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RESEARCH ARTICLE

EFFECT OF LOW LEVEL LASER ON BACTERIAL ISOLATIONS FROM THE BILE.

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Abstract

Objective: Using laser irradiation to attenuate or kill pathogenic bacteria isolated from the human gallbladder to prepare a vaccine consists of all the bacteria isolated from the bile to use it in immunizing the patients who experts problems in their bile system.

Background: A variety of microorganisms isolated from the bile responsible for causing Cholecystitis and a large number of remedies and antibiotics used to prevent the infection or to treat it.

Materials & Methods: Five microorganisms isolated from forty samples taken from patients underwent cholecystectomy operations, the bacteria isolated were (*Staphylococcus aureus , Listreia monocytogenes , Escherichia coli , Shigella sonnei & Salmonella typhi*) ,they were killed and attenuated using diode laser, the wavelengths used were , 820 nm respectively. Forty four rabbits underwent the vaccination program, forty rabbits of them were divided in to 5 groups with 8 rabbits each and each group was further subdivided in to two subgroups with 4 rabbits each, the first subgroup was inoculated with the live attenuated vaccine while the second one was inoculated with the killed vaccine of each of the five bacterial types, the remaining 4 were injected with the mixed (killed and attenuated vaccine of all the bacterial types) .

The humeral immune response was studied using radial immunodifussion test, to determine the immunoglobulin's concentration, for the IgM, IgG and IgA, then the animals of all the groups were injected with the challenge dose.

Results: Immunoglobulin concentration rates were higher in the immunized subgroup animals compared with those of the control ones. The live attenuated vaccine induced highly immune response as compared with killed vaccine. The challenge dose was given to all the animals; the control subgroup animals died while the immunized animals remain alive and healthy.

Conclusions: Live attenuated and killed vaccines inoculated intraperitonial induced high immunity against the infection, live attenuated vaccine was better than the killed one in inducing immunity responses.

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Introduction:

The pathogenic microorganism is defined as one of the causes or is capable of causing disease. Virulence factors are those characteristics of a bacterium that enhances it`s pathogenicity, i.e. it`s ability to cause disease, (1). Presence of gallstones in the gallbladder may lead to acute cholecystitis; an inflammatory condition characterized by retention of bile in the gallbladder and often secondary infection by intestinal microorganisms, predominantly E.coli and Bacteroides species. Typhoid fever bacteria can live on gallstones in people who carry the disease without showing any symptoms' (2).

Bacterial infections are resistant to several types of antimicrobial agents, therefore, other means of treatment of such bacteria are needed, (3).

The liver (the gall bladder included) has more complicated metabolism and it`s malfunction leads to vastly more complex derangement, (4), lasers with red and near-infrared wavelengths have low energy which provide no biomolecular ionization, they direct cold (subthermal) light toward body cells without injuring or damaging them in any way,(5) , at the same time they are thought to work by targeting the bacterium responsible for causing diseases. This bacterium can absorb various lasers and light, which cause the bacterium to burst and become destroyed leading to an improvement in the patient`s condition. Laser treatment can be an effective way to reduce the infection and inflammation, (6).

Due to the importance of optimization of the available drug preparations through preparation of various drug combinations or giving these preparations with other methods of therapy, low level lasers used widely in such preparations, (7).

Live attenuated and killed bacteria administered as vaccines, when introduced into the body act on inducing immunity, killed vaccines produce relatively less immunity. The live vaccines contain major immunizing antigens and hence, more and longer antigenic stimulation results in prolonged immunity as compared to killed vaccines, (8).

Materials and Methods:

Isolation of the bacteria: The gallbladders of 40 patients lying in Al - Hussein Teaching Hospital – Al-Muthanna Province – Iraq, suffering from Cholecystitis were collected after cholecystectomy operations.

The Bacteria isolated from the bile using one of two methods:

- Brain heart infusion broth method; stones in the gallbladder may lead to acute cholecystitis, the stones transmitted to sacrotubes containing 25 ml of Brain heart infusion broth, mixed thoroughly and incubated at 37 C^{o} for 24 hr, the mixture then cultured on Blood and MacConky agar, then incubated for ten days.
- Cold centrifuge method; the bile evacuated from the gall bladder and transmitted to a sterile test tube to be placed in a cold centrifuge system at a speed of 10000 rpm for 30 min , the sediment cultured on the Blood agar, MacConky agar, Nutrient broth, Trypton soya broth and other media.

