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Cellular immune responses of pregnant Guinea pigs Immunized with live attenuated *Rhodococcus equi*

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Abstract

Rhodococcus equi (R. equi) remains significant causes of severe pneumonia in neonatal foals. And considers as an opportunistic pathogen for compromised cellular immunity people. The potential to increase passive transfer of specific *R. equi* cellular immunity to newborn by preparturient vaccination of their dams was evaluated in Pregnant Guinea pigs as a pilot study. Attenuated autogenous vaccine was prepared from a Congo red negative (CR-) *R. equi* local isolate mixed with adjuvant (potassium alum sulphate), tested for sterility, safety and potency before vaccination. Two groups of pregnant G. pigs were used. The first group was vaccinated twice subcutaneously (S.C) with the prepared vaccine at five and three weeks prior expected parturition. Similarly, the second group was inoculated with adjuvant plus phosphate buffer saline (PBS) twice s.c and kept as control. Cellular immune response in vaccinated animals was detected by skin test which measured at 24, 48 and 72 hours post intradermal (i.d) inoculation of *R. equi* soluble antigen. Offspring from both vaccinated and control dams were assessed for cellular immune responses specific to *R. equi* by in vivo delayed-type hypersensitivity (DTH) test and in vitro extraction of specific *R. equi* transfer factor (TF) from their spleen. Delayed-type hypersensitivity responses to *R.equi* were detected only for offspring of vaccinated dams, specific *R. equi* TF was extracted from offspring of vaccinated dams but not from offspring of nonvaccinated dams. The results revealed that vaccination of pregnant G. pigs with the prepared attenuated vaccine was safe and efficient method to stimulate cell mediated immunity which transferred to their offspring and participated in the protection against experimental challenge.

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Introduction

Rhodococcus equi previously named *Corynebacterium equi* is facultative, intracellular, Gram-positive pleomorphic bacterium that survives and replicates within

macrophages causing granulomatous inflammation (Hondalus and Mosser, 1994).It is initially described as a veterinary pathogen that caused a severe respiratory disease in young foals between 1 and 5months of age (Al-Salihi *et al*., 2013; Vázquez-Boland *et al.,* 2013; Horowitz *et al*., 2001; Bain, 1963). Because of the facultative intracellular nature of *R. equi*, cell-mediated immune plays an important role in acquired resistance to infection. A large part of the knowledge of cell-mediated immunity to *R. equi* infections comes from observations in a mouse model (Kanaly *et al.,* 1996). The TF was known to be able to increase CMI and induce protection against microbial pathogens.In addition, its activity was evaluated against numerous viral, bacterial, protozoal and parasitic diseases (Kirkpatrick, 1996). Effective immunization against intracellular pathogens such as mycobacteria requires the use of live organism rather than killed one to promote immunity. The unclear reasons might be related to the requirement for antigen persistence, the activation of different pathways within phagocytic cells or least likely, the secretion of specific protective antigens by live organisms (Collins, 1988). As the Congo red reaction provides a simple and efficient means of screening virulence, ability to bind Congo red appeared to be encoded by virulence associated plasmid (Berkhoff and Vinal, 1986) and loss of virulence which accompanied loss of pigmentation appeared to be related to deletion of DNA from plasmid (Maurellie *et al*., 1984). Takai *et al* (1991) have demonstrated the association of large plasmids and 15 to 17 kDa antigens with the virulence of *R. equi* in mice and foals, while mutants cured of the large plasmids, lacked the antigens and showed a loss of virulence. Passive interference and complications of the newborn's maturing immune system enable the widely practicing of maternal vaccination than newborn vaccination (Bandrick *et al*., 2008).

