Studies on the effect of clindamycin on experimental infection with *Staphylococcus aureus* in relation to dose and treatment with special emphasis to antibiotic bacterial resistance

Amnah A.H. Rayes

Faculty of Applied Sciences. Umm Al- Qura University Makkah Saudi Arabia
Amnaa_rayes_50@yahoo.com

Abstract: present study aimed to investigate and search about the most common causes of bacterial resistance to antiboiotics which are the dose and method of treatment using Clindamycin on experimental infected mice with *Staphylococcus aureus.* ATCC 25923 and study the effect of antibiotic on immune response humeral and cellular. A total number of 100 adult albino male mice were divided into 5 groups (20 mice each.). All groups were injected intra peritoneal I/P with *Staphylococcus aureus* 3 x10^9 cuf. 24 hours after bacterial injection all groups were treated with Clindamycin 500 mg/8h for 8 days. 1st group: control group.: animals apparently healthy untreated.orally received saline sol., 2nd group: orally received single dose of defined prescribed dose of Clindamycin, 3rd group: orally received 5 times the defined dose of Clindamycin then decreased,4th group: orally received 1/5 of defined dose Clindamycin then increased and 5th group: orally administrated the initial dose of Clindamycin along the experiment. Animals were sacrificed, blood samples were collected and divided into 2 portions; first one added to it anticoagulant for determination of phagocytic index and the second one without anticoagulant for Serum collection then keep in -20°C till used for determination of anti body titer in experimental infected mice. Blood samples were collected 6 hours after antibiotic administration for 8 days. Determination of antibotic concentration in blood, determination of antibody titer in serum and phagocytic index in blood for detection of the effect of antibiotic dose on the immune response of experimental infected mice with *S.aureus*. At the end of treatment survived mice scarified and isolation of *S. aureus* was performed, the isolated strain was identified as *Staphylococcus aureus.* ATCC 25923 (same injected strain) using API20E system. Antibacterial susceptibility performed using disk diffusion method, the results displayed that the concentration of anti Clindamycin in serum was correlated with anti body titer and phagocytic index, all the obtained results were discussed.

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Key words: Clindamycin; *Staphylococcus aureus;* immune response; humeral; cellular; antibody titer; phagocytic index; disk diffusion

Introduction:

Disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the major public health concerns, particularly if it is included pathogenic organisms (**Drusano, 2004; Finley, *et al.*,2013).** Nowadays, about seventy percent of the bacteria that cause infections are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs (**Handel, *et al*.,2009**).

Although there were low levels of preexisting antibiotic-resistant bacteria before the widespread use of antibiotics **(Caldwell & Lindberg 2011; Nelson 2009**) evolutionary pressure from their use has played a role in the development of multidrug resistance varieties and the spread of resistance between bacterial species.(**Hawkey & Jones, 2009,**) In medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics. (**WHO**, **2002).**

In some countries, antibiotics are sold over the counter without a prescription, which also leads to the creation of resistant strains. Other practices contributing towards resistance include the addition of antibiotics to livestock feed. (**Okeke, *et al*.,1999; Ferber, 2002; Mathew, *et al*.,2007**) The use of antimicrobials in farming (**Witte, 2000; Teuber, 2001**), together with the practice of raw sewage discharge into receiving waters, has resulted in a significant increase of antibiotic resistant bacteria in aquatic environments. Household use of antibacterials in soaps and other products, although not clearly contributing to resistance, is also discouraged (as not being effective at infection control) (**CDC,2009**) Unsound practices in the pharmaceutical manufacturing industry can also contribute towards the likelihood of creating antibiotic-resistant strains.

(**Larsson and Fick, 2009** ;**Novo, *et al*.,2013**). Present study aimed to investigate and search about the most common causes of bacterial resistance to antiboiotics which are the dose and method of treatment using clindamycin on experimental infected albino mice with *Staphylococcus aureus* and study the effect of antibiotic on immune response humeral and cellular.

Material and methods:

Bacterial strain:

Staphylococcus aureus. ATCC 25923, lyophilized strains Kindly, supplied from King Fahd Hospital (armed forces) in Jeddah.

Antibiotics:

Antibiotic used in the experiment was purchased from local pharmacy in Jeddah, commercial Clindamycin with defined dose 500 mg /8 h. for 8 days. Clindamycin is a good choice to treat a variety of *Staphylococcus aureus* infections.

experimental design:

A total number of 100 adult albino male mice (55 - 65 gm) body weight were divided into 5 groups (20 mice each.). All groups were injected intra peritoneal I/P with *Staphylococcus aureus* 3 x10^9 cuf. 24 hours after bacterial injection all groups were treated as in the table 1.

