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## Comparative study of *Klebsiella pneumoniae* in mice immunized with two types of antigens

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### Abstract

This study was designed to explore, detect the humoral immune response by using Elisa technique, to detect the cellular immune response by Delayed Type Hypersensitivity "DTH" (skin test). And to study histopathological changes in laboratory mice organs which immunized with two prepared Ags, and challenged later with viable virulent *K. pneumoniae*. The immunized groups (WSK Ag & WHKK Ag) revealed significant increase ( $p < 0.05$ ) in the mean values of (O.D) & serum (Ab.) titer level as compared with control negative group, while showed non-significant increase ( $p > 0.05$ ) in mean values of (WSK Ag) as compared with (WHKK Ag).

All groups of mice (immunized & control negative) were conducted in (DTH) skin test at (zero time, 24, 48 & 72 hrs.) and both immunized (SWHKK & SWSK Ags) groups showed positive reaction after (24, 48 & 72 hrs.) of skin test as compared with control negative group which give negative reaction.

The pathological examination of a control group was infected with *Klebsiella pneumoniae* appeared more lesions than the groups that were immunized with (WSK & WHKK) Ags. It was concluded that the (WSK Ag) was provided a good immune response better than (WHKK Ag) against the *K. pneumoniae* infection, also the encapsulated *K. pneumoniae* has the ability to dissemination and colonization of most internal organs and the inflammation was rapidly convert from acute to chronic stages.

**Keywords:** Comparative, *Klebsiella pneumoniae*, Mice Immunized, antigens

### Introduction

*K. pneumoniae* is an opportunistic, colonizing pathogen<sup>[1]</sup>, existed asymptotically in the skin, upper respiratory and gastrointestinal tracts of healthy human and animals that can cause respiratory and urinary tract infections (U.T.Is) as well as septicemia especially in immunocompromized hosts who suffer from severe diseases such as diabetes mellitus, chronic obstructive pulmonary diseases, liver disease and renal failure<sup>[2, 3]</sup>.

*K. pneumoniae* had many resistance factors that include CPS, LPS, siderophore, adhesins and antibiotics resistance<sup>[4]</sup>, in addition to biofilm that resist the host immune response by prohibition of mechanical barriers and phagocytosis lead to immune response depression, infections persistence and increase drug resistance ability<sup>[5]</sup>.

*Klebsiellae* typically express two types of surface antigens (Ag): O- Ag which is considered as a lipopolysaccharide (L.P.S) antigen and K-Ag which represents the capsular polysaccharide (C.P.S). Both of (C.P.S) and (L.P.S), are mainly utilizing for an anti *Klebsiella* vaccine and produce protective against a disease via both humoral and cellular immunity<sup>[6]</sup>.

Histopathological staining mostly special stains are used extensively in medicine especially in the study of abnormal tissue to aid treatment. In recent years, for the importance of *K. pneumoniae* as a Zoonotic, Nosocomial and Community Acquired infections in human and animal as well as resistance to antibiotics which are related to Biofilm formation in *Klebsiella*, so scientists have tended to produce vaccines or antigens against *K. pneumoniae* infections such as some researchers in the world,<sup>[7]</sup> and in Iraq, so the present study aims to:

1. Preparation of two Antigens: (Sonicated & Heating Ags) from *K. pneumoniae*.
2. To detect the humoral immune response by using Elisa technique.
3. To detect the cellular immune response by Delayed Type Hypersensitivity "DTH" (skin test).
4. Study histopathological changes in laboratory mice organs which immunized with two prepared Ags and challenged later with viable virulent *K. pneumoniae*.

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## Materials and Methods

### 1. Strain of microorganism

The isolate strain used in this study were obtained from the Unit of Zoonotic Diseases, College of Veterinary Medicine/ Baghdad University, which was isolated from urine sample of patients suffered from U.T.I and this strain was confirmed by microscopical and biochemical tests (Analytical Profile Index) (API 20-E) for *Enterobacteriaceae* that consists of 20 microtubes, to be sure it was *K.pneumonia*, and using the Mouse *K.pneumoniae*-Elisa kit/ MyBioSource /USA for Elisa assay.

