



The Efficacy of Prepared Specific *Pseudomonas aeruginosa* Transfer Factor to Protect Mice against Experimental Challenge

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Abstract | This study was conducted for the preparation and extraction of specific *Pseudomonas aeruginosa* transfer factor (TFt) from rats immunized with this pathogen and to evaluate its efficacy to protect the recipient mice against challenge with virulent *P. aeruginosa*. Two groups of rats (each one of six rats) were used, the first group was immunized subcutaneously (S.C) twice in two -week intervals with the whole sonicated antigen, the second group (control) was inoculated S.C with phosphate buffer saline (PBS). The immunized and control rats were monitored daily for the appearances of any clinical signs along one week post each immunization, and checked for the delayed hypersensitivity (DTH). Only the immunized rats revealed positive skin test reaction. The TFt were extracted from the spleen cells of the immunized rats, tested for sterility and safety. To evaluate the efficiency of the prepared TFt, three groups of mice (each of ten) were used. The first group was injected intraperitoneally (I/P) with 0.5 mL of TFt equivalent to 5×10^8 cells/mL, the second group (positive control) was immunized S.C with 0.5 mL of sonicated *P. aeruginosa*, while the third group injected S.C with 0.5 mL PBS (negative control). Later on, all the mice were tested by DTH – skin test. The immunized and TFt recipient mice showed a positive skin reaction, while the control group did not reveal any skin reaction. One week later, all the mice were challenged by I.P inoculation of 0.5 mL containing 5×10^7 CFU of *P. aeruginosa*, the survival rates were 90%, 80% and 10% in vaccinated, TFt recipient and control mice respectively. The results of the current study demonstrated the ability of the TFt to transport the specific DTH- skin reaction to the recipient non sensitized mice in addition, to its efficacy as immunotherapy to protect them against experimental challenge with *P. aeruginosa*.

Keywords | *Pseudomonas aeruginosa*, transfer factor, Immunotherapy, DTH- skin test, Cellular immunity

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INTRODUCTION

Pseudomonas aeruginosa is a common serious pathogen, which can cause a variety of difficult to treat infection in human and animals. It is Gram negative, aerobic, coccobacillus bacterium with unipolar motility and found in soil, water and skin flora (Markey et al., 2014). In veterinary medicine, this pathogen is responsible for many infections such as mastitis in dairy animals; wound infection, metritis in equine, corneal ulcer, otitis, abscesses and urinary tract infections in canine (Radostits et al., 2007). Infections caused by *P. aeruginosa* have been particularly difficult to treat due to the organism's resistance to many antimicrobial agents (Cholley et al., 2010; Kato et al., 2015), this re-

sistance attributed to the existence of numerous drug efflux pumps and little permeability of the main outer membrane porins (Mesaros et al., 2007). This has led researchers to employ various immunotherapeutic techniques to prevent and treat *P. aeruginosa* infections by using passive or active immunization methods (Cripps et al., 1994; Alwan, et al., 2010), immunotherapeutic modalities have been proposed as a potential means to improve the host resistance to this organism (Doring and Pier, 2008; Al-Graibawi, 2015). Many antigens of *P. aeruginosa* have been extracted and utilized as vaccine candidates such as flagella, pili, lipopolysaccharide (LPS) and O- polysaccharide, mucoid exopolysaccharide, exoenzyme and enzymes, outer membrane protein, live and dead *P. aeruginosa* and whole cell soni-

