Determination the titer antibodies against LPS extracted from *Pseudomonas aeruginosa* isolated from eye infection

تحديد عيارية الاجسام المضادة لمتعدد السكريد الشحمي المستخلص من بكتريا *Pseudomonas aeruginosa* المعزولة من خمج العين

Jinan M. Hasan **Rasmyia A. Abu-risha** **May T. Flayyih**
College of Science/ University of Baghdad
جنان محمد حسن **رسمية عبد أبو ريشة** **مي طالب فليح**
كلية العلوم/ جامعة بغداد

Abstract

From September 2008 to May 2009, 122 specimens were collected by sterile cotton swaps from patients suffering from eye infection at Ibn Al-Haitham Teaching Eye Hospital, 12 of them (9.83% isolation percentage) were diagnosed *as Pseudomonas aeruginosa* isolates by using biochemical tests, API 20 E system: they were named (P(1), P(2),……..P(12)). The agglutination sera for the grouping of *P. aeruginosa* test show that all the isolates belong to the *P. aeruginosa* strains (6, 9, 12, 16). The result show that P10 isolate have a greater ability to adhesion when all *P. aeruginosa* isolates were tested on Congo red agar medium and adherence to smooth surfaces. The chemical analysis of crude LPS that was extracted from P10 by using digestive enzyme and hot phenol water method showed that the percentage of carbohydrates was 7.3 and the percentage of protein was 1.3. No nucleic acids were found while the chemical analysis of partially purified LPS that was made by gel-filtration chromatography by using Sephacryl 200 S showed that the percentage of carbohydrates in the partial purified LPS was 19.5% and the percentage of binding proteins was 0.006%. No nucleic acids were found. When the molecular weight of LPS was measured by gel-filtration chromatography by using Sepharose C1- 6B- 200 it was found to be 630957 Dalton. The sera were prepared by using two wild type rabbits (weight 2-2.5 kg). They were injected with five doses over 70 days of different concentrations of partially purified LPS that have been extracted from *P. aeruginosa* P (10) isolate. One rabbit was used as a control that has been injected with PBS. The titer of antibodies in sera was determined by passive haemagglutination and it was in the immunized rabbit's sera 160, and the best concentration of LPS was 10µg.

المستخلص

جمعت 122عينة للفترة من أيلول 2008 لغاية أيار 2009 من مرضى يعانون من التهاب العين من مستشفى ابن الهيثم التعليمي للعيون بواسطة مسحات قطنية معقمة . باستخدام نظام الAPI 20 E شخصت 12 عينة من هذه العينات و التي تمثل 9.83% من العينات انها بكتريا *Pseudomonas aeruginosa* . في حين اظهر التشخيص المصلي باستخدام اختبار التلازن المصلي لمجاميع ا لـ *P. aeruginosa* أن العزلات تعود للسلالات الآتية (16,12,9,6) و قد أظهرت النتائج ان العزلة P10 لها القابلية الأكثر على الالتصاق عند اختبار قابلية جميع العزلات على الالتصاق على الأسطح الملساء و اغار احمر الكونغو . تم استخلاص متعدد السكريد الشحمي الخام من العزلة P10 باستخدام طريقة الفينول الساخن حيث كانت نسبة التحليل الكيميائي للكاربوهيدرات 7.3% وللبروتينات 1.3% على التوالي في حين لم توجد أي أحماض نووية ، في حين اظهر التحليل الكيميائي لمتعدد السكريد الشحمي المنقى جزئيا بواسطة كروماتوكرافيا الترشيح الهلامي باستخدام هلام الـ Sephacryl 200 S أن نسبة الكاربوهيدرات كانت 19.5% ونسبة البروتينات المرتبطة ك انت

