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Transfer of *Rhodoccocus equi* Immunity in Guinea Pigs by Mean of Sensitized Spleen Cells

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Abstract: This study was performed to transfer the immunity against *R. equi* by using sensitized spleen cells in guinea pigs. Firstly, two groups of guinea pigs were used, the first injected i.m with formalin killed antigen prepared from *R. equi* mixed with in complete Fruend's adjuvant at two weeks interval. While the second was inoculated with the adjuvant plus Phosphate Buffer Saline (PBS) and kept as control. The immune response, both humoral and cellular were monitored using tube agglutination test, passive haemagglutination test, skin test, E-rosette and migration inhibitory factor in treated groups. Injection of animals induced both types of immune response, the treated and control groups considered as donors for sensitized and non-sensitized spleen cells, respectively. Secondly, three groups were used as recipients, the first one injected i.p with 2 mL of $5X10^8$ cell mL^{-1} sensitized spleen cells, the second group similarly injected with non-sensitized cells and the third one injected with PBS only then kept as control. The immune response was monitored in the recipients which revealed specific *R. equi* antibodies and developed Delayed Type Hypersensitivity (DTH). Statistical analysis was done using one way analysis of variance and F-test which revealed a high significant differences among treated and control groups ($p<0.05$). The results suggest that sensitized spleen cells of guinea pigs has an effect on other recipients and concluded that transfer of sensitized cells were effective in R.equi specific immune response in comparison to the control.

Key words: *Rhodoccocus equi*, guinea pigs, spleen cells, statistical analysis, PBS, DTH

INTRODUCTION

Rhodococcus equi is one of the most important causes of foal's diseases between 1-6 months of age, since 1923 the organism has been identified world wide. Magnusson (1938) found that all isolates from pyaemic foals were agglutinated by the same antiserum. The disease characterized by chronic abscessating pneumonia of foals (Bain, 1963). The most common manifestation of *R.equi* is a chronic suppurative bronchopneumonia with extensive abscessation and associated suppurative Lymphadenitis. Foals with low intake of colostrums or colostrums with low level of immunoglobulins concentration predisposed to *R. equi* infection in early age (Ardans *et al*., 1986).

The local isolate was diagnosed in Iraq due to pneumonic case leading to death of one foal. An epidemiological study in the state explained that 50% of healthy foals showed low titers of antibodies to *R. equi* in their sera (AL-Salihi, 1993). Early recognition of *R. equi* cases prevent the spread of virulent organism, farms with enzootic disease or that used for breeding horses for many years which appeared to be more heavily infected with virulent organism than those where the disease was not present (Prescott *et al*., 1989; Takai *et al*., 1991). Studies of immunity to *R. equi* infection are limited by the availability of appropriate seronegative animals and by the cost of housing sufficient number of experiments and suitable laboratory animals such as guinea pigs, a valuable approach to studies involving this and other equine infectious diseases (Ross *et al*., 1996).

The ability of *R. equi* to resist phagocytosis and intracellular killing by macrophages is the basis of

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its pathogenicity; macrophages may be the best cells for *in vitro* assessment of virulence and pathogenic mechanism of *R. equi* (Hondalus and Mosser, 1994). Cell-mediated Immune response CMI plays an important role in acquired resistance to *R. equi* (Ross *et al*., 1996; Madarane *et al*., 1997). The main object of this study is to examine the effect of sensitized spleen lymphocytes with *R. equi* killed vaccine on the cellular and humoral immune response in comparison to unsensitized by using guinea pigs.

MATERIALS AND METHODS

Experimental animals: Thirty of apparently healthy guinea pigs from the same colony,5-6 months of age and weighing about 400-500 g were brought to the College of Veterinary Medicine, University of Baghdad, AL-America, Baghdad, Iraq and kept at the experimental animals house. They were divided into five groups, six animals each, reared in separated cages and fed concentrated pellets with alfalfa and drank clean water, in addition to healthy furniture, then left for four weeks to adapt prior starting the experiment.

Serological tests Passive Hemaagglutination Test (PHA) and Tube A gglutination Test (TAT) were done to detect any response to *R. equi* prior to vaccination and transfer of cells. In addition to skin test was conducted on these animals to detect CMI, fecal samples were taken several times for bacterial isolation and prove that these animals were free of *R. equi* and not exposed to it before.

Bacterial isolates: *Rodococcus equi*, local isolate recultured twice on 5-7% sheep blood agar media and tested for virulence. Colonies stained red on Congo red staining media (Berkhoff and Vinal, 1986) were described as Congo Red positive (CR+) and were used for vaccine preparation.

Culture media: Different types of culture media were used including:

- Blood agar media: Prepared by adding 5-75 of sheep blood to the base media (Oxoid).
- Trypticase Soy broth (TSB). (Oxoid).
- Trypticase Soy Agar (TSA) (Oxoid).
- Nutrient broth (Fluca).
- Nutrient agar (Fluca).
- Brain heart infusion broth. (BHIB) (Mast).
- Brain heart infusion agar. (BHIA) (Mast).
- Triple Sugar-Iron agar. (TSI) (Oxoid).

