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Original Research Article

Evaluation of Peritoneal Macrophages Migration Inhibition and Lymphocytes Mitotic Index in *Shigella Dysenteriae* Type1 Immunized Guinea Pigs

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Abstract

Background and objective: To determine the performance and functional activity of macrophage and lymphocytes after immunization with phenol killed *Shigella dysenteriae* type 1.

Materials and Methods: phenol killed *Shigella dysenteriae* type 1 (16×10^3bacteria/ml) was injected subcutaneously(S/C) in 10 guinea pigs at a dose of 0.5 ml .At the third week animals given a 1st booster dose of (1 ml) of killed bacteria. At fifth week, a second booster dose (1ml) was given (S/C). Five animals were used as control group and injected with phosphate buffer saline. After isolation of peritoneal macrophage and Blood Lymphocytes, macrophage migration inhibition (MMIT) and lymphocytes, transformation (LTT) tests have been applied.

Results: The level of inhibition of migration decreased gradually, while reducing the concentration of the PHA antigen. Migration inhibition coefficient was (0.477±0.268) when PHA antigen (400 µg/ml), (0.610±0.181) when PHA (40 µg/ml), (0.641±0.145) when PHA antigen (4 µg/ml). No significant difference in migration inhibition coefficient was detected between immunized groups ($p>0.05$) at different concentration of PHA antigen. The control group did not show any migration inhibition for macrophages at different concentrations of PHA antigen. Significant difference was detected between control groups ($p<0.05$) at different concentration of PHA antigen. Significant differences($p<0.01$), ($p<0.05$), ($p<0.01$)were detected between immunized and control groups at 4 µg / ml, 40 µg / ml, 400 µg / ml, concentration of PHA antigen respectively . A clear response of the lymphocytes toward PHA was detected in lymphocyte transformation test. The mitotic index (Ml) of the lymphocytes exposed to the (0.1mg/ml) PHA antigen was (11.56 ± 3.22); for (5mg/ml) PHA was (41.49 ± 26.298) and for control was (1.18±0.516). Significant difference in mitotic index was detected between groups ($p<0.01$) according to the dose of PHA antigen.

Conclusions: MMIT and LTT can be used effectively to evaluate and monitoring of cellular immunological responsiveness to *shigella dysenteriae* type 1 antigen .Both MMIT and LTT proportionally associated with pre sensitization dose and duration of exposure to antigen.

Keywords: *Shigella dysenteriae*, macrophage migration inhibition, lymphocyte transformation

1.Introduction

Diarrheal disease continue to exist as these condition leading cause of mortality among children under five years of age[1]. Shigella, a highly virulent pathogen that causes bacterial dysentery, is one of the leading causes of diarrheal disease and contributes significantly to the burden worldwide[2] *Shigella dysenteriae* strain have ability to cause epidemic and severe diarrhea (shigellosis)mostly in poor and crowded countries [3]. Low bacterial dose (10^1-10^2) via contaminated food or water have the ability to induce clinical disease mainly in children older than 2 years due to immaturity of intestinal immune system[4]. Shigella causes more than 150 million diarrhoea episodes and more than one million deaths annually[3]. The key step in the *S. dysenteriae* type 1 pathogenesis is bacterial invasion of the colonic mucosa and translocate effector proteins from the bacterial cytoplasm to the membrane and cytoplasm of the

host cell via type three-secretion apparatus (T3SA), a needle-like structure[5] and formation of micro abscess in the wall of the large intestine, followed by mucosal ulcerations and pseudomembrane formation[6]. Wild type *Shigella* infection causes a severe alteration of the barrier function of a small intestinal mucosa cells and might contribute (along with enterotoxins) to the induction of watery diarrhea. Increasing evidence reveals the ability of pathogens to modulate host immunity, preventing the induction of an efficient immune response that may contribute to the clearance of primary infection and/or protection from reinfection[7]. Shigella creates a strong immunosuppressive environment in the course of infection and have the ability to divert functions of Dendritic cells (DCs) and B and T lymphocytes, a direct targeting of the adaptive immune system[8]. Antibody-mediated protection arises only after several episodes of infection, is of short duration, and is poorly efficient in limiting reinfection, particularly in young children [7].

