**ANIMAL RESEARCH ARTICLE**

**Immune response and pathophysiological features of Klebsiella pneumoniae in mice**

Nasma Maged Elemary¹, Mohamed Mahrour Emara², Amin Abd Elhady Tahoun³, Walied Abdob Sobhy⁴, Ramadan Ahmed Eloomany⁵

**Abstract**

**Objective:** To assess the bacterial colonisation of mice organs and faeces infected with 3 strains of Klebsiella pneumoniae, to measure levels of tumour necrosis factor-alpha, tumour necrosis factor-beta and interleukin-6 in mice serum, and to evaluate immune response of mice infected with Klebsiella pneumoniae.

**Method:** The animal study was conducted at Kafreslsheikh University, Egypt, in 2021, and comprised mice 5-7 weeks old who were infected with 3 strains of Klebsiella pneumoniae; K80 uge+ (uri, kfu+, mrkD+; K68 gyrA+(gyrase A), mrkD+; and K84 ube+, kfu+, mrkD+). They were monitored for 14 days. The bacterial colonisation of mice livers, lungs, spleens and faeces were determined using culture on MacConkey agar. The percentage of neutrophils detected as cluster of differentiation 11b+ and cluster of differentiation 45+ in the mice serum was determined by flow cytometry. Levels of tumour necrosis factor-alpha and tumour necrosis factor-beta were measured using enzyme-linked immunosorbent assay.

**Results:** There were 4 sets of female mice [1 control and 3 infected groups for which 3 K. pneumoniae strains (K80 uge+, kfu+, mrkD+; K68 gyrA+, mrkD+; and K84 ube+, kfu+, mrkD+)] weighing 13-24gm was used. Colonisation of mice organs and faeces was high after 24 hours then declined rapidly after 3 days, 10 days and 14 days in case of infection with capsulated and non-capsulated strains of bacteria. Livers, lungs and spleens showed maximum inflammation after 24 hours, then declined rapidly. Both cytokine production and organ inflammation increased after one day of infection. There was a significant correlation between the produced cytokines and histopathological changes in liver, lung and spleen. The neutrophils increase in case of infection with K84 and K80 was more than non-capsulated K68.

**Conclusion** Neutrophils were found to play an important role in the clearance and treatment of Klebsiella pneumoniae.

**Keywords:** Mice, Interleukin, Neutrophils, Spleen, Cytometry, Lung, Inflammation, Liver, Faeces, Enzyme, Immunity.

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**Introduction**

The hazardous pathogen Klebsiella (K.) pneumoniae frequently causes sepsis, pneumonia and urinary tract infections (UTIs) in both healthy and immunocompromised people.¹ It accounts for 5-20% of gram-negative sepsis cases,²,³ leading to high mortality ranging from 27.4% to 37%.⁴ Moreover, 48.9% mortality was observed in intensive care unit (ICU) patients infected with K. pneumoniae.⁵ In the past few years, strong drug-resistant extended-spectrum β-lactamases as well as carbapenemase-producing K. pneumoniae isolates have been reported.⁶,⁷ Additionally, the prevalence of extremely virulent and invasive K. pneumoniae (hvKp) infections has been continuously rising.⁸,⁹ As a consequence, using antibiotics to treat K. pneumoniae infections is extremely difficult. To overcome this challenge, the prevention and treatment of K. pneumoniae infections need new effective strategies. However, the progress of these strategies requires a fuller comprehension of K. pneumoniae's pathogenic mechanism.¹²

The kfu (Klebsiella ferric iron uptake) gene is a pathogenic gene corresponding to an iron uptake system. LPS (Lipopolysaccharide) protect bacteria from complement mediated lysis. Its production is regulated by the uridine diphosphate galacturonate 4 epimerase (uge) gene. The type 3 fimbriae of K. pneumoniae are composed of the major fimbrial subunit (MrkA) and adhesin (MrkD) ( type 3 fimbrial adhesion gene subunits) which previously has revealed to mediate binding to collagen.¹³

