component that highly participates in progressing pneumonia infection is crucial for developing treatment or/and active immunization against such a major cause of bacterial nosocomial infections. The present project was designed to investigate the role of CPS and LPS antigens in the development of pneumonia disease in rat model pneumonia.

MATERIALS AND METHODS

Bacterial isolates

Thirty-nine K. pneumoniae isolates were obtained from nosocomial infections at the Sulaimaniyha hospitals, all Klebsiella species were cultivated on MacConkey agar and identified as a Klebsiella pneumoniae using API 20E system.

Determination of total polysaccharide and lipopolysaccharide

The extraction of all isolated K. pneumoniae strains was achieved by heating method. Total polysaccharide was extracted from boiling 2 mL of 10^{10} CFU mL^{-1} K. pneumoniae at 100 °C for 30 min. Then, suspension was centrifuged for 10 min at 2000 xg while the supernatant and the pellet were used for the extraction of CPS and lipopolysaccharide. For extraction of CPS, 2.5 mL of absolute ethanol was mixed with the supernatant and kept at - 4 °C for 2 hrs. Then, centrifuged at 7000 xg in cool centrifuge for 10 minutes at -4°C and precipitated polysaccharide was dissolved in 0.5ml of distilled water and used for inoculation. For each 0.5 ml of the extracted CPS, 0.5 ml from phenol solution (5%) was added and mixed. Next step was the addition of 0.5 ml of concentrated (100%) Sulfuric acid (H_2SO_4) which was left to cool at 25 °C. Finally, the concentration of CPS in the samples were measured by spectrophotometer at 490 nm. And total polysaccharide concentration expressed as µg per 10^{10} CFU/ ml based on standard glucose curve.

Preparation and inoculation of antigens

Whole bacterial antigens were prepared form 5 ml of bacterial culture with 10^{10} CFU/ml by centrifugation for 30 min at 6000 rpm, where the pellet was then washed twice by normal saline before being resuspended in 5 ml normal saline for injection. Capsular polysaccharide (K-antigens) was extracted and prepared as described above. Lipopolysaccharide (O-antigens) were prepared by boiling of 5ml bacterial culture with 10^{10} CFU/ml for 2 h to remove heat-labile flagella, capsule and fimbrial antigens but retain heat-stable polysaccharide O antigens. Boiled culture centrifuged for 30 min at 2000 xg, before suspending the sediment in 5ml of normal saline and resuspended in fresh saline.

Inoculation of animals for pneumonia model

To understand the role of each K-antigen and O-antigen in developing pneumonia in rate model. The albino laboratory rat (female) strain Rattus norvegicus of age between 70-80 days and weighing between 230 and 250 g were kept in a suitable room at the Animal House supervised by the College of Science in the Department of Biology at University of Sulaimaniya, where ethics was evaluated and approved. Rats got unlimited supply of food and water during the experiments. The symptoms of infection such as being hunched, lethargic and having starry coat were closely observed and reported at regular intervals from the start to the end of the animal experiments.

Experimental design

Design of experiment was made up of sixteen rats distributed to four groups (four rats each). Before inoculation with CPS and LPS-antigens, 100 µl of blood was taken from the rats' tail veins and then spread on blood agar in order to see if the rats suffered from other bacterial infection or not. Rats were then anesthetized by chloroform inhalation and inoculated intratracheally with a blunt-ended feeding needle.[15] For the chronic pneumonia model, rats were intratracheally administrated with K-antigen, O-antigen, and whole bacterial cells approximately 10×10^{10} CFU per rat in 100 µl. To rule out any contamination and potential secondary bacterial infection during lung dissection, another group of rats was inoculated with normal saline alone as a control. The rats were monitored regularly for the signs of infection. Then, at the end of the experiment at day 14, five rats were sacrificed, cardiac blood sample were collected using sterile syringes (5cc) under sterile conditions & lungs were removed aseptically for histopathological examination.

