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The Innovative Method for Vaccine Preparation Against Multidrug Resistant and Virulence *Acinetobacter baumannii* Iraqi Isolates

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Abstract:
The expanding of the medically important diseases created by multidrug-resistant *Acinetobacter baumannii* warrants the evolve a new methodology for prevention includes vaccination and treatment. Totally of forty-five clinical isolates identified as *A.baumannii* were obtained from hospitalized patients from three hospital in Baghdad City during the period from February 2016 to August 2016. Followed by diagnosing using different methods. Every strain was tested for susceptibility testing also some important virulence factorswere detected. Two isolates were chosen for the immunization and vaccine model, the first one remittent for most antibiotics except one are too virulence (strong) and the second is less virulent and resistance (weak).Enzyme-linked immunosorbent assaywas used for assessments of Toll like receptor 4,and Toll like receptor 2 concentrations in mouse serum at 14, 21 and 28 days of immunization. Results proved that the strong isolate showed resistance to all antibiotics except one and positive to all virulence factors except one, while the weak isolate resistance to Ceftriaxone, Cefotaxime, positive to tow virulence factors. Mice were intramuscular inoculated with strong and weak isolate. There are high significant differences when using strong *A.baumannii* strong in the level of TLR4 and there was not an important variation among the use of strong and weak isolation in the level of TLR2.Finaly,the yield refers to the TLR4 plays a key role in innate sensing with multidrug resistance isolate immunization, whereas TLR 2 shows it gives the same level of stimulation during immunization with both strains but lesser concentration than TLR4, so the inactivated with MDR isolate has a potential for development as a candidate vaccine for strong protection against MDR isolate infections.

Keywords: Vaccine; *Acinetobacter baumannii*; TLR4, TLR2; Virulence factors; MDR.

Introduction:

This bacterium is considered as a low virulence microorganism except when isolated in immune compromised patients. These microorganisms are most correlated with hospital acquired infection more than society acquired infections [1].A thin, slimy film of bacteria that adheres to a surface that is called biofilm-like; other bacteria make it protected from hostile environments [2]. Also, the biofilm formed in that part of liquid and air is called ‘‘pellicle’’, also stay on top that needs more organizations due to the loose solid surface for initial attachment [3]. The connection between biofilm with antibacterial agent reluctance is of considerable interest to Biomedical Researchers. Beside what is worth to mention, many researches show a few antibacterial agents ambidextrous to stimulate biofilm formation, that give us an idea how that thin film is regulated by global react for outer Stresses, including antibiotics [4]. Multiple virulence factors are required for the pathogenesis of infected with *A. baumannii*, consisting of capsule, bacterial phospholipases, penicillin-binding proteins, secreted outer membrane vesicles, with slimy film production [5].

The resistance to multiple antibiotics besides slimy film on different surfaces that form a significant way in the pathogenicity of *A. baumannii* previous reported clinical isolates of this bacteria has been related to the multidrug-resistant (MDR) phenotype, which is a consequence of different resistance mechanisms against different antibiotics, such as permeability defects, expression

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of multidrug efflux pumps, production of antibiotic degradation - modification enzymes and alteration of drug-targeting sites [5]. Generally, the aim of this study 1-isolated*A.baumannii* to prepare local vaccine against *A.baumannii* Iraqi isolate. 2-to clarify mechanisms involving in natural immunity and the role of these receptors as the parameter for protective immunity.

Materials and Methods

Isolation and Diagnosis *A. baumannii*:

This bacterium was isolated from the duration between Feb. 2016 to Aug. 2016, and specimens were collected from patients suffering from urinary tract infections (UTIs), infected wounds, and sputum. Then all bacterial isolates were diagnosed by three different methods, including Chromagar media, Vitek 2 system and Genotype diagnosis by PCR [6].