Also, the immunopathogenesis of mouse infection contain both tumour necrosis factor-beta (TNF-β), and tumour necrosis factor-alpha (TNF-α).¹⁴,¹⁵ Additionally, the innate immune sensors play a promising role in stopping both local and systemic infections.¹⁶ In the early stages of infection, neutrophils are primarily sent to the infection site to destroy the pathogens through both oxidative and non-oxidative mechanisms.¹⁷ On the other hand, macrophages play a significant role in both innate and acquired immunity in the respiratory system, and are crucial for lung
infection defence.\(^\text{18}\) Alveolar macrophages are resident mononuclear phagocytes that are situated near the lung’s contact with the outside environment. They have the ability to identify and eliminate pathogens before neutrophil recruitment occurs after infection.\(^\text{20,22}\). However, the complement system also has the ability to use opsonophagocytosis to destroy invasive infections.\(^\text{23}\). Additionally, the effective host defence in the lung against bacterial infection, to a large extent, depends on the rapid elimination of the pathogens from the respiratory tract, whereas the failure may result in prolonged infection and the dissemination of bacteria.\(^\text{24}\). Some K. pneumoniae serotypes are highly prevalent in community-acquired liver abscesses with severe extrahepatic metastasis, including septic endophthalmitis.\(^\text{25}\). Accordingly, research is required to determine how these serotypes affect liver inflammation and innate immune responses.

The current study was planned to investigate in depth the bacterial colonisation of mice organs and faeces infected with 3 strains of K. pneumoniae, to measure levels of TNF-α, TNF-β and interleukin-6 (IL-6) in mice serum, and to evaluate immune response of mice infected with K. pneumoniae.

### Subjects and Methods

The animal study was conducted at Kafreslsheikh University, Egypt, in 2021. After approval from the institutional ethics review committee, mice 5-7 weeks old were acquired and subsequently divided into 1 control and 3 infected groups for which 3 K. pneumoniae strains (K80 uge\(^{-}\), kfu\(^{-}\), mrkD\(^{-}\); K68 gyrA\(^{-}\), mrkD\(^{-}\); and K84 uge\(^{-}\), kfu\(^{-}\), mrkD\(^{-}\)) were collected from the Mansoura University Hospital. K68 and K84 were isolated from urine, and K80 was isolated from sputum. The isolates were grown in Luria-Bertani (LB) broth, under constant agitation at 37°C. All the mice were also infected with an overnight culture of K. pneumoniae, which had been centrifuged at 27,000g for 15min, and the pellet produced resuspended in a comparable volume of phosphate-buffered saline (PBS). The required density for infected mouse (10\(^6\) CFU/100μl of K. pneumoniae in 100-μl single dose of K. pneumoniae in 2% sucrose-PBS solution by using a pipette tip. Control mice were orally gavaged with 2% sucrose-PBS solution by using a pipette tip. Mice were scuffed without any anaesthesia.

To estimate colonisation density, the mice were transferred to isolation containers. Faecal pellets (0.02 g; roughly 2 pellets) were generated and placed, together with at least two glass beads, in a 2ml screw-capped tube. The samples were diluted to 1:10 (weight/volume) in PBS. The faecal pellets (1 min) were homogenised (Homogenizer OS40-Pro Laboratory LCD (Liquid crystal display) digital overhead stirrer mixer, United States). The bigger material was then pelleted out of the tubes by spinning them in a minicentrifuge. On suitable selective plates, 10-fold serial dilutions were plated from the supernatant and incubated for an overnight period at 30°C. Bacterial count (30-300 CFU) was observed in the plated dilution.

The colonisation density was determined in the lung, liver and spleen by removing the organs under sterile conditions immediately following euthanasia after anaesthesia by inhaled halothane. The organs were weighed and transferred to 15ml conical tubes. For liver and lung, equal weight-to-volume of PBS was added, and then the mixture was homogenised at power setting of 2 for 30s. For spleen, 10 times the volume of 1× PBS was added to the weight of the organ and then homogenised. The samples were also plated by the same procedure. The observed detection limit for both the lung and liver was 33 CFU/ml, whereas for the spleen it was 10\(^2\) CFU/ml.