Macrophage migration inhibitory factor (MIF or MMIF) also known as glycosylation inhibiting factor (GIF), L-dopachrome isomerase, or phenylpyruvate tautomerase, is a protein that in humans is encoded by the MIF gene[9]. MIF is an important regulator of innate immunity[10].Bacterial antigen stimulate white blood cells(WBCs) to release MIF into the blood stream. The circulating MIF binds to CD74 on other immune cells to trigger an acute immune response[11].MIF is classified as an inflammatory cytokine. WBCs release MIF in response to glucocorticoids stimulation, thus MIF partially counteracts the inhibitory effects that glucocorticoids have on the immune system. Trauma also stimulate anterior pituitary gland to produce MIF[12]. The MIF gene product MIF encodes a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation[13,14]. MIF plays a role in the regulation of macrophage function in host defence through the suppression of anti-inflammatory effects of glucocorticoids[14-16].

Lymphocyte transformation test (LTT);has other synonyms(Lymphocyte activation test; Lymphocyte proliferation test; Lymphocyte stimulation test) is currently the only laboratory method for the determination of a specific cellular sensitization[17]. It is based on the principle of the specific antigen (allergen) induced lymphocyte cell division following contact with the corresponding antigen. A positive reaction proves the existence of antigen specific lymphocytes (memory cells) in the patient's blood[18]. The newly optimized LTT variants have attained an enhanced sensitivity and specificity through the addition of a genetically manipulated interferon-alpha to the cell culture. The spectrum of LTT indications encompasses four important areas of medical diagnostics: Diagnosis or exclusion of: - Cell mediated sensitization (Type IV Allergy) - Defects and function disorders of the immune system -Tissue compatibility/incompatibility (Transplantation medicine) - Lymphocyte reactivity toward infectious agents, to monitor cancer, occupational exposure to dust and other antigens, and other environmental antigens and mitogens[19-21].

The objective of current study is to determine performance and functional activity of macrophage and lymphocytes after exposure to *Shigella dysenteriae* type 1 antigenic stimulation due to great importance in the production of vaccines.

2. Materials and methods

2.1. Isolation of *Shigella dysenteriae* type 1:

Stool samples were collected from children with diarrhoea during January 2014 to march 2014after obtaining assigned approval of ethical committee at college of medicine Diyala University, Iraq. Submitted samples transported via transport media (Phosphate buffered saline) in to laboratory and inoculated on MacConkey agar and Selenite-F broth for enrichment and incubated at 37^{o}C for 24 hours in aerobic environment. Selenite-F broth, was subcultured on Salmonella-Shigella agar (Oxoid) after overnight incubation[22]. Conventional biochemical reactions (urea, citrate, triple sugariron, indole, motility, oxidase test, catalase test) used for identification of colonies morphologically suggestive of Shigella species. Polyvalent shigella antisera for slide agglutination tests from pro lab diagnostic used for typing[23].Non-serotypable isolates were further checked by API 20 E (Bio Murex, France)[24].

2.2. Immunization of guinea pigs

Fifteen guinea pig males purchased from pharmaceutical and toxicological investigations department –Ministry of health-Iraq used in experiment. Approval of ethical committee at college of veterinary medicine, Diyala University was obtained for starting the experiments.

Immunization was conducted through a subcutaneous injection of (10) animals at a dose of 0.5 ml of phenol killed *Shigella dysenteriae type 1* suspension contain (16×10^3) bacteria per ml. At the third week of the initial injection animals given a first booster dose of one ml of phenol killed *Shigella dysenteriae type 1*. At the fifth week, a second booster dose of one ml was given subcutaneously.

Five animals were used as control group and injected with phosphate buffer saline (PBS)[25].