Preparation of tissues for histopathological examination

Lungs were aseptically removed and prepared for histological sections depended on standard methods. Specimens were subjected to 10% formalin, and then dehydrated and cleared before embedding with paraffin wax. The, 5 µm thickness sections were carefully made for H&E staining and evaluated via light microscopy where final images were prepared by a digital camera (Nikon, Tokyo, Japan).

RESULTS

Quantitative determination of total polysaccharide content

Most of K. pneumoniae with mucoid and more viscous colonial properties, on the other hand revealed to the amount of polysaccharide content in the capsule. Obtained results shown in (table 1) indicated that clinical isolates of K. pneumoniae with highest polysaccharide content ranging from (29-80 µg / 10^{10} CFU/ ml). These variations among different K. pneumoniae may come from genetic variation between them that playing main role in capsule production. The results showed that the highest polysaccharide production was from K. pneumoniae isolated from blood samples while other strains of the same species from other isolation sites like sputum, urine, burn, wound, and environmental samples exhibited different degree of concentrations (table 1). The highest polysaccharides concentration of K. pneumoniae was isolated from blood. Isolated K. pneumoniae form sputum contained the second highest of CPS content (65 µg / 10^{10} CFU/ ml). On the other hand, lowest polysaccharide content was obtained from isolated environmental species.

TABLE 1: total polysaccharide concentration in K. pneumoniae.

Site of isolation	Number of isolates	Amount of total CSP µg / 10^{10} CFU/ ml
Blood	2	75-**80**$^{(**)}$
Burn wound	16	35-52
Urine	5	58-64
Floor	5	29-37
I.C.U.	4	34-40
Sputum	1	65
Bed	1	37
Dressing utensil	1	38
Bath room	2	36- 37
Surgical wound	2	39- 41
Total	39	

I.C.U.: Intensive Care Unit, (**) used for infection and extraction of K-antigen and O-antigen

Characterization pneumonia in rats caused by whole cell of K. pneumoniae, CSP (K-antigen), and LPS (O-antigen)

To inspect the cellular events prior to pneumonia infection and to examine the influence of K & O-antigens in those events, the host reactions were investigated under histological study. It included whole bacterial cells, K and O-antigens. In addition, challenge pneumonia infection rats by utilizing a large polysaccharide content of clinically isolated K. pneumoniae from blood. Approximately 10^{10} CFU/ ml were used for each of whole bacterial cells, extracted K-antigens and O-antigens proved that the variations seen among the whole bacterial cell, CPS, and O-antigen were not due to the inoculums. And rats were found equally challenged intratracheally with the same dose 10 international units (I.U.).

Histological characterization caused by whole bacterial cell

To assess K. pneumoniae ability to translocate to the lungs causing a lethal infection post intratracheal inoculation in rats which were recorded by developing positive blood cultures and documenting survival profiles. Analysis of survival indicated that all animals survived along with negative bacterial growth on blood and lung tissues of these animals regardless of any pneumonic reactions. Obtained results of macroscopic analysis of lungs tissues revealed important areas of emphysema, focal hepatization and suppuration (fig-1-I), when compared with lungs from healthy control animals (fig-1-II).