Antimicrobial susceptibility testing:

All isolates were tested for antimicrobial susceptibility with Amikacin (30 µg) (AK), Gentamicin (10µg) (GEN), Ceftazidime (30µg) (CAZ), Ciprofloxacin (5µg) (CIP), Ampicillin-sulbactam (10/10µg) (A/S), Imipenem (10µg) (IPM), Meropenem (10µg) (MEM), Piperacillin (100µg) (PI), Ticarcillin (75µg) (TI), Tetracycline (30µg) (TE), Cefepime (30µg) (CPM), Ceftriaxone (30µg) (CRO), Cefotaxime (30µg) (CTX), Levofloxacin (5µg) (LEV), Trimethoprim-sulfamethoxazole (1.25/23.75µg) (SXT) antibiotic agents (Bioanalysis ,Turkey) All of the inoculated plates were aerobically incubated at 37°C for 18-24 hr. in an aerobic atmosphere. Resultswere interpreted based on the instruction provided by Clinical Laboratory and Standard Institute (CLSI 2014) Guidelines, and used *Pseudomonas aeruginosa* ATCC® 27853 and *Escherichia coli* ATCC® 25922 as a quality control for trimethoprim-sulfamethoxazole [7].

Virulence factor detection assays:

Virulence factor phenotypic detection of *A. baumannii* isolates was done in order to detect the ability of biofilm formation followed the method described by [8] and another ten virulence factors. Which were Capsule [9], Biofilm and Motility [10], Twitching motility [11], Heamolysin [12] and Pellicle formation [13].

Vaccine preparation:

In order to prepare local vaccine against *A. baumannii* Iraqi isolate, we is selected two isolates the first one is highly virulence multidrug isolate while the second one is less virulence and resistant to few antibiotics used in this study. The vaccine was prepared in a modified manner from the original method [14] by growing the *A. baumannii* isolate (strong) on Mueller-Hinton broth and washed 3 times in phosphate buffer saline (PBS) , pH=7 before inactivation in 3.5% formalin for 20 hr. Complete inactivation of the bacteria was confirmed by plating on blood agar. The concentration of inactivated cells was adjusted to 1×10 cells/ml and combined 1:1 (v:v) with the aluminum phosphate adjuvant.

Mice immunization schedule:

Male BALB/C mice age ranged between 6 to 8 weeks and their weight ranged from 20-25 grams obtained from the Biotechnology Research Center at Al-Nahrain University and housed under specific pathogen-free conditions thatwere used in a vaccination model by intramuscular injection of 100µl of the vaccine into each quadriceps muscle (total dose = 1×10^{8} inactivated cells) on days 14 and 21. The animal experiments wereperformed according to the protocols and guidelines approved by Al-Nahrain University Animal Care, The mice were randomly divided into 4 groups. Mice of groups 1, 2 and 3 were vaccinated, then bled almost every other day (days 14, 21, and 28) and sacrificed at the end of the experiment. The control group 4 mice were similarly inoculated with a mixture of PBS and adjuvant [15].

Mouse model of *A. baumannii* infection:

A murine model of disseminated sepsis was used for bacterial challenge. *A. baumannii* strains were grown for 18hr. at 37℃ in Mueller-Hinton broth and adjusted to the appropriate concentration in physiologic saline. Inoculate was prepared by mixing the bacterial suspensions 1:1 (v:v) with PBS. Mice were injected intraperitoneally with (0.5 ml) of inoculate and bacterial concentrations were determined by plating on blood agar and survival was monitored for 7 days [15].

Active and passive immunization:

For active immunization studies, mice were challenged on day 28 after receiving immunizations on days 14 and 21. In passive immunization studies, 200µl of serum was collected at 28 days from vaccinated mice then administered subcutaneously 3hr. before the challenge [15].

Assessment level of Toll like receptor 4 and Toll like receptor 2 by ELISA kit:

Serum was collected of 1ml at 14, 21, and 28 days in an assessment concentration of Toll like receptor 4 and Toll like receptor 2 by ELISA Kit Assay procedure of TLR4 and TLR2 all Reagent Preparation before starting. It is recommended that

all Standards and Samples be added in duplicate to the Microtiter plate according to manufacturing companies (USbiological).

Data analysis

Statistical analysis was performed by analysis of mono way variance where appropriate using (SPSS VERSION 21) Values are expressed as mean _ SEM... A,b.c LSD (Least Significant Difference) for rows, similar letters mean the absence of significant differences and The opposite is true.