2.3. Peritoneal macrophages migration inhibition test (PMMI)

PMMI was conducted for the immunized and control group, three weeks after the injection of the second booster dose (at 8th week of experiment).Each animal were injected with 30 ml of liquid paraffin intraperitoneally then 72 hours later each animal was anesthetized and injected intraperitoneally with 150 ml of RPMI-1640 solution provided with heparin 50 IU / ml and 20% fetal calf serum (Gibco®)[26].Then conducts the process of massage quiet for each animal and surgical incision of 7 cm length at the midline of the abdomen was done for collection of Peritoneal fluid in a sterile bottle and left to settle down and then centrifuged at 3000 rpm / min at 5co for three consecutive times[25].Cells suspension was diluted with RPMI-1640(Gibco®GlutaMAX™ media)[27]containing 15% of fetal calf serum and 100 IU of penicillin and 100 µg /ml of streptomycin per were added the a capillary tube was filled with proceeded and shut down one of the slots and centrifuged at 2000 rpm / min for 5 minutes and cut the capillary tube between the cells layer and the liquid and set within the drilling special tissue culture dishes then put the PHA antigen in the drilling using three concentrations of a 4 µg/ ml and 40 µg / ml and 400 µg / ml and then RPMI-1640.Tissue culture dishes were incubated at 37Co with the presence of 5 %CO2 for 24 hours. The percentage of phagocytic cells migrating was calculated according to following equation [25]

$$Migrationpercentage = \frac{migration\ \ area\ of\ cells\ withpresenceofantigen}{migration\ \ area\ of\ cells\ withoutantigen} \times 100$$

2.4. Lymphocyte transformation test:

Five adult guinea pigs, were enrolled in immunization program and five unimmunized. From each animal 2 ml of blood were withdrawn and placed in a tube containing 20 units heparin / ml bloodand diluted with normal saline by 2:1[28, 29]. Lymphoprep™ was mixed thoroughly before use by inverting the bottle several times. Three ml of Lymphoprep™ was added to a tube[30]. Three ml of Blood was diluted with an equal amount of phosphate-buffered saline (PBS) plus 2% fetal bovine serum (FBS). Blood was layered carefully on top of Lymphoprep™ to minimize mixing of blood with Lymphoprep™. Then centrifuged at 3000 rpm for 30 minutes at room temperature (15 - 25°C) with brake off. Upper plasma layer was removed and discarded without disturbing the plasma-Lymphoprep™ interface. Mononuclear cell layer at the plasma-Lymphoprep™ interface was removed and retained without disturbing erythrocyte/granulocyte pellet. Mononuclear cells then washed by normal saline solution and centrifuged at 2000 rpm / min for five minutes for three consecutive times [30,31].

Taking the cellular sediment and mixed with prepared (RPMI-1640)[27] containing 15% of fetal calve serum, 100 IU of penicillin and 100 µg/ml of streptomycin. Three ml of RPMI-1640 containing lymphocytes was distributed equally in five sterile tubes with a lid for three groups. For the first group 0.1 mg/ml of phytohaemagglutinin *(PHA*) was used[32], In the second group 5 mg / ml (PHA)and the third group was control. Tubes were incubated at 37 CO with 10% CO_2 for (7-5) days then all tubes were centrifuged at 2000 rpm/minute for 10 minutes and smears on slides were made from cellular sediments and left to dry then slides were stained by Lieshman's stain and examined under the microscope. Mitotic index was calculated according to the following equation[28]:

$$Mitoticindex(MI) = \frac{numberofcellsindivisionstage}{numberofundividedcells} \times 100$$

2.5. Statistical analysis

All collected data were entered in Statistical Package for Social Sciences version 17.0 (SPSS v.17) and MS Excel (2010), were used for statistical analysis of data. All results of continuous data were expressed as Mean± Standard Deviation (SD). ANOVA and t-tests were used for detection of differences between groups which considered significant at $p<0.05$, $p<0.01$ levels.

3. Results

The results of the PMMI test revealed inhibition of migration for the peritoneal macrophages in tissue culture dishes, 24 hours after the addition of three different concentrations of Phytohaemagglutinin (PHA) antigen as shown in (Table 1).The level of inhibition of migration decreased gradually, while reducing the concentration of the PHA antigen. Migration inhibition coefficient was (0.477± 0.185) when using PHA antigen concentration (400 µg / ml) (Figure1-A), migration inhibition coefficient was (0.6106± 0.163) when using PHA antigen concentration (40 µg / ml), migration inhibition coefficient was (0.641± 0.13) when using PHA antigen concentration (4 µg / ml). No significant difference in migration

inhibition coefficient was detected between immunized groups ($p>0.05$) at different concentration of PHA antigen. Migration inhibition coefficient in term of (mean ± SD) inversely associated with dose of PHA antigen among immunized group.

