Research Article

Some Effects of Prepared vaccine from Purified Extrcellular Toxic Complex (ETC) Produced by *Klebsiella pneumonia* K2 local strain

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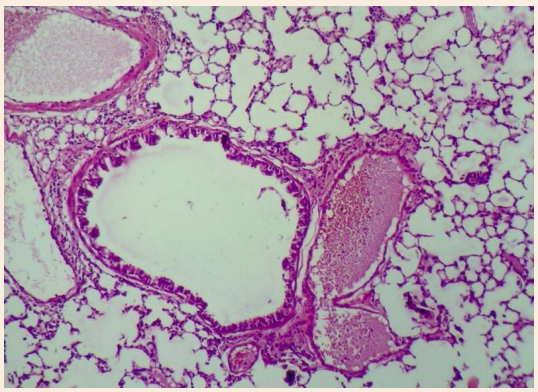
Abstract

Fifty clinical samples collected from sputum of patients who suffered from pneumoniae in Ibn-Balady hospital and the hospital in Baghdad city during the period from November 2012 to May 2013 for the isolation and identification of *Klebsiella pneumoniae*, one of the important causative agents of infection occurs in the lungs. Sputum samples were subjects to the standard laboratory procedures including identification by biochemical test and VIETK system. The isolates were examined to produce extracellular toxic complex (ETC) it was found that the isolate named K_2 was the higher production. The study involved the histopathological changes were noted, abundant mononuclear infiltrate of inflammatory cells with necrosis of lung parenchyma. The second group of mice injected with ETC (0.05 ml) that contain protein (1.085mg/ml**)** represented as sub lethal dose. Histopathological changes were noted showing near of the normal appearance of alveoli and alveolar space, with presence of congestion of blood vessels. The third group of mice inject with (0.5 ml from Tris –base buffer only) represented control showed normal alveoli and alveolar space with presence of bronchial.

In the immunological test the sample ETC examined with ELISA and given IgG titer (189.68±50.70 ng/ml**)** compared with control (46.78±12.45**).** This titer of IgG tested with Double immune diffusion assay and gave precipitation line with antigen compared with control. The study can concluded that the ETC induced immunological response and production antibody in the animal.



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Introduction

An alternative to the use of antibiotics is to attempt immunological control of *Klebsiella* infections which performed by vaccination of patients at risk or by immunotherapy. Anti-capsular polysaccharide antibodies were found to provide a high degree of protection against corresponding capsular serotypes [1]. A good antibody response was observed with this vaccine in patient victims of acute trauma, with nine out of ten patients responding to at least 18 of the 24 antigens [2]. The 24-valent vaccine was used to immunize 2000 healthy volunteers (simultaneously with a *Pseudomonas* vaccine) to obtain a hyperimmune intravenous immunoglobulin preparation, which was tested in a randomized clinical trial in intensive care unit patients [3].

The incidence and severity of infections caused by *Klebsiella* strains with K-types included in the vaccine was not affected by the immunization. It was later found that the hyperimmune immunoglobulin persisted longer than expected in the serum, which may be of interest from a clinical point of view, but difficult to explain [4]. Vaccination

against the *Klebsiella* O- antigen variation is attractive for several reasons. First, it is much less variable than the K-antigen, with only nine distinct serotypes [5 , 6] and with serotype O1 representing a large fraction of the isolates (e.g., nearly 40% of the isolates in the study of [7]. Second, in spite of the presence of the thick capsule, the O-antigens are exposed to the surface, and anti-O1 monoclonal antibodies were shown to be protective in experimental models [8]. Other candidate vaccines against *Klebsiella* infection or its pathogenic effects include a cytotoxin toxoid [9], a conjugate made of O-polysaccharide and iron regulated cell surface proteins [10], and hepta- or monovalent bacterial lysates [11]. The *Klebsiella* outer membrane protein A (KpOmpA) was shown to have properties of a carrier protein when conjugated to *Streptococcus pneumoniae* polysaccharides [12] and is suitable for nasal immunization [13]. KpOmpA interacts with antigen presenting cells by activating them, triggering cytokine production by macrophages and dendritic cells [14]. Most *Klebsiella* infections are acquired during hospital stays and account for 5 to 7.5% of all nosocomial infections.