Results and Discussion

In our study, result out of 55 gram negative bacterial isolates only 45 were proved as *A. baumannii* after conforming identification by phenotypic and genotypic methods [16].

Data accessible in Table 1 shows the resistance number and percentage of *A. baumannii* isolates to the antibiotics used in update study. We discovered that the antibiotic resistance result showed a high level resistance of *A.baumannii* clinical isolates to 14 from 15 antibiotics. Current study revealed that All *A.baumannii* isolates had (97.78%) resistance to Cefotaxime, and Cefotaxime variable percentage of resistance to Piperacillin (82.22%), Ticarcillin (86.66%), Cefepime (77.78%), Imipenem(11.11%), Meropenem (26.67%), Amikacin (2.22%), Levofloxacin(93.33%) according to Clinical and Laboratory Standards Institute guidelines 2014 depending on a diameter of inhibition zone mm. while Sensitive of isolates to antibiotics as follow Tetracycline (8.89%), Amikacin (84.44%), Meropenem(66.66%),Gentamicin and Imipenem (88.87%),Cefepime and Piperacillin (11.11%), Levofloxacin (6.67%), Ampicillin- sulbactam (100%), Ticarcillin (13.33%) and low level of Intermediate isolate to Imipenem, Ceftriaxone, Ciprofloxacin and Cefotaxime (2.22%), Meropenem, and Piperacillin (6.67%), Cefepime (11.11%), and Amikacin (13.33%). Table.1 and Fig. 1 list the number and percentage of all isolates.

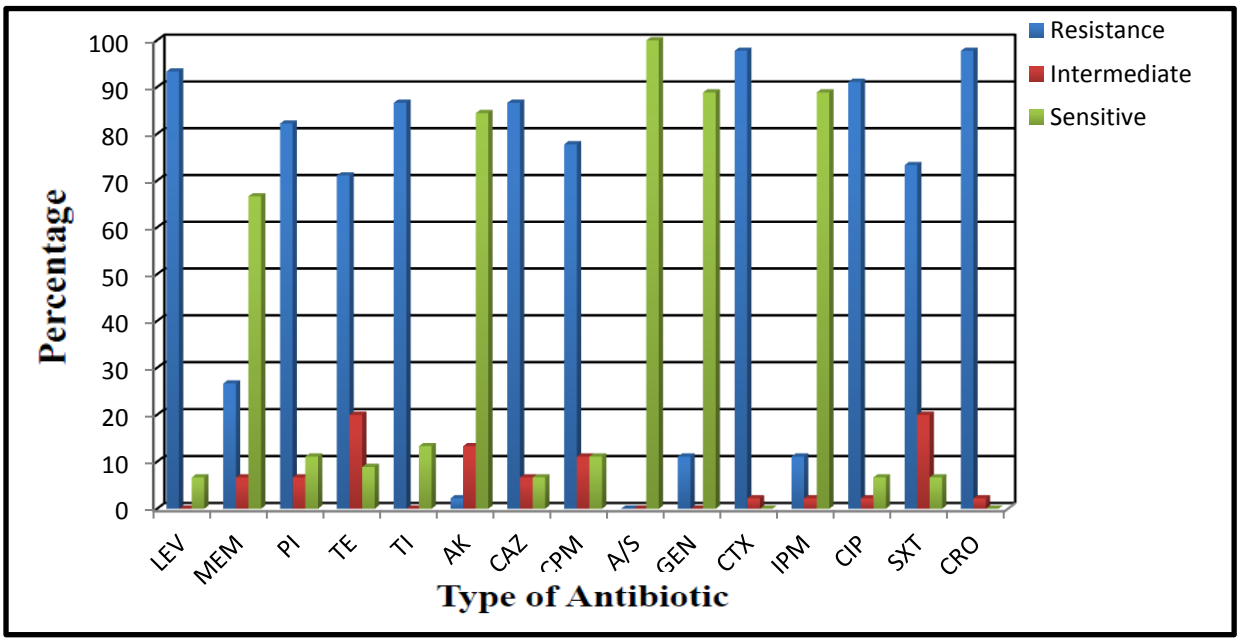


Figure 1. Antibiogram profile of *A.baumannii* isolates by disk diffusion method.