Media and solutions were sterilized by autoclaving at 121°C UNDER 15lb/inch2 for 10-15 min. Gram stain and biochemical tests were used to find out the characters of the organism used to prepare the vaccine. The organism was identified according to the biochemical features, characteristics of colonies in blood agar, nature of growth on liquid media gram staining, catalase and oxidase reaction, nitrate test, urease production and fermentation of sugars as the organism was identified according to the biochemical features, characteristics of colonies in blood agar, nature of growth on liquid media gram staining, catalase and oxidase reaction, nitrate test, urease production and fermentation of sugars as described by Nakazawa, (1980).

Preparation of antigens:

Whole cell antigen: This antigen was prepared according to Nakazawa, (1980), *R. equi* was inoculated on nutrient agar at 37°C for 24 h, then harvested and washed using Phosphate Buffer Saline (PBS) pH 7.2 and resuspended in PBS.

The suspension was subjected to 121°C UNDER 15 lb/inch2 for 2.5 h using autoclave, washed twice in PBS. The antigen adjusted to a concentration equivalent to McFarland opacity tube No. 3 (900×106 bacteria mL^{-1} and kept at 4°C until used).

Soluble antigen: It was prepared according to Prescott *et al.* (1979) the procedure summarized, *R. equi* inoculated on nutrient agar for 24 to 48 h at 37°C, culture harvested by suspending the colonies from the surface of medium using PBS. The growth washed three times with PBS centrifuged at 10000 rotation per minute (rpm) for 30 min, sediment resuspended and sonicated for 30 min interval in water-cooled sonicater oscillator 15-20KHZ sec^{-1} (MSE-150-watt-ultrasonic distingrator GE.MK-2). The homogenate suspension centrifuged twice at 15000 rpm for 15 min to remove cellular debris, the resulted supernatant was filtered through Millipore filter size 0.45 μm2. The supernatant distributed in sterile tubes and kept at -20°C until used. This soluble antigen is used for Passive Hemagglutination test (PHA), skin test or Delayed Type Hypersensitivity reaction (DTH) and Macrophages migration Inhibition Factor (MIF). Protein concentration of antigen was determined in the filtered supernatant using the of Lowry *et al.* (1951).

Vaccine preparation: Animals sensitized by using formalin killed vaccine, according to Prescott *et al.* (1979). *R. equi* inoculated on BHIA, incubated for 58 h at 37°C, the organism recultured in 20 mL bottle size containing 5 mL of BHIB and incubated at 37°C for 18-24 h, Gram stain was prepared to examine presence of contaminants. Bacterial growth was harvested in PBS with 0.6% formalin (Formal PBS) washed with centrifugation of the organism, the bacteria resuspended in formol PBS 0.6% and incubated at 37°C to kill the organism till the next day. The organism washed twice using centrifugation, resuspended in PBS to prepare the vaccine and adjusted to concentration of (10)9 × (10)10 bacteria mL^{-1} using spectrophotometer. Drops of resulted suspension furnitured on blood agar medium and incubated aerobically and anaerobically to examine the sterility of it. Potency of the vaccine also was tested after the mixture of the suspension with incomplete Fruend's adjuvant with equal volume in apparently healthy guinea pigs intramuscularly (i.m) with 2 mL of the vaccine and tested for humoral and cellular immune response. Also safety test was done to the vaccine using two apparent healthy guinea pigs i.m with double dose then observed for any clinical signs and pathological changes for one week.

Lymphocytes preparation

Sensitization of animals: Animals were vaccinated with the prepared vaccine to obtain sensitized lymphocytes (Donors) i.m twice at two weeks intervals; the other group of guinea pigs was injected i.m with PBS plus adjuvant as control and to obtain normal cells, sensitization was confirmed by skin test and MIF as *in vivo* and *in vitro* test.

Preparation of lymphocytes: Spleen from sensitized and non-sensitized(control) obtained, cut into pieces and grinded in RPMI-1640 media supplied with 10% Fetal Calf Serum (FCS) twice and cells were made free from red blood cells using 0.83% ammonium chloride, then washed in PBS until complete removal of platelets. The viability of cells was determined by trypan blue dye exclusion test using 0.1% trypan blue (Hudson and Hay, 1980). Cells were resuspended in PBS (pH 7.2) at the concentration of 5×10^{8} cell/mL. Spleens from control animals were treated as in previous to normal cells.

Sensitized leucocytes activity assay: To evaluate the presence of *R. equi* specific immunological of the sensitized cells, two group of animals, *R. equi* free, group-3 (Recipients) were inoculated with 2 mL of sensitized cells intraperitoneally (i.p), group-4 received normal cells i.p as control.Gtoup -5 contained six animals injected i.p with PBS only as control. Treated animals were inoculated intradermally (id) with 0.1 mL of various concentrations of soluble antigen at 33.75, 3.375 and 0.337 $\mu g\ mL^{-1}$ in separated sites in clean shaved left flank of each animals and injected 0.1 mL of PBS as control. Skin test was recorded 24, 48 and 72 h after i.d injection of soluble antigen. All animals were punctured from the heart to collect blood for antiserum weekly, serum was kept in -20°C until used. Animals were divided in five groups as follows:

Group-1 (Vaccinated guinea pigs) Six animals were vaccinated twice at the first week with 2.5 mL of formalin killed vaccine of local isolate containing equivalent to 10^{9}-10^{10} bacteria mL^{-1} with adjuvant i.m and boosted with 1.5 mL of the same dose at third week (Al Azzawi, 1995). Group-2, six animals were injected PBS with adjuvant i.m to have normal cells (non-sensitized). Group-3 (Recipients) six animals were injected i.p with 2 mL containing 5×10^{8} cell mL^{-1} sensitized cells i.p. Group-4, six animals received the same dose of normal cells obtained from group-2 and used as control of recipients and Group-5, six animals were injected only with PBS i.p as control.

