ORIGINAL ARTICLE **Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ**

Evaluation of prepared specific *Pseudomonas aeruginosa* transfer factor against experimental challenge

Mawlood Abbas Ali Al-Graibawi *
Zoonotic diseases unit, College of Veterinary Medicine, University of Baghdad, Iraq

ARTICLE INFO
Received: 10.07.2015
Revised: 25. 07.2015
Accepted: 27.07.2015
Publish online: 10. 08.2015

***Corresponding author:**
Email address:
algraibawi_57@yahoo.com

Abstract
This study was designed to evaluate the protective efficacy of prepared specific *P. aeruginosa* transfer factor (TF). The first experiment was performed to prepare TF into two laboratory experimental groups. Each group contained six rats. The first group was immunized subcutaneously (S.C) with a sub lethal dose (5x104 CFU/ ml) of *P. aeruginosa* twice at two-week intervals. This group considered as a donor of specific *P. aeruginosa* transfer factor (TFt). The second group was injected S.C with phosphate buffer saline (PBS) pH 7.2 and considered as a control and source of TFn. The cell-mediated immunity (CMI) of the immunized and control rats was assessed by delayed type hypersensitivity-skin test (DTH-skin test). Only immunized rats gave positive DTH-skin reaction, in comparison with control rats. The second experiment was including the extraction of transfer factors from the splenocytes of the immunized (TFt) and control (TFn) rats. Thirty rats were divided randomly and equally into three groups, to evaluate the efficiency of the prepared TF against experimental challenge. The first group was inoculated S.C with 2 ml of TFt twice one-week interval, similarly the second group was inoculated S.C with two doses of TFn while, the third group inoculated with PBS pH 7.2. Later on, all the recipient rats were examined by DTH-skin test. Only the TFt recipient rats gave positive DTH-skin test reactivity that indicate the passive transfer of CMI. Seven days later, all the recipients were challenged with one ml containing 2x107 CFU of virulent *P. aeruginosa* intraperitoneally. The survival rate of TFt recipient rats was 80 % in compare to 20% and 10% of TFn and PBS recipient rats, respectively. In conclusion, the result of this study revealed that specific *P. aeruginosa* transfer factor plays an important role as a biological substance for therapy or supplementary therapy for infections caused by *P. aeruginosa*.

To cite this article: Mawlood Abbas Ali Al-Graibawi, (2015). Evaluation of prepared specific *Pseudomonas aeruginosa* transfer factor against experimental challenge. MRVSA. 4 (2), 17-27. DOI: 10.22428/mrvsa. 2307-8073.2014. 00423.x

Keywords: *Pseudomonas aeruginosa*, transfer factor, DTH, Rats, immunotherapy.

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative rod, non- spore forming, strict aerobic and motile microorganism. It is widespread in the environment of animals. *Pseudomonas aeruginosa* is responsible for a variety of the difficult treated infections in veterinary medicine including: wound infection, mastitis in dairy animals, metritis in

horses, otitis, corneal ulcer, in addition to abscesses and urinary tract infections in dogs (Radostits *et al.,* 2007; Markey *et al.,* 2013). It is also a major cause of morbidity and mortality in compromised patients, such as those with burns, cystic fibrosis, chronic bronchitis and cancer (Kato *et al.,* 2015). This bacterium has a high incidence of antimicrobial resistance due to the presence of multiple drug efflux pumps and low permeability of the major outer membrane porins (Mesaros *et al.,* 2007). This has led researchers to evaluate immunotherapeutic methods based on either passive or active immunization to prevent and treat *P. aeruginosa* infections. Priebe *et al.,* (2003) reported that a combination of cellular and humoral immune responses is needed for protection against *P. aeruginosa* infections. William *et al.,* (2010) revealed that adoptive transfer of specific immune T lymphocytes can significantly enhance the resistance to *P. aeruginosa* infection in granulocytopenic mice. Cell-mediated immune responses play an important role in host defenses against infection with *P. aeruginosa* (Buret *et al.,* 1993). Transfer factor (TF) also called dialyzable leukocyte extract, is a low molecular weight (less than 10.000 Daltons) substance extracted from the T-cells of mammals (including human) and bird. TF has been described for the first time by Lawrence (Lawrence, 1949; and Xu *et al.,* 2013). It is a natural, non-species specific extract of leukocytes that can transferring cell-mediated immunity from an immune donor to a nonimmune recipient, and act as an immunomodulator agent (Kirkpatrick, 1996). The chemical structure, molecular weight and the exact mechanism of function of TF are still not fully known, albeit was discovered over 65 years ago. However, TF has been used to improve the clinical responses in infectious diseases, allergies, cancer, and immunodeficiency (Fabar, 2004; Levine *et al.,* 2011; Viza *et al.,* 2013 and Zhou *et al.,* 2015). So far, the reviewing of the available recent literatures, reveals no any report concerning specific transfer factor as immunotherapy in *P. aeruginosa* infection. The current study was designed to prepare TF specific to *P. aeruginosa* and to evaluate its immunological and protective efficacies by the DTH- skin test and immune protection tests respectively.

