Preparation of Vaccine for pathogenic bacteria causing abortion using low level diode laser

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البحث مستل من رسالة الطالب a

Abstract:

The objective of this study was investigate the effect of Low Level diode laser on the bacterial isolates (*Listeria monocytogenes, Enterobacter cloacae, Klebsiella pneumonia, Staph aureus, Esherichia coli and Pseudomonas aeruginosa*) which used in preparation of vaccine and to determine the immune responses using the RID kit. The antibacterial agent's sensitivity before irradiation was performed by using twelve antibiotics, these strains showed high resistance for these antibiotics except amikacin. But after irradiation the sensitivity was increased. Some features were studied such as bacterial count which decreased and lost of blood hemolysis and biocyanine stain from p.aeruginosa according to increasing the time and power of irradiation. Bacterial growth killed and attenuated to prepare vaccine using diode laser (660) nm of wavelength having (50 and 250) mw of power and the frequency (1-10) kHz. The experiment was conducted on 24 adult white New Zealand male rabbits with (1.5-2) Kg body weight each, they were divided into sixth groups with 4 rabbits each and inoculated with killed and attenuated vaccine and one group was used as control. After (35day) of immunization, determination was done for (IgM, IgG, IgA in addition to C3 and C4). The Ig and Co. of immunized animals were higher (P>0.05) compared with control animals, also the live attenuated vaccine induced highly immune response as compared with killed vaccine. The control group died after challenge dose while the immunized animals not.

Key words: - Bacteria, vaccine, laser.

الخلاصة

تهدف الدراسة الى التحري عن تاثير الليزر الواطئ الطاقة على العزلات البكتيرية (*L.monocytogenes, E.cloacae, K.pneumonia, Staph aureus, E.coli and P.aeruginosa*) المستخدمة في تحضير اللقاح وقيست الاستجابة المناعية ضد اللقاح بطريقة الانتشار المناعي في هلام الاكاروز . فحصت الحساسية البكتيرية لـ 12 مضاد حيوي و جميعها اظهرت مقاومة لها عدا amikacin ، كما درست بعض الخصائص الاخرى بعد التشعيع بالليزرذو الطول الموجي 660 نانومتر وطاقة خرج قصوى (250 و50) ملي واط وتردد من (1-10) كيلو هيرتز وقد اظهرت النتائج تاثيرا واضحا لليزر على الحساسية البكتيرية وصولا الى قتل البكتريا وايضا على العد البكتيري الحي والذي تناقص بزيادة التعرض للاشعاع (بكتريا مضعفة) حتى انعدام العد (بكتريا مقتولة) ولوحظ ايضاً اختفاء

صبغة البايوسيانين المفرزة من بكتريا p.aeruginosa بزيادة التعرض للاشعاع . اجريت التجربة على 24 ارنب نيوزلندي من الذكور البيضاء تتراوح اوزانها من 1.5-2 كيلوغرام وقسمت الى 6 مجاميع بواقع 4 ارنب في كل مجموعة تمهيدا لحقنها باللقاح الحي المضعف واللقاح المقتول . وبعد انتهاء فترة التمنيع (35) يوم، تم قياس الكلوبيولينات المناعية والمتمم والتي ازدادت بصورة ملحوظة. عالية احصائيا بمستوى معنوية ($P > 0.05$) للحيوانات الممنعة مقارنة بحيوانات السيطرة كذلك اظهر اللقاح الحي المضعف تفوقا على اللقاح المقتول وبعد اعطاء جرعة التحدي لكل حيوانات التجربة فقد هلكت حيوانات السيطرة ولم تحدث اية هلاكات في المجاميع الممنعة.