Cold centrifuge method; the bile evacuated from the gall bladder and transmitted to a sterile test tube to be placed in a cold centrifuge system at a speed of 10000 rpm for 30 min , the sediment cultured on the Blood agar, MacConky agar, Nutrient broth, Trypton soya broth and other media.

Five species of bacteria isolated, the number of isolates of each bacterium was as the followings; five isolates of *Escherichia coli,* two isolates of *Staphylococcus aureus* and *Listeria monocytogenes* and just one isolate from both *Salmonella typhi* and *Shigella sonnei.*

Laser treatment:

One milliliter of each isolate`s dilution 1.5×10^{8} cell / ml, transferred to a sterile ependorftube, the transferred bacteria were treated with diode laser at the room temperature in a dark place. Samples were exposed to laser irradiation with wavelength 820 nm and output of 50 –100 mW (0.1 W – 0.050 W) , exposure time of 10 – 15 minutes (600 – 900 sec) , frequency 10 KHz and beam dimension of 0.125 cm^2 .The power density was 0.40 – 0.8 W / cm^2 and the energy density 240 – 360 J/cm^2 and / cm^2 480 – 720J respectively.

Vaccines preparation:

Pure culture of bacteria prepared on blood agar for each species isolate**.** 6 ml from normal slain added on the plate surface to be mixed with the growth using a glass rod. The suspension mixed with vortex for 3 min. 5 ml of the suspension form each species was centrifuged (cold centrifuge) at 6000 rpm for 10 min.

The fluid thrown and the sediment was suspended twice again using normal saline, at the second time , suspension mixed with vortex, and suspended once again in 5 ml of normal saline , to be compared with Macferland solution.

The suspension irradiated with laser to obtain live attenuated and killed vaccines. Live attenuated mix vaccine prepared by adding 0.5 ml (dilution 1.5×10^8 cell / ml) of the live attenuated vaccine of each of the five bacteria species, the same thing done to prepare the mix vaccine of the killed bacteria from the five species.

Immunization program:-

Forty four rabbits underwent the vaccination program, forty rabbits of them were divided in to 5 groups with 8 rabbits each and each group was further subdivided in to two subgroups with 4 rabbits each, the first subgroup was inoculated with the live attenuated vaccine while the second one was inoculated with the killed vaccine of each of the five bacterial types, the remaining 4 were injected with the mixed (killed and attenuated vaccine of all the bacterial types) , Table ;1.

Table 1:- Vaccination program.

groups	Total No. in each group	Subgroups	Animals Number
1st. group S. aureus group	8	Live attenuated	4
		Killed	4
2nd. group L. moncytogenes	8	Live attenuated	4
		Killed	4
3rd. group S. typhi	8	Live attenuated	4
		Killed	4
4th. group S. sonnei	8	Live attenuated	4
		Killed	4
5th. group E.coli	8	Live attenuated	4
		Killed	4
6th. group Control group	4	Injected normal slain	4

The humeral immune response was studied using radial immunodifussion test, to determine the immunoglobulin's concentration, for the IgM, IgG and IgA, then the animals of all the groups were injected with the challenge dose.

Blood Sampling:

After one month from the date of the vaccine inoculation blood samples taken from all the rabbits , 3 ml of blood taken from the left marginal vein to determine the level of the Immunoglobulins IgM, IgG and IgA in the serums of the rabbits which inoculated with the live attenuated and killed vaccines and those of the control subgroups using radial immunodifussion (RID) to explain the relationship between the antigen concentration and the half diameter of the precipitating ring shown on the agar indicated the specific antibodies reaction against the antigens.

Challenge dose:-

Each group then was inoculated with 1 ml of the bacterial suspension colony forming units of all the bacterial species IP, to measure the lethal dose (LD50), while the sixth group was inoculated with a mixture of all the five bacterial species, (9).