Clinical manifestations: The treated animals have been examined during the first week daily and for any appearance of clinical signs including body temperature, pulse and respiration.

Collection of samples: Blood samples were collected from the five groups at zero time and weekly during the period of the experiment till the depression of immune response. Sera were were separated and kept at -20°C until used.

Evaluation of vaccine efficiency: Guinea pigs treated previously were subjected to serological tests to find out the immune responses.

Humoral immune response was detected using two methods:

Tube Agglutination Test (TAT): This test was done according to (Nakazawa, 1980), briefly serial two fold s mixed dilutions (0.5 mL) were mixed with an equal volume of autoclaved whole cell antigen, shaked and kept at 37°C for 18-24 h.

Antibodies titers were expressed as the reciprocal of highest dilution showing a clear degree of agglutination.

Passive Hemagglutination test (PHA): This method was used as described by (Prescott *et al*., 1979). Then fold diluted sera and equal volume of treated chicken RBCs 50 μL of each, dilutions beginning from 1:10 ending at 1:10240 into microtiter plates 8 rows of 12 wells, 50 μL of equal volume titrated and read after 2 h under room temperature, then left at refrigerator overnight at 4-8°C and read again to compare the results

Cellular immune response: Was detected using three methods include:

Delayed Type Hypersensitivity reaction (DTH): Area of the left flank of each guinea pigs was clipped and shaved carefully, the cleaned area was divided into four parts, inoculated i.d with 0.1 mL of soluble a ntigen at the concentrations of 33.75, 3.375 and 0.337 μ mL^{-1} and the fourth part was injected with 0.1 mL of PBS Ph 7.2 two weeks after the boosting dose. Also DTH reaction was done to the recipients after i.p injection of sensitized cell after the boostering dose. The diameter of skin reaction was m easured at 24, 48 and 72 h according to Ellenberger *et al*. (1984).

Erythrocytes rosettes formation (*E. rosette*): This test was done using peripheral blood leucocytes of guinea pigs at first, third and eighth weeks for all animals during the study. Sheep erythrocytes were used with cells according to Braganza *et al*. (1975) the method.

Macrophages Migration Inhibition Factor (MIF): This test was done according to the method described by (Weir, 1973), which was modified by Mayo *et al*. (1977). Animals were injected i.p with 15-20 mL of sterile paraffin oil to stimulate peritoneal reaction.

Resulted macrophages were resuspended twice in sterile PBS and they were packed into capillary tubes, placed in duplicates in chambers plates, cultured with various concentrations of bacterial antigens (33.75, 3.375 and 0.337 μg mL^{-1}), on the other hand, cells were packed in high- molecular weight antigens (Mitogen) (Phytohemagglutinin PHA) and Concovalin-A (Con-A) in addition to others left without antigens as control in the same animals as described by Chess *et al*. (1974).

The Migration Index (MI) was calculated:

$$MI = \frac{\text{Mean area of migration in presence of antigen}}{\text{Mean area of migration in absence of antigen}}$$

Animals showing MI less than 0.80 were considered responsive and more than 0.80 as non-responded.

Statistical analysis was conducted according to principles of statistics (Al-Mohmmed *et al*., 1986). Two way analysis of variance and t-test were done to analyze the data obtained from vaccinated (donors) and non-vaccinated (recipients) groups, also between groups and weeks.

RESULTS

Experimental guinea pigs: Clinical observations post vaccination: Vaccinated guinea pigs became slightly depressed and restless manner after 48 and 72 h of vaccination. Temperature was elevated to (39.4±0.3°C), pulse rate (170±4 beat min^{-1}) and respiration rate (90±4.2 breath min^{-1}) were recorded for the first week and returned to normal in all inoculated after six days post vaccine administration. Local swelling at the sites of injection was detected after 48-72 h and disappeared after 10 days. Means of temperature, pulse and respiration rates were not changed from the normal ranges in the control animals.

Humoral immune response: Both groups of vaccinated and control animals had no antibody titer to *R. equi* before vaccination, specific antibodies were detected at second week post first vaccination ranged from 10-20 in TAT and 0-20 in PHA, titer reached the peak level three weeks later after the boostering dose, then declined later. Control animals did not show any titers to *R. equi* (Fig. 1 and 2).

Cellular immune response:

Delayed type hypersensitivity reaction: Animals did not react to *R. equi* prior vaccination. They reacted later and positive skin test at 24, 48 and 72 h after post intradermal inoculation with various concentrations of *R. equi* antigen. Vaccinated animals responded and the site of injection appeared erythematous and indurated in shape which was clear during the first 48 h, while control animals did not react to the injectable antigens (Table 1).