This study intended to determine the stimulation and passive transfer of cellular immunity in the pregnant G. pigs immunized with attenuated *R. equi* to protect their offspring against experimental challenge with virulent *R. equi.*

MATERIALS AND METHODS

Bacterial isolates

Rhodococcus equi was isolated previously from suppurative bronchopneumonia of foals in *R. equi* endemic farm in Iraq. The isolate was identified as described previously (Nakazawra, 1980). Colonies stained red on Congo red containing media (Berkhoff and Vinal, 1986) were described as Congo red positive strain (CR+) and were used for challenge and antigen preparation. Its lethal dose$_{50}$ (LD_{50}) in mice was $2x10^7$ bacteria by intravenous (IV) route. The attenuated, Congo red negative *R. equi* (CR-) isolate kindly provided by Dr. Wisal A. Al-Azzawi - College of Veterinary Medicine / University of Baghdad, its attenuation was previously described (Al-Azzawi *et al.,* 2000). This isolate was used to prepare attenuated autogenous vaccine as previously described in details (Al-Graibawi *et al.,* 2013).

Antigen preparation

Soluble antigen used for DTH -skin test was prepared from CR+ *R. equi* isolate according to Prescott *et al.,* (1979). Briefly the *R. equi* was cultured on nutrient agar for 48 hours at 37° C. The culture was harvested by suspending the growth from the agar surface in PBS. The growth was washed three times by centrifugation at 10.000 rotations per minute (rpm) for 30 minutes with sterile PBS pH.7.2. The sediment was sonicated for 30 min. at intervals in a water-cooled sonicator oscillator at 15 KHZ/sec. (MSE- watt-ultrasonic disintegrator GE.MK_2). The homogenate was centrifuged twice at 12000 rpm for 30 min. each time to remove cellular debris. Protein content was determined in the supernatant, using the method of Lowry *et al* (1951) thereafter, filtered through 0.22 µm Millipore filter and stored at -20℃ .

Experimental animals

Two groups of apparently healthy pregnant guinea pigs of similar age (6-9 months) weighing about 400 grams were housed in the animal experimental house at the College of veterinary medicine. It kept there for three weeks for acclimatization before starting the experiment. The animals were examined with skin test for the detection of CMI to *R. equi* prior to vaccination. Several fecal samples were taken from each guinea pig for bacterial isolation to confirm that these guinea pigs were *R. equi* free and not exposed previously to this organism. They were reared in the separated cages and fed commercial assorted pellets and alfalfa. This study was approved by the Ethical and Research Committee of the College of Veterinary Medicine – University of Baghdad.

A-First group (vaccinated group)

Twelve pregnant guinea pigs vaccinated s.c twice at fifth and third weeks prior the expected parturition date with attenuated vaccine prepared from a CR- *R. equi*, the first dose 1ml containing $1X10^9$ bacteria \ ml and boosted two weeks later with 0.5 ml of the same vaccine containing $1X10^9$ bacteria\ml (Al-Azzawi, 1995). Only offspring from dams which parturited 2-3 weeks after booster vaccine were used.

B- Second group (control)

Six pregnant guinea pigs injected s.c twice with PBS instead of antigen plus adjuvant only. The offspring of vaccinated and control groups were challenged intra-pulmonary with twenty LD50 of CR+ *R. equi* three weeks after birth.

Clinical examination

Vaccinated and control groups were examined daily for the appearances of clinical signs including temperature, pulse and respiration and any observation of distress or discomfort along seven days later after vaccination. Also the offspring of both groups were examined daily till two weeks post challenge.

Evaluation of vaccine efficiency

Vaccine was evaluated for cellular immune response in the dams and their offspring by delayed-type hypersensitivity (DTH) test and extraction of specific *R. equi* transfer factor as follow:

Delayed-type hypersensitivity -Skin test

Two weeks post the 2nd dose of the vaccination, the left flank region of each dam was prepared by clipping and shaving carefully to avoid abrasions the shaved area was divided into four parts injected i.d with 0.1ml of soluble antigen at various concentrations of 33.75µg /ml, 3.37 µg /ml and 0.33 µg /ml and the fourth part was injected with 0.1 ml of BPS ph 7.2. Similarly skin test was performed for their offspring at three weeks of age. The diameter of skin reaction was measured at 24, 48, and 72 hours post injection by ruler according to method described previously (Ellenberger *et al.,* 1984).

Transfer factor assay

As a further assessment of passive transfer of *R .equi* cellular immune responses, transfer factor was extracted from the spleen of offspring born from the vaccinated and control dams, and evaluated in vivo by intraperitoneal (i.p) administration to the recipient (non-sensitized) G. pigs (Ross and Kirkpatrick, 1992) .