1- Introduction:

Abortion defined as the termination of pregnancy at any stage that does not end with live birth, though it is often technically defined as the termination of pregnancy by the removal or expulsion from the uterus of a fetus or embryo before fetal viability, (1). *L.monocytogenes* is an important pathogen of humans and animals due to its capability for invasion of nonphagocytic cells and its replication in the cytosol of these cells, (2). *K.pneumonia* is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics and showing characteristic radiographic abnormalities due to a severe pyogenic infection, which has a high fatality rate if untreated, (3). According to the CDC, *E.cloacae* is responsible for bacteremia, endocarditis, osteomyelitis, septic arthritis as well as infections in the skin, respiratory tract and urinary tract. Many of these infections are nosocomial, (4). *P. aeruginosa* is an opportunistic pathogen does not infect healthy individuals, but does cause a wide variety of severe infections in immunocompromised patients. For that reason, *P.aeruginosa* categorized as an opportunistic pathogen, (5). *E.coli* defined as the one of several types of bacteria that normally inhabits the intestine of humans and animals. Their pathogenisity is due to their ability to produce many virulence factors including many enzymes and toxins which help bacteria to avoid immune response and resist antibiotics treatment, (6). *S.aureus* is a frequent pathogen in nosocomial infections and limited outbreaks in hospitals. *S.aureus* and *E.coli* are among the most frequent causal organisms in human bacterial infections, (7).

The word laser is an acronym for the words; Light Amplification by Stimulated Emission of Radiation. It is a light source but it is very highly different from many traditional light sources, (8). The energy level is the quantum state of an atom or molecule, which ranges from a base or ground level to a high level in which this species driven to a state of excitation, (9). Susceptibility of bacteria to laser light varied from one Genus to another ranging from highly effect on their metabolic mechanisms reaching killing by laser treatment, (10). He-Ne laser (632.8 nm) used to cure DNA plasmid of *E.coli*, and it was done by this wavelength. In the current study, chopped 660 nm

Diode laser used to investigate its effect on bacterial growth, which used in preparation of vaccine in this study.

Vaccine is a biological preparation that improves immunity to a particular disease. Vaccine typically contains an agent that resembles a disease-causing microorganism, and often made from weakened or killed forms of the microbe or its toxins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of the microorganisms that it later encounters (11). There are several types of vaccines in use such as Killed, Attenuated, Toxoid, Subunit and Conjugate vaccine. These represent different strategies used to try to reduce risk of illness, while retaining the ability to induce a beneficial immune response, (12).

2-Materials and Methods:-

2-1-Bacterial isolates:

Six isolates of bacteria were obtained from aborts women. The samples identified according to Berge's manual. The identification tests including cultural, morphological characteristics, microscopic examination by using Gram stain, Hemolysin Detection, CAMP test and biochemical characteristics was done for each isolate, in adding to Antibiotics Sensitivity Test and Identification by using API system (13).

2-2-Laser Device and Irradiation Setup:

Omega diode laser was used in this study, the device emits the wavelengths (660 nm), the out put power (50 and 250) mw with different frequencies (1kHz, 5kHz**,** 10kHz) and exposure time adjusted to (5, 10, 15, 20 , 30 and 50) min. The set up of diode laser is illustrated in Figure (1).

2-3-Irradiation of Samples and Preparation of vaccine:

One isolate of each bacterium was chosen to study laser effect on bacterial growth. Six isolates of bacteria cultured on blood agar at 37˚C for 24hr. harvesting the surface of the plates with normal saline using glass rods. And using cold centrifugation at 6000 rpm for 10 min, Cell pellets were washed twice with physiological saline then mixed by vortex and re suspended in 5 ml of normal saline (pH=7.2) and compared with McFarland solution. The bacterial suspension irradiated with laser to obtain live attenuated and killed vaccines. The bacterial suspension was examined by culturing on blood agar to confirm the sterility of the antigen, (14). 24 healthy rabbits randomly divided into six groups and inoculated with killed and attenuated vaccine and using one group as control.

3-Result and discussion:

In this study we obtained six isolates of bacteria from aborts women in sammawah city, and we found that the laser effect on bacterial count and

hemolysin production which is decreased until lost by increasing dose of irradiation. Also The susceptibility of isolates to antibiotics which were resistant before irradiation but they render sensitive after irradiation, as figure (2), which shows the inhibition zone diameters of *L.monocytogenes,* before and after irradiation at, (50mw, 10kHz, 15min).

Laser irradiation may effect in one of these ways, it may affect the pumping system, which mainly responsible for multidrug resistance including beta lactams and aminoglycoside group (amikacin), or alter the target site of these antibiotic. These results have a good agreement research, (15). One of the most important ways of laser efficiency is the irreversible inhibition of plasmid DNA activity after laser irradiation which may destroy them and unable the plasmid to produce beta–lactamase enzyme, Increasing outer membrane permeability which is also associated with other antimicrobial agents such as the beta–lactams. Increase bacterial sensitivity to amniglycosides group may be due to increase the penetration of antibiotic by reducing the lipopolysaccaride layer (LPS) which plays a role in resistance processes (16). The resistance can be both chromosomal or plasmid mediated. Laser irradiation can break strand DNA plasmid thus the bacteria may loss their ability to resist such type of antibiotic, (17).