0.006% و لا يحتوي على احماض نووية . عند قياس الوزن الجزيئي لمتعدد السكريد الشحمي بواسطة كروماتوكرافيا الترشيح الهلامي باستخدام هلام ال - Sepharose C1- 6B 200 أظهرت النتائج أن الوزن الجزيئي لمتعدد السكريد الشحمي يبلغ 630957 دالتون . حضرت المصول بحقن أرنبين من النوع البري (2-2.5 كيلو غرام) بخمس حقنات على مدى 70 يوما و بتراكيز مختلفة من متعدد السكريد الشحمي المستخلص من بكتريا ال- *P. aeruginosa* العزلة P10 والمنقى جزئيا مع استخدام أرنب سيطرة واحد حيث تم حقنه بمحلول دارئ الفوسفات . حددت عيارية الا جسام المضا دة في المصول باستخدام تفاعل التلازن الدموي المنفعل و قد كانت العيارية لمصول الأرانب الممنعة ضد متعدد السكريد الشحمي 160 والتركيز الأمثل لمتعدد السكريد الشحمي هو 10 مايكروغرام .

Introduction

Pseudomonas aeruginosa is the most commonly encountered gram-negative species that is not a member of the family *Enterobacteriaceae* [1]. *Pseudomonas aeruginosa* can colonize normal humans in whom *Pseudomonas aeruginosa* is saprophyte and cause disease in humans with abnormal host defenses [2].

The virulence factors of *P. aeroginosa* include: exotoxins, endotoxins, and a variety of cytotoxic substances including proteases, phospholipases, rhamnolipids and the blue – green pigment pyocyanin, an alginate- like exopolysaccharide is responsible for the mucoid phenotype [3], in addition to pili and intrinsic resistance to many antimicrobial agent [1]. Lipopolysaccharide (LPS) is the major component of the outer membrane of gram – negative bacteria; it protects the pathogenesity from host defenses and mediates the entry of the bacteria into eukaryotic cells [4]. Keratitis caused by *P. aeroginosa* is one of the most rapidly developing and destructive diseases of the cornea. Once the bacteria infect the cornea, complex host tissue reactions occur, including inflammation, cellular and humeral immune responses and degradation of stromal proteins [5]. It is very uncommon in a normal eye and usually only develops when the ocular defense has been compromised [6]. *P. aeroginosa* keratitis following trauma to the cornea may result in blindness [7]. During corneal infection LPS has been proposed to be a ligand responsible for the invasion of the cornea [8]. This study aims to:

Isolation of *P. aeruginosa* from patients suffering from corneal infection, extracting and partially purifying Lipopolysaccharide from the isolate of *P. aeroginosa* that has the greater ability of adhesion and antisera preparation against LPS and antibodies titer determination in rabbit serum using passive haemagglutination.

Methods

1. Isolation and Identification of bacteria

From September 2008 to May 2009, one hundred and twenty two specimens were collected from patients suffering from eye infection from Ibn Al-haithem Teaching Eye Hospital. Specimens were collected by cotton swaps. The collected specimens were inoculated on MacConkey agar then incubated at 37^{o}C for 24 hr. The pale non lactose fermentor colonies were selected then a single colony was inoculated on Blood agar for the activation and detection of bacterial ability to lyses red blood cells (β-hemolysis). Isolated colonies were inoculated on Cetrimide 0.03% medium, incubated at 37^{o}C for 24 hr. Then single colony inoculated on King A agar and King B agar to determine their ability to produce pigments. Single colonies were inoculated on Nutrient agar to carry out other biochemical tests that confirmed the identification

of bacterial isolates [1]. More confirmation methods was carried out using biochemical tests, API 20 E system and agglutination sera test for the grouping of *P. aeruginosa* (Sanofi Diagnostic Pasteur).

2. Detection of bacterial ability to produce slime layer

2.1.Congo red agar method

Congo red agar was inoculated with single colony with streaking, incubated at 37^{o}C for 24 hr.; a positive result was indicated by black colonies with a dry crystalline consistency refer to positive result, while non-slime producers usually remained pink [9].