Preparation of leucocytes extract

Spleens of offspring from vaccinated and control dams were collected aseptically and soaked for 10 min in PBS containing 1000 U penicillin/ ml and 1000 μg streptomycin/ml. After washing twice with sterile PBS, the spleen tissue was pressed through a stainless-steel screen and suspended in RPMI 1640 medium supplied with 10% fetal calf serum. The cells were made free from red blood cells by treatment with 0.83% ammonium chloride then washed repeatedly with PBS till complete removal of platelets, the viability was determined by trypan blue dye exclusion using 0.1% trypan blue, the viable cells were always more than 80%, the cells were adjusted at $5x10^8$ cells /ml and subjected to repeated freezing- thawing ten times. The leucocytes extract, thus obtained was pooled and stored at -20 ℃ (Al – VarezThull and Kirkpatric , 1996) .

Extraction of transfer factor

The cells extract was centrifuged at 12000 rpm for 30 minutes using cold centrifuge to remove cellular debris. The supernatant was filtered through Amicon 10 filter (Amicon Inc Lexington, Mass) to obtain the lysate containing TF. The lysate was filtered through 0.22 µm millipore filter and referred as TFt and TFn for transfer factor

extracted from offspring of vaccinated and control dams respectively. Both TF were aliquoted and stored at -20^{0}C for later use. Sterility was confirmed by culturing a few drops of the final product on blood agar plates and incubated at 37℃ for 48 hours (Kirkpatrick, 1996).

Transfer factor activity assay

Eighteen G. pigs (*R. equi* free and were not exposed previously to this organisms) were divided equally in three recipient groups. The first recipient group inoculated i.p. 2 ml (equivalent to 5 x 10^8 cell/ml) of TFt. Similarly, the second and third recipient groups were inoculated with TFn and PBS respectively. Seven days post TF administration; all the recipients were tested for DTH test as previously described.

Results

Clinical examination

Following vaccination, the pregnant guinea pigs were slightly depressed and listless. Transient elevation of temperature (39.2 ± 0.2 C), respiration rate (88 ± 4 breath/min) and pulse rate (160 ± 5 beat /min) were recorded for 3-5days. These clinical parameters returned to normal range in the 6th day post vaccination. 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, *R. equi* CR- was isolated from its contents. There were no adverse effects in vaccinated pregnant guinea pigs which gave normal and healthy offspring. The means of the body temperature, pulse and respiration rates remained within the normal ranges in the control group. Localized small nodule swelling was detected during palpation at the sites of injection of adjuvant and PBS. These nodule was disappeared within 10 days and no bacteria were isolated from its content.

Cellular immune response of dams

Vaccinated pregnant guinea pigs had positive skin test reactivity at 24, 48 and 72 hours post i.d inoculation with various concentrations of *R. equi* soluble antigen. The positive skin reaction was detected as edematous, erythematous and indurated areas at the sites of inoculation. The reaction was more evident during the first 48 hours. The control pregnant dams did not show any reaction to the injected antigens (Table1).

Cellular immune response of offspring

Skin test

The two weeks old Offspring from vaccinated dams were reactive to i.d inoculation of soluble *R .equi* antigen at 24 and 48 hours later, while the offspring of control dams did not show any reaction (Table 2).

Transfer factor

The recipient (non-sensitized) guinea pigs that received TFt prepared from the spleens of the newborn guinea pigs from vaccinated dams gave positive skin test reactivity at 24, 48 and 72 hours after i.d inoculation with *R. equi* antigen, while those received PBS and TFn prepared from spleens of newborn guinea pigs from control dams did not react to the *R. equi* antigen (Table 3).

Clinical observation after challenge

Body temperature, pulse and respiratory rates were within normal ranges during the first three weeks of life of the offspring born from both vaccinated and control dams. After challenge, the offspring of vaccinated group showed slight increase in body temperature (39.1±0.21°C). Pulse rate (167± 3.3 beat/min) and respiration rate (88± 2.9 breath/min) with mild signs of illness without death .The offspring of control dams showed marked increase in the body temperature (40 ± 0.3° C), pulse rate (180 ± 6.04 beat/min) and respiratory rate (98 ± 2.1 breath/min) and decreased physical activity, depressed and anorexic by 36 hours post challenge, then showed hunched posture, weight loss, rough hair coat and rapid labored breathing by day four post challenge. All the offspring of the control dams died during 5-16days after challenge. At necropsy the predominant lesions were restricted to the lungs animals. The affected lungs were extensively consolidated with multiple yellowish creamy exudates. Liver, spleen, kidney and lymph nodes were enlarged and congested.