The effect of laser on the bacterial chromosome may lead to loss their hemolysin production and virulence of bacteria, (18). The results showed that hemolysin production was lost or decrease at wavelength 660 nm with different times and power of the irradiation. The results of irradiation showed a significant decrease in the bacterial viability as the dose increase. Bacterial irradiation with laser may induce cell wall, protein synthesis, membrane function, nucleic acid and metabolic processes inhibition. Table (1) shows the results of irradiation with diode laser using wavelength 660nm, power (50 and 250) mW, frequency 10 KHz and time (5, 10, 15, 20, 30 and 50) min.

When the rabbit is exposed to the organism (also called antigen) its response is that the body immune system will be stimulated to produce antibody, which is directed specifically against that antigen. The body immune system has the capacity to remember (memory cells) specific organisms so that with subsequent exposures, antibodies will be produced at a higher and faster rate. Generally, the more invasive vaccine is enhance the generated protection. The negative side of this immunization is that the reaction may be too strong and cause rabbit to become ill or actually die from the vaccine, (19). The results showed that all non-immunized challenged animals died at (2-10) day post inoculation with mixture viable of *l.monocytogenes, k.pneumonia* and *E.cloacae*, in vaccine (A), and *P. aeruginosa*, *E.coli* and *S.aureus* in vaccine (B), these results indicate that the animals were exposed to infective dose of highly virulent bacterial isolates which overcome innate immunity and rapidly replicated and disseminated from the site of inoculation to the internal organs that cause bacteremia, septicemia, septic shock and death of infected mice. This observation supported the results of previous

studies who explained that endotoxemia of organism can initiate septic shock, the process began with the proliferation of microorganism at a site of infection, and invade the blood stream directly or may proliferated locally and release various substance in the blood stream, these substance include endotoxin in turn stimulate the release of plasma precursors or cells (monocyte, macrophages, endothelial cells, neutrophils and others) of endogenous mediators of sepsis which lead to failure of multiple organs system, disseminate intravascular coagulation and death, (20).

The results of clinical signs and bacterial isolation indicate that immunized animals with killed and attenuated vaccine of bacterial mixture led to sufficient immune response protecting rabbit from lethal infection with virulent viable bacterial mixture, mild to moderate bacterial isolation from internal organs of immunized animals suggested that microorganism spread from the site of inoculation to internal organs particularly reticuloendothelial system, during this time, activated phagocytic cell to killed most of them. This evidence supported by previous worker who revealed that the host defense mechanism against intracellular pathogen in normal rabbit is the phagocytosis by TNF-α producing macrophages which can activated by INF-γ producing by NK cells and CD4+ Th1 cells, (21).

According to our immunological, bacterial isolation we speculated that humoral immunity response play a role in the protection of the immunized rabbite against experimental infection by bacteria which used in this study. These findings were agreed with some reports which showed that the phagocytic cells, (22), complement, (19) and immunoglobulins, (23), play an important role in host defense against bacterial infection. Hyperplasia of spleen white pulp and lymphocytic aggregation around blood vessel of internal organs may be correlate with a good acquired immunity. This evidence was agreement with previous results which reported by, (24) who explained that the development of lymphoid tissue hyperplasia may be reflecting the development of a good acquired immunity.

Radial immunodifussion (RID) method used to determine C3,C4 and immunoglobulin concentration IgG, IgM and IgA, there were an increased in the level of C3,C4, IgM, IgG and IgA in the immunized animals comparing with the control ones, there were significant variations $P > 0.05$ for all the laboratory animals. Figure (3) showed the concentration of Complement and immunoglobulins for the animals inoculated with vaccine (A) before challenge dose. Compared with the control groups, there were significant variations $P > 0.05$, when compared with control animals results.

Figure (4) show the concentration level of C3, C4 and Immunoglobulin IgM, IgG and IgA after challenge with vaccine dose (A) compared with the control subgroup, there were significant variations $P > 0.05$.