cated antigen (Sedlak-Weinstein et al., 2005). The greatest challenge to any vaccine is that its activity is not compromised due to the presence or occurrence of hypermutable *P. aeruginosa* strains (Grimwood et al., 2015). The Cell mediated immunity (CMI) plays vital roles in the host defences against *P. aeruginosa* infection (Colizzi, et al., 1982; Buret et al., 1993; William et al., 2010). Since CMI is critical for controlling infectious diseases, as well as immunodeficiency, autoimmune diseases, and allergies, transfer factor have been used in the prevention and treatment of these diseases (Arnaudov and Kostova, 2015). Transfer factor also named dialyzable leukocyte extract (DLE) is a small molecular weight extract was known to be able to transfer the CMI and induce protection against microbial pathogen; its activity was evaluated against many viral, bacterial, protozoal and fungal diseases (Kirkpatrick, 1996; Al-Graibawi, et al., 2000; Xu et al., 2013; Arnaudov and Kostova, 2015). Transfer Factor exists in the lymphocytes of birds, mammals and fish, and it seems able to transport the antigen-specific information to T-lymphocytes (Lawrence, 1949; Kirkpatrick, 2000). The present study was conducted to prepare specific *P. aeruginosa* transfer factor and to evaluate its immunological and protective efficacy as immunotherapy to protect recipient mice against challenge with this pathogen.

MATERIALS AND METHODS

BACTERIA AND ANTIGENS PREPARATION

The origin and characterization of the *P. aeruginosa* isolate used for the preparation of sonicated and soluble antigens as well as for experimental challenge was previously described in details (Al-Graibawi, 2015). The isolate was retested by assessing the colony morphology, Gram staining and performing various biochemical tests (Markey et al., 2014), its mean lethal dose 50 (LD50) was detected in the inoculated mice (Reed and Muench 1938). The *P. aeruginosa* antigens employed in the current study were prepared as described by Al-Graibawi (2015) with some modifications; the *P. aeruginosa* was streaked on trypticase soya agar and incubated at 37 °C for 48 hours. The culture was harvested by sterile PBS, and washed three times with PBS by centrifugation at 6.000 rotations per minute (rpm) for 20 minutes; the sediment was resuspended to a concentration of 2×10^9 bacteria/ mL in PBS (Miles and Misra 1938). The suspension was subjected to sonication for 40 minutes at intervals with a water-cooled sonicator oscillator at 15 KHZ/sec. (MSE- watt-ultrasonic Disintegrator GE.MK2), the entire techniques were performed in the Biosafety level 2 cabinet. The sonicated bacterial suspension was tested for sterility by streaking onto blood agar plate, and divided into two parts, the first part was used as whole sonicated antigen for the immunization. The second part was centrifuged twice at 12000 rpm for 45 minutes each time to exclude the sediments, the protein content in

the supernatant was determined according to Lowry et al. (1951), filtered through a 0.22µm Millipore filter and used as soluble antigen for DTH skin testing.

EXPERIMENTAL ANIMALS

Twelve Albino Swiss rats, five rabbits and 90 mice used in the present study, were housed in the laboratory animal house at the College of Veterinary Medicine / Baghdad. The ages of the experimental animals were 6-8 weeks. Several faecal specimens were taken from the animals for bacterial isolation to confirm that these animals were free from *P. aeruginosa*. They were reared in a separate plastic cage and fed commercial assorted pellets and alfalfa. The current study was approved by the Ethical and Research Committee of the Veterinary Medicine College / Baghdad University.

IMMUNIZATION AND CLINICAL EXAMINATION

Two groups of rats (6 in each group) were used for the preparation and extraction of the TF from donor rats. The first (immunized group) was injected S.C twice at two week intervals with 1 mL of the *P. aeruginosa* whole sonicated antigen, while the second (control group) was inoculated S.C twice with 1 mL of sterile PBS. Along one week post each immunization, the rats of the control and immunized groups were monitored daily for the appearances of any clinical signs.

DELAYED-TYPE HYPERSENSITIVITY -SKIN TEST

Two weeks post the 2nd dose of the immunization; the left flank region of each rat was clipped and shaved cautiously. The flank area was divided into three parts. The first part was inoculated I.D with 0.1 mL of BPS as a control, while the second and third parts were inoculated with 0.1 mL of various concentrations of the soluble antigen, 40 µg /mL, and 4µg / mL respectively. The diameter of skin reaction was assayed by the ruler at 24, 48, and 72 hours after inoculation (Campa et al., 1982).