Materials and Methods

Bacteria

The *P. aeruginosa* bacterium used in this study was isolated from the patient, who was admitted to Al-Yarmouk Teaching Hospital in Baghdad/ Iraq. The isolate was confirmed as *P. aeruginosa* by assessing the colony morphology, Gram stain, oxidase test, production of blue/green pigment and conducting the different biochemical tests (Markey *et al* 2013). It was inoculated into trypticase soy broth and incubated for 24 hours at 37°C. Later on, the bacteria were pelleted by centrifugation, washed three times in sterile PBS pH 7.2. The viable bacterial count was measured by culturing serial dilutions overnight on trypticase soy agar (Milesand Misra, 1938). The Mean lethal dose 50 (LD50) and sublethal doses were detected in the inoculated mice (Reed and Muench, 1938). In mice, the LD50 and the sublethal dose were 4x106 CFU and 5x104 CFU respectively.

Preparation of soluble antigen

The soluble antigen used in DTH-skin reaction test was prepared from *P. aeruginosa* isolate according to the method described previously (Campa *et al.,* 1982). Briefly, the *P. aeruginosa* was plated and grown on trypticase soya agar for 48 hours at 37 °C. The

growth was harvested by sterile PBS pH 7.2. The harvest was washed three times by centrifugation at 5.000 (rpm) for 40 minutes. The sediment was sonicated for 35 minutes at intervals in a water-cooled sonicator oscillator at 15 KHZ/sec. (MSE- watt-ultrasonic Disintegrator GE.MK2). The homogenate was centrifuged with cool centrifuge twice at 10000 rpm for 45 minutes each time to remove cellular debris. The protein content of the supernatant was assayed according to Lowry *et al.,* (1951). Later on, the extracted supernatant was sterilized through 0.45 μm Millipore filter and stored at -20°C.

Experimental laboratory animals

Forty-two Albino Swiss rats and five rabbits were obtained from the College of Veterinary Medicine in Baghdad University. The ages of the experimental animals were 5-8 weeks. The animals were kept for two weeks for acclimatization prior to starting of the experiment. Several faecal samples were taken from animals for microbial isolation to confirm that these animals were free from *P. aeruginosa*. They were reared in a separate plastic cage and fed alfalfa and commercial assorted pellets. This study was approved by the Ethical and Research Committee of the College of Veterinary Medicine / University of Baghdad.

First experiment

Immunization and clinical examination

This experiment was performed for preparation and extraction of the TF from donor rats. Twelve rats were divided randomly and equally into two groups. The first group (immunized group) was injected subcutaneously (S.C) twice at two weeks intervals with a sub lethal dose (5x104 CFU) of *P. aeruginosa*. While, the Second group was injected twice with sterile PBS pH 7.2 and acted as control. Along ten days post each immunization, the immunized and control rats were checked daily for the appearances of clinical signs.