The control group did not show any migration inhibition for macrophages at different concentrations of PHA antigen in tissue culture dishes (figure 1-B), the mean migration inhibition coefficient was more than 1,000. Significant difference was detected between control groups ($p<0.05$) at different concentration of PHA antigen. Significant difference ($p<0.01$),($p<0.05$),($p<0.01$)was detected between immunized and control groups at 4 µg/ml, 40 µg/ml, 400 µg/ml, concentration of PHA antigen respectively.

Lymphocyte transformation test showed a clear response of the lymphocytes toward phytohaemagglutinin (PHA).As shown in Table 2, the mitotic index (Ml) of the lymphocytes exposed to the (0.1mg/ml) PHA antigen was (11.56±3.22) and for lymphocytes exposed to (5mg/ml) PHA was (41.49±26.298) and for control (1.18±0.516).Figure (2) shows the obvious response of lymphocytes to the antigen through a high degree of cells division. Significant difference in mitotic index was detected between groups ($p<0.01$) according to the dose of PHA antigen.

Table 1: Peritoneal macrophage migration inhibition factor in immunized guinea pigs and control group

Group	Animal No.	PHA Antigen concentration			ANOVA (p value)
		4 µg / ml	40 µg / ml	400 µg / ml	
		migration inhibition coefficient	migration inhibition coefficient	migration inhibition coefficient	
Immunized group	1	0.725	0.725	0.544	p>0.05
	2	0.632	0.591	0.429	
	3	0.786	0.786	0.671	
	4	0.759	0.740	0.740	
	5	0.681	0.681	0.640	
	7	0.713	0.702	0.409	
	7	0.441	0.287	0.160	
	8	0.392	0.374	0.230	
	9	0.611	0.570	0.480	
	10	0.671	0.650	0.474	
Mean ± SD		0.641± 0.13	0.6106± 0.163	0.477± 0.185	
Control group	1	2.050	1.000	0.833	p<0.05
	2	1.790	1.120	1.000	
	3	4.220	2.140	1.140	
	4	2.990	1.850	1.560	
	5	2.680	2.478	1.759	
Mean ± SD		2.7460± 0.953	1.717±0.641	1.258±0.388	
T test (P value)		0.00759	0.017049	0.008278	

Figure (1): peritoneal macrophage migration inhibition test:
1-A: full migration out of capillary tube noticed
1-B: inhibition of macrophage migration noticed, cell s reside inside capillary tube

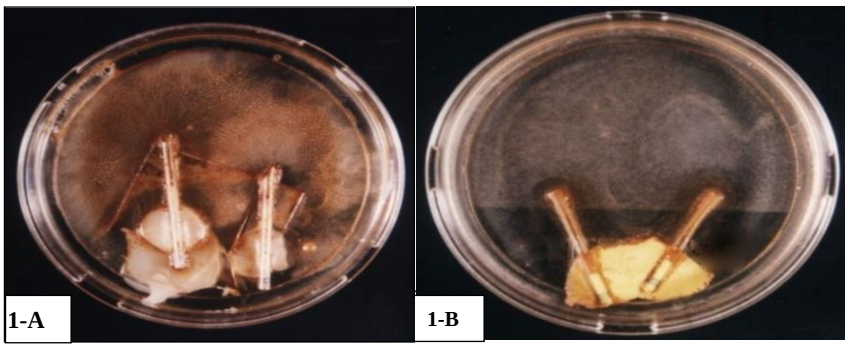
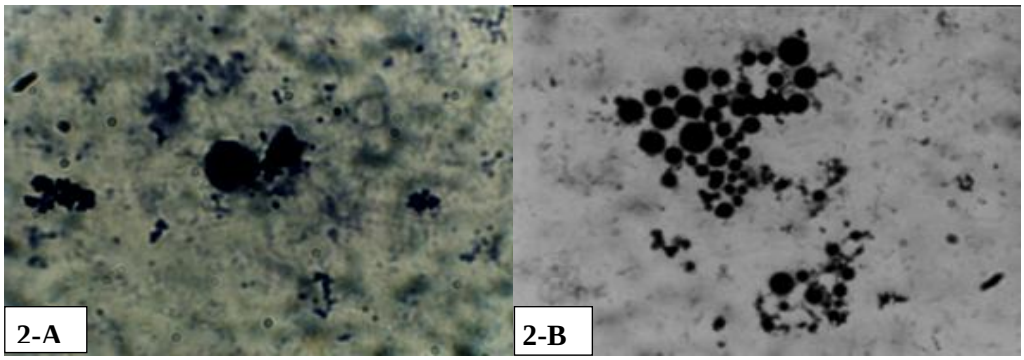