Livers were gathered under sterile conditions from the infected mice by K. pneumoniae that were infected orally with hvKP. Liver samples were cut into 6.5mm sections and added to 7.5% formalin (7.5 parts formalin: 1 part tissue). After about 24-48h, the samples were transferred into ethanol (70%) and stored at 4°C till they were treated for haematoxylin and eosin (H&E) staining as well as for gram staining.\(^\text{26}\)

For lungs, the lungs of various groups were fixed for 48h in 4% paraformaldehyde and then transferred into paraffin oil. Sections were prepared by cutting 5μm thick and they were then stained using H&E and prepared for gram staining.\(^\text{16}\)

Spleen from different mice was dissected and transferred into 10% formalin (neutral buffered). After about 24h, the obtained tissues were transferred into 70% ethanol and then stored till paraffin embedding. These tissues were further sectioned and stained using H&E.\(^\text{27}\)
Whole blood was collected and allowed to clot by being left at 25°C unattended for roughly 15-30 minutes. The clot was eliminated via centrifugation at 1,000-2,000 x g for 10 min in a refrigerated centrifuge. The obtained supernatant was categorized as serum. Enzyme-linked immunosorbent assay (ELISA) kits (Bio vision, USA) were used to determine the levels of cytokines in serum by following the manufacturer’s guidelines, and were reported as pg/ml. Flow cytometry was used to determine the neutrophils % in the mice serum, as reported previously. Foetal bovine serum was used to block the serum for 15 min and then stained by using fluorophore-conjugated antibody cocktails for about 30 min at 4°C. Surface antibody markers included cluster of differentiation (CD)45 monoclonal antibody (IBL-5/25, FITC, Cat #MA5-17961, Bio-Legend, San Diego, CA, USA). The neutrophils in the serum were categorized as CD45+CD11b cells. All samples were analysed on Accuri c6 Necton Dickinson™ flow cytometer.

Mean values and the standard error of the mean values were used to present the findings. One-way analysis of variance (ANOVA) was used to determine the statistical significance of data. P<0.05 was considered statistically significant.

Results

There were 4 sets of female mice 1 control and 3 infected groups for which 3 K. pneumoniae strains (K80 uge+, kfu+, mrkD+; K68 gyrA+, mrkD+; and K84 “uge”, kfu”, mrkD”) weighing 13-24 gm were used. Overall, 1(%) mouse died, in the K80 group, after 12 days, while the rest survived till, they were euthanised.

The plated dilutions revealed that bacterial colonisation was significant after 24h then declined rapidly after 3 days, 10 days and 14 days in capsulated and non-capsulated strains of bacteria (Figure 1A-1D).

Regarding histopathological findings, on the 1st dpi (days post inoculation), the lungs of control group showed normal alveolar and bronchial tissues. The lung of the infected animals showed severe perivascular and peribronchial reaction associated with inflammatory cell infiltration, and peribronchial necrosis of the neighbouring alveoli. The damage was more prominent in animals infected with K84, followed by K80 and then K68. The liver of control animals showed normal hepatocytes. The liver of infected animals showed mid-zonal hepatic necrosis and subcapsular abscesses. The hepatic lesions were more severe in K84 group, moderate in K80 and mild in K68 group (Figure 2A). The spleens of the control group showed normal red and white pulps. The infected groups revealed lymphoid necrosis, especially in K84 and K80 groups, while K68 group showed mild lymphoid depletion. A considerable number of bacteria were found in the sinusoids of the liver sections that had been taken, and these bacteria were identified as gram-negative bacilli. After 3 days, pneumonia was more severe in infected groups K84 and K80. The pulmonary lesions were in the form of bronchopneumonia in K68 group. The size and number of abscesses within hepatic tissues were increased in all infected groups, especially in K84 and K80 groups. K68 group showed significant increase of hepatic lesions in comparison with control group, and significant decrease in comparison with K84 group. The magnitude of splenic necrosis was more pronounced in this sacrifice, with more aggravated lesions in K84 and K80 groups than K68 (Figure 2B). On 3rd and 4th sacrifice at 10th and 14th dpi, respectively, the lungs showed decreased pneumonia in all the infected groups. Similarly, the hepatic necrosis and lymphoid depletion were decreased in a time-dependent manner relative to the earlier lesions (Figure 2C). After 14 days, degenerative and necrotic changes were observed within the different organs, mostly in K84 group. The lungs showed mild to moderate bronchopneumonia, the liver exhibited small granuloma and the spleen demonstrated moderate lymphoid depletion (Figure 2D). K80 and K68 groups showed marked decline in the inflammation within the examined organs and mostly within the normal limits.