Table 1: Antibiotic Susceptibility of 45 *A. baumannii* isolates.

CRO	SXT	CIP	IPM	CTX	GEN	A/S	CPM	CAZ	AK	TI	TE	PI	MEM	LEV	Antibiotics
44	33	41	5	44	5	45	35	39	1	39	32	37	12	42	R
97.78	73.33	91.11	11.1	97.7	11.1	0	77.7	86.6	2.22	86.6	71.1	82.2	26.6	93.3	N(%)
1	9	1	0	1	0	0	5	3	6	0	9	3	3	0	I
2.22	20	2.22	0	2.22	0	0	11.1	6.67	13.3	0	20	6.67	6.67	0	N(%)
0	3	3	40	0	40	45	5	3	38	6	4	5	30	3	S
0	6.67	6.67	88.8	0	88.8	100	11.1	6.67	84.4	13.3	8.89	11.1	66.6	6.67	N(%)
45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	Total
100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	N(%)

R=Resistance , S=Sensitive , I=Intermediate (moderate) , N%=Number of Percentage
PI=Piperacillin 100µg ,TI=Ticarcillin 75 µg , A/S=Ampicillin-Sulbactam 10/10 µg, CAZ=Ceftazidime 30 µg,
CPM=Cefepime 30 µg , CRO=Ceftriaxone 30µg, CTX=Cefotaxime 30µg ,IPM=Imipenem 10 µg
MEM=Meropenem 10 µg , AK=Amikacin 30µg, GEN=Gentamicin 10µg, TE=Tetracycline 30µg, CIP=Ciprofloxacin 5µg
LEV=Levofloxacin 5µg , SXT=Trimethoprim-sulfamethoxazole 1.25/23.75µg

The current search shows that *A. baumannii* may be silently spread with high level, Table 2 in a hospital and health care facility that threat of undetected reservoirs. However, the source of infection may include the environment and health care device can involve with transport these bacteria among staff and patients [17].

Results from Fig.1 show that higher resistant percentage was found for Ticarcillin .This result partly agrees with a pervious study by [18].All isolates are resistant to Ticarcillin (91.6%) and similar to the study achieved by [19] who found this bacterial strains were reluctance to Piperacillin (100%), *A. baumannii* were High reluctance for that antibiotic because widespread use of these

antibiotics in Baghdad hospitals; also high level of resistance to cephalosporins: Cefotaxime (97.78%), and (77.78%, 97.78%) for cefepime and Ceftriaxone receptively [20] .

Resistance to this bacteria strain to Cefotaxime and cefepime was (100%) that is higher than our study result. High level of reluctance to 3 generation cephalosporins could be attributed to the production of ESBLs, since it mediates reluctance to wide spectrum cephalosporins (e.g.: Ceftriaxone and Cefotaxime) [21].

Results from Fig.1 show that reluctance to meropenemwas (26.67%). This result is lower than that reported in pervious search contacted in Turkey, which reported that the resistant for this bacteria strain collected from clinical samples to meropenem was (53.3%) [22].

While in another study in UK [23] found that only tow isolates of this bacteria are reluctant to this antibiotic. Meropenem is more active against gram negative bacteria [24]. Lower accessibility of this antibiotic in Baghdad health care facility that reason of lower percentage of resistance to Meropenem.

The results in this study reveal that Amikacin, was more effective (84.44%) than other aminoglycosides. This result was parallel with the other studies worldwide, as with [25] In Turkey and another study in Europe [26] who found that resistance against aminoglycosides were (5.4%) to Amikacin, lower than get in our result .

As shown in Fig.1, our results show that resistance percentage to levofloxacin (93.33%) higher than the study achieved by [27]. Percentages of resistance of isolates to the remaining antibiotics were higher than previous studies in Brazil [28] and India this is because multi-resistance plasmid harboring *A. baumannii* [29].Mentioned that unsuitable and wrong ways to give antibiotics with poor control of infect all that leading to raised reluctance for these bacteria to available antibiotics.