TRANSFER FACTOR EXTRACTION

Three weeks after the booster dose, TF was extracted from the spleen of the immunized rats as an additional test of the host immune system responsiveness to the antigen. The splenocytes were collected aseptically as described previously (Rozzo and Kirkpatrick, 1992), briefly, the spleen was pressed through a stainless-steel screen and suspended in RPMI 1640 medium. The suspension was made free from red blood cells by lysis with 0.83 % ammonium chloride. The cells were washed repeatedly with sterile PBS to exclude the platelets. Then the viability of the leukocytes was detected by staining with 0.1% trypan blue. The viable cells were about 80%, then, the cells were adjusted at 5×10^8 cells /mL and subjected ten times for repeated freeze- thawing. The obtained leukocyte extract were centrifuged with a cold centrifuge at 10000 rpm for 50 minutes to remove

cellular debris. The supernatant was filtered through, centrifugal filter units (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, IRL) to get the lysate containing TF. This lysate was sterilized by 0.45 µm millipore filters and referred as TFt and were aliquot and stored at -20 C°. The sterility test was carried out by spreading a few drops of the lysate on the blood agar plates and incubated at 37 for one week. The plates were regularly monitored during the incubation period to ensure their sterility.

PILOT EXPERIMENT

To evaluate the safety and immunological activity of the prepared TFt to induce DTH- skin reaction, five rabbits were inoculated I.P with 2 mL (equivalent to 5 x 10⁸ cells / mL) of TFt as a pilot experiment, they were observed daily for the occurrences of any clinical signs and tested for DTH- skin test one week later.

SECOND EXPERIMENT

Thirty mice were divided randomly and equally into three groups, to determine the immunological and protective efficiency of the prepared transfer factor. The first group was injected S.C with 0.5 mL (equivalent to 5 x 10⁸ cells /mL) of TFt. Similarly, the second groups were inoculated S.C with 0.5 mL of the prepared sonicated antigen. While the third control group was injected S.C with 0.5 mL of BPS. Two weeks later, all the mice were tested by DTH- skin test, the diameter of skin reaction was determined at 24, 48, and 72 hours, after I.D inoculation of the soluble antigen by the caliper (Campa et al., 1982). One week later, all the mice were challenged intraperitoneally with five LD₅₀ of *P. aeruginosa* at a dose of 0.5mL containing (2x10⁷ CFU/ mL). After the challenge, all the mice were monitored daily for the surviving and appearances of clinical signs.

RESULTS

CLINICAL SIGNS

Post immunization, There were no adverse effects in immunized rats, they were slightly listless and depressed. Small localized swelling was detected during palpation at the sites of inoculation post 72 hours. This swelling was disappeared within two weeks, the clinical signs in the control rats remained within the normal values.

Table 1: Mean diameter of skin reaction of immunized and control rats post I.D injection with various concentrations of *P. aeruginosa* soluble antigen

Antigen conc. (µg/mL)	Mean diameter of skin reaction (mm)					
	Immunized rats (n= 6)			Control rats (n= 6)		
	24h	48h	72h	24h	48h	72h
40	6.4	7.8	6.6	0	0	0
4	2.4	4.1	3.6	0	0	0
PBS	0	0	0	0	0	0

DELAYED TYPE HYPERSENSITIVITY – SKIN TEST

The rats of immunized group revealed positive skin test reactivity, after I.D insertion of various concentrations of *P. aeruginosa* soluble antigen at 24, 48 and 72 hours. The positive skin reaction was detected as edematous, erythematous areas at the sites of inoculation, the rats in the control group did not show any reaction to the injected antigens (Figure 1 and Table 1).



Figure 1: Positive skin reaction test of the TFt recipient rat post intradermal inoculated of soluble *P. aeruginosa*

PILOT STUDY

The result of the of TFt inoculation into the rabbit in a pilot study revealed that this preparation was safe, and able to induce a specific DTH-skin test, which manifested as an edematous, erythematous area on the sites of antigen inoculation (Figure 2).