Table (1) Skin reaction of pregnant guinea pigs post i.d inoculation with various concentrations of *R. equi* soluble antigen

Antigen conc. (µg/ml)	Mean diameter of skin reaction (mm)					
	Vaccinated dams (n=9)			Control dams (n=5)		
	24h	48h	72h	24h	48h	72h
33.75	9.2 *(5-16)	13.5 (8-20)	7.1 (3-10)	**0	0	0
3.37	4.5 (3-7)	5.5 (5-8)	2.6 (1-5)	0	0	0
0.33	2.5 (2-4)	2.8 (2-5)	1.2 (1-3)	0	0	0
PBS	0	0	0	0	0	0

*Values given in parentheses are the range of skin reaction diameter (mm).
** Skin reaction was observed at six hours post i.d inoculation and considered to be an immediate type of hypersensitivity.

DISCUSSION

The use of vaccine for the prevention or control of microbial pathogens is preferential alternative to treatment. *Rhodococcus equi* is considered one of the most common causes of respiratory disease in foals at 3 weeks to 6 months of age and is responsible

for severe or chronic pyogranulomatous pneumonia (Al-salihi 2013; Hines 2007). Affected foals require costly and prolonged antibiotic treatment that is not always successful (Giguère *et al*., 2011), and foals that recover may have decreased potential of starting a racing career (Ainsworth *et al.,* 1998, Dawson *et al.,* 2010).So far, no vaccines are available, although several immunization strategies have been tested to prevent rhodococcosis (Giguère *et al.,* 2003).

Table (2) Skin reaction in the offspring of vaccinated and control guinea pigs post i.d inoculation with *R .equi* antigen.

Antigen conc. (µg/ml)	Mean diameter of skin reaction (mm)					
	Offspring of vaccinated dams			Offspring of control dams		
	24h	48h	72h	24h	48h	72h
33.75	3.3 *(2-6)	2.4 (2-3)	0	**0	0	0
3.37	2.3 (2-3)	1.4 (1-3)	0	0	0	0
0.33	1.3 (1-2)	0	0	0	0	0
PBS	0	0	0	0	0	0

*Values given in parentheses are the range of skin reaction diameter (mm).
** Skin reaction was observed at six hours post i.d inoculation and considered to be an immediate type of hypersensitivity.

Table (3) Skin reaction of guinea pigs received TFt and TFn from spleen of offspring from vaccinated and control dams.

Antigen conc. (µg /ml)	Mean diameter of skin reaction (mm)						
	TFt recipients			TFn recipients			PBS recipients
	24h	48h	72h	24h	48h	72h	
33.75	8.4	11.2	5.9	0	0	0	0
3.37	4.1	5.2	2.4	0	0	0	0
0.33	2.1	1.8	0	0	0	0	0
PBS	0	0		0	0	0	0

Host defense against virulent *R. equi* is thought to be cell-mediated, similar to its defense against facultative intracellular bacteria, such as *L. monocytogenes* (Berche *et al.,* 1987) *Mycobacterium tuberculosis* (Shinnick *et al.,* 1995) and *Salmonella typhimurium* (Buckmeier and Heffron,1991) In the present study the immunogenicity and protective potential of a CR – *R. equi* vaccine in pregnant G. pigs were investigated. As the Congo red staining provides a simple and efficient means of screening virulence, ability to bind Congo red appeared to be encoded by virulence