### 2. Preparation Antigens

**a. Whole Killed / Heating *K.pneumoniae* Antigen (WKHKAg):** This antigen was prepared according to [8].

**b. Preparation of Whole Sonicated *K.pneumoniae* Antigen (WSKAg):** This antigen was prepared according to [9] with some modification.

### 3. Design of experimental study

Sixty white Swiss BALB /C mice both genders aged (6-8 wks), divided into four groups equally and were treated as following:

1. The 1st group (15 mice) was immunized S/C with (0.25 ml) WKHKAg, two doses with 2 wks intervals at ( $7.5 \times 10^4$  CFU/ml) and (2 mg/ml) protein concentration.
2. The 2nd group (15 mice) was immunized S/C with (0.25 ml) WSKAg, two doses with 2 wks intervals at ( $7.5 \times 10^4$  CFU/ml) and (2 mg/ml) protein concentration.
3. The 3rd group (15 mice) was inoculated S/C with (0.25 ml) PBS, two doses with 2 wks intervals and served as

control negative group.

4. The 4th group (15 mice) was served as positive control group.

At (27-30) days post immunization, the skin test was applied and blood samples were collected from seven mice of each( 1st, 2nd and control negative) groups then the serum was separated and stored at (-20oC) for serological test (Elisa test). Study macroscopical examination of sacrificed mice and internal organs pieces were collected, then fixed in (10% NBF) for histopathological examination. At 28 days after immunization, the remained animals from the first three groups (1st, 2nd & 3rd) with the 4th group (positive control) were inoculated (I/P) in the lower part of abdomen with (0.25 ml) of the bacterial suspension as a challenge dose containing ( $7.5 \times 10^4$  CFU/ ml) of virulent *K.pneumoniae*. All inoculated animals were killed after 48 hrs from challenge dose, blood samples were taken for Elisa test, internal organs and tissues macroscopically were examined, then pieces from internal organs (lung, kidney, liver, spleen, heart, intestine and stomach) were collected and fixed in (10% NBF) for histopathological examination.