Concerning combination of carbapenem drugs, our result show (0%) resistance for *A. baumannii* isolated from Baghdad, but in different nationwide surveillance study [30], we demonstrated the distribution of the different isolates and also showed the resistance and the carbapenemase gene distributions among these isolates. There were 3 main classes of *A. baumannii* identified, which showed the higher antimicrobial resistance percentage to Ampicillin/Sulbactam was (5%).

Furthermore, the detection of virulence factor has been revealed that each isolate has more than one virulence factor as seen in Table 2 and Fig.2. All *A. baumannii* isolates are non motile and capsulated. Otherwise, all isolates were pellicle producer and found to be positive in twitching motility, but only 25(55.56 %) out of 45 isolates were found to be positive in biofilm production. The results are listed in Table 2.

Table 2. Virulence factors percentage of *A.baumannii* isolate

V. Factor / Result	Pellicle	Biofilm	Twitching Motility	Motility	Capsule
Positive	45 (100%)	S 5(11.12%) M 10(22.22%) W10(22.22%)	45(100%)	0(0%)	45(100%)
Negative	0 (0%)	20 (44.44%)	0(0%)	45(100%)	0(0%)
Total	45(100%)	45(100%)	45(100%)	45(100%)	45 (100%)

S= Strong biofilm forming, **M**=Moderate biofilm forming, **W**=Weak biofilm forming