Table 1: showing experimental groups administrated clindamycin

groups	treatment
1st group	control group: animals apparently healthy untreated.orally received saline solution.
2nd group	orally received single dose 500mg/8h. of defined prescribed dose of clindamycin *
3rd group	orally received 5 times the defined dose of clindamycin then decreased **
4th group	orally received 1/5 of defined dose clindamycin then increased ***
5th group	orally administrated the 500mg/8h. of c clindamycin along the experiment (+ control)

*Normal dose 500 mg/8h.

**Initial dose 2500 mg decreased to 2000 mg, 1500mg,1000mg,500mg,50mg,25mg and10mg

***Initial dose 50 mg increased to 100 mg, 200mg,300mg,400mg,500mg,600mg and700mg

Sampling:

Animals were sacrificed, blood samples were collected and divided into 2 portions with first one added to it EDTA as anticoagulant for determination of phagocytic index and the second one without anticoagulant and placed in a slope in 4°C for 24 h. then centrifuged 1000 xg for 10 min at 4°C. Serum was collected and keep in -20°C till used for determination of anti body titer in experimental infected mice. Blood samples were collected 6 hours after antibiotic administration for 8 days.

determination of antibodies concentration in blood:

Serum samples containing unknown concentrations of antibiotic were diluted 1:2, 1:4, and 1:5 in sterile pooled human serum. Four seeded agar plates were used, each with two discs saturated with the reference standard and two discs saturated with the patient's undiluted or diluted serum. For maximal accuracy, duplicate plates were set up with discs saturated with diluted serum. The plates were incubated for 4 hr at 37 °C. Zone diameters of the reference standards and of the patient's serum were measured and averaged. Correction of the zone diameter was accomplished by adding or subtracting the difference between the mean zone diameter of the reference standard obtained in the assay plate and the zone diameter of the correction point of 2.0 µg/ml in the standard curve. The final result, in micrograms per milliliter, was extrapolated from the standard curve and multiplied by the dilution of the original specimen. **(Warren, *et al*.,1972)**

Determination of antibody titer:

Antibody titer determined using Thermo Scientific Easy-Titer Antibody Assay Kits (23300) which, enable accurate determination of antibody concentration in mice serum in brief Prepare standards (5 to 500 ng/mL) by diluting purified antibody in Kit Dilution Buffer. Prepare samples by diluting at least 20-fold in Dilution Buffer to within assay range (8 to500 ng/mL).Vortex vial of microsphere beads to create homogeneous suspension. Pipette 20µL of bead suspension and 20µL of each sample and standard into 96-well microplate wells. Incubate microplate for 5 minutes with vigorous mixing. Add 100 µL of Kit Blocking Reagent. Incubate microplate for 5 minutes with vigorous mixing. Measure absorbance on standard plate reader (340 nm). Plot standard curve and interpolate samples to determine concentration.

Phagocytic assay:

Macrophage monolayers were obtained and prepared it were harvested from the peritoneal cavities of mice and were resuspended in Hanks' balanced salt

solution (HBSS) (Sigma Chemical Co., St. Louis, MO). The number of viable cells was determined by trypan blue dye exclusion and the coverslips were washed with HBSS. *Candida albican* particles were added to the monolayers in a 5:1 (particle: macrophage) ratio and the coverslips incubated at 37°C in humidified atmosphere. After 30 min, the coverslips were washed with HBSS, fixed in methanol, and stained with Giemsa. After drying, the coverslips were mounted on glass slides and examined microscopically. The percentage of cells with ingested particles was multiplied by the average number of particles per macrophage to calculate phagocytic index. At least 100 macrophages were counted per cover slip (**Belline, *et al*.,2004)**

Antibacterial susceptibility and disk diffusion method:

Before and after clindamycin treatment course each experimental group, mice were scarified and bacterial strain was isolated from liver and spleen in nutrient broth incubated at 37°C for 24h. isolated strain was identified using API20E Antimicrobial resistance patterns of isolates were determined by the agar disk diffusion method **(Bauer, *et al*., 1996**). *S. aureus* was suspended in sterile saline to a turbidity to match a McFarland No. 2 standard (bioMe´rieux, Marcy l'Etoile, France), diluted 1:20, and streaked on Mueller-Hinton agar (Difco Laboratories, Detroit, MI). Clindamycin disks. Plates were incubated at 37 °C for 24 h. Characterization of strains as sensitive, intermediate or resistant was based on the mean size of the inhibition zones around each disk according to the National Committee for Clinical Laboratory Standards criteria (**NCCLS, 1999).**

Statistical:

The mean values of antibiotic concentration in serum and phagocytic index in blood. All the experimental groups comparing to the control (5th group orally administrated the inital dose of clindamycin along the experiment) were subjected to statistical analysis, using the "F" test.