Figure (5 and 6) show the concentration level of C3, C4 and Immunoglobulin IgM, IgG and IgA before and after challenge with vaccine dose (B) compared with the control subgroup, with significant variations $P > 0.05$.

The result shows increment of IgM, IgG and IgA levels in the animals as a whole which were inoculated with live attenuated vaccine more than those received killed vaccine because the attenuated vaccine stimulate immune system more than killed vaccine and this agreed with, (25), who referred that the mice infected with microbial antigens stimulate the formation of Lymphopoiesis and B–cells which can produce large amount of IgM after vaccination of the rabbits. IgM also called macroglobulin; it is found mainly in the blood and lymph fluids and is the first to be made by the body to fight a new infection. (26) founded a raise in the IgM after immunization of the rabbits with antigens of *E.coli* (Killed antigens).

IgG is the major serum immunoglobulin, the most abundant type of antibody, is found in all body fluids and protects against bacterial and viral infections, it passes through the placenta providing a natural passive immunity to the newborn, these founding compatible with those of, (27 and 28) who injected the laboratory animal with lipopolysaccharied to stimulate the B-cell to produce the IgG. IgA is found in high concentration in the mucosal membranes, particularly those lining the respiratory passages and gastrointestinal tract, as well as in saliva and tears, (29). The same figure show a raise in IgA levels in the serum of the irradiated animals compared with those of the control ones agreeing with the results of, (27 and 30) who referred to increasing IgA level after vaccination the animals with killed and attenuated.

4-Conclusions:-

The sensitivity of bacterial isolates to antibiotics increased with increment of powers of the laser output with respect to the frequencies of diode laser while bacterial growth was completely stopped in line with the increase of power output, frequencies and exposure time. The bacterial count decreased with increasing of powers the laser output until lost. Live attenuated and killed vaccines inoculated intraperitonial induced high immunity against the infection, live attenuated vaccine was better than the killed one in immunity response. IgG gives higher rate of immunoglobulin concentration.

5-References:-

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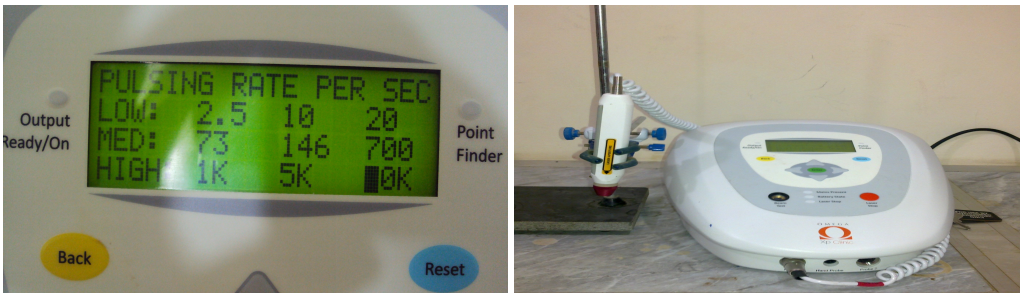
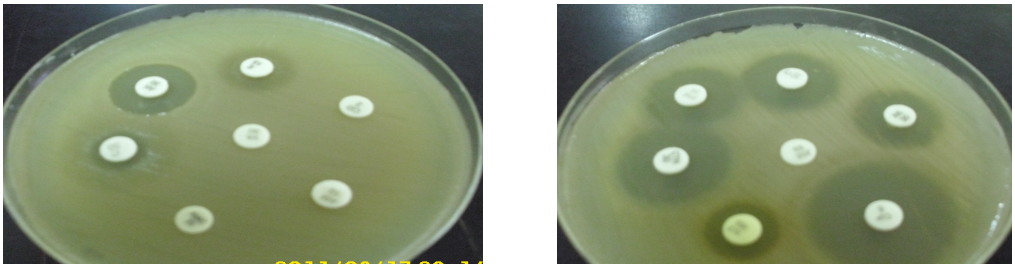


Fig. (1): The setup of irradiation method by Diode laser.



Before irradiation After irradiation

Fig. (2): The susceptibility of bacteria using Diode laser.

Table (1): Determination lethal times of irradiation using wavelength 660 nm.