Table 1: Skin reaction of vaccinated and controls guinea pigs post i.d inoculation with various concentrations of *R. equi* antigen

	Mean diameter in (mm) vaccinated group (n = 6) donors			Control group (n = 6 animals)		
Antigen Conc. μ mL^{-1}	24 h	48 h	72 h	24 h	48 h	72 h
33.75	10.8 *(7-15)	14.8 *(9-20)	10.5 *(5-15)	0	0	0
3.37	5.8 *(3-9)	8.2 *(5-12)	3.2 *(1-5)	0	0	0
0.33	3.5 *(1-5)	5 *(2-7)	2.7 *(1-4)	0	0	0
PBS	0	0	0	0	0	0

Table 2: Migration of peritoneal macrophages received from immunized (Donors) guinea pigs (Group1)

			Presence of antigen			Presence of PHA and Con-A				
						PHA			Con-A	
Animal No.	Migration measurements	Absence of antigen	33.75 μg mL^{-1}	3.37 1/10	0..33 1/100	10 μg μL^{-1}	0.1 μg μL^{-1}	0.05 μg μL^{-1}	1 μg $L\mu^{-1}$	10 μg μL^{-1}
1	Area of Migration	18.64	1.54	1.39	1.92	C.I	3.93	4.76	C.I	C.I
	Mig. %		8%	7%	10%	0	21%	25%	0	0
	Mig.Index		0.083	0.075	0.103	0	0.211	0.255	0	0
2	Area of Migration	19.24	3.56	4.12	5.61	C.I	0.211	0.255	C.I	C.I
	Mig. %		18.5%	21%	29%	0	29.7%	33.4%	0	0
	Mig.Index		1.85	0.214	0.352	0	0.297	0.334	0	0
3	Area of Migration	17.50	4.05	3.11	6.16	C.I	5.64	4.04	C.I	C.I
	Mig. %		23%	17.8%	35%	0	32%	23%	0	0
	Mig.Index		0.231	0.177	0.352	0	0.322	0.231	0	0
4	Area of Migration	14.06	4.25	3.60	4.90	C.I	3.41	4.86	C.I	C.I
	Mig.%		30%	25.6%	34.8%	0	24%	34.6%	0	0
	Mig.Index		0.302	0.260	0.349	0	0.243	0.346	0	0

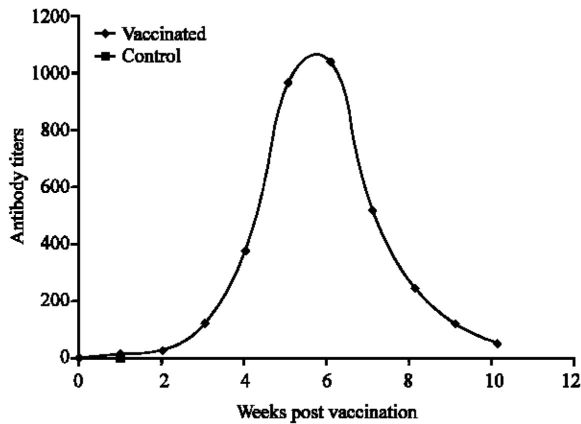

Fig. 1: Antibody titers in the sera of gulinea pigs following vaccination using TAT

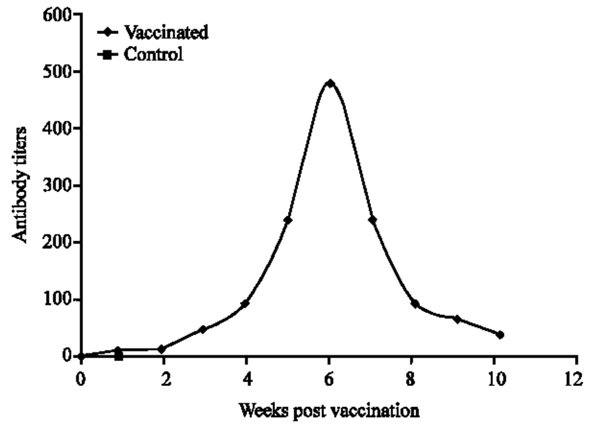

Fig. 2: Antibody titers in the sera of vaainated guinea pigs using PHA

Erythrocytes rosettes (*E. rosettes*): Sensitized blood leucocytes with an equal volume of sheep erythrocytes resulted in E-rosettes formation, in the first week did not have a high percentage of formation which reached 0.70±1.33 then increased in the third week to reach 0.78±2.86. The total leucocytes rates in the same period ranged from 0.7±1.3 and 0.8±1.2 later, the *E. rosettes* percentages elevated after five weeks and were found 0.83±9.15 and 0.73±1.7, respectively, in addition to the total cells 0.83±1.17 and 0.73±1.27.

Control group did not show any increase in E-rosettes percentages, total cells recorded from 0.72±1.28 and 0.67±1.33 in the same first and third weeks post inoculation, after five and eight weeks recorded 0.5±1.5 and 0.53±1.47, respectively.