Challenge dose of the half number of the total animals after the bacterial counting was as following: *E. coli* 50×10^4 CFU / ml. L. *monocytogenes* 3×10^7 CFU / ml., Staph .*aureus* 3×10^7 CFU / ml., *Shigella sonnei* 20×10^6 CFU / ml. and *Salmonella typhi* 3×10^4 CFU / ml.

Results:-

Effect of laser on Antimicrobial susceptibility of bacteria:-0

Some parameters affected the sensitivity of the isolates to some antibiotics which were resistant before irradiation but they render sensitive after irradiation, as occurred with Ampicilline. Table ;2 shows times required to attenuate or kill each species of the isolated bacteria using 820 nm wavelength diode lasers; power 50mW and 100 mW frequency 10 KH .

Determination of Laser Lethal Times of the bacterial Isolates:-

The results of irradiation show a significant decrease in the bacterial viability of all species as the dose increase. It is clear to see that the survival rates of the cells decreased as the laser exposure time increased.

E. coli irradiated with diode laser using wavelength 820 nm ; the results was weak growth at power output 50 mW , time 10 minutes and frequencies of 1, 5 and 10 KHz respectively , in power output 100 mW , killing occurred at 10 KHz for 10 min , the same thing occurred with L. monocytogenes.

Irradiation of S. aureus with diode laser using wavelength 820 nm, power output 50 mW, frequencies 1, 5 and 10 KHz for 10 minutes gave weak growth. It showed weak growth at 100 mW frequencies, 1, 5 for 10 min. while the growth disappeared on the time used was 15 minutes and the frequency 10 KHz.

S.sonnei exposed to diode laser 820 nm , showed no-growth when power 50 mW , frequency 10 KHz for 15 minutes and 100 mW , frequency 10 KHz for 10 minutes.

In case of *S .typhi* diode laser 820 nm wavelength, killed the bacteria colonies (no-growth) at 100 mW power output , frequency (10) KHz for 10 minutes , Table;3.

The wavelengths ; (660 nm at 50 mW power, frequency 10 KHz for different times) and (915 nm , frequency 10 KHz , power 100 mW and different times) used also to irradiate the bacterial isolates and showed different responses as shown in Table; 4.

Immunoglobulins concentration:-

Radial immunodifussion (RID) method used for determination immunoglobulin's concentration IgM , IgG and IgA in the rabbits serum that inoculated with live attenuated and killed vaccines, there were an increasing in the level of IgM ,IgG and Table 4: Immunoglobulins concentration (M ± SD) for the both types of vaccine , (live attenuated and killed) of each bacterial species and the mixed vaccine.

IgA for all the animals comparing with the control ones , there were significant variations $P > 0.05$, for all the laboratory animals , Table ; 4 and Figures;1 , 2 and 3 show the Immunoglobulins concentration of for live attenuated , killed and control subgroups.

There were significant variations $P > 0.05$ when the Immunoglobulins concentration (M ± SD) of IgM, IgG and IgA in the animal`s serum inoculated with live attenuated and killed vaccines of each bacterium independently and the mix vaccine compared with that of the control subgroups.

Table 2**:** Times required to attenuate or kill each species of the isolated bacteria using 820 nm wavelength diode lasers power 50mW and 100 mW frequency 10 KH.

