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A novel method of preparation attenuated *Klebsiella pneumoniae* vaccine isolated from respiratory tract infections by using low power laser diodes

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ABSTRACT

Klebsiella species are important pathogens in nosocomial infections. The bacteria overcome host immunity through several means. Capsular polysaccharide is the main determinant of their pathogenicity. They cause destructive changes, Necrosis, inflammation, and hemorrhage occurs within lung tissue. The organisms gain access to the host aspirates colonizing oropharyngeal microbes into the lower respiratory tract. Thus this study aimed to produce a vaccine with low cost and effective, throughout uses low power laser light. The result of ID50 of virulent *K. pneumoniae* using (10^8 ,10^6 , 10^5,10^3) cell\ml, when inoculated intra-nasal and mouth, show, the infection and grossly lesions viewed have occurred after two days of inoculation time in all groups of an experiment. Whereas, the results of the same experiment for plotted isolate low power laser light with using methylene blue, Rhodamine 110, and Anthocyanin show no pathogenic lesion grossly viewed. As well as for that experiment related to a formulated vaccine. Challenge test shows the activity of mice, and the anatomical section with clear appearance, in comparison with control groups that elicit severe purulent inflammation of pleura and abscesses loci in the lung with congestion. These finding may be related directly to the elevating of INF-γ and IL8, and obviously increasing of IgA level.

Keywords: *Klebsiella pneumoniae*, Low power laser light, Methylene blue, Rhodamine 110, Anthocyanin.

1. INTRODUCTION

Klebsiella species belongs to the family Enterobacteriaceae. They are generally facultative anaerobes [1]. The genus consists of over 77 capsular antigens (K antigens), leading to different serogroups [2]. *K. pneumoniae* is an opportunistic pathogen and causative agent of many diseases. Characterized by luxurious capsular polysaccharide (K antigen)and lipopolysaccharide O side chain (O antigen) are two of the most important virulence factors [3]. They serve to protect the bacterium from phagocytosis by the host. As well as biofilms are formed in vivo protect the pathogen from attacks of the host immune responses and antibiotics [4], and often display multidrug-resistance phenotypes that are commonly caused by the presence of extended-spectrum β-lactamases or carbapenemases, making it difficult to choose appropriate antibiotics for treatment [5]. It has been considered a respiratory pathogen that causes pneumonia, the symptoms include a toxic presentation with sudden onset, high fever, and hemoptysis. Diagnosis through chest radiograph looks for abnormalities such as bulging inter lobar fissure, and cavity abscesses [6]. In addition to the K and O antigens, it has several virulence factors depending on the sites of infection such as type 1 and 3 fimbriae (pili), serum resistance, siderophores, bacteriocin and extended spectrum β-lactamases (ESBLs) [7]. *K. pneumoniae* opportunistically infects a variety of mucosal surfaces including the genitourinary tract and lower respiratory tract (LRT) [8]. Pneumonia caused by this bacterium is a fatal disease with mortality rates up to 22.7% [9]. The incidence of *K. pneumoniae*

pneumonia is more commonly associated with the nosocomial infection rather than environmental sources [11]. The most prevalence virulence factors that mediating the virulence of *K. pneumoniae* in the lung, including a capsular polysaccharide, LPS, enterobacterial common antigen, OmpA, OmpK36, the AcrAB efflux pump, and the biofilm-related factor YciI, [12]. In last two decades, laser light with different wavelength has been used widely around the world in many medicinal and industrial procedures. Moreover, diode lasers are now commonly used for photodynamic therapy (PDT) and have the additional advantages of being relatively low cost and use normal mains voltage as a power source. Therefore this study is aimed to find a new method of attenuating an encapsulated pathogenic bacterium isolated from severe cases of respiratory tract infections, and as an alternative method instead of classical techniques such as (heat, chemicals, and others).

2. MATERIALS AND METHODS

Depending on Al Saadi and coworkers [13], the most resistant *K. pneumoniae* isolate for most usable antimicrobial disks was selected for further uses in the experiments.

2.1 Determination of infectious dose 50(ID_{50}) of selected virulent isolate [14]

By using McFarland (tube 0.5), different bacterial isolate concentrations (10^8, 10^6, 10^5, 10^3) cell\ml are prepared from the stock growth of overnight activated bacterial isolate, using Brain heart infusion broth / Himedia (India).