## Results

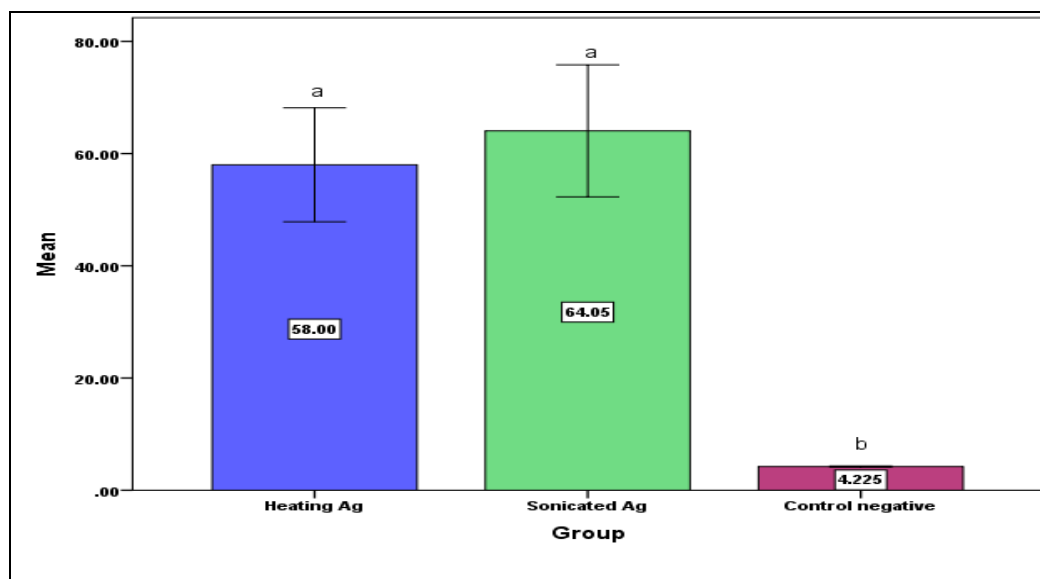
### Humoral immune response

#### 1. Enzyme Linked Immuno Sorbent Assay Test (ELISA):

The immunized groups (WSK Ag & WHKK Ag) revealed a significant increase ( $p < 0.05$ ) in the mean values of (O.D) & serum (Ab.) titer level as compared with control negative group (PBS) which give negative results, as displayed in (table1) & (fig.1) while showed non-significant increase ( $p > 0.05$ ) in mean values of (WSK Ag) as compared with (WHKK Ag).

**Table 1:** The mean & Standard Error of antibody titer levels in the serum of immunized and control negative group.

Groups	Mean±SE
Whole Heating Killed <i>K.pneumoniae</i> Ag Mice Group	58.00±8.07a
Whole Sonicated <i>K.pneumoniae</i> Ag Mice Group	64.05±5.88a
Control negative Mice Group	4.22±0.04b
LSD	13.333



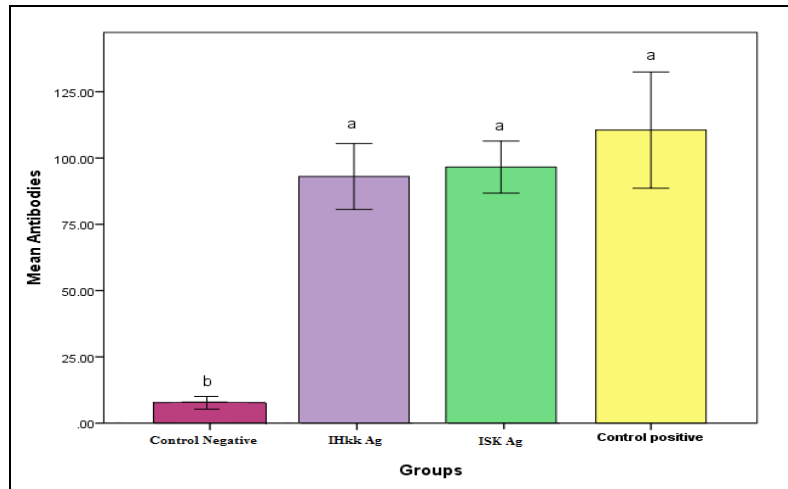
**Fig 1:** The results of the mean of antibody titer levels in the serum of immunized & control negative group.

Also the peak of (O.D) & serum (Ab.) titer level ( $p < 0.05$ ) were significantly observed in the positive control & both immunized infected groups as compared with control negative and non-infected immunized mice groups. The mean value

results of the control positive and the both infected immunized (IWSK & IWHKK Ag) mice groups showed non-significant ( $p > 0.05$ ) gradual increase among them respectively, as clarified in (table 2) & (fig.2).

**Table 2:** The mean & Standard Error of antibody titer levels in the serum of control positive & both immunized infected mice groups.

Groups	Mean ± SE
Infected Whole Heating Killed <i>K. pneumoniae</i> Ag (IWHKK Ag) group	93.05 ± 6.20a
Infected Whole Sonicated <i>K. pneumoniae</i> Ag (IWSK Ag) group	96.58 ± 4.90a
Control positive (CP) group	110.53 ± 10.96a
LSD	27.822
Control negative (CN)	4.22 ± 0.04b



**Fig 2:** The results of the mean antibody titer levels in the serum of control positive & infected immunized (IWHKK & IWSK Ag