Toxin produce by *Klebsiella pneumoniae* possessing various degrees of virulence in rats and mice were examined for their *in vitro* production of an extracellular toxic complex (ETC). The ETC has been shown to be lethal for and produce extensive lung pathology in mice. This compound has been shown to be composed of capsular polysaccharide, lipopolysaccharide, and a small amount of protein [15] and the compound that it was of 63% CPS, 30% LPS, and 7% protein. the production of an extracellular toxic complex ETC that appeared to be responsible for lethality and the extensive lung pathology produced by *K. pneumoniae* in animal model [16]. The ETC is probably released as a natural product of cell growth and not due to cell lysis[17] . Since the ETC from *K. peumonia* Immunization of experimental animals with sub lethal doses of the ETC was protective against both homologous and heterologous strains, and this protection was due to antibody production. An examination of the various phases of growth of K. *pneumonia* showed that there was extracellular release of the component parts of the ETC occurring during all phases of growth. The presence of the ETC in the supernatant fluids was due to actual release of this material as opposed to cell lysis. Antibodies to the lipopolysaccharide portion (which has been shown to possess the observed toxicity) of the ETC were protective against the homologous bacterium [15].

Studies employing radial immunodiffusion examining the sera of mice infected with this organism demonstrated *in vivo* production of the complex at levels sufficiently high to produce death. However, the possibility that the toxicity may be associated with a protein has not yet been excluded. If, however, it is the LPS portion that is toxic, it is not entirely clear how LPS is capable of killing experimental animals. When LPS is administered to animals intravenously, a prompt and transitory hypertensive state results, followed by a progressive, severe hypotension [18]. Tissue perfusion decreases, and death is thought to occur as a result of circulatory collapse The cause of the vascular changes which result in circulatory collapse have not been fully determined. The role that the CPS portion of the ETC plays in the toxicity of the compound is not yet clear. The aim of this study is production vaccine from Extracellular toxic complex of *Klebsiella pneumoniae* K2.

Materials and Methods

Production and Purification of the ETC from the *Klebsiella pneumonia* K2 isolate

The production of extracellular toxic complex (ETC) by klebsiella pneumonia K2 isolated according to Al-Sudane [19] and the purification of ETC according to Al-Jumaily and Al-Soundany [20] using two step column chromatography, ion exchange DEAE-cellulose and gel filtration (Sepharose-4B).

Toxin assay *in vivo*

It was determined according to method described by Straus *et al.*,[16]. Three group of mice ,(20 – 25gm) in the first group of mice was injected intrapertonally with 0.5ml of purified ETC toxin that contain (**10.875 mg/ml**) protein and represent lethal dose. The second group inject with (0.05 ml of ETC + 0.45 ml Tris –base buffer) that contain protein (**1.085mg/ml)** represented as sub lethal dose. Third group inject with (0.5 ml from Tris –base buffer only)

represented control. The second and third group of mice killed after 72 hours, the thoracic cavity was opened, and the lungs were aseptically removed, samples of lung tissue were excised from the affected area. Similar area was taken from control mouse lung, and fixed immediately in (10%) formalin.

Histopathological Examination

Lung tissues have been prepared for histopathological examination according to the method described by Junqueira *et al.*[21] using paraffin sections technique.

Immunological Studies

The antigen used in this experiment was ETC which was prepared and purified as described previously, the antigen was dialyzed against distilled water over night ,and protein concentration was estimated as described . The preparation of the first dose of the immunization and other dose was done following the method described by Sikarwar and Batran [22] as follows:-For first dose, equal amounts of Freund's complete adjuvant (1ml) were mixed before immunization with (1ml) of purified ETC contain (800 µg/ml) protein was injected to the experimental animal group, or phosphate buffer saline which was injected to the control animal group.

In the other dose, which consist of (1ml) of incomplete Friend's adjuvant with (1ml) of purified ETC contain (800 µg/ml) protein was injected to the experimental animal or mix with (1ml) of phosphate buffer saline to immunize the control animal group.

