Journal of Medical Bacteriology

Determination of Immunological Properties of *Pseudomonas aeroginosa* PA103, by Serum Bactericidal Assay & ELISA

Shahrzad Aliniay Sharafshadehi [1#], Neda Shadvar [2#], Seyyed Nematollah Seify [3], Mohammad Reza Mohammadi [4], Marziyeh Taher Sarjam [5], Saina Najafi [6], Nahal Hadi [7], Javad Fathi [7*], Mahdi S. Sadati [8*]*

1 Department of Microbiology, Faculty of Advanced Science And Technology, Tehran Medical Science, Islamic Azad University,Tehran, Iran.

2 Department of Microbiology and Parasitology, School of Medicine,Bushehr University of Medical Sciences, Bushehr,Iran.

3 Department of Medical Microbiology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran.

4 Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

5 Molecular cell biology_Microbial Biotechnology, Islamic Azad University Rasht Branch, Iran.

6 Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

7 Department of Medical Bacteriology and Virology, Faculty of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

8 Department of Microbiology, Faculty of Biological Sciences, Islamic Azad University Tehran-North Branch, Tehran, Iran.

ARTICLE INFO

Article type:
Research Article

Article history:

Received	20	Dec	2024
Revised	24	Dec	2024
Accepted	17	Jan	2025
Published	16	Feb	2025

Keywords:
Assay, Bactericidal, Exotoxin A, Pseudomonas aeruginosa, Serum.

ABSTRACT

Background: Exotoxin A (ExoA) is one of the most known and important virulence factors of *Pseudomonas aeruginosa*. This toxin is the cause of ADP-ribosylation in eukaryutic Elongation Factor-2 (EF-2), which results in the protein synthesis inhibition. Recent studies had shown immunostimulational characteristics of detoxified the ExoA. Our aim in this study was to evaluate the immunological properties of detoxified ExoA by Serum Bactericidal assay, in comparison with ELISA.

Methods: The production of ExoA was done on the ExoA-producing strain, *Pseudomonas aeruginosa* PA103, provided by Pasteur institute of Iran. After culture in semi-industrial scale, it was detoxified and purified by dialysis. The dialysate was injected to mice and rabbit. After 3weeks, the total sera were collected. Serum bactericidal assay & ELISA were performed.

Results: The results had shown a significant increase of antibodies against detoxified ExoA of 1/16 and antibody in ELISA method. Also, this has shown more antigenecity & immunogenosity by SBA method.

Conclusion: To conclude the study that has been done here, SBA has much higher advantages than ELISA, in determining the immunological properties of Exo A. Furthermore, since Exo A could act

*Corresponding Authors: Saina Najafi: Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. *Tel*: +98-21-5211527, *E-mail:* najafsaina4@yahoo.com

as a hapten, many other subunits from other bacteria or viruses could be conjugated on Exo A and form multi-target vaccines.

- ***Please cite this paper as:*** Aliniay Sharafshadehi S, Shadvar N, Seify SN, Mohammadi MR, Taher Sarjam M, Najafi S, Fathi J, Sadati MS. Determination of Immunological Properties of *Pseudomonas aeroginosa* PA103, by Serum Bactericidal Assay & ELISA. *J Med Bacteriol.* 2025; **13** (1): pp.40-45. DOI: 10.18502/jmb.v13i1.18046

Introduction

Pseudomonas aeruginosa is a gram negative, reknown opportunistic, and one of the most prevalent multi-drug resistant pathogens (1, 2). This bacteria has a very low infection dosage (3), therefore prevention and early detection are crucial for eradiction. Pathogenic strains posses type III secretion system that delivers toxins to the cytoplasm of the host, directly. Exotoxin A (exoA is delivered by this pathway it could ADP-ribosylate eukaryutic elongation factor-2 (EF-2 and inhibit protein synthesis (4, 5). It consists of two main fragments; A & B. The fragment A is catalytic, and fragment B is a carrier and attaches to eukaryutic cell receptors (3). Moreover, a third fragment had also been discovere but the function has remained unknown (6). Innate immunity has a key role in defense against *Pseudomonas aeruginosa* attacks (7, 8). Although, it has to be noted that the best candidates for vaccine develoment against this pathogen should induce both of the innate and adaptive immunities.

Previous studies had shown detoxified exoA inducing innate (9, 10). Although, in order to investigate Exo A efficacy as a candidate for vaccine, many methods have been implemented. The two most important methods used for this purpose are: enzyme-linked immunosorbent assay (7), & Serum Bactericidal Assay (11, 12). Many studies have led to the production of commercially available ELISA kits for *P. aeruginosa* exoA and less often studies had been performed by SBA.