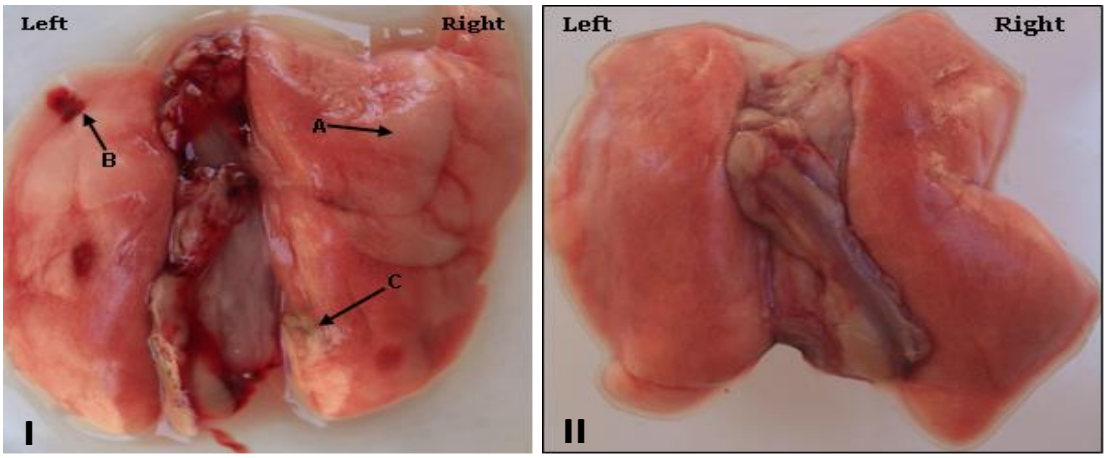

FIGURE 1: I- Morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) 10×10^{10} CFU of K. pneumoniae per rat (A- Emphysema areas on lungs, B-Focal areas of hepatization on the left lung, C-Area of suppuration on the diaphragmatic lobe of the right lung). II- Normal morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) of normal saline.

Further histopathological examination of the rat lung lesions demonstrated multifocal area of emphysema, sever blood vessels congestion and perivascular inflammatory cells infiltration (macrophages & granulocytes), mucus exudates in bronchiolar lumina & peribronchiolar and interstitial tissues infiltration by inflammatory cells. Moreover, some alveolar spaces are filled with neutrophilic granulocytes and macrophage (fig-2-I) and interalveolar hemorrhagic effusions were seen in some area (fig-2-II) when compared with normal histology in (fig-2-III).