Bacteria	Power mW	Frequency (kHz)	Time (min)	Results
S.aureus	50 250 250	10 10 10	20 30 40	growth attenuated killed
L.monocytogenes	50 250 250	10 10 10	10 20 50	growth attenuated killed
E.coli	50 250 250	10 10 10	5 10 15	growth attenuated killed
P.aeruginosa	50 250 250	10 10 10	20 30 50	growth attenuated killed
K.pneumonia	50 250 250	10 10 10	20 30 40	attenuated attenuated killed
E.cloacae	50 250 250	10 10 10	15 20 30	attenuated attenuated killed

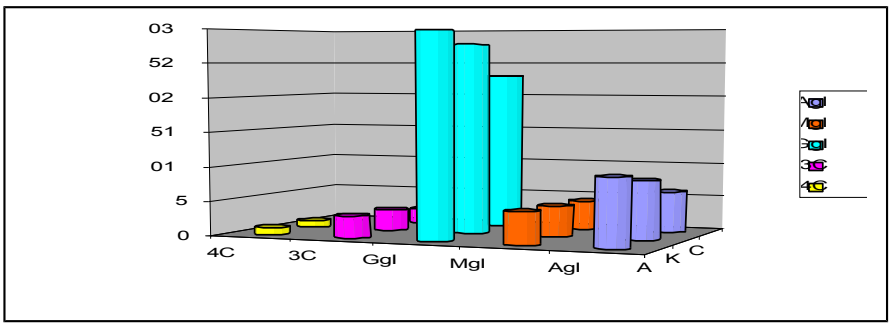


Figure (3): C3, C4 and Immunoglobulin concentration (M ± SD) of vaccine (A) befor challenge dose.

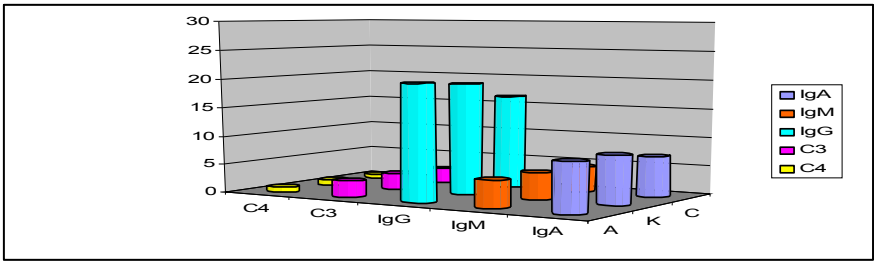


Figure (4): C3, C4 and Immunoglobulin concentration (M ± SD) of vaccine (A) after challenge dose.

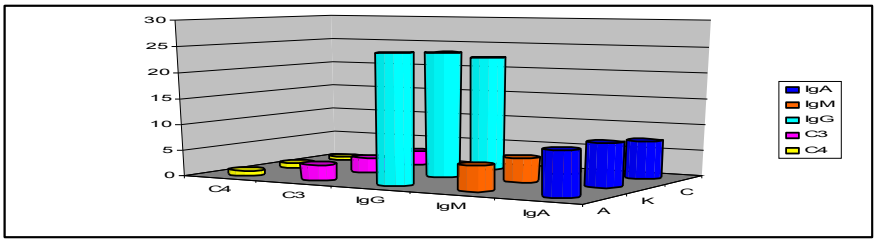


Figure (5): C3, C4 and Immunoglobulin concentration (M ± SD) of Vaccine type (B) before challenge dose.

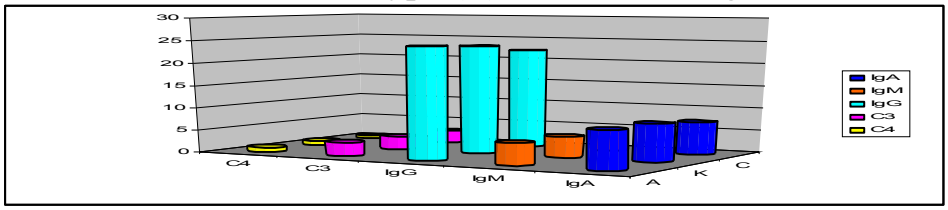


Figure (6): C3, C4 and Immunoglobulin concentration (M ± SD) of vaccine type (B) after challenge dose.