Research Article

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Detection of Lethal Dose 50 of Biofilm-producing Methicillin-resistant *Staphylococcus aureus* Local Isolate From Mastitis

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Abstract

It is very important, before starting the manufacture of any vaccine from any microorganism estimation of LD_{50} of that microorganism to determine their pathogenicity and virulence. Estimated LD_{50} was very important to be used in challenge tests later to estimate the protection level of the manufactured vaccine in experimental animals. So, this study was aimed to estimate LD_{50} of local methicillin-resistant Staphylococcus aureus (MRSA) bacterial isolate. A pilot study has been done to determine approximately LD_{50} of used MRSA in the study by using different bacterial concentrations of MRSA to determine approximate LD_{50} that can be able to kill half numbers of animals used in the study to be used later in the estimation of exact LD_{50} by using of Up-and-Down method. Ninety Wistar albino rats have been used for this purpose, eighty-four animals which divided into fourteen groups by six animals for each group (for pilot study) and remained six animals for (Up-and-Down method). The results showed that 9 X 10^{10} CFU/ml was lead to killing half numbers of animals used in the study, this dose has been used as starting dose in the Up-and-Down method to estimation of the exact LD_{50} dose. The results showed that 5.526 X 10^{10} CFU/ml was the exact LD_{50} of local MRSA isolate, which will be used later in the challenge test to estimate the protection level of a locally prepared vaccine against MRSA isolate.

Keywards: LD_{50} , Pilot study, *Staphylococcus aureus*, Biofilm-producing, Methicillin-resistant,Vaccine

الخلاصة

من المهم جدا قبل البدء في عملية تصنيع اي لقاح من اي كائن مجهري حي قياس الجرعة القاتلة لنصف عدد الحيوانات LD_{50} لتحديد إمراضيه وفوعة ذلك الكائن الحي . ومن المهم ايضا استخدام الجرعة المحسوبة والقاتلة لنصف عدد الحيوانات في اختبار التحدي لاحقا لتحديد مستوى الحماية التي توفرها اللقاح المصنع من ذلك الكائن الحي في الحيوانات المختبرية نتيجة حقن تلك الجرعة . اجريت دراسة مصغرة لتحديد الجرعة التقريبية القاتلة لنصف عدد الحيوانات لجرثومة المكورات العنقودية الذهبية والمعزولة محليا والقادرة على قتل نصف عدد الحيوانات المستخدمة في التجربة لاستخدامها لاحقا في حساب الجرعة القاتلة لنصف عدد الحيوانات LD_{50} باستخدام طريقة الصعود والنزول. تم استخدام اربع وثمانون جرذا في الدراسة المصغرة وقسمت الى اربعة عشرمجموعة ثانوية بواقع ستة جرذان لكل مجموعه .تم حقنهم بتراكيز مختلفة من العزلة الجرثومية لتحديد الجرعة التقديرية القادرة على قتل نصف عدد الحيوانات المستخدمة في التجربة. بعدها استخدمت ستة جرذان اضافية في تحديد الجرعة الدقيقة القاتلة لنصف عدد الحيوانات بطريقة الصعود والنزول. اثبتت النتائج ان الجرعة التقريبية القادرة عل قتل نصف عدد الحيوانات كانت 9 X 10^{10} CFU/ml وعند استخدام هذه الجرعة في طريقة الصعود والنزول كجرعة الانطلاق اثبتت النتائج ان الجرعة الدقيقة القاتلة لنصف عدد الحيوانات لجرثومة المكورات العنقودية الذهبية المحلية كانت 5.526 X 10^{10} CFU/ml والتي سوف يتم استخدامها لاحقا في اختبار التحدي لقياس مستوى الحماية التي توفرها اللقاح المصنع من العترة الجرثومية لعزلة المكورات العنقودية الذهبية المحلية .

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Introduction

Renewed trials have been effort all over the world to control many infectious diseases by vaccination, among them, *Staphylococcus aureus* is considered an important pathogen both in animals and humans (1). Many trials every year effort to develop new vaccines against *S. aureus* that cause many diseases both in humans and animals, bovine mastitis is considered an important disease that causes more economic loss due to their effect on the mammary glands and dairy industry by decrease milk quality, and bad milk and milk products (2). vaccination is important method to control *S. aureus* infections (3).Lethal dose 50 (LD_{50}) defined as a dose lead to dying 50 % of exposed animals to infective microorganism (4). While minimum lethal dose is defined as a lower dose of infective microorganism lead to the observation of mortality (5). LD_{50} was used to illation for infectivity of microorganisms (6). It is very important to know LD_{50} in any process of new vaccine development against any microorganism. Classical methods such as reed and Muench or recently modified methods were used to estimate the number of CFU in bacterial infection or viral concentration in viral infection to determine their virulence also estimate their immunogenicity (7). From 1920 after the development of LD_{50} estimation procedure to now several modifications done to it which included arithmetical modifications done by Reed and Muench 1931, Karber 1931, Lorke,1983, or graphical modifications done by Miller and Tainter 1944, Litchfield and Wilcoxon 1949, revised Up-and-Down procedure OECD 1987 (8). So we aimed by this study to estimation of LD_{50} of a local isolate of MRSA bacterial strain from bovine mastitis to use it later in vaccine preparation against it.