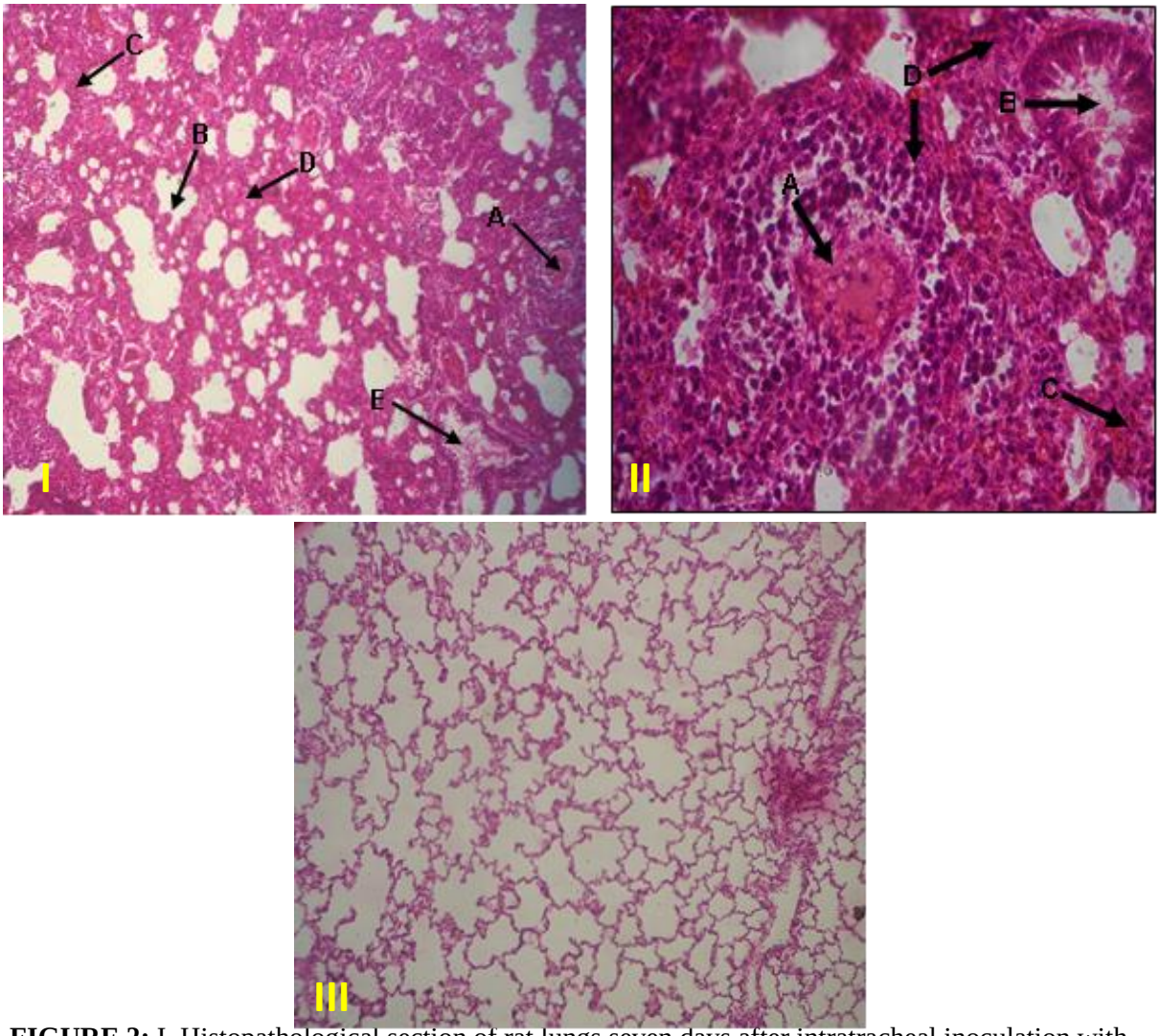

FIGURE 2: I. Histopathological section of rat lungs seven days after intratracheal inoculation with (100 µl) 10×10^{10} CFU of K. pneumoniae per rat. Lung (paraffin section x400) shows: A-Severe congestion of the alveolar blood vessels, B- Mucus exudates in the bronchiolar lumina, and sloughing of the bronchiolar epithelium lining, C-Infiltration of (Polymorphonuclear & Mononuclear leukocytes) inflammatory cells in the interstitial tissues and interalveolar septa, with diffusion (red blood cells) through lung tissues, D- Perivascular and peribronchiolar infiltration of inflammatory cells). II. Histopathological section of rat lungs seven days after intratracheal inoculation with 10 (I.U.) K. pneumoniae. Lung (paraffin section x100) shows:(A- sever blood vessels congestion and perivascular inflammatory cells infiltration, B-Multifocal areas of emphysema, C-Thickening of interstitial tissue related to infiltration of large numbers of inflammatory cells (PMN & MN) leukocyte, D-Occluding of many alveoli by inflammatory exudates, E-Mucus exudates in the bronchiolar lumen, with sloughing of the bronchiolar epithelium lining). III. Normal morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) of normal saline.