2.2 *In vivo* test

Fifty albino mice weight (25-30)g are used after adaptation for three days in animal house / Biotechnology center /University of Al-Nahrain. In determining the ID50 of selected pathogenic K. pneumoniae. Lab. animals are divided into five groups, four of which are used for tested bacterial isolate concentrations, and the fifth one is represented as the control group. Each group of treated mice inoculated with 0.1ml of activated virulent bacterial isolate inoculums and the control is treated with normal saline only throughout mouth and nose. All groups were monitored during a period of one week. After that, the lab animals are anesthetized by inhalation using chloroform (BDH), and then the animals are sacrificed, and a surgical section was done in the thoracic cavity to investigate about pathological changes that have been occurred.

2.3 Selection of the suitable conditions for attenuating isolates

Three different photosensitized dye s are used, Methylene blue (Lambda Physik, USA), Rhodamine-110 (Lambda Physik, USA) and Anthocyanin (plant source (beetroot)/Iraq-Biotech.dept.). And according to [13], three plan tubes included activated bacterial isolate are mixed well with 10-4 of photosensitized dye and plotted to one of laser light using CW Diode Laser (Aiwa /China) at 650nm for Methylene blue (BDH/ England), and DPSS Laser /Diode Pumped Solid state laser (Aiwa /China) at 532 nm. for Rhodamine-110 and Anthocyanin. The bacterial isolate that elucidated some variation in several phenotypes that related to the virulence factors was selected.

2.4 Determination of infectious dose 50 (ID50) using bacterial isolate plotted with low power laser light

Depending on the changes that have been occurred in several bacterial phenotype parameters due to plotting to low power laser light such as antimicrobial sensitivity, colony morphological, and biochemical test, that previously characterized the virulence selected *K. pneumoniae* [13]. Determination of ID50 of selected plotted *K. pneumoniae*, was done by using 30 albino mice divided into 5 groups according to [14], each group included 6 mice demonstrated as; Rhodamine-110 (6p), Anthocyanine (3p), Methylene blue (6p), and activated virulent *K. pneumoniae* respectively, the last group is represented as control group. The inoculum of the bacterial isolate was 108 cell\ml, and the procedure was done by inoculating 0.1ml of each prepared plotted isolate in mouth and nose (each was inoculated with 0.05ml) of mice and monitored for 1week. After the period was ended, lab animals were anesthetized by inhalation using chloroform (BDH). Blood samples were pooled from the heart using 3ml syringe (Set®inject/Germany) and transferred to plane tubes, by centrifugation at 2000 rpm for 10min the serum was separated, collected, and stored at a temperature(-16°C), for further tests such as antibody titers and cytokines (IL4, IL8, IL12, INFγ, and IgA) level measurement. Simultaneously, the anatomical section of mice thoracic cavity was done.

2.5 Preparation of formulated vaccine: According to the Stanley *et al.,*[15] the vaccine was prepared. The following procedure was done in Al-Razi center for kits preparation\ Ministry of an industry.

2.6 Preparation of inoculums: Depending on the McFarland (0.5) 10^8 cell/ml, attenuated plotted *Klebsiella pneumoniae* isolate was prepared as a suspension and then precipitated by centrifugation at 8000 rpm /10 min.

2.7 Preparation of stabilizer: Stabilizer is one of the important materials that the vaccine must be composed, the following materials were suggested to compose in the vaccine.

Ascorbic acid PROLABO/French phrase	0.01 g/L
Lactose BDH/England	0.1 g/L
Sucrose BDH/England	0.026 g/L
Glycine BDH/England	0.0014 g/L

Each one of this material is dissolved in 0.5ml of phosphate buffer saline (PBS) and sterilized by closed system filtration with Millipore filter paper 0.45µm. (Milliporecorp / USA)

2.8 Preparation of preservative

Because the suggested vaccine was attenuated bacteria, therefore the usable preservative must be unusual with the common bacterial vaccine, thus we selected the antimicrobial agent that exhibited no effect on the selected bacterial isolate (highly resistant), in addition, characterized as a wide spectrum antimicrobial agent, hence Erythromycin is represented as a preservative in cons. 0.015g\0.5ml of PBS. Erythromycin is the drug of choice for those suffering from penicillin allergy as it has antibiotic spectrum quite similar to penicillin [16]. All the components of stabilizers and preservative were mixed together and added to the pellet of bacterial isolate inoculums, and mixed by a vortex (Whirlimixer / England) for 2 minutes. Then the mixture was distributed in several sterile vials each one included 0.5ml and stored for 1day in deep freeze (-70 C°), and lyophilized for 2-3 days, finally stored in low temperature at -16 C° .