Immunization of rabbit with the extracellular toxic complex (ETC)

The immunization of rabbits was done following the method of Sikarwar and Batran [22] as follows: - Eight Newzealand rabbits about(2.5 kg) in weight were used in this experiment, their age range between (6-7 months), these rabbits were divided into two groups shown below : -

The first group (controls) was immunized subcutaneously with phosphate buffer saline with either Freund's complete adjuvant (in the first dose) or Freund's incomplete adjuvant in (other dose).

The second group (experimental) immunized subcutaneously of the (1ml) purified ETC of *K.pneumoniae* contain (800 µg/ml) protein Antigen was given subcutaneously with (1ml) of complete Freund's adjuvant. Five doses of same amount of antigen were given at seven days interval each with incomplete Freund's adjuvant. Antibody titer and specificity of hyper immune sera against purified ETC was checked by ELISA test and double immune diffusion assay.

Collection of serum

After 10 days from the boosting dose , blood was collected from all groups blood sampling and serum preparation were started as described by Al-Hayali [23] as following:-

Five milliliter of blood was collected from rabbit (either control or experimental) by ear vein puncture in sterile test tubes, which were put inclined position at room temperature for 1 hr to induce clotting .The adhesion between the clot and the walls of test tube was -removed carefully by needle , and the test tubes were left in refrigerator for 24 hrs to enhance clot shrinkage and isolated from serum. Serum samples were centrifuged at (3000xg) for 5minutes then the supernatants stored in the refrigerator for further usage.

Immunological test

An enzyme –linked immunosorbent assay (ELISA).

It was prepared according to the kit by Easy-titer mouse IgG and rabbit IgG assay (PIERCE Company) and the absorbance read at 405nm. The standard curve was generated and the sample concentration determined from standard curve (**Figure 1).**

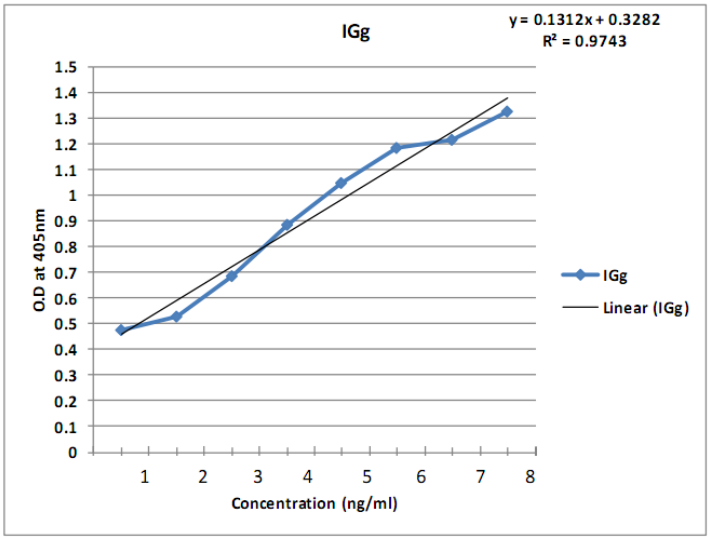

Figure 1 A standard curve of IgG

Double immune diffusion Assay

Immunodiffusion assay was done following the method of Ouchterlony [24], the method based on antigen-antibody diffusion reaction in semi hard media like agarose.

Statistical Analysis

The statistical analysis system, SAS [25] was used to study the effect of different factors in the study parameters least significant difference-LSD test to compare between means in the study.

Results and Discussion

The effects of purified toxin of *K.pneumoniae* K2 on lung tissue of mice *in vivo*

After injection of purified ETC of *K. pneumoniae* K2 i.p with lethal dose (0.5 ml) of purified ETC toxin to the first group that contain (10.875 mg/ml) protein , photographs of Hematoxylin and eosin – stained sections of lung after 4 hours, are shown in **figures (2 and 3)**. Toxin caused many pathological effects in the lung .the section of lung showing abundant mononuclear infiltrate of inflammatory cells with necrosis of lung parenchyma.