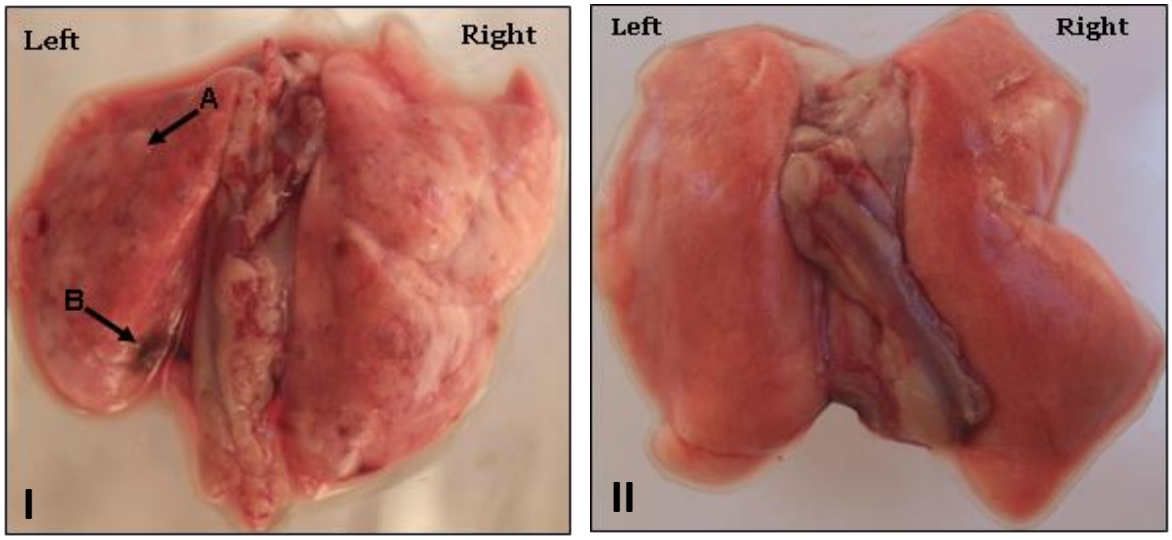

FIGURE 3: I. Morphological appearance of rat lung seven days after intratracheal inoculation with (100µl) capsular polysaccharide extracted from K. pneumoniae: Note the presence of (A-Emphysema (multifocal areas of emphysema), B-Associated with variable sized areas of congestion). II- Normal morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) of normal saline.

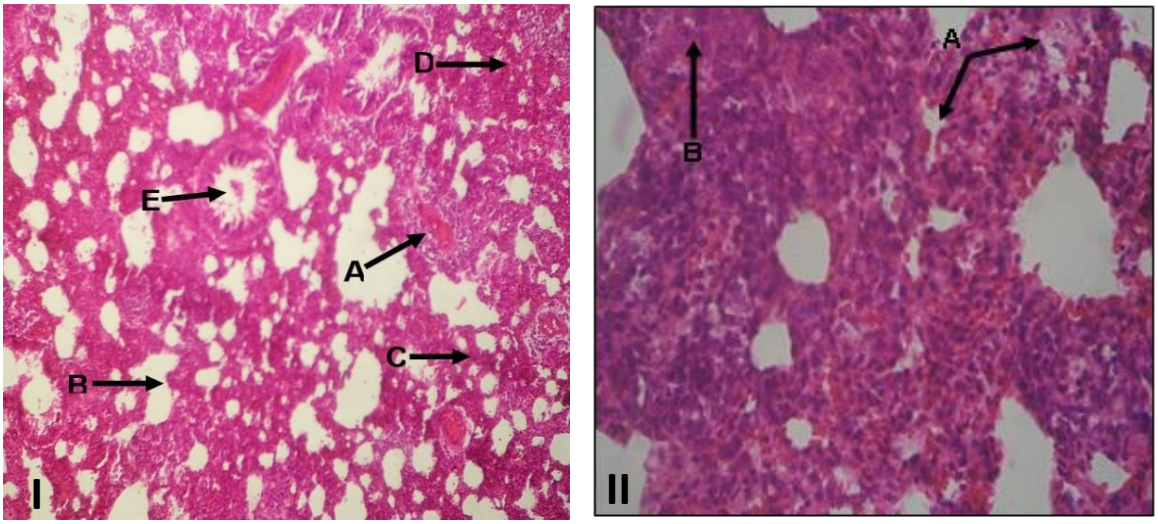

FIGURE 4: I. Histopathological section of rat lungs seven days after intratracheal inoculation with (100 µl) capsular polysaccharide extracted from K. pneumoniae. Lung (paraffin section x100) shows: (A- sever blood vessels congestion and perivascular inflammatory cells infiltration, B-Multifocal areas of emphysema, C-Infiltration of large number of inflammatory cells (PMN&MN) leukocytes to the interstitial tissue and interalveolar septa, D- Occluding of alveolar lumina by inflammatory exudates, E- Mucus exudates in the bronchiolar lumen, sloughing of the bronchiolar epithelium lining). II. Histopathological section of rat lungs seven days after intratracheal inoculation with (100 µl) capsular polysaccharide extracted from K. pneumoniae. Lung (paraffin section x400) shows: (A- Mucus exudates in some space of alveoli and some free red blood cells in the alveolar lumina, B- Infiltration of polymorphonuclear & mononuclear inflammatory cells in the interalveolar septa).