**2. Delayed Type Hypersensitivity skin test (DTH)**

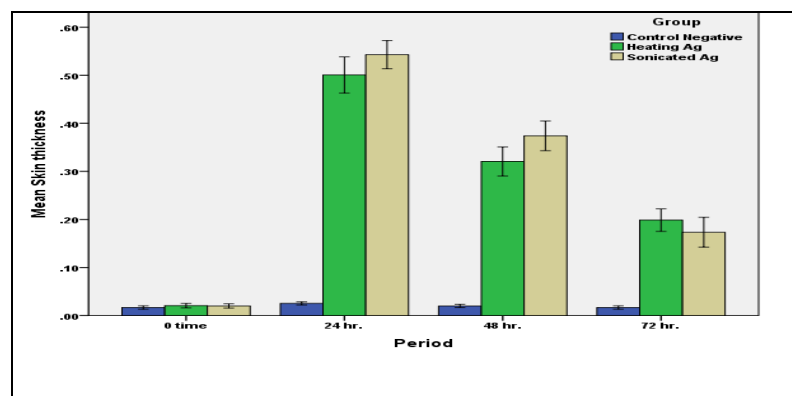
All groups of mice (immunized & control negative) were conducted to (DTH) skin test at (zero time, 24, 48 & 72hrs.) and both immunized (SWHKK & SWSK Ags) groups showed positive reaction after (24, 48 & 72 hrs.) of skin test as compared with control negative group which give negative reaction. The increasing skin thickness of footpad

with(SWSK Ag) at ( 24 & 48 hr.) revealed a significant ( $p \leq 0.05$ ) increase ( $0.54 \pm 0.01$ ) & ( $0.37 \pm 0.01$  mm) respectively than the (SWHKK Ag) group that showed ( $0.50 \pm 0.01$ ) & ( $0.32 \pm 0.01$  mm) respectively with LSD (0.0305), whereas at (72 hr.) post examination, the value was decline in group (1) to ( $0.19 \pm 0.01$  mm) and in group (2) to ( $0.17 \pm 0.01$  mm) as displayed in the table (3) & Fig.(3).

**Table 3:** The Mean and Standard Error of skin thickness of immunized and non-immunized animals (control negative group) before & after (24, 48 & 72hr.) of injection.

Groups	0 time (M±E)	24 hr. (M±E)	48 hr. (M±E)	72 hr. (M±E)
Group1 (Heating Ag)	D 0.02±0.002	A 0.50±0.01a	B 0.32±0.01a	C 0.19±0.01a
Group 2 (Sonicated Ag)	D 0.02±0.002	A 0.54±0.01a	B 0.37±0.01a	C 0.17±0.01a
Group 3 (Control Negative)	A 0.01±0.001	A 0.02±0.001b	A0.02±0.001b	A0.01±0.001b
LSD	0.0305			

Means with different small letters in the same column significantly different ( $P < 0.05$ ).  
 Means with different capital letters in the same row significantly different ( $P < 0.05$ ).



**Fig 3:** Results of mean skin thickness of both immunized & control negative animal groups.

**Histopathological Study**

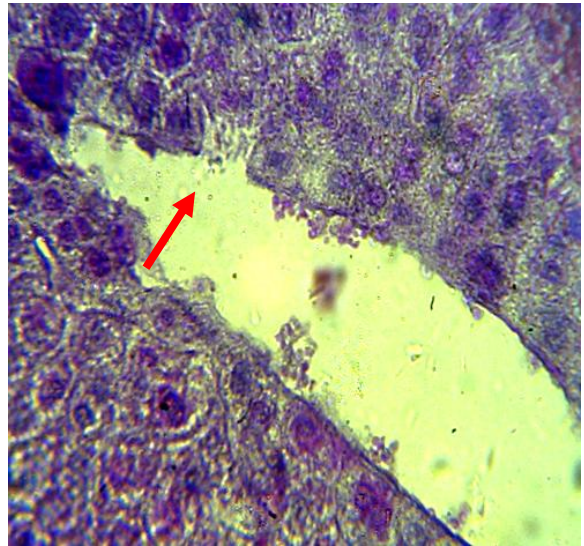
The pathological examination of control group that infected with *Klebsiella pneumoniae* that stained with Hematoxillin & Eosin stain exhibited in lung of control group showed congested blood vessels and inflammatory cells infiltration

like macrophage and neutrophile (fig 4) and in some sections multiple variable size granulomatous lesions (fig 5). Sections of control group when using PAS stain revealed aggregation of *Klebsiella pneumoniae* in the interstitial tissue of the liver (Fig.6), and sections stained by Warthin Starry stain shows