2.9 Schedule of immunization

Depending on the results of ID50 of plotted isolates, immunization procedure was carried out according to the method described by Sikarwar, and Batra, [17], which was achieved as mentioned in ID50 determination, using inoculum at 108 Cell/ml, and the period of monitoring are 2 weeks instead of 1 week .

2.10 Challenge test

After immunization period, the mice were re-inoculated with activated virulent Klebsiella pneumoniae, then monitored for 1week subsequently. After that, mice were anesthetized by using chloroform. Blood samples were pooled from the heart and separate the serum by centrifugation at 2000 rpm for 10min, and then collected the serum samples for each group separately, and stored at a temperature(-16C°), For further tests such as IgA and cytokines (IL4, IL8, IL12, INFγ, and IgA) level measurement.

2.11 Quantitative determination of mice serum cytokines (4, 8, 12), INFγ and Ig:

According to the company instructions (AVIVA SYSTEMS BIOLOGY/ USA) the procedure of Interleukins (4, 8, 12), INF γ, and IgA were done.

3. RESULTS AND DISCUSSION

3.1 Determination of infectious dose 50(ID50) of *Klebsiella pmeumoniae*

Infectious dose (ID50) is that quantity of pathogenic microorganisms that will produce obvious infection in 50 percent of the test subjects. The result of ID50 experiments using (108,106, 105,103) cell\ml, when the inoculum is applied locally (mucous membrane of nose and mouth), show, the infection occurs after two days of inoculation time in all groups of an experiment, the lab. animals grossly suffered from increasing in body temperature, with loss of appetite, accelerate in breathing and dullness. After the period is ended, the anatomical results of the animal chest region in all groups, exhibit accumulation of pus in the thoracic cavity, with miscellaneous spots of lung abscesses and purulent inflammation of pleura (Figure1).These results were agreed with [18], who reported that inoculation of the mice into the lungs with K. pneumoniae, which has a thick capsule, caused an expensive, voluminous pneumonia characterized by thickening of alveolar septa with infiltration of inflammatory cells and accumulation of bacteria in alveolar spaces. Whereas disagreed with Won-Hee Lee *et al.,* [19] who determining the lethal dose of K. pneumonia infections was 1×106 when injected intraperitoneally into mice. The disagreement may be due to the difference in the route of administration which was used. Thus we can suggest that any bacterial isolate concentration led to infected mice, and simultaneously reflects the high bacterial pathogenicity (Figure 1).

3.2 Determination of ID50 by using selected plotted isolates to low power laser light

The results of inoculation mice with selected exposed isolates show, slightly hyperthermia with dullness for the first three days of inoculation, then all mice returned to normal state with no mortality, the periodic raising of temperature indicated to the response of immune system against infection particularly K. pneumoniae included endotoxin(LPS) in the cell wall act as pyrogenic agent, also the temperature is one of body defense mechanism due to secretion of several interleukins such as IL-8, and other pro-inflammatory cytokines as Chen and coworkers [20]mentioned. After a week of inoculation, the blood samples were pooled and the anatomical section was applied in the thoracic cavity. The result is revealed, no changes occurred in all organs and membranes of thoracic cavity for all tested groups of mice (Figure 2).

3.3 Vaccination by using formulated plotted isolates

Depending on the results of previous ID50 experiments, the three isolates which are selected according to [13], The results of inoculation with formulated selected exposed isolates using intranasal and orally inoculations show as in experiment before, slight hyperthermia but beyond one day of inoculation, then returned to normal state. After two weeks, re-inoculation of mice with activated virulent Klebsiella pneumoniae as a challenge test the results show, afterward one week of the second inoculation the mice remain active, and the anatomical section of thoracic cavity of mice for (Rhodamine-110, Anthocyanine, and Methylene blue) groups, exhibit clear thoracic cavity (Figure3 A, B, C), in comparison with control group that show severe purulent inflammation of pleura and abscesses loci in the lung with congestion (Figure 1).