Evaluation of immunization efficiency

The cell mediated immunity was evaluated in the experimental rats by DTH - skin test and extraction of specific *P. aeruginosa* transfer factor as follows:

Delayed-type hypersensitivity -Skin test

Two weeks after the booster dose of the immunization, the right flank region of each rat was clipped and shaved carefully. The flank area was divided into three parts. The third part was injected intradermal (I.D) 0.1 ml of BPS pH 7.2 and considered as control. While the first and second parts were injected with 0.1 ml of different concentration of *P. aeruginosa* soluble antigen, 30 μg /ml, and 3μg / ml respectively. The diameter of skin reaction was measured by the ruler at 24, 48, and 72 hours post injection (Campa *et al.,* 1982).

Extraction of transfer factor

Twenty one day post the second immunization dose, TF were extracted from the spleen cells of the rats of both experimental and control rats. The cells were collected aseptically and according to method described previously (Kirkpatrick, 1996). After washing three times with sterile PBS pH 7.2, the spleen was pressed through a stainless-steel screen and suspended in RPMI 1640 medium with 10% fetal calf serum. The suspension was made free from erythrocytes with 0.83% ammonium chloride. The cells were repeatedly washed with sterile PBS pH 7.2 to remove the platelets. The viability was detected by staining with 0.1% trypan blue. The viable cells were about 85%. Later on, the cells were adjusted at 5x108 cells /ml and subjected to repeated freezing- thawing ten times. The obtained leukocyte extract were centrifuged at 12000 rpm for 45 minutes using a cold centrifuge to remove cellular debris. The supernatant was filtered through, centrifugal filter units for concentration and purification of biological solution (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, IRL) to get the lysate containing TF. The lysate was sterilized by 0.22 μm millipore filter and referred as TFt and TFn for transfer factors extracted from spleens of immunized and control rats respectively. The TF products were aliquot and stored at -20o C. The sterility test was performed by culturing a few drops of the final product on blood agar plates and incubated at 37℃ for one week. The plates were regularly examined during the incubation period to ensure their sterility.

Pilot study

A pilot experiments was done in rabbit, to evaluate the immunological activity of the prepared TFt to elicit DTH- skin reaction. Five rabbits were injected I.P with 2 ml (equivalent to 5 x 108 cells /ml) of TFt. One week later, all rabbits were assayed for DTH- skin test.

Second experiment

To evaluate the immunological and protective efficacy of the prepared transfer factor, thirty rats were divided randomly and equally into three recipient groups. The first group was inoculated S.C with 2 ml (equivalent to 5 x 108 cells /ml) of TFt twice one week interval. Similarly, the second groups were injected with TFn which was extracted from control rats .While the third group were injected I.P with 2 ml of BPS pH 7.2 and acted as control. One week post, TF injection and all the recipients were examined by DTH-skin test. The diameter of DTH- skin reaction was measured at 24, 48, and 72 hours post injection by the ruler (Campa *et al.,* 1982). Then all the recipients were challenged with five LD50 of *P. aeruginosa* at dose of 0.5ml containing (2x107 CFU) intraperitonally.

Clinical examination

Post the challenge, all the recipient rats were checked daily for the surviving and appearances of clinical signs.

Results

Post immunization clinical signs

Following immunization, the rats were slightly depressed and listless. Localized swelling was detected during palpation at the sites of injection after 48 hours. Some experimental animals developed small abscesses. These abscesses was disappeared within two weeks, however, *P. aeruginosa* was isolated from its contents. There were no adverse effects in immunized rats. The clinical signs in the control rats remained within the normal ranges.

Delayed type hypersensitivity – skin test

The rats of immunized group revealed positive skin test reactivity at 24, 48 and 72 hours, post I.D inoculation with various concentrations of *P. aeruginosa* soluble antigen. 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 (Table1).

Table (1): 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
30	5.6	8.3	6.1	*0	0	0
3	3.4	4.3	2.7	0	0	0
PBS	0	0	0	0	0	0

*Skin reaction was observed at five hours, post I.D inoculation and considered to be an immediate type of hypersensitivity.

Pilot study

The result of the DTH-skin test in rabbits revealed an edematous, erythematous area at the sites of inoculation in all the rabbits received the Tft (Fig.1).