Table 2: Indicators of lymphocytes division

Animal No.	Mitotic Index %			ANOVA P value
	Control group	PHA concentration 0.1 mg/ml	PHA 5mg/ml	
1	1.50	11.88	20.28	(p<0.01)
2	1.80	16.22	36.35	
3	0.70	9.60	75.80	
4	1.30	12.45	14.27	
5	0.60	7.68	60.78	
Mean ± SD	1.18± 0.516	11.56± 3.22	41.49± 26.298	

Figure (2): lymphocyte transformation test:
A: Obvious lymphocytes mitotic activity in response to antigen noticed (200x)
B: lymphocytes mitotic activity in response to antigen after 5 days incubation noticed (400x)



4. Discussion

The capillary tube technique for assaying macrophage migration has been widely used in studies on cellular immunity and has allowed a better understanding of lymphocyte-macrophage interaction associated with the expression of delayed hypersensitivity[11]. The sensitive lymphocyte on exposure to specific antigen which in current study PHA, stimulate the production of soluble factor has called migration inhibitory factor (MIF)[33]. MIF inhibits the migration of the peritoneal macrophage. T lymphocytes, macrophages/monocytes, endothelial cells (ECs), eosinophils, polymorphonuclear neutrophils (PMNs), epithelial cells, smooth muscle cells, synovial fibroblasts, and anterior pituitary cells have been shown to secret MIF and express specific MIF receptors such as the cell surface form of the class II invariant chain (CD47)[34], the chemokine receptors CXCR2 and CXCR4 which are functional receptors for MIF[11,35], these fact suggests that MIF is involved in a wide array of physiological and pathophysiological processes. In current study, the rate of inhibition of macrophage migration in immunized animals with phenol killed *shigella dysenteriae type* 1 inversely associated with dose PHA antigen (4µg/ml, 40µg/ml, 400µg/ml) as well as duration period of experiment that was sufficient for priming various immune cells for response to antigen. Migration inhibition coefficient in term of (mean ± SD) inversely associated with dose of PHA antigen among immunized group. Presensitization with *shigella dysenteriae type 1* lipopolysaccharide (LPS) as well as the proinflammatory molecules produced in response to injection of killed bacterial suspension like TNF-α, IL-5, IFN-γ, transforming growth factor β, have all been shown to stimulate MIF mRNA expression and protein secretion[11,36,37]. The complement-activated product C5a promotes MIF release from PMNs in vitro and during sepsis[38]also upon exposure to pathogen or its antigenic fragments ,Toll-like receptor also expressed for antigen bindings like in case of Toll-like receptor 4 (TLR4) is known to induce the MIF secretion[11]. After recognition of immune complex, macrophages produce MIF and express MIF receptors. In turn, the secreted MIF acts as an autocrine/paracrine enhancer of TNF production[39]. MIF play vital role in leukocytes recruitments via increase expression of adhesion molecules such as intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM)-1, and chemokines, including IL-8 (CXCL8) and CCL2;[40] induces adhesion and migration of monocyte-lineage

cells in post capillary venules, and that function is mediated by the chemokine CCL2 (MCP-1), which is induced in endothelial cells by MIF, itself, increase endogenous MIF production via vascular endothelial cells[41]. The pleiotropic nature of MIF cytokine is illustrated by the numerous mechanisms implicated in its effects, including activation of mitogen-activated protein kinase (MAPK) signalling[18], MIF upregulated of proinflammatory mediators TNF-α, IL-1, IL-6, IL-8, and IL-12 from macrophages[11,33] Counter regulation of endogenous glucocorticoids and inhibition of cellular apoptosis[11]. Experiments utilizing exposure of guinea pig macrophages to MIF-containing supernatants provided evidence for a dose, time, and temperature-dependent adsorption of MIF soluble mediator to the peritoneal macrophage via receptors. The interaction of MIF secreted from different cells including peritoneal macrophages argues for selectivity of lymphocyte modulation of macrophage populations mediated by specific receptor (CD74) and increase the phagocytic activity of macrophages beside production of various cytokines and molecules in response to antigen challenges