The second group of mice inject with (0.05 ml of ETC + 0.45 ml Tris –HCl buffer) that contain protein (1.085 mg/ml**)** represented as sub lethal dose, photographs of Hematoxylin and eosin – stained sections of lung after 72 hours shown in figure (4 and 5) Section of the lung showing near of the normal appearance of alveoli and alveolar space with presence of congestion of blood vessels. Third group of mouse inject with (0.5 ml from Tris –HCL buffer only) represented control photographs of Hematoxylin and eosin – stained sections of lung after 72 hours, are shown in figures (6 and 7).Section of control lung showing normal alveoli and alveolar space with presence of bronchial. Straus et *al.*[16] mention when injection purified ETC was placed in a distal bronchus of the lungs of six mice in

0.5ml of phosphate-buffered saline. This dose represented about 42% of the LD50 for mice. Histological examination of the lung tissue showed extensive lung pathology in the presence of *K. pneumoniae* ETC .This progressive destruction of lung tissue continued up to day 3 post inoculation, when the study was exminated. The lung tissue damage produced by the ETC alone was similar to the destruction produced by an active lobar pneumonia in the rodent lung. Cryz *et al.* [1] Histological examination of lung tissue from infected control animals showed pronounced inflammatory cellular infiltrate in the alveolar spaces, intra- and per bronchial inflammation, and tissue necrosis. In contrast, pathological changes noted in lungs from immunized animals were restricted to infrequent intra- and per bronchial involvement.

Al-Jumaly., *et al* [26] The biological effect of purified toxin of *K. pneumoniae* K8 has been examined *in vivo* by injection of 0.1 ml of toxin that contain protein (35mg/100 ml) transtracheally in a distal bronchus. The exposure time was 72 h, after which the animal was autopsied. Histopathological changes were noted, the alveoli were inflated, the bronchioles were damaged, and the area of frank necrosis was evident.

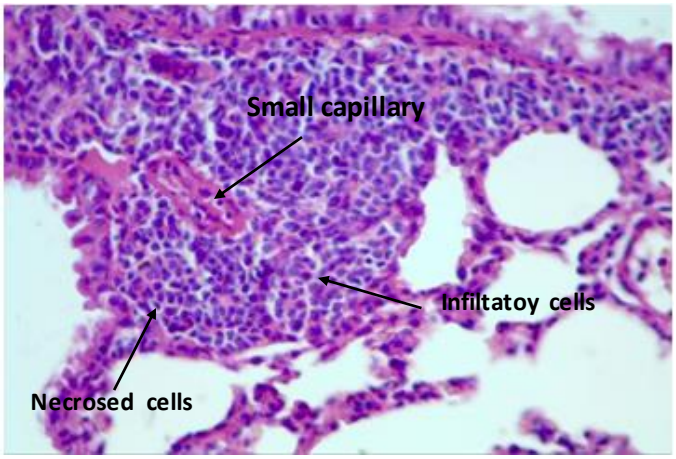

Figure 2 The section of lung treated by lethal dose of pure toxin showing abundant mononuclear infiltrate of inflammatory cells with necrosis of lung parenchyma and *emphysem. X400 (H and E)*

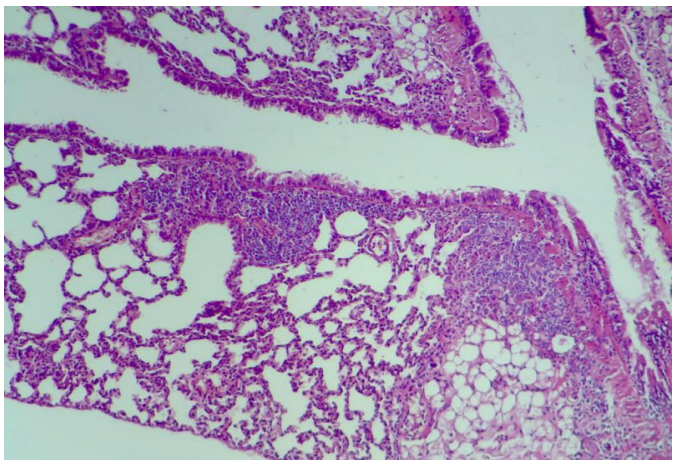

Figure 3 The section of lung treated by lethal dose of pure toxin showing abundant mononuclear infiltrate of inflammatory cells with necrosis of lung parenchyma and emphysema.X100 (H and E)