Adoptive transfer of the DTH-skin test

The recipient (non-sensitized) rats that received TFt prepared from the spleens of the rats immunized with *P. aeruginosa* revealed positive skin test reactivity at 24, 48 and 72 hours after I.D inoculation with *P. aeruginosa* soluble antigen, whilethose received PBS and TFn prepared from spleens of control rats did not react to the *P. aeruginosa* soluble antigen (Table 2).

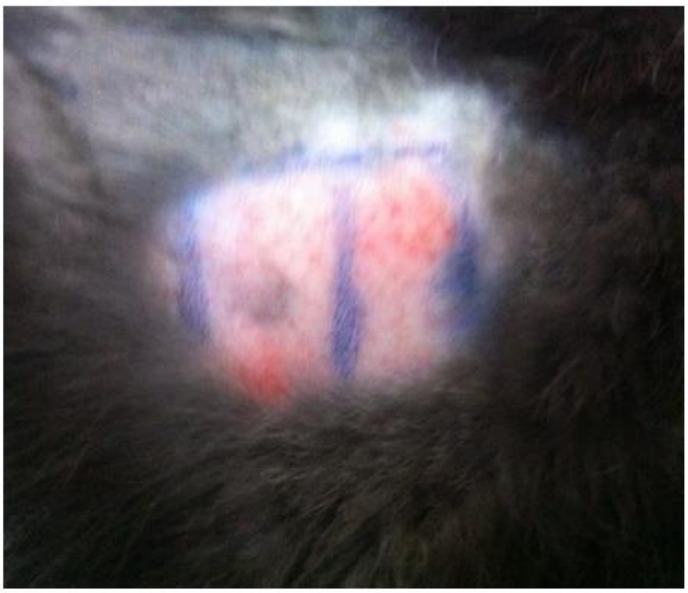

Figure (1) Skin reaction test of rabbit received prepared transfer factor.

Table (2): Skin reaction of rats received TF post I.D injection with various concentrations of *P. aeruginosa* soluble antigen.

Antigen conc. (µg/ml)	Mean diameter of skin reaction (mm)				
	TFt recipients (No. 10)			TFn (No. 10) recipients	PBS (No. 10) recipients
	24h	48h	72h		
30	5.1	7.8	3.8	0	0
3	3.1	3.8	2.3	0	0
PBS	0	0	0	0	0

Post challenge clinical observations

Prior to the challenge, all the recipient rats showed normal clinical parameter. After the challenge, the TFt recipient's rats showed moderate signs of illness, while the control and Tfn recipient's rats showed marked decreased physical activity, depressed and anorexic by 36 hours. Then, rats showed hunched posture, weight loss, rough hair coat and rapid laboured breathing by day four post challenge. The TFt recipient rats revealed a high rate of protection (80%) against challenge with *P. aeruginosa* compared to 20% and 10% protection rate in the TFn and PBS recipient rats, respectively (Table. 3).

Table (3). Survival rate of recipient groups post challenge with *P. aeruginosa*.

Recipient group	Number of rats	Survival number	Survival rate (%)
TFt- group	10	8	80%
TFn- group	10	2	20%
PBS- group	10	1	10%

Discussion

Pseudomonas aeruginosa is an opportunistic pathogen. It rarely affects healthy individuals. *P. aeruginosa* can cause a wide range of infections, particularly in those with a weakened immune system e.g: cancer patients, new-borns and people with severe burns, diabetes mellitus or cystic fibrosis. In veterinary medicine, *P. aeruginosa* is not a prominent pathogen but is responsible for a variety of difficult-to-treat infections (Haenni *et al.,* 2015). This has led researchers to investigate immunotherapeutic methods to prevent and control *P. aeruginosa* infections, based on either passive or active immunization (Cripps *et al.,* 1994), a number of various vaccines and several immunotheraputical procedures have been used in the last decades against *P. aeruginosa* infections (Priebe *et al.,* 2003; Pier, 2005; Doring and Pier, 2008).