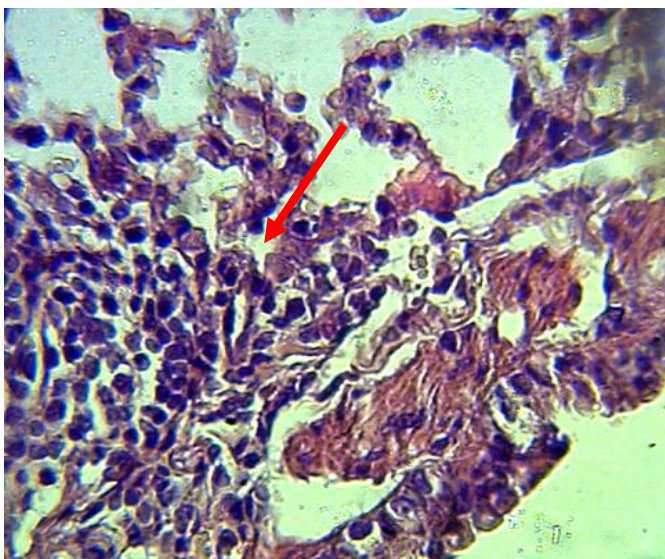
the aggregation of *Klebsiella pneumoniae* in the interstitial tissue in the liver (Fig.7).

(WHKAg) that infected with *Klebsiella pneumoniae* after immunized with Ag. And infected by *K. pneumoniae* stained with Hematoxillin & Eosin stain noticed in kidney showed infiltration of inflammatory cells in the interstitial tissue, desquamation of epithelial lining degenerative cells of renal tubules, mesangial cell proliferation and lymphocytes (Fig. 8) liver shows marked inflammatory cell aggregation around central veins and in the interstitial tissue like lymphocytes and macrophage (Fig. 9).

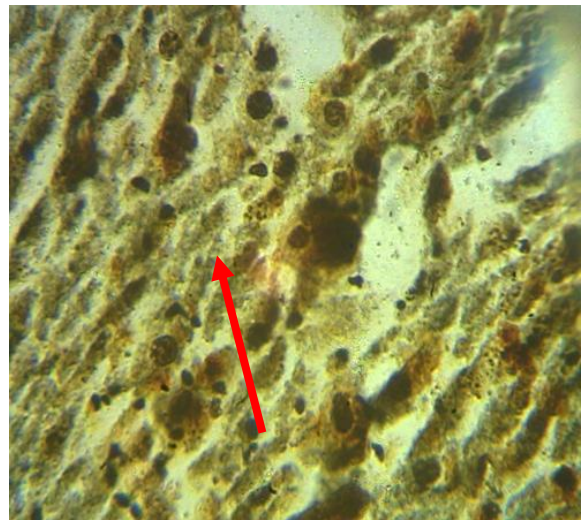
(WSKAg) group that were infected with *Klebsiella pneumoniae* after immunized with Ag. And infected by *K. pneumoniae* stained with Hematoxillin & Eosin stain showed increased thickness of intra alveolar septa of the lung and inflammatory cells infiltration include macrophage and lymphocytes in lung (Fig. 10), and exhibited aggregation of *Klebsiella pneumoniae* in the parenchyma but less than of control group in the organs of immunized animals like liver when stained with PAS stain (Fig. 11).