Serum bactericidal assay (11) is one of the most important methods in order to determine the humoral immunity of a bacterial vaccine. This method was initially introduced as a gold standard for all *Neisseria meningitidis* serotypes by NCCLS (CLSI) (13). Soon the further application of this method on other bacteria, such as *Salmonella typhi*, revealed how essiential the innate immunity is in the rapid response to a bacterial pathogen invasion. The determination of such a rapid response is extensively important, since this response could lead to the detaining and/or clearance of a bacterial pathogen by the T cells.

Our aim in this research was to study the immunostimilational effect of *P. aeruginosa* Exo A by two methods, ELISA & SBA as a candidate for vaccine against this pathogen (7, 10).

Materials and Methods

Bacterial Strain

The strain used in this study was *Pseudomonas aeruginosa* PA103, provided from bacterial cell bank of pasteur institute of iran (CSBPI). This strain is pigment-less and produces high concentrations of exo A. This strain has also been used as to challenge lab animals.

Seed Culture

Seed culture was done by the culture of *Pseudomonas aeruginosa* PA103 on 20 tubes containing brucella agar and incubated in 37 degrees celcius for 24 hours. Then the colonies formed were washed by phosphate saline buffer

(pH=7.2). The seed culture solution was then collected in a jar.

Fermentation

The seed culture was added to a fed batch culture of trypticase soy broth in a fermentor (Novo-paljas, Netherlands). The temperature was set to 36±1 °C. The pH was set 7.2, and aggetation on 450 rpm.

Exotoxin A Harvesting

After 24 hours, ammonium acetate 1N was added to the fermentation system and a dark-coloured percipitation was formed immediately. All of it was collected and quality control tests were performed.

Purification & Detoxification of Exo A

The Exo A obtained was added to 0.2N NaOH and boiled at 100 degrees celcius for 2 hours. The pH was set to 7. It was then dialyzed (cut-off= 20KDa) against distilled water for 3 days and following dialyze, gel chromatography was done by sepharose 4B-CL and the Exo A was purified.

Abnormal Toxicity Test

5 BALB/c mice (2-2.3g) & 3 guinea pigs were each injected by 50 micrograms of Exo A. The animals were all monitored for a week and there were no signs of weight loss.

Pyrogenicity

50 micrograms of exo A were injected to 3 rabbits and their body temperature were monitored for 3-5 days and showed no change.

Gel Diffusion

An agar plate with two separated holes (about 3 cm) were chosen. Exo A was filled in the first well and anti- Exo A serum was filled in the second well and left at room temperature for 30 minutes, then incubated for 24 hours in 37 degrees celcius. Following incubation, a white bond indicating the formation of antigen (exo A)-antiserum in the plate was visualized.

Immunization Program

Immunizations of rabbits were executed by considering 3 groups of 3 white newzealand rabbits. Group 1 was injected by 50 micrograms of Exo A toxoid. Group 2 was injected by 25 micrograms of *Brucella abortus* lipopolysaccharide as sham expose. Finally, group 3 was injected by 50 micrograms of normal saline as negative control. The injections were done three times, every two weeks. On the second injection, *Pseudomonas aeruginosa* PA103 was injected to all groups as challenge. Two weeks after the last injection, the total blood for all groups were drawn and the sera were separated.

BALB/c Mice Spleen Colony Count

Five BALB/c mice that were previously challenged by *Pseudomonas aeruginosa* PA103, were sacrificed and their spleen were removed and disintigrated in 1ml saline. The spleen concentration was diluted to 1:10, 1:100, & 1:1000 then steak cultured on trypticase soy agar and incubated at 37 degrees celcius. After a week, the colonies formed were counted.

ELISA

ELISA was done by plate checkerboard (cross-titration table) method, in order to determine the optimal titres of serum. Exo A was first dissolved in carbonate buffer (100ml of 0.05M carbonate

buffer (pH=9.6)) and 100 microliters of solution was added to each well of the microplate and incubated overnight at 4 degrees celcius. The plate was washed three times by washing buffer (3% sodium azide and 0.05% tween 20 in 0.01M PBS (pH=7.2)) and the sera previously diluted were added to each well, in the next day. The microplate was incubated at room temperature for 6 minutes. Goat anti-mouse antibodies were diluted 1:1000 in PBS and 100 microliters were added to each well and incubated for 1 hour at room temperature, after rewashing the wells by buffer. Horseradish peroxidase-conjugated goat anti-IgG (diluted 1:3000) was added to the wells and incubated. After adding the substrate and another incubation period of 15 minutes, stopping buffer (1M H_2SO_4) Was added and the absorbance at 450 nanometers was read.