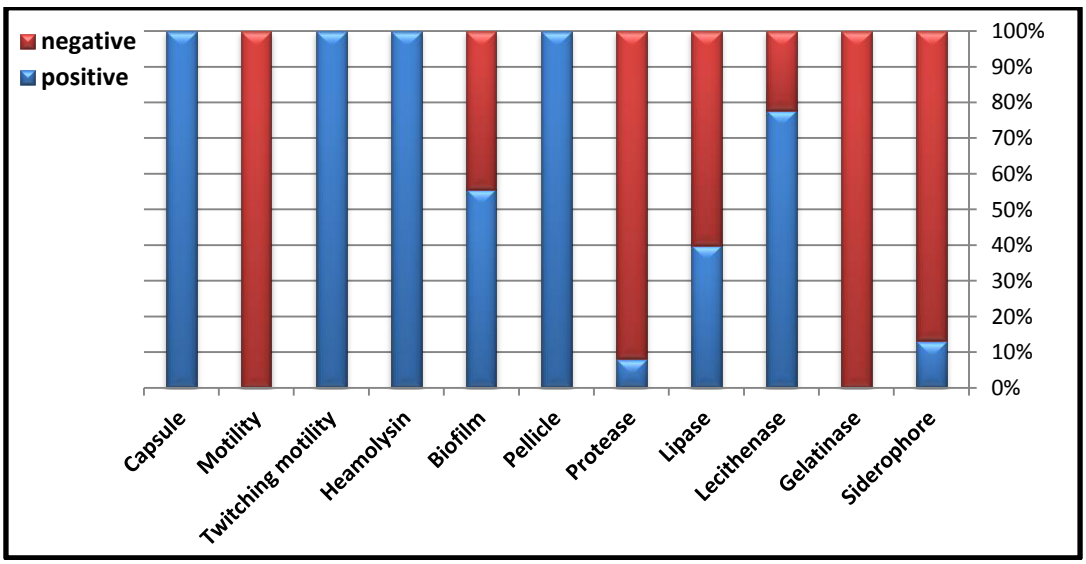


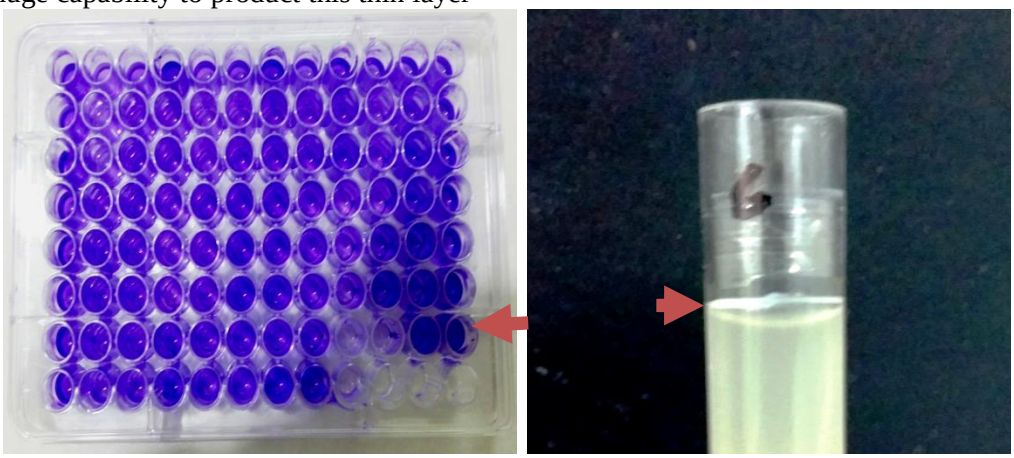
Figure 2. Percentage of virulence factors to *A. baumannii* isolates.

In our search, a total of 25 isolates produced biofilm, and 20 isolates non-biofilm produce. The results of quantitative assay for biofilm formation percentage are shown in Table 2 andFig.2 10 (22.22%) isolate weak biofilm produce, 10 (22.22%) isolate moderate biofilm produce, 5(11.12%) isolate strong biofilm produce, and 20 (44.44%) were non biofilm produce .That result is higher than that obtained in the previous study [31] That showed 3(6.67%) weak biofilm produce, 5(11.11%) moderate biofilm produce, 37(82.22%) strong biofilm produce, and 27(37.4%) non biofilm produce the variation in biofilm formation is possibly related to the Variations in *csuA/BABCDE* genes of the tested isolate, because these genes have been considered as the most common important factors that can influence slimy film product among different isolates [32].

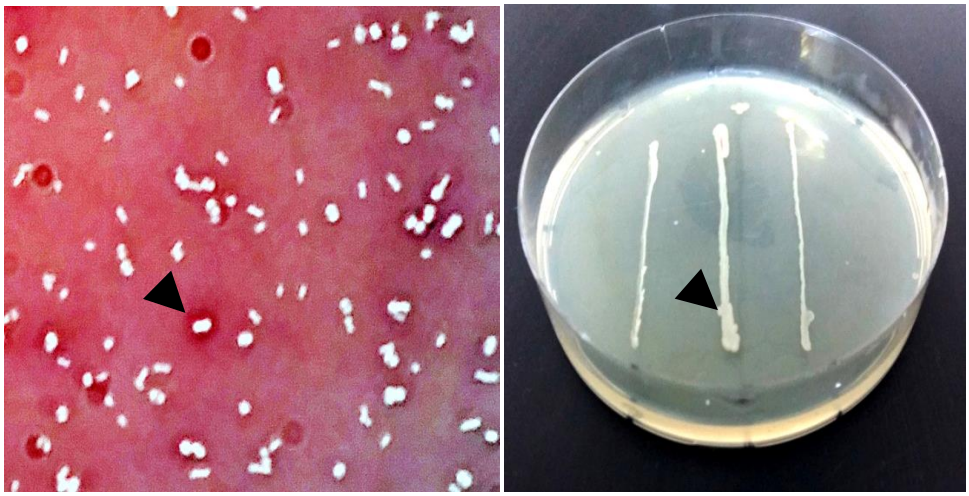
From Fig. 2 the result of pellicle forming all 45(100%) isolate of *A. baumannii* was positive which is similar to the result of the study which reported that the members of the *A. baumannii* strain have huge capability to product this thin layer more than other species that help infect mechanism to host body via *A. baumannii*, and probably contributing to the increased risk of clinical infection [33].

While the result of Clinical strain showed twitching motility in all isolates (100%) while, motility (0%) as shown in Fig.2 This result is in agreement with the study of [34] who reported that isolates that twitching did not motile and this bacteria strain is motile, but did not twitch. The reason behind that PilA appeared huge level from amino acid sequence conservation within twitching isolates, indicating that type IV pili may play a role in motility in this species.

The study result about virulence factor capsule showed all isolates of *A. baumannii* 45(100%) positive capsule that are in agreement with the results of the study achieved by [35]. The acquisition capsules of bacteria that can resist the non- suitable status like heat and drought, and assist those bacteria in survive on living and non-living objects in hospitals [36]. It is show in Fig.3.