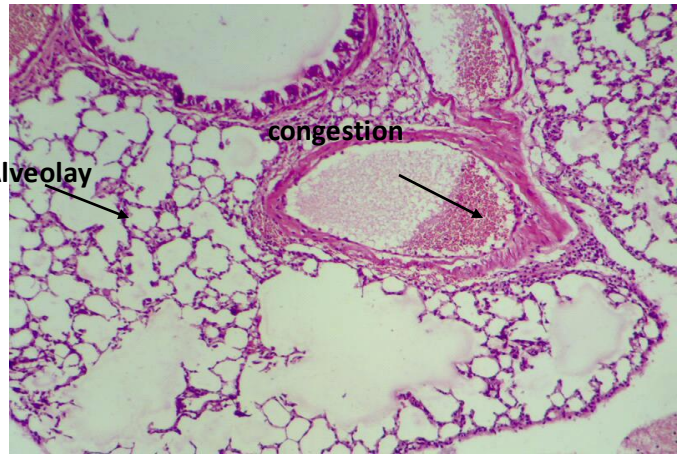

Figure 4 The section of lung treated by sublethal showing near the normal appearance of alveoli and alveolar spacy with presence of congestion of blood vessels . X250 (H and E)

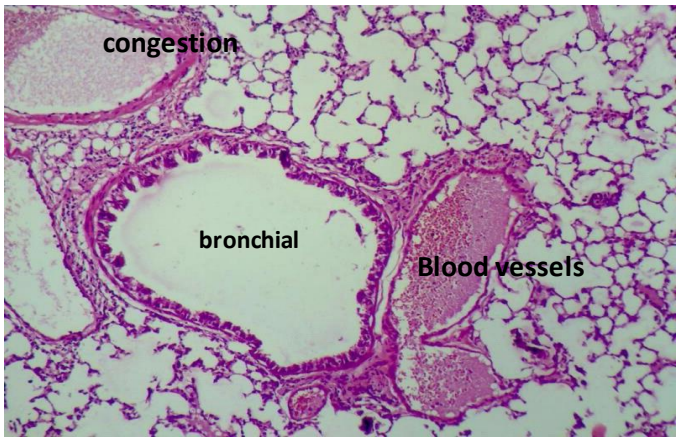

Figure 5 The section of lung treated by sub lethal dose showing near the normal appearance of alveoli and alveolar spacy with presence of congestion of blood vessels. X250 (H and E)

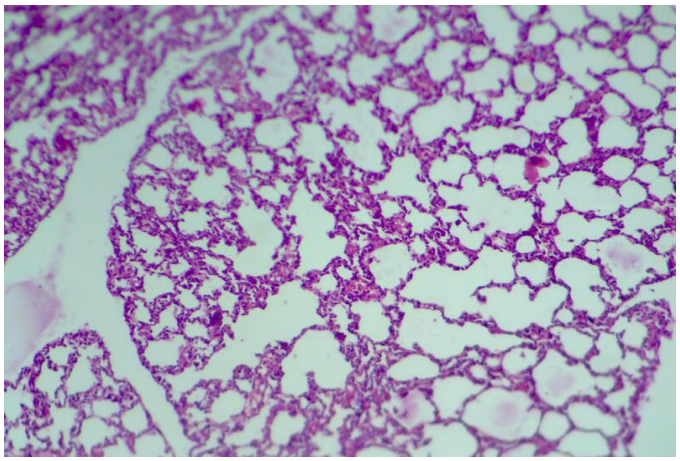

Figure 6 The section of control lung of mice showing normal alveoli and alveolar space with presence of bronchial X250(H and E).