The results of this study revealed that the immunization of the rats with the sub lethal dose of *P. aeruginosa* did not induce systemic adverse reactions. However, loss of appetite, listless and depressed were observed on the animals for 96 hours, which might be due to immunological and inflammatory reactions (Tizard, 2004).The positive results of DTH- skin test of the immunized rats indicated that the sublethal dose of *P. aeruginosa* stimulated a CMI response. These results are in agreement with those reported previously (Colizzi, 1982; Markham and Powderly, 1988). The positivity of the post intradermal inoculation of soluble antigen depends on existing of specific memory T cells previously exposed to this antigen. Moreover, local inflammation post 48–72 hours occurred due to the recruitment of leukocytes (lymphocytes, monocytes) to the site of inoculation (Tizard 2004).

The DTH-skin test is a standard method to detect the in *vivo* cell mediated immune responses, and it is mediated by CD4+ T cell and CD8+ T cell cytokine production (Colizzi 1982). The CD8+ T cells act as principal effector cells in the intraperitoneal *P. aeruginosa* infection of mouse model (Markham *et al.,* 1984). Markhan and Powderly, (1988) reported that the transfer of the T cells obtained from BALB/c mice immunized with 102 live *P. aeruginosa* protected granulocytopenic recipient mice from *P. aeruginosa* infection and inhibit the *in vitro* growth of *P. aeruginosa*. Meanwhile, Powderly *et al.,* (1986) demonstrated that transmission of specific immune T lymphocytes can significantly elevate the resistance of granulocytopenic mice to *P. aeruginosa* infection. The stimulation of both neutrophil recruitment and activity has been noticed to be a central effector procedure of protective immune response post intestinal immunization, this indicated that cell mediated responses play important role in host defences against pulmonary infection with *P. aeruginosa,* (Pier and Markham, 1982; Buret *et al*., 1993).

Many studies have revealed the role of T cells in the transfer of CMI to protect laboratory animals against *P. aeruginosa* infection (Markham *et al.,* 1985). The most important restriction on the application of Immunotherapy through T-cell is graft-versus-host reaction which induced from immune-mediated attack of recipient tissue by donor T cells (Schroeder, 2002; Ferrara *et al.,* 2010; Schroeder and DiPersio, 2011). Munster *et al.,* (1974) suggested the use of transfer factor or xenogeneic cells in adoptive transfer of CMI to avoid graft-versus-host reactions. The TF is non- antigenic, and did not elicit any immune reaction in the recipients due to its very small molecules, in contrary to the viable T cells (Kirkpatrick, 1996). In this study, the ability of specific TF was evaluated in passive transferring of *P. aeruginosa* cellular immune responses to recipient non sensitized animals (rats and rabbits of pilot experiment). However, only the TFt recipient rats and rabbits were revealed positive DTH-skin test post I.D inoculation of soluble antigen. This result indicated the efficacy of the prepared TF in the passive transmission of cellular immune responses- DTH-skin reaction to non-sensitized recipient animals. In addition, this result may offer an encouraging approach in improving recovery from *P. aeruginosa* infection, and is compatible with previous study (Al-Graibawi *et al.,* 2000). The classical response to TF treatment is the transfer of cutaneous hypersensitivity to previously non sensitized individuals (Lawrence, 1949).

Post challenge, the high level of protection in the TFt recipient group in comparison with those of Tfn and PBS recipient groups indicating the efficacy of the prepared specific TF in protection against infection with *P. aeruginosa*. Transfer factors has been successfully used clinically in the treatment of viral, bacterial, fungal and parasitic infections, as well as immune deficiency diseases associated with defects in cellular immunity (Al-Graibawi *et al.,* 2000 ; Levine *et al.,* 2011; Viza *et al.,* 2013 and Zhou *et al.,* 2015). Cellular immunity play important role in the host defences against pulmonary infection with *P. aeruginosa* (Pier and Markham, 1982; Buret *et al.,* 1993). The high level of protection in the TFt recipient group in comparison with those of Tfn and PBS recipient groups is indicated the efficacy of the prepared specific TF in protection against *P. aeruginosa* challenge infection. The accurate mechanism of action of TF is remained unknown although these factor was discovered over 65 years ago. However, and for the first time, Zajícová *et al.,* (2014) reported the role of TF in the enhancement of the expression of the IL-17. These functions may represent the main mechanism of immunotherapeutic and immunomodulatory effects of TF. In conclusion, this study approved that the specific *P. aeruginosa* transfer factor has the ability to transport specific *P. aeruginosa* CMI. The TF could protect the recipient hosts against *P. aeruginosa* challenge infection and revealed a high level of protection.