Serum Bactericidal Assay

SBA was performed by serial two-fold dilution of the pooled sera in DPBS, by adding 50 micrograms of the first well, containing 100 micrograms of undiluted serum to the 1:2 well and serially diluted to 1:128. Then, 50 micrograms of *Pseudomonas aeruginosa* PA103 with 10 times 5 concentration was added in each well as challenge. many contols were also considered, such as negative serum, cellular control, positive control, complement control, & bacterial control. The microplate was then incubated for 30 minutes in 37 degrees celcius. After incubation, 1 micrograms of the contents of each well was streak cultured on trypticase soy agar plates and incubated at 37 degrees celcius for 24 hours. Finally, the colonies were counted and by reference to control plates, any plate countless or equal to 50% of the colonies were considered as positive.

Results

Result of Mice Spleen Colony Count

Exo A was has reduced the colony forming units in BALB/c mice challenged by *Pseudomonas aeruginosa* PA103, which was 5113 CFU in the negative control, compared to 4267 CFU in challenged mice.

Table 2. The titre of anti-Exo A-IgG by ELISA (Pvalue<0.01).

	Two weeks after first injection	Two weeks after second injection	Total
Exo A	56.2±7	93.6±9	163.4±11
Negative	0.4±0.02	0.1±0.02	0.1±0.05

Determination of the Bactericidal Activity of Serum by SBA

As it could be seen in the results, the final bactericidal titer of anti-IgG antibody against Exo A in the first injection is 1:16 and in the last injection had raised to 1:32. In order to understand the SBA results, the results for ELISA has also been given in Table-1 and 2.

Table 1. Bactericidal Activity of Serum Induced by Exo A.

	1/2	1/4	1/8	1/16	1/32	1/64	1/128
FI	+	+	+	+	-	-	-
SI	+	+	+	+	+	-	-

FI: first injection, SI: Second injection.

Discussion

The strain used in this study was *Pseudomonas aeruginosa* PA103, producing no blue-green pigments, and produces high concentrations of Exo A, as it was mass produced in our research, as well.

Post-mortem analysis showed extensive necrosis in the inner organs of the mice especially lungs, liver, stomach, & small intestine, based on previous discription of pure exotoxin A. Our study is in comply with them, accordingly (3, 14).

The role of detoxified Exo A in innate immunity, confronting infections caused by *Pseudomonas aeruginosa* is well understood. There are frequent researches on the detection of *Pseudomonas aeruginosa* by ELISA & SBA in patients serum, and the immunological data of *Pseudomonas aeruginosa* are mostly regarding to ELISA (15, 16). However, our research was performed, based on SBA for exo A as the candidate.

By the rise of multi-drug resistant *Pseudomonas aeruginosa*, it has became one of the dominant infections in burn, cystic fibrosis, immunocomprimised, & intensive care unit patients (12). Therefore, it is crucial to consider a safe factor such as Exo A, or more factors as a combined or conjugated vaccine, either prophylactic or therapeutic, for this nosocomial infection.

Eventhough Exo A is known to induce innate (humoral) immunity (17), it is also a key factor that has the potential to induce cellular immunity as well, since it can bridge between both immune system pathways (18, 19). This means evaluation of opsonophagocytosis induced by Exo A should also be done.

With both methods used to assess the immunological properties of exo A, which are SBA and ELISA, ELISA is more routine than SBA (4, 13, 20). Although, SBA is more reliable than ELISA, SBA could measure the antibody that could be achieved by a vaccine (or vaccine candidate (21, 22). SBA could determine both of the antigenecity and the bactericidal titre of the serum, coincidently. However, ELISA could just quantitate an antibody against an antigen.

The colony count of the BALB/c mice spleen had shown Exo A could reduce *Pseudomonas aeruginosa* colony forming units, which is fairly good for only one subunit. In studies done on many other subunits, it had been shown less effectiveness in the reduction of colonies by one subunit (13).

Conclusion

To conclude the study that has been done here, SBA has much higher advantages than ELISA, in determining the immunological properties of Exo A. Since, Exo A is the most important pathogenecity factor in *Pseudomonas*, it is essiential to consider it as the main candidate for vaccine development against this pathogen. Moreover, the efficacy of a vaccine against *Pseudomonas aeruginosa* could also be enhanced by using more subunits and pathogenecity factors, as they might have synergistic effects by together. Furthermore, since Exo A could act as a hapten, many other subunits from other bacteria or viruses could be conjugated on Exo A and form multi-target vaccines.

Acknowledgements

I would like to thank all of my colleagues in AlgoTech Startup group in conducting such a valuble research and also Pasteur Institute of Iran.

Funding Information

This study received no form of funding from any organization. It was funded by the authors.