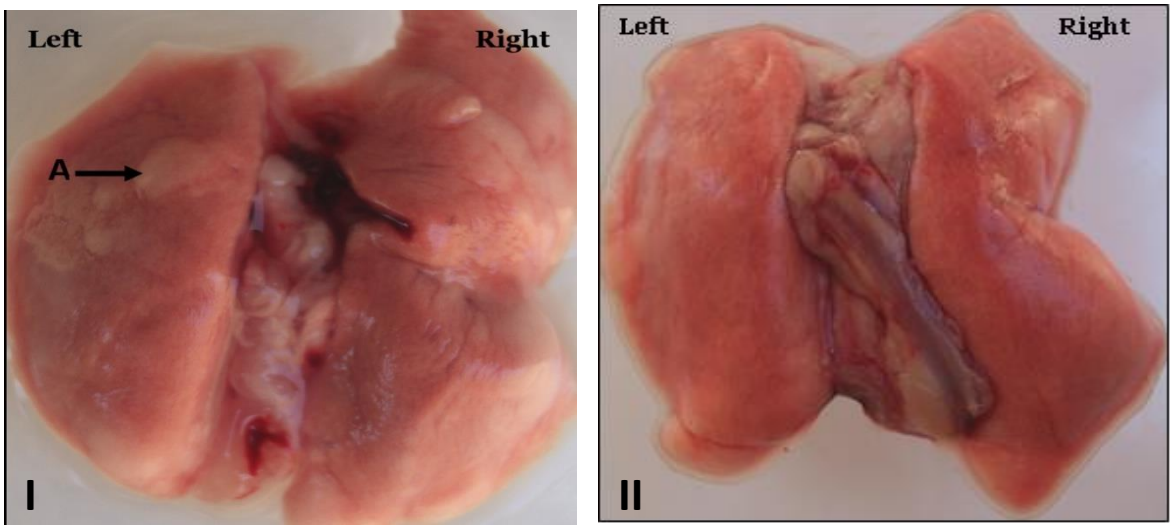

FIGURE 5: I. Morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) of O-antigen extracted from K. pneumoniae (Note the presence of Emphysema areas on lungs). II- Normal morphological appearance of rat lungs seven days after intratracheal inoculation with (100 µl) of normal saline.