2.2. Adherence to smooth surfaces

Glass tubes containing 10 ml of Tryptic soy broth were inoculated with single colonies of tested bacterial strain by sterile loop; negative control was made by adding 10 ml of Tryptic soy broth to a glass culture tube. The tubes were inoculated at 37^{o}C for 24-48 hr. After that the tubes content was decanted and 10 ml of 0.1% safranin stain solution was added to all tubes including negative control. Each tube was then gently rotated to ensure uniform staining of any adherent material on the inner surface and the contents was gently decanted. The tubes were then placed upside down to drain. A positive result was indicated by the presence of an adherent layer of stained material to the inner surface of the tube [10].

3. Extraction and Partial Purification of LPS of *Pseudomonas aeruginosa*

The extraction of LPS was carried out according to [11] by hot water phenol method and the purification by gel- filtration chromatography by using Sephacryl 200 S.

4. Chemical analysis of LPS

Protein determined according to [12], while carbohydrates were estimated according to [13]. Ashwell method was used to determine nucleic acids [14].

5. Preparation of antisera

According to [15] antisera were prepared in rabbits. Two rabbits 2-2.5kg were hyperimmunized over a period of 70 days by five injections, the first two injections were given intramuscularly with olive oil as an adjuvant, on day 0, one ml of 10 µg of LPS in olive oil (1:1) and on day 14, one ml of 20 µg of LPS in olive oil, the third, fourth and fifth injections were given intravenously on days 28, 40 and 56 respectively, one ml of 50 µg of LPS. One control rabbit was injected PBS instead of LPS. On day 70, blood was collected from immunized and control rabbits by heart stabbing and the sera were pooled.

6. Passive hemagglutiation

Indirect passive hemagglutination was used according to [16] in order to measure antibodies titers against LPS antigens of *Pseudomonas aeruginosa* in immunized and control rabbits' s era.

6.1. Solutions used in passive hemagglutination

Solution (1): 0.025 M PBS, pH 7.2.

Solution (2): Al-Sever' s solution

This was prepared according to Herbert [16], by dissolving 2 gm of dextrose, 0.8 gm of sodium citrate, 0.42 gm of sodium chloride and 0.54 gm of citric acid in small volume of D.W. pH was adjusted to 6.1, and the volume was completed to 100 ml by D.W., and then sterilized by autoclaving for 10 min.

Solution (3): Sheep red blood cells solution

Solution 2 was used in collecting sheep blood; this was collected in a volume of 1:1.2 (blood volume: Al-Sever´s solution volume) in a sterile container, after that blood was preserved at 4^{o}C in refrigerator and used for only three weeks post collection.

6.2. Sheep Red blood cells preparation

Sheep red blood cells were separated from plasma and Al-Sever's solution by centrifugation (1500 rpm for 10 min); the cells were washed three times with solution 1, then suspended in solution 2 for 10 min, after that suspended in PBS in a concentration of 10%.

7. Breaking down the complement factor of sera

Complement factor of immunized and control rabbits sera was broken down by incubating the sera in a water bath at 56^{o}C for 30 min [16].

8. Adsorption of sera

In order to get rid of natural heterophilic antibodies that may exist in rabbits' sera against sheep red blood cells (anti-sheep) which may lead to unspecific reactions, adsorption of sera must be done. This can be done by conduct a reaction between these sera and red blood cells solution. If the result is positive, it means that the anti-sheep antibodies exist and they must be diminated by making absorption of the sera, as follows:

1/10 dilution of rabbit serum was made, mixed with sheep red blood cells solution in a ratio of 1: 0.1 (volume of sera /volume of red blood cells solution). The tubes were shaken, incubated in a water bath at 37^{o}C for 10 min, then the red blood cells were precipitated by centrifugation for 10 min at 1500 rpm and the sera were pooled in clean tubes. To ensure that the sera do not contain anti-sheep antibodies, a pre test must be done between these sera and red blood cells solution. 0.05 ml of sera was added in the well of microtiter plate, to which 0.05 ml of red blood cells solution was added. The microtiter plate was incubated at 37^{o}C for 1 hr.; then at 4^{o}C for 18 hr. [16]. If a positive result occurs, the adsorption of sera must be repeated by the same previous method in order to make sure that the sera do not contain any anti-sheep antibodies that may give unspecific reactions.