Materials and methods

Bacterial strain

Local biofilm-producing methicillin-resistant S. aureus MRSA isolated from bovine mastitis was used in the study to estimation of it is LD_{50}. The bacterial isolate was obtained from central research laboratory, College of Veterinary Medicine, University of Basrah and confirmed as MRSA by (9)

Bacterial concentrations :

Different bacterial concentrations were prepared according to McFarland standards, these concentrations were estimated by spectrophotometer at OD600 and the results were recorded.

Animals

Ninty male Wistar albino rats were used, 84 of them were used in a pilot study which they were divided into 14 groups that were treated by different concentrations and volumes of bacterial strain as shown in table (3), whereas remained 6 animals were used in Up-and-down method to estimation LD_{50} of the bacterial strain

McFarland standards

McFarland standards depend on chemical reaction occurred between each of 1 % barium sulfate ($Ba2Cl.2H2O$) and 1 % of sulfuric acid ($H2SO4$) after mixing them well, this reaction lead to formation of turbidity as a result of barium sulfate precipitation, this turbidity can be compared visually with certain concentration of bacterial suspention. It can be prepared by mixing different volumes from both of them with optimized concentrations for each one in every dilution as shown in table (2) leading to formation of of different degrees of turbidities representing different degrees of bacterial concentrations with specific optical density for each concentration. (10)

Table (1) Show the component of the McFarland standards and their conc.

McFarland scale	McFarland turbidity as CFU/ml	1 % Ba Cl2.2H2O	1 % H2SO4
0.5	1.5×10^8	0.05	9.95
1	3×10^8	0.1	9.9
2	6×10^8	0.2	9.8
3	9×10^8	0.3	9.7
4	1.2×10^9	0.4	9.6
5	1.5×10^9	0.5	9.5
6	1.8×10^9	0.6	9.4
7	2.1×10^9	0.7	9.3
8	2.4×10^9	0.8	9.2
9	2.7×10^9	0.9	9.1
10	3.0×10^9	1	9.0

Spectrophotometer

Lovibond® trade mark spectrophotometer type (spectrodirect) was used in the study to measure bacterial concentration using OD600 wavelength.

Up-and-down method (11,12)

In this method after administration of a dose to the experimental animal, it can be reduced if the animal was died or elevating the dose if the animal was survived and does not respond or exhibited clear clinical signs, allowing to optimum dose titration to get the perfect dose that gives the best results. Clinical signs exhibited by affected animals include increasing respiration rates, abdominal type respiration, abdominal debilitation, sluggishness, absence of response to reflexes, convulsion, tremors, severe muscle spasms, severe lacrimation, cyanosis, ataxia, and depression.

Pilot study :

A pilot study has been done, 84 male Wistar albino rats at eight weeks of age and 200-225 grams/animal body weight has been used to estimate approximately lethal dose 50 (LD_{50}) for the MRSA bacteria, they were divided into equal 14 groups by six animals for each group, different volumes and concentrations were prepared from the local MRSA, each group of rats were injected by different prepared concentrations with different volumes of MRSA strain by intrapretonial rout I/P as shown in table (3). All animals were breaded as follow :

1- Every 6 animals were kept in one cage with labeling each of them with in their cage.

2- All the ideal environmental conditions for rearing were set such as temperature, darkness, feeding on concentrated feed, and water-saving.

3- Animals were kept under this condition for one week to check the health of animals before starting the study.

4- Animals were injected by intraperitoneal I/P route with different bacterial concentrations according to pilot study design.

5- Animals were observed for 72 hours and results were recorded.

Results and Discussion :

Bacterial concentrations:

Different bacterial concentrations were prepared according to McFarland standards turbidity, then these prepared concentrations were estimated by spectrophotometer at OD600 wave length, table (2) illustrate different OD600 values for different bacterial concentrations prepared according to McFarland standards turbidity

Table (2): Different OD600 values that equal for different bacterial concentrations according to McFarland standards

Bacterial concentrations according to McFarland standards turbidity	Spectrophotometer values at OD600 wavelength
1.2×10^7	0.007
1.2×10^8	0.136
1.2×10^9	0.976
1.2×10^{10}	2.238
3×10^{10}	2.782
6×10^{10}	3.020
9×10^{10}	3.496

Pilot study results

The result shows that 9×10^{10} CFU/ml bacterial concentration of local isolate MRSA lead to killing 3 of 6 injected animals, while 6×10^{10} CFU/ml of bacterial concentration killed 2 animals of total 6 injected animals, finally, 3×10^{10} of bacterial concentration killed only 1 of total 6 injected animals after 24 hours. All other concentrations do not cause any death of injected animals as shown in table(3).

