Production of polyvalent and monovalent diagnostic antibodies against *Pseudomonas aeruginosa*

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Key words: Antibodies , monovalent, polyvalent, *Pseudomonas aeruginosa*, *immuno Absorbance test, ELISA.*

Abstract

Recents studies focus on production and purification of antibodies for rapid diagnosis, or passive therapy for treatment bacterial, viral and cancer disease.The present study is an attempt to prepare and produce polyvalent and monovalent diagnostic antibodies against Pseudomonas aeruginosa. Immuno absorbance method was dependent for polyvalent antisera purification to get monovalent antibodies against P. aeruginosa. Animal model were rabbits for hyperimmunization to get polyvalent antibodies. Slide agglutination for preliminary rabbit serum assay against P.aeruginosa antigen. ELISA was done to confirm monovalent antibodies purity. The prepared monovalent antisera was highly sensitive (96%) and highly specific (95%) to detect P. aeruginosa.

انتاج اضداد تشخيصية متعددة واحادية التكافؤ ضد بكتريا الزوائف الزنجارية *Pseudomonas aeruginosa*

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مفتاح الكلمات: الاضداد, احادية التكافؤ, متعددة التكافؤ, الزوائف الزنجارية, اختبار الاليزا , الامتصاص المناعي المرتبط بالانزيم

الخلاصة
الدراسات الحديثة تركز على إنتاج وتنقيه الأجسام المضادة للتشخيص السريع ، أو للعلاج المناعي لعلاج البكتيريا اوالفيروسية اوامراض السرطان. هذه الدراسة هي محاولة لتحضير وإنتاج الأجسام المضادة متعددة التكافؤ والأحادية التكافؤ لغرض التشخيص ضد الزوائف الزنجارية. حيث اعتمدت طريقه الامتصاص المناعي والتي تعتمد علي تنقيه المضادات متعددة التكافؤ للحصول علي الأجسام المضادة الاحاديه التكافؤ ضد الزوائف الزنجارية. وكان النموذج الحيواني هو الأرانب للحصول على الأجسام المضادة متعددة التكافؤ. التقدير المناعي الاولي لمصل الأرانب المضادة للزوائف الزنجارية تم بواسطة اختبار تلازن الصفيحة الزجاجية. تم استخدام اختبار الاليزا لتاكيد نقاء الأجسام المضادة الاحاديه التكافؤ. معدل الحساسية التشخيصية للمصول المحضرة لتشخيص العزلات الموجبة كانت (96%) ومعدل خصوصيتها لتحديد العزلات السالبة كانت (95 %).

Introduction

The Gram-negative bacilli of the genera *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter,*and *Proteus* are members of the normal intestinal flora of humans and animals and may be isolated from a variety of environmental sources. With the exception of *Proteus*, they are sometimes collectively referred to as the coliform. Many disease syndromes produced by these organisms are among the most common infections in humans requiring medical intervention(1, 2).

Pseudomonas aeruginosa still a strict infectious in human patients, including with malignancy and those getting chemotherapy, persons with burns, in coronary care units, and adolescent with cystic fibrosis and HIV (3, 4, 5). Morbidity and mortality from disease with *P. aeruginosa* stay high though antibiotics availability to which the organism is susceptible.

The major antigens of coliforms are referred to as H, K, and O antigens. The coliforms and *Proteus* are divided into serotypes on the basis of combinations of these antigens(6).

The outer envelope of Enterobacteriaceae include outer membrane and lipopolysaccharide (LPS). The serology of the LPS has been investigated, especially the somatic surface antigen. Antisera against heatstable antigen have agglutination and rise type-specific protection.In addition, another antigens present in Gram-negative bacteria, which is the flagellar or **H** antigen. In some organisms, like *Klebsiella pneumoniae* and *Escherichia coli.* The third antigen, K antigen, which act as polysaccharide at exterior to the outer layer(7,8,9). There is no available vaccine to infection with the organism, and trails vaccines against a various of epitopes have been accused by toxicity and/or poor or inconsistent immunogenicity in risky people (10). Passive prophylaxis antibodies using monoclonal antibodies (MAb) against protective *P. aeruginosa* epitopes are strategies for preventing *P. aeruginosa* infection, but mice MAb are not in choice for humans. Polyclonal human antisera from donors have different antibodies titers, and human MAb are difficult to make(11, 12).

Many patients sensitive to pseudomonas are immunocompromised, do not respond to immunization, and may not make high-avidity. Others may require rapid dose of high-titer immunization to prevent infection after acute and heavy exposure to pseudomonas in burn units or intensive care units (ICU), hence, immunization with the delay in the stimulation of antibodies may not be preferred (13, 14, 15).

P. aeruginosa strains have high-molecular-weight PS (high MW PS) portions of the LPS O chains 06ad (International Typing System [IATS]), 011 (IATS), 016 (IATS 02a,b,e), 170003 (IATS 02a,b), and PAO1 Holloway (IATS 05). It is the main epitopes on *P. aeruginosa*(16).