Macrophage Migration Inhibitory Factor (MIF): Peritoneal macrophages of sensitized and normal animals were collected and subjected to various concentrations (33.75, 3.375 and 0.337 μg mL^{-1}). Table 2 and 3 show different degree of inhibition for immunized

Table 3: Migration of peritoneal macrophages obtained from control guinea pigs (Group2)

Animal No.	Migration measurements	Absence of antigen	Presence of antigen 33.75 μg mL⁻¹	3.37 1/10	0..33 1/100	Presence of PHA and Con-A: PHA 10 μg μL⁻¹	0.1 μg μL⁻¹	0.05 μg μL⁻¹	Con-A 1 μg Lμ⁻¹	10μg μL⁻¹
1	Area of Migration	20.49	18.71	19.1	18.9	C.I*	9.05	18.54	C.I	C.I
	Mig. %		91%	93%	92%	0	44%	90.5%	0	0
	Mig.Index		0.913	0.933	0.922	0	0.442	0.905	0	0
2	Area of Migration	18.05	15.12	17.0	16.6	C.I	8.05	14.51	C.I	C.I
	Mig. %		83.8%	94%	92.5%	0	44.6%	80%	0	0
	Mig.Index		0.838	0.943	0.925	0	0.446	0.804	0	0
3	Area of Migration	22.13	18.23	20.0	16.29	C.I	9.50	14.60	C.I	C.I
	Mig. %		82%	90.5%	73.6%	0	42.9%	65.9%	0	0
	Mig.Index		0.823	0.905	0.736	0	0.429	0.659	0	0
4	Area of Migration	16.30	14.69	12.91	15.10	C.I	7.98	13.28	C.I	C.I
	Mig. %		90%	79%	92.6%	0	48.9%	81.5%	0	0
	Mig.Index		0.901	0.792	0.926	0	0.489	0.815	0	0

Table 4: Skin reaction of recipients and control guinea pigs post i.d inoculation with various concentration of *R. equi* antigen

Antigen conc μg mL⁻¹	Mean diameter in (mm) Recipient group (3) N = 6: 24 h	48 h	72 h	Control group (4) N = 6: 24 h	48 h	72 h	Control group (5) N = 6: 24 h	48 h	72 h
33.75	8.3 (4-13)	10.4 (6-15)	5.7 (3-8)	0	0	0	0	0	0
3.37	5.1 (2-8)	7.8 (4-12)	3.8 (2-7)	0	0	0	0	0	0
0.33	4.4 (2-7)	6.3 (4-9)	2.9 (1-5)	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0	0

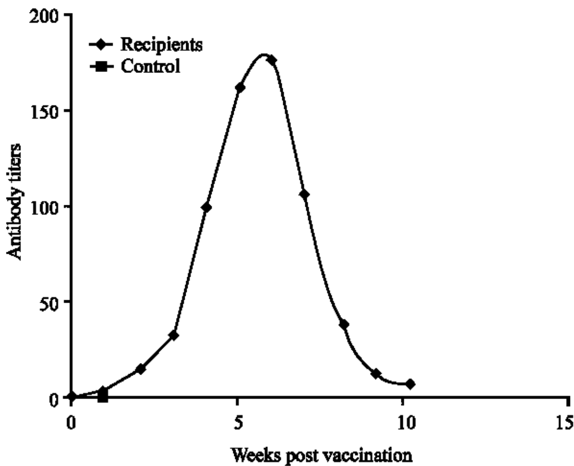

Fig. 3: Antiboday titers of recipients and control guinea pigs using TAT

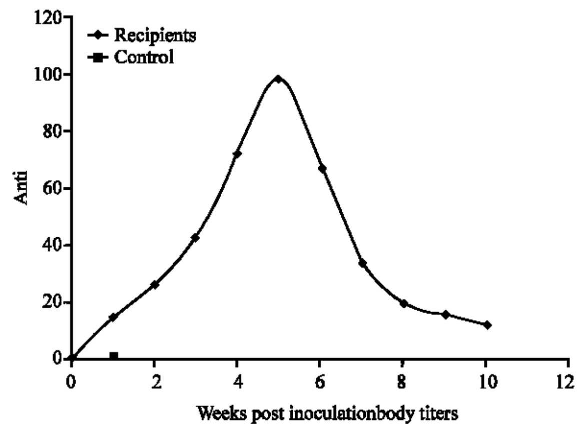

Fig. 4: Antiboday titers of recipients and control using PHA test

and non-immunized animals, lesser area of migration in the presence of antigen reached 1.39 mm² with migration percentage of 7% at concentration (3.375 μg mL⁻¹) compared to areas without antigen for the same animals which was found to reach 18.64 mm², in comparison to other antigen concentrations. Control group (Table 3) show large area of migration in presence of same concentration (3.375 μg mL⁻¹) by the mean of 17.27±2.74 and percentage 8 9.13±5.99 nearly to migration without the antigen which was found 19.24±2.24 in comparison with previous migration area. Phytohemagglutinin (PHA) and Conconavalin (Con-A) 10 μg mL⁻¹ to first with 1, 10 μg mL⁻¹ to second resulted in complete inhibition to the immunized animals. Cells subjected to concentrations 0.05 and 0.1 μg mL⁻¹ of PHA had lower migration areas. The peritoneal cells of control treated as before with PHA and Con-A resulted in complete inhibition.