K84 group showed that the levels of TNF-β reached a maximum after 3 days then declined. In K80 group, it rose suddenly after 1 day then declined rapidly. In K68 group, it rose after 1 day and continued to rise. However, the observed levels of TNF-β and TNF-α were in the order K84 >K80 >K68. The levels of TNF- α increased and reached the maximum after 3 days of infection, then declined rapidly in K84 and K80 groups. Moreover, in K68 group, TNF-α gradually increased and reached the maximum after 7 days,
Figure 1B: Bacterial colonisation in mice livers in case of infection with 3 Klebsiella (K.) pneumoniae strains after says 1, 3, 7, 10 and 14.

*P<0.05, **P<0.01, ***P<0.001, ns: Not significant.

Figure 1C: Bacterial colonisation in mice lungs in case of infection with 3 Klebsiella (K.) pneumoniae strains after says 1, 3, 7, 10 and 14.

*P<0.05, **P<0.01, ***P<0.001, ns: Not significant.

Figure 2: Pathological changes in control and infected mice with different strains of Klebsiella (K.) pneumoniae at the 1st dpi. The 1st pannel shows the lungs of different groups. Bronchopneumonia was more evident in K84 group (A indicates alveoli and B indicates bronchi; bar=50µm). Quantitative scoring of pulmonary lesions was performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group, b P<0.001 versus K84 group. The 2nd pannel shows hepatic tissues of control group, K84, K80 and K68 infected groups (H indicates normal hepatocytes, CV indicates central vein and arrowheads indicate abscesses; bar=50µm). Quantitative scoring of hepatic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 3rd pannel shows spleen of control group, K84, K80 and K68 infected groups (WP indicate white pulp; bar=50µm). Quantitative scoring of splenic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group.

Figure 2B: Pathological changes in control and infected mice with different strains of Klebsiella (K.) pneumoniae at the 3rd dpi. The 1st pannel shows the lungs of different groups, severe necrotising pneumonia in K84 infected animals (A indicates alveoli, B indicates bronchi and BV indicates blood vessels; bar=50µm). Quantitative scoring of pulmonary lesions was performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 2nd pannel shows hepatic tissues of control group, K84, K80 and K68 infected groups (H indicates normal hepatocytes, CV indicates central vein, and arrowheads indicate abscesses; bar=50µm). Quantitative scoring of hepatic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 3rd pannel shows marked lymphoid necrosis in K84 and K80 groups and moderate necrosis in K68 group (WP indicates white pulp, and arrowheads indicate necrosis; bar=50µm). Quantitative scoring of splenic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group.
Figure 2C: Pathological changes in control and infected mice with different strains of Klebsiella (K.) pneumoniae at the 10th dpi. The 1st panel shows the lungs of different groups, marked interstitial pneumonia in K84 infected animals (A indicates alveoli, B indicates bronchi and BV indicates blood vessels; bar=50µm). Quantitative scoring of pulmonary lesions was performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 2nd panel shows hepatic tissues of control group, K84, K80 and K68 infected groups (H indicates normal hepatocytes, CV indicates central vein, and arrowheads indicate abscesses; bar=50µm). Quantitative scoring of hepatic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 3rd panel shows marked lymphoid necrosis in K84 and moderate necrosis in K80 group and mild necrosis in K68 group (WP indicates white pulp, and arrowheads indicate necrosis; bar=50µm). Quantitative scoring of splenic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group.

Figure 2D: Pathological changes in control and infected mice with different strains of Klebsiella (K.) pneumoniae at the 14th dpi. The 1st panel shows the lungs of different groups, perivascular infiltration of inflammatory cells in K84 and K80 infected animals and normal lung in K68 group (A indicates alveoli, B indicates bronchi, BV indicates blood vessels, and arrows indicate infiltration of inflammatory cells; bar=50µm). Quantitative scoring of pulmonary lesions was performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 2nd panel shows hepatic tissues of control group, K84, K80 and K68 infected groups (H indicates normal hepatocytes, CV indicates central vein, and arrowheads indicate focal infiltration of mononuclear cells; bar=50µm). Quantitative scoring of hepatic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. The 3rd panel shows moderate lymphoid depletion in K84 and normal spleen in K80 and K68 groups (WP indicates white pulp, and arrowheads indicate necrosis; bar=50µm). Quantitative scoring of splenic lesions was performed using one-way ANOVA, followed by Tukey-Kramer multiple comparison test. a P<0.001 versus control group. Total 3 replicates were used. The control gave the same result in all days.