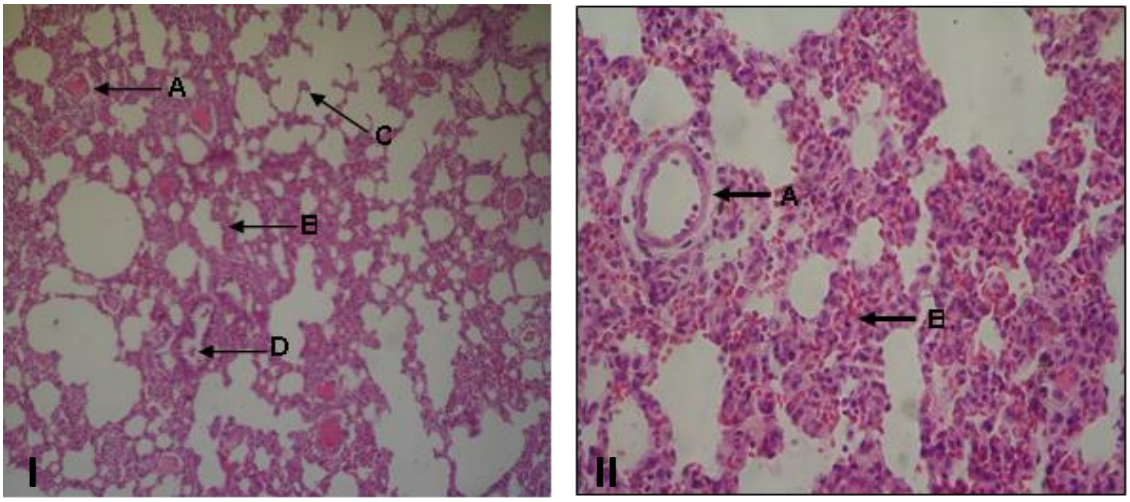

FIGURE 6: I. Histopathological section of rat lungs seven days after intratracheal inoculation with (100 µl) of O-antigen extracted from K. pneumoniae. Lung (paraffin section x100) shows: (A-Congestion of pulmonary blood vessels, and moderate perivascular infiltration of inflammatory cells, B-Alveolar capillaries associated with infiltration of inflammatory cells in the interalveolar septa resulting in thickening of them, C- Emphysematous regions are also evident, E- Mucus exudates in the bronchiolar lumina, and sloughing of the bronchiolar epithelium lining). II. Histopathological section of rat lungs seven days after intratracheal inoculation with (100 µl) of O-antigen extracted from K. pneumoniae. Lung (paraffin section x400) shows: (A-Moderate perivascular edema, B-Slight infiltration of inflammatory cells and some free red blood cells (hemorrhage) in interstitial tissue and interalveolar septa).

Histological characterization caused by somatic antigens (O-antigens)

Since the O-antigens of Klebsiella strains contain a number of molecules composed of repeating subunits of carbohydrate associated with the core antigen of LPS (11), the role of LPS O antigens in the development of pneumonia was investigated. In contrast with the pathological changes seen in the lungs of the rats caused by whole bacterial cells and CPS, the morphology of

rats' lungs infected with the O antigens as shown in (fig-5-I) appeared with slight abnormality (fig-5-II). Further histopathological examination of the lungs showed congestion of pulmonary blood vessels with edema and accompanied by perivascular as well as interalveolar septa infiltration by inflammatory cells, and emphysematous regions (fig-6-I) and (fig-6-II). Bacterial cells did not recover neither from the blood or the lung homogenates at the end of the experimental period.

DISCUSSION

Most of K. pneumoniae colonies with mucoid and more viscous properties, appeared to have large amount of polysaccharide content in the capsule. K. pneumoniae tends to produce largest quantities of CPS as exhibited by the consistent formation of mucoid colonies.[21] In addition, the degree mucoidity was seen strongly associated with the establishment of infection.[22] The various CPS contents measured in this study from different K. pneumoniae clinical isolates ranging from (29-80 µg / 10^{10} CFU/ ml) may be due to genetic variation, which plays a role in capsule production in those strains. This explanation was previously suggested [23] who isolated rmpA2 gene from plasmids of a K. pneumoniae strain. This gene was found to encode an activator for CPS synthesis.

The highest polysaccharides concentration of K. pneumoniae was obtained from blood isolates. This finding is consistent with the previous study [17] that described that the well-developed CPS play essential role in the protecting K. pneumoniae against phagocytosis and complement-associated serum killing. Isolated K. pneumoniae form sputum contained second highest CPS content, which may aid strains to resist dust cell found in lower respiratory tract and antimicrobial compound that presented in the airway liquid. [23] Lowest polysaccharide content was recorded in environmental isolates, which might be related to bacterial use of CPS as source of nutrition and utilizing the sugars when energy sources are low. [24] Or simply, these amounts of polysaccharide concentration might be enough to resist unfavorable environmental conditions. It was reported that the functional activation of RmpA2 during opportunistic infection by K. pneumoniae may assist the later to adapt its metabolic carbon flow upon exposure to various environmental conditions, whether in parasitic or free-living stages. [21]