Results:

concentrations of antibiotic in serum:

present study revealed that in the 2nd group which subjected for one dose only of clindamycin 500 mg/12 h for 8 days, the antibiotic concentration decreased from the 1st day to be 0 concentration at day 7 and 8, while the 3rd group which subjected for 5 times the normal prescribed dose the concentration decreased along 8 days of the experiment but did not rich to concentration zero. 4th Group which subjected to1/5 defined dose then decreased the antibiotic nearly found along the course in suitable high concentration but not constant concentration the higher concentration in the day 5 and the lowest concentration in the day 8. Group 5 which subjected to 500 mg/8h for 8 days, concentration of clindamycin was nearly constant along 8 days of treatment. Concerning the mortality percent in all treated groups, the highest mortality was in 2nd group 46% (orally received single dose of defined prescribed dose of clindamycin) while the lowest mortality was in 5th group 3% (orally administrated the inital dose of clindamycin along the experiment) table 2.

Table 2: Showing concentrations of clindamycin in serum during 8 days after experimentally infected mice with S. *aureus* (mg) and mortality%

group	0 +6h.	12+6h	24+6h	36+6h	48+6h	60+6h	72+6h	84+6h	Mort.
1st gp	00	00	00	00	00	00	00	00	00.00
2nd gp	465±23	411±19	373±18	216±11	134±8.3*	11±0.5*	00±00*	00±00*	46%*
3rd gp	2224±89*	1732±86*	1234±61*	789±39	471±23	211±12	56±2.8*	21±1.0*	33%*
4th gp	46±2.5*	92±4.6*	164±8.2*	215±10	377±17	283±14	76±3.8*	35±1.7*	13%*
5th gp	478±21	495±24	488±22	495±20	499±25	479±19	498±19	497±21	3%

Mean of 2 samples ± SD * significance

Antibody titer serum of infected mice with *S.aureus*:

Results showed that the antibody titer in 2nd group was the highest titer 1/64 was in day 5 then decreased to 1/16 in the day 8 while 3rd group showed that the highest titer was in day 2 and 3. 4th group displayed that the highest titer was in day 3 while 5th group showed that the highest titer was in day 8 and lowest titer was in day1 to day 7 table 3.

Table 3: showing antibody titer of experimentally infected mice with *S.aureus*

group	0+6h.	24+6h	36+6h	48+6h	60+6h	72+6h	84+6h	96+6h
1st gp	00	00	00	00	00	00	00	00
2nd gp	1/8	1/16*	1/32*	1/32*	1/64*	1/32*	1/16	1/16*
3rd gp	¼ *	1/8	1/8	¼*	½*	½*	½*	00*
4th gp	1/16*	1/16*	1/32*	1/16	1/8	1/8	¼*	00*
5th gp	1/8	1/8	1/8	1/16	1/8	1/8	1/16	1/32

* significance

Phagocytic index of infected mice with *S.aureus:*

Present study displayed that the phagocytic index in 2nd group was low then increased, the highest phagocytic index was in day 8 while the lowest was in day1. 3rd group revealed that the highest phagocytic index was in day 8 while the lowest was in day 6 in comparison 4th group showed that the highest phagocytic index was in day1 while the lowest one was in day8. Group 5th showed that the highest phagocytic index was in day 6 while the lowest was in day 8 but the phagoctic index nearly in the same level in this group table 4.