Biofilm test of *A.baumannii* (red arrow) **Pellicle forming of *A.baumannii* (red arrow)**



Capsule of *A.baumannii* (black arrow) **Twitching motility of *A.baumannii* (black arrow)**

Figure 3. The most virulence factors to *A. baumannii* isolates

The results of statistical analysis in Table 4 and Fig.4 show high significant differences when using strong isolation of level TLR4 (p value = 0.001) in 14 days and subsequently decreasing concentration in 21 and 28 days, whereas Table 5 and Fig.5 show no important variation among the use of strong and weak isolations of level TLR2 concentration and almost the same level within 14 to 21 days and decreasing in 28 day statically p value of least of (0.05 value) was considered statistically significant. as shown below.

Table 4. Concentration TLR4 Weak and Strong isolate in serum of mice during 14, 21 and 28 day.

Strong isolate TLR4			
	Conc.	**ng/ml**	
Sample	Test	Control	P value
day 14	6.4±0.53 a	0	0.001
day21	0.5±0.13 b	0	0.05
day 28	0.47±0.09 b	0	0.05
P VALUE	0.01		
LSD	0.87		
Weak isolate TLR4			
	Conc.	**ng/ml**	
Sample	Test	Control	P value
day 14	0	0	NS
day21	0.75±0.25	0	0.001
day 28	0	0	NS
P value	0.05		

Table 5. Concentration TLR2 Weak and Strong isolate in serum of mice during 14, 21 and 28 day.

Strong isolate TLR2				
	Con.	**ng/ml**		
Sample	Test	Control	Expected value	P value
day14	17.11±0.98 a	0	6.85	0.001
day21	18.21±0.64 a	0	6.85	0.001
day28	15.53±0.72	0	6.85	0.001
LSD	1.4			
P value	0.001			
Weak isolate TLR2				
	Conc.	**ng/ml**		
Sample	Test	Control	Expected value	P value
day 14	18.86±0.57 a	0	6.85	0.001
day21	17.39±0.49 b	0	6.85	0.001
day 28	15.53±0.71 c	0	6.85	0.001
LSD	1.12			
P value	0.01			

Similar letters mean the absence of significant differences and the opposite is true.

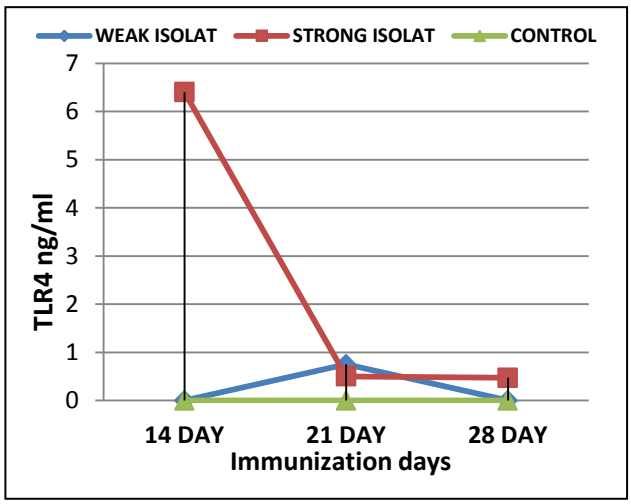


Figure 4. TLR4 Concentration in mice infection with Strong and weak *A.baumannii* from 14 to 28 day.

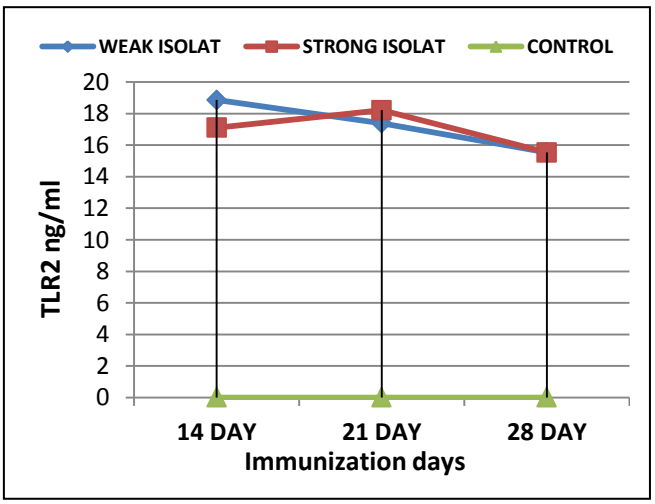


Figure 5. TLR2 Concentration in mice infection with strong and weak *A.baumannii* from 14 to 28 day.