Recipient guinea pigs: Clinical observations post i.p administration of sensitized cells Clinical findings after inoculation included depression and restless especially at first 72 h. Temperature was calculated and found of (39.8±0.2°C), pulse rate (174±5 beat min⁻¹) and respiration

(91±4 breath min^{-1}) were recorded during the first week and returned to normal in recipients by the end of the first week. The means of body temperature, pulse and respiration rates remained within the normal ranges in the control group which received normal cells.

Humoral immune response: Both recipients and control animals had no antibody titer to *R. equi* prior to sensitized cells administration. Later, antibodies detected four weeks post i.p injection of cells, reached valuable titers ranged f rom (10-320) (10-160) in TAT and PHA tests respectively, then declined by the seventh week (Fig. 3 and 4) and control animals did not show any rise in antibodies titers.

Cellular immune response: Delayed Type Hypersensitivity reaction (DTH): Animals received cells i.p had positive skin test reactivity at 24, 48 and 72 h post inoculation with various concentrations of *R. equi* soluble antigen. Area with erythema appeared more clearly during first 48 h, while control group did not react to the antigen in comparison to recipients (Table 4).

Erythrocytes-rosettes formation: The capacities of T-lymphocytes to form rosettes with sheep red blood cells were detected after incubation of recipients T-cells, the results recorded were 0.70±1.6 and 0.73±1.8, respectively for the first and second weeks post i.p injection, control animals did not form rosettes compared with recipients which reached 0.66±6.9 and 0.67±5.9 in the same previous period. The total active cells were found 0.70±1.30 and 0.73±1.27 in addition to control 0.66±1.34 and 0.67±1.33 in respective reading. The frequencies of rosettes increased five and eight weeks after i.p inoculation reach 0.85±1.0, 0.83±1.4 with total active cells of 0.85±1.15, 0.83±1.17 compared with control of 0.70±6.4 and 0.61±6.0 rosettes formation.

Macrophages inhibition migration factor: for macrophages received from recipients (Table 5), migration area of 1.98 mm^3 with percentage of 13% at the concentration (3.375 µg mL^{-1}) compared to chamber without antigen which reached 15.22 mm^3 and low migration indexes. In control group normal cells had a large area of migration at concentration (3.375 µg mL^{-1}) of mean area 16.70±3.94 and percentage 87.4±3.29 as mean. (Table 6 and 7). Peritoneal cells incubated in presence of PHA and Con-A 10 µg mL^{-1} to the first and 1, 10 µg mL^{-1} to the second appeared in complete inhibition, other cells incubated in low concentrations 0.05 and 0.1 µg mL^{-1} of PHA has alow level of migration indexes. Cells from control animals subjected to both PHA and Con-A were completely inhibited, also group-5 used as control injected only PBS i.m never deviated and showed no any raise in immune response (Table 6). The one way analysis of variance and F-test values for vaccinated and non-vaccinated groups compared with control groups revealed a highly significant ($p<0.05$) differences between groups (vaccinated and non-vaccinated) and between weeks within each group.

Table 5: Migration of peritoneal macrophages of recipients (Group -3)

			Presence of antigen			Presence of PHA and Con-A				
						PHA			Con-A	
Animal No.	Migration measurements	Absence of antigen	33.75 µg mL^{-1}	3.37 1/10	0..33 1/100	10 µg μL^{-1}	0.1 µg μL^{-1}	0.05 µg μL^{-1}	1 µg $L\mu^{-1}$	10 µg μL^{-1}
1	Area of Migration	14.87	1.85	2.02	2.26	C.I*	3.86	4.83	C.I	C.I
	Mig. %		12%	13.6%	17%	0	25.9%	32.5%	0	0
	Mig.Index		0.124	0.136	0.172	0	0.259	0.325	0	0
2	Area of Migration	15.22	1.60	1.98	2.17	C.I	4.10	5.05	C.I	C.I
	Mig. %		10.5%	13%	14%	0	26.9%	33%	0	0
	Mig.Index		0.105	0.130	0.143	0	0.269	0.332	0	0
3	Area of Migration	14.71	2.07	3.65	4.41	C.I	2.12	4.57	C.I	C.I
	Mig. %		14%	24.8%	29.9%	0	28%	31%	0	0
	Mig.Index		0.141	0.248	0.299	0	0.280	0.311	0	0
4	Area of Migration	16.19	2.84	2.90	4.59	C.I	3.82	4.40	C.I	C.I
	Mig. %		17.5%	17.9%	28%	0	23.6%	27%	0	0
	Mig.Index		0.175	0.179	0.284	0	0.236	0.272	0	0
5	Area of Migration	13.89	3.13	3.51	C.I	C.I	4.17	5.09	C.I	
	Mig. %		22.5%	25%	0	0	30%	36.6%	0	
	Mig.Index		0.225	0.253	0	0	0.300	0.366	0	