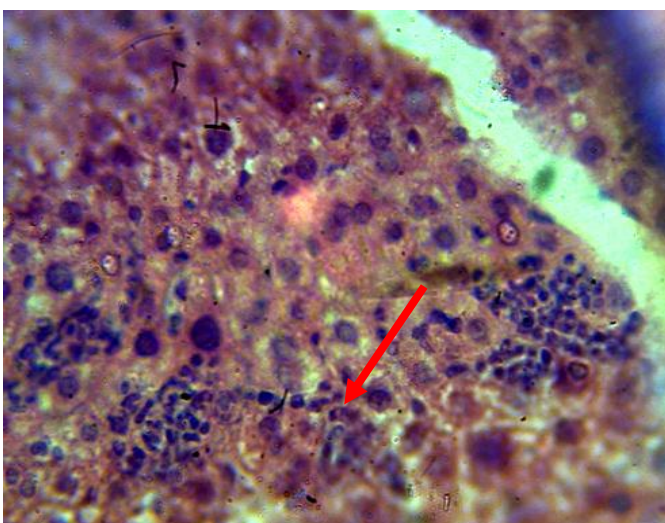
**Fig 6:** Histopathological section of liver of control group shows the aggregation of *Klebsiella pneumoniae* in the interstitial tissue and in lumen of central vein (←) (PAS stain X400)



**Fig 4:** Histopathological section in lung of control group animal infected with *K. pneumoniae* showed congested blood vessels and inflammatory cells infiltration like macrophage and neutrophile (↔) (H&EX400).



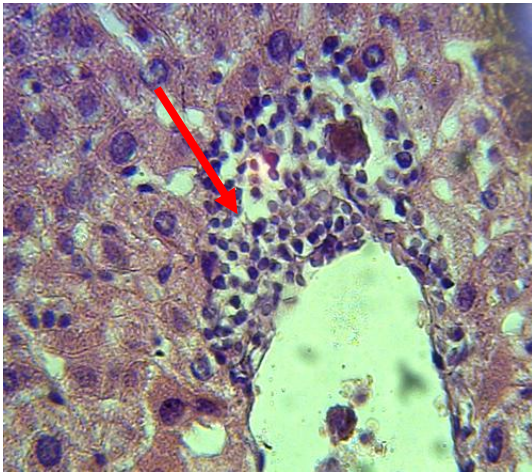
**Fig 7:** Histopathological section of liver of control group shows the aggregation of *Klebsiella pneumoniae* in the interstitial tissue (←) (Warthin Starry stain X400)



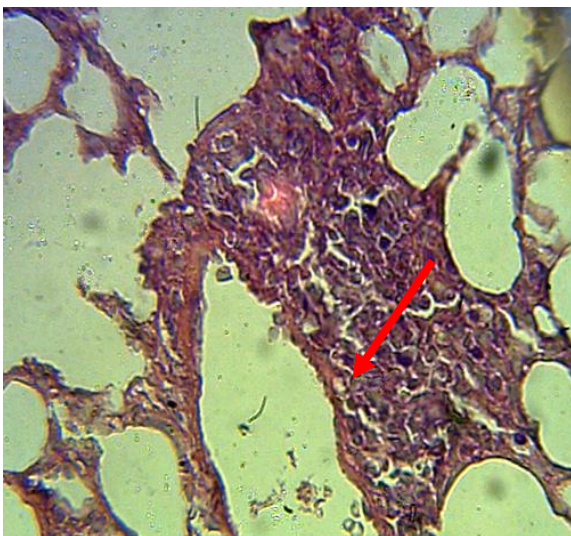
**Fig 5:** Histopathological section in liver of control group animal infected with *K. pneumoniae* showed multiple variable size granulomatous lesions (←) around congestion of blood vessels also scatter throughout the liver (H&EX400).



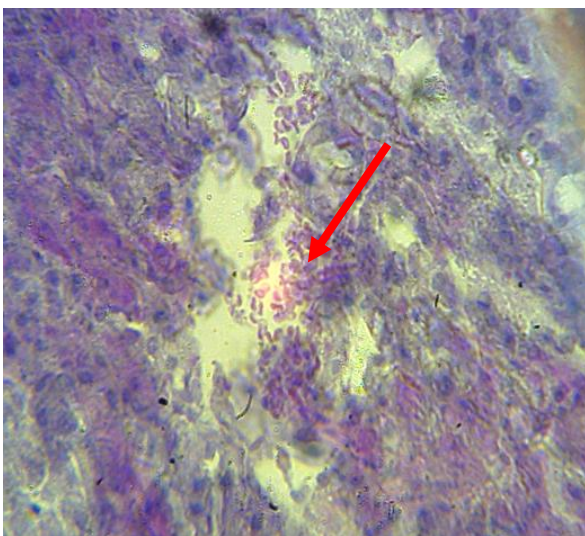
**Fig 8:** Histopathological section in kidney of animal immunized with WHKK Ag. And infected by *K. pneumoniae* bacteria showed infiltration of inflammatory cells in the interstitial tissue, desquamation of epithelial lining degenerative cells of renal tubules, mesangial cell proliferation and lymphocytes (H&EX400).