Analysis of survival indicated that all animals survived along with negative bacterial growth on blood and lung tissues of these animals regardless of any pneumonic reactions. The clearance of whole bacterial cell in the blood might be due to that explained by researchers. [25-27] They reported that capsular type containing repeating specific sequences of mannose or L-rhamnose -α -2/3- mannose or L-rhamnose are readily recognized by a surface lectin on macrophages. Those macrophages are able to ingest and eventually kill Klebsiella serotypes that contain the CPS specific repeating sequences. This observation is consistent with [15] a study demonstrated that human alveolar macrophage were able to phagocytose K. pneumoniae. Our results of macroscopic analysis on lungs tissues infected with whole bacterial cells revealed distinct areas of emphysema, focal hepatization and suppuration when compared with lungs from healthy control animals. Further histopathological examination of the rat lung lesions demonstrated multifocal area of emphysema, sever blood vessels congestion and perivascular inflammatory cells infiltration (macrophages and granulocytes), mucus exudates in bronchiolar lumina and peribronchiolar and interstitial tissues infiltration by inflammatory cells. Moreover, some alveolar spaces are filled with neutrophilic granulocytes and macrophage and interalveolar hemorrhagic effusions were seen in some area when compared with normal histology. These changes were previously documented previously the authors reported that in response to K. pneumoniae in the alveolar exudates.[28]

Animal's inoculated intratracheally with the CPS had pneumonia with characteristics relatively comparable to the ones detected with pneumonia infections caused by the administration of the whole bacterial cells. Infected rats also developed abscesses and multifocal area of emphysema in their lungs compared to normal morphological lung appearance. Furthermore, histopathological study of the lesions demonstrated congestion of blood vessels manifested with perivascular, interstitial tissues and interalveolar septa infiltration of inflammatory cells, and some alveolar lumina exhibited occluding by inflammatory exudates. It was clearly observed that CPS was essential for developing pneumonia

in rats, and as mentioned that capsule-host immune system interactions often determine the outcome of infection. [19] The histological effects and tissue damage caused by high amount of CPS present at the site of infection could be mainly due to infiltration of various immune cells as part of immune response to fight such an important microbial antigen. Researchers working on Streptococcus pneumoniae found that CPS antigen stimulated the immune system to cause infiltration of leukocyte to the site of presence CPS and release of other immune components including (interleukins, cytokines, complement proteins) that might lead to tissue destruction. The researchers concluded that capsular polysaccharide determine infiltration of Neutrophile extracellular trap (NET) to the site of infection. Their findings have shed some light on the significant impact of pneumococcal capsule size which can determine the response by innate immune systems including the formation of NETs which may result in the severity of pneumonia. [29]

Since the O-antigens of Klebsiella strains contain a number of molecules composed of repeating subunits of carbohydrate associated with the core antigen of LPS. [11] The role of LPS O antigens in the development of pneumonia was investigated. In contrast with the pathological changes seen in the lungs of the rats caused by whole bacterial cells and CPS, the morphology of rats' lungs infected with the O antigens appeared with minor abnormality. Further histopathological examination of the lungs showed congestion of pulmonary blood vessels with edema and accompanied by perivascular as well as interalveolar septa infiltration by inflammatory cells, and emphysematous regions were also observed. Bacterial cells did not recover neither from the blood or the lung homogenates at the end of the experimental period. This observation was consistent with a study [29] previously observed influx of PMN into the lung tissues in response to administration of LPS O side chain in mice. Lung edema in our study were observed by a study[30] reported that LPS from K. pneumoniae interaction with epithelium submucosal membrane, which followed by inhibition of Na^+ absorption leading perhaps to hypersecretion in the respiratory airways. Similar results were obtained by other investigators [11] who demonstrated that, CPS but not O antigen plays far important role in the pathogenesis and virulence of Klebsiella spp.. Furthermore, it has been reported by molecular approach study that CPS is more essential than LPS in developing pneumonia infection. Essentially, the lack of CPS, rather than LPS O side chain, had a stronger impact on K. pneumoniae capacity of causing pneumonia.[15] It can be concluded that CPS was more important in developing pneumonic reaction than O antigens which played a moderate role when compared to that observed from whole bacterial inoculation.

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Data Availability Statement

The data supporting this study's findings are available from the corresponding authors upon reasonable request.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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