Each humoral immune response to an infection requires a specific cellular immune response with clonal proliferation of various antigen-specific lymphocyte subpopulations. Of central importance here are antigen-specific T helper lymphocytes ($CD4^+$ TH cells). In addition to effector T cells, long-lived T and B memory lymphocytes are formed. In the presence of antigen-presenting cells and protein antigens, specific CD4+ T memory cells also proliferate in vitro. The lymphocyte transformation test (LTT), also known as the lymphocyte proliferation or lymphocyte activation test, is based on this principle[42].

In human, Functional deficits in T lymphocytes can be congenital and manifest themselves very early, but also delayed (e.g., between 20 and 35 years of age). However, they also occur quite often as part of existing underlying systemic diseases (tumors, autoimmune diseases, chronic inflammations, etc.). They can in addition result from chemotherapy or radiotherapy as part of cancer treatment or even prolonged pharmacotherapy (immunosuppressants, antibiotics, etc.).These functional deficits are generally not detected in the differential blood count or even in the quantitative immune profile.[43]

In current study, the Lymphocyte transformation test (LTT) used to measure the ability of lymphocytes to proliferate in response to phytohaemagglutinin stimulation i.e. immunocompetence. Phytohaemagglutinin is a plant lectin is carbohydrate-binding proteins that bind to lymphocyte surface receptors and activate cells in an antigen independent manner[44]. Most people will respond to at least one of several common microbial antigens like *shigella dysenteriae type* 1[44]. Lymphocyte proliferation normally occurs early in an immune response. Lymphocyte transformation assay test the integrity of the early proliferative response using either nonspecific mitogens (phytohaemagglutinin) or specific antigens to induce blastogenesis. Antigen induced lymphocyte proliferation also correlates with previous exposure to antigen and acquisition of cellular immunity in current study guinea pigs exposed to phenol killed *shigella dysenteriae type*1cause priming of blastogenesis process when tested with nonspecific mitogen, phytohaemagglutinin stimulation with obvious difference in mitotic index compared with control group. In current study, Lymphocytes Blastogenesis and transformation index in term of (mean±SD) appear to be dose deponent.

In conclusion MMIT and LTT can be used effectively to evaluate and monitoring of cellularimmunological responsiveness to *shigella dysenteriae type*1 antigen and both MMIT and LTT proportionally associated with pre sensitization dose of antigen, duration of exposure to antigen.

References

[1] AL-Ezzy AIA, Abood WN. Immuno-Microbiological Diagnosis of Co-Infections with Rota Virus, Giardia lamblia, Entamoeba histolytica And Salmonella enterica As A Possible Pathological Strategy For Acute Diarrhea Among Iraqi Children. *International Journal of Advanced Research* 2013; 1: 216-24.

[2] Ali AI, Abood WN. Possible correlation between O-antigen serogroup of Enteropathogenic/Enteroinvasive E. coli and co infection with Giardia G. lamblia and Entamoeba histolytica as a possible pathological strategy for acute diarrhea among children of Baghdad governorate. *Diyala Journal for pure science* 2010; 6: 206-26.

[3] Kabayiza JC. Acute gastroenteritis in Rwandan children under five years of age investigated by real-time PCR. *Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy Gothenburg*: University of Gothenburg; Sweden 2014.

[4] Binet R, Deer DM, Uhlfelder SJ. Rapid detection of Shigella and enteroinvasive Escherichia coli in produce enrichments by a conventional multi-

plex PCR assay. *Food microbiology* 2014; 40: 48-54.

[5] Parsot C. Shigella type III secretion effectors: how, where, when, for what purposes? *Curr Opin Microbiol* 2009; 12: 110-6.

[6] Cruz CBNd, Souza MCSd, Serra PT, et al. Virulence Factors Associated with Pediatric Shigellosis in Brazilian Amazon. *BioMed research international* 2014; 2014.