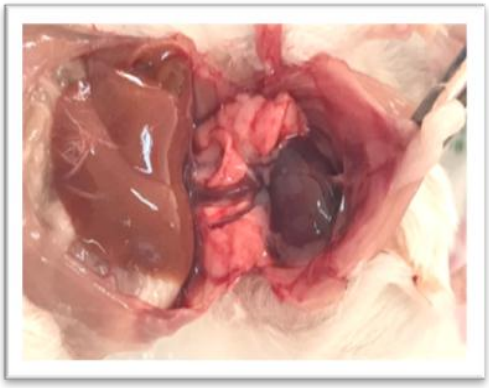
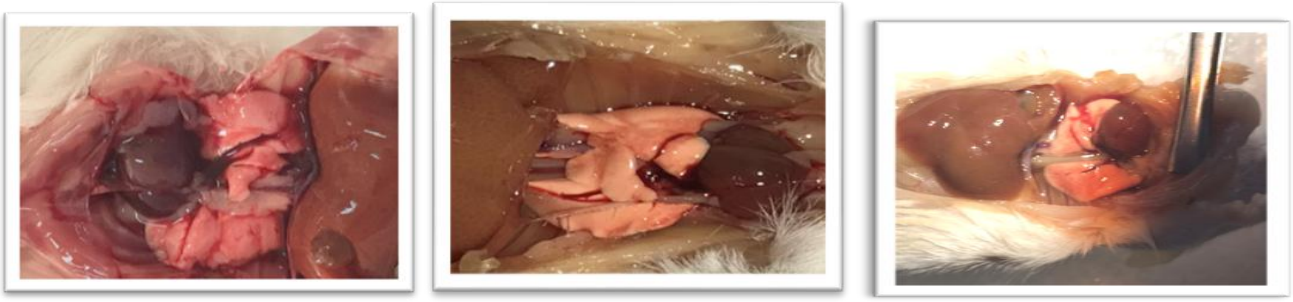


Figure 2: The anatomical section of mouse thoracic cavity inoculated with exposed *K. Pneumoniae* to photosensitized dye and laser light.



A-Anthocyanine B- Rhodamine-110 C- Methylene blue

Figure 3: The anatomical section of mice thoracic cavity treated with formulated *K. pneumoniae* exposed to photosensitized dyes and low power laser light.

3.4 Determination of some immune parameters

Interferon-γ (Type II) is synthesized mostly by Th1 lymphocytes and by NK cells after an antigenic stimulation [21]. Interestingly, natural killer cells can furthermore function as "adaptive effectors" once activated by T cell-induced INF-γ or when IgG eventually elicits antibody-dependent cell cytotoxicity. INF-γ secreted to activate macrophages and to induce their microbicidal functions, On the other hand, this cytokine is very important in the stimulation of both immune responses (innate and adaptive), throughout the macrophages which acts as antigen presenting cells then stimulate Th1 [22]. Attenuated preparation of bacteria that induce an acute infection generally have been highly effective at generation CTL responses as Stanley et al., [15] mentioned. The results of INF-γ in the present study show a significant elevation in all tested groups in compared with control group, meanwhile there were significant differences among treated with attenuated isolate groups, all groups show significant elevation except that related with methylene blue in compared with control group, the difference among these groups may be related with the wavelength that correlated with type of photosensitized dye, in addition to the bacterial susceptibility to laser dye [15]. As well as all vaccinated groups elicit elevation in the conc. of this cytokines particularly that related to methylene blue and anthocyanin in comparison with control, that reflected a good formula has been used in the preparation of vaccine, especially when uses the bacterial components as an adjuvant, thus we can suggest that there is a synergistic effect has occurred among the components of the vaccine. Whereas IL-4 is a cytokine that participates in the regulation of the immune system at multiple levels [23]. It is a growth and survival factor for lymphocytes, as well as participates in the differentiation of naïve CD4+ T cells into the Th2 type and is important for the production of allergen-specific IgE [24]. The recent results, (table 1) showed a variation among tested groups ranged from no change to a significant elevation compared with control group. Because IL-4 drives the alternative activation of macrophages, which has been shown to increase allergic lung inflammation in mouse models and to be correlated with asthma severity in asthma patients as Dasgupta and coworkers [25] mentioned. Thus the recent result was considered excellent exclusively for using attenuated bacteria alone or formulated (vaccine), which revealed no effect on the level of IL-4 for Rhodamine-110 and Methylene blue groups, in compared with that of Anthocyanine and control group .while IL-8 (CXCL8), is a critical inflammatory mediator. It was originally identified for its role in chemo-attraction of neutrophils, for which it was named neutrophil chemotactic factor (NCF) and neutrophil activating protein (NAP-1) [26]. The results in Table1, show a significant elevation in the level of this cytokine in all groups except that related with methylene blue (attenuated bacterial group), in comparison with