Approximately ten serogroups of *P. aeruginosa* consider a dominant of human isolates of clinical origin (16), suggesting a minimum number of components in a multivalent preparation. The reconstitution of mice IgG2 limits the subclasses of produced IgG, though isotype switching or new IgG mice reconstituted with other constant regions will produce antibodies of different IgG subclasses(14).

Serological typing system of *P. aeruginosa* would be of great assessment in puplic health studies.

Recently, many techniques for manufacturing human MAb of an appropriate isotype and directed against selected antigens has become available against *P aeruginosa* and others microorganisms(16).

The present study an attempt to prepare polyvalent and monovalent antibodies against *P. aeruginosa* to be as rapid diagnostic antisera.

Materials and Methods:

Bacteriology:

All media were prepared as recommended by manufacturer instructions.

Isolates of Bacteria:

All bacterial isolates were obtained from scientific laboratory in department of clinical laboratory sciences which obtained originally from various patients of general Basrah hospital .

Bacterial Diagnosis:

The diagnosis of isolates were confirmed and identified according to bacterial growth, cultural characteristics, Grams staining and biochemical test(12). The identified bacteria were *Escherchia coli, Staphyllococcus aureus, Staphyllococcus epidermidis, Pseudomonas aeruginosa*, and *Proteus.*

Processing Of Bacterial Antigen :

For pool bacterial antigens absorbance, many bacterial isolates were identified and cultivated. They were *P. aeruginosa, E coli, Staph. aureus, Staph. epidermidis*, and *Proteus.* The isolates were cultivated in brain-heart infusion in 100ml conical flask at 37°C for 18 hour in shaker incubator (16); each isolate cultivated separately.

The different isolates were centrifuged (3000 g for 20min). The bacterial precipitate were filled with distilled water. The process of centrifugation and suspend were repeated 3 times. The bacterial concentration of each isolates were measured to be $4*10^6$ cell per ml(16).

Antigen Sonication:

Antigen of different isolates (*E. coli, Staph. aureus, Staph. epidermidis, P. aeruginosa*, and *Proteus*) were boiled in water bath for 1hr to kill it, then destroyed by freezing and sonication (ultrasonicator

VCX) for 2 min three times. The sonicated bacterial antigens were stored at -20 ℃ for immunization and serological test(13).

Immunization:

Rabbits model were selected as source of antisera. Fourteen rabbits (males) as duplicate immunization for each bacterial antigen. Two rabbits were selected as control. Bacterial antigen (1 ml) of *P. aeruginosa, E. coli, Staph. aureus, Staph. epidermidis,* and *Proteus* were injected between shoulder. The interval of injection was weekly for two times. The third booster injection was 2ml of soluble antigen at marginal ear vein (16). Three days after booster injection , the blood were collected.

Agglutination test:

Control and immunized rabbits blood were clotted by left it for 1hr at room temperature. The clotted blood was centrifuged (2000 g for 10 min).

Slide agglutination was dependent as preliminary test for prepared polyvalent and monovalent antisera. The bacterial antigens were extracted from overnight bacterial culture grown on nutrient agar. The bacterial were mixed with normal saline as emulsifying agent then mixed with diluted antiserum (1:10)with distilled water. Granular agglutination after mixing and titling indicator for positive test(13).

ELISA test:

Hepatitis kit was modified to be diagnostic ELISA test for detection sensitivity of prepared *P. aeruginosa.* The modification included printing of somatic O antigen of *P. aeruginosa* instead of hepatitis virus antigen. Checker board was applied to determine the optimum concentration of antisera, antigen and conjugate.

The cut-off point was determined by equation:

Cut-off point = mean O D of 3 positive samples / 4

this done according to screening of many bacterial isolates to evaluate sensitivity and specificity of the test.

Sensitivity rate = correctly recognised positive isoltes / total isolates *100
Specificity rate = correctly recognised negative isoltes / total isolates *100

Purification of Antisera:-

The absorbance of polyvalent antisera was adopted for purification from cross reacted antibodies to get monovalent antibodies. The ratio 1:1 (*P. aeruginosa* polyvalent antisera : whole bacterial other species antigens; *E. coli S. aureus, S. epidermidis,* and *Proteus*. The above mixture was incubated in

water bath with shaking for 10 min. at 37 °C then centrifuged at 3000 g for 20min. Above absorbence repeated more than time till getting purified antisera without any cross reacted antibodies(13).

Results and discussion:

All bacterial isolates (*P. aeruginosa, E. coli, S. aureus, S. epidermidis,* and *Proteus*) related with present study were identified by cultural, microscopically, and biochemical tests(12). The species *E. coli, S. aureus, S. epidermidis,* and *Proteus* are frequently appeared in cultures isolted from human beside *P. aeruginosa* , therefore were selected to prevent suspicious diagnosis with *P. aeruginosa.*

Serological test:

Prepared sera in laboratory animals against unheated *P. aeroginosa* also contain antibodies against different inadequately heat-labile bacterial constituents, but trails to apply of these for serological typing system have given confusing results(17,18). Hence , the present study dependent on immuno absorption test for monovalent antisera preparation.