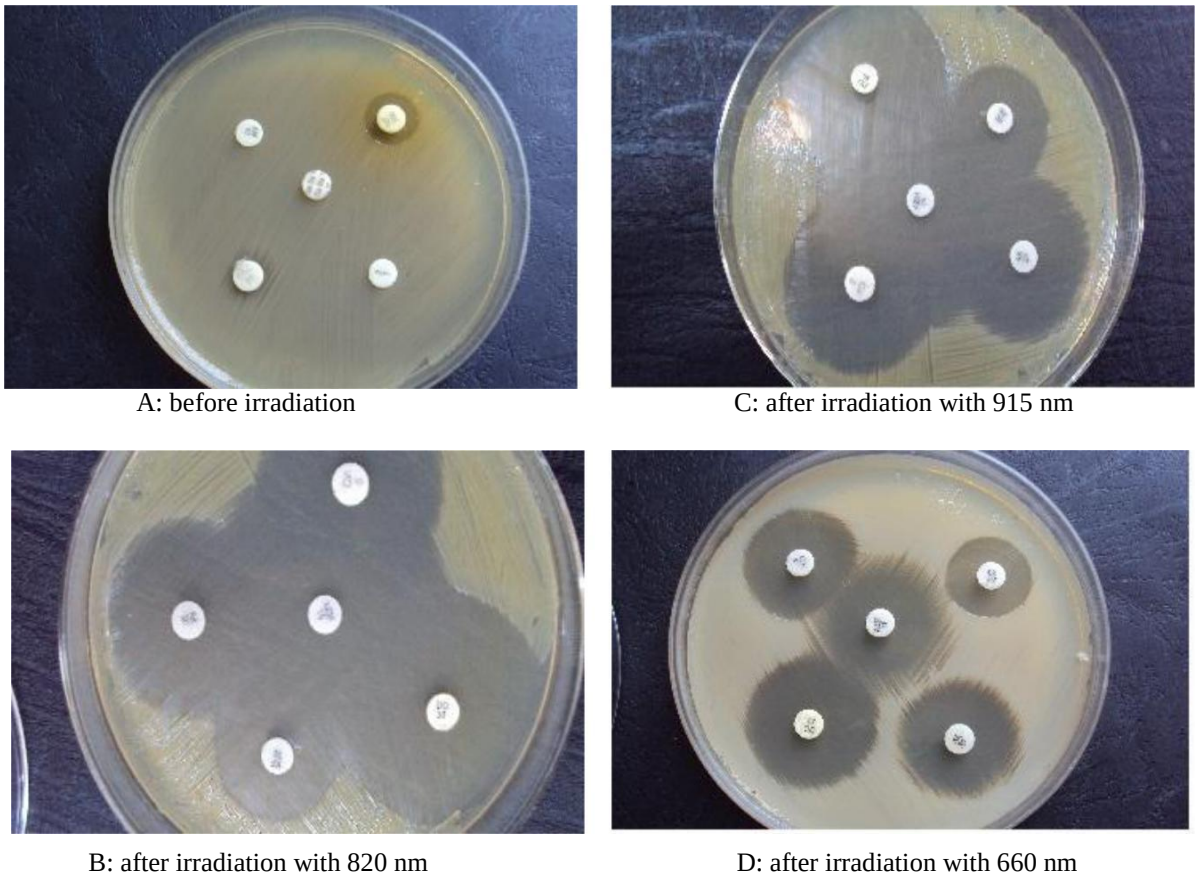
Power (W)	Time (min)		Frequency (KHz)	Result
		E. coli		
0.050W	15 min		1 KHz	Weak growth
	15 min		5 KHz	Weak growth
	15 min		10 KHz	No growth
0.1 W	15 min		1 KHz	Weak growth
	15 min		5 KHz	No-growth
	10 min		10 KHz	No-growth
		L. monocytogenes		
0.050W	15 min		5 KHz	Weak growth
	15 min		10 KHz	Weak growth
0.1 W	15 min		1 KHz	Weak growth
	10 min		5 KHz	Weak growth
	10 min		10 KHz	Weak growth
	15 min		10 KHz	No-growth
		Staph. aureus		
0.050W	15 min		5 KHz	Weak growth
	15 min		10 KHz	Weak growth
0.1W	15 min		1 KHz	Weak growth
	10 min		5 KHz	Weak growth
	10 min		10 KHz	Weak growth
	15 min		10 KHz	No-growth
		S. sonnei		
0.050W	15 min		1 KHz	Weak growth
	10 min		5 KHz	Weak growth
	10 min		10 KHz	Weak growth
	15 min		10 KHz	No-growth
0.1W	15 min		1 KHz	Weak growth
	10 min		5 KHz	No-growth
	10 min		10 KHz	No-growth
		S. typhi.		
0.050W	15 min		5 KHz	Weak growth
	10 min		10 KHz	Weak growth
0.1W	15 min		1 KHz	Weak growth
	10 min		5 KHz	Weak growth
	15 min		5 KHz	No-growth
	10 min		10 KHz	No-growth

Table 3: Effect of laser diode wavelengths 660 nm, frequency 10 KHz, power 50 mw and 915 nm , frequency 10 KHz, power 100 mW and different times on bacterial species.