**Fig 9:** Histopathological section in liver of animal immunized with WHKK Ag. And infected by *K. pneumoniae* shows marked inflammatory cells aggregation around central veins and in the interstitial tissue like lymphocytes and macrophage (←) (H&EX400).



**Fig. 10:** Histopathological section in lung of animal immunized with WSK Ag. showed increased thickness of intra alveolar septa of the lung and inflammatory cells infiltration include macrophage and lymphocytes (←) (H&EX100)



**Fig 11:** Histopathological section of liver of WSK Ag group shows aggregation of *Klebsiella pneumoniae* in the parenchyma (←) (PAS stain X400)

## Discussion

The results showed that *K. pneumoniae* – derived WHKK and WSK antigens elicited both humoral & cell mediated immunity in immunized mice that were agreed with [10].

For each immunized animal group, the skin reaction at the injection site of SWHKK and SWSK antigens was suggested to be mediated by cellular immune response summarized by differentiation of naïve T-cells into memory cells and effector cells (T helper) which in turn produce cytokines that activate / attract the immune cells to the site of infection [11].

Results also showed that the mean values of DTH and serum Ab titer were slightly increase in mice immunized with WSK Ag ( $0.54 \pm 0.01$ ,  $64.05 \pm 5.88$  pg/ml) respectively as compared to those immunized with WHKK Ag ( $0.50 \pm 0.01$ ,  $58.00 \pm 8.07$  pg/ml) respectively. This result was coincided with those observed by [12]; and [10] who emphasized that Th-1 immune response play a fundamental in the eradication of *K. pneumoniae*.

Survival of WHKK / WSK- Immunized mice with after infection with *K. pneumoniae*, indicated that both antigens (especially WSK Ag) enhanced the immunity against *K. pneumoniae*, this result was consistent with [13, 14], who stated that WSK Ag could be used as subunit vaccine (consisting of CPS, LPS and type III fimbriae) which stimulates immunity against *K. pneumoniae* infections.

However, infection of all examined organs of non-immunized animals may indicated that *K. pneumoniae* caused a wide spectrum of severe pathological lesions which upheld by [15] who demonstrated that *K. pneumoniae* was accompanied with massive pathological changes such as suppurative infections in lung, liver, urinary tract and brain, disseminated all over the world. The inflammatory cells infiltration (mainly neutrophils) in examined organs may indicated that *K. pneumoniae* infection activated the pro-inflammatory cytokines production such as IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$  which have prime role in inflammatory cells attraction [16], and [17] revealed that the Enterobacteriaceae bacteria showed that proliferation of mononuclear cells aggregation around central veins as well as congestion of the blood vessels with infiltration of inflammatory cells in the lumen.

Post infection with *K. pneumoniae* can regulate adhesion molecules such as endothelial intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1). However, neutrophils in an acute inflammation are rapidly recruited to inflamed area, releasing their toxic substances, killing the causative agent, and finally initiation of healing process [18].

## Conclusion

The peak of (O.D) & serum (Ab.) titer level ( $p < 0.05$ ) was significantly observed in the control positive & both immunized infected groups as compared with control negative and non-infected immunized mice groups. skin test as compared with control negative group which give negative reaction. post examination, the immunized (SWSK Ag) group revealed significant ( $p \leq 0.05$ ) increase in the mean of skin thickness of footpad against SWSK Ag than the value of immunized (SWHKK Ag) group only, and from histopathological examination appeared that SWSK Ag and SWHKK Ag. Groups protect the experimental animal body against virulence *K.pneumoniae*.

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