The varying concentrations of Toll Like Receptor 4 and Toll Like Receptor 2 that appeared originally in our search to get best knowledge about roles of Toll- like receptor 4 and Toll Like Receptor 2 in host receptor for this bacteria. Our results show that TLR4 perform significant function in innate sensing for *A. baumannii* by whole cell resulting in active removal of the bacteria from sepsis infection. Because the main ingredient of *A. baumannii* is LPS, it is considered the main ligand for TLR4 to prove the TLR4 actually crucial receptors during *A. baumannii* sepsis infection in vivo that agree with the result of previous study [37].

However, other receptor sense lipid, peptide, glycan, and combination among them, that are main ingredient of gram positive bacteria but, to a lesser degree, are also found in gram-negative bacteria. To provide first aspect to function of this receptor in *A.*

baumanni sepsis TLR2, variance TLR4, has received attention primarily as an important pattern recognition receptor for gram positive bacteria; although it might also share in host natural immunity versus gram negative bacteria. This also agrees with the study of [38].

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طريقة مبتكرة لتحضير لقاح ضد *A. baumannii* ذات مقاومة متعددة وضارية لعزلات عراقية

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الخلاصة :

ان التوسع في الاهمية السريرية للامراض التي تسببها *A.baumannii* ذات المقاومة المتعددة يضمن تطوير منهجيات جديدة للوقاية تشمل التلقيح والعلاج. تم الحصول على خمسة واربعين عزلة سريرية تم تحديدها على انها *A.baumannii* من مرضى في ثلاث مستشفيات في مدينة بغداد خلال الفترة من فبراير 2016 الى اغسطس 2016. ثم شخصت باستخدام طرق مختلفة. تم فحص جميع العزلات لاختبار الحساسية للمضادات الحيوية، كما تم الكشف عن بعض عوامل الفوعة الهامة. تم اختيار اثنين من العزلات للتمنيع ونموذج اللقاح الاولى مقاومة لجميع المضادات الحيوية ماعدا واحد هي ضارية جدا (قوية) والثانية اقل ضراوة ومقاومة (ضعيفة). تم استخدام تقنية ELISA لتقييم تراكيز TLR4,TLR2 في مصل الفئران في 14، 21، 28 يوما من التمنيع. بينت النتائج ان العزلة القوية اظهرت مقاومة لجميع المضادات الحيوية باستثناء واحد وايجابية لجميع عوامل الضراوة ماعدا واحد، في حين ان مقاومة العزلة الضعيفة لمضادي السيفترياكسون و والسيفوتاكسايم وايجابية لعاملين ضراوة. تم تلقيح الفئران بالعضلة باستخدام العزلة القوية والضعيفة. وكان هناك فروق معنوية عالية عند استخدام العزلة القوية لتركيزTLR4ولم يكن هناك فرق معنوي كبير بين استخدام العزلة القوية والضعيفة لتركيزTLR4، وتشير نتائجنا الى ان TLR4 تلعب دوراً رئيسياً في الاستشعار الطبيعي ضد العزلة الممنعة ذات المقاومة المتعددة، في حين انTLR2تبين انه يعطي نفس المستوى من التحفيز اثناء التمنيع الى السلالتين ولكن بتركيز اقـل من TLR2 وبـذلك يكون التمنيع بهذه الطريقة جيد لعمل لقاح مناسب للحصول على حماية قوية ضد الاصابة بهذه العزلات.

الكلمات المفتاحية : لقاح، *A.baumannii*، TLR4,TLR2، عوامل الفوعة ، مقاومة متعددة للمضادات.