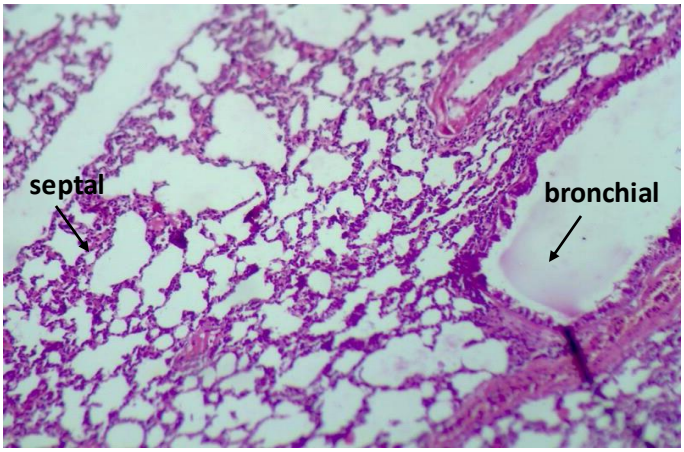

Figure 7 The section of control lung of mice showing normal alveoli and alveolar space with presence of bronchial X250(H and E)

An enzyme –linked immunosorbent assay (ELISA).

The ELISA test was used to determine the titer specific of anti-body in rabbit antiserum results are displeased in table (1) the titer IgG with rabbit treated by purified ETC was shown higher than the titer IgG with the control rabbit ,(Mean 198.68 ng/ml and 46.78 ng/ml) respectively(figure 8). Straus [15] was examined the antibody ETC pattern *in vivo* test against ETC toxicity (mice) and it was found that 80% was protected and Immunization with the ETC of *K. pneumoniae* elicited the production of mouse protective antibodies in rabbits. These antibodies neutralized the toxicity of the ETC and protected mice (probably by opsonisation) against challenge with the homologous organism.

Cryz *et al.* [1] referred the elicited IgG response to vaccination with *Klebsiella* CPSs since this class of anti-CPS antibody has been shown to afford good protection against experimental *Klebsiella* infection. Fung., *et al* [27] referred to LPS induce TNF-∞ Also the capsular polysaccharide (CPS) has ability to stimulate antibody production against *Klebsiella pneumonia*.

Table 1 ELISA test to determine the titer of IgG in rabbit anti serum

	Control			**Treated**	
No. Rabbit	**OD 405 nm**	**ng/ml**	**No. rabbit**	**OD 405 nm**	**ng/ml**
1	0.922	47.889	5	1.224	228.75
2	0.953	55.401	6	1.143	152.5
3	0.901	43.0062	7	1.19	197.5
4	0.892	40.8465	8	1.172	180
	SD	24.910		SD	101.409
	SE	12.455		SE	50.704
	Mean+SE	A 46.78±12.45		Mean+SE	B 189.68±50.70

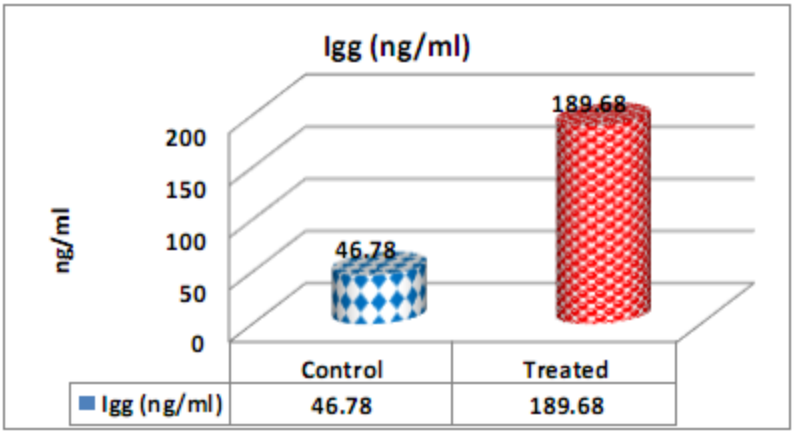

Figure 8 Comparative between control rabbit IgG and treated rabbit with purified ETC.

Double Immune diffusion Assay

The antigen that used in this experiment was a concentrated purified ETC with a protein concentration of (0.5mg/ml) result shown in **figure 9** indicate the presence of the precipitation line between anti- ETC and purified ETC in peripheral wall .No preprecipition line were indicate between the ETC anti body and control serum. This test used in the detection, identification and quantification of antibodies and antigens, such as immunoglobulins. Robert *et al* [28] found that (0.4mg /ml) of CPS of *Klebsiella pneumoniae* type2 given the best precipitation line.

Figure 9 Double immune diffusion assay of purified ETC with anti –ETC antibody

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