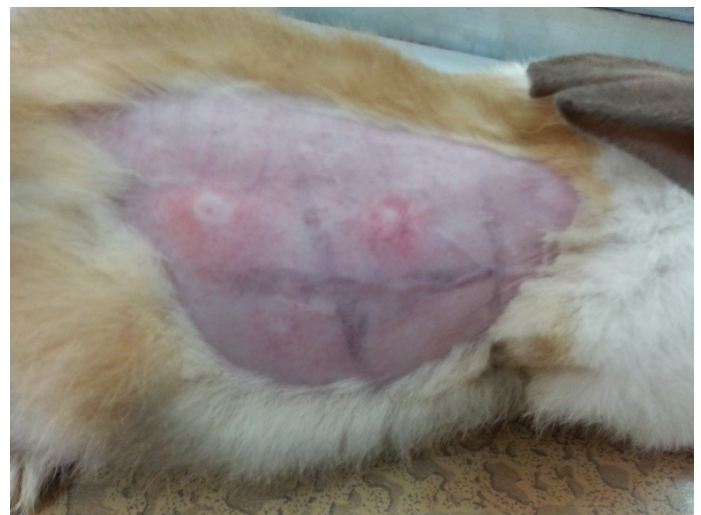


Figure 2: positive skin reaction test of the TFt recipient rabbit post I.D inoculated of soluble *P. aeruginosa*

ADOPTIVE TRANSFER OF THE DTH-SKIN TEST

The recipient (non-sensitized) mice that received TFt and the mice immunized with the sonicated antigen showed positive skin test reactivity in the footpads after I.D injection.

tion with *P. aeruginosa* soluble antigen, while the control mice did not react to the soluble antigen (Table 2).

Table 2: Mean skin thickness in footpads of immunized, TFt recipient and control mice post injection of *P. aeruginosa* soluble antigen

Antigen conc. (µg/mL)	Mean thickness of skin reaction in footpads (mm)						
	Immunized mice (n= 10)			TFt recipient mice (n=10)			Control (n=10)
	24 h	48 h	72 h	24 h	48 h	72 h	
40	2.87	3,78	2.66	2.75	3.63	2.46	1.88
PBS	1.89	1.88	1.87	1.88	1.87	1.87	1.87

POST CHALLENGE CLINICAL OBSERVATIONS

Post the challenge, the immunized and TF recipient mice demonstrated only moderate signs of illness, while the control mice revealed marked decreased physical activity, depressed and anorexic by 24 hours. Then, mice revealed hunched posture, weight loss, rough hair coat and rapid laboured breathing. The immunized and the TFt recipient mice showed a higher survival rate (90%) (80%) respectively against challenge with *P. aeruginosa* compared with 10% of the control mice, most of the mice in the control group were died during the first 24-96 hrs post challenge (Table 3).

Table 3: The survival rates of the TFt recipient, immunized and control mice post challenge

Group	Number of mice	Survival number	Survival rate (%)
Immunized	10	9	90%
TF recipient	10	8	80%
Control	10	1	10%

DISCUSSION

Pseudomonas aeruginosa is an important opportunistic bacterium, this pathogen was associated with a numerous infections in human and animals, especially in those with a weakened immune response. (Haenni et al., 2015). Numbers of various vaccines and several immunotherapeutical procedures have been used in the last decades against *P. aeruginosa* infections (Priebe et al., 2003; Pier, 2005; Dorning and Pier, 2008; Grimwood et al., 2015).

The results of the current study showed that the immunization of the rats with the whole sonicated *P. aeruginosa* antigen was safe and did not induce systemic adverse reactions. However, lack of appetite, listless and depressed were observed in the rats for 48 hours, this may be associated with immunological and inflammatory reactions as reported by Tizard (2004). The sonication technique is widely used for the antigen preparation due to its efficacy to produce higher proteins than other methods (Sangdee

et al., 2012). The DTH-skin test is a classical method for the in vivo evaluated of CMI, and it is mediated by the interaction of antigen presenting cell with CD4+ T helper cell, CD8+ T cell cytokine production, leading to the local inflammation post 48–72 hours due to the mobilization of leukocytes (lymphocytes, monocytes) to the site of I.D inoculation of antigen (Yates and Deshazo, 2001).