[7] Nothelfer K, Arena ET, Pinaud L, et al. B lymphocytes undergo TLR2-dependent apoptosis upon Shigella infection. *The Journal of experimental medicine* 2014; 211: 1215-29.

[8] Sansonetti PJDS, James P. Debugging how bacteria manipulate the immune response. *Immunity* 2007; 26: 149-61.

[9] Savaskan NEF-R, Gnter; Buchfelder, Michael; Eypoglu, Ilker Y;. Brain miffed by macrophage migration inhibitory factor. *International journal of cell biology* 2012; 2012.

[10] Roger TD, Julie; Chanson, Anne-Laure; Giddey, Marlyse; Le Roy, Didier; Calandra, Thierry. Macrophage migration inhibitory factor deficiency is associated with impaired killing of gram-negative bacteria by macrophages and increased susceptibility to Klebsiella pneumoniae sepsis. *Journal of Infectious Diseases* 2013; 207: 331-9.

[11] Kasama TO, Kumiko; Sato, Michihito; Takahashi, Ryo; Wakabayashi, Kuninobu; Kobayashi, Kazuo,. Macrophage migration inhibitory factor: a multifunctional cytokine in rheumatic diseases. *Arthritis* 2011; 2010.

[12] Larson D, Horak K. Macrophage migration inhibitory factor: controller of systemic inflammation. *Crit Care* 2006; 10: 138.

[13] Chen PL, YL; Wang, W et al. ISO-1, a macrophage migration inhibitory factor antagonist, inhibits airway remodeling in a murine model of chronic asthma". *Mol Med* 2010; 16: 400-8.

[14] Takahashi KK, K; Linge, HM; Zhang, Y; Lin, X; Metz, CN; Al-Abed, Y; Ojamaa, K; Miller, EJ. Macrophage CD74 contributes to MIF-induced pulmonary inflammation. *Respir Res* 2009; 10 33.

[15] Al-Abed Y MC, Cheng KF, et al. Thyroxine is a potential endogenous antagonist of macrophage migration inhibitory factor (MIF) activity". *Proc Natl Acad Sci USA* 2011; 108: 8224-7.

[16] Fan HK, Wenping; Yang, Yuan H; Gu, Ran; Harris, James; Fingerle Rowson, Gnter; Bucala, Richard; Ngo, Devi; Beaulieu, Elaine; Morand, Eric F,. Macrophage Migration Inhibitory Factor inhibits the antiinflammatory effects of glucocorticoids via glucocorticoid induced leucine zipper. *Arthritis & Rheumatology* 2014.

[17] Von Baehr V, Doebis C, Volk H-D, von Baehr R. Suppl 1: The Lymphocyte Transformation Test for Borrelia Detects Active Lyme Borreliosis and Verifies Effective Antibiotic Treatment. *The open neurology journal* 2012; 6: 104.

[18] Kano Y, Hirahara K, Mitsuyama Y, Takahashi R, Shiohara T. Utility of the lymphocyte transformation test in the diagnosis of drug sensitivity: dependence on its timing and the type of drug eruption. *Allergy* 2007; 62: 1439-44.

[19] Texas BCaBSo. Lymphocyte transformation test. Texas, 2003.

[20] Pichler Wa, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy* 2004; 59: 809-20.

[21] von Baehr VM, Wolfgang; Liebenthal, Christa; von Baehr, Rdiger;Bieger, Wilfried;Volk, Hans-Dieter,. Improving the in vitro antigen specific T cell proliferation assay: the use of interferon-alpha to elicit antigen specific stimulation and decrease bystander proliferation. *Journal of immunological methods* 2001; 251: 63-71.

[22] Winn WCK, Elmer W. *Koneman's color atlas and textbook of diagnostic microbiology*. Lippincott Williams & Wilkins, 2006.

[23] Diagnostics Pl. Shigella Slide Agglutination Antisera Retrieved from: http://www.prolab.com/inserts/Shigella.pdf. 2012.

[24] Zafar AS, Nasim; Bhutta, Zulfiqar A,. Frequency of isolation of Shigella serogroups/serotypes and their antimicrobial susceptibility pattern in children from slum areas in Karachi. *Journal - Pakistan Medical Association* 2005; 55: 184.