Table 6: Migration area and indexes of peritoneal macrophages derived from control guinea pigs (Group-4)

Animal No.	Migration measurements	Absence of antigen	Presence of antigen 33.75 μg mL^{-1}	3.37 1/10	0..33 1/100	Presence of PHA and Con-A: PHA 10 μg μL^{-1}	PHA 0.1 μg μL^{-1}	PHA 0.05 μg μL^{-1}	Con-A 1 μg Lμ^{-1}	Con-A 10 μg μL^{-1}
1	Area of Migration	21.34	18.64	19.45	20.80	C.I*	9.47	17.21	C.I	C.I
	Mig. %		87%	91%	97.5%	0	44%	80.6%	0	0
	Mig.Index		0.873	0.911	0.975	0	0.444	0.806	0	0
2	Area of Migration	18.05	15.42	17.03	16.27	C.I	11.01	14.98	C.I	C.I
	Mig. %		85%	94%	90%	0	61%	83%	0	0
	Mig.Index		0.854	0.943	0.901	0	0.609	0.829	0	0
3	Area of Migration	22.15	19.90	20.02	4.41	C.I	8.48	16.15	C.I	C.I
	Mig. %		89.8%	90%	949%	0	38%	73%	0	0
	Mig.Index		0.898	0.904	0.949	0	0.383	0.729	0	0
4	Area of Migration	19.79	17.86	18.04	18.87	C.I	10.13	15.23	C.I	C.I
	Mig. %		90%	91%	95%	0	51%	77%	0	0
	Mig.Index		0.902	0.912	0.953	0	0.512	0.769	0	0
5	Area of Migration	12.90	8.02	9.13	9.87	C.I	4.20	7.70	C.I	C.I
	Mig. %		62%	70.8%	76.5%	0	32.6%	59.7%	0	0
	Mig.Index		0.622	0708	0.765	0	0.326	0.597	0	0

Table 7: Migration areas and indexes of peritoneal macrophages derived from control guinea pigs (PBS)

Animal No.	Migration measurements	Absence of antigen	Presence of antigen 33.75 μg mL^{-1}	3.37 1/10	0..33 1/100	Presence of PHA and Con-A: PHA 10 μg μL^{-1}	PHA 0.1 μg μL^{-1}	PHA 0.05 μg μL^{-1}	Con-A 1 μg Lμ^{-1}	Con-A 10 μg μL^{-1}
1	Area of Migration	18.205	17.032	18.021	15.863	C.I*	16.038	8.453	C.I	C.I
	Mig. %		93.5%	98.9%	87%	0	88%	46%	0	0
	Mig.Index		0.935	0.989	0.871	0	0.881	0.464	0	0
2	Area of Migration	21.068	18.951	20.090	20.861	C.I	19.740	11.311	C.I	C.I
	Mig. %		89.9%	95%	98.9%	0	93.7%	53.7%	0	0
	Mig.Index		0.899	0.953	0.989	0	0.937	0.537	0	0
3	Area of Migration	19.691	17.064	18.125	18.980	C.I	17.304	10.051	C.I	C.I
	Mig. %		86.6%	92%	96%	0	87.8%	51%	0	0
	Mig.Index		0.866	0.920	0.963	0	0.878	0.515	0	0
4	Area of Migration	22530	20.219	21.219	21.863	C.I	20.541	11308	C.I	C.I
	Mig. %		89.7%	94.7%	97%	0	91%	50%	0	0
	Mig.Index		0.897	0.947	0.970	0	0.912	0.502	0	0
5	Area of Migration	20.123	18.630	19.042	18.901	C.I	18.982	9.905	C.I	C.I
	Mig. %		92.6%	94.6%	93.9%	0	94%	49%	0	0
	Mig.Index		0.926	0946	0.939	0	0.943	0.492	0	0

DISCUSSION

Since *R. equi* is thought to be an intracellular bacterium, cellular immunity plays major role in *R. equi* infection than antibody mediated (Carter and Hylton, 1974). Later research done by Prescott *et al.* (1979) demonstrated the presence of cell-mediated immunity to *R. equi* infection from transferring of peripheral blood lymphocytes in foals. On the other hand, antibodies to the organism may contribute to immunity in horses by blocking the initial stages of cellular infection, altering the route by which bacteria enter the macrophages and decreasing the bacterium?s ability to inhibit phagosome-lysosome fusion (Hines *et al.*, 1997). Intracellular inoculation of the guinea pigs with the prepared vaccine did not induce adverse systemic reactions with the exceptions of transient elevation of body temperature, accompanied by increased pulse and respiration rates, which may be due to the expression of immunological and inflammatory reactions in which these results were in compatible with (Prescott *et al.*, 1979; Al-Azzawi, 1995).