Acknowledgments

I would like to thank Prof. Dr. Afaf A. Yousif and Dr. Abdulkarim Jafar karim (Collage of Veterinary Medicine, Baghdad University) for providing insightful comments.

References

Al-Graibawi MA, Al-Izzi SA, and Khalifa AK. (2000). Role of the transfer factor in in the protection against *Rhodococcus equi*. Iraqi J. Agric. 5 (4): 162-167.

of intestinal induced immunity to *Pseudomonas aeruginosa* in the rat lung; Role of neutrophils and leukotriene B4. Infect. Immun. 61: 671-9.

Campa M, Toca L, Lombardi S, Garzelli C, Colizzi V and Falcone G. **(1982).** Cell - mediated immunity and delayed-type hypersensitivity in *Pseudomonas aeruginosa*-infected mice. Med Microbiol Immunol. 170(3):191-9.

Cholley P, Gbaguidi-Haore H, Bertrand X, Thouverez M, Plesiat P and Hocquet D. (2010). Molecular epidemiology of multidrug-resistant *Pseudomonas aeruginosa* in a French university hospital. J Hospital Infect. 76(4): 316–9.

Colizzi V, Garzelli C, Campa M, Toca L and Falcone G. (1982). Role of specific delayed-type hypersensitivity in Pseudomonas aeruginosa-infected mice. Immunology: 47, 337-344.

Cripps AW, Dunkley ML and Clancy RL. (1994). Mucosal and systemic immunizations with killed *Pseudomonas aeruginosa* protect against acute respiratory infection in rats. Infect. Immun. 62: 1427.

Doring G and Pier GB. (2008). Vaccines and immunotherapy against *Pseudomonas aeruginosa.* Vaccine. 26:1011-1024.

Faber R A. (2004). Transfer factor as immunotherapy and supplement of chemotherapy in experimental pulmonary Tuberculosis. Clin. Exp. Immunol.136 (2):215-23.

Ferrara J, Reddy P, and Paczesny S. (2010). Immunotherapy through T-cell receptor gene transfer induces severe graft-versus-host disease. Immunotherapy. 2 (6): 791–794.

Haenni M, Hocquet D, Ponsin C, Cholley P, Guyeux C, Madec JY and Bertrand X. **(2015).** Population structure and antimicrobial susceptibility of Pseudomonas aeruginosa from animal infections in France.BMC Veterinary Research Bacteriology. 11:9: 1-5.

Kato I, Fujimoto F, Higurashi Y, Yamaguchi R, Takayama K, Suzuki M, Okugawa S, Okazaki M and Moriya K. (2015). Antibiotic Susceptibilities of *Pseudomonas aeruginosa* Isolated from Blood Samples and Antibiotic Utilization in a University Hospital in Japan. Infect Dis Ther. 4 (2): 213-8

Kirkpatrick CH. (1996). Activities and characteristics of transfer factor. Biotherapy. 9:13-16.

Lawrence HS. (1949). The cellular transfer factor of cutaneous hypersensitivity to tuberculin in man. Proc. Soc. Exp. Biol. Med. 71:516-22.

Levine PH, Pizza G, Ajmera K, De Vinci C and Viza D. (2011). Transfer factor in virus-associated malignancies: an underestimated weapon in the prevention and treatment of cancer. Adv. Tumor Virol. 2, 7-20.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. (1951). Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry. Vol. 193, 1, p. 265-275.

Markey BK, Leonard FC, Archambault M, Cullinane A and Maguire D. (2013). Clinical Veterinary Microbiology. 2nd ed. Mosby Elsevier, 275-288.