Table 4: Showing phagocytic index of experimentally infected mice with *S.aureus*

group	12+6h.	24+6h	36+6h	48+6h	60+6h	72+6h	84+6h	96+6h
1st gp	00	00	00	00	00	00	00	00
2nd gp	23±1.14*	16±0.90	43±2.15*	56±2.80*	77±3.84*	89±4.45*	94±4.72*	98±4.61*
3rd gp	12±0.60	23±1.15*	31±1.55*	11±0.51	8±0.70*	5±0.25*	45±2.25*	54±2.64*
4th gp	87±4.35*	73±3.65*	61±3.05*	63±3.14*	46±2.30*	32±1.40*	13±0.65	5±0.33
5th gp	9±0.45	12±0.60	11±0.55	10±0.60	19±0.96	14±0.63	11±0.33	6±032

Mean of 2 samples ± SD * significance

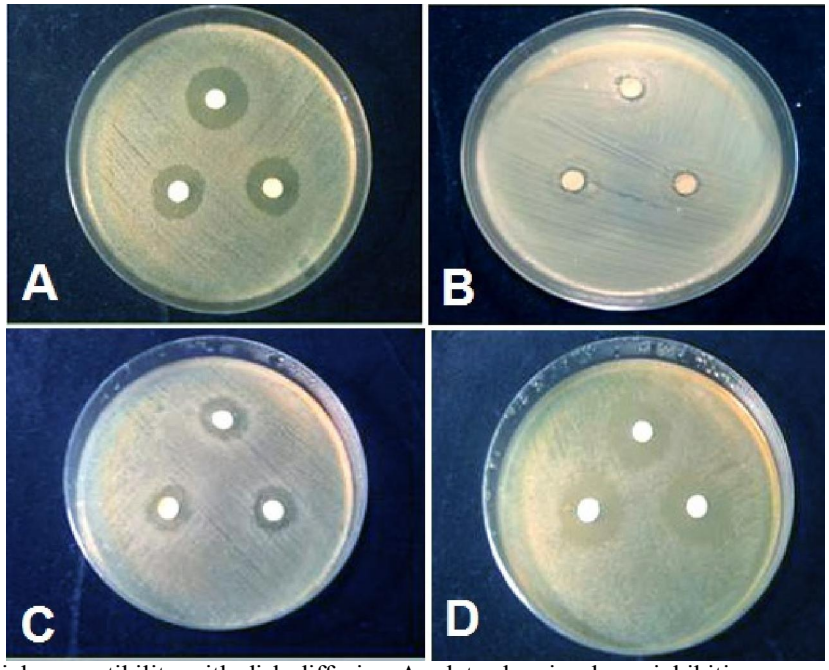

Figure1: Antibacterial susceptibility with disk diffusion A. plate showing large inhibition zone of *S. aureus* against clindamycin before experiment, B. plate showing small inhibition zone of *S. aureus* isolated from group 2(orally received single dose of defined prescribed dose of clindamycin) C. plate showing relatively small inhibition zone of *S. aureus* isolated from group 3 (orally received 5 times the defined dose of clindamycin then decreased) D. plate showing large inhibition zone of *S. aureus* isolated from group 5 (orally administrated the initial dose of clindamycin along the experiment).

Disk diffusion method and antibacterial susceptibility:

Isolated bacterial strain was identified as *S. aureus* and tested for antibiotic resistance using disk diffusion method and inhibition zones was measured for each group it is expressed as mean for 3 replicates ±SD. Present study revealed that the highest inhibition zone was for test before experiment and the lowest group was the second group (orally received single dose of defined prescribed dose of clindamycin) followed by 4th group (orally received 1/5 of defined dose clindamycin then increased) then 3rd group an 5th group table, 5 fig, 1.

Table 5: showing inhibition zone in different experimental group before and after treatment of *S.aureus* with clindamycin (3 replicates±SD)

group	Inhibition zone (mm)
Before treatment	19± 0.95*
2nd group	3±0.15*
3rd group	9±0.45
4th group	7±0.35
5th group	12±0.60

* significance

Discussion:

present study aimed to investigate and search about the most common causes of bacterial resistance to antiboiotics which are the dose and method of treatment using clindamycin on experimental infected albino mice with *Staphylococcus aureus* and study the effect of antibiotic on immune response humeral and cellular.

One of the most terrible problems in medicine is the emergence and spread of antibiotic resistance, which is becoming a serious menace to modern societies. the major problem of the emergence of resistant bacteria is greatly due to misuse and overuse of antibiotics. (**WHO, 2002)**. An increasing resistance to antibiotics, which are in daily use for the treatment of infections in both humans and animals, is well documented. Many bacteria have become resistant to several types of antibiotics and there are hardly any new antibiotics available to combat multiresistant bacteria of this kind. It is important to make sure that antibiotics continue to be an effective treatment for the generations to come, but incorrect and excessive use of antibiotics can increase the occurrence of resistant microorganisms.