Many researchers demonstrated the important roles of cellular immune responses in *P. aeruginosa* infection, Markham et al. (1984) demonstrated that the CD8+ T cells act as principal effector cells in the intraperitoneal *P. aeruginosa* infection of mouse model. Markham et al., (1985) reported the role of T cells in the transfer of cellular immunity to protect laboratory animals against challenge with *P. aeruginosa*. Powderly et al. (1986) reported that the transport of specific immune T cells can significantly increase the resistance of mice with granulocytopenic to experimental infection with *P. aeruginosa*. The results of Markhan and Powderly (1988) indicated the role of the T cells obtained from BALB/c mice sensitized with the 10² CFU of live *P. aeruginosa* to protect granulocytopenic recipient mice against experimental challenge with this pathogen and inhibit the *in vitro* multiplication of *P. aeruginosa*.

Meanwhile, the most important restriction related to the transfer of the T-cell from the donor to the recipient is the graft-versus-host reaction which occurred due to the immune-mediated attack of recipient tissue by the donor T cells (Schroeder, 2002; Ferrara et al., 2010; Schroeder and DiPersio, 2011), to avoid this reaction, Munster et al. (1974) suggested the use of transfer factor or xenogeneic cells in adoptive transfer of the cellular immunity. The TF is non- antigenic, and did not stimulate any immune reaction in the recipients due to its small molecule size, in contrary to the viable T cells (Kirkpatrick, 1996). In the current study, the efficacy of the prepared *P. aeruginosa* TF was evaluated in passive transferring of *P. aeruginosa* cellular immune responses to recipient non sensitized animals (mice and rabbits). Only the immunized mice and the TFt recipient rabbits and mice were demonstrated positive DTH-skin test after I.D injection of the soluble antigen. These results demonstrated the efficacy of the prepared TFt in the passive transport of the DTH-skin reaction to non-sensitized recipient individuals. The classical response to TF treatment is the transmission of DTH- skin reactivity to previously non sensitized individuals (Lawrence, 1949; Zhou et al., 2015).

The current study revealed, high level of protection in the immunized and TFt recipient mice compared with the high mortality in the control mice, The death of control mice during 24-96 hrs post challenge with *P. aeruginosa* indicate the multiplication of this pathogen which, overcome the innate immunity and distributed to the inter-

nal organs, leading to bacteremia, septicemia, and death, similar results were reported by Allan et al. (2005). While, higher survival rate in the TFt recipient mice confirmed the ability of the prepared TFt to protect the mice against challenge with this pathogen. Transfer factor is an immunoregulatory agent has been successfully used in the human and veterinary medicine as immunotherapy against the infections caused by various types of bacterial, viral, parasitic and fungal pathogens, as well as the diseases associated with defects in the cellular immune responses (Al-Graibawi et al., 2002; Levine et al., 2011; Viza et al., 2013 and Zhou et al., 2015). The cellular immune responses play vital role in the host defences against pulmonary infection with *P. aeruginosa* (Pier and Markham, 1982; Buret et al., 1993).

The exact mechanism of action and biological properties of TF is remained unknown, although this factor was discovered over 65 years ago. However, and for the first time, Zajíčová et al. (2014) mentioned the vital role of TF in stimulation the expression of the IL-17. Xu et al. (2014) demonstrated that the immunoregulatory effect of the IL-17 may enhance the bacterial clearance and increase the survival rate via increasing neutrophil recruitment, and performing protective role in the early stage of *P. aeruginosa* infection in mice, other researchers demonstrated the immunoregulatory actions of the IL-17 in the infection, inflammation and autoimmune diseases (Miossec and Kolls, 2012; Zhu and Qian, 2012), these findings may explain the efficacy of the prepared TFt to protect the recipient mice against challenge in the current study.

In conclusion, this study revealed the ability of the specific TFt to transport the specific DTH- skin reaction to the recipient non sensitized mice in addition, to its efficacy as immunotherapy to protect them against experimental challenge with *P. aeruginosa*, this result may propose an encouraging method in enhancing recovery from infection with this pathogen.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Both authors contributed equally in all the details of the

current manuscript.

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