The specific *R. equi* antibodies were detected post the first dose and elevated to (160-1280) in the vaccinated guinea pigs and (40-160) in the recipients after the boostering dose and inoculation of sensitized cells which I n agreement with those of previous studies (Al-Azzawi, 1995; Al-Graibawi, 1998), they reported that parentral immunization of guinea pigs with killed and attenuated *R. equi* resulted in high titers of *R. equi* antibodies. The duration of effect of treatment with Transfer Factor (TF) has been documented in every case. In a study using TF as preventive measure, for

Varicella-Herpes virus infection (Steele *et al*., 1980), the TF treated patients had active CMI for 17 months, as detected *in vitro* and *in vivo* by skin test. The ability of TF prepared from spleen of sensitized guinea pigs to induce DTH in recipients non-sensitized animals and protect then against experimental challenge with virulent *R. equi* indicated the role of TF in increasing cellular immunity in *R. equi* infection (Al-Graibawi, 1998), since CMI plays an important role in the control of R.equi infection. The results of this study indicate that sensitized spleen cells can be used in transfer of immune response to *R. equi* in guinea pigs and this will be accordance with findings of (Wilks *et al*., 1982; Madarane *et al*., 1997). Control animals did not show any rise in antibodies to be considered.

Several *in vivo* and *in vitro* tests used to demonstrate cell-mediated immune response such as DTH (Wilson, 1955; Ellenberger *et al*., 1984) lymphocyte blastogenic assay and transfer factor assay (Al-Graibawi, 1998), MIF, E-rosette and reduction of NBT (Braganza *et al*., 1975; Felsburg *et al*., 1977; Al-Azzawi, 1995). Detection of CMI to *R. equi* for both vaccinated and recipient guinea pigs was the important result. Delayed-type hypersensitivity reaction developed after intradermal inoculation with *R. equi* soluble antigen and appeared in wide areas of skin reaction after 24, 48 and 72 h of inoculation. The positive skin test in this study was in similar manner with those reported by (Wilson, 1995; Ellenberger *et al*., 1984; Al-Salihi, 1993; Al-Azzawi, 1995; Al-Graibawi, 1998). The efficacy of intraperitoneal administration with sensitized leucocytes was in accordance with results of Al-Azzawi, (1995) while A-Graibawi (1998) used TF to transfer the immune response.

Moreover, the use of highly T-lymphocytes to form spontaneous E-rosette characterized by rapid (5 min) formation with sheep erythrocytes (SRBCs) was measured in treated guinea pigs after they were skin tested with *R. equi* soluble antigen (Felsburg *et al*., 1977). In addition to rapid formation, no similar consistent changes in populations of total T-cells, characterized by longer (60 min) rosette formation with sheep erythrocytes. In this study, the results obtained from immunized and recipient guinea pigs demonstrated the sensitization to the microbial antigen as evidenced by arise in the E-rosette formation which correlates with the report of (Braganza *et al*., 1975). Variations between groups treated appeared in aclear E-rosette formation and especially to animals injected i.m with the vaccine and showed high significant percentage of rapid and total cells similar to results reported by (Fundenberg *et al*., 1975; Al-Azzawi, 1995). These results suggest the increase in circulating T-cells may represent immunologically active T-cells.

The *in vitro* macrophages migration inhibitory factor was used to detect the development of delayed type hypersensitivity in guinea pigs immunized with the prepared vaccine and other received sensitized spleen cells. The activity of the MIF was demonstrated by it's ability to inhibit the migration of peritoneal exudates cells from capillary tubes by use of *R. equi* antigen and mitogens (PHA and Con-A) in which indicated the ability of them to inhibit the migration of peritoneal macrophages (Weir, 1973; Mayo*et al*., 1977). Experimental data presented here showed that the prepared *R. equi* antigen could elicit the production of specific migration inhibition of peritoneal cells derived previous. However when derived cells were initially tested, there was greater percentage of migration for the immune cells rather than normal cells (Mayo *et al*., 1977; Al-Azzawi, 1995). These observed results are in agreement with the finding of (Smith and Bigley, 1972).

Recent studies of immune response to *R. equi* infection in mice given spleen cells showed a significant CMI compared with mice given normal cells (Madarame *et al*., 1997). A final point is that of the important role of guinea pigs lymphocytes for the resistance to *R. equi* infection with respect to the mitogens and the essential effect of macrophages in the induction of inflammation and regulation of immune response to Rhodococcal infections which studied by Giguere and Prescott, (1998), using BALB-mouse, there was detected cytokines observed following sensitization with virulent and a virulent *R. equi*. There was great percentage of migration inhibition on the immune cells rather than normal cells. The results of MIF test to sensitized and recipient guinea pigs were near to each, both groups showed insignificant Migration Index (MI) which was less than 0.80 in comparison to normal cells derived from control groups which had MI more than 0.80 and known as unresponsive cells at the same concentrations in the same direction of (Kumar *et al*., 1974). These observed differences between MI in the case of presence and absence of antigens indicate the important of its role and the efficacy to induce inhibition for the subject cells.

The documentation that inherited diseases of young foals characterized by lymphopenia, lack of (Ig) synthesis, absence of cell-mediated immunity, thymic hypoplasia and marked reduction in the number of splenic cells and lymph node lymphocytes, resulted in highly susceptible foals to secondary infections, which cause death by three months of age just like inherited combined immunodeficiency in Arabian foals which led to many studies of immune response in horses (Radostits *et al*., 1994).

The results presented in this study demonstrate that transferred sensitized spleen cells can induce CMI which play an important role in development of protection against *R. equi* infection.

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