Figure 3A: Levels of cytokines tumour necrosis factor-beta (TNF-β) in case of infection with 3 Klebsiella (K.) pneumoniae strains and the control along 3 days (3 replicates were used each day for each strain). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 3B: Levels of cytokine tumour necrosis factor-alpha (TNF-α) in case of infection with 3 Klebsiella (K.) pneumoniae strains and the control along 3 days (3 replicates were used each day for each strain). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 3C: Levels of cytokine cluster of differentiation (CD)11b+ in case of infection with 3 Klebsiella (K.) pneumoniae strains and the control along 3 days (3 replicates were used each day for each strain). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 3D: Levels of cytokines cluster of differentiation (CD)45+ in case of infection with 3 Klebsiella (K.) pneumoniae strains and the control along 3 days (3 replicates were used each day for each strain). * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 3E: Flow cytometry charts for control, K84, K80 and K68 groups for cluster of differentiation (CD)45 and CD11b. Each sacrifice had 12 mice; 3 for each strain and control.
then declined rapidly (Figures 3A-B).

For CD45, level of neutrophils was higher in K80 >K84 >K68 in comparison with the control group. It increased with time. For CD11b, the levels of neutrophils in the serum were higher in K84 group, followed by K80, and then K68. It also increased with time. However, CD45 was higher than CD11b in comparison with the control group (Figures 3C-D). Flow cytometry supported the important role of neutrophils (Figure 3E).

**Discussion**

Only 1 mouse died and it was infected with the K80 strain, while all other mice survived till, they were subjected to euthanasia. An earlier study reported high mortality. For viable bacterial counts in mice faeces and organs, plated dilutions revealed that bacterial colonisation was high after 24h then declined rapidly after 3 days, 10 days and 14 days in capsulated and non-capsulated strains of bacteria. Another study revealed that the rate of bacterial burden peaked after 48 hours of infection, and gradually decreased thereafter.

In the current study, cytokines rose after 1 day or 3 days, then declined rapidly, and the inflammation of mice organs was the highest after 1 day or 3 days, then declined, showing a direct correlation. After 1 day, the livers showed mid-zonal hepatic necrosis and subcapsular abscesses. This agrees well with a previous study. The lungs showed severe perivascular oedema and alveolar necrosis. The spleens showed lymphoid necrosis and lymphoid hyperplasia, as reported previously. H&E staining of liver sections revealed that many bacteria were distributed in the sinusoids, and they were identified as gram-negative bacilli. This agreed with a previous study. After 3 days, the inflammation in the livers declined rapidly, and the necrosis was mild. The lung had moderate pneumonia. The spleen had mild lymphoid depletion and necrosis. After 10 days, the lungs and the spleens were mildly affected as the livers had small granuloma, while the spleen was normal. After 14 days, the inflammation declined in the organs and the spleen was normal. This decline in response was compatible with those reported by previous studies.

It was noticed in the current study that the levels of TNF-β in case of infection with K84 reached the maximum after 3 days, then declined. For K80, it rose suddenly after 1 day, then declined rapidly. For K68, it rose after 1 day and continued to rise. These results are close to those reported elsewhere. The levels of TNF-α increased and reached the maximum after 3 days of infection, then declined rapidly in K84 and K80 groups. In case of infection with K68, it reached the maximum after 7 days, then declined rapidly, while it did not show changes at various time points compared to the control group, as reported earlier. This shows that K68 was the least virulent as it is un-capsulated, while the other strains were capsulated.

The level of neutrophils was measured as CD45+ and CD11b+ in the current study. The levels of neutrophils increased with infection. This reveals that neutrophils play an important role in the clearance of K. pneumoniae. The neutrophils increased in case of infection with K84 and K80 more than K68, suggesting that capsulated strains caused more infection in the mice.

**Conclusion**

Neutrophils were found to play an important role in the clearance and treatment of K. pneumoniae. Cytokine production had a positive relation to the histopathological changes in liver, lung and spleen of the mice studied.

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**References**