control group. Since IL-8 is responsible for the chemotactic migration and activation of immune cells as monocytes, lymphocytes, basophils, and eosinophil at sites of inflammation as Guillermo Duque and his partner [27]mentioned, therefore increasing the level of this cytokines led to the determination of infection, as well as counteract the dissemination of infection and eliminated locally. Interleukin-12 (IL-12) is a pivotal regulatory cytokine that preferentially activates natural killer (NK) and Th1 cells to produce interferon gamma (INF- γ), both cells are related against infections and tumor cells, in bone marrow and peripheral organs like lung and spleen. Because the lung is an organ in contact with airborn pathogens and the site of inflammatory disorders triggered by the respiratory environment. Therefore lung NK cells differ in phenotypic and functional characteristics. Indeed the lung macrophages have the ability to influence the cytotoxicity of NK cells by cell-to-cell contact. This suggests that the differences of NK cell subsets are in part due to a modulation by the organ environment [18]. The results in this study show, decreasing in the level of this cytokine in treated groups, this results may indicate to the changing has been occurred in plotted isolates led to declines in stimulation of NK cells that led to decreasing in IL12 level [28].

On the other hand, Immunoglobulin A (IgA) is the most abundant antibody isotype separated in the body and has an important role in the immune responses at mucosal surfaces such as the gastrointestinal (GI), the respiratory, and the reproductive tracts [29]. The present results of IgA elucidated significant increasing in all treated groups in comparison with control group, this finding directed toward the effect of low power laser light and its dyes on exposed isolate to stimulate humoral immunity to produce IgA, and because there are two types of IgA (IgA1 and IgA2), the first one is related to upper part secretions of the body, while the second with the lower part secretions [15], therefore we suppose, IgA1 was over produced than IgA2, and neutralized the extracellular isolate that inoculated through nose and mouth in challenge test, and then counteract the ability of virulent K. pneumoniae to produces infection, as it obviously appeared in anatomical sections of thoracic cavity of mice.

Table 1: Some immune parameters levels for formulated and non-formulated exposed *K. pneumoniae*

Inculcation composition	INF-γ	IL-4	IL-8	IL-12	IgA
Cont. virulent	108.78± 2.31	69.5 ± 1.52	34.09 ± 2.51	485 ± 1.78	60 ± 3.25
Cont. 6pass.	129.65±1.77	49.8 ± 2.15	16.14 ± 2.43	428.3 ± 2.1	81 ± 2.01
Rhodamine-110 formulated vaccine	198.35± 0.33*	49.2 ± 1.75	158.2 ± 1.95*	171.67± 1.7	222 ± 1.72*
Rhodamine-110 attenuated	198.78 ± 2.13*	49.8 ± 1.34	151.36 ± 1.39*	245 ± 1.52	214 ± 1.45*
Anthocyanine formulated vaccine	338.8 ±1.56*	126 ± 1.2*	168.6 ± 1.8 *	353.3 ± 1.2	310.33 ± 1.32*
Anthocyanine attenuated	312 ± 1.63*	137.6 ± 1.02*	82.27 ± 1.36*	150.83± 1.3	320.3 ± 1.74*
Methylene blue formulated vaccine	497.04 ± 4.23*	54.4 ± 2.81	76.82 ± 1.92*	428.33± 1.9	280.33 ± 1.78*
Methylene blue attenuated	104 ±1.23	74 ±1.72	25 ±1.35	499.2 ±1.6	237 ±1.50*

*Significant value at P≤ 0.05

Thus we can conclude, attenuated isolate by using low power laser light with different photosensitized dyes (anthocyanine, Rhodamine-110, and Methylene blue), either formulated or not, are protected directly from *K. pneumoniae* infection. This protection was achieved through an elevation in the levels of INF-γ and IL8, which emerged obviously by increasing in IgA level.

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