Bacterial species	Wavelength	Growth		
		5 min	10 min	20 min
E .coli	660 nm	growth	growth	growth
L monocytogenes		growth	growth	Weak growth
S. aureus		growth	growth	growth
S .sonnei		growth	growth	Weak growth
S .typhi		growth	Weak growth	Weak growth
Bacterial species	915 nm	Growth		
		10 min	20 min	30 min
E . coli		growth	growth	Weak growth
L monocytogenes		growth	growth	Weak growth
S. aureus		growth	Weak growth	Weak growth
S .sonnei		growth	Weak growth	No-growth
S .typhi		growth	Weak growth	No-growth
Bacterial species	Wavelength	Growth		
		5 min	10 min	20 min
E .coli	660 nm	growth	growth	growth
L monocytogenes		growth	growth	Weak growth
S. aureus		growth	growth	growth
S .sonnei		growth	growth	Weak growth
S .typhi		growth	Weak growth	Weak growth
Bacterial species	915 nm	Growth		
		10 min	20 min	30 min
E . coli		growth	growth	Weak growth
L monocytogenes		growth	growth	Weak growth
S. aureus		growth	Weak growth	Weak growth
S .sonnei		growth	Weak growth	No-growth
S .typhi		growth	Weak growth	No-growth

Table 4: Immunoglobulins concentration (M ± SD) for the both types of vaccine, (live attenuated and killed) of each bacterial species and the mixed vaccine.

Groups	Immunoglobulin concentration rate (M ± S.D)		
	IgM	IgG	IgA
E. coli.			
Attenuated	424.3±19.9	3075.9±84.3	484.5±20.7
Killed	386.55±8.93	2398±140	430.6±24.5
Control	198.68± 8.56	1075.4± 40.1	171. 63± 3.95
L. monocytogenes			
Attenuated	476.7±29.8	3293 ± 179	504.2±14.8
Killed	383.18± 9.47	2723 ± 118	439.9±27.6
Control	198.68± 8.56	1075.4± 40.1	171.63 ± 3.95
S. aureus			
Attenuated	428.9±24.5	3108.7 ± 93.3	490.85 ± 9.96
Killed	379.0± 17.5	2574 ± 159	419.38 ± 9.70
Control	198.68± 8.56	1075.4± 40.1	171.63 ± 3.95
S. sonnei			
Attenuated	446.3±28.5	3013±204	475.4±20.7
Killed	322.6±18.0	2367±107	448.1±31.0
Control	198.68± 8.56	1075.4± 40.1	171.63 ± 3.95
S .typhi			
Attenuated	445.3± 28.1	2979 ± 118	493.1 ± 23.9
Killed	364.3 ± 19.1	2825.8 ± 55.1	419.5 ± 12.9
Control	198.68± 8.56	1075.4± 40.1	171.63 ± 3.95
Mixed vaccine			
Attenuated	429.8±23.6	3109.6 ± 92.5	491.82 ± 9.98
Killed	378.0± 17.7	2575 ± 158	420.40 ± 9.72
Control	197.66± 8.55	1074.4± 41	170.60 ± 3.93



A: before irradiation C: after irradiation with 915 nm

B: after irradiation with 820 nm D: after irradiation with 660 nm

Figure (1):- Antimicrobial sensitivities for S. aureus isolate before and after irradiation.