Ethics approval and consent to participate

All procedures performed in this study were in accordance with the ethical standards of national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Tang Y, Ali Z, Zou J, et al. Detection methods for Pseudomonas aeruginosa: history and future perspective. *Rsc Advances* 2017; **7**(82):51789-800.
2. Baltch A, Franke M, Smith R, et al. Serum antibody concentrations of cytotoxin, exotoxin A, lipopolysaccharide, protease, and elastase and survival of patients with *Pseudomonas aeruginosa* bacteremia. *Clin Infect Dis* 1996; **23**(5):1109-16.
3. Humada TA, Abdullah HI. Quantitative determination of endogenic human *Pseudomonas aeruginosa* exotoxin A (PEA) concentrations in serum of renal failure patients by ELISA. *Tikrit Med J* 2015; **20**(1).
4. Schultz WW, Phipps TJ, Pollack M. Enzyme-linked immunosorbent assay for *Pseudomonas aeruginosa* exotoxin A. *J Clin Microb* 1979; **9**(6):705-8.
5. Khan A, Tauseef I, Aalia B, et al. Age-related variations in the in vitro bactericidal activity of human sera against *Pseudomonas aeruginosa*. *Central Eur J Immunol* 2018; **43**(1):18-25.
6. Morlon-Guyot J, Méré J, Bonhoure A, et al. Processing of *Pseudomonas aeruginosa* exotoxin A is dispensable for cell intoxication. *Infect Immun* 2009; **77**(7):3090-9.
7. Farrant KV, Spiga L, Davies JC, et al. Response of *Pseudomonas aeruginosa* to the innate immune system-derived oxidants hypochlorous acid and hypothiocyanous acid. *J Bacteriol* 2020; **203**(2):10.1128/jb. 00300-20.
8. Alhazmi A. NOD-like receptor (s) and host immune responses with *Pseudomonas aeruginosa* infection. *Inflam Res* 2018; **67**:479-93.
9. Cigana C, Lorè NI, Riva C, et al. Tracking the immunopathological response to *Pseudomonas aeruginosa* during respiratory infections. *Sci Rep* 2016; **6**(1):21465.
10. Priebe GP, Goldberg JB. Vaccines for *Pseudomonas aeruginosa*: a long and winding road. *Exp Rev Vaccines* 2014; **13**(4):507-19.
11. Ryskjær J, Deacon CF, Carr RD, et al. Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbAlc levels, but is not acutely affected by food intake. *Eur J Endocrinol* 2006; **155**(3):485-93.
12. Tramper-Stranders GA, van der Ent CK, Slieker MG, et al. Diagnostic value of serological tests against *Pseudomonas aeruginosa* in a large cystic fibrosis population. *Thorax* 2006; **61**(8):689-93.
13. Gotschlich E. Development of polysaccharide vaccines for the prevention of meningococcal diseases. *Monogr Allergy* 1975; **9**:245-58.
14. Galloway D, Hedstrom R, Pavlovskis O. Production and characterization of monoclonal antibodies to exotoxin A from *Pseudomonas aeruginosa*. *Infect Immun* 1984; **44**(2):262-7.
15. Mauch RM, Jensen PØ, Moser C, Levy CE, Høiby N. Mechanisms of humoral immune response against *Pseudomonas aeruginosa* biofilm infection in cystic fibrosis. *J Cystic Fibrosis* 2018; **17**(2):143-52.
16. Mahmood AS. DNA extraction protocol for rapid pcr detection of pathogenic *Pseudomonas aeruginosa* and detection CD4 and CD8 in Iraqi burn case. *Biochem Cell Archives* 2019; **19**(1).
17. Grumelli S. Host-pathogen interaction in the lung of patients infected with *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa*-an armory Within: London, IntechOpen; 2019.
18. Nabonee MA, Rahman A, Mishu NJ, et al. Immunization with formaldehyde inactivated whole cell vaccine against multidrug resistant *Pseudomonas aeruginosa* produces protective IgG antibodies in mice model. *Arch Microbiol Immunol* 2023; **7**:230-5.

19. Lovewell RR, Patankar YR, Berwin B. Mechanisms of phagocytosis and host clearance of *Pseudomonas aeruginosa*. *Am J Physiology* 2014; **306**(7):L591-L603.
20. Szu SC, Taylor DN, Trofa AC, et al. Laboratory and preliminary clinical characterization of Vi capsular polysaccharide-protein conjugate vaccines. *Infect Immun* 1994; **62**(10):4440-4.
21. Holst J, Feiring B, Fuglesang J, et al. Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. *Vaccine* 2003; **21**(7-8):734-7.
22. Stratton CW. Serum bactericidal test. *Clin Microbiol Rev* 1988; **1**(1):19-26.