Calculation of LD_{50} by using of Up-and-down method :

According to the Up-and-down method, the last concentration that killed the half number of animals was suggested as a default concentration to be used as start dose in the Up-and-down method to calculation LD_{50} according to (13) as follow :

According to table 1 and 2 approximately LD_{50} dose of local MRSA bacterial isolate was 3.496 OD600 that equal to 9×10^{10} which was used as a starting dose in the Up-and-down method, this used dose can be reduced if the animal was died or elevated if the animal was survived by ± in dose reach to 2×10^2. It has been used 6 animals in this method and the results were as in table (4):

The Up-and-Down method nowadays was used as one of the important methods that take into account the issue of animal welfare because it reduces the number of animals killed (11). It is very important to know approximately lethal dose 50 to start from it by increasing or decreasing 30 % from it to estimate exact LD_{50} . It is nowadays one of excellent method can be used to estimate both lethal and or infective doses for tested microorganism. Pathogens of high values LD_{50} mean that it has high pathogenicity and need for high doses of vaccine (14). Our result proved that LD_{50} of local isolated MRSA was 5.526×10^{10} CFU/ml by using of Up-and-Down method and this is differ something from each of Saganuwan (7) who was referred that LC50 of *Staphylococcus aureus* (ATCC 29123) strain was 1.75×10^{10} CFU/ml in rats and also Jankie *et al*. (11) who showed that the LD_{50} of *S. aureus* (ATCC 29123) strain was 1.75×10^{10} CFU/ml in rats also, this small variation between our results and other authors may explained to a difference in the strain of *S. aureus* used in the study in which we used local MRSA isolate strain in contrast to standard strain use by other authors.

Senna *et al*.(15) revealed that LD_{50} of *S. aureus* with Pc1. neo antigens were 1.1×10^8 CFU/ml when injected I/P in the mouse, while Gaudreau, *et al*.(16) referred that *S. aureus* with fibrinogen antigens was 1×10^7 CFU/ml when the microorganism injected intravenous I/V in mouse also, Haghighat, *et al*.(17) showed LD_{50} of *S. aureus* with endotoxin-free PBS antigens was 5×10^8 CFU/ml when injected intraperitoneal route I/P in mice. The reason for this difference in the results may due to many factors that affect LD_{50} determination such as: whether the determination method is *in vitro* or *in vivo*, pathogenicity degree

of the used microorganism, titration of antigen and antibody levels, a rout of microorganism administration was an important factor effect on the result of LD_{50} (18). The pathogenicity of any pathogen can change depending on the of the animal used in the experiment (19).

Conclusion

It was very important to estimate the LD_{50} of any microorganism before preparing of vaccine from it. Up and down method was one of the methods concerned with animal welfare as it leads to killing the least number of animals and provides compassion for animals, provided conditional to know the approximate LD_{50} to start from it to know exact LD_{50} .

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Conflict of Interest

The authors state that there is no conflict of interest.

Table(3)Show different bacterial concentrations and volumes prepared from local MRSA with their killing effect on different groups

Group	No .	McFarland standards	Volume of inj.	Death	Live	Death/Live
G1	6	1.2 X 10^7	0.5 ml/animal	0	6	0/6
G2	6	1.2 X 10^8	0.5 ml/animal	0	6	0/6
G3	6	1.2 X 10^9	0.5 ml/animal	0	6	0/6
G4	6	1.2 X 10^{10}	0.5 ml/animal	0	6	0/6
G5	6	3 X 10^{10}	0.5 ml/animal	0	6	0/6
G6	6	6 X 10^{10}	0.5 ml/animal	0	6	0/6
G7	6	9 X 10^{10}	0.5 ml/animal	0	6	0/6
G7	6	1.2 X 10^7	1 ml/animal	0	6	0/6
G8	6	1.2 X 10^8	1 ml/animal	0	6	0/6
G9	6	1.2 X 10^9	1 ml/animal	0	6	0/6
G10	6	1.2 X 10^{10}	1 ml/animal	0	6	0/6
G11	6	3 X 10^{10}	1 ml/animal	1	5	1/5
G12	6	6 X 10^{10}	1 ml/animal	2	4	2/4
G14	6	9 X 10^{10}	1 ml/animal	3	3	3/3

Table (4) Show bacterial concentrations used in the Up-and-down method with their effects on used animals to estimate LD_{50} of MRSA

No.	concentration	Result X or O	Symbols that estimate from table values
1-	$9 X 10^{10}$	X ↓	X
2-	$7 X 10^{10}$	X ↓	X
3-	$5 X 10^{10}$	O ↑	O
4-	$7 X 10^{10}$	X ↓	X
5-	$5 X 10^{10}$	O ↑	O
6-	$7 X 10^{10}$	X	X

O : Remaining live with no effect X: Killing effect (Death)

According to Up-and-down method LD_{50} calculated as follow :

LD_{50} value = xf + K d, Xf =The last dose used

K = table value, d = (± in dose)

Symbols XXOXOX from table value was (- 0.737), $LD_{50} = 7 X 10^{10} + (- 0.737) (2 X 10^{10})$

$= \mathbf{5.526 X} 10^{10}$

$5.526 X 10^{10}$ was equel to 2.941 OD 600 at spectrophotometer

$7 X 10^{10}$ was equel to 3.185 OD 600 at spectrophotometer

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