associated plasmid (Berkhoff and Vinal, 1986). The loss of virulence is accompanied by the pigmentation loss appeared to be related to deletion of DNA from plasmid (Maurelli *et al.,* 1984). Takai *et al*. (1991) have demonstrated the association of large plasmids and 15 to 17 kDa antigens with the virulence of *R.equi* in mice and foals, while mutants cured of the large plasmids, lacked the antigens and showed a loss of virulence. The Congo red negative isolate used in the present study for vaccination probably lost its virulence associated antigen and plasmids as indicated by its pathogenicity in guinea pigs. In other facultative intracellular pathogens Congo red staining has been associated with virulence (Prpic *et al.,* 1983; Maurelli et *al.,* 1984).
Vaccination of pregnant guinea pigs with prepared vaccine did not induce adverse systemic reactions except slight depression and listless for 72 hours and transient elevation in body temperature, increased pulse and respiratory rates for 3-6 days, which might be the expression of immunological and inflammatory reactions and was consistent with previous studies (Chirino- Trejo *et al.,* 1987; Martens *et al.,* 1989 and Al-Azzawi *et al.,* 2000). The localized swelling at site of vaccine injection was probably attributable to the local action of adjuvant that was clear in the control group, whereas S.C inoculation with live *R.equi* caused abscessation (Ellenberger *et al.,* 1984). The vaccination procedure appeared safe for the pregnant guinea pigs and without any adverse effects.
Skin test for DTH performed as an *in vivo* test was used to assess CMI in vaccinated pregnant guinea pigs. The positive results of skin test in this study were in agreement with those of others (Al-Salihi, 2011; Ellenberger *et al.,* 1984). Al-Azzawi *et al.,* (2000) reported that vaccination of guinea pigs orally and parenterally with attenuated *R. equi* isolate elicited a skin reaction at 24, 48 and 72 hours post i.d inoculation. Delayed-type hypersensitivity test is defined as a hypersensitive response mediated by sensitized TDTH cells that release various cytokines. The response generally takes 2 to 3 days to develop (Kuby, 1994), and it has been considering as a useful diagnostic and epidemiological tool for several infectious agents like tuberculosis and glanders for many years (Al-Salihi, 2011). Protection against such intracellular pathogens depends on subtle and complex coordination of the CMI response, while it is poorly understood (Prescott, 1991). Effective immunization against intracellular pathogens such as mycobacteria requires the use of live organism rather than killed one to promote cellular immunity. The reasons are unclear but may be related to the requirement for antigen persistence, the activation of different pathways within phagocytic cells or least likely, the secretion of specific protective antigens by live organisms (Collins, 1988). The detection of DTH response and TF in the offspring born from vaccinated dams only, indicated the passive transfer of cellular immunity to the newborn G. pigs. Immunity in the neonatal animal is primarily maternally derived, either by lymphocytes that pass into the newborn across the placenta or following colostrum ingestion (Bandrick, et.al 2008)**.** Reviewing the available recent literatures, no any report concerning specific transfer factor from newly born animals has been published. The classical response to TF treatment is the transfer of cutaneous hypersensitivity to previously non-sensitized individuals (Kirkpatrick, 1992). The ability of TFt to induce DTH in recipient G.pigs indicated its role in increasing cellular immunity and may offer an encouraging approach in improvement of *R. equi* infection. The offspring of vaccinated dams appeared sick for four to six days post challenge,

they exhibited rise in body temperature, pulse and respiratory rates. These offsprings withstood challenge and returned to normal condition from the second week onwards, and did not excrete the organisms in their feces beyond ten days. These clinical findings were in agreement with those reported by Al-Azzawi (2000).While the systemic response was more severe and of longer duration in offspring from control dams. These offspring continued to excrete challenge organisms until death which usually occurred during (5-16) days post challenge. Similar results were observed in guinea pigs infected with *R.equi* (Ishino *et al.,* 1987). The isolation of *R. equi* from the visceral organs of dead offspring can be explained on the basis of the bacteriaemic nature of the *R. equi* infection (Al-Salihi *et al.,* 2013; Giguère *et al.,* 2011; Al-Salihi, 1993). It appeared that vaccination of pregnant guinea pigs with attenuated CR - *R. equi* vaccine was safe, immunogenic and capable of inducing cellular immunity as detected by DTH response and transfer factor induction. Moreover, it protects their offspring against the challenge with virulent *R. equi* .

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Conflict of interest statement

The authors of this paper have no financial or personal relationships with people or organizations that could inappropriately influence or bias the content of this paper.

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