The outer envelope of Gram-negative bacteria contain of the outer membrane and lipopolysaccharide (LPS). The immunology of the LPS of the Enterobacteriaceae has been thoroughly investigated, especially the somatic or O antigen. Antibodies against this heatstable antigen have agglutinating properties and give type-specific protection (19)

Prepared polyvalent antisera against *P. aeruginosa* were tested by slide agglutination and ELISA test. The results of the first and second absorption agglutination were summarized in table (1).

The table (1) show obvious cross reactivity among *P. aeruginosa* and *E. coli S. aureus, S. epidermidis,* and *Proteus.*

Table (1): Comparison of pre and post absorption agglutination of polyvalent antisera against *P. aeruginosa* by slide agglutination and ELISA test.

Polyvalent Anti-P. *aeruginosa*	Type of Test	Sonicated Whole Antigen				
		P. aeruginosa	*E. coli*	*S. aureus*	*S. epidermidis*	*Proteus Spp.*
Pre Absorption	Slide agglutination	+ve	+ve	+ve	+ve	+ve
	ELISA	+ve	+ve	+ve	+ve	+ve
2nd	Slide agglutination	+ve	+ve	-ve	-ve	-ve

Absorption	ELISA	+ve	+ve	+ve	+ve	+v
3rd Absorption	Slide agglutination	+ve	-ve	-ve	-ve	-ve
	ELISA	+ve	-ve	-ve	-ve	-ve

+ve: positive

-ve :negative

The present study confirmed and agree with others studies (20,5,10) that *P. aeruginosa* have cross reactivity with *E. coli S. aureus, S. epidermidis,* and *Proteus.* The cross-reactivity is the main source of flase-positive among different serological microorganism diagnosis wether by simple slide agglutination or by complicated test like ELISA or RIA.

Many absorption sera were done to purify polyclonal antisera aginst *P. aeruginosa* to be monovalent diagnostic antisera. The purification was done to reduce cross reactivity antibodies against *E. coli, S. aureus, S. epidermidis,* and *Proteus a*s shown in table (1). Both test of simple slide agglutination and enzyme linked immunosorbent assay (ELISA) were used to confirm antisera purity from cross reactivity of common antigens with others bacterial species. The first absorption test for prepared antisera were positive in both test (slide agglutination and ELISA) for all bacterial species that selected in the present study.

At the second absorption , also was positive in both test except those in *Stpaph. epidermedis* and *Proteus spp.* were negative in slide agglutination while positive in ELISA test which that belong to highly sensitivity of ELISA that detect traces of cross reactive antibodies.

At the third absorption test, all cross reacted antibodies were absorbed completely that appear negative result in both slide agglutination and ELISA test.

The outcome clarified a complex relations among the bacterial species according to immunogenicity, O-polysaccharide as high-molecular-weight, serogroup, antigenicity, and density of subtype epitope.

Cross-reactions between bacterial species and *Pseudomonas aeruginosa* were studied by different quantitative immunoelectrophoretic process. Antigen complex of *Pseudomonas aeruginosa* and a relted rabbit antiserum. Antigens of *Pseudomonas aeruginosa* were cross-reacted with antigens from many other bacterial species, gram-negative and gram-positive. Absorption of antibodies the degree of cross-reactivity was found to be 25–100%. The members of the Enterobacteriaceae also cross-reacted fairly extensively with *Pseudomonas aeruginosa*(21,22).

Sensitivity and Specificity Rates:

In order to evaluate the prepared antisera against P. *aeruginosa*, sensitivity rate aquation(23) were applied among 25 isolates that correctly diagnosed by biochemical test. All isoltes were positive by slide agglutination that mean sensitivity rate is equal 96% as described belwo:

Sensitivity rate = correctly recognised positive isoltes / total isolates *100
=24/25 *100= 96%

The specificity rate were evaluated by deviding number of correctly recognised as negative over total number of tested isolates.

Specificity rate = correctly recognised negative isoltes / total isolates *100
=38/40 *100= 95%

Many factors influence on the sensitivity and specificity rate like mutation, personal error and the stability of the prepared antisera.

Hence, the prepared diagnostic monovalent antisera is rapid , sensitve and specific. It is also reduced cost significantly than bacterial identifcation that depend on cultural and biochemical test. The important advantage of serological diagnosis, it is require only seconds to get the positive or negative results.
These findings (95% and 96%) indicate that the current serogroup of *P. aeruginosa* are probably insufficient to clarify the full range of LPS antigens required to obtain total serology to a broad range of medical isolates.
The present study recommend to study serotypes variation of *P. aeruginosa* that present in Iraq contary that increase the sensitvity and specificity of the serological diagnosis. Also recommend to generalize serological test for all clinical bacterial pathogens.

Acknowledgment:
Thanks to the central laboratory of Primary Health Care in Basrah directory of health. Also, best regards to lecturer Eiman A. Saed that support and assist me to accomplish this work.

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