[25] Rose N, Friedman H. Manual of clinical immunology American Society for Microbiology. *Washington, DC* 1980: 213.

[26] LifeTechnolgies. Fetal Bovine Serum, Qualifed (Canada)Product code:1248302-1248302 Retrieved from http://www.lifetechnologies.com/order/catalog/product/12483020. 2014.

[27] Life Technolgies. GlutaMAX™ media, RPMI-1640, Retrieved from https://www.lifetechnologies.com/content/applications/cell-culture/pdf.par.28095., 2014.

[28] Openheim JaS, B. *Lymphocyte transformation in manual of clinical immunology* Washington, USA 1980.

[29] Verma AP, Kashi N; Singh, Aloukick K; Nyati, Kishan K; Gupta, Rakesh K; Paliwal, Vimal K,.

Evaluation of the MTT lymphocyte proliferation assay for the diagnosis of neurocysticercosis. *Journal of microbiological methods* 2010; 81: 175-8.

[30] Axis-Shield. Density Gradient Medium(Lymphoprep™). 2014.

[31] Ting A, Morris P. A Technique for Lymphocyte Preparation from Stored Heparinized Blood1. *Vox sanguinis* 1971; 20: 561-3.

[32] Cruickshank RD, J.P; Mormion, B.P; Swain, R.H., *Medical Microbiology.Vol.2*. London: hurchil Livingston. 1975.

[33] Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity". *Nat Rev Immunol* 2003; 3: 791-800.

[34] Leng LCNM, C.; N. Metz. MIF signal transduction initiated by binding to CD74. *Journal of Experimental Medicine* 2003; 197: 1467-76.

[35] Bernhagen JKRKR. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nature Medicine* 2007; 13: 587-96.

[36] Daryadel AG, Remo Filippo; Simon, Hans-Uwe; Yousefi, Shida. Apoptotic neutrophils release macrophage migration inhibitory factor upon stimulation with tumor necrosis factor. *Journal of Biological Chemistry* 2006; 281: 27653-61.

[37] Larson DF, Horak K. Macrophage migration inhibitory factor: controller of systemic inflammation. *Critical Care* 2006; 10: 138.

[38] Riedemann NC, Guo R-F, Gao H, et al. Regulatory role of C5a on macrophage migration inhibitory factor release from neutrophils. *The Journal of Immunology* 2004; 173: 1355-9.

[39] Paiva CNA, Rosa H; Magalhes, Elisabeth S; Alves, Letcia S; Lessa, Luiz Paulo; Silva, Maria Helena; Ejzemberg, Regina; Canetti, Cl‹udio; Bozza, Marcelo T,. Migration inhibitory factor (MIF) released by macrophages upon recognition of immune complexes is critical to inflammation in Arthus reaction. *Journal of leukocyte biology* 2009; 85: 855-61.

[40] Gregory JLM, Eric F; McKeown, Sonja J; Ralph, Jennifer A; Hall, Pamela; Yang, Yuan H;McColl, Shaun R;Hickey, Michael J. Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2. *The Journal of Immunology* 2006; 177: 8072-9.

[41] Cheng QM, Sonja J; Santos, Leilani; Santiago, Fernando S; Khachigian, Levon M; Morand, Eric F; Hickey, Michael J,. Macrophage migration inhibitory factor increases leukocyte endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. *The Journal of Immunology* 2010; 185: 1238-47.

[42] von Baehr VD, Cornelia; Volk, Hans-Dieter; von Baehr, Rdiger,. Suppl 1: The Lymphocyte Transformation Test for Borrelia Detects Active Lyme Borreliosis and Verifies Effective Antibiotic Treatment. *The open neurology journal* 2012; 6: 104.

[43] Berlin IfMD. Determination of immunocompetence using the lymphocyte transformation test (LTT Immune Function). In: GbR IfMDB-PM, ed. Berlin-Germany, 2014.

[44] Adachi TT, Hayato; Funakoshi, Takeru; Hirai, Hiroyuki; Hashiguchi, Akihiko; Amagai, Masayuki; Nagao, Keisuke,. Comparison of basophil activation test and lymphocyte transformation test as diagnostic assays for drug hypersensitivity. *Clinical and Translational Allergy* 2014; 4: 1.