9. Determination of the best concentration of LPS for adsorption on the surfaces of SRBc

In order to determine the best concentration of LPS of *Pseudomonas aeruginosa* for adsorption on the surface of sheep red blood cells, consecutive concentrations of LPS were prepared by using PBS: (1, 10, 25, 50, 100)µg/ml (these concentrations were depend on LPS contents of carbohydrates), 1 ml of each concentration was incubated in water bath at 100^{o}C for 1 hr., left for cooling, then 1 ml of red blood cells solution was added to each concentration in clean tubes, mixed gently. The tubes were then placed in a water bath at 37^{o}C for 1 hr., with shaking upside down to ensure complete mixture. The red blood cells, that were covered with LPS antigens, were precipitated by centrifugation for 10 min at 1500 rpm, the cells were washed twice by PBS, then suspended in PBS in a concentration of 1% in order to use them in titration reaction, within 24 hr [16].

10. Titration

Two folds dilutions of 1/10 rabbit sera dilutions were made in PBS. After that, 50µl of each dilution were added in each well of a microtiter plate, then 0.05 ml of sensitized red blood cells with LPS in all previously prepared concentrations were added. The microtiter plate was incubated at 37^{o}C for 1 hr.; then at 4^{o}C for 18 hr. At the same time the control sample was made by adding 50µl from unsensitized red blood cells to the microtiter plate; 50µl of immunized and control sera were added. Another control sample was made by adding 50µl of unsensitized red blood cells with 0.05 ml of PBS. The third control sample was made by adding 50µl of sensitized red blood cells with 50µl of PBS. The microtiter plate was incubated at 37^{o}C for 1 hr., then at 4^{o}C for 18 hr. then the result was read.

Results and Discussion

1. Isolation and Identification

One hundred and twenty two specimens were collected from patients suffering from eye infection. Twelve specimens were identified as *Pseudomonas* isolates. The results of agglutination sera test for the grouping of *P. aeruginosa* are shown in Table (1).

Table (1): O- antigen grouping of *P. aeruginosa* isolates according to agglutination sera test.

Isolate number	O- antigen groups number
***P. aeruginosa* (1)**	P6
***P.aeruginosa* (2)**	P16
***P. aeruginosa* (3)**	P12
***P.aeruginosa* (4)**	P12
***P.aeruginosa* (5)**	P12
***P. aeruginosa* (6)**	P9
***P.aeruginosa* (7)**	P16
***P.aeruginosa* (8)**	P9
***P.aeruginosa* (9)**	P16
***P. aeruginosa* (10)**	P16
***P.aeruginosa* (11)**	P16
***P.aeruginosa* (12)**	P12

The results demonstrated that the local clinical isolates of *P. aeruginosa* from corneal infections belong to the following O- antigen groups (6, 9, 12, 16) [7], *P. aeruginosa* isolates belong to O- antigen groups (1, 2, 3, 4, 5, 6, 7); these isolates were selected for their ability to damage mouse corneas. Corneal isolates of *P. aeruginosa* belong to groups 11and 6 in [17]. [18] used groups (6, 8, 10 , 11) of *P. aeruginosa* clinical isolates from corneal infection. The differences of the O-antigen group's number between the previous studies and our study may be due to the differences among *P. aeruginosa* corneal isolate O-antigen group, differences in the time and place of isolates collection and differences in the identification method for the grouping of *P. aeruginosa* isolates.

2. Adhesion tests

P. aeruginosa isolates were tested for their ability to produce slime layer by the Congo red agar method and adhesion to smooth surfaces method (Christensen method) to select the isolate that have a greater ability for adhesion. Positive result appeared as black colonies with a dry crystalline consistency on Congo red agar (+++)

while red color gave ++ or + result. In the Christensen method the isolates that gave large surface area of adhesion of slime layer on tubes surface were gave +++ result and the isolates that gave small surface area were gave ++ or + result.