Markham RB, Goellner J and Pier GB.(1984). *In vitro* T cell-mediated killing of *Pseudomonas aeruginosa* 1.Evidence that a lymphokine mediates killing. Immunol.132: 962-8.

Markham RB, Pier GB, Goellner JJ and Mizel SB. (1985). *In vitro* T cell mediated killing *of Pseudomonas aeruginosa* II. The role of macrophages and T cell subsets in T cell killing. Immunol.1985:134:4112-17.

Markham RB and Powderly WG. (1988). Exposure of mice to live *Pseudomonas aeruginosa* generates a protective cell-mediated immunity in the absence of an antibody response. Immunol. 140; 2039-45.

Mesaros N, Nordmann P, Ple´ siat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F and Lebesque P. (2007). *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millenium. Clin Microbiol Infect 13, 560–578.

Miles AA and Misra SS. (1938). The estimation of bactericidal power of the blood. J. Hygiene. 38:732-49.

Munster AM, Leary AG, Spicer SS and Fisher MW. (1974). Effect of lymphocytotherapy on the course of experimental Pseudomonas sepsis. Ann Surg. 179 (4): 482-488.

Pier G. (2005). Application of vaccine technology in prevention of Pseudomonas aeruginosa infections. Expert Review of Vaccines .4 (5): 645-56

Pier GB and Markham RB. (1982). Induction in mice of cell-mediated immunity to *Pseudomonas aeruginosa* by high molecular weight polysaccharide and vinblastine. Immunol. 128: 121-5.

Powderly WG, Pier GB and Markham RB. (1986). T Lymphocyte-mediated Protection against *Pseudomonas aeruginosa* Infection in Granulocytopenic Mice. J. Clin. Invest. 78:375-380.

Priebe GP, Meluleni GJ and Coleman FT. (2003). Protection against Fatal *Pseudomonas* aeruginosa *Pneumonia* in Mice after Nasal Immunization with a Live, Attenuated *aroA* Deletion Mutant. Infect. Immun. Vol. 71 no. 3 1453-1461.

Radositis OM, Gay CC, Hinchliff KW and Constable PD (2007). Veterinary Medicine, Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats.10th ed. New York, Oxford, Philadelphia, Saunders Elsevier, pp: 1081-1085.

Reed L J and Muench LH. (1938). A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27: 493-497.

Schroeder ML. (2002). Transfusion-associated graft-versus-host disease. Br J Haematol. 2002; 117(2): 275–287. Good review of the clinical and pathophysiologic aspects of transfusion-associated graft-versus-host disease. [PubMed: 11972509]
Schroeder ML and DiPersio JF. (2011). Mouse models of graft-versus-host disease: advances and limitations. Disease Models & Mechanisms 4, (3): 318-333

Tizard IR. (2004). Veterinary Immunology: An Introduction; 7th Ed., W.B. Saunders Company, Philadelphia. pp 1:3-9.

Viza D, Fudenberg HH, Palareti A, Ablashi D, De Vinci C and Pizza G. (2013). Transfer factor: an overlooked potential for the prevention and treatment of infectious diseases. Folia Biol. 59, 53-67.

Williams BJ, Dehnbostel J and Blackwell TS. (2010). *Pseudomonas aeruginosa*: host defence in lung diseases. Respirology. 15: 1037– 1056.

Xu Y , Zhang Q, Zhan X, Xie D, Dai G and Yang H.(2013). Preparation and immunological evaluation of oral solution of egg yolk-derived hepatitis B virus-specific transfer factor. J. South Med. Univ. 33(12): 1827-1830.

Zajícová A, Javorková E, Trošan P, Chudíčková M, Krulová M and Holáň V. (2014). A low-molecular-weight dialysable leukocyte extract selectively enhances development of $CD4^{+}ROR\gamma t^{+}$ T cells and IL-17 production. *Folia Biol (Praha* 60 (6): 253-60.

Zhou J , Kong C, Wang X, Shao J, Feng L and Zhang Z.(2015). Preparation and identification of transfer factor specific to *Staphylococcus aureus* in vitro. Biotechnol Appl Biochem. 62 (1):112-20.