Present study indicated that un- proper dose and treatment with clindamycin; high then lowed, low then high and single dose along the course of treatment, all make disturbance of clindamycin concentrations in serum along the treatment course, the results nearly coincide with (**Golub, *et al*.,1990; Labro, 2000; Barrie, 2012).**

Antibiotics are typically dosed at levels below the minimum inhibitory concentration (MIC) so as to reduce the bacterial resistance. For instance, some antibiotics, when administered at levels above the MIC inhibit phagocyte function. These effects seem to be independent of their antibacterial effect (**Tacconelli, *et al*., 2009 and CDC, 2009**)

Concerning the effect of antibiotics and immune response. present study displayed that the immune response of experimental infected mice with *S, aureus* both arms of immune response humeral and cellular immunity were affected,the results indicated that antibody titer of all experiment was Results showed that the antibody titer in 2nd group was the highest titer 1/64 was in day 5 then decreased to 1/16 in the day 8 while 3rd group showed that the highest titer was in day 2 and 3. 4th group displayed that the highest titer was in day 3 while 5th group showed that the highest titer was in day 8 and lowest titer was in day1 to day 7,this meaning that there antagonistic effect of antibody titer and dose of clindamycin the results nearly agree with that obtained by **Woo, *et al*. (1999)** reported that clarithrtiomycin and doxycycline suppress the antibody response induced by tetanus toxoid, pneumococcabiol polysaccharide vaccine,

Concerning cellular immunity and effect of clindamycin, present study displayed that the phagocytic index in 2nd group was low then increased, the highest phagocytic index was in day 8 while the lowest was in day1. 3rd group revealed that the highest phagocytic index was in day 8 while the lowest was in day 6 in comparison 4th group showed that the highest phagocytic index was in day1 while the lowest one was in day8. Group 5th showed that the highest phagocytic index was in day 6 while the lowest was in day 8 but the phagoctic index nearly in the same level in this group the results nearly agree with the obtained results by **Williams, *et al*. (2005**) reported that quinolones, including ciprofloxacin and moxifloxacin, are nalidixic acid analogue antibiotics, which exert their bactericidal effect by inhibiting DNA gyrase activity. Ciprofloxacin has been shown previously to have immunomodulatory effects, recently reviewed by **Dalhoff and Shalit (2003)**, and although moxifloxacin is a relatively new drug, it too has been shown to be immunomodulatory in both animals and humans. (**Shalit, *et al*., 2001; Williams, *et al*., 2001**) Clarithromycin is a semi-synthetic acid-stable member of the broad-spectrum macrolide family and has been shown to modulate release of several cytokines.4,5 T helper lymphocytes are important for both cell-mediated and humoral immunity. (**Mosmann and Sad 1999**).

The immune system is natural defense mechanism against illness. It allows your body to fight against the invasion by bacteria, viruses, yeast, fungus etc. Taking antibiotics reduces the level of bacterial infection, but your immune system still has to completely finish fighting the infection (**Shalit, *et al*.,2001**).Once you have a particular infection, and your body fights it without the use of antibiotics, your immune system will develop 'memory T cells' (**Williams, *et al*.,2001)**. The next time you contract the same infection, these memory T cells "remember" the previous infection and mounts an immediate immune response to fight it. With the use of antibiotics you are giving the responsibility of fighting infection to the antibiotics instead of your body's immune system. So overtime, and with the overuse of antibiotics your immune system can become less effective (**Morikawa, *et al*.,1996**).

Regarding the antibacterial susceptibility and antibiotic resistance disk diffusion method was used for determination of *S. aureus* resistance for clindamycin, after treatment course, present study revealed that isolated bacterial strain was identified as *S. aureus* and tested for antibiotic resistance using disk diffusion method and inhibition zones were measured for each group. Present study revealed that the highest inhibition zone was for test before experiment and the lowest group was the second group (orally received single dose of defined prescribed dose of clindamycin) followed by 4th group (orally received 1/5 of defined dose clindamycin then increased) then 3rd group an 5th group. The results nearly agree with **Messi, *et al*., (2005**) and **Novo, *et al*.,(2013).**

From present study it was concluded that treatment with antibiotics must be carful and with proper dose and duration to avoid antibiotic resistance which may result from taking antibiotics with wrong way or when they are not needed.. When bacteria survive after a course of antibiotics, another antibiotic must be used with proper dose for complete course even after treatment, to avoid creation of resistant bacteria to antibiotic. During infection using of antibiotics suppress the immune response humeral and cellular so that infected patients must be use probiotics as alternative to antibiotics.

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