Table (2): Result of adhesion tests of *P. aeruginosa* isolates.

No. of isolates	Congo red method	Christensen method
***P. aeruginosa* (1)**	+	+
***P. aeruginosa* (2)**	+	+
***P. aeruginosa* (3)**	+	+
***P. aeruginosa* (4)**	+	+
***P. aeruginosa* (5)**	+	+
***P. aeruginosa* (6)**	+ + +	+ +
***P. aeruginosa* (7)**	+ +	+ +
***P. aeruginosa* (8)**	+ +	+ +
***P. aeruginosa* (9)**	+ +	+ +
***P. aeruginosa* (10)**	+ + +	+ + +
***P. aeruginosa* (11)**	+ +	+ +
***P. aeruginosa* (12)**	+ +	+ +

+++ represent black colonies with a dry crystalline consistency in Congo red agar method, large surface area of adhesion of slime layer on tubes surface in the Christensen method
++ or + represent red color in Congo red agar method, small surface area of adhesion of slime layer on tubes surface in the Christensen method

The results show that P 10 isolates have the greater ability for adhesion in both Congo red agar methods Figure (1) and in Christensen method Figure (2). Both methods described here are based on the enhancement of exopolysacharide production by using enriched media, Tryptic Soy Broth (TSB) in the Christensen method [10], while the Congo red agar method also requires the use of a highly nutritious medium Brain Heart Infusion broth with 5% sucrose supplementation. Congo red stain was chosen because it has been used as a stain for showing the presence of the exopolysaccharide of aquatic gram negative bacilli [9].

Figure (1): *P. aeruginosa* P (10) isolate in Congo red method, showing the black colonies with a dry crystalline consistency.

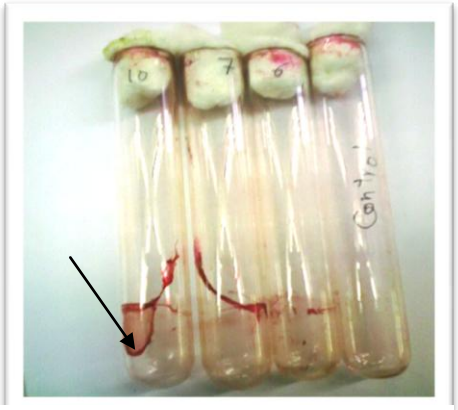

Figure (2): Christensen method showing that P 10 isolate have a great ability for adhesion (→).

Adherence properties vary from strain to strain and the attachment matrix used. The first step in the pathogenesis of pseudomonal infections is the bacterial attachment and colonization [19]. However, four main groups of adhesins have been identified which

mediate the attachment of *P. aeruginosa* to host tissues and surfaces; these are: mucoid exopolysaccharide (alginate), pili, mucin binding outer membrane (OM) protein F, surface lectins [20]. The outer-core polysaccharide portion of the lipopolysaccharide (LPS) in the outer membrane of PA is commonly considered to be the bacterial ligand for adherence to corneal epithelium and bacterial internalization [21].

3. Extraction and Partial Purification of LPS

0.9 gm of dried cell was obtained from 3 liters of bacterial culture in TSB medium. LPS was obtained from P10 isolate by lysozyme method in which cells were subjected to lysozyme and nuclease treatment before phenol extraction. The carbohydrate amount in the crude LPS was determined according to [13] it was 7.3% and the protein amount was determined according to [12]; it was 1.3%, no nucleic acids were found as demonstrated in Table (3).

Table (3): The percentages of carbohydrates, protein and nucleic acids in the crude and partial purified LPS

LPS Extraction	Carbohydrate Percentage	Protein Percentage	Nucleic Acids Percentage
Crude	7.3%	1.3%	Zero
Partial purified	19.5%	0.006%	Zero

Figure (3) show that at 490 nm four peaks were separated. The first two peaks and the last peak are minor while the third peak is major and at 280 nm there are three small peaks in the first two peaks and the last peak of carbohydrate, and one major peak in the third peak of carbohydrates this demonstrated that there is a small amount of protein bound to the LPS and it is difficult to separate it from the LPS. This result relates to the previous result recorded in by Al-Azzawi [22], and it also related to the results recorded in [11, 23, 24].

The carbohydrates percentage in partially purified LPS was 19.5, protein percentage was 0.006% and no nucleic acids were found, as shown in Table (3). We notice the rising of carbohydrate percentage in the partial purified LPS, i.e., 19.5%, while it was 7.3% in the crude LPS. This is evidence of the removing of some cellular components that contaminated LPS extraction and raised the purity of LPS. The LPS preparation was designated pure according to: elution as single symmetrical peaks of gel filtration system and absence of detectable ribose or deoxyribose [14].

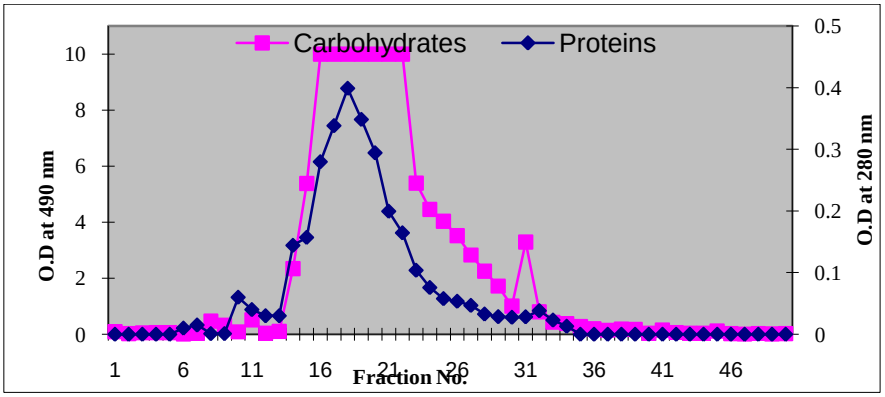

Figure (3): Gel-filtration chromatography on Sephacryl 200 S column (75×2) cm. fraction volume 5 ml, flow rate 75ml/hr., elution buffer (PBS 0.025 M, pH 7.2)

4. Detect the Ability of LPS to Induce the Production of Antibodies

Rabbits were used in order to detect the ability of LPS to induce antibodies. After 70 days, blood was collected from the control and immunized rabbits (those immunized with 5 injections of partially purified LPS extraction from P (10) isolate) by heart stabbing. Sera was obtained by centrifugation and kept at -20^{o}C. Passive Haemagglutination Reaction was used in order to determine the antibodies titer in rabbit sera and for the determination of the optimal antigen (LPS) concentration. The results show there was no positive reaction in control mouse serum as shown in Figure (4) while in immunized rabbit sera the titer of antibody was 160, as shown in Figure (5).

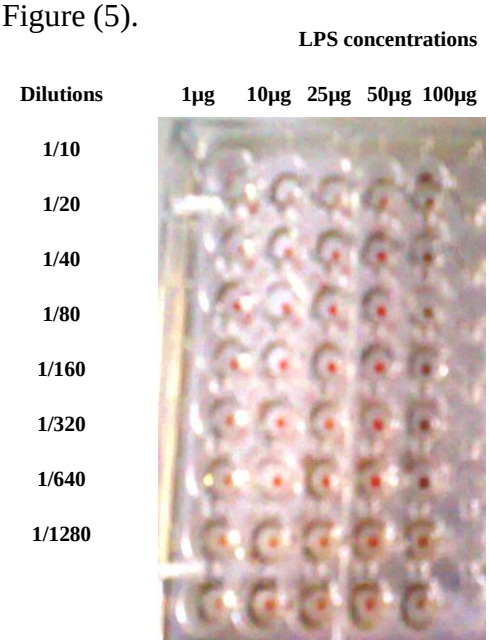

Figure (4): Passive haemagglutination reaction of double dilutions (1/10, 1/20, 1/40, 1/80, 1/160, …..) of control rabbit serum injected with 0.025 M PBP, pH 7.2 with SRBCs that coated with different concentration (1, 10, 25, 50 and 100 µg/ml) of LPS extraction from *Pseudomonas aeruginosa* P (10) isolate, showing negative reaction.

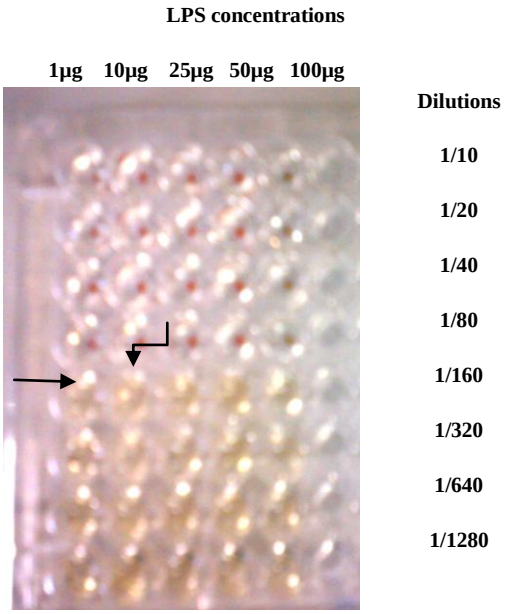

Figure (5): Passive haemagglutination reaction of double dilutions (1/10, 1/20, 1/40, 1/80, 1/160, …….) of immunized rabbit serum immunized with LPS extraction from *Pseudomonas aeruginosa* P (10) isolate with SRBCs coated with different concentrations (1, 10, 25, 50 and 100 µg/ml) of LPS, showing the optimal antigen concentration (10 µg/ml) (⌐→) and the antibody titer in immunized rabbit 160(→).

The titer of antibody is the convert of the highest dilution of sera that gave positive results with the antigen concentration and the optimal antigen (LPS) concentration was 10 µg/ml, as shown in Figure (5). The optimal concentration is the concentration that gave positive reaction with the highest dilution of immunized sera. Red blood cells have been found to be extremely convenient passive carriers of antigen, and sheep cells have proved satisfactory in practice [16]. The agglutination of a particulate antigen by its specific antibody is, classically, the simplest way of estimating the quantity of that antibody in a serum on the other hand red blood cells are not only possible to coat almost any antigen on their complex surfaces, but the coated cells are amongst the most sensitive available indicators of antibody [16]. According to [25] LPS isolated from several strains of *P. aeruginosa* was derived either from the phenol or water phase, and was found to be highly immunogenic and protective in mice with

doses as low as 0.001 µg; the level of protection correlated with anti-LPS antibody titers.

References

1. Forbes, B. A.; Sahm, D. F. and Weissfeld, A. S. (2007). Bailey and Scott's Diagnostic Microbiology. 12th ed. Mosby, Inc. China. pp. 340-350.
2. Brooks, G. F.; Butel, J. S.; Carroll, K. C. and Morse, S. A. (2007). Jawetz, Melnick and Adelberg's Medical Microbiology. 24th ed. The McGraw-Hill Companies, Inc. USA.
3. Govan, J. R. W. (2005). Pseudomonas and non-fermenters. In: Medical Microbiology. 16th ed. (Greenwood, D.; Slack, R. C. B. and Peutherer, J. F. (eds)). Churchill Livingstone. China. pp. 282-287.
4. Brandenburg, K. and Wiese, A. (2004). Endotoxins: Relationships between structure, function and activity. Curr. Top. Med. Chem. 4(11): 1127-1146.
5. Hazlett, L. D.; McClellan, S.; Kwon, B. and Barrett, R. (2000). Increased severity of *Pseudomonas aeruginosa* corneal infection in strains of mice designated as Th1 versus Th2 responsive. IOVS. 41(3): 805-810.
6. Kanski, J. J. (2007). Clinical Ophthalmology. 6th ed. Elsevier Limited. China. pp. 250-260.
7. Gerke, J. R. and Nelson, J. L. (1977). Oral vaccination and multivalent vaccine against *Pseudomonas aeruginosa* keratitis. IOVS. 16(1): 76-80.
8. Van Delden, C. (2004). Virulance factors in *Pseudomonas aeruginosa.* In: *Pseudomonas*: Virulance and Gene Regulation. Vol.2. (Ramos, J. L. (ed)). Plenum Publishers, New York. PP. 3-46.
9. Freeman, D. J.;Falkiner, F. R. and Keane, C. T. (1989).New method for detecting slime production by coagulase negative Staphylococci. J. Clin. Path. 42: 872-874.
10. Christensen, G. D.; Simpson, W. A.; Bisno, A. L. and Beachey, E. H. (1982). Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect. Immun. 37(1): 318-326.
11. Johnson, K. G. and Perry, M. B. (1975). Improved techniques for the preparation of bacterial lipopolysaccarides. CAN. J. Microbiol. 22: 29-34.
12. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951). Protein measurment with folin phenol reagent. J. Biol. Chem. 193(1): 265-275.
13. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. and Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. Anal. Chem. 28(3): 350-356.
14. Ashwell, G. (1957). Colorimetric analysis of sugar. In: Methods in Enzymology. 3: PP.73-105.
15. Cryz, S. J.; Fűrer, E. and Germanier, R. (1983). Passive Protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. 40(2): 659-664.
16. Herbert, W. J. (1978). Passive haemagglutination with special reference to the tanned cell technique. In: Handbook of Experimental Immunology.1 (3): pp. 20.1 – 20.19.
17. Preston, M. J.; Gerceker, A. A.; Koles, N. L.; Pollack, M. and Pier, G. B. (1997). Prophylactic and therapeutic efficacy of immunoglobulin G antibodies to

Pseudomonas aeruginosa lipopolysaccharide against murine experimental corneal infection. IOVS. 38(7): 1418-1425.

18. Zaidi, T. S.; Priebe, G. P. and pier, G. B. (2006). A live-attenuated *Pseudomonas aeruginosa* vaccine elicits outer membrane protein-specific active and passive protection against corneal infection. Infect. Immun. 74(2): 975-983.

19. Pollack, M. (2000). *Pseudomonas aeruginosa*. In: Principles and Practice of Infectious Diseases. 5th ed. (Mandell, G. L.; Bennett, J. E. and Dolin, R. 5. pp. 2310-2327.

20. Pitt, T. L. and Simpson, A. J. H. (2006). *Pseudomonas* and *Burkholderia* spp. In: Principles and Practice of Clinical Bacteriology. 2 .pp. 427-435.

21. Zaidi, T. S.; Fleiszig, S. M.; Preston, M. J.; Goldberg, J. B. and Pier, G. B. (1996). Lipopolysaccharide outer core is a ligand for corneal cell binding and ingestion of Pseudomonas aeruginosa. IOVS. 37: 976-986.

22. Al-azzawi, R. H. M. (1998). Extraction and partial purification of the local *Pseudomonas aeruginosa* lipopolysaccharide. Msc Thesis. College of Science/ University of Baghdad.

23. Pier, G. B.; Sidberry, H. F.; Zolyomi, S. and Sadoff, J. C. (1978). Isolation and characterization of a high molecular weight polysaccharide from the slime of *Pseudomonas aeruginosa.* Infect. Immun. 22(3): 908-918.

24. Darveau, R. P. and Hancock, R. E. W. (1983). Procedure for isolation of bacterial lipopolysaccharides from smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. 155(2): 831-838.

25. Cryz, S. J.; JR; Pitt, T. L.; Fűrer, E. and Germonier, R. (1984). Protection against fatal *Pseudomonas aeruginosa* burn wound sepsis by immunization with lipopolysaccharide and high-molecular-weight polysaccharide. Infect. Immun. 43(3): 795-799.