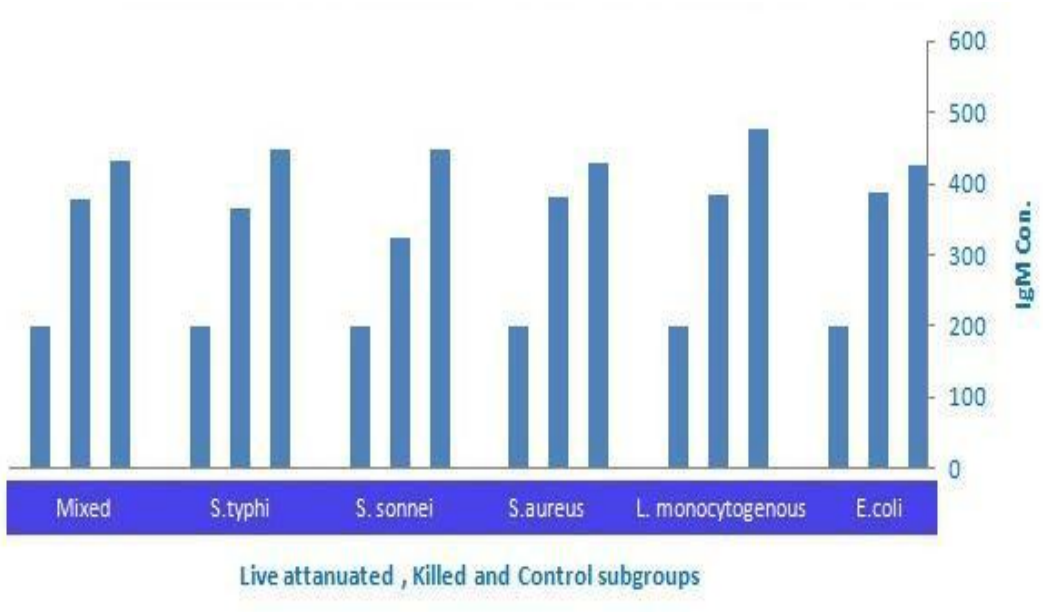


Figure (2):- Concentration of IgM in attenuated, killed and control subgroups.

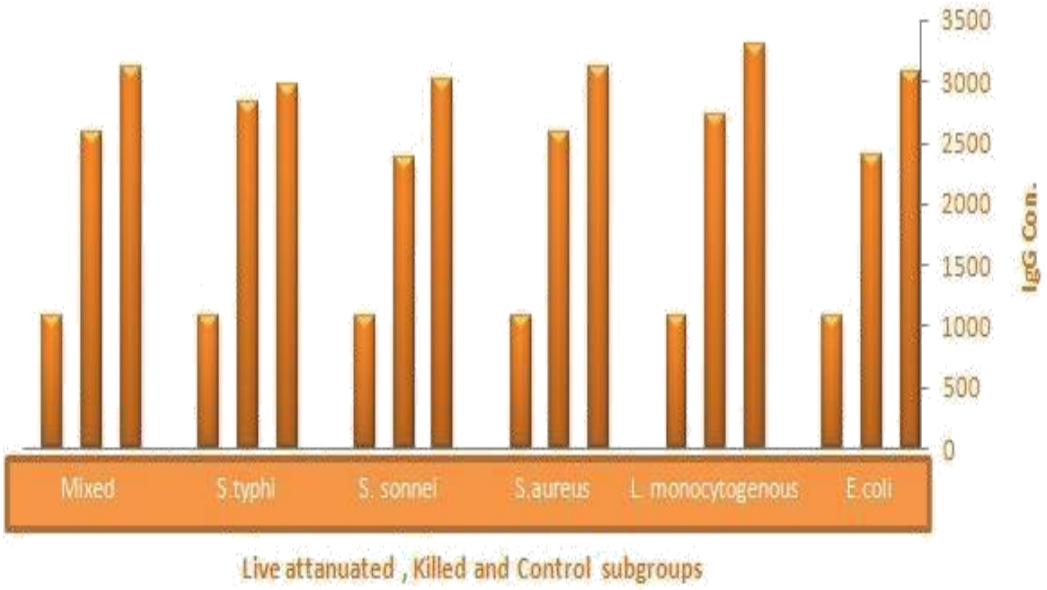


Figure (3):- Concentration of IgG in attenuated, killed and control subgroups.

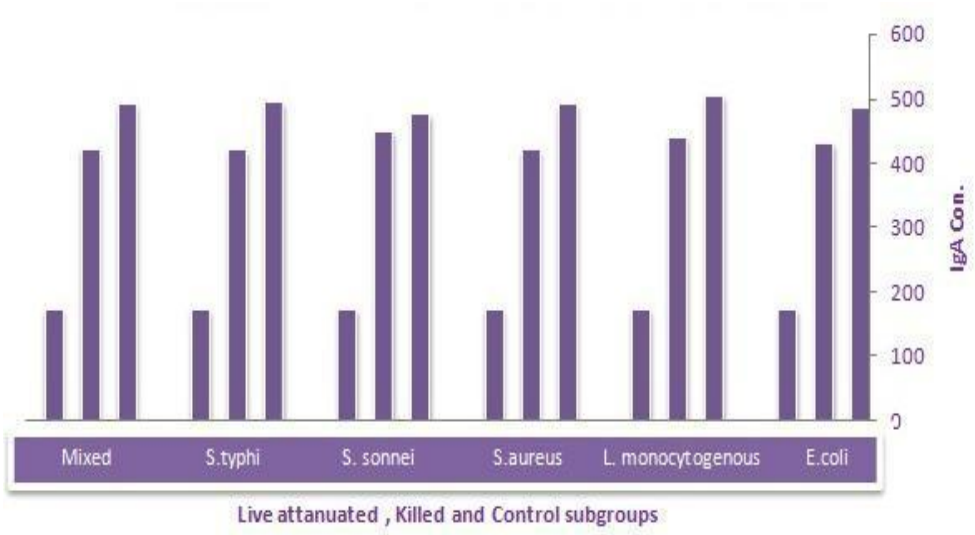


Figure (4):- Concentration of IgA in attenuated, kille**d** and control subgroups.

Challenge dose:-

The animals of the control subgroups of all the groups which were injected with normal saline died at (4 – 8 days) after inoculation with the challenge dose (Lethal dose), prepared from each bacterium colonies and the mix bacterial colonies. No one of the vaccinated subgroup`s animals died when injected with the challenge dose, Table 5

Table 5: The number of deaths in the experimental animals after injection of the challenge dose.

No.	Animals groups	Animals number	Death numbers
1	First group (S. aureus group)	8	No-death
2	Second group (L.monocytogenes)	8	No-death
3	Third group (S. typhi group)	8	No-death
4	Fourth group (S. sonnei group)	8	No-death
5	Fifth group (E. coli group)	8	No-death
6	Sixth group (control group) injected with the challenge dose	4	All the animals of the group were died.

Discussion:

E. coli , L .monocytogenes and *S. aureus* lost hemolysin production post irradiation , hemolysin production lose in the isolates is rare , in the current study it occurred may be due to a fact that laser irradiation effect hemolysin production in these bacteria which were mostly – encoded by chromosome. The effect of laser on the bacterial chromosome may loss their hemolysin production being unable to produce hemolysin and lost their virulence, (10).

The challenge dose of the half number of the total animals after the bacterial counting used in the current study agree with those obtained by, (11 & 12). Laser irradiation can break strand DNA plasmid thus the bacteria may loss their ability to resist the antibiotics, (13).

Live, attenuated vaccines contain a version of the living microbe that has been weakened in the lab. so it can't cause disease. Because a live attenuated vaccine is the closest thing to a natural infection, got by attenuating the isolates losing the bacterial ability of the blood hemolysis due to inability on hemolysin production after irradiation by laser, (14).

Attenuation is the operation of removing the factors which play essential role in the virulence of the bacteria (not killing the bacteria) to stimulate the immune system for production antibodies, ,immunoglobulin is defined as animal origin protein endowed with known antibody activity,(15 & 16).

Cellular receptors found on membranes of lymphocytes (B & T), they bind to foreign antigens, B- cell recognition molecules are called immunoglobulines or antibodies. The main immunoglobulin classes are IgG, IgM, IgE, IgA & IgD, IgM is the main immunoglobulin produced early in the primary response while the IgG is the predominant antibody in the secondary immunological response and constitutes an important defence against bacteria and viruses, (17).

Innoculation of the attenuated and killed vaccines in to the lab. animals activate the Prostoglandines and the histamines which in tern trigger the synthesis of the Immunoglobulin by plasma cells and also by the lymphocytes, (18). Table ;4 show increasing in concentration levels IgM , IgG and IgA of serum of the laboratory animals which give the live , attenuated and killed vaccines when compared with animals of control subgroup , founding high significant variations $P > 0.05$ when compared with the control animals results.

Also there was a raising in IgG level in the test animal`s serum when compared with control animals , these foundings compatible with those of , (19, 20 & 21) ' who injected the laboratory animal with lipopolysaccharied to stimulate the B-cell to produce the IgG . The increase in the level of IgM, IgG and IgA for the animals inoculated with of mix kind five from bacteria in comparison with the control one, there were significant variations